A NEW SPECIES OF *PRISTIMANTIS* (ANURA: STRABOMANTIDAE) FROM THE PACIFIC COAST OF THE DARIEN PROVINCE, PANAMA, WITH A MOLECULAR ANALYSIS OF ITS PHYLOGENETIC POSITION

ANDREW J. CRAWFORD[1,2,3,6], MASON J. RYAN[4], AND CÉSAR A. JARAMILLO[1,2,5]

[1]*Smithsonian Tropical Research Institute, Apartado 0843-03092, Balboa, Ancón, Republic of Panama*
[2]*Círculo Herpetológico de Panamá, Apartado 0824-00122, Panamá, Republic of Panama*
[3]*Departamento de Ciencias Biológicas, Universidad de los Andes, AA 4976, Bogotá, Colombia*
[4]*University of New Mexico, Albuquerque, NM, USA*
[5]*Universidad de Panamá, Facultad de Medicina, Departamento de Histología y Neuroanatomía Humana, Panamá, Republic of Panama*

ABSTRACT: We describe a new species of *Pristimantis* (Anura: Strabomantidae) from the Pacific coast of the Darién Province, Panama. The type locality is on Cerro Piña, in the Serranía de Sapo, at 700–800 m elevation. This new species is readily distinguished from all other known congeners from the region based on external morphology. Despite the small size of the two type specimens, histological analysis of the gonads shows these individuals were potentially reproductive males. Molecular phylogenetic analyses based on the ND2-WANCY mitochondrial gene region reveal that this new species is genetically distinct. Molecular and morphological data place the new species in the *Pristimantis* (*Hypodictyon*) *ridens* species series, and suggest that *P. cerasinus* be moved to this group as well. Molecular analyses also reveal a potential synapomorphy of the genus *Pristimantis* relative to the other five genera sampled here: the loss of the D-stem of the tRNA^{CYS} gene. Our finding of another endemic frog from eastern Panama lends further support to the concept of the Darién as a center of endemism and not just a conduit between continents.

RESUMEN: Describimos una nueva especie del género *Pristimantis* (Anura: Strabomantidae) de la costa del Pacífico de la Provincia de Darién en Panamá. La localidad tipo es Cerro Piña a 700–800 metros sobre el nivel del mar. Con base en la morfología externa esta nueva especie es fácilmente distinguible de todas las otras especies conocidas en la región. A pesar del pequeño tamaño de los dos especímenes tipo, análisis histológicos de sus gónadas muestran que estos individuos eran machos potencialmente reproductores. Los análisis de filogenia molecular basados en la región de ADN mitocondrial ND2-WANCY revelan que la especie nueva es genéticamente distinta. Los datos moleculares y morfológicos sugieren asignar tentativamente a la nueva especie dentro de la serie de especies "*Pristimantis* (*Hypodictyon*) *ridens*" y que la especie, *P. cerasinus*, debe ser incluida en este grupo también. Los análisis moleculares también revelan una posible sinapomorfía del género *Pristimantis* en comparación con los otros cinco géneros incluidos en este análisis: la pérdida de la "rama D" del gen ARNt^{CYS}. El descubrimiento y descripción de otra especie endémica del oriente de Panamá da más apoyo a nuestro concepto del Darién como un centro de endemismo y no sólo como una vía entre dos continentes.

Key words: Gonads; Histology; Molecular phylogenetics; *Pristimantis*; Species description; Sperm development; tRNA secondary structure

THE DARIÉN region of eastern Panama is part of the Tumbes–Chocó–Magdalena global biodiversity hotspot (Mittermeier et al., 1999; updated information available at http://www.biodiversityhotspots.org), and a particularly endangered portion of this diversity comprises amphibians (Stuart et al., 2004). Panama hosts 197 species of amphibian as of 2008 (AmphibiaWeb, 2008). Across the border in the Colombian Chocó region of the Pacific coast there are 139 named species of amphibians in the area below 800 m elevation (Lynch and Suárez-Mayorga, 2004).

Eastern Panama is home to roughly 20 named species of direct-developing anurans in the clade Terrarana sensu Hedges et al. (2008), also known as brachycephalids (Frost et al., 2006). Terraranan frogs have been a continual challenge for taxonomists because of substantial homoplasy and concomitant dearth of autapomorphies to identify species and unambiguous synapomorphies to identify clades (e.g., Campbell and Savage, 2000; Lynch and Duellman, 1997). Molecular phylogenetic analyses have revealed that tradi-

[6] CORRESPONDENCE: e-mail, andrew@dna.ac

tional taxonomy often does not reflect relationships within or among the major lineages of Terrarana (Crawford and Smith, 2005; Heinicke et al., 2007). Herein we describe a new species in the genus *Pristimantis* based on morphological characters, and provide a DNA sequence–based analysis of its distinctness and phylogenetic position. We also uncover an unusual feature of the inferred secondary structure of a mitochondrial tRNA gene, which may help in resolving higher-level relationships.

MATERIAL AND METHODS
Morphological Analyses

Frogs were collected in the field, photographed by A. J. Crawford in the field on the day following collection, and euthanized with dilute chloretone. Specimens were fixed in 10% formalin and stored in 70% ethanol (Pisani, 1973). We examined comparative material from the Círculo Herpetológico de Panamá (CH), the Instituto de Ciencias Naturales of the Universidad Nacional, Bogotá, Colombia, and recent field collections by A. J. Crawford (Appendix 1). The terminology for morphological descriptions follows mainly Lynch and Duellman (1997), Savage (2002), and Savage and Villa (1986). Morphometric abbreviations used throughout the text are as follows: snout–vent length (SVL), length of tibia (tibia), length of head (measured from the tip of snout to posterior of mandible; HL), greatest head width (HW), interorbital distance (IOD), distance from eye to nostril (E–N), distance from the corners on eyes (eye length), and distance between nostrils (ND). All measurements were taken using dial calipers accurate to the nearest 0.1 mm. Observations on the color of the frogs in life were based on field notes and color slides of specimens. The holotype (MVUP 2255, AJC 0922) resides in the Museo de Vertebrados de la Universidad de Panamá and the paratype (CH 8132) resides in the CH.

Histological Methods

Gonads were extracted and processed using standard paraffin-embedding techniques (Luna, 1968). Four-micrometer sections were cut with a rotary microtome and stained using hematoxylin and eosin (Luna, 1968). Identification of histological features was confirmed by comparison with published accounts (Rugh, 1951; Uribe A., 2002).

Molecular Phylogenetic Analyses

Fresh liver samples were preserved in a NaCl-saturated buffer containing 0.25 m ethylenediaminetetraacetic acid and 20% dimethyl sulphoxide (Seutin et al., 1991), and stored in the natural history collection of the CH. We conducted molecular phylogenetic analyses of 39 frog specimens comprised of 36 terraranids and three outgroup taxa (Appendix 2). These outgroups have been shown to be outside of Terrarana but within the larger Nobleobatrachia (a.k.a. Hyloidea) clade (Darst and Cannatella, 2004; Roelants et al., 2007). Analyses included molecular data from the holotype and paratype of the new species described herein. A segment of 1450 bp of contiguous mitochondrial DNA sequence containing the complete ND2 gene, five complete tRNA genes, small portions of the COI gene, and a sixth tRNA gene were used in molecular analysis of each sample. However, no third-position sites from ND2 or COI were used in the analyses (see below). Twenty-two samples were taken from Crawford and Smith (2005) and 14 were taken from Wang et al. (2008) and detailed molecular laboratory techniques are provided in these two publications. The new species described here was referred to as "*P.* sp. nov. A" in Wang et al. (2008). Two terraranid sequences (*Oreobates quixensis* and *Pristimantis altamazonicus*) and one outgroup sequence (*Ceratophrys cornuta*) are published for the first time here. In addition to polymerase chain reaction and sequencing primers listed in Table 2 of Crawford and Smith (2005), we used three previously unpublished internal primers for sequencing: TRPf.J (L5551) 5′-AGACCAARARCCTTCAAAGC-3′, ALAf.A (L5603) 5′-AAGACTTGCAGGACATTAACC-3′, and ND2r.F1 (H4980) 5′-ATCTTCCGGATTTGTGTTTGATT-3′.

