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Toads, tall mountains and taxonomy: the *Rhinella granulosa* group (Amphibia: Anura: Bufonidae) on both sides of the Andes

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Abstract. A toad in the *Rhinella granulosa* group has been recognized as present on Trinidad since 1933. In 1965, the Trinidadian population was described as a subspecies of *Bufo granulosus*, *B. g. beebei*. It has its type locality on the island and was eventually raised to species status as *B. beebei* (Beebe's toad). Recently Beebe's toad was synonymized with *Rhinella humboldti*, a species with a type locality in the Magdalena Valley of western Colombia. The Magdalena Valley is separated from the Orinoco Basin by the Eastern and Merida Cordilleras. These ranges have peaks that exceed 5,000 m and an almost continuous altitude at about 3,000 m. Here we examine the morphology, advertisement calls, and mtDNA from several populations of these lowland toads to test whether the western Colombian *R. humboldti* and the Orinoco-Trinidad *R. beebei* are conspecific and form a single taxon that occurs on both sides of the Andes. The morphological, molecular, and advertisement call analyses suggest that *R. humboldti* and *R. beebei* are distinct taxa composed of independent evolving lineages. *Rhinella beebei* is therefore resurrected from the synonymy of *R. humboldti* for the Trinidad and some of the adjacent mainland Orinoco populations in both Venezuela and Colombia. This increases the number of described species in the clade to fourteen. *Rhinella humboldti* and its sister *R. centralis* (Panama) are the only members of the *R. granulosa* group to occur west of the Andes, and our molecular results suggest the TMRCAs for *R. beebei* and *R. humboldti* at about 9 Mya, a time when the Eastern Cordillera was much lower in altitude than it is today and the Merida Cordillera was in its early stages of formation.

Key words. Cryptic species, Janzen's hypothesis, palaeoelevation, speciation, systematics, topographic barriers.

Introduction

The timing of the Andean uplifts and related processes are critical to understanding Neotropical biogeography. Fossil fish of the genera *Brachyplatystoma*, *Lepidosiren*, *Arapaima*, *Phractocephalus*, and *Colossoma* and fossils of the Colombian matamata turtle, *Chelus colombiana*, suggest that an ancient Amazonian-Orinoco fauna once occurred on both sides of the Andes (LUNDBERG 2005, CADENA et al. 2008). Prior to the existence of the Andean Eastern Cordillera, central Colombia was part of the palaeo-Amazon-Orinoco system (HOORN et al. 1995, 2010, LUNDBERG et al. 1998). The north-flowing palaeo-Amazon-Orinoco

drained the basin east of the developing Andean mountain chain from Bolivia to Venezuela. However, during or after the uplift of the Eastern and Northern Andes, the new drainage divide isolated the trans-Andean Magdalena Valley, and much of the Amazonian and Orinoco fauna disappeared from the region (LUNDBERG 2005). Today, the Eastern Cordillera (Colombia) and the Cordillera de Merida (Venezuela) form barriers between the Orinoco drainage to the east and the Magdalena and Maracaibo drainages to the west. These barriers have peaks that exceed 5,700 m and altitudes that are almost continuous at 3,000 m above sea level. Resolving estimates of barrier uplift and river drainage shifts with molecular-clock es-

timates of genetic divergence times for various Andean and Amazonian populations is important for understanding South America's spectacular biodiversity (JAVADI et al. 2011).

Two species of toads in the genus *Rhinella* are present in the Republic of Trinidad and Tobago. The marine toad, *R. marina*, is a large, widespread species known to reach at least 240 mm in body length and possibly the largest and best-studied toad in the world. It is locally known as the crapaud and has played a role in the islands' ecosystems as well as the culture of Trinidad and Tobago (KENNY 1969, MURPHY 1997). A second, smaller (< 60 mm) species of *Rhinella* is also present on Trinidad but not Tobago. This toad's presence on the island has been known since at least 1933 when PARKER (1934) included it in his list of Trinidadian anurans. It has been known to science as *Bufo granulatus*, *B. granulatus beebei*, *B. beebei*, *Rhinella beebei*, and most recently as *R. humboldti* (KENNY 1969, MURPHY 1997, NARVAES & RODRIGUEZ 2009).

A short historical summary of the *Rhinella granulosa* group nomenclature

SPIX (1824) described *Bufo granulatus* from Brazil, DUMÉRIEUX & BIBRON (1841) described *B. dorbignyi* from Uruguay, and PARKER (1935) considered *dorbignyi* a subspecies of *granulosa*. Following the trends of consolidating populations and names and recognizing polytypic species, MÜLLER & HELLMICH (1936) described *B. g. major* and considered *B. granulatus* to be composed of three subspecies: *B. g. granulatus*, *B. g. dorbignyi*, *B. g. major*. However, other authors suggested the subspecies might be distinct at species level (SCHMIDT & INGER 1951). MYERS & CARVALHO (1952) described *B. pygmaeus* from Rio de Janeiro, a species they believed to be closely related to *B. granulatus*. GALLARDO (1957) described *B. g. fernandezae* from Buenos Aires, Argentina, and later GALLARDO (1965) reviewed the group, recognizing 14 subspecies of *B. granulatus* that were distributed from the north coast of South America (including Trinidad and the Isla de Margarita) to Uruguay. Adding nine new taxa to those already described, he wrote,

"...one can see in *B. granulatus* a clear distribution of the subspecies according to the hydrographical systems, a distinct subspecies belonging to the basin of each of the following rivers: Magdalena, Orinoco, Upper and Middle Amazon, Tocantins, and Araguaia, Sao Francisco, Rio de la Plata..."

GALLARDO went on to comment that other distinct taxa were found in Guyana (*Rhinella merianae*), on the Isla de Margarita (*R. barbouri*), and that the Trinidad population was the same as the one represented in the Orinoco drainage (*R. beebei*). He designated a specimen from Trinidad as the holotype for *Bufo granulatus beebei*, and an additional 13 specimens as paratypes, all with the same locality data (Churchill-Roosevelt Highway, Trinidad)

Since GALLARDO's work, the polytypic species concept has been replaced with a lineage-based species concept (DE QUEIROZ 1998) and advances in molecular technology

have led to the resurrection of many old names and the discovery of many new species (SANTOS et al. 2009). The family Bufonidae was revised by FROST et al. (2006) and found to be a worldwide clade with 35 genera. The cosmopolitan, polyphyletic genus *Bufo* was subsequently divided into several genera, most of which follow the principles of biogeography. *Rhinella* FITZINGER, 1826 was the oldest available name for the clade of Neotropical toads that included both the *R. granulosa* and *R. marina* groups.

