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The cane or marine toad, *Rhinella marina* (Anura, Bufonidae): two genetically and morphologically distinct species

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Abstract

Rhinella marina is a Neotropical toad that has been introduced widely worldwide. Its toxic effects to frog-eating predators threaten the native and domestic fauna of some regions where it has been introduced. Despite previous studies suggesting two genetically distinct cryptic species within *R. marina*, one east and one west of the Andes, its taxonomic status remained unresolved due to the absence of morphological complementary evidence. For the first time, data from two mitochondrial genes (ND3 and CR) and 23 morphometric landmarks are combined to evaluate the taxonomic status of this species. Our results support the hypothesis of two separate evolutionary lineages within *R. marina* and demonstrate that these lineages have significantly diverged in skull shape. We identified two distinct morphotypes, one eastern and one Andean western, with no overlapping morphospaces. The geographic pattern of genetic variation was consistent with a stable structured population with no evidence of recent demographic or geographic expansions. The concordance between the observed geographic patterns in morphometric and genic traits calls for the recognition of two species under *R. marina* name.

Key words: biological control, mitochondrial DNA, morphometric geometry, phylogeny, taxonomy, Venezuela

Resumen

Rhinella marina es un sapo neotropical que se ha esparcido globalmente. Los efectos tóxicos que produce la ingesta de sus adultos en sus depredadores han convertido a esta especie en una amenaza para la fauna silvestre y para los animales domésticos en algunas regiones en donde esta especie ha sido introducida. A pesar de que estudios genéticos sugieren que *R. marina* incluye al menos dos especies crípticas, una al este y la otra al oeste de los Andes, su estatus taxonómico sigue sin resolverse debido a la ausencia de evidencia morfológica que complemente a la genética. Con base en el análisis de dos genes mitocondriales (ND3 y CR) y de 23 hitos morfométricos evaluamos el estatus taxonómico de poblaciones de *R. marina*. Nuestros resultados apoyan la hipótesis de la existencia de dos linajes evolutivamente independientes en *R. marina* y demuestran que ambos linajes han divergido significativamente en la forma del cráneo. Identificamos dos morfotipos, uno al este y otro al oeste de la cordillera Andina, cuyos morfoespacios no se solapan. Encontramos una estructura geográfica de la variación genética concordante con la de dos poblaciones estables sin evidencias de expansiones demográficas recientes. La coincidencia en la variación geográfica entre las características morfométricas y genéticas de estas poblaciones abogan por el reconocimiento de dos especies de *R. marina.*

Introduction

The marine or cane toad, *Rhinella marina* (formerly *Bufo marinus*) (Linnaeus), is one of the most cosmopolitan amphibian species of the world. Although its natural range extends from South Texas to Central Amazonia, cane

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toads have also established populations in central United States, Australia, Japan, Philippines, Taiwan, Papua New Guinea and several islands across the Caribbean and the Pacific, as a result of translocations (Easteal 1981, 1985; Lever 2001). Due to the toxicity of adult frogs to predators and their potential for large-scale impact on many wild and domestic animals, cane toads are recognized by the International Union for the Conservation of Nature (IUCN) and the Invasive Species Group (ISG) as one of the 100 worst invasive species of the world (Global Invasive Species Database 2005).

Despite being among the most studied amphibian species (*e.g.* regarding its physiology and ecology) (Shine 2010), the taxonomic identity of populations under *R. marina* name remains unresolved. Its widespread natural distribution has suggested a complex of cryptic species, but the delimitation of these species has been difficult. The thirty-one synonyms proposed along its taxonomic history (Frost 2015) has evidenced the taxonomic complexity of this taxon. Morphological traits can be uninformative in recognizing species boundaries (*i.e.* cryptic species) if i) species have evolved under a strong stabilizing selection that reduces morphological variations or ii) mate recognition relies on non-morphological characteristics (*e.g*. vocalizations) as most often occurs in anurans (Bickford *et al.* 2007; Borkin *et al.* 2004; Stuart *et al.* 2006). Among anurans, the genus *Rhinella* has been considered as morphologically conserved (Graybeal 1997; Pramuk 2006). Although unique unreversed synapomorphies can be used to diagnose some South American species groups of toads (*Rhinella margaritifera*, *Rhinella marina*, *Rhinella granulosa*, *Rhaebo guttatus* and *Incilius valliceps*), the paucity of variation in osteological characters have challenged taxonomist attempting to separate some species within these groups (Pramuk 2006).