Resulting DNA sequences were aligned using the inferred amino acid translations for the ND2 and COI gene regions, whereas the tRNA genes were aligned using the inferred secondary structure based on the work of J.

Robert Macey and colleagues (e.g., Macey et al., 1997a–c; Weisrock et al., 2001). We ran three molecular phylogenetic analysis of our aligned dataset: a partitioned metropolis-coupled Monte Carlo Markov Chain (MCMC) Bayesian phylogenetic analysis (Rannala and Yang, 1996; Yang and Rannala, 1997), a maximum likelihood analysis (ML; Felsenstein, 1981) and a maximum parsimony (MP; Camin and Sokal, 1965) nonparametric bootstrap evaluation of nodal support (Felsenstein, 1985).

For the Bayesian model–based analysis, we partitioned the data into four groups: one partition for each codon position (of the ND2 gene plus the small fragment of the COI gene) and the combined tRNA genes. We tested the total data and each data partition for significant departure from the assumption of stationarity using a χ^2 test implemented in PAUP* 4.0b10 (Swofford, 2000). For each of our four data partitions, we used a Bayesian information criterion as implemented in the perl script, DT-ModSel (version: 13-Aug-02) by Minin et al. (2003) to select the most appropriate model among the 56 commonly used models of molecular sequence evolution. Model selection procedures included only ingroup taxa.

We performed Bayesian phylogenetic analyses using the software, MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). We ran two simultaneous analyses, each using four metropolis-coupled MCMC chains with the heating parameter set at 0.08, and all other parameters and prior distributions left at their default values. Runs lasted five million generations, with trees sampled every 1000 generations. For comparison, we ran an ML analysis on the unpartitioned data set using PAUP*, starting from a neighbor-joining tree and using a heuristic search with subtree pruning–regrafting branch-swapping.

For comparison with Bayesian marginal posterior probability (mpp) support values for each clade, we also conducted a MP nonparametric bootstrap analysis using PAUP*. MP bootstrap support is regarded as a more conservative estimate of clade support than mpp, but under certain conditions it may be less accurate (e.g., Alfaro et al., 2003). We employed heuristic searches with tree bisection–reconnection branch-swapping and 50 random-addition sequence replicates on each of 2000 bootstrapped data sets. Nodal support was estimated from the proportion of pseudoreplicates that contained a given node.

RESULTS

Morphological characters and molecular phylogenetic analyses (see below) suggest that populations from Serranía del Sapo represent a new species that belongs to the *Pristimantis* (*Hypodictyon*) *ridens* species series. This group is characterized by expanded digital disks, toe V longer than toe III, absence of supernumary tubercles under fingers and toes, absence of toe webs, a coarsely areolate venter, and tympanic membrane and annulus usually distinct (Hedges et al., 2008; Lynch, 1976; Savage, 2002). We name this species below.

SPECIES ACCOUNT

Pristimantis adnus, *sp. nov.*
Fig. 1

Holotype.—An adult male (see histology results below; MVUP 2255; collector number AJC 0922) collected by A. J. Crawford, accompanied by Carolina Polanía, Dr. Chris Jiggins, and other members of the laboratory of Dr. Eldredge Bermingham of the Smithsonian Tropical Research Institute. This frog was found along a trail approximately 700 m in elevation at the foot of Cerro Piña in the Serranía del Sapo, above the Río Piña on the Pacific coast of the Darién Province, Republic of Panama (Fig. 2). Global positioning system (GPS) coordinates were not taken at this point, but elevation was estimated using a wristwatch altimeter. GPS points were taken both below the collecting site (07.67314, −078.20639, datum WGS 84, 546 m elevation) and above it (07.69080, −078.19803, datum WGS 84, 1150 m elevation). The collecting site was reached by following a ridge up from our base camp located at the coordinates 07.682, −078.2022 (elevation by GPS: 85 m) next to the Río Piña, 6.2 km upstream from Bahía Piña. The holotype was collected in the leaf litter on the ground on 13 May 2003, at 1310 h. Liver and testes were removed from holotype through a ventrolateral incision for genetic and histological analyses, respectively.
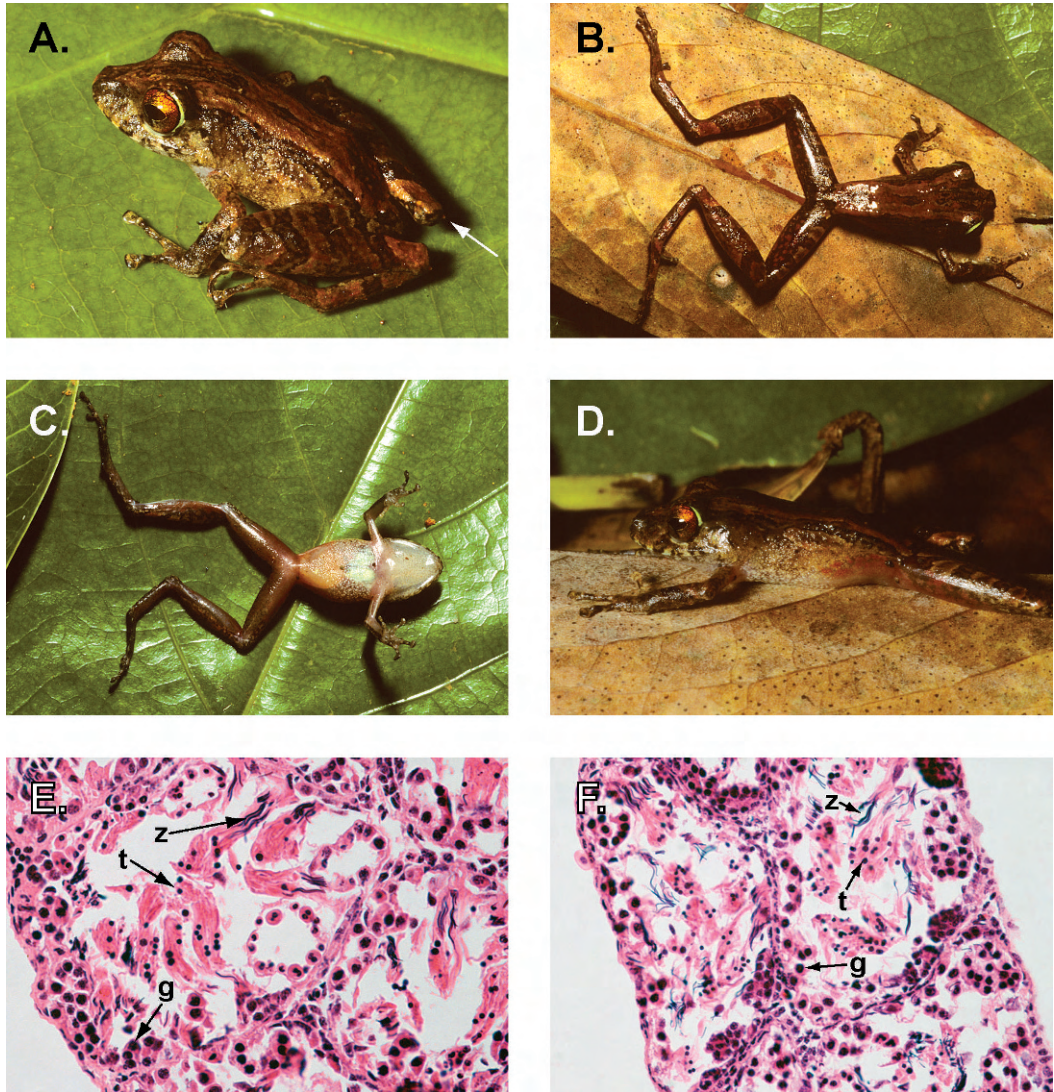
FIG. 1.—Photographs A–D are of holotype specimen MVUP 2255 (collector number AJC 0922) taken during the daytime. (A) profile, with white arrow indicating the enlarged heel tubercle on the right leg. (B) Dorsum and posterior thighs (note spots on posterior thighs). (C) Venter (note silver-colored musculature posterior to pectoral girdle). (D) Lateral view including groin (note slight red coloration in groin). Images (E) and (F) are histological sections of (E) MVUP 2255 and (F) CH 8132 (collector number AJC 0924), demonstrating the sex and reproductive status of these males. Letters indicate the following structures: spermatogonia (g), spermatids (t), and spermatozoa (z).