NARVAES & RODRIGUES (2009) re-examined the *Rhinella granulosa* group, using a Canonical Discriminant Analysis and raised many of GALLARDO's subspecies of *granulosa* to species level, synonymized others, and described a new species (*R. centralis*) from Panama, based on specimens GALLARDO had considered to be *Bufo granulatus humboldti*. The end result was 12 species in the *R. granulosa* group. They viewed members of the *granulosa* group as not necessarily associated with drainages, but with open habitats with high rainfall, noting that most species (*R. azarai*, *R. bergi*, *R. centralis*, *R. dorbignyi*, *R. fernandezae*, *R. humboldti*, *R. major*, *R. mirandaribeiroi*, *R. pygmaea*) are found below 300 m above sea level. This is relevant to the Orinoco Basin-Trinidad populations because they regarded *B. g. beebei* GALLARDO as a junior synonym of *B. g. humboldti* GALLARDO.

On the basis of biogeography alone, the synonymous status of *R. beebei* in *R. humboldti* is surprising. The straight-line distance between Girardot, Colombia and Trinidad is about 1,580 km. Keeping in mind that toads of the *granulosa* group are for the largest part lowland, savannah species, the topography between the type locality for *humboldti* and Trinidad includes the Cordillera Oriental, the Serranía de Perijá, the Cordillera de Mérida, the Coastal Cordillera of Venezuela, as well as a thousand kilometres or more of savannah. Thus, the evolution of the South American landscape has supplied multiple historic opportunities for corridors as well as multiple barriers to gene flow before, during, and after the formation of the Andes.

GALLARDO (1965: 114) described *Bufo granulatus beebei* based on AMNH 55774 (as well as 11 other specimens with the same locality data). The toads were collected along the Churchill-Roosevelt Highway in Trinidad at an altitude of about 30 m. Three pages later he described *B. g. humboldti* based upon a male specimen (MCZ 24882) from the municipalities of Girardot and Gualanday, Department of Tolima, Colombia (~4°16'N, 74°81'W, altitude ~326 m a.s.l.) and a female paratype MCZ 8978 from the municipality of Fundación, Department of Magdalena, Colombia (~10°29'N, 74°12'W, altitude ~60 m a.s.l.): both localities are in the Rio Magdalena drainage basin, but from opposite ends of the basin and more than 700 km apart.

Authors writing about this toad in Trinidad prior to GALLARDO's work called it *Bufo granulatus* (PARKER 1934). Post 1965, the commonly used name was *Bufo granulatus beebei* (KENNY 1969) until RIVERO et al. (1986) elevated the subspecies to species level. MURPHY (1997) followed using the name *B. beebei* and considered the toad an Orinoco/Trinidad endemic.

NARVAES & RODRIGUES (2009: 43) chose the name *humboldti* over *beebei* "...because it is associated with continental populations while *beebei* was named after insular specimens obtained at Trinidad and Tobago." The ICZN code (RIDE 1999) does not recognize page priority, thus this is an acceptable decision, assuming the two names actually represent a single operational taxonomic unit.

GUERRA et al. (2011) examined the advertisement calls of seven species within the *granulosa* group and found the calls consisted of long trills, composed of notes with a variable pulse number (2–8). TORRES-SUAREZ & VARGAS-SALINAS (2013) reported on the call of *R. humboldti* from the Reserve of Wisirare, municipality of Orocué, Department of Casanare, Colombia (4°54'40"N, 71°26'12"W, altitude 126 m a.s.l.). This location lies within the Orinoco drainage, and the toad's call has four pulses per note.

All GenBank sequences from Trinidad and the east side of the Andes have been labelled *Rhinella humboldti* (KP685211.1, KP685210.1, KP685174.1, KP685173.1, KP685153.1, KP685131.1, KP685099.1, KP685058.1, KP685025.1, KP685025.1, KP684965.1, KP684964.1, KP149216.1, KP149488.1, KP149473.1, DQ158276.1, DQ158434.1, DQ158358.1, EF532287.1, EF532269.1, EF532251.1). The molecular analysis and phylogeny of the *R. granulosa* group by PEREYRA et al. (2015) found it consisted of 13 species distributed throughout open habitats of South America and Panama. They performed separate phylogenetic analyses under direct optimisation (DO) of nuclear and mitochondrial sequences and recovered the *R. granulosa* group as monophyletic. However, they found a topological incongruence that they explained as the result of multiple processes of hybridisation and introgression. All the sequences they used from *R. humboldti* were from the east side of the Andes. None were from the type locality of *R. humboldti*, nor from the west side of the Andes.

Aims of this study

Here we examine toads of the *R. granulosa* group from Trinidad, Venezuela, and Colombia to resolve the correct name for the island and Orinoco Basin populations and attempt to identify whether the species present on Trinidad is a widespread species found across northern South America on both sides of the Andes as hypothesized by NARVAES & RODRIGUEZ (2009) or an Orinoco Basin/Trinidad endemic as suggested by GALLARDO (1965) and MURPHY (1997).

Methods and materials

Molecular methods

DNA extraction, purification and amplification protocols follow JOWERS et al. (2014). DNA was extracted from three liver samples of *Rhinella humboldti* adult males from Trinidad (Republic of Trinidad and Tobago, close to Trincity Central Road, (UWIZM.2012.27.72.1-3) and from three Co-

lombian individuals from two localities east of the Colombian Andes (ICN 55784), Department of Casanare, Municipality of Trinidad, Vereda La Cañada, Finca La Palmita (5°19'11.4"N, 71°20'51"W) (ICN 55776), Department of Casanare, Municipality of Paz de Ariporo, Vereda La Colombina, Finca El porvenir (6°2'36.5"N, 71°5'34.2"W) and west (CZUT-A 1717), Department of Tolima, Municipality of Prado, Vereda El Caiman, Hydroelectric dam "Hidropardo", Eco-Hotel Palo de Agua, 03°44'55.6"N, 74°50'23.5"W, altitude 376 m a.s.l.). The primers for the 12S rDNA: 12SA 5'-AAACTGGGATTAGATACCCACTAT-3', 12SB 5'-GAGGGTGACGGGCGGTGTGT-3' (KOCHER et al. 1989) and 16S rDNA: 16SL 5'-GCCTGTTTATCAAAA-CAT-3', 16SH 5'-CCGGTCTGAACTCAGATCAGT-3' (PALUMBI 1996) amplified an approximately 430 and 550 base-pair (bp) fraction, respectively. Templates were sequenced on both strands, and the complementary reads were used to resolve rare, ambiguous base-calls in Sequencher v. 4.9. After removing PCR primers and incomplete terminal sequences, 347 and 481 bp of the 12S and 16S rDNA gene fraction, respectively, 836 bp total, were available for analyses and aligned with the larger *Rhinella* data set (~2,450 bp in length 12S and 16S rDNA) from PEREYRA et al. (2015) to improve support of internal branches.