Few genetic studies have shown evidence of cryptic species within *R. marina*. Based on sequence analyses of the *ND3* gene and the flanking *tRNA* genes, Slade & Moritz (1998) detected a phylogenetic break within *R. marina* separating populations east and west of the Venezuelan Andes. Additional evidence based on the 12S rRNA, 16S rRNA and cytochrome *b* (Cytb) mitochondrial genes summed to the increasing evidence of two paraphyletic groups of *R. marina*, one from Central America and Ecuador (west of the Andes) and those from the Amazon Basin (east of the Andes) (Vallinoto *et al.* 2010). However, the lack of complementary evidence on nuclear genes or morphological diagnostic characters have postponed the reevaluation of the taxon because mitochondrial phylogenies may not always reflect the species phylogeny (Avise *et al.* 1983). For example, conflicting signals between the mitochondrial and nuclear-based phylogenies of *R. marina* populations across the Amazon river have been associated with recent hybridization with closely related species (Sequeira *et al.* 2011).

The development of geometric morphometry in the last few decades has substantially improved the statistical power for distinguishing shapes, offering new avenues for the study of systematics of organisms using morphology (Adams *et al.* 2004; Adams *et al.* 2013; Rohlf & Marcus 1993). Based on the use of landmarks, these methods overcome some of the limitations of the classic morphometry based on linear distances, *e.g.* the high correlation existing between linear measurements and size, by removing the non-shape variation of scale, position and orientation of the landmark configuration (Kendall 1977). Despite its widespread use in the analysis of complex cranial morphologies, a feature commonly used for studying the systematics of bufonids (Pramuk 2006), landmark-based geometric morphometry has not been widely used in taxonomy of anurans, although it has proved useful in complementing molecular evidence for phylogenetic inferences (Kaliontzopoulou 2011)

Using a fine-scale sampling and combining evidence from two mitochondrial genetic markers and from the shape of cranial structures, we reevaluate the taxonomic status of populations of *R. marina* east and west of the Andean cordillera in Venezuela.

Materials and methods

Ethics statement. All animal procedures were conducted in accordance with international guidelines. Animal transport followed guidelines for transport of live animals by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and euthanasia was conducted according to the recommendations of the American Association for Veterinary Medicine (AMVA). The study was approved by the Comisión Interministerial para el Acceso a Recursos Genéticos del Ministerio del Poder Popular para el Ambiente (Venezuela), a governmental office that grants access to genetic resources and evaluates bioethic issues related with the use of wildlife. The study did not involve protected or endangered species. *Rhinella marina* is currently listed by the International Union for the Conservation of Nature (IUCN) in the *Least Concern* category.

TABLE 1. Locality, geographic coordinates, region, source, GenBank accession numbers of mitochondrial haplotypes, and sample size used in this study.

Locality	Geographic coordinate	Region	Source	GenBank Accession Numbers (Number of samples)	
				ND3	CR
VE1	8.500828 N 71.392431 W	West	This study	KP704669–KP704677 (9)	KP995007–KP995014 (8)
VE2	8.612631 N 71.662886 W	West	This study	KP704678–KP704684 (7)	KP995015–KP995020 (6)
VE3	8.62375 N 71.650028 W	West	This study	KP704685–KP704691 (7)	KP995021–KP995026 (6)
VE4	8.566028 N 71.367583 W	West	This study	KP704692–KP704693 (2)	KP995027–KP995028 (2)
VE5	11.866508 N 70.031294 W	West	This study	KP704694–KP704696 (3)	KP995029–KP995030 (2)
VE6	7.8655 N 72.4321 W	Southern depression	This study	KP704697–KP704698 (2)	KP995031–KP995032 (2)
VE7	10.575506 N 64.228825 W	East	This study	KP704699–KP704670 (2)	KP995033–KP995034 (2)
VE8	10.3775 N 68.7725 W	East	This study	KP979778 (1)	KP995035 (1)
VE9	7.5287 N 68.236783 W	East	This study	KP979779–KP979781;KP704701–KP704713 (16)	KP995036–KP995042 (7)
VE10	7.835372 N 68.917461 W	East	This study	KP704714–KP704723 (10)	KP995043–KP995053 (11)
VE11	8.930939 N, 68.044225 W	East	This study	KP704724 (1)	KP9955054 (1)
VE12	10.015892 N 69.677606 W	Northern depression	This study	KP704725–KP704734 (10)	KP995055–KP995062 (8)
VE13	Lake Maracaibo	West	(Slade & Moritz 1998)	(2)	
VE14	Higuerote	East	(Slade & Moritz 1998)	(2)	
VE15	Güiria	East	(Slade & Moritz 1998)	(1)	
Texas, USA		West	(Slade & Moritz 1998)	(1)	–
Mexico		West	(Slade & Moritz 1998)	(5)	–
Costa Rica		West	(Slade & Moritz 1998)	(3)	
Panama		West	(Slade & Moritz 1998)	(1)	–
French Guyana		East	(Slade & Moritz 1998)	(2)	–
Peru		East	(Slade & Moritz 1998)	(1)	–