Paratopotype.—CH 8132 (collector number AJC 0924). Collection data as above by A. J. Crawford, but at approximately 800 m elevation and at 1220 h. Liver and testes were removed from paratopotype through a ventrolateral incision for genetic and histological analyses, respectively.

Diagnosis.—A member of the *Pristimantis* (*Hypodictyon*) *ridens* species series with the following qualities: (1) skin on dorsum is shagreen with scattered enlarged granules; small supratympanic ridge present; venter areolate; dorsolateral fold absent; (2) tympanic membrane not concealed; tympanic annulus faintly visible; (3) canthus rostralis distinct and concave; nostrils protuberant; area between nostrils concave; (4) enlarged supraocular tubercle absent; series of superciliary tuber-

cles on edge of upper eyelid weakly developed; cranial crests absent; (5) vomerine odontophores not observed; (6) vocal slits and vocal sac absent; (7) finger II longer than finger I; disks expanded; (8) fingers lacking lateral fringes; (9) ulnar tubercles absent; (10) heel bearing single enlarged but modestly sized tubercle; a small elongate inner tarsal, tuberculate fold present; (11) inner metatarsal tubercle elongate, ovoid and elevated; outer metatarsal tubercle small, conical and round; a small elongate inner tarsal, tuberculate fold present; (12) lateral fringes on toes absent; webbing absent; subarticular tubercles on toes projecting; supernumary tubercles absent; plantar tubercles absent; third toe longer than fifth; (13) posterior thigh dark brown to red, with lighter colored red or orange spots; pale red or orange spots in groin and anterior surface of thighs; (14) SVL in males 19–20 mm; females unknown.

Among Panamanian species, *P. adnus* is superficially similar to *P. ridens*, *P. caryophyllaceus*, and *P. cruentus* of the *Pristimantis (Hypodictyon) ridens* species series; *P. cerasinus* of the *Pristimantis (Hypodictyon) rubicundus* species series; and *P. taeniatus* of the *Pristimantis (Pristimantis) frater* species group (Hedges et al., 2008; Savage, 1981). However, none of these aforementioned species has spotting on the posterior thigh, whereas *P. adnus* shows obvious spotting (Fig. 1B,D). In *P. adnus* and *P. cerasinus* the tip of toe V does not reach the distal subarticular tubercle of toe IV. *Pristimantis cruentus* and *P. caryophyllaceus* have a pointed or enlarged supraocular tubercle whereas *P. adnus* and *P. cerasinus* lack a pointed or enlarged supraocular tubercle. In *P. adnus* the series of superciliary tubercles along the edge of the upper eyelid is weakly developed (strongly developed in *P. cerasinus*). The canthus rostralis of *P. adnus* is sharper (more angular) than that of *P. cerasinus* and is longer than that of *P. cruentus*. The tip of the rostrum of *P. adnus* is rounded (distinctly pointed in *P. caryophyllaceus*). The supertympanic stripe in *P. adnus* is faint or lacking (dark and distinctive in *P. taeniatus* of Panama). Although *P. adnus* and *P. ridens* can be readily distinguished using the above characteristics, these two species do

resemble one another superficially in terms of dorsal coloration, small size, and silvery musculature visible through the skin on the ventral side of the body (posterior to the pectoral girdle in males). *Pristimantis adnus* possesses an enlarged pointed heel tubercle, but in *P. adnus* the heel tubercle is less obvious than that found in *P. cruentus*, *P. caryophyllaceus*, and *P. cerasinus*. Our review of specimens of *P. ridens* and *P. taeniatus* showed that these frogs sometimes have a small pointed heel tubercle as well, suggesting that this character may be of limited value in identifying *P. adnus* in Panama.

