Systematics of the *Rhinella margaritifera* complex (Anura, Bufonidae) from western Ecuador and Panama with insights in the biogeography of *Rhinella alata*

Sueny P. dos Santos¹, Roberto Ibáñez²,³, Santiago R. Ron¹

¹ Museo de Zoología, Departamento de Ciencias Biológicas, Pontificia Universidad Católica del Ecuador, Av. 12 de Octubre y Roca, Aptdo. 17–01–2184, Quito, Ecuador ² Smithsonian Tropical Research Institute, Apartado Postal 0843-03092, Panama, República de Panama ³ Departamento de Zoología, Universidad de Panama, Panama, República de Panama

Corresponding author: Sueny P. dos Santos (santiago.r.ron@gmail.com)

Academic editor: F. Andreone | Received 17 September 2014 | Accepted 2 April 2015 | Published 30 April 2015

http://zoobank.org/CBFCB838-C07B-4329-AEEA-F61F8F12C16C

Citation: dos Santos SP, Ibáñez R, Ron SR (2015) Systematics of the *Rhinella margaritifera* complex (Anura, Bufonidae) from western Ecuador and Panama with insights in the biogeography of *Rhinella alata*. ZooKeys 501: 109–145. doi: 10.3897/zookeys.501.8604

Abstract

The *Rhinella margaritifera* species group consists of 17 species of toads distributed in tropical and subtropical South America and eastern Central America. The identity of some of its species is poorly understood and there are numerous undescribed cryptic species. Among them, the status of *Rhinella margaritifera* is one of the most problematic. Its range includes lowland rainforests separated by the Andes, the Chocoan rainforest to the west and the Amazonian rainforest to the east. This distribution is puzzling because the Andes are an old and formidable barrier to gene flow and therefore should generate vicariant speciation between disjunct lowland populations. Herein we clarify the taxonomy of populations of the *R. margaritifera* complex from Central America and the Chocó region of South America. The morphological and genetic variation of *R. margaritifera* was examined from 39 populations from Chocó, 24 from the upper Amazon region of Ecuador, and 37 from Panama, including the holotype of the Panamanian *R. alata*. Phylogenetic analyses were performed based on mitochondrial genes 12S rRNA, 16S rRNA, and cytochrome c oxidase I (COI) and the nuclear gene Tyrosinase (Tyr). The genetic and morphological data show that Panamanian and Chocoan populations are conspecific. In the phylogeny, populations from Chocó and Panama form a well-supported clade. The morphology of the holotype of *R. alata* falls within the variation range of Panamanian and Chocoan populations. Based on all this evidence, we assign the populations from western Ecuador and Panama to *R. alata* and demonstrate that the unusual distribution pattern of "*R. margaritifera*" on both sides of the Andes was an artifact of incorrectly defined species boundaries.
Keywords
Andes, Biogeography, Chocó, Morphology, Panama, Phylogeny, *Rhinella alata*

Introduction

*Rhinella* is a genus of bufonid frogs distributed from southern Texas, through southern Sonora (Mexico), south tropical Mexico, Central America, and South America. There are 87 recognized species of *Rhinella* (Frost, 2014) among which 17 belong to the *R. margaritifera* species group (Lavilla et al. 2013, Moravec et al. 2014). Thirteen of these species are distributed throughout the Amazon Basin, the Guyanas and Central America, while *R. hoogmoedi* Caramaschi & Pombal, 2006 occurs in the Brazilian Atlantic Forest, *R. scitula* (Caramaschi & Niemeyer, 2003) and *R. ocellata* ( Günther, 1858) in the Brazilian Cerrado, and *R. paraguayensis* Ávila, Pansonato & Strüssmann, 2010 in the Brazilian Pantanal (Caramaschi and Niemeyer 2003, Caramaschi and Pombal 2006, Lima et al. 2007, Fouquet et al. 2007a, Ávila et al. 2010, Frost 2014). They inhabit the forest floor and their cryptic coloration mimics the forest leaflitter. Morphologically they have been characterized by the presence of hypertrophied supra and postorbital crests, especially in females. Putative synapomorphies for the group are the expansion of the posterior ramus of the pterygoid and nasals that articulate laterally with the preorbital process of the maxilla (Pramuk 2006).

The *R. margaritifera* species group (formerly *Bufo typhonius* or *Bufo margaritifer* group) has one of the most complex histories in the systematics of Neotropical anurans (Hoogmoed 1986, 1989, 1990, Hass et al. 1995, Fouquet et al. 2007b). The boundaries among its species member are poorly understood as a result of a highly variable intraspecific morphology and scant morphological differentiation between some species. In addition, some of the type material is unavailable or poorly preserved and several species descriptions lack details. Despite recent progress in the systematics of the group (i.e. Vélez-Rodríguez 2004, Pramuk 2006, Fouquet et al. 2007b, 2012b, Ávila et al. 2010, Lavilla et al. 2013, Moravec et al. 2014) a number of cryptic species still need to be identified, specially among Amazonian populations (Hoogmoed 1990, Hass et al. 1995, Vélez-Rodríguez 2004, Pramuk 2006, Fouquet et al. 2007b, Lavilla et al. 2013, Moravec et al. 2014).

Two species of the *R. margaritifera* group have been reported west of the Andes (Chocó region, humid forests west of the Andes in Colombia and Ecuador) and in eastern Panama: *R. alata* and *R. margaritifera*. *R. alata* was described by Thominot (1884) as *Bufo alatus*, based on an adult male collected at Obispo, Isthmus of Panama. Boulenger (1885) considered it a junior synonym of “*B. typhonius*”, and Hoogmoed (1986, 1989) suggested that it was, possibly, a synonym of *B. acutirostris* (Spix, 1824). La Marca (1997) reported populations of *R. alata* from northern Venezuela. Gorzula and Señaris (1999) suggested that *R. margaritifera* only occurs in southern Venezuela and *R. alata* north of the Orinoco. However, Barrio-Amorós (1999 “1998”, 2004) disagreed with both reports and considered that *R. alata* was not distributed in Venezuela.
Rhinella margaritifera was described by Laurenti in 1768. It occurs in eastern Panama (Frost 2014), the Chocóan lowlands of western Ecuador and western Colombia (e.g. Anderson 1945, Miyata 1982, Ruiz-Carranza et al. 1996, Ortega-Andrade et al. 2010, Ortiz et al. 2013, Ron et al. 2014), Amazonia and vicinities in Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru, Surinam and Venezuela (Lavilla et al. 2013). A genetic study by Fouquet et al. (2007b), using two mitochondrial genes (12S and 16S) and the two nuclear genes (Tyrosinase and 18S), showed that R. margaritifera was paraphyletic and contained up to 11 cryptic species. Populations from the Chocó region have been widely referred as R. margaritifera although Solis et al. (2010) remarked that populations from the Ecuadorian Chocó might belong to a separate species. Unfortunately, they did not provide further details.

The distribution of R. margaritifera in the humid lowlands west and east of the Andes is intriguing because, particularly for amphibians, the Andes represent a formidable barrier to gene flow (e.g. Santos et al. 2009). Despite similar environmental conditions, only four amphibian species are shared between the lowland rainforests of the Amazon basin and the Chocó: R. margaritifera, R. marina, Hypsiboas boans and Trachycephalus typhonius. Moreover, there is genetic and morphological evidence suggesting that populations on each side of the Andes of R. marina and Trachycephalus typhonius represent separate species (Slade and Moritz 1998, Ron and Read 2011). Thus, the distribution of R. margaritifera is suggestive of either an unusual biogeographic history or the existence of cryptic species.

Herein, genetic and morphological information were integrated to clarify the taxonomy of the populations of R. margaritifera from Panama and the Chocóan region. Populations from the western and eastern Andean slopes were compared to test the role of the Andes as a dispersal barrier in shaping the evolution of the R. margaritifera species complex.

**Methods**

**Population sampling**

Populations from Panama, the Ecuadorian Chocó, and the Amazon basin were sampled (Figs 1 and 2). Specimens examined morphologically are listed in Appendix 1; specimens analyzed genetically are listed in Table 1.

Morphometric analyses were based on 120 adult specimens of R. margaritifera from Panama (14 specimens from 10 populations), Ecuadorian Chocó (74 specimens, 37 populations), and the Ecuadorian Amazon (32 specimens, 18 populations). Qualitative morphological characters were examined in the same specimens and 28 additional individuals from 27 Panamanian populations (Figs 1 and 2; Appendix 1). Genetic analyses were based on newly generated sequences of R. margaritifera from 32 individuals and 19 populations: R. margaritifera from the Ecuadorian Chocó (12 individuals, 7 populations); R. margaritifera from Panama (3 individuals, 2 popula-
tions) and *R. margaritifera* from the Amazon basin (17 individuals, 10 populations), and six sequences for the outgroups (see Table 1). Sequences of eight *R. dapsilis* were generated, including all available homologous sequences for the *R. margaritifera* species group from GenBank (http://www.ncbi.nlm.nih.gov/genbank; Table 1). *R. marina, R. chavin, R. nesiotes* and *R. festae* were included as outgroups. The morphometric and genetic analyses were based on the same individuals, when possible. Several specimens used in the morphological analyses lacked tissues and were not included in the genetic analyses. However, their identification was unambiguous based on geographic distribution and morphological characters.

Examined specimens are deposited at the Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ, Quito, Ecuador), the American Museum of Natural History (AMNH, New York, USA), Círculo Herpetológico de Panamá (CH, Panama, Panamá), Centro de Ornitología y Biodiversidad (CORBIDI, Lima, Perú) and Museo de Vertebrados de la Universidad de Panamá (MVUP). We also examined photographs of the holotypes of *R. alata* from Musée National d’Histoire Naturelle (MNHN, Paris, France). Tissues were obtained from the QCAZ and CH collections. Tissues (liver or thigh muscle) were stored in 95% ethanol.

**Morphological analyses**

Morphological terminology and abbreviations follow Vélez-Rodriguez (2004) and Narvaez and Rodrigues (2009). Sexual maturity was determined by the presence of nuptial pads in adult males and convoluted oviducts or mature eggs in gravid females. Specimens from the QCAZ collection were euthanized with the anesthetic spray Roxicaine, fixed in 10% formalin, and preserved in 70% ethanol.