Preliminary analyses were performed with the complete mtDNA data set of the *R. granulosa* group. However, because the aim of our study was not to assess the overall phylogenetic relationships within the group, but rather to assess the relationship between *R. humboldti* and *R. beebei*, we only included two haplotypes per species for the final analyses. As suggested by PEREYRA et al. (2015), *R. bernardoi* mtDNA sequences showed signs of introgression, likely through hybridisation, and were therefore excluded from the analyses. Additionally, BLAST searches were conducted in GenBank and matches with high genetic affinity (~98%) were downloaded and included in the alignment, including shorter *R. humboldti* sequences of either the 12S or 16S rDNA gene fragments, but not available for both. Sequences were aligned in Seaview v. 4.2.11 (GOUY et al. 2010) under ClustalW2 (LARKIN et al. 2007) default settings. Genetic p-distances and standard errors (% ± SE) were calculated using MEGA v. 6 (TAMURA et al. 2011).

We used the software BEAST v. 1.5.4 (DRUMMOND & RAMBAUT 2007) to estimate coalescence times between species (TMRCA). We ran the analyses under a speciation (Yule process) prior to using a strict molecular clock model. Inspection of trace files indicated that a strict-clock model could not be rejected (the parameter 'Coefficient of Variance' in relaxed clock analyses abuts zero), and thus, we report results based on this model only. Dates of divergence estimated for 16S rDNA-corrected distances adopted for other anurans have been estimated to correspond to 0.39–0.40% divergence per million years (MARTINEZ-SOLANO et al. 2004, MANZANILLA et al. 2007) and are in accordance with rates assumed for the 16S and 12S rRNA genes in other amphibians such as newts and true salamanders (CACCONI et al. 1997, VEITH et al. 1998), ranging between 0.4 and 0.7% per million years and 0.44–0.54% for the 12S

rDNA in *Rana* (SUMIDA et al. 2000). Thus, in the absence of a reliable prior on the substitution rate or a well-dated calibration point, we specified a normal prior on the clock rate with a mean value of 0.0022 substitutions/site per lineage per million years for the combined 12S and 16S rDNA gene fragments. This calculation was obtained by considering the mutation rate of each gene fragment of 12S and 16S rDNA and the length of each in the alignment. We do stress however that this is just an estimated approximation of divergence to assess and date possible observed patterns to allopatric events in the study area and hence caution needs to be exercised in interpreting any molecular clock estimates. Analyses were run for 100,000,000 generations, and posterior distributions of parameter estimates were visually inspected in Tracer v. 1.5 (RAMBAUT & DRUMMOND 2007). Ten percent of the resulting topologies and parameter values were discarded as burn-ins in TreeAnnotator v. 1.8.2. The most appropriate substitution model for the Bayesian Inference (BI) analysis was determined by the Bayesian Information Criterion (BIC) in jModeltest v. 2 (POSADA 2008). The tree was constructed using the BI optimality criteria under the best-fitting substitution model (GTR+I+G). MrBayes was used with default priors, Markov chain settings, and random starting trees. Each run consisted of four chains of 100,000,000 generations and was sampled every 1,000th generation. A plateau was reached after few generations with 25% of the trees resulting from the analyses discarded as burn-ins. Phylogenetic relationships between haplotypes for each locus were estimated using a Maximum Likelihood (ML) approach, as implemented in the software RAxML v. 7.0.4 (SILVESTRO & MICHALAK 2010), using the default settings. The 50% bootstrap consensus tree was built in PAUP v. 4 (SWOFFORD 2002). All analyses were performed through the CIPRES platform (MILLER et al. 2010).

Morphological methods and call analysis

We examined museum material related to *R. humboldti* and *R. beebei*. External morphological data were collected for 149 museum specimens (Appendix 1). Measurements taken included SVL (snout–vent length), femur length, tibia length, horizontal orbit diameter, head width, head height (depth), foot length, and were taken to the nearest 0.1 mm using dial callipers. Statistical analyses were conducted with Microsoft Excel QI Macros, and a PCA was performed using Data.

Figure 1 illustrates the locations from which we have examined specimens as well as the type localities for *R. g. beebei* (GALLARDO), *R. g. barbouri* (GALLARDO), and *R. g. humboldti* (GALLARDO). HYACK et al. (2001) detail the problems associated with obtaining repeatable measurements on anurans, particularly preserved specimens that have not been carefully prepared. We agree that obtaining measurements that can be duplicated is at best problematic. Thus we use the morphometrics to test our position only in the broadest sense and ask the reader to look mainly at the ranges reported.

Advertisement call analyses were recorded by MORLEY READ (Trinidad). Trans-Andean *R. humboldti* were recorded at Department of Tolima, municipality of Coello by SIGIFREDO CALVIJO GARZON and loaned by the Herpetology, Eco-physiology and Ecology Research Group from the Universidad del Tolima. The call from Hato, Guarico (Colombia), was available on the Internet (https://www.youtube.com/watch?v=9_vzpEdtLqY, accessed 30 October 2014). Call recordings were analysed in PRAAT v. 6.0.14.

The largest samples of specimens examined were from Puerto Ayacucho, Venezuela on the Orinoco River, about 580 km southwest of Trinidad, and Camarata, Bolivar,

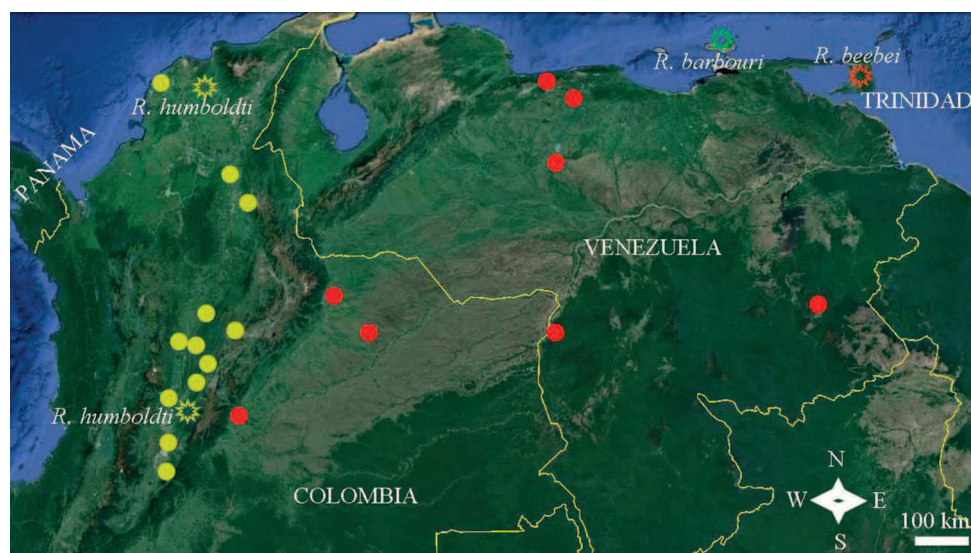


Figure 1. Type localities (coloured wheels) and localities from which members of the *Rhinella granulosa* group were examined. Yellow localities are trans-Andean, red localities are Orinoco-Trinidad.