Among Colombian species, *P. adnus* is most similar to *P. thectopternus* from the Cordillera Occidental of Colombia (Lynch, 1975), a member of the *Pristimantis (Pristimantis) conspicillatus* species group (Hedges et al., 2008). However, these two species can be distinguished as follows: In *P. thectopternus* the spots on the posterior thighs are distinctly white (dark red-orange in *P. adnus*), the tympanum is half as wide as the eye (one-third as wide in *P. adnus*), limb bars on the dorsal surface of the legs are one-fourth the width of the gray interspaces (bars and interspaces have the same width in *P. adnus*), and a supratympanic stripe is dark and distinct (faint to lacking in *P. adnus*). *Pristimantis roseus* from the Pacific lowlands of Colombia resembles *P. ridens* but has "posterior thigh cream peppered with brown and marbled with brown" (Lynch, 1980). Both *P. ridens* and *P. roseus* have concealed tympana (distinct in *P. adnus*). *Pristimantis adnus* may be distinguished from western Colombian members of the *Pristimantis (Hypodictyon) rubicundus* species series as follows: *Pristimantis lanthanites* has vocal sac and slits (absent in *P. adnus*), and a uniform posterior thigh (Lynch, 1975; spotted in *P. adnus*). *Pristimantis labiosus* from the Pacific lowlands of southern Colombia and Ecuador has vocal slits (absent in *P. adnus*), a conical tubercle on upper eyelid (absent in *P. adnus*), and very broad finger and toe disks (Lynch, 1994; less broad in *P. adnus*). *Pristimantis orpacobates* from the northern end of the Cordillera Occidental of Colombia (1140–2000 m elevation) has one to three conical tubercles on upper eyelid (absent in *P. adnus*) and large finger and toe
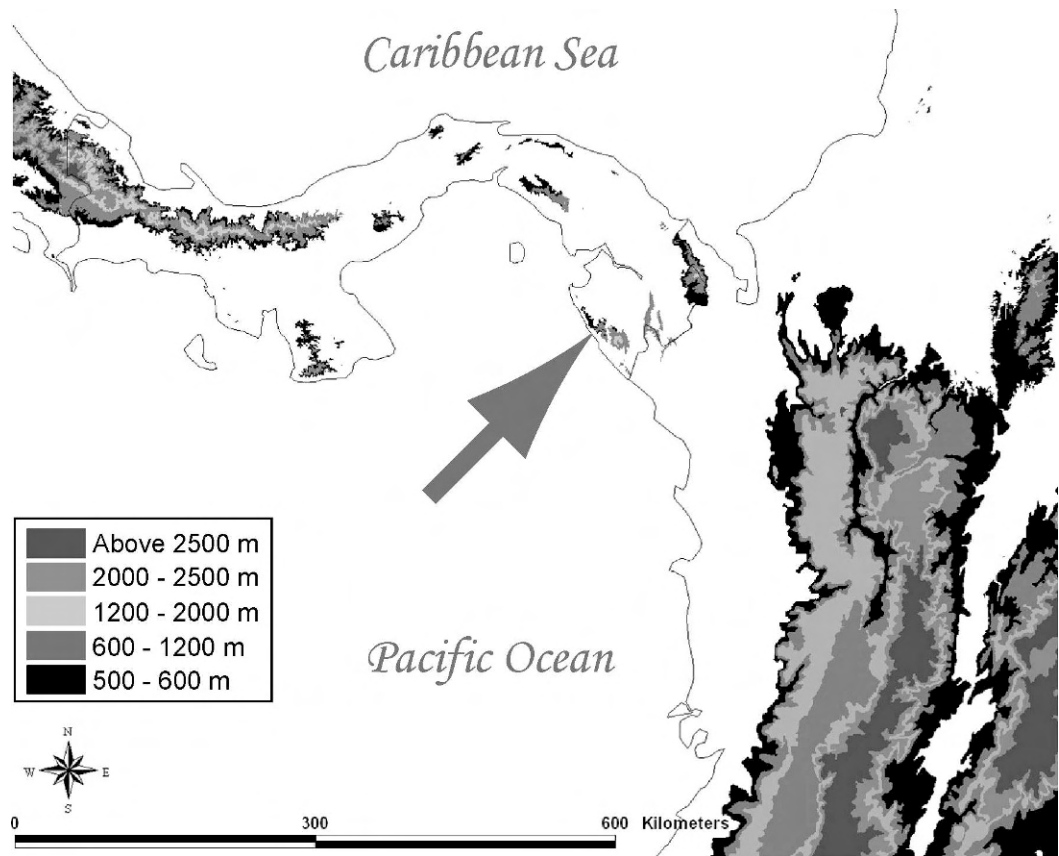
FIG. 2.—Map of Panama and northwestern Colombia, with arrow indicating the type locality of *Pristimantis adnus*. Elevation in meters indicated by shading. On the western edge of the map lie the Talamanca Mountains of Costa Rica, and the Colombian Andes lie on the eastern edge.

disks (smaller in *P. adnus*), and tiny white flecks on posterior thighs (Lynch, 1994; posterior thighs have red-orange spots in *P. adnus*). *Pristimantis ocellatus* from southern Pacific Colombia lacks a heel tubercle (Lynch and Duellman, 1997; present in *P. adnus*). *Pristimantis w-nigrum* has prominent black spots on flanks, groin, and concealed surfaces (no prominent black spots in *P. adnus*); the posterior thigh color is black with white spots (brown with red-orange spots in *P. adnus*, and males have vocal slits (absent in *P. adnus*).

Description of the holotype.—A small frog, 19.0 mm SVL; head wider than body; head longer than wide; canthus rostralis distinct and concave; nostrils protuberant; area between nostrils concave; nostrils directed laterally and slightly posteriorly; eyes directed laterally; eye length greater than ND; vocal slits and vocal sac absent; enlarged supraocular tubercle absent; series of superciliary tubercles on edge of upper eyelid weakly developed, with faint white spots; upper eyelid rugose but lacking an enlarged superocular tubercle; eyes do not extend beyond jaw in dorsal view; head round in dorsal view and truncate in profile; tympanum distinct, ovoid, not concealed, with annulus faintly visible; small supratympanic ridge present; one round postrictal tubercle; interobital distance greater than width of upper eyelid; distance of eye to tympanum almost equal to distance from eye to lip; finger length formula I < II < IV < III; tip of finger IV reaches beyond distal subarticular tubercle on finger III; tip of finger II barely reaches distal subarticular tubercle of finger III; disks on fingers: I and II expanded and even; III and IV

fan shaped; disk pads on fingers: I rounded; II, III, IV broadened; a raised lateral keel extending from base of finger disks to digit; subarticular tubercles distinct and projecting, pungent, and round; supernumary tubercles absent; accessory palmar tubercles absent; thenar tubercle elongate and wide, (0.5 mm) one-third length of finger I; palmar tubercle deeply lobed U; toe length formula I < II < III < V < IV; tip of toe V reaches distal subarticular tubercle on toe IV; tip of toe III reaches medial subarticular tubercle on toe IV; disks on toes: I and II expanded and even, III and IV fan-shaped, V truncate; pads on toes: I, II, and III broadened, IV and V truncate; subarticular tubercles on toes distinct and projecting, ovoid and pungent; supernumary tubercles absent; plantar tubercles absent; inner metatarsal tubercle elongate, ovoid and elevated, almost half the length of toe I; lateral fringes absent; outer metatarsal tubercle small, conical and round; a small elongate inner tarsal, tuberculate fold present; a pointed heel calcar; a small lateral skin fold anterior to cloacal opening, much smaller than opening.

Dorsum is shagreen with some scattered enlarged granules; interocular fold absent; a pair of postocular folds extend posteriorly from eye to back; throat texture granular with faint black pigmentation under scattered white spots; venter coarsely areolate with scattered white spots and sparse dark pigmentation.

Histology.—Histological analysis of the gonads confirmed that both type specimens are males. Both individuals were potentially reproductive adults at the time they were collected, as evidenced by the spermatozoa (Fig. 1E,F).

Color in preservative.—Holotype: The originally reddish-brown dorsum (Fig. 1) turned a light ashen gray in preservative; extremities lighter than trunk dorsally; posterior thighs reddish-brown with pale, slightly orangish spots; dorsal surface of legs with barred with brown and gray; venter pale with fine dusting of brown pigmentation on anterolateral of gular area.

Color in life.—The following are taken from the field notes of A. J. Crawford written within 0–3 days of capture. For holotype (AJC 0922): Ventral abdomen coated in fine white dots,

ventral skin see-through (transparent); anterior 40% of abdomen shows silvery-white chest musculature. Gular ashen grey with mild white flecking. Blotchy red-orange on groin and anterior thighs, plus some on ventral thighs and ventral calves. Posterior thighs are dark brown with a few light dots showing red-orange color. Suprascapular "W" pattern of tubercular ridging. No supraocular tubercles. Calcars tiny but clearly visible on heels. Protuberant nares and big head are reminiscent of *P. cerasinus*. Iris more maroon than gold-colored. Tissued 16 May 2003.

A. J. Crawford field notes for paratype (CH 8132): Ventral abdomen covered in fine white flecks but still see-through (transparent); anterior one-third of abdomen shows silvery chest muscles. Reddish orange coloration (or is it orangish-pink?) found in groin, anterior thighs, ventral calves, and even a tiny spot in armpits. Posterior thighs have same color, too, but localized to lots of dots amid a dark brown background color; dark brown color localized to posterior thighs. Tiny calcar tubercles and tiny supercilliary tubercles. Sharp canthus rostralis forming a concave area between it and upper lip. Nares prominent. Iris is a rosy gold color. Pickled 16 May 2003.

Measurements of holotype (and paratype) in millimeters.—SVL: 19.1 (20.0); tibia: 10.8 (12.3); HL: 7.5 (7.8); HW: 6.9 (7.2); IOD: 2.0 (2.0); E–N: 2.0 (2.4); eye length: 2.3 (2.5); IND: 1.7 (1.6); width of finger IV disk: 0.7 (0.8); width of finger III: 0.6 (0.8). ratios (×100%) of holotype: SVL/tibia = 56; SVL/HL = 39 IOD/HW = 28; HL/HW = 92.