The goal of the morphological analyses was to compare three geographic regions: (1) Chocó (2) Panama, and (3) upper Amazon basin. Because the phylogeny showed that Panama and Chocó populations are conspecific, we also compared Chocó + Panama vs. upper Amazon. Morphometric analyses were based on adult and well-preserved specimens (Simmons 2002). We measured the following variables: (1) SVL (snout-vent length, from the tip of snout to the mid-vent); (2) TL (tibia length, from the outer edge of flexed knee to the heel); (3) FL (femur length, from the mid-venter to the outer edge of flexed knee); (4) HL (head length, from the posterior margin of tympanum to the tip of snout); (5) HW (head width, between knobs at angles of jaws, if present); (6) STCH (supratympanic crest height, the distance between the angle of the jaw and the highest point of the ridge above of the tympanum); (7) SOCH (supraorbital crest height, the distance between the angle of jaw and the highest point of the ridge at the mid-orbit); (8) NSD (nostril-snout distance, from the nostril to the tip of the snout); (9) IND (inter-nostril distance, distance between nostrils); (10) TD (tympanum diameter, from the posterior to the anterior edge of the tympanum); (11) FT (foot length, from the posterior edge of the metatarsal tubercle to the tip of the toe IV). Measurements were taken with digital calipers (to the nearest 0.01 mm). Two qualitative mor-
phological characters were also analyzed: (1) vertebral apophyses (present/absent) and (2) bony knob at angle of jaws (present/absent).

Principal Components Analysis (PCA) and Discriminant Function Analysis (DFA) were used to assess morphometric differentiation between Chocó, upper Amazon, and Panama. To remove the effect of body size (SVL), the MANOVA and PCA were applied to the residuals from the linear regressions between the measured variables and SVL, for males and females separately. For the PCA, only components with eigenvalues > 1 were retained. All measurements were first subjected to the Shapiro-Wilk normality test for normal distribution (Shapiro and Wilk 1965). Data not normally distributed were log-transformed. Levene’s test was used to determine if variables were homoscedastic (Levene 1960). Number of analyzed specimens were (1) Chocó: 43 males and 31 females, (2) Panama: 6 males and 8 females, (3) upper Amazon basin: 16 males and 16 females. All analyses were performed using JMP® 9.0.1 (SAS Institute 2010).

DNA extraction, amplification, and sequencing

Total DNA was extracted from muscle or liver tissue preserved in 95% ethanol or tissue storage buffer using standard guanidine thiocyanate protocol (M. Fujita, unpublished) with modifications. Polymerase Chain Reaction (PCR) was used to amplify the mitochondrial genes 12S rRNA, 16S rRNA, cytochrome c oxidase I (COI) and nuclear gene Tyrosinase (Tyr). PCR amplifications were carried out under standard protocols. Using standard primers developed by Bossuyt and Milinkovitch (2000), Goebel et al. (1999), Pauly et al. (2004), and Meyer et al. (2005). Amplicons were sequenced by Macrogen Inc., Seoul, Korea.

Phylogenetic analyses and genetic distances

Preliminary sequence alignment was done with Geneious Pro 5.4.6 (Drummond et al. 2011). The sequence matrix was imported to Mesquite 2.75 (Maddison and Maddison 2011) and the ambiguously aligned regions were adjusted manually to produce a parsimonious alignment. Phylogenetic trees were obtained using Bayesian Inference (BI) in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) and Maximum Likelihood (ML) in Garli 2.0 (Zwickl 2006). The best-fit models of sequence evolution were selected under the Akaike information criterion (AIC) and the best partitioning scheme for the combined nucleotide data set and the models of character evolution for the BI and ML were estimated with PartitionFinder 1.0.1 (Lanfear et al. 2012). We ran three analyses: (1) the complete multi-locus data set, (2) only mitochondrial genes, (3) only the nuclear gene.

The Bayesian search consisted of two parallel runs each with $130 \times 10^6$ generations with four Markov chains. The convergence of the runs was assessed with Tracer 1.5 (Rambaut and Drummond 2007) evaluating the effective sample sizes and stopping
Figure 1. Localities of the *Rhinella margaritifera* group from Chocó (triangles) and Amazon (squares). Gray for specimens analyzed morphologically, black for specimens analyzed both genetically and morphologically. Specimens (listed in Appendix 1 and Table 1) are deposited at the Museo de Zoología of Pontificia Universidad Católica del Ecuador (QCAZ), Centro de Ornitología y Biodiversidad (CORBIDI), and National Museum of Natural History (USNM).
Figure 2. Panamanian populations of the *Rhinella margaritifera* group included in this study. White crosses for specimens analyzed morphologically, black crosses analyzed both morphologically and genetically. The type locality of *R. alata* is shown with a triangle. Specimens (listed in Appendix 1 and Table 1) are deposited at American Museum of Natural History (AMNH), Muséum National d’Histoire Naturelle du Paris (MNHN), Círculo Herpetológico de Panama (CH), and the Museo de Vertebrados de la Universidad de Panama (MVUP).
Table 1. GenBank accession numbers for DNA sequences used in the phylogenetic analysis.

| Museum No. | Species      | Country     | Locality                  | GenBank Accession No. | GenBank Accession No. | GenBank Accession No. | GenBank Accession No. | Reference         |
|------------|--------------|-------------|---------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-------------------|
| QCAZ10253  | R. alata     | Ecuador     | Reserva La Chiquita       | KR012523              | KR012524              | KR012525              | KR012526              | This study        |
| QCAZ10254  | R. alata     | Ecuador     | Reserva La Chiquita       | KR012524              | KR012526             | KR012527              | KR012528              | This study        |
| QCAZ10255  | R. alata     | Ecuador     | Reserva La Chiquita       | KR012525              | KR012526             | KR012527              | KR012528              | This study        |
| QCAZ11598  | R. alata     | Ecuador     | Reserva La Chiquita       | KR012526              | KR012527             | KR012528              | KR012529              | This study        |
| QCAZ13882  | R. alata     | Ecuador     | Manta Real                | KR012527              | KR012528             | KR012529              | KR012530              | This study        |
| QCAZ13896  | R. alata     | Ecuador     | Manta Real                | -                     | DQ158471             | DQ158471             | -                     | Pramuk 2006       |
| QCAZ14607  | R. alata     | Ecuador     | Borbón                     | KR012528              | KR012529             | KR012530              | KR012531              | This study        |
| QCAZ37244  | R. alata     | Ecuador     | Valle Hermoso              | KR012539              | KR012540              | KR012541              | KR012542              | This study        |
| QCAZ37248  | R. alata     | Ecuador     | Valle Hermoso              | KR012540              | KR012541              | KR012542              | KR012543              | This study        |
| QCAZ23161  | R. alata     | Ecuador     | San Lorenzo               | KR012534              | KR012535              | KR012536              | KR012537              | This study        |
| QCAZ25023  | R. alata     | Ecuador     | La Tortuga                 | KR012536              | KR012537              | KR012538              | KR012539              | This study        |
| QCAZ25025  | R. alata     | Ecuador     | La Tortuga                 | KR012537              | KR012538              | KR012539              | KR012540              | This study        |
| QCAZ25032  | R. alata     | Ecuador     | La Pedorrera               | KR012538              | KR012539              | KR012540              | KR012541              | This study        |
| CH9104     | R. alata     | Panama      | Cana, Boca Cupé           | KR012507              | KR012508              | KR012509              | KR012510              | This study        |
| MVUP2299   | R. alata     | Panama      | Rio Chico Masambi, Parque Nacional Soberanía | KR012511 | KR012512 | KR012513 | KR012514 | This study |
| CH9192     | R. alata     | Panama      | Parque Nacional Soberanía  | KR012521              | KR012522              | KR012523              | KR012524              | This study        |
| QCAZ11597  | R. alata     | Ecuador     | Reserva La Chiquita        | -                     | DQ15872              | DQ15872              | -                     | Pramuk 2006       |
| 104MC      | R. castaneotica | French Guyana | Tibourou                | EF364355              | EF364356            | EF364357             | EF364358             | Fouquet et al. 2007b |
| 110PG      | R. castaneotica | French Guyana | Moint Saint Marcel       | EF364353              | EF364354            | EF364355             | EF364356             | Fouquet et al. 2007b |
| QCAZ38477  | R. dapsilis  | Ecuador     | Villano B                 | KR012513              | KR012514              | KR012515              | KR012516              | This study        |
| QCAZ38512  | R. dapsilis  | Ecuador     | Villano BII               | KR012514              | KR012515              | KR012516              | KR012517              | This study        |
| QCAZ38560  | R. dapsilis  | Ecuador     | Villano B                 | KR012515              | KR012516              | KR012517              | KR012518              | This study        |
| QCAZ38621  | R. dapsilis  | Ecuador     | Villano K4                | KR012516              | KR012517              | KR012518              | KR012519              | This study        |
| QCAZ38688  | R. dapsilis  | Ecuador     | Villano K4                | KR012517              | KR012518              | KR012519              | KR012520              | This study        |
| QCAZ38755  | R. dapsilis  | Ecuador     | Villano BII               | KR012518              | KR012519              | KR012520              | KR012521              | This study        |
| QCAZ38892  | R. dapsilis  | Ecuador     | Comunidad Kutintza 2      | KR012519              | KR012520              | KR012521              | KR012522              | This study        |
| Museum No. | Species        | Country   | Locality        | GenBank Accession No.          | Reference                  |
|-----------|----------------|-----------|-----------------|-------------------------------|----------------------------|
| QCAZ38998 | *R. dapsilis*  | Ecuador   | Comunidad Kurintza 3 | KR012520, KR012641, KR012590, KR012549 | This study                 |
| MTR191999 | *R. hoogmoedi* | Brazil    | Bahia, Camacan  | -                             | Fouquet et al. 2012a       |
| 112BM     | *R. lescurei*  | French Guyana | Litany        | EF364343, EF217473, EF364279  | Fouquet et al. 2007b       |
| 3027T     | *R. lescurei*  | French Guyana | Mitaraka       | JN692065, JN63405, EF364279   | Fouquet et al. 2012b       |
| 108MC     | *R. margaritifera* | French Guyana | Kaw            | EF364333, EF364292, EF364266  | Fouquet et al. 2007b       |
| 136MC     | *R. margaritifera* | French Guyana | Crique Margot | EF364335, EF364292, EF364266  | Fouquet et al. 2007b       |
| 389MC     | *R. margaritifera* | French Guyana | Camp Canopé   | JN692029, - , -               | Fouquet et al. 2007b       |
| 374MC     | *R. margaritifera* | French Guyana | Régina         | JN692038, JN691389, JN690782  | Fouquet et al. 2007b       |
| 390MC     | *R. margaritifera* | French Guyana | St Georges    | JN692037, JN691388, JN690781  | Fouquet et al. 2007b       |
| 2559T     | *R. margaritifera* | French Guyana | Pic Matecho   | JN690780, JN691387, JN690780  | Fouquet et al. 2012b       |
| 4482T     | *R. margaritifera* | French Guyana | Angouëlème    | JN692042, JN691379, JN690772  | Fouquet et al. 2012b       |
| 163BM     | *R. margaritifera* | French Guyana | Guatemala     | EF364320, EF364292, EF364266  | Fouquet et al. 2007b       |
| 164BM     | *R. margaritifera* | French Guyana | Montagne des Singes | EF364321, EF364292, EF364266 | Fouquet et al. 2007b       |
| 176BM     | *R. margaritifera* | French Guyana | Crique Grand Leblond | EF364323, EF364292, EF364266 | Fouquet et al. 2007b       |
| 195MC     | *R. margaritifera* | French Guyana | Kaw            | EF364325, EF364292, EF364266  | Fouquet et al. 2007b       |
| 2034AT    | *R. margaritifera* | French Guyana | Nouragues     | JN692033, EF364292, EF364266  | Fouquet et al. 2007b       |
| 204MC     | *R. margaritifera* | French Guyana | Saul           | EF364328, EF364295, EF364269  | Fouquet et al. 2007b       |
| 217MC     | *R. margaritifera* | French Guyana | Grant Santi   | EF364329, EF364299, EF364273  | Fouquet et al. 2007b       |
| 225MC     | *R. margaritifera* | French Guyana | Road St. Elie | EF364330, EF364292, EF364266  | Fouquet et al. 2007b       |
| 284MC     | *R. margaritifera* | French Guyana | St Elie       | EF364336, EF364292, EF364266  | Fouquet et al. 2007b       |
| 288AG     | *R. margaritifera* | French Guyana | St Georges    | JN692021, JN691380, JN690773  | Fouquet et al. 2012b       |
| 294MC     | *R. margaritifera* | French Guyana | Camp Canopé   | JN692029, EF364292, EF364266  | Fouquet et al. 2012b       |
| 2BM       | *R. margaritifera* | French Guyana | Cisame        | EF364313, EF364293, EF364267  | Fouquet et al. 2007b       |
| 307PG     | *R. margaritifera* | French Guyana | Lac Toponowini | JN692022, EF364292, EF364266  | Fouquet et al. 2012b       |
| 361MC     | *R. margaritifera* | French Guyana | Lucifer       | JN692031, EF364292, EF364266  | Fouquet et al. 2012b       |
| 408PG     | *R. margaritifera* | French Guyana | Mont Kotika   | JN692023, EF364292, EF364266  | Fouquet et al. 2012b       |
| 66MC      | *R. margaritifera* | French Guyana | Monts Bakra   | EF364334, EF364298, EF364272  | Fouquet et al. 2007b       |
| 74AF      | *R. margaritifera* | French Guyana | St Georges    | JN692020, EF364266, EF364292  | Fouquet et al. 2012b       |
| Museum No. | Species | Country | GenBank Accession No. | Reference |
|-----------|---------|---------|----------------------|-----------|
| TYR       | R. margaritifera | French Guyana | EF364314, EF364315, EF364316 | Fouquet et al. 2003 |
| 16S       | R. margaritifera | French Guyana | EF365275             | Wiens et al. 2005 |
| COI       | R. margaritifera | French Guyana | EF364317, EF364318, EF364319 | Fouquet et al. 2012 |
| 125       | R. margaritifera | France | EF364320, EF364321, EF364322 | Fouquet et al. 2012 |