Venezuela about 480 km south of Trinidad. We also examined smaller numbers of toads from Maracay and Aragua, Venezuela. The Colombian specimens examined were mostly from the west side of the Andes, but also included specimens from Meta on the east side of the Andes.

Results

Molecular results

The three Trinidadian samples differed by 1 bp difference, a transversion (G–A) in the 16S rDNA gene fragments between frogs 1 (UWIZM.2012.27.72.1) and 2 (UWIZM.2012.27.72.2) and frog 3 (UWIZM2012.27.72.3) (A–G). Both eastern Colombian samples were identical with the only exception of one indel insertion. GenBank BLAST searches of the sequences obtained from the Trinidad and east Colombian frogs matched *Rhinella humboldti* for 99%. The 12S rDNA sequence from Trinidad frog 3 matched *R. humboldti* (KP685211 and DQ158434) for 100% and for 99% with all *R. humboldti* with the 16S rDNA gene fragment. The other Trinidad individual was identical to *R. humboldti* from Trinidad (DQ158434) for both gene fragments. The eastern Colombian *R. humboldti* matched for 100% both 12S and 16S rDNA gene fragments of *R. hum-*

boldti (KP685211 and KP149366). Lack of *R. humboldti* 12S rDNA sequences from west of the Andes resulted in a 99% match to its sister taxon, *R. centralis*. The 16S rDNA fraction matched *R. humboldti* for 99% (1 bp difference) and *R. centralis* with 6 and 7 bp differences.

The best-fitting model for the BI tree was the GTR+I+G (-lnL=6655.89206, BIC=13686.875302). All analyses recovered a well-resolved monophyletic clade (BPP: 1) with *R. humboldti* (i.e., *R. beebei*) from Trinidad, Venezuela and Colombia and sister clade to *R. merianae* (BI BPP: 1.00, ML: 78%, Beast BPP: 1.00). More weakly supported in the BI and RAxML analyses but recovered in all three analyses is the grouping of our sequenced frog from west Colombia with another *R. humboldti* from the same side of the Andes, ~ 500 bp of the 16S rDNA, which thus explains the moderate support, except in the Bayesian analyses with a 0.96 BPP. This grouping forms a sister clade to *R. centralis* from Panama, with a 0.91 and 1.00 BPP in the BI and Beast tree respectively and with a 64% ML bootstrap support. The *R. humboldti* (west Andes) + *R. centralis* sister relationship to *R. humboldti* (east Andes) + *R. merianae* is well supported in all analyses (BPP: 0.81, in the BI tree and 1.00 BPP in the Bayesian analyses) (Fig. 2).

Our results suggest an old divergence between *R. humboldti* from each side of the Andes (mean value for the TM-

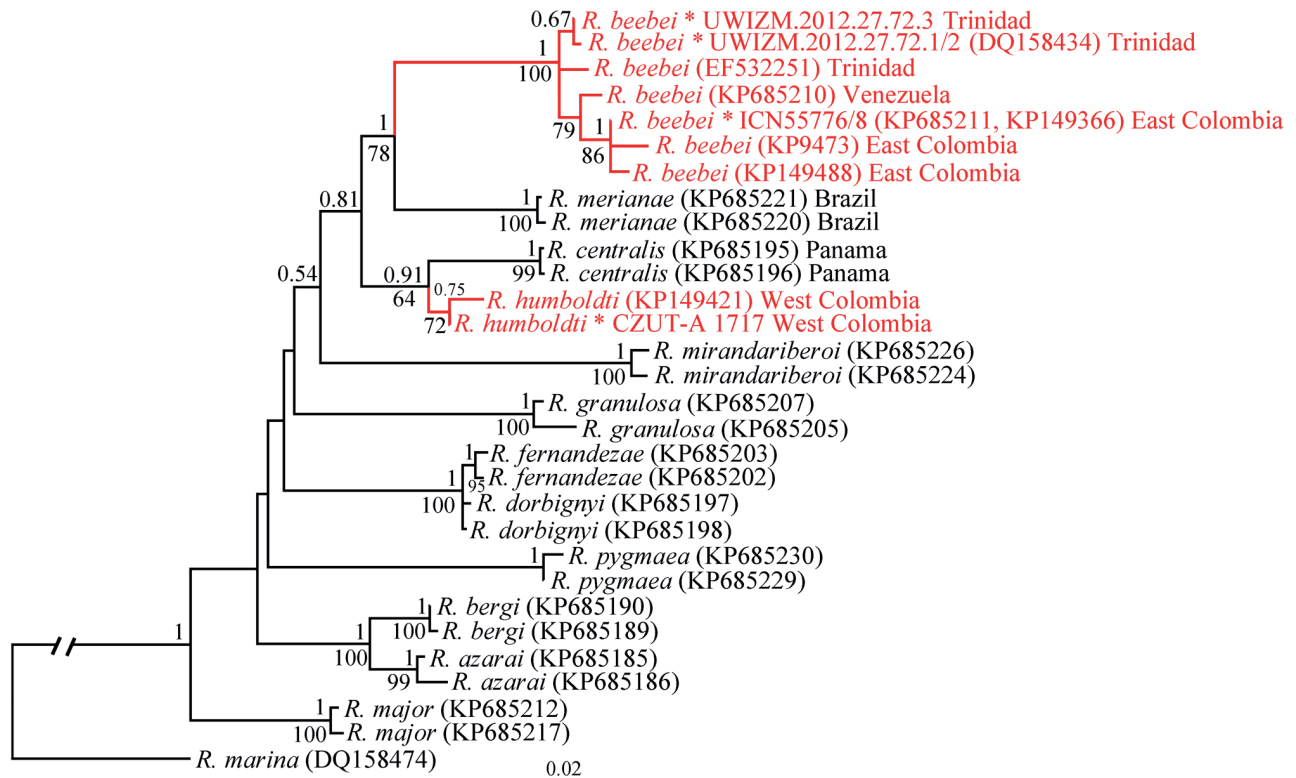


Figure 2. Best ML tree for the 12S and 16S rDNA data set of the *Rhinella granulosa* species group. Taxonomic assignment follows *R. beebei* (east of the Andes) and *R. humboldti* (west of the Andes). Values on and under nodes are posterior probabilities recovered from Bayesian inference analyses and the 50% majority rule consensus tree, respectively. The three specimens with asterisks were sampled and sequenced for this study. *Rhinella beebei* and *R. humboldti* are shown in red.