Etymology.—The name of this small species refers to the Spanish acryonym, ADN, for ácido deoxyribonucleico, meaning deoxyribonucleic acid (DNA) in English. This eponymous reference to DNA in Spanish refers to the great potential for these data-rich molecules to accelerate phylogenetic inference, species discovery, and species identification, especially in species-rich clades of morphologically homoplastic Neotropical amphibians, such as Terrarana.

Remarks.—The molecular and morphological analyses reported here suggest that this new species be assigned to the *Pristimantis* (*Hypodictyon*) *ridens* species series (Hedges et al., 2008). This species is currently known

only from the type locality, located in World Wildlife Fund ecoregion NT0122, the eastern Panama montane forest (Olson et al., 2001). Photographic evidence hints that this species might also occur on the Pacific coast of Colombia or on the Serranía Tacarcuna to the northeast, but these reports cannot be verified at this time. Both examples of this species were found in the leaf litter during the day, but we do not yet know whether this species should be considered diurnal. Searches at this elevation were limited to a single afternoon, so we cannot comment on the relative abundance of this species. Limited taxonomic sampling of molecular characters suggests that the closest relative of *P. adnus* is *P. cerasinus* (Fig. 4).

Molecular Phylogenetic Results

Although we found some length variation among DNA sequences for the ND2-WANCY region, we inferred that all data came from mitochondrial DNA rather from than pseudogenes transferred to the nucleus because (1) we did not observe nonsense mutations in the ND2 or COI genes; (2) the light strand shows strongly biased nucleotide frequencies as observed previously in animal mtDNA (Macey et al., 1998; Sperling and Hickey, 1994), especially in third position sites (see below); and (3) all tRNA genes appeared to code for tRNA molecules with functional secondary structures. However, an unusual secondary structure was observed in all samples of *Pristimantis*. These samples appeared to be missing 4, 7, or 9 bp from the region of the D-stem of the tRNA^{CYS} gene, relative to all other samples, including *Craugastor*, which showed the standard secondary structure (Fig. 3). The standard D-stem of the tRNA^{CYS} gene is 9 bp long. Two *Pristimantis* sequences (GenBank accession numbers EU443188 and EU443184) showed a distinct 5-bp sequence in this gene region that could potentially form a tiny stem structure. However, these two 5-bp sequences show no similarity with each other and no apparent homology with the other Terrarana sequences (Fig. 3), and may represent novelties.

The null hypothesis of stationarity of nucleotide frequencies among taxa was rejected for the combined data set ($P = 0.0012$) and for third position sites alone ($P = 0.0000$), but not for first ($P = 0.9849$) or second position sites ($P = 1.0$) or for the combined tRNA genes ($P = 1.0$). Therefore, we removed third position sites from all phylogenetic analyses (Bayesian, ML, and MP bootstrap). We also removed all gapped sites as well as the hypervariable 3′ end of the ND2 gene, which was difficult to align even at the amino acid level because of the high level of sequence and length variation (Crawford and Smith, 2005). All analyses, therefore, included 968 characters, of which 521 were parsimony informative and 124 were "singletons."

Based on our model selection results we applied a six-parameter general time-reversible (GTR) model (Tavaré, 1986) + Γ (Yang, 1994) to the three partitions included in our analysis (positions 1 and 2, plus tRNA genes). In the ML analysis we adopted a single GTR + Γ model for all sites, as chosen by DT-ModSel. Likelihood topology and MP bootstrap consensus tree topology were identical to the Bayesian consensus tree topology (Fig. 4), suggesting that the inferred relationships are robust to method of inference. In addition, most nodes on the tree were resolved with very high statistical support as measured by both MP bootstrap and mpp (Fig. 4). Key to this study, *P. adnus* came out sister to *P. cerasinus* with high nodal support in both analyses. The placement of *Oreobates* relative to other genera was not resolved in this analysis.

DISCUSSION

The high level of species diversity of the Darién and northern Chocó may be explained in part by the mixing of Central and South American faunas during the Great American Biotic Interchange (Marshall, 1988; Savage, 1982, 2002; Stehli and Webb, 1985; Vanzolini and Heyer, 1985). *Pristimantis* most likely originated in South America, where it is most diverse (Duellman, 1999; Heinicke et al., 2007; Lynch, 1971), and spread into Central America as far north as Honduras (Duellman, 2001; Savage, 1982) in the Pliocene with the completion of the Isthmus of Panama, or perhaps earlier (Wang et al., 2008).

In addition to its role as a conduit, the Isthmus of Panama has also fostered its own endemic amphibian fauna, perhaps fostered in