**Species Data:**

- **TYR**
  - **Species:** R. margaritifera
  - **Country:** French Guyana
  - **GenBank Accession No.:** EF364314, EF364315, EF364316
  - **Reference:** Fouquet et al. 2003

- **16S**
  - **Species:** R. margaritifera
  - **Country:** French Guyana
  - **GenBank Accession No.:** EF365275
  - **Reference:** Wiens et al. 2005

- **COI**
  - **Species:** R. margaritifera
  - **Country:** French Guyana
  - **GenBank Accession No.:** EF364317, EF364318, EF364319
  - **Reference:** Fouquet et al. 2012

- **125**
  - **Species:** R. margaritifera
  - **Country:** France
  - **GenBank Accession No.:** EF364320, EF364321, EF364322
  - **Reference:** Fouquet et al. 2012

**Locality Data:**

- **TYR**
  - **Species:** R. margaritifera
  - **Country:** French Guyana
  - **GenBank Accession No.:** EF364314, EF364315, EF364316
  - **Reference:** Fouquet et al. 2003

- **16S**
  - **Species:** R. margaritifera
  - **Country:** French Guyana
  - **GenBank Accession No.:** EF365275
  - **Reference:** Wiens et al. 2005

- **COI**
  - **Species:** R. margaritifera
  - **Country:** French Guyana
  - **GenBank Accession No.:** EF364317, EF364318, EF364319
  - **Reference:** Fouquet et al. 2012

- **125**
  - **Species:** R. margaritifera
  - **Country:** France
  - **GenBank Accession No.:** EF364320, EF364321, EF364322
  - **Reference:** Fouquet et al. 2012

**Species List:**

- **TYR**
  - **Species:** R. margaritifera
  - **Country:** French Guyana
  - **GenBank Accession No.:** EF364314, EF364315, EF364316
  - **Reference:** Fouquet et al. 2003

- **16S**
  - **Species:** R. margaritifera
  - **Country:** French Guyana
  - **GenBank Accession No.:** EF365275
  - **Reference:** Wiens et al. 2005

- **COI**
  - **Species:** R. margaritifera
  - **Country:** French Guyana
  - **GenBank Accession No.:** EF364317, EF364318, EF364319
  - **Reference:** Fouquet et al. 2012
**Systematics of the Rhinella margaritifera complex (Anura, Bufonidae)**

| Museum No.   | Species       | Country    | Locality                  | GenBank Accession No. | Reference         |
|--------------|---------------|------------|----------------------------|-----------------------|-------------------|
| MNKA9691     | *R. cf. paraguayensis* | Bolivia    | -                          | -                     | Jansen et al. 2011 |
| ESTR00173    | *Rhinella* sp.     | Brazil     | Amazonas, Carolina         | -                     | Fouquet et al. 2012a |
| AF7275337    | *Rhinella* sp.     | Brazil     | Mato Grosso, APM Manso     | -                     | Fouquet et al. 2012a |

**Outgroup**

| Museum No.   | Species       | Country    | Locality                  | GenBank Accession No. | Reference         |
|--------------|---------------|------------|----------------------------|-----------------------|-------------------|
| QCAZ50698    | *Rhinella* marina | Ecuador    | Puerto Cayo                | KR012508              | This study        |
| QCAZ50702    | *Rhinella* marina | Ecuador    | San Andrés de Rocafuerte   | KR012509              | This study        |
| QCAZ18203    | *Rhinella* festae | Ecuador    | Estación Biológica Jatun Sacha | KR012510             | This study        |
| KU217501     | *Rhinella* festae | Ecuador    | Pastaza                    | -                     | Pramuk 2006       |
| MTD43789     | *Rhinella* chavin | Peru       | Palma Pampa                | -                     | Pramuk 2006       |
| UTA53310     | *Rhinella* nesiotes | Bolivia    | La Paz                     | -                     | Pramuk 2006       |
when all post burn-in values were greater than 200. The first 10% of the sample was discarded as burn-in (Castañeda and Queiroz 2011).

For the ML analysis, we carried out 20 replicate searches and increased the setting “genthreshfortopoterm” until all searches resulted in similar likelihood values, indicating an efficient search (Zwickl 2006; final value was 200,000). Ten replicate searches started from stepwise trees and ten from random trees. The setting “limsprrange” was set to 10 (default = 6). Node support was assessed with non-parametric bootstrapping (Felsenstein 1983) with 100 pseudoreplicates with the same settings of the stepwise full search but with a single replicate per search. The 50% majority rule consensus for the bootstrap trees was obtained with Mesquite 2.75 (Maddison and Maddison 2011).

Uncorrected pairwise (p) genetic distances were obtained for gene 16S using software Mesquite 2.75 (Maddison and Maddison 2011). Missing and ambiguous sites were excluded. Genetic distances comparisons were based on gene 16S because it has been widely used as a barcode standard in amphibians (e.g. Vences et al. 2005). We assumed that genetic distances > 3% are suggestive of interspecific differentiation (Fouquet et al. 2007c). Genetic distances thresholds are problematic because they can lead to both false negatives and false positives in species identifications (Collins and Cruickshank 2013). We used the threshold only as a working hypothesis that was tested with morphological comparisons.

**Results**

**Phylogenetic analyses**

The complete matrix contained up to four genes and 3045 bp for 92 samples. For the complete data set, PartitionFinder chose seven partitions as the best strategy (best model in parenthesis): 12S (GTR + I + G), 16S (GTR + I + G), COI 1st position (TIMef + G), COI 2nd position (TVM + I + G), COI 3rd position (TrN + G), Tyr 1st and 2nd position (TrN + G), Tyr, 3rd position (TrN + I + G). For the mitochondrial analyses, the same five partitions were chosen, one for each ribosomal RNA gene and each codon position in COI. For the nuclear analysis, two partitions were chosen: Tyr, 1st and 2nd position and Tyr, 3rd position.

The tree topologies for the Maximum likelihood and Bayesian phylogenies were similar except for weakly supported nodes (posterior probability < 0.95 and bootstrap < 75). The Maximum Likelihood tree (Fig. 3) shows a basal divergence of *R. castaneotica*, which is sister to two clades containing the remaining species of the *R. margaritifera* species group. One clade is strongly supported in the Bayesian consensus (posterior probability = 1) although it has low bootstrap support (= 63). It contains three groups: Panama (posterior probability = 1.0, bootstrap = 100), Chocó (posterior probability = 1.0, bootstrap = 86) and upper Amazon (posterior probability = 1.0, bootstrap = 68). Chocó and Panama form clade sister to the upper Amazon clade. Both clades, which are on opposite sides of the Andes, are separated by pairwise genetic distances (uncor-
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Figure 3. Maximum Likelihood phylogram depicting relationships within the Rhinella margaritifera species group. The phylogram was derived from the analysis of 3045 bp of mitochondrial (12S, 16S, COI) and nuclear (Tyr) genes. Numeric codes on terminals are individual collection numbers (associated data listed in Table 1). Posterior probabilities (above) and bootstrap values (below) are shown on branches except when they are < 0.50 and 50%, respectively. Abbreviations are: EC = Ecuador, FG = French Guyana, BR = Brazil, BO = Bolivia, PE = Peru, PA = Panama. Outgroups are not shown.