RCA: 9 million years, with the 95% HPD interval ranging between 7.3 and 11 Mya). However, it is important to keep in mind that these estimates represent divergence times between haplotypes and thus necessarily precede population divergence times (although the magnitude of this offset between haplotype and population divergence is unknown). The topologies resulting from BEAST analyses are fully concordant with those resulting from MrBayes and RAXML analyses, including support for the positioning of *R. humboldti* and *R. beebei* with their likely sister species, *R. merianae* and *R. centralis* (Figs 2 and 3).

Genetic divergences between Trinidad and Venezuela (0.60% ± 0.02) were similar to the differences encountered between eastern Colombia (0.60% ± 0.07) and much higher than within Trinidad localities (0.31% ± 0.14). Within *R. beebei*, the genetic divergence was relatively low (0.5% ± 0.06). Comparison within *R. humboldti* was not possible due to the lack of the 12S rDNA gene fragment (from KP149421, 16S rDNA: 0.21%). The combined 12S and 16S rDNA divergence between *R. beebei* and *R. humboldti* was 1.44% ± 0.06, i.e., slightly higher than that between *R. centralis* and *R. humboldti* (1.23% ± 0.26) and lower than between *R. centralis* and *R. beebei* (2.39% ± 0.21) and between *R. merianae* and *R. beebei* (2.71% ± 0.18). Because of the 3% cut-off divergence estimated for taxonomical species delimitations inferred from the 16S rDNA gene (VIETES et al. 2009), we here report on genetic divergence based only on this gene for comparative reasons: *R. beebei* and *R. humboldti* (1.98% ± 0.06); *R. centralis* and *R. humboldti* (1.0% ±

0.09); *R. centralis* and *R. beebei* (2.4% ± 0.01); and *R. merianae* and *R. beebei* (2.7% ± 0.04).

Morphological and call analysis results

The examination of toads from Trinidad, several locations in Venezuela, and several locations in Colombia suggests multiple morphs to exist within *R. humboldti*. Two distinct morphs at Puerto Ayacucho suggest possibly sympatric species.

Tibia/femur and femur/SVL ratios tested with two sample t-tests assuming equal variance could not distinguish Trinidad and Venezuelan Camarata males ($p > 0.05$). However, the tests readily distinguished the femur/SVL and tibia/femur ratios of the Puerto Ayacucho, the Trinidad-Orinoco Basin populations, and the trans-Andean population ($p < 0.05$) from each other (Fig. 4). These traits and three others were tested with a PCA (details in Appendices 2–4).

Advertisement calls: Figure 5 compares spectrograms and oscillograms for calls from a Trinidad specimen, an Orinoco Basin specimen (Venezuela), and a Trans-Andean Colombian specimen. Frequency information was as follows: Trinidad – mean frequency 3,150 Hz, lowest frequency 2,790 Hz, maximum frequency 3,540; Orinoco Basin – mean frequency 2,815 Hz, lowest frequency 2,400 Hz, maximum frequency 3,350 Hz; Colombia – mean frequency 2,750 Hz, lowest frequency = 2,270 Hz, maximum frequency = 3,380 Hz.

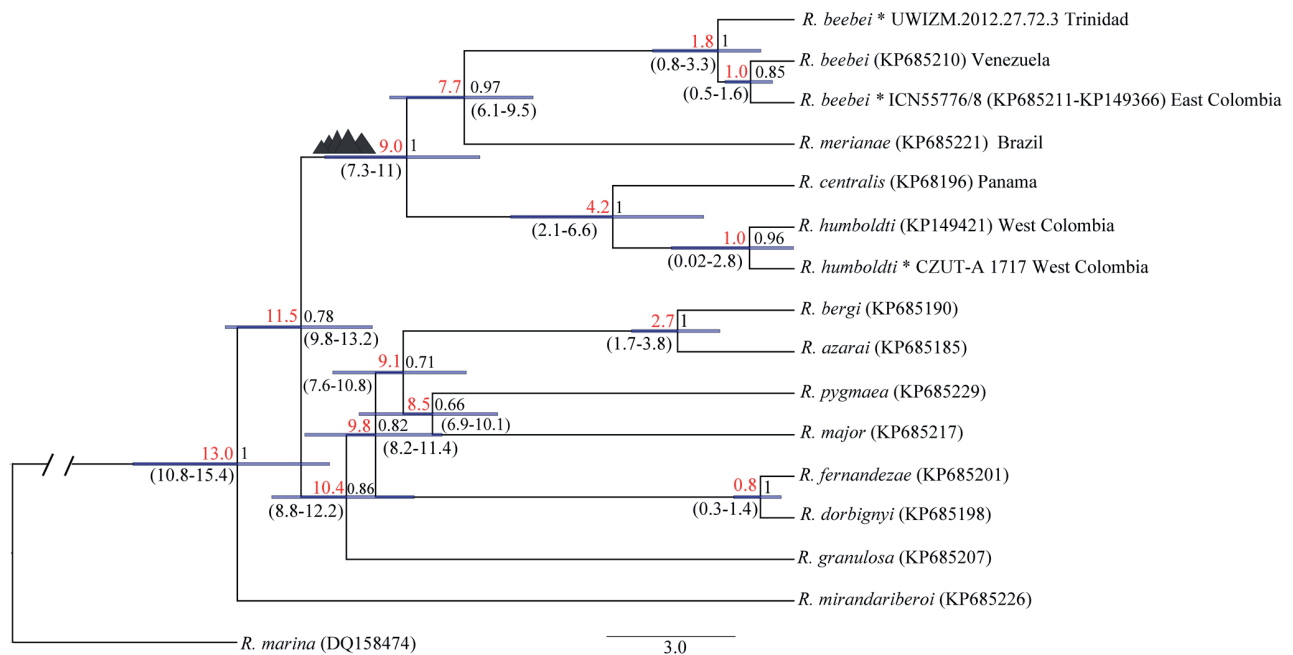


Figure 3. Bayesian inference tree obtained from BEAST of all major *Rhinella granulosus* group lineages. Naming follows *R. beebei* (east of the Andes) and *R. humboldti* (west of the Andes). Values at nodes are posterior probabilities recovered from the analyses; 95% HPD intervals are shown as bars and ranges. Red values are the mean time since the most recent common ancestor (TMRCA, in million years). Species denoted with an asterisk were specimens sampled and sequenced for this study. The drawing in the tree represents the Andes mountain range.