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C. augusti AY273109           AAGCCCCGGCAGGAA---TTAGCTGC-CTCTTGGAGTTTGCAACCCCACGTGTAACACCCCGGGGCC
C. bocourti AY273110          AAGCCCCGGCAGGAA---CAAACTGC-GTTTTGGAGTTTGCAACTCCACGTGTGACACCCCTGGGAC
C. bransfordii AY273140       AAGCCCCGGCAGGAA----TATTCTGC-TCTTGGAGGTTGCAACTCCATGTGTGACACCCCAGGGCT
C. daryi AY273107             AAGCCCCGGCAGAAACTTTCTTCTGC-TTCTCGGAGCTTGCAATTCCGCGTGTAACACCCCAGGGCC
C. gollmeri AY273124          AAGCCCCGGCAGGA--CTATTCTGC-TTCTTGGGGGTTGCAACCCCACGTGTAACACCCCAGGACC
C. megacephalus AY273111      AAGCCCCGGCAGGAA---TTATCTGC-GTTTTGGGGGTTGCAACCCCACGTGTGACACCCCAGGACC
C. noblei AY273127            AAGCCCCGGCAGAAAA-TTTTTCTGC-TTCTTGGGGGTTGCAACCCCACGTGTAACACCTCGAGGCC
C. podiciferus AY273135       AAGCCCCGGCAGAAA---TCTTCTGC-TTCTCGGAAGTTGCAATTCCGTGTGTGACACCCCAGAGCC
C. pygmaeus AY273119          AGGCCCCGGCAGAAA---TAATCGGC-GGTTTGGGGTTTGCAACCCCATGTGGAGCACTTCAGGGCC
C. ranoides AY273112          AAGCCCCGGCAGAAA--TTACTCTGC-GTTTTGGGGTTGCAACCCCACGTGTGACACTTCCGGGACC
C. rhodopis AY273131          AAGCCCCGGCAGAAA--CTCTCCTGCTTTTTCGAGGTTTGCAACCTCACGTGTGACACCCCAGAGCC
C. sartori AY273121           AAGCCCCGGCAGGAA---TTAGCTGC-GTTTTGGGATTTGCAAGCCCACGTGTGACACCCCAGAGCC
C. tabasarae AY273115         AAGCCCCGGCAGAAA---TTACTCTGC-GTTTTGGGGTTGCAACCCCACGTGTGACACCCCGGGACC
C. trachydermus AY273106      AAGCTCCGGCAGAAGA-TTTTTCTGC-TTCTTGGGAATTGCAAGCCCACATGTAACACCCCAGAAACC
Ceratophrys cornuta LSU 17416 AAGCCCCGGCAGAAATATTATTCTGC-TTCTCGAGATTTGCAATCTCGCGTGTGACACCCCAGAGCC
P. adnus AJC0922 EU443191     AAGCCCCGGCAGA-AT-TTCTTCTGC-TTCTAGAGGTTTGCGGTCTCA-ATT--------CAGGGCC
P. adnus AJC0924 EU443192     AAGCCCCGGCAGA-AT-TTCTTCTGC-TTCTAGAGGTTTGCGGTCTCA-ATT--------CAGGGCC
P. altae EU443185             AAGCCCCGGCAGG--TATTACCCTGC-TGCTCGAGGTTTGCAGACTCT-ACT--------CAGGGCC
P. altamazonicus LSUMZ H 15467 AAGCCCCGGCAGA-ATCTA-CTCTGC-TGCTTAAGATTTGCAGTCTTT-ATT--------CAGAGCC
P. cruentus EU443176          AAGCCCCGGCAGA--AAATAATCTGC-TTGTGGAGGTTTGCGGTCTCA-GTC--------CAGGGCC
P. cruentus EU443186          AAGCCCCGACAGA--AATTATTCTGT-TAGTTGAAATTTGCGGCCTCG-ATT--------CTAGACC
P. cruentus EU443188          AAGCCCCGGCAGA--AACTAAATCTGCTTTGTCGAGGTTTGCGGACTCG-A-TTCAA--GCAGGGCCC
P. sp. C, AJC 0601 EU443184   AAGTCCCGGCAGA-TGC-TATTCTGC-TTATAGAGGTTTGCAGCCTCA---AAGTT----CGAAACC
P. museosus AY273103          AAGCCCCGGCAGA--AATAAAATTTGC-TTATTGAGAATTGCGGGCTCG-GTT-------CGCGGGCT
P. pardalis AY273102          AAGCCCCGGCAGG--TA-TTACCTGC-TGCTTGAGGTTTGCAGACTCT-ATT--------CAGGACC
P. pirrensis EU443190         AAGCCCCGGCAGGAATTA---CCTGC-TGCTTGAGGTTTGCAAACTCT-ATT--------CGAGGCC
P. ridens EU443159            AAGCCCCGGCAAA--AAATCTTTTGCTTT-CTGAGGTTTGCAAACTCT-GTA--------CAAGACC
P. ridens EU443154            AAGTCCGGGCAAA--ATTTCTTTTGC-GTCTTGAGGCTTGCAAACTCT-ATG--------CCGGACC
P. ridens EU443164            AAGCCCCGGCAAA--AAATCTTTTGCAATTTTGAGGTTTGCAAACTCT-GTA--------CAAGACC
P. ridens AY273101            AAGTCCGGGCAAAAT---TTCTTTGC-TTCTTGAGGTTTGCAAACTCT-ATG--------CCGGACC
P. ridens EU443165            AAGCCCAGGGCA-A--ATTTC-TTTGC-TTCTTGAGGTTTGCAAACTCT-GTG--------CTGGGCC
P. sp. nov. B, EU443193       AAGCCCCGTCAGAAA--TTTC-TT-GTGAGGTTTGCGGTCTCG-GT-T-------CAGGGCC
Euhyas pantoni AY273104       AAGCCCCGGCAAA----CTATTTGC-TTCTTGAGGGTTGCAACCTCACGTGTAACACCTCGGGGCC
Oreobates quixensis LSU12784  AAGCCCCGGCAGGAA--TTCTCCTGC-TTCTCGAGGTTTGCAATCTCGCGTGTGGAAACACCCCGAGGC
L. labialis AY273100          AAGCCCCGGCAGA-GT-TTCCTCTGC-TTCTTGGGATTTGCAATCCCACGTGTGACACCCCAAGGCC
L. melanonotus AY273099       AAGCCCCGGCAGA-TT-TTCCTCTGC-TTCTTGGGATTTGCAATCCCACGTGTGACACCCCGGAACC
Syrrhophus pipilans AY273105  AAGCCCCGGCAATAA---TTATTTGC-TTCTTGAAGTTTGCAACTTCACGTGGAACACCTCGGGACC
                              *<<<<<AA<<<<T#######<<<<T#####<<<AC  Cvs   <<<AC <<<D <<<D  <<<<<AA
```

Fig. 3.—DNA sequence alignment of the tRNA[CYS] gene based on inferred secondary structure, illustrating the inferred absence of the D-stem structure in *Pristimantis*, relative to other samples used in this study. Secondary structure follows Macey et al. (1997*a–c*). Structural annotations are indicated on the bottom line: amino acid stem (AA), amino acid acceptor base (*), D-stem (D), T-stem (T), and anti-codon (Cys). The length of a given stem structure is indicated by the less-than symbol (<) to the left of the corresponding annotation. (This gene is encoded on the light strand, and would be transcribed from right to left.) To aid in visualization, the T-stem and D-stem positions are underlined, and the anti-codon positions are highlighted in bold. The nucleotides in the D-stem region of two *Pristimantis* samples could potentially form a diminutive 2-bp stem structure. Note that our phylogenetic analyses excluded all gapped sites, the D-stem, and positions annotated here with the # symbol. The tRNA[CYS] gene sequence was unavailable for two individuals: *Craugastor fitzingeri* (FMNH 257745) and *P. cerasinus* (FMNH 257713).

part by the relatively low capacity for migration or dispersal and relatively high population structure among amphibians (Beebee, 2005; Crawford, 2003; Vences and Wake, 2007). In addition to *P. adnus*, other endemics include montane frogs such as *Strabomantis laticorpus*, *Atelopus glyphus*, and *Rhinella acrolopha* (Frost, 2009). Although the isthmian land bridge was not completed until approximately 3 million yr ago (Ma; Coates and Obando, 1996), the inchoate Darién began to appear around 12 Ma or earlier (Kirby et al., 2008), forming long, narrow islands by 6 Ma (Coates et al., 2004). These islands may have allowed colonization of the Darién from the north or south in advance of the completion of the land bridge in the Pliocene (Wang et al., 2008;