Sample collection. We selected 14 localities around the Andean cordillera; six on the east side, six on the west side, one on the northern depression and one on the southern depressions (Figure 1). Geographic coordinates for all localities are given in Table 1. Seven to ten adults were collected at each locality during 2010, totalizing 80 specimens. All specimens were collected under permit No. 394 issued on January 2010 to Margarita Lampo by the Venezuelan Ministry for the Environment. Toads were transported alive to the laboratory in plastic containers with water and ventilation holes. In the laboratory, they were euthanized using intradermal benzocaine injection (182mg/kg) (Birchard 2002). From each specimen, 50g of muscle tissue were cut from the inguinal area and stored in 95% ethanol. The specimens were fixed in formalin and stored in 70% ethanol for subsequent morphometric analyses. All voucher specimens were deposited at Museo de Historia Natural La Salle (MHNLS) in Caracas.

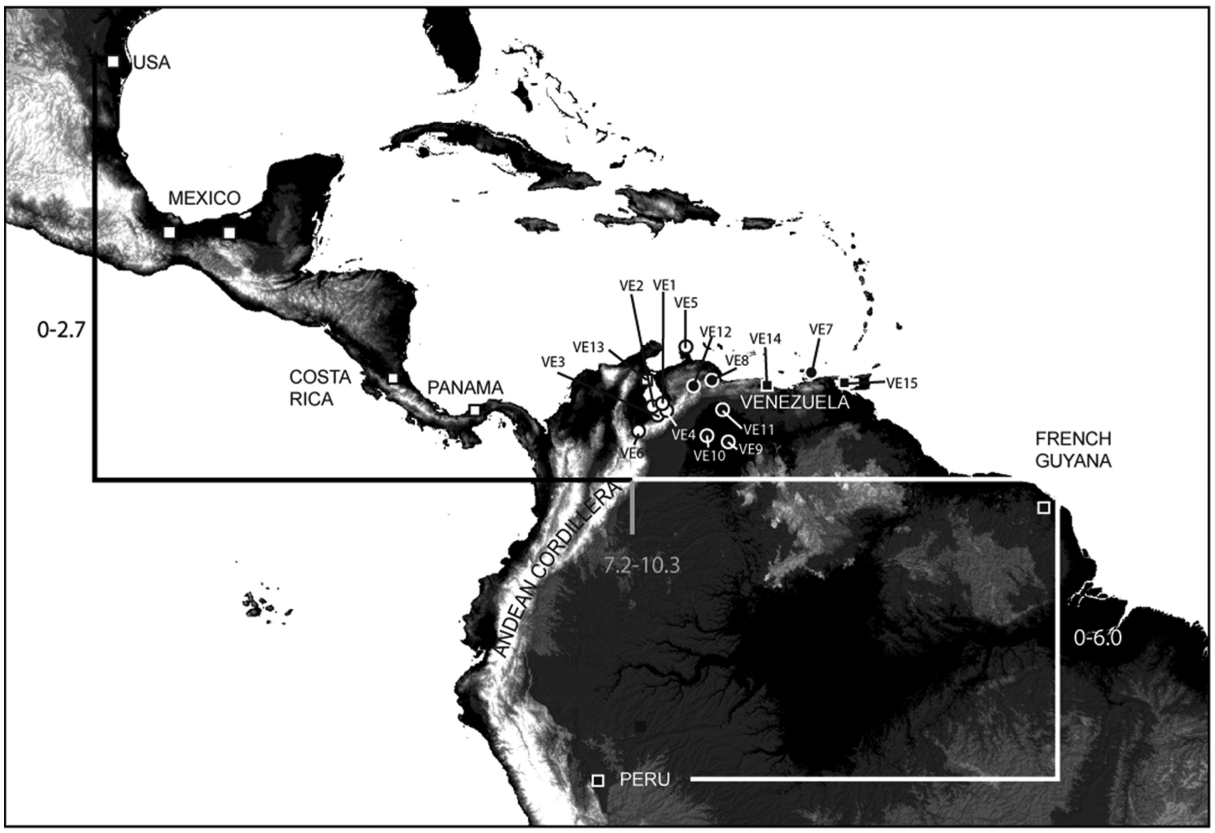

FIGURE 1. Geographic distribution of *Rhinella marina* populations sampled for two mitochondrial markers and morphometric landmarks across the Andean cordillera in northern Venezuela. The circles indicate locations of specimens sequenced in this study and the squares indicate locations associated to previously published sequences used in this study. Black circles and squares correspond to eastern populations and white circles and squares to western populations. The geographic coordinates of localities are given in Table 1. The genetic distances between western and eastern populations (grey) are greater than between western populations (black) or between eastern populations (white) (see text for details).

DNA extraction and PCR amplification. DNA was extracted from tissue samples using phenol-chloroform with ethanol precipitation (Sambrook & Russel 2001) and stored at -20ºC. We selected DNA fragments for the subunit III of NADH dehydrogenase (ND3) and the Control Region (CR) using conventional PCR assays. We used primers ND3.1, ND3.2, MVZ25-L (Moritz *et al.* 1992, Slade & Moritz 1998), Cyt*b*A-L and ControlP-H (Goebel *et al.* 1999).

Polymerase chain reaction for ND3 and CR amplification were carried out in a final volume of 25 µL volumes, each containing 2µL of DNA, 0.2µL of *Taq* polymerase, 1.25 µL $MgCl_2$ 50mM, a total of 0.5µL of dATP+dGTP+dTTP+dCTP, 0.25 µL of each primer, 2.5µL 10x buffer and distilled water to 18.05µL. Amplifications were performed in a MJ Research thermocycler programmed as follows. Initial denaturation cycle at 95º C (30 sec), annealing at 50º C (1 min) and extension at 72º C (2 min) for 41 cycles. Amplification products