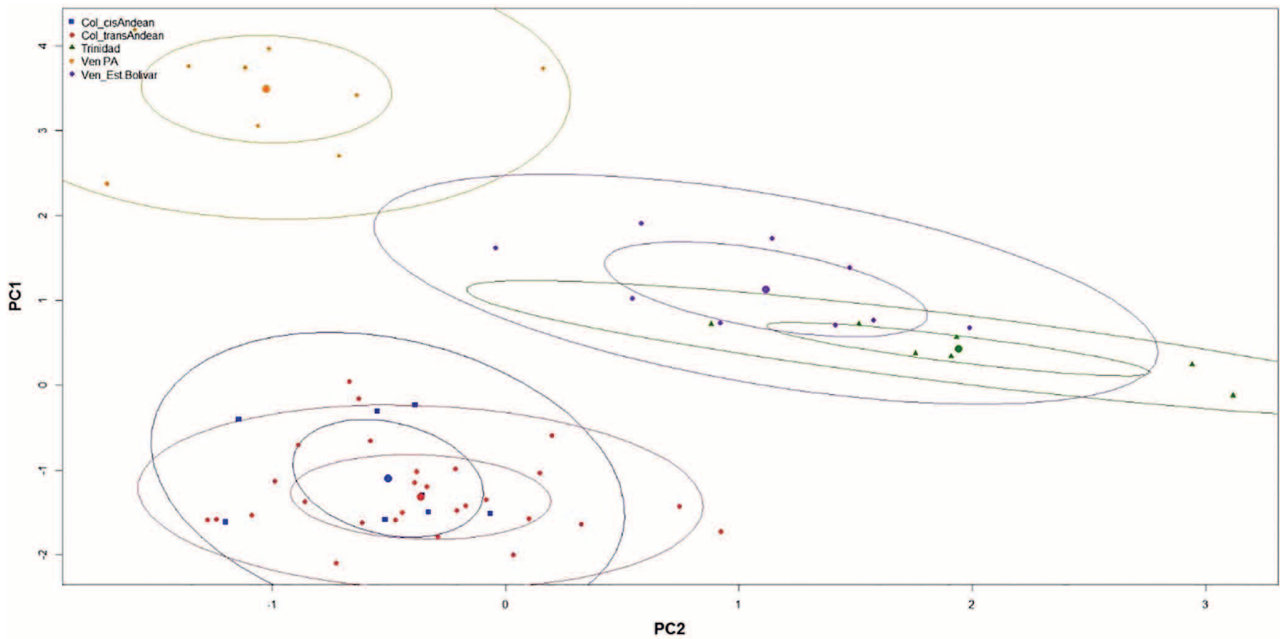


Figure 4. A PCA of ratios calculated from members of the *Rhinella humboldti* group. Three distinct groups: green triangles and purple diamonds – Trinidad, Orinoco Basin, Bolivar Venezuela cluster; orange diamonds – Puerto Ayacucho, Venezuela cluster; and the blue squares and the red diamonds form the cis-Andean/trans-Andean Colombia cluster. Ratios included were femur:tibia, femur:SVL, orbit length:SVL, head width:head length, parotoid length:SVL, foot length:SVL. The eigenvalues are given in Appendix 2.

Calls from Trinidad and the Orinoco Basin (Venezuela) specimens are similar in structure with four pulses per note, with the second and third the strongest, with similar amplitude and three notes per 1/10 of a second. The call from the Tolima population (Colombia) is divergent with only three pulses per note, with the second the strongest, and lower amplitude with four notes per 1/10 of a second.

Discussion

The barriers to understanding speciation created by the polytypic species concept and subspecies was recognized and discussed by CRACRAFT (1983). Genetic divergences of the 16S rDNA gene between *Rhinella beebei* and *R. humboldti* (1.98%) are less than the 3.0% cut-off suggested by VIEITES et al. (2009) to identify species level delimitation. However, those authors further qualify this value with a statement that the uncorrected pairwise genetic divergences in the 16S rRNA gene to all other described species is in most cases 3%, but that it is sometimes only 1–2%. They furthermore state that the lower molecular differentiation can be augmented by a qualitative difference in advertisement calls, or a diagnostic difference in at least one morphological character known to be generally species-specific. Moreover, applying the 3% cut-off threshold would result in the lumping of *R. beebei*, *R. humboldti*, *R. merianae* and *R. centralis* in one species complex. We believe that the assignment of a certain accumulation of genetic divergence to establish taxonomic relationships should be treated with caution, as different taxa and evolutionary processes will affect the accumulation and or loss of genetic diversity differently.

Our small sample size of vocalizations shows the cis-Andean *R. beebei* has four pulses per note, while the trans-Andean *R. humboldti* has three pulses per note. The number of pulses per note in the *R. granulosa* group was found to be species-specific by GUERRA et al. (2011).

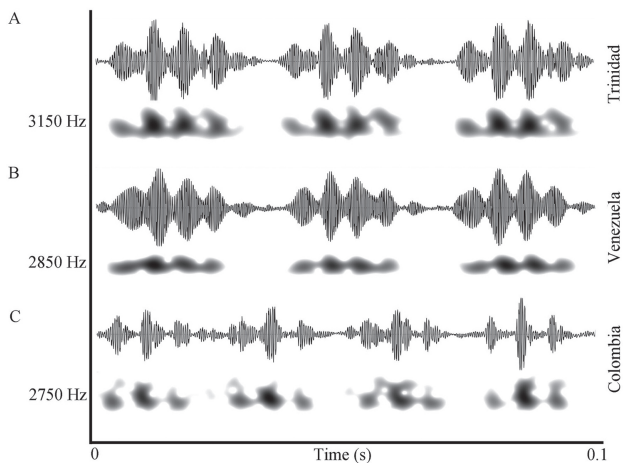


Figure 5. Comparative spectrograms and oscillograms of the advertisement call of *Rhinella humboldti* and *Rhinella beebei* from (A) Trinidad, (B) Guarico, Venezuela, and (C) Tolima, Colombia.

The morphological ratios used in our PCA, as well as such qualitative traits as the beaded ocular crests in *R. beebei* and the smooth ocular crests in *R. humboldti*, are additional morphological evidence (Fig. 6) for the independent evolution of the populations on separate sides of the Andes and concur with the suggestions by VIEITES et al. (2009).