Weigt et al., 2005). The increasing number of known Darién endemic species of amphibians (e.g., Ibáñez and Crawford, 2004) and reptiles (e.g., Myers, 2003) supports the hypothesized old age of the fauna of eastern Panama.

Comparison of our phylogeny with Hedges et al. (2008) is not straightforward because on the one hand the tree presented here has relatively scant sampling, whereas on the other hand our tree includes some species not sampled by Hedges et al. (2008). Hedges et al. (2008) recovered a clade containing *P. ridens*, *P. cruentus*, and other members of the *P. ridens* series as sister to a clade containing members of the *Pristimantis* (*Hypodictyon*) *rubicundus* species series. We sampled one presumed member of the *P. rubicundus*
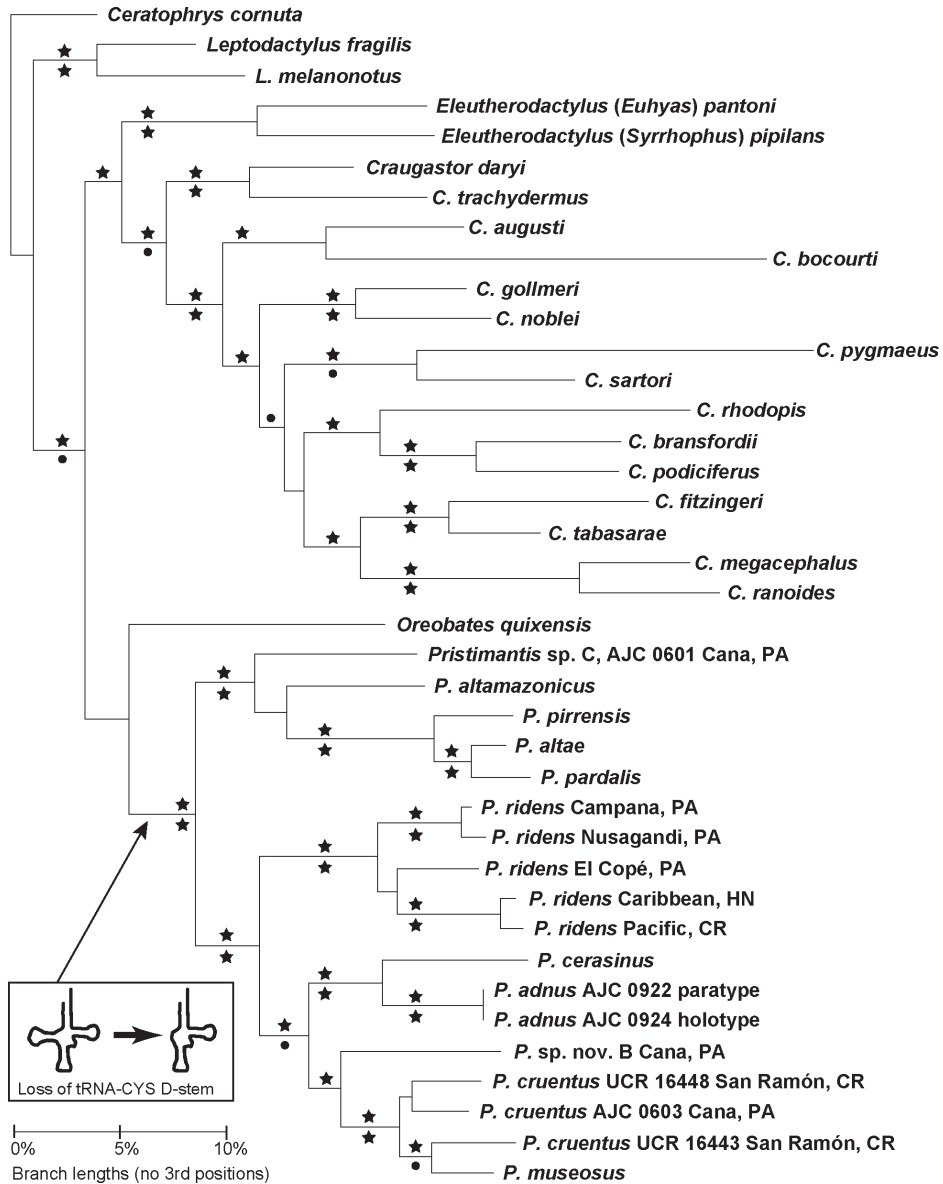
FIG. 4.—Bayesian consensus phylogram of four genera of terraranid frogs and two outgroup genera based on 968 bp of mtDNA comprised of first and second position sites of *ND2*, five tRNA genes, and flanking sites. Bayesian marginal posterior probabilities (mpp) for a given node are indicated above each branch, with maximum parsimony bootstrap support (bss) indicated below the branch. A star indicates 1.0 mpp or >94% bss. A circle indicates 0.95–0.99 mpp or 84%–94% bss. Note that *Pristimantis cruentus* is paraphyletic with respect to *P. museosus*.

series, *P. cerasinus*, which was not sampled by Hedges et al. (2008). In our analysis, *P. cruentus* is more closely related to *P. cerasinus* than to *P. ridens*, a result that would appear to conflict with that of Hedges et al. (2008), assuming that the samples are correctly

allocated to species series. To restore monophyly to these taxonomic groups, we recommend that *P. cerasinus* be moved to the *P. ridens* series, along with *P. adnus*. Moving *P. cerasinus* to the *P. ridens* series makes *P. achatinus* the only member of the *P. rubi-*

cundus series that enters Central America and restricts the *P. rubicundus* series to the eastern side of the Panama Canal.

Possible Synapomorphy of Pristimantis

The loss of the D-stem of the tRNA^{CYS} gene was observed previously in acrodont lizards and was suggested to be a synapomorphy of the clade (Macey et al., 1997*b*), but has also been observed to occur independently in other squamate clades (Macey et al., 1997*c*). We can readily infer that the absence of the D-stem of the tRNA^{CYS} gene also represents the derived condition within the phylogeny presented here (Fig. 4). Although the taxonomic sampling in the present study is limited, this structural feature of the mitochondrial genome is a potential synapomorphy of the genus *Pristimantis*, as well. We suggest that changes in secondary structure and other higher-level features of mitochondrial DNA may serve as a source of important characters useful for resolving deeper-level relationships among Terrarana and other anurans.

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Appendix I

Specimens Examined

Pristimantis adnus *(holotype and paratype).*—PANAMA: *Darién Province:* Serranía del Sapo, above the Río Piña, 6.2 km upstream from Bahía Piña, 700 m, MVUP 2255 (AJC 0922); 800 m, CH 8132.

Pristimantis cruentus *(five specimens).*—PANAMA: *Coclé Province:* Caño Sucio, Santa María, 95 m, CH 5369; Parque Nacional General de División Omar Torrijos Herrera, 700 m, AJC 0568, CH 0387. *Comarca Kuna Yala:* Nusagandi, 420 m, CH 3452. *Darién Province:* Cana, Pirre high camp, 1550 m, AJC 0597.

Pristimantis cerasinus *(nine specimens).*—PANAMA: *Bocas del Toro Province:* Culubre, 170 m, CH 6519; Río Changuinola, Guayacán, 245 m, CH 6571. *Coclé Province:* San Miguel Arriba, 400 m, CH 5098; Parque Nacional General de División Omar Torrijos Herrera, 800 m, CH 6588, 6591. *Colón Province:* Parque Nacional Soberanía, Quebrada Juan Grande, 30 m, CH 7108-9. *Comarca Kuna Yala:* Nusagandi, 420 m, CH 7102. *Panama Province:* Chilibre District, Altos de Cerro Azul, 600 m, AJC 0971.

Pristimantis ridens *(six specimens).*—PANAMA: *Coclé Province:* Tres Hermanas, Río Indio, 25 m, CH 4968. *Colón Province:* Parque Nacional Soberanía, Río Limbo, 50 m, CH 4418; Río Coclé del Norte, Los Almendros, 10 m, CH 5224; Sherman, 70 m, CH 3850; Sierra Llorona, 200 m, CH 4836. *Panama Province:* Cerro Trinidad, 875 m, CH 4278.

Pristimantis taeniatus *(five specimens).*—PANAMA: *Darién Province:* Trail to Serranía de Pirre, 700 m, CH 5529. *Panama Province:* Altos de Cerro Azul, 800 m, CH 3663, 6796; Lago Alajuela, Quebrada Tranquilla, 80 m, CH 3859; Río Chagres, Estación de la ACP en Río Chico, 200 m, CH 6854.

APPENDIX II

Species names, institutional voucher numbers, field tag numbers, locality information, and GenBank accession numbers for all frog samples used in this study. Most DNA sequences were previously published in Crawford and Smith (2005) or Wang et al. (2008). Three DNA sequences are newly published here: those from *Ceratophrys cornuta*, *Pristimantis altamazonicus*, and *Oreobates quixensis*.