were separated by gel electrophoresis on 2% agarose gels in TAE buffers and PCR products were visualized after staining with ethidium bromide (Sambrook & Russel 2001). Prior to sequencing, PCR products were purified using the commercial kit Concert GIBCO®. Sequences were resolved using an automated DNA sequencer (ABI3130XL) by the Unit for Genetic and Forensic Studies at Instituto Venezolano de Investigaciones Científicas (IVIC).

Genetic divergence. Alignment of sequences was performed with the ClustalW algorithm using no gap penalty implemented in Bioedit v.7.0.5.1, and adjusted manually. The number of segregating sites (*S*), haplotipic diversity (*Hd*), and nucleotide diversity (*p*) were estimated using the program DnaSP v.5.10.01 (Librado & Rozas 2009) for the concatenated ND3+CR fragment. Tajima´s *D* (Tajima 1989) and Fu´s *F* tests (Fu 1997) were used to examine the selective neutrality of mitochondrial fragments and detect departures from the mutation-drift equilibrium due to of recent demographic expansions. Significance of these indices was assessed using confidence limits generated under a coalescent model assuming constant population size and the number of segregating sites (Rozas *et al.* 2003). A hierarchical analysis of molecular variance (AMOVA) of the concatenated (ND3+CR) fragment was also performed using Arlequin 3.5 to partition the genetic differentiation attributable to differences between regions (eastern vs. western) (F_{CT}), to differences among localities within regions (F_{SC}), and to variance among localities in both regions (F_{ST}). We also examined the percent genetic distances among populations for ND3, including sequences previously published (Slade & Moritz 1998), using the k2p model implemented in program MEGA v.5.0.

Phylogenetic and haplotype network reconstructions. Saturation indices were estimated using DAMBE 2.2.13 in order to assess whether particular positions or classes of substitutions needed to be weighted or excluded prior to the phylogenetic analyses. Phylogenetic analyses were conducted for the separated ND3 and CR markers using maximum parsimony (MP) and maximum likelihood (ML) algorithms in PAUP* v.4.0b10 and the Bayesian analysis in MrBayes v.3.1. Trees were rooted on *Rhinella granulosa* for ND3 and on *Rhinella rubescens* and *Rhinella crucifer* for CR. The MP analyses were performed after excluding non-informative characters using heuristic searches with TBR branch swapping and 1000 random addition sequence replicates (Swofford & Olsen 1990). Support for each branch was assessed for the MP, ML and BI analyses by bootstrap proportions based on 1000 pseudoreplicates. We used the Akaike Information Criterion (AIC) and the Bayesian inference criterion (BIC) implemented in software Jmodeltest v.0.1.1(Posada 2008) to choose the substitution model that best fit our data. Each Bayesian analyses consisted of 5 x 10^6 generations with a random starting tree and four Markov chains sampled every 1,000 generations. Median-joining networks were reconstructed for the combined data sets (ND3 + CR) by program Network v.4.6 (Bandelt *et al.* 1999).