Given the molecular, morphological, and vocal evidence that the trans-Andean population is distinct from the cis-Andean populations, we remove *Bufo granulosa beebei* GALLARDO from the synonymy of *Rhinella humboldti* (GALLARDO), and recognize *Rhinella beebei* as the valid name for the Trinidad-Orinoco Basin member of the *R. granulosa* group. Our findings that two distinct lineages exist on either side of the Andes Mountains fully supports previous work (GUARNIZO et al 2015) on the importance of the Andes mountain range as a key factor in allopatric speciation in *R. humboldti* and other anurans.

Furthermore, GenBank BLAST search results of western Colombian *R. humboldti* matched the only sequenced *R. humboldti* from the west of the Andes, with a 99% affinity and only 1 bp difference. Further evidence arises from the sister clade relationship to *R. centralis* from Panama, the only other species within the *R. granulosa* group found west of the Andes. This finding was also supported by the lower genetic p-distances (12S and 16S rDNA)

from *R. humboldti* (1.23%) than between *R. humboldti* and *R. beebei* (1.44%). The sister relationship between *R. meriana* and *R. beebei*, both from the east side of the Andes, further corroborates the taxonomic distinction between *R. humboldti* and *R. beebei*.

The timing of Andean uplifts and the establishment of a continuous topographic barrier in western South America remain critical to understanding Neotropical biogeography. HORTON (2014) noted that recent advances would allow independent geologic estimates of barrier uplift and river drainage shifts that can be compared with molecular-clock calculations of genetic divergence times for various Andean and Amazonian populations.

Central to this discussion is JANZEN'S (1967) hypothesis that mountain passes are "higher in the tropics." JANZEN'S climatic model predicts tropical mountain passes would be more effective barriers to a species' dispersal than temperate-zone passes at equivalent altitudes. He predicted that tropical lowland organisms were more likely to encounter a mountain pass as a physical barrier to dispersal, which should in turn favour smaller distribution ranges and an increase in species turnover along altitudinal gradients (JANZEN 1967, GHALAMBOR et al. 2006).

Evidence suggesting the palaeoelevation of the Eastern Cordillera was lower in the past was discussed by GREGORY-WODZICKI (2000). She reviewed the quantitative palaeoelevation estimates for the Central and Colombian Andes and suggested the Eastern Cordillera was 35–40% of the modern altitude (2,820 m above sea level) 5–4 Mya, which would correspond to about 1,000 m. Additionally, JAVADI et al. (2011) notes that the Merida Andes were uplifted in two phases. The first phase was in the Late Miocene (7–5 Mya) from the NW–SE convergence of the Panama Arc and western South America. The second phase in the Pliocene/Quaternary (5–1 Mya) resulted from the convergence of the Maracaibo Block and the Guyana Shield. Once these two ranges were established near or at their present altitudes, they formed an effective barrier that prevented lowland amphibians from moving across the mountains. Our approximate mean estimate of 9 Mya as the date of divergence is well before the 5 Mya date for the Eastern Cordillera uplift that would place the altitude at about 846 m, and is near the bottom of the time range for the uplift of the Meridian Andes (7–5 Mya). Divergence between *R. beebei* + *R. meriana* and *R. humboldti* + *R. centralis* dates to the recession of the floodbasin system after the Miocene. The uplift of the Andes in the latter Miocene would have separated both clades. Similarly, a dendrobatid phylogeography (SANTOS et al. 2009) concluded that most extant Amazonian species dispersed into the Amazonian Basin after the Miocene floodbasin receded and immigration occurred on either side of the Andes around the Miocene-Pliocene transition.

The combined barriers of the Eastern Cordillera and the Merida Andes with modern altitudes of 3,000–5,000 m are effective in preventing lowland species from moving east or west across these ranges. ROJAS-MORALES (2012) has provided the example of a forest-dwelling dipsadid snake,

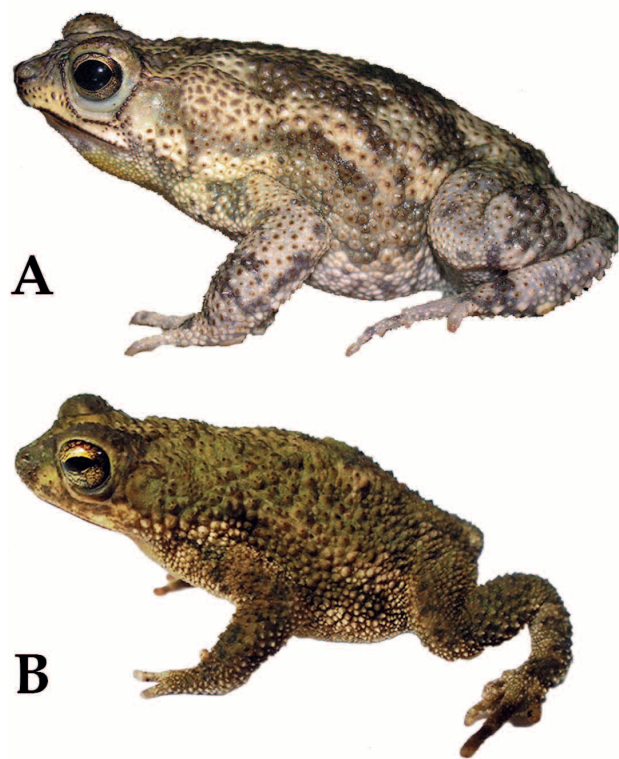


Figure 6. (A) *Rhinella beebei* from Trinidad. Photo by JOANNA M. SMITH; (B) *R. humboldti* from Antioquia, Colombia, municipality of Caucaasia. Photo by GUSTAVO GONZALEZ.

Rhinobothryum, that apparently crossed the Andes from west to east and speciated in the Amazon basin to produce *R. lentiginosum*. In the case of the toads under discussion here, they likely moved west and speciated in the Magdalena Valley (*R. humboldti*) and adjacent Panama (*R. centralis*).

The confusion of *R. humboldti* and *R. beebei* parallels the situation of four other anurans reported to occur in the humid lowlands on both sides of the Andes: *Rhinella margaritifera*, *R. marina*, *Hypsiboas boans* and *Trachycephalus typhonius*. There is genetic and morphological evidence that *R. marina* and *Trachycephalus typhonius* populations on either side of the Andes represent separate species (SLADE & MORITZ 1998, RON & READ 2011). SANTOS et al. (2015) assigned the populations of *R. margaritifera* from western Ecuador and Panama to *R. alata* and demonstrated that the unusual distribution pattern of *R. margaritifera* on both sides of the Andes was an artefact of incorrectly defined species boundaries.