Figure 3. Maximum Likelihood phylogram depicting relationships within the Rhinella margaritifera species group. The phylogram was derived from the analysis of 3045 bp of mitochondrial (12S, 16S, COI) and nuclear (Tyr) genes. Numeric codes on terminals are individual collection numbers (associated data listed in Table 1). Posterior probabilities (above) and bootstrap values (below) are shown on branches except when they are < 0.50 and 50%, respectively. Abbreviations are: EC = Ecuador, FG = French Guyana, BR = Brazil, BO = Bolivia, PE = Peru, PA = Panama. Outgroups are not shown.

The genetic distances and the morphological differences (see next section) between the Chocó-Panama clade and the upper Amazon clade suggest that they are separate species. The 16S genetic distances between the Chocó and Panama clades range from 1.26 to 1.99% (average = 1.63, SD = 0.19). The relatively low genetic distances and the lack of morphological differences between their populations (see next section) indicate that they are conspecific. The Chocó populations further segregate latitudinally in two well-supported clades. One includes the populations in northern Ecuador (e.g. Reserva La Chiquita and Borbón) while the other includes central and southern populations (e.g. Manta Real and Valle Hermoso, Fig. 3).
Figure 4. Maximum Likelihood phylogram depicting relationships within the *Rhinella margaritifera* species group. The phylogram was derived from the analysis of 2495 bp of mitochondrial gene fragments (*12S*, *16S*, *COI*). Numeric codes on terminals are individual collection numbers (associated data listed in Table 1). Bootstrap values appear above branches. The branches without numbers have bootstrap values < 50%. Abbreviations: EC = Ecuador, FG = French Guyana, BR = Brazil, BO = Bolivia, PE = Peru, PA = Panama. Outgroups are not shown.

The sister clade to Chocó-Panama + Upper Amazon has weak support and includes other members of the *R. margaritifera* group (*R. dapsilis, R. hoogmoedi, R. lesurei, R. martyi, R. ocellata, R. paraguayensis* and “*R. margaritifera*”) from the Guiana region and...
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Amazonian Brazil, Ecuador and Peru. Relationships among them are weakly supported on most branches.

The Maximum Likelihood tree based on mitochondrial genes (Fig. 4) has similar topology to the Maximum Likelihood tree derived from the analysis of the complete data set (Fig. 3). The Bayesian consensus tree, derived from the Tyrosinase gene, has definitely lower resolution (Appendix 2).

**Morphological analyses**

*Morphometric comparisons.* Morphometric data from adults are summarized in Table 2. In the examined series, Amazonian males and females were significant larger than their counterparts from Chocó (Fig. 5; males Student’s *t* = -10.32, DF = 57, *p* < 0.001; females *t* = -13.12, DF = 45, *p* < 0.001) and Panama (males *t* = -8.7, DF = 22, *p* < 0.001; females *t* = -4.43, DF = 20, *p* < 0.001). There are no significant differences in SVL between Chocoan and Panamanian populations (males *t* = 1.37, DF = 47, *p* = 0.91; females *t* = -1.58, DF = 37, *p* = 0.06).

Significant differences were observed in relative crest size between the Chocó-Panama and upper Amazon clades (Fig. 6). In the former, female supratympanic crest height had a range between 51.6 to 63.5% of head length (n = 39); in the later, range was 68.6 to 95.5% (n = 16). Ranges did not overlap and differences were significant (Wilcoxon’s *Z* = -5.77, *p* < 0.001). Male supratympanic crest height had a range between 49.3 to 59.8% of head length in Chocó-Panama (n = 49); in upper Amazon, range was 50.6 to 78.4% of head length (n = 16). Ranges overlapped but differences were significant (Wilcoxon’s *Z* = 3.11, *p* = 0.0018).

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**Figure 5.** Box and whisker plots showing snout-vent length variation in adult *Rhinella margaritifera* (upper Amazon) and *R. alata* (Chocó and Panama). The central bar indicates the median, the interquartile range is shown by the box length, and the range is shown by the short horizontal lines (whiskers). **SVL** = snout-vent length. The black cross is the holotype of *R. alata*.
### Table 2.
Descriptive statistics for morphometric measurements of adults from *Rhinella margaritifera* from Amazonian Ecuador and *R. alata* from Chocó and Panama. Mean ± SD is given, with the range below. Abbreviations are: **SVL** = Snout-Vent Length; **TL** = Tibia Length; **FL** = Femur Length; **HL** = Head Length; **HW** = Head Width; **STCH** = Supratympanic Crest Height; **SOCH** = Supraorbital Crest Height; **NSD** = Nostril-Snout Distance; **IND** = Inter-Nostril Distance; **TD** = Tympanum Diameter; **FT** = Foot Length. All measurements are in mm.

|                     | *R. margaritifera* |                      | *R. alata* |                      |
|---------------------|--------------------|----------------------|------------|----------------------|
|                     | Amazon             | Chocó                | Panama     | combined             |
|                     | Males (n = 16)     | Females (n = 16)     | Males (n = 64) | Females (n = 8) | Males (n = 49) | Females (n = 39) |
| **Morphometric**     |                    |                      |            |                      |                |                |
| **measurements**     |                    |                      |            |                      |                |                |
| **SVL**              | 45.6 ± 4.11        | 68.90 ± 8.26         | 36.66 ± 2.42 | 44.82 ± 4.42 | 38.03 ± 0.59 | 42.38 ± 3.82 |
|                     | (54.36–39.88)      | (77.97–55.42)        | (43.25–31.84) | (56.19–38.55) | (39.20–37.54) | (49.69–37.78) |
| **TL**               | 18.73 ± 1.97       | 29.36 ± 2.97         | 15.98 ± 1.14 | 18.26 ± 1.24 | 15.86 ± 1.16 | 17.79 ± 0.75 |
|                     | (23.13–15.14)      | (34.26–24.01)        | (18.72–13.69) | (20.73–16.22) | (18.12–15.09) | (18.99–16.41) |
| **FL**               | 19.67 ± 1.97       | 29.33 ± 3.67         | 15.69 ± 1.34 | 18.16 ± 1.72 | 16.39 ± 0.37 | 17.46 ± 0.67 |
|                     | (23.84–16.15)      | (35.34–22.75)        | (19.28–13.09) | (20.04–15.18) | (17.01–16.03) | (18.72–17.62) |
| **HW**               | 16.9 ± 1.59        | 25.88 ± 2.73         | 12.57 ± 0.95 | 15.10 ± 1.6 | 12.98 ± 0.17 | 14.90 ± 1.12 |
|                     | (19.93–14.77)      | (30.69–21.01)        | (15.14–10.31) | (18.94–12.49) | (13.3–12.8) | (17.23–13.79) |
| **HL**               | 14.6 ± 1.28        | 22.27 ± 2.71         | 11.61 ± 0.8 | 13.67 ± 1.19 | 11.85 ± 0.21 | 13.18 ± 1.12 |
|                     | (17.44–13.27)      | (26.51–17.94)        | (13.88–10.29) | (16.84–11.54) | (12.2–11.54) | (15.45–11.77) |
| **SOCH**             | 9.46 ± 0.86        | 15.43 ± 2.02         | 7.71 ± 0.59 | 9.28 ± 0.86 | 8.39 ± 0.21 | 9.13 ± 0.49 |
|                     | (11.38–8.19)       | (18.33–12.06)        | (8.87–6.45) | (11.40–7.77) | (8.67–8.2) | (9.87–8.53) |
| **STCH**             | 8.78 ± 1.55        | 17.73 ± 3.26         | 6.27 ± 0.54 | 7.96 ± 0.68 | 6.59 ± 0.31 | 7.38 ± 0.39 |
|                     | (12.27–6.78)       | (22.7–13.25)         | (7.97–5.36) | (9.71–6.63) | (6.99–6.31) | (8.01–6.99) |
| **NSD**              | 2.08 ± 0.44        | 2.45 ± 0.42          | 1.63 ± 0.29 | 1.70 ± 0.21 | 1.47 ± 0.17 | 1.66 ± 0.16 |
|                     | (2.64–1.41)        | (3.37–1.79)          | (2.23–1.05) | (2.08–1.25) | (1.60–1.18) | (1.89–1.35) |
| **IND**              | 3.35 ± 0.35        | 3.12 ± 0.37          | 2.50 ± 0.33 | 2.80 ± 0.43 | 2.41 ± 0.11 | 2.42 ± 0.23 |
|                     | (3.89–2.70)        | (3.73–2.59)          | (3.23–1.86) | (3.98–2.16) | (2.59–2.31) | (2.63–2.08) |
| **TD**               | 3.48 ± 0.24        | 4.14 ± 0.21          | 3.34 ± 0.47 | 3.46 ± 0.59 | 3.38 ± 0.20 | 3.79 ± 0.25 |
|                     | (3.99–3.18)        | (4.48–3.65)          | (4.03–1.95) | (4.45–2.5) | (3.60–3.13) | (4.05–3.31) |
| **FT**               | 16.87 ± 2.145      | 24.87 ± 3.64         | 13.46 ± 1.12 | 15.33 ± 1.52 | 13.72 ± 0.68 | 14.96 ± 0.76 |
|                     | (21.85–13.76)      | (28.86–19.13)        | (15.88–11.43) | (19.40–13.15) | (14.70–12.82) | (16.54–14.39) |
|                     |                    |                      | 13.48 ± 1.07 | 15.88–11.43 | 15.25 ± 1.39 | (19.4–13.15) |
Figure 6. Box and whisker plots showing relative size of supratympanic crests for adult *Rhinella margaritifera* (upper Amazon) and *R. alata* (Chocó-Panama). The central bar indicates the median, the interquartile range is shown by the box length, and the range is shown by the short horizontal lines (whiskers). STCH = supratympanic crest height, HL = head length. The yellow cross is the holotype of *R. alata*.