Geometric morphometry. Landmark configuration. Morphometric analyses were based on digital mammogram images (Mamoray Agfa HT300) of preserved carcasses of 65 males with snout-vent lengths between 15 and 18 cm. All specimens were exposed to 30 kilovolts/7 milliamps for 0.8 seconds at a focal distance of 105mm. A 5x5 cm plastic underlying mesh was used as a reference scale. We selected 23 landmarks on anatomical homolog structures of the cranial area of all specimens (Figure 2). These were informative sites frequently used in amphibian taxonomy (Duellman & Trueb 1994; Zelditch *et al.* 2004) (Table 2).

Shape variation analysis. We performed a generalized Procrustes analysis (GPA) to separate size and shape components of form variation (Bookstein 1991). Shape coordinates were computed by standardizing each configuration to a unit centroid size and by minimizing differences in translation and rotation of all specimens using a least-square algorithm implemented in tpsDig software (v.2.12). To plot the data on a Euclidean space the resulting coordinates were projected orthogonally onto a plane tangential to the shape space using the software tpsRelwarp v.1.46 (Rohlf 2008). A consensus shape configuration for each group (east and west) was calculated by averaging the coordinates for each group, and these were used to derive a series of Principal Warps (PW). We initially used unweighed PWs (α=0), and then assigned a greater weight to distant PWs (α=1) (Rohlf & Marcus 1993) to explore shape changes at larger scales as suggested by (Bookstein 1989). A matrix of Partial Warps scores was generated by projecting each specimen configuration onto the Principal Warps.

A principal component analysis and a canonical variate analysis implemented in MorphoJ (v. 1.02) were performed on the matrix of Partial Warp scores, to obtain the RelativeWarps (RW) and their associated scores, and to test the hypothesis of shape differences between groups (east vs. west) (Klingenberg 2011). Percent misclassifications were estimated based on the identification function. Each group was plotted onto the RW axes, and thin-plate spline (TPS) deformation grids were obtained using tpsRelwarp v1.46 (Bookstein 1991; Zelditch *et al.* 2004).

TABLE 2. Anatomical landmarks used for the geometric morphometry analysis of skulls of adult males of *Rhinella marina* from northern Venezuela.

Landmark		Anatomical structure
1	Left	Maximum point in squamosal bone
2	Left	Nasal-maxilla posterior joint
3	Left	Maxilla-quadratojugal joint
4	Left	Pre maxilla
5	Left	Nasal-maxilla anterior joint
6	Right	Nasal-maxilla anterior joint
7	Right	Pre maxilla
8	Right	Maxilla-quadratojugal joint
9	Right	Nasal-maxilla posterior joint
10	Right	Maximum point in squamosal bone
11	Left	Nasal bone-anterior rim of eye joint
12	Right	Nasal bone-anterior rim of eye joint
13	Left	Superior edge of postorbital crest
14	Left	Occiptal condyle
15	Right	Occipital condyle
16	Right	Superior edge of postorbital crest
17	Left	Squamosal-occipital suture
18	Left	Frontoparietal-squamosal suture
19	Left	Nasal-frontoparietal junction at the orbit
20	Central	Nasal-frontoparietal junction along midline
21	Right	Nasal-frontoparietal junction at the orbit
22	Right	Frontoparietal-squamosal suture
23	Right	Squamosal-occipital suture

Results

Genetic divergence and phylogenetic relationships. We obtained 70 sequences of 429 bp for ND3 and 56 sequences for CR, although amplification fragments for CR differed significantly in their lengths between eastern (707 bp) and western (737 bp) samples due to the presence of gaps. Geographic coordinates of collection sites, GenBank accession numbers for sequences obtained in this study and the source for previously published sequences are given in Table 1.