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- (75°20'39.73"N, 5°50'25.32"W) ICN 45215–16; Caldas Norcasia vía a la casa de maquinás, después del Río La Miel. (74°54'22.7"N, 5°32'26.15"W) ICN 40261; Caldas Samaná km 15.6 carretera La Victoria-carretera central (74°56'40.8"N, 5°21'36.27"W) ICN 34711, 34713; Casanare, Paz de Ariporo Vd. La Colombina, Finca El porvenir (6°2'36.5"N, 71°5'34.2"W) ICN 55776, ICN 55777, ICN 55779–80; Trinidad, Vd. La Cañada, Finca La Palmita (5°19'11.4"N, 71°20'51"W); ICN 55784; ICN 55781 (5°19'11.4"N, 71°20'51"W); ICN 55776 Paz de Ariporo Vd. La Colombina, Finca El Porvenir (6°2'36.5"N, 71°5'34.2"W); ICN 55777–80. Cesar La Gloria Ciénaga de Morales, población Las Puntas (73°45'2"N, 8°32'42"W) ICN 37312; Huila, Campoalegre 75°19'32"N, 2°41'W) ICN 9367; Villavieja (75°12'56.76"N, 3°12'53.11"W) ICN 11819, 11821; Meta, San Martín Vereda La Castañeda, Cultivo de Palma Palma Sol S.A (3°31'45"N, 73°32'30"W) ICN 55782–3; ICN 55785 Cumaral, Hacienda La Cabaña (4°18'N, 73°21'W); ICN 55786 Villavicencio. Vd. Santa María baja (4°13'N, 73°38'W); Tolima Honda Hacienda Tupinamba (74°47'30.75"N, 5°8'15.16"W) ICN 43838; ICN 43948; Venadillo Finca Paloballo, km. 40 vía Alvarado-Venadillo (74°56'17.8"N, 4°36'50.1"W) ICN 43168–69; Venadillo 4 km al S de Venadillo (74°56'6.77"N, 4°41'40.4"W) ICN 52093, 52098–99; Tolima (75°9'16.34"N, 4°5'33.6"W) ICN 17636; Mariquita, (~5°14'N, 74°55'W) FMNH 81833–34, Tolima no specific locality FMNH 54182, ICN 3848, 17636, 43168–69, 43938, 52093, 52098–99; Trinidad: BMNH 22, 116, 546–47, 1547–48; FMNH 218784, 251214.
- Venezuela: Aragua (~10°07'N, 67°58'W) FMNH 69780, 176430; Estado Bolívar, Camarata, (~5°38'N, 62°18'W) BMNH 149–151, 154–155, 157–159; Maracay (10°14'N, 67°35'W) FMNH 125405, 174359; FMNH Puerto Ayacucho (~27°58'N, 67°35'W) FMNH 175307–088, 175311, 175313–14, 175319, 175322, 175325, 175327, 175338, 175342.

Appendix 2

Results of the two sample t-tests assuming equal variances

(A) Comparison of tibia/femur ratios from four populations of *Rhinella beebei/humboldti*, alpha set at 0.05; (B) Comparison of femur/SVL ratios from four populations of *Rhinella beebei/humboldti*, alpha set at 0.05. Bold typeface indicates null hypothesis rejected.

| A | Llanos, Venezuela | Bolivar, Venezuela | Tolima, Colombia |
|----------|----------------------|--------------------|----------------------|
| Trinidad | p=0.00, df=13 | p=0.098, df=14 | p=0.00, df=13 |
| llanos | | p=0.00, df=16 | p=0.00, df=15 |
| Bolivar | | | p=0.00, df=15 |
| B | Llanos, Venezuela | Bolivar, Venezuela | Tolima, Colombia |
| Trinidad | p=0.00, df=14 | p=0.67, df=14 | p=0.114, df=13 |
| llanos | | p=0.00, df=16 | p=0.00, df=14 |
| Bolivar | | | p=0.00, df=15 |

Appendix 1
Specimens examined

Colombia: Bolívar, Santa Rosa (10°27'N, 75°20'W) ICN 2167, ICN 2208; Boyacá, Puerto Boyacá Inspección, Puerto Romero, vereda La Fiebre, finca La Barrilera (74°20'29.47"N, 5°50'13.12"W) ICN 38501, ICN 38504, ICN 38509; Vereda el Ocal, La Fiebre, Km. 29–30 carretera a Otanche (75°20'39.73"N, 5°50'25.32"W) ICN 38511; Puerto Boyacá, Inspección Puerto Romero, vereda a Fiebre, finca La Barrilera (74°20'29.47"N, 5°50'13.12"W) ICN 44707; Quebrada La Cristalina, Km. 38–39 carretera a Otanche (74°19'16.87"N, 5°49'40.7"W) ICN 45208–10; Campamento Technint. (74°29'33.9"N, 5°57'17.32"W) ICN 45211; Boyacá Puerto Boyacá Vereda el Ocal, La Fiebre, Km. 29–30 carretera a Otanche

Appendix 3

The communalities and eigenvalues from the PCA

Ratios derived of measurements included femur:tibia (*f/t*), femur:svl (*f/SVL*), orbit length:SVL *ol/SVL*, head width:head length (*hw/hl*), parotoid length:SVL (*pl/SVL*), foot length:SVL (*ft/SVL*).

| | ratios | PC-1 | PC-2 | PC-3 | PC-4 | PC-5 | PC-6 |
|---|---------------|---------|---------|----------|---------|---------|---------|
| 1 | <i>f/t</i> | 0.5190 | 0.1867 | 0.07682 | 0.2052 | 0.5145 | 0.6189 |
| 2 | <i>f/SVL</i> | 0.5051 | 0.1328 | 0.3075 | 0.3123 | 0.1364 | -0.7188 |
| 3 | <i>ol/SVL</i> | -0.4350 | 0.1240 | 0.09886 | 0.8717 | -0.1099 | 0.1174 |
| 4 | <i>hw/hh</i> | 0.1301 | 0.8580 | -0.09063 | -0.1157 | -0.4693 | 0.07183 |
| 5 | <i>pl/SVL</i> | -0.2675 | 0.1199 | 0.9099 | -0.2632 | 0.05241 | 0.1190 |
| 6 | <i>ft/SVL</i> | 0.4448 | -0.4262 | 0.2317 | 0.1338 | -0.6940 | 0.2594 |
| | eigenvalues | 3.160 | 1.208 | 0.8709 | 0.4893 | 0.1969 | 0.07556 |

Appendix 4

Percentages of variation for each PC

Numbers correspond to those in Appendix 3.

| PC | % variation | total variation |
|----|-------------|-----------------|
| 1 | 53.42 | 53.42 |
| 2 | 20.3 | 73.73 |
| 3 | 14 | 87.73 |
| 4 | 8.22 | 95.95 |
| 5 | 3.31 | 99.26 |
| 6 | 0.74 | 100 |