Three components with eigenvalues > 1.0 were extracted from the PCA for females (Table 3). The three components accounted for 67.3% of the total variation. The highest loadings of the PCA for females were supratympanic and supraorbital crest height, and tibia length for PC I, inter-nostril distance and tympanum diameter for PC II, and nostril-snout distance and inter-nostril distance for PC III. Three components with eigenvalues > 1.0 were extracted from the PCA in males (Table 3). The three components accounted for 63.3% of the total variation. The highest loadings for the PCA for males were head length and head width for PC I, inter-nostril distance and tympanum diameter for PC II, and tibia length and foot length PC III. The morphometric space of the Chocoan, upper Amazon, and Panamanian populations broadly overlaps in both males and females (Fig. 7).

In the DFA classification for females, 51 out of 55 females were assigned correctly to their geographic region. The four misclassified females from Ecuadorian Chocó were assigned to Panamanian populations. All specimens from the upper Amazon were correctly classified. In the DFA for males, 56 out of 65 males were correctly classified. The eight misclassified males from Ecuadorian Chocó were assigned to Panamanian populations and only one from upper Amazon to Panamanian populations. All males and females from Panama were correctly classified. The DFA analyses indicate that populations from the Ecuadorian Chocó are morphometrically very similar with those from Panama, both groups being markedly different from *R. margaritifera* from the upper Amazon.

Finally, evidence of sexual dimorphism was found in relative crest size: females have larger cephalic crests than males (Fig. 6). The ratio supratympanic crest height/
Table 3. Character loadings and eigenvalues for Principal Components (PC) Analysis. The analysis was based on ten size-corrected morphometric variables measured in Amazonian, Chocoan and Panamanian populations of the *R. margaritifera* species group. Abbreviations are: **TL** = Tibia Length; **FL** = Femur Length; **HL** = Head Length; **HW** = Head Width; **STCH** = Supratympanic Crest Height; **SOCH** = Supraorbital Crest Height; **NSD** = Nostril-Snout Distance; **IND** = Inter-Nostril Distance; **TD** = Tympanum Diameter; **FT** = Foot Length. Bold figures indicate highest loadings.

| Variable  | PCA Females |  |  | PCA Males |  |  |
|-----------|-------------|---|---|-----------|---|---|
|           | PC I        | PC II | PC III | PC I        | PC II | PC III |
| FL        | 0.330       | 0.165 | 0.167 | 0.272       | 0.159 | 0.322 |
| FT        | 0.334       | 0.214 | 0.418 | 0.061       | 0.038 | **0.661** |
| HL        | 0.350       | -0.065 | 0.153 | **0.448**   | -0.268 | -0.078 |
| HW        | 0.343       | 0.132 | -0.288 | **0.446**   | -0.222 | -0.045 |
| IND       | -0.203      | **0.381** | **0.512** | 0.280       | **0.502** | -0.142 |
| NSD       | 0.217       | 0.155 | **-0.580** | 0.262       | 0.386 | -0.186 |
| SOCH      | **0.368**   | -0.067 | 0.190 | 0.423       | -0.071 | -0.082 |
| STCH      | **0.411**   | -0.154 | -0.039 | 0.409       | -0.290 | -0.045 |
| TD        | 0.071       | **0.817** | -0.159 | 0.099       | **0.557** | -0.128 |
| TL        | **0.368**   | -0.200 | 0.232 | 0.134       | 0.228 | **0.610** |
| Eigenvalue| 4.411       | 1.192 | 1.128 | 2.800       | 1.947 | 1.585 |
| Cumulative variance (%) | 44.11 | 56.03 | 67.31 | 28.00 | 47.47 | 63.32 |

head length (STCH/HL) was significantly different between males and females in the Chocó-Panama clade (Wilcoxon’s *Z* = 5.15, *p* < 0.001) and the upper Amazon clade (Wilcoxon’s *Z* = -4.35, *p* < 0.001).
Qualitative morphological characters

The upper Amazon clade differs from the Chocó-Panama clade in having protruding vertebral apophyses in the dorsum and bony knobs at angle of jaws (both absent in the Chocó-Panama clade; Figs 8–10). The Chocó-Panama clade differs from other species of the *Rhinella margaritifera* complex by a combination of an absence of vertebral apophyses, an absence of bony knob at angle of jaws, low cranial crests, and the tympanum rounded or ovoid (see Systematic account section). A large number of specimens were examined (see Populations sampling section) and all conform to this characterization. Thus, it seems unlikely that there are additional species of the group in the Chocoan and Panamanian regions.

The holotype of *R. alata* (Thominot, 1884) (Fig. 11) is an adult male with an SVL of 39.2 mm. It has poorly developed supratympanic crests and lacks bony knobs at the angle of jaws. The vertebral apophyses are inconspicuous. These characters and the location of its type locality (within 6 km of one of our examined populations) lead us to conclude that it is conspecific with the Panamanian and Chocoan populations examined herein.

Systematic account of *Rhinella alata*

*Rhinella alata* (Thominot, 1884)

*Bufo alatus* Thominot, 1884. Holotype: MNHN 84285, adult male from Obispo, Panama.

Diagnosis. *Rhinella alata* is a small-sized (Table 2; Figs 8 and 9) species of *Rhinella* having the following combination of characters: (1) average SVL of females 44.25 mm (SD = 4.36, n = 39), males 36.83 mm (SD = 2.31, n = 49); (2) bony knob at angle of jaws absent, corner of mouth angular; (3) supraorbital crests low and thick, continuous with preorbital crests; usually with crenulate texture on vertical surfaces; (4) supratympanic crests concave and small; their posterior edge usually next to the anterior border of parotoid glands; (5) *canthus rostralis* present but inconspicuous, sometimes continuous with preorbital crests; (6) parietal crests usually present, ill-defined; (7) heel reaching posterior margin of eye when hindlimbs adpressed; (8) vertebral apophyses no protruding; (9) snout subacuminate in dorsal view, from rounded to protruding in profile; (10) skin on dorsum bearing a mixture of warts, pustules, and minute tubercles; (11) mid-dorsal line from snout to vent often present; (12) spiculate tubercles on external border of shank, evident especially on females; (13) dorsolateral row of sharply pointed, conical tubercles between posterior border of parotoid glands and groin; (14) tympanic membrane and tympanic annulus distinct; moderately large, ovoid to round; (15) parotoid glands small, elongated posteriorly; (16) upper eyelid warty; (17) tarsal fold absent; (18) digits slender and long, with small knobs at tip; lateral fringes present; finger lengths 3 > 4 > 2 > 1; toe lengths 4 > 5 > 3 > 2 > 1; (19) nuptial pads present.
Figure 8. Dorsolateral and ventral views of *Rhinella alata* from the Chocó region. A and C QCAZ 50568 (SVL 40.37 mm), adult female, La Concordia, Santo Domingo Province, Ecuador B and D QCAZ 37248 (SVL 40.23 mm), adult male, Valle Hermoso, El Oro Province, Ecuador. Not shown at the same scale. Photos by S.R. Ron.

*Rhinella alata* is most similar to *R. acutirostris*. Both species differ from other members of the *R. margaritifera* group by the absence of protruding vertebral apophyses, canthus rostralis not raised, snout projected, and low cranial crests. *Rhinella acutirostris* differs from *R. alata* in having a bony knob at the angle of jaws (bony knob absent in *R. alata* [Hoogmoed 1986, Lötters and Köhler 2000]). *Rhinella alata* differs from the holotype of *R. proboscidea* (ZSM 1145/0) in having a less protruding snout and skin on dorsum bearing a mixture of warts, pustules, and minute tubercles (smooth skin in *R. proboscidea*). *Rhinella dapsilis* is much larger than *R. alata* (*R. dapsilis* holotype SVL = 77 mm, adult male; Myers and Carvalho 1945) and has a fleshy proboscis in the snout (proboscis absent in *R. alata*). *Rhinella alata* differs from *R. yunga* in having tympanic membrane and annulus distinct (tympanic membrane and annulus absent in *R. yunga*; Moravec et al. 2014). *Rhinella hoogmoedi, R. magnussoni, R. martyi, R. paraguayensis, R. scitula, R. sclerocephala, and R. stanlaii* have a bony knob at angle of jaws (Caramaschi and Pombal 2006, Lima et al. 2007, Fouquet et al. 2007a, Ávila et al. 2010,
Figure 9. Dorsolateral views of *Rhinella alata*. A Cerro Azul, Parque Nacional Chagres, Panama Province, Panama. Photo by Ángel Sosa B Cerro Bruja, Parque Nacional Portobelo, Colón Province, Panama. Photo by Ángel Sosa C Gamboa, Colón Province, Panama. Photo by Roberto Ibáñez.
Figure 10. Dorsolateral views of Rhinella margaritifera from the Ecuadorian Amazon. Females: A QCAZ 55930 (SVL 80.15 mm) B QCAZ 55914 (SVL 72.49 mm), Lorocachi, Pastaza Province, Ecuador; males: C QCAZ 52343 (SVL 37.59 mm) D QCAZ 52344 (SVL 36.66 mm), Cascada San Rafael, Sucumbíos Province, Ecuador. Photos by S.R. Ron. Not shown at the same scale.

Rhinella alata differs from R. castaneotica, R. margaritifera (sensu stricto) and R. roqueana, by the absence of protruding vertebral apophyses (present in R. castaneotica [Caldwell 1991], R. margaritifera [Lavilla et al. 2013], and R. roqueana [Melin 1941]).

Rhinella alata is most closely related to populations of R. margaritifera from the upper Amazon basin in Ecuador and Peru. They can be easily distinguished by differences in body size (Fig. 5; see morphometric comparisons section) and relative size of cranial crests (Fig. 6).

Holotype. The holotype is an adult male with SVL = 39.2 mm (Fig. 11). Descriptions of the holotype have been provided by Leavitt (1933) and Hoogmoed (1989). The bony knob at angle of jaws and vertebral apophyses are absent. The crests are low and thick. There is a dorsolateral row of conical tubercles from the posterior border of the parotoid gland to the groin. There is a clear mid-dorsal line from the snout to the vent. The tympanum is rounded.