Species	Institutional voucher number[a]	Field collection number[b]	Collection locality[c]	Longitude	Latitude	GenBank accession no.
Ceratophrys cornuta	LSUMZ H-17416	JPC 14732	PE Guajara-Mirim, Rondônia, BR, ? m	−64.55	−10.316	GU168781
Craugastor augusti	MVZ 226839	RCS 12112-A	Bitter Lake NWR, Roswell, New Mexico, US, 1085 m	−104.4104	33.59909	AY273109
Craugastor bocourti	UTA A-55235	GAR 181	Purulha, Baja Verapaz, GT, ? m	−90.1644	15.2475	AY273110
Craugastor bransfordii	FMNH 257700	AJC 0213	Nusagandi, Kuna Yala, PA, 420 m	−78.9833	09.3167	AY273140
Craugastor daryi	UTA A-55251	MEA 2248	Sierra de Xucaneb, Alta Verapaz, GT, ? m	−89.78	15.65	AY273107
Craugastor fitzingeri	FMNH 257745	AJC 0102	EB Las Cruces, Puntarenas, CR, 1100 m	−82.975	08.783	AY273117
Craugastor gollmeri	FMNH 257561	AJC 0230	EB Fortuna, Chiriquí, PA, 1100 m	−82.217	08.75	AY273124
Craugastor megacephalus	FMNH 257714	AJC 0072	EB La Selva, Heredia, CR, 100 m	−84.025	10.417	AY273111
Craugastor noblei	FMNH 257616	AJC 0333	PN Omar Torrijos, Coclé, PA, 800 m	−80.592	08.667	AY273127
Craugastor podiciferus	FMNH 257653	SJA 23703	EB Las Cruces, Puntarenas, CR, 1100 m	−82.975	08.783	AY273135
Craugastor pygmaeus	UTA A-55246	ENS 9595	Sierra Madre del Sur, Oaxaca, MX, 1245 m	−97.0975	16.1942	AY273119
Craugastor ranoides	MVZ 207277	DAG 3071	Vulcán Cacao, Guanacaste, CR, 1100 m	−85.45	10.9333	AY273112
Craugastor rhodopis	UTA A-55231	ENS 10024	Jacaltepec, Oaxaca, MX, 150 m	−96.2388	17.8638	AY273131
Craugastor sartori	UTA A-51105	ENS 8328	Aldea La Fraternidad, San Marcos, GU, ? m	−91.8815	14.9297	AY273121
Craugastor tabasarae	SIUC H-06964	KRL 8918	PN Omar Torrijos H., Coclé, PA, 800 m	−80.592	08.667	AY273115
Craugastor trachydermus	UTA A-48500	ENS 6751	Livingston, Izabal, GU, 935 m	−89.24	15.71	AY273106
Pristimantis adnus	MVUP 2255	AJC 0922	Río Piña, Puerto Piña, Darién, PA, 800 m	−78.2022	07.682	EU443191
Pristimantis adnus	CH 8132	AJC 0924	Río Piña, Puerto Piña, Darién, PA, 700 m	−78.2022	07.682	EU443192
Pristimantis altae	UCR 16472	AJC 0398	MNH La Paz, San Ramón, Alajuela, CR, 1230 m	−84.55855	10.18223	EU443185
Pristimantis altamazonicus	LSUMZ H-15467	JPC 14628	Rio Ituxi, Amazonas, BR, ? m	?	?	GU168782
Pristimantis cerasinus	FMNH 257713	AJC 0071	EB La Selva, Sarapiquí, Heredia, CR, 75 m	−84.00700	10.43033	EU443194
Pristimantis cruentus	none yet	AJC 0603	Cana, PN Darién, Darién, PA, 1600 m	−77.71667	7.77111	EU443188
Pristimantis cruentus	UCR 16448	AJC 0458	San Ramón, Alajuela, CR, 960 m	−84.59698	10.21877	EU443176
Pristimantis cruentus	UCR 16443	AJC 0463	San Ramón, Alajuela, CR, 960 m	−84.59698	10.22100	EU443186
Pristimantis museosus	SIUC H-06970	KRL 8881	PN Omar Torrijos H., Coclé, PA, 800 m	−80.59167	8.66667	AY273103
Pristimantis pardalis	FMNH 257675	AJC 0188	Fortuna, Chiriquí, PA, 1000 m	−82.21667	8.75000	AY273102
Pristimantis pirrensis	CH 5641	AJC 0594	Cana, PN Darién, PA, 500 m	−77.68405	7.75607	EU443190
Pristimantis ridens	FMNH 257833	AJC 0336	Cerro Campana, Panama, PA, 900 m	−79.92738	8.68564	EU443159
Pristimantis ridens	FMNH 257697	AJC 0211	Nusagandi, Panama, PA, 400 m	−78.98330	9.31670	EU443164
Pristimantis ridens	MVUP 1787	KRL 0692	PN Omar Torrijos H., Cocle, PA, 800 m	−80.59167	8.66667	EU443165
Pristimantis ridens	UTA A-57017	ENS 10727	Agalta, Olancho, HN, 1080 m	−86.14800	14.95900	EU443154
Pristimantis ridens	FMNH 257746	AJC 0103	Las Cruces, Puntarenas, CR, 60 m	−82.97500	8.78333	AY273101
Pristimantis sp. nov. B	none yet	AJC 0580	Cana, PN Darién, PA, 1300 m	−77.72225	7.76358	EU443193
Pristimantis sp. C	none yet	AJC 0601	Cana, PN Darién, PA, 500 m	−77.68405	7.75607	EU443184
Euhyas pantoni	USNM 327872	SBH 103516	Hardwar Gap, St. Andrew Parish, JM, 50 m	−76.72	18.08	AY273104
Leptodactylus fragilis	UTA A-48666	ENS 7104	Puerto Barrios, Izabal, GT, 50 m	−88.71	15.6	AY273100

APPENDIX II
Continued.

Species	Institutional voucher number[a]	Field collection number[b]	Collection locality[c]	Longitude	Latitude	GenBank accession no.
Leptodactylus melanonotus	UTA A-53817	ENS 9368	San Rafael, San Marcos, GT, 600 m	−91.9	14.87	AY273099
Oreobates quixensis	LSUMZ H-12784	JPC 10264	Cuyabeno, Sucumbíos, EC, ? m	?	?	GU168783
Syrrhophus pipilans	UTA A-51050	JAC 19141	Nenton, Huehuetenango, GT, ? m	−91.842	15.761	AY273105

[a] CH = Círculo Herpetológico de Panamá, Panamá City, Panama; FMNH = Field Museum of Natural History, Chicago, Illinois, USA; LSUMZ = Lousiana State University Museum of Zoology (Natural Sciences), Baton Rouge, Louisiana, USA; MVUP = Museo de Vertebrados de la Universidad de Panamá, Panamá City, Panama; MVZ = Museum of Vertebrate Zoology, Berkeley, California, USA; SIUC = Southern Illinois University at Carbondale, Illinois, USA; UCR = Museo de Zoología, Universidad de Costa Rica, San Pedro, Costa Rica; USNM = National Museum of Natural History, Washington, DC, USA; UTA = University of Texas at Arlington, Texas, USA.

[b] AJC = Andrew J. Crawford; DAG = David A Good; ENS = Eric N. Smith; GAR = Rony García Anleu; JAC = Jonathan A. Campbell; JPC = Janalee P. Caldwell; KRL = Karen R. Lips; LDW = Larry David Wilson (Randy McCranie, collector); MEA = Manuel E. Acevedo; RCS = Robert C. Stebbins; SBH = S. Blair Hedges; SJA = Stevan J. Arnold (AJC, collector).

[c] MHN = Monumnento Histórico Natural; PE = Parque Estadual; PN = Parque Nacional; NWR = National Wildlife Refuge. Countries are indicated by their ISO 3166 two-letter codes. Final numbers indicate elevation in meters.