Variation. Variation in dorsal and ventral coloration of preserved specimens is shown in Figures 12 and 13. Background dorsal coloration varies from light gray (QCAZ 37244, AMNH 88689), light brown (QCAZ 14607, AMNH 104454) to dark gray (QCAZ 6733) or dark brown (QCAZ 11598, AMNH 52744), with irregular black and yellowish marks (QCAZ 4444, AMNH 88690). Some specimens
have nearly uniform brown dorsum without marks (QCAZ 31603, 10296, AMNH 10296). A clear mid-dorsal line is often present (e.g. QCAZ 3502, QCAZ 12233).

Ventral surfaces of preserved specimens have a cream to yellowish-cream background color with irregular darker marks arranged in diverse patterns; marks can
be light gray (QCAZ 6734, AMNH 88689), light brown (QCAZ 6732, AMNH 104454), dark gray (QCAZ 31606) or dark brown (QCAZ 6733, AMNH 89459), and vary from being restricted to the anterior half of the body (QCAZ 31604, AMNH 89459) to being present over the entire venter (QCAZ 4445, AMNH 88694). A longitudinal mid-ventral cream thin stripe can be present in the gular region (QCAZ 31602, 31606) or from the gular region to the mid-venter (QCAZ 6731, 11598).

Head shape in dorsal view varies from elongated (QCAZ 11598, AMNH 89459) to subtriangular (QCAZ 4447, AMNH 55475); in lateral view it varies from rounded (QCAZ 31605, AMNH 52749) to protruding (QCAZ 11393, AMNH 55475). Canthal region coloration varies from light gray or light brown to dark gray or dark brown. In some individuals the area below the eye and tympanum is yellowish cream (QCAZ 4441, AMNH 20896), to gray (QCAZ 31606) or brown (QCAZ 31602, AMNH 88695). Cloacal tubercles vary from yellowish cream (QCAZ 4441, AMNH 20896), to gray (QCAZ 31606) or brown (QCAZ 31602, AMNH 88695).

**Color in life.** Based on digital photograph of an adult female QCAZ 50568 (Fig. 8). Dark brown dorsum with irregular light brown and yellowish marks; there is a clear mid-dorsal line. Dorsal surfaces of tights and shanks are dark brown with transversal brown bands. Dorsal surfaces of forelimbs are dark brown with irregular light brown marks. Dark brown tubercles are abundant on the dorsum. Ventral surfaces vary from light brown to dark brown, with some irregularly distributed white and orange spots. The fingertips and the subarticular tubercles on fingers and toes are red-orange. Canthal region and tympanum are dark brown; iris greenish yellow with black reticulation.

**Figure 13.** *Rhinella alata* from Panama showing variation in dorsal and ventral coloration of preserved specimens. Left to right, male: AMNH 89459 (SVL 37.54 mm); females, AMNH 88694 (SVL 41.21 mm), AMNH 55476 (SVL 41.19 mm), AMNH 104454 (SVL 49.69 mm), AMNH 88689 (SVL 42.75 mm), AMNH 20896 (SVL 42.98 mm). See Appendix 1 for locality data. Not shown at the same scale.
Based on a digital photography of an adult male QCAZ 37248 (Fig. 8). Light brown dorsum with black spots and light brown and light gray marks. Dorsal surfaces of tights, shanks and forelimbs are light brown with transversal dark brown bands. Brown tubercles are abundant on the dorsum. Ventral surfaces are dark brown with irregularly distributed yellowish marks; the posterior part of the venter is cream. The subarticular tubercles of palms, soles, and fingertips are red-orange. Canthal region and tympanum are dark brown; iris greenish yellow with black reticulation.

**Distribution and ecology.** *Rhinella alata* has been recorded at 37 localities in the Ecuadorian Chocó (Cañar, Carchi, El Oro, Esmeraldas, Manabí, Pichincha, and Santo Domingo Provinces; Fig. 1), one locality in the Colombian Chocó (Barbacoas, Nariño; see **Taxonomic remarks**) and 35 localities in Panama (Comarca Guna Yala and Provinces Cocle, Colón, Darién and Panama; Fig. 2). It has a wide elevation range, from 19 to 1500 m above sea level.

The examined specimens from Chocoan populations contain 21 gravid females (average SVL = 45.37 mm, SD = 4.05 mm): QCAZ 4262, QCAZ 4441, QCAZ 4442, QCAZ 4443, QCAZ 7065, QCAZ 10296, QCAZ 11597, QCAZ 11598 collected in January; QCAZ 50568 collected in February; QCAZ 11392, QCAZ 31601, QCAZ 31603, QCAZ 31605 collected in April; QCAZ 25023 collected in June; QCAZ 10439 collected in August; QCAZ 14607 collected in November; QCAZ 10301 collected in December. This suggests year round reproductive activity with a peak between January and April, a period that corresponds to the rainy season in the Ecuadorian Chocó.

In Panamanian populations gravid females were found in January (AMNH 104454), September (AMNH 55461), November (AMNH 88689), and December (AMNH 53699). In central Panama, *R. alata* breeds in ponds and pools along permanent streams or swamps. Reproduction is explosive and most takes place from the middle of the rainy season to early dry season (Wells 1979, Ibáñez et al. 1999). Choruses last less than 24 hours with males usually calling at night and oviposition occurring by day, especially in the early afternoon (Wells 1979). Otherwise, individuals are primarily diurnal, found active on the leaf litter of the forest floor during daytime, and often found asleep on leaves of low vegetation at night (Ibáñez et al. 1999). Diet is specialized on ants (Toft 1981).

Most of the Ecuadorian specimens are from Reserva Mayronga and Reserva Ecológica Cotacachi-Cayapas. They were found in the leaf litter of secondary forest and in agricultural lands. Some adults were observed at night within the forest in vegetation above the ground and some were found in amplexus (QCAZ 10271, QCAZ 10274, QCAZ 10275 in November 1996, and QCAZ 31604, QCAZ 31605 in February 1996). All the specimens collected in Reserva Ecológica Cotacachi-Cayapas were found in secondary forest. At some collecting sites, the forest has been cleared for cacao plantations (QCAZ specimen database).

According to the classification of Sierra et al. (1999) the vegetation types for Ecuadorian localities are: (1) Lowland Evergreen Forest of Coastal Range, characterized by abundant epiphytes, climbers and herbaceous plants, with a canopy of 30 m (e.g. Reserva La Chiquita, Durango); (2) Semideciduous Lowland Forest of Coastal Range, defined by
the presence of broad canopy trees up to 20 m and curved shafts; the tree stratum is characterized by the presence of spiny, deciduous species with epiphytes while the forest floor has herbaceous plants (e.g. Bilsa, La Tortuga); (3) Evergreen Foothill Forest of Coastal Range, characterized by a canopy that can reach 30 m or more and trunks of trees covered with orchids, bromelias, ferns and aroids (e.g. Manta Real, Alto Tambo); (4) Deciduous Lowland Forest of Costal Range, characterized by losing leaves during part of the year with a great variety of cactus and thorny plants; the most conspicuous trees are the family Bombacaceae have curved trunks and broad crown. (e.g. El Progreso); (5) Semideciduos Foothill Forest of Coastal Range, characterized by having slightly dispersed vegetation, with trees over 20 m and dense herbaceous layers of ferns (e.g. Valle Hermoso).

The main vegetation types for Panamanian localities are (following Hogan 2010): (1) Isthmian-Atlantic Moist Forests, characterized by tall tropical evergreen forest with buttressed canopy trees reaching 40 m and with an extremely rich epiphyte flora (e.g. Cruces Trail, Punta Rincón); (2) Eastern Panamanian Montane Forest, at elevations from 500 to 1800 m above sea level, includes marshes, swamp forests, semi-deciduous tropical moist forests, premontane wet forest, cloud forests and elfin forests (e.g. Cana, Cerro Tacarcuna); (3) Chocó-Darién Moist Forests, at elevations between 0 and 1000 m above sea level, between the Pacific Ocean and the western range of the Andes (e.g. Dad Nakue Dubpir, Udirbi).

**Taxonomic remarks.** Based on morphological characters, Vélez-Rodriguez (2004) ascribed four populations from Panama and Colombia to *R. alata*: Isthmus of Panama (Panama; 15 males, 10 females); Parque Nacional Los Katíos (Colombia; 12 males, 15 females); Gorgona and Güape Island (Colombia; 7 males, 8 females); Municipio Restrepo (Colombia; 7 males, 8 females). Based on data from Vélez-Rodriguez (2004), these populations differ from the holotype of *R. alata* and populations of *R. alata* in Ecuador and Panama (in parentheses) in having: (1) a *canthus rostralis* protruding in females and ill-defined in males (inconspicuous in males and females), (2) parietal crests well defined in females, ill-defined in males (ill-defined in males and females), (3) vertebral apophyses slightly visible externally (absent). The differences suggest that those specimens are not *R. alata* and may belong to a different species. Alternatively, differences between *R. alata* described by Vélez-Rodriguez (2004) and our study could be an artifact resulting from the use of distinct terminology for similar character states.

In contrast, Mueses-Cisneros and Moreno-Quintero (2012) reported two species of the *R. margaritifera* group form Barbacoas, Nariño, Colombia (*Rhinella* sp. 9 and *Rhinella* sp. 10). Two photographs of live individuals (pp. 45) show morphological features that fall within the observed variation of *R. alata*. We tentatively assign them to *R. alata* but direct specimen examination is required to confirm this identification.

**Discussion**

The taxonomic status and phylogenetic position of populations traditionally ascribed to *R. margaritifera* (= *Bufo typhonius*; e.g. Anderson 1945, Miyata 1982, Ortega-An-
drade et al. 2010) from western Ecuador and Central America were reviewed. The examination of the holotype of *R. alata* in combination with the morphological and genetic information from 72 populations from the Chocó region and Panama indicate that those populations should be referred to *R. alata*. The similarity between Chocoan and Panamanian populations was previously noted by Hoogmoed (1990).

**Systematics and morphology**

Hoogmoed (1990), Lescure and Marty (2000) and Fouquet et al. (2007b) considered that *R. margaritifera* from French Guyana, with hypertrophied crests, corresponds to *R. margaritifera* sensu stricto. In a recent review, however, Lavilla et al. (2013) assigned a neotype with the type locality in “Humaitá, State of Amazonas, Brazil”. In our phylogeny (Fig. 3), the sister clade of *R. ocellata* include the closest localities to the new type locality for *R. margaritifera* and are likely to contain populations of *R. margaritifera* sensu stricto. Our phylogeny and previous reviews (e.g. Fouquet et al. 2007b) indicate that species diversity in the *R. margaritifera* group is greatly underestimated. In our phylogeny, two *R. margaritifera* from the southern Amazon in Ecuador (QCAZ 18241 and QCAZ 23917) are more closely related to *R. margaritifera* from French Guyana and *R. dapsilis* than to other *R. margaritifera* from Amazonian Ecuador. They probably represent an undescribed species, characterized by the presence of vertebral apophyses, bony knobs at the angle of jaws, and poorly developed crests. More studies are needed to define the status of these populations, as well as that of *R. cf. paraguayensis* from Bolivian and Brazilian Amazon and *R. cf. hoogmoedi* from Brazilian Atlantic Forest.

The identity of the upper Amazon clade (Ecuador-Peru) remains unresolved. It was not possible to ascribe it unequivocally to any described species of the *R. margaritifera* species group and it is unlikely to be *R. margaritifera* sensu stricto (as defined by Lavilla et al. 2013). Thus, these populations may belong to an undescribed species characterized by having prominent supratympanic crests, conspicuous vertebral apophyses in the dorsum and bony knobs at angle of jaws (Fig. 10). We refrain from describing this species until genetic samples of *R. margaritifera* sensu stricto are available and a comprehensive review of the group is carried out. For now, we suggest that these populations are referred as *R. margaritifera* sensu lato.

These results raise some rather interesting questions. For instance, the complete distribution range of *R. alata* is yet to be determined. Extensive and explicit studies are necessary to reveal whether the species is continuously distributed from Ecuador to Panama or if it consists one, two (or more) disjoint population nuclei. This would be an indispensable step before planning further studies on the evolutionary history or conservation status of the species. Moreover, future studies including a larger number of samples, more representative of the geographic range of each species within the *R. margaritifera* group, from Colombia, Venezuela and Suriname, will help to clarify their evolutionary identity. It will also be necessary to re-evaluate, using molecular, mor-
phological, ecological, behavioral, and phylogenetic analyses, the taxonomic status of species that have been previously described only morphologically such as *R. acutirostris*, *R. magnussoni*, *R. proboscidea*, *R. roqueana*, *R. sclerocephala*, *R. scitula* and *R. stanlaii*. Integrative approaches like the one we pursued in this study will help to disentangle the complex evolutionary history, systematics, and taxonomy of this species group.

**Biogeographic implications**

Because all species in the *R. margaritifera* species group are distributed in South America, it is reasonable to assume that the presence of *R. alata* in Central America is the result of a single dispersal event from South America. The genetic distances between Chocoan and Panamanian populations are low (range 1.2–1.9%) and suggest that their divergence was recent and occurred after the closure of the Panamanian isthmus during the late Pliocene. Assuming a rate of evolution of the gene *16S* of 0.00249–0.00277 substitutions per site per lineage per Myr (Evans et al. 2004; Lemmon et al. 2007), the divergence between these populations occurred ~ 2.16 to 3.42 Myr ago (under the 0.00277 rate) or ~ 2.41 to 3.81 Myr ago (under the 0.00249 rate). Thus, it is likely that the divergence between Panama and Chocó took place after the completion of the Panamanian Isthmus (~ 3.5 Myr ago; Coates et al. 1992, Coates and Obando 1996). These estimates of time of divergence, however, should be considered with extreme caution because they assume a molecular clock at a rate estimated for species in different families. Further explicit studies will be necessary to estimate divergence times with more confidence.

*Rhinella alata* is sister to populations of *R. margaritifera* from the Ecuadorian and Peruvian Amazon and the eastern Andean slopes, up to 2000 m of elevation, forming altogether a robust clade. The two lineages are highly divergent from each other (uncorrected *p* distances 3.0–5.5%, mitochondrial gene *16S*) and are morphologically distinctive. Therefore, both clades clearly represent separate species. Previously, *R. margaritifera* was considered to occur on lowland rainforests east and west of the Andes of Ecuador. This distribution was atypical because out of 174 amphibian species inhabiting the Amazonian rainforests of Ecuador below 600 m of elevation, only three also occur in the rainforests of the Chocó region west of the Andes: *Hypsiboas boans*, *Rhinella marina* and *Trachycephalus typhonius* (Ron et al. 2014). Despite having similar environmental conditions and being geographically close (as low as 100 km of airline distance), rainforests on both sides of the Andes share few amphibian species, a result of the barrier effect of the Andes. Our results showing that *R. margaritifera* only occurs on the eastern side demonstrate that their unusual distribution was an artifact of the incorrect delimitation of species boundaries. We suspect that the same problems could explain the disjunct distributions of *Rhinella marina*, *Trachycephalus typhonius* and *Hypsiboas boans*. Therefore, tropical rain forests of the Amazon and the Chocó may not share amphibian species.
Acknowledgments
This work was funded by Secretaría Nacional de Educación Superior, Ciencia, Tecnología e Innovación (Arca de Noé initiative) and Pontificia Universidad Católica del Ecuador. RI was supported by the Sistema Nacional de Investigación de Panama. We are particularly thankful to Annemarie Ohler, the Curator of Muséum National d’Histoire naturelle, who provided data and photos of the holotype of *R. alata*. David Buckley for valuable comments on the text. Ángel Sosa and Mario Yánez-Muñoz shared photographs of *R. alata*. Darrel Frost, Curator of AMNH, loaned specimens of *R. alata* from Panama. Pablo Venegas, curator of CORBIDI, provided a tissue sample from Peru. Nadia Páez for help in taking measurements of specimens. We are thankful to Andrea Manzano and María Ordoñez for carrying laboratory work. For specimen collection and locality data, we are indebted to Néstor Acosta, Silvia Aldás, Alejandro Arteaga, Fernando Ayala, Alvaro Barragán, Paola Buitrón, David Cannatella, Edwin Carrillo, Luis Coloma, Rafael de Sa, Alexandra Endara, Jorge García, Stella de la Torre, Mireya Dimas, Sehoya Harris, César Jaramillo, Fidel Jaramillo, Diego Lombeida, Alfredo Lopez, Luis E. Lopez, Fernando Nogales, Giovanni Onore, Aida Ortiz, Alexandra Quiquango, Fabián Sáenz, Frank Solís, Samuel Sucre, Italo Tapia, Queti Tapia, Eduardo Toral, J. M. Touzet, Ana M. Velasco, and Tania Villegas.

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Appendix 1

Examined material. Numbers in bold indicate specimens analyzed genetically and morphometrically.

*Rhinella alata.* — ECUADOR: PROVINCIA CANAR: Manta Real, Río Patul (2.5679°S, 79.3666°W), 350–400 m (QCAZ 3437, 3551, 4757–758); Manta Real (2.5537°S, 79.3642°W), 500 m (QCAZ 12778–779). PROVINCIA CARCHI: Vía Zumba–El Chota, 1500 m (QCAZ 12233). PROVINCIA EL ORO: Valle Hermoso, Parroquia Bella María (3.5019°S, 79.8172°W), 379 m (QCAZ 37244, 37248); El Progreso, vía Pasaje–Pan de Azúcar (3.2883°S, 79.7581°W), 180 m (QCAZ 10366). PROVINCIA ESMERALDAS: Lagarto, Mayronga Reserve (1.042°S, 79.28°W), 100 m (4262–4264, 4441–4451, 4709–4717, 6637–6642); Reserva Ecológica Bilsa (0.6202°S, 79.931°W), 534 m (QCAZ 6731–6743); Corriente Grande, Río Cayapas (0.6895°S, 78.9589°W), 70 m (QCAZ 10271, 10274–281, 10289, 10290, 10292, 10295–299, 10299, 10301); Reserva Ecológica Cotacachi Cayapas, Charco Vicente (0.6962°S, 78.9109°W), 60 m (QCAZ 3338–3339, 11391–396); Pichiyacu, Comunidad Chachi, Río Cayapas (0.9081°S, 78.998°W), 260 m (QCAZ 31602–609); Reserva Ecológica Cotacachi–Cayapas o Playa de Oro (0.8285°S, 78.722°W), 179 m (QCAZ 49381–382, 49387, 49391); Las Golondrinas near Río Canandé (QCAZ 12651–652); Durango, Río San José (1.054°S, 78.625°W), 33 m (QCAZ 24968–978); Río Onzole (0.712°S, 79.092°W), 110 m (QCAZ 10440–443); Comunidad Loma Linda, Río Onzole (0.8754°S, 79.0511°W), 95 m (QCAZ 10439); La Concordia (0.0022°S, 79.4105°W), 144 m (QCAZ 50573, 50568); San Lorenzo, Protectora La Chiquita (1.2333°S, 78.76°W), 60 m (QCAZ 10253, 10254–255, 11597,
11598); San Lorenzo, La pera del Guarapo (1.2684°S, 78.8067°W), 253 m (QCAZ 23161); La Pedorrera (0.4667°S, 79.9833°W), 53 m (QCAZ 25032); La Tortuga (0.591°S, 79.957°W), 86 m (QCAZ 25023); Borbón (1.0667°S, 79.05°W), 70 m (QCAZ 14607); Viche (0.6615°S, 79.5387°W), (QCAZ 4674); Durango (1.0427°S, 78.6245°W), (QCAZ 8549, 35250); 7 km western of Durango (1.0133°S, 78.6682°W) 220 m, (QCAZ 23164, 23623); Viruela, Río Cayapas (1.1142°S, 78.9936°W), 45 m (QCAZ 10289); Al Tambo (0.9169°S, 79.5662°W) 253 m, (QCAZ 21138); El Milagro, La Mayronga (1.003°S, 79.326°W). PROVINCIA MANABÍ: El Carmen (0.274°S, 79.459°W). 300 m (QCAZ 7038–7039, 7065). PROVINCIA PICHINCHA: Reserva Forestal ENDESA (0.1667°S, 79.1667°W), 720 m (QCAZ 1659); Río Canoi (0.075°S, 79.051°W), 570 m (QCAZ 2745); 1 km E of Pedro Vicente Maldonado (0.0833°S, 79.039°W), 670 m, (QCAZ 2752); San Miguel de los Bancos (0.0166°S, 78.8833°W), (QCAZ 3813, 3815–818); San Miguel de los Bancos, Río Pitzará, 130 m (QCAZ 50846); km 9 San Miguel de los Bancos–Puerto Qui-to road (0.072°S, 78.9599°W), (QCAZ 5860); Puerto Qui-to, ENDESA (0.098°S, 79.117°W), (QCAZ 36827). PROVINCIA SANTO DOMINGO: Bosque Protector La Perla (0.057°S, 79.359°W), (QCAZ 3500–504); km 8 road to Santo Domingo (0.2005°S, 79.1924°W), 528 m (QCAZ 23621). PANAMA: COMARCA GUNA YALA: Dad Nakue Dubpir, Río Ogandí (9.2477°N, 78.1744°W), 150 m (CH 8842); Udiribi, Reserva Forestal (9.3167°N, 78.9833°W), 342 m (CH 1706); PRO-VINCIA COCLÉ: La Mina, Río Indio (8.9382°N, 80.1469°W), 48 m (CH 4922); near Río Tife cascade, Parque Nacional General de División Omar Torrijos Herre-ra (8.7065°N, 80.6352°W), 460 m (CH 0065); Obispo (9.1167°N, 79.6833°W) (MNHN 84285); Quebrada La Tiburcia, Cascajal (8.7158°N, 80.4605°W), 180 m (CH 5042); Quebrada La Varona, near Palmarazo (8.7342°N, 80.6565°W), 125 m (CH 5139). PROVINCIA COLÓN: Chitra, Santa Isabel (9.5186°N, 79.1534°W), 90 m (CH 7783); El Limón, Río Indio (8.9919°N, 80.1701°W), 19 m (CH 4967); Rinconcito, Punta Rincón (9.0135°N, 80.6884°W), 52 m (CH 1412); Río Caimi-to, Petaquilla (8.9706°N, 80.671°W), 54 m (CH 5476); Río Boquerón (9.3857°N, 79.4826°W), 150 m (AMNH 89459); Río Frijoles, Camino del Oleoducto, Parque Nacional Soberanía (9.1523°N, 79.7347°W), 67 m (CH 0307); road to Piña, after the represa Gatú (9.2603°N, 79.94°W), 34 m (CH 1679); Sta. Rosa and Guayabalito (9.1833°N, 79.65°W), 36 m (AMNH 55475); PROVINCIA DARIÉN: between Dos Bocas de Antaral and campsite on Serranía de Jingurudó (7.6564°N, 77.9986°W), <675 m (CH 4641); Cerro Tacarcuna, Río Pucuro (8.0011°N, 77.4852 °W), 640 m (AMNH 104454); Cana, trail to Boca de Cupé, Pinogana (7.7661°N, 77.6752°W), 518 m (CH 9104); Estación Pirre, Río Peresénico (8.0192°N, 77.7325°W), 90 m (CH 4057); Laguna Purriche (7.7222°N, 77.6555°W), 475 m (CH 6376); PROVINCIA PANAMA: Altos de Majé (AMNH 88689–8690, 88694); Barro Colorado (9.1636°N, 79.8378°W), 79 m (AMNH 20896, 5274, 55461–462); Parque Nacional Soberanía, Ancón (9.0764°N, 79.6594°W), 130 m (CH 9192); Chiva Chiva Road, Parque Na-cional Camino de Cruces (9.0284°N, 79.5899°W), 41 m (CH 0491); Cruces trail (9.0453°N, 79.5892°W), 77 m (AMNH 55460); Finca Santa Bárbara, Nuevo Eme-
Rhinella margaritifera.

— ECUADOR: PROVINCIA ORELLANA: Parque Nacional Yasuní, Estación Científica Yasuní (0.6772°S, 76.4012°W), 230 m (QCAZ 8415, 17736, 17740, 41011); Parque Nacional Yasuní, Bloque 31 (0.942°S, 75.905°W), (QCAZ 11909); Parque Nacional Yasuní, Río Yasuní (0.9248°S, 75.9152°W), 206 m (QCAZ 11940); Parque Nacional Yasuní, Via Pompeya-Iro (0.6536°S, 76.4536°W), 287 m (QCAZ 17216, 17329, 43011, 22401); Parque Nacional Yasuní, Apaika (0.8656°S, 75.9245°W), (QCAZ 33545); Estación Biológica Tiputini (0.0639°S, 76.1493°W), 250 m (QCAZ 10207); Nuevo Rocafuerte (0.8967°S, 75.437°W),186 m (QCAZ 39466); Añangu (0.5249°S, 76.3844°W), 255 m (QCAZ 43952–953); Chiroisla (0.58°S, 75.9177°W), 207 m (QCAZ 44318–319; Huiririma (0.7116°S, 75.6239°W), 194 m (QCAZ 44563–565). PROVINCIA PASTAZA: Río Bobonaza (1.8056°S, 77.3313°W), 250 m (QCAZ 10650); Kapawi Lodge (2.5387 °S, 76.8583°W), 239 m (QCAZ 25476, 25488–489); Pomona (1.625°S, 77.9072°W), 846 m (QCAZ 25631). PROVINCIA SUCUMBIOS: Reserva Limoncocha (0.4062°S, 76.6195°W), 261 m (QCAZ 43104, 43108); Pañacocha (0.4712°S, 76.0667°W), 255 m (QCAZ 44098–099). PROVINCIA NAPO: Reserva Yachana (0.8333°S, 77.1667 °W), 350 m (QCAZ 42269); Cascada de San Rafael (0.1036°S, 77.5808°W), 1300 m (QCAZ 31708). PROVINCIA MORONA SANTIAGO: Plan de Milagro (3.0011 °S, 78.5052°W), 1950 m (QCAZ 48242).
### Appendix 2

| 10226MSH Rₚ, aff. margaritifera Anavihanas AM BR | 10359MSH Rₚ, aff. margaritifera Anavihanas AM BR | 1085C Rₚ, margaritifera Kaw2 FG |
| 111AF Rₚ, martyi Brownsburg FG | 1386C Rₚ, margaritifera Crique Margot FG |
| 13872MTR Rₚ, margaritifera Lourenço AP BR | 13875MTR Rₚ, margaritifera Lourenço AP BR |
| 13875MTR Rₚ, margaritifera Lourenço AP BR | 13875MTR Rₚ, margaritifera Lourenço AP BR |
| 143p9 Rₚ, margaritifera Kaw2 FG | 156MC Rₚ, martyi Trionfection FG |
| 163BM Rₚ, margaritifera Guatemala FG | 164BM Rₚ, margaritifera Montagne des Singes FG |
| 176BM Rₚ, margaritifera Trinite FG | 195MC Rₚ, margaritifera Kaw2 FG |
| 2034T Rₚ, margaritifera Nouragues FG | 204MC Rₚ, margaritifera Saul FG |
| 217MC Rₚ, margaritifera Grant Santi FG | 226MC Rₚ, margaritifera St Elie FG |
| 2559T Rₚ, margaritifera Pic Matecho FG | 284MC Rₚ, margaritifera St Elie FG |
| 288AG Rₚ, margaritifera St Georges FG | 294MC Rₚ, margaritifera Camp Canope FG |
| 307FG Rₚ, margaritifera Toponowini FG | 307MC Rₚ, margaritifera Lucifer FG |
| 3274MC Rₚ, margaritifera Regina FG | 338MC Rₚ, margaritifera Apetou FG |
| 390MC Rₚ, margaritifera St Georges FG | 408PG Rₚ, margaritifera Mont Kolika FG |
| 4482T Rₚ, margaritifera Angouleme FG | 66MC Rₚ, margaritifera Monts Bakra FG |
| 74AF Rₚ, margaritifera St Georges FG | 92bm Rₚ, margaritifera Cisame FG |
| QCAZ17775 Rₚ, margaritifera Indanza EC | QCAZ18241 Rₚ, margaritifera Shaime EC |
| QCAZ23917 Rₚ, margaritifera Gualaquiza EC | QCAZ38755 Rₚ, dapsilis VillanoBi EC |
| QCAZ38892 Rₚ, dapsilis VillanoKurinta EC | QCAZ38892 Rₚ, dapsilis VillanoKurinta EC |
| 104mc R_castaneotica Tibou relaxed | 110pp R_castaneotica Mont Saint Marcel FG |
| MRT6313 R aff margaritifera Serra do Kukoinhokoen AM BR | MRT6317 R aff margaritifera Serra do Kukoinhokoen AM BR |
| 12bm R_lescurei Litany FG | 30271 R_lescurei Mitaraka FG |
| CORDID5840 R margaritifera Madre de Dios PE | QCAZ10253 R margaritifera La Chiquita EC |
| QCAZ10254 R margaritifera La Chiquita EC | QCAZ10255 R margaritifera La Chiquita EC |
| QCAZ14468 R margaritifera Borbon EC | QCAZ11598 R margaritifera La Chiquita EC |
| QCAZ13882 R margaritifera Manta Real EC | QCAZ17989 R margaritifera Jatun Sacha EC |
| QCAZ17990 R margaritifera Jatun Sacha EC | QCAZ17991 R margaritifera Jatun Sacha EC |
| QCAZ23161 R margaritifera Santi Lorenzo EC | QCAZ23632 R margaritifera 7Kmn Cosanga EC |
| QCAZ25023 R margaritifera La Tortuga EC | QCAZ25025 R margaritifera La Tortuga EC |
| QCAZ25025 R margaritifera La Tortuga EC | QCAZ37244 R margaritifera Valle Hermoso EC |
| QCAZ49968 R margaritifera PN Soberania PA | QCAZ49960 R margaritifera PN Soberania PA |
| QCAZ49959 R margaritifera PN Darien PA | QCAZ38477 R dapsilis VillonoB EC |
| QCAZ38621 R dapsilis VillanoK4 EC | QCAZ38686 R dapsilis VillanoK4 EC |
| QCAZ38686 R dapsilis VillanoK4 EC | QCAZ38998 R margaritifera VillanoKurinta EC |
| QCAZ38512 R dapsilis VillanoBi EC | QCAZ38560 R dapsilis VillanoB EC |

Bayesian consensus phylogram depicting relationships within the *Rhinella margaritifera* species group. The phylogram was derived from the analysis of 550 bp of nuclear gene Tyrosinase. Museum catalog numbers are shown in Table 1. Abbreviations are: EC = Ecuador, FG = French Guyana, BR = Brazil, BO = Bolivia, PE = Peru, PA = Panama. Outgroups are not shown.