Saturation indices for the two markers (0.1862 for ND3 and 0.3267 for CR) were significantly lower than the critical values ($p<0.001$). For the concatenated fragment, haplotype diversity was high while nucleotide diversity was low (Table 3). Non-significant Tajima's *D* and Fu's *F* indicated no evidence of an excess of low-frequency haplotypes in any of the lineages that could suggest a recent demographic expansion. The AMOVA attributed the largest variance component to differences between regions (eastern vs. western) (97.54%). Variations among populations within regions and within populations only accounted for a small fraction of the variance component (1.05 and 1.42%, respectively) (Table 4). The k2p genetic distances estimates indicated large percent divergences of 7.2—10.3 between regions compared to 0–6 within regions (Figure 1). Uncorrected p-distances were slightly lower (6.3–8.7 between regions and 0–5.6 within regions) but showed similar variation pattern. The haplotype network for the combined markers (ND3+CR) depicted a clear separation between two haplo-groups, eastern and western, with no common haplotypes between these groups (Figure 3). Haplotypes from both depressions were also unique, and grouped with different haplo-groups. The northern depression haplotypes (Hap21 and Hap22) grouped with the eastern haplo-group (<1% divergence), while those from the southern depression (Hap7) grouped

with the western haplo-group (<1% divergence). In the one hand, we could not identify a contact zone where both haplotypes were present. On the other hand, we found no evidence of star-like structures with frequent haplotypes connected to low frequency haplotypes that could suggest recent demographic expansions.

TABLE 3. Sample size (*N*), number of segregating sites (*S*), haplotype diversity (*Hd*), nucleotide diversity (*p*), Tajima's (*D*), Fu's (*F)* and raggedness index (*r*) for eastern and western lineages of *Rhinella marina* from Venezuela based on the concatenated ND3 +CR datasets.

Lineage	N	S	Hd	π	D	F
Eastern	30	36	0.926	0.00805	-0.0989	-0.518
Western	26	12	0.927	0.00270	-0.0491	-0.539

*p<0.05

TABLE 4. Analysis of Molecular Variance (AMOVA) based on 821 informative sites for the concatenated ND3+CR mitochondrial markers of *Rhinella marina* collected east and west of the Venezuelan Andes.

Source of variation	df	Variance components	Percent variation	F_{ST}
Between regions	1	143.366	97.54	0.975**
Among populations within regions	9	1.536	1.05	0.425**
Within populations	45	2.082	1.42	0.985**
Total	55		100.0	

***p*<0.05

The ML and BI phylogenetic reconstructions produced similar topologies: a phylogenetic break within *R. marina* separating populations into two major clades, an eastern and a western clade. The ND3 and CR optimal trees were congruent in terms of the split of the eastern and western clades (Figure 4). The ML analysis for the CR fragment yield six trees with a score of 77 steps, consistency and rescaled consistency indices of 0.8617 and 0.8584 respectively, and high support bootstrap values for both clades (Figure 4a). For the ND3, the analysis produced nine trees with a score of 94 steps, consistency and rescaled consistency indices of 0.8731 and 0.8420 respectively, although bootstrap values were lower than for CR. The inclusion of previously published ND3 sequences from Central America and other South American countries resulted in the grouping of samples from Panama, Costa Rica, Mexico and United States with the Venezuelan western clade and samples from Peru and the French Guyana with the eastern clade (Figure 4b).

Morphological divergence. The first two PCA axes summarized 74 per cent of the total amount of variation in the data set. Thus, between-group variation described from here refers to the morphospaces delimited by these two axes. The resulting polygons defining the morphospaces of the groups showed no overlap (Figure 5b). The first two CVA axes produced an unequivocal separation of the eastern and western groups (λ= 0.1372, χ^2=79.4456, *df* =42, *p*=0.00042) with no misclassifications. The thin-plate splines grids showed deformations for each group in opposite directions mainly around the pre-maxillar and nasal bones (landmarks 4, 5, 6, 7, 11, 12, 19, 20, 21) and the occipital regions (landmarks 13, 14, 15, 16, 17, 18, 22, 23) (Figure 5a). These landmarks allow for the unequivocal separation of morphotypes (*i.e.* no misclassifications) and can be used as diagnostic characters. Landmarks 5 and 6 on the anterior joint of the nasal bone with the upper maxilla, 2 and 9 on the posterior joints between the nasal bone and maxilla, and 14 and 15 on the occipital condyles showed the largest contributions to the observed shape changes. In western populations, a forward displacement of the occipital and the pre-maxilla regions results in a shortening of the occipital region. The longitudinal distance between the maxilla-quadratojugal joints (landmark 3 and 8) and the occipital condyles (landmarks 14 and 15) is significantly shorter in western populations compared with eastern populations (Student-*t*=5.187, *df*=51.6, *p*=0.0000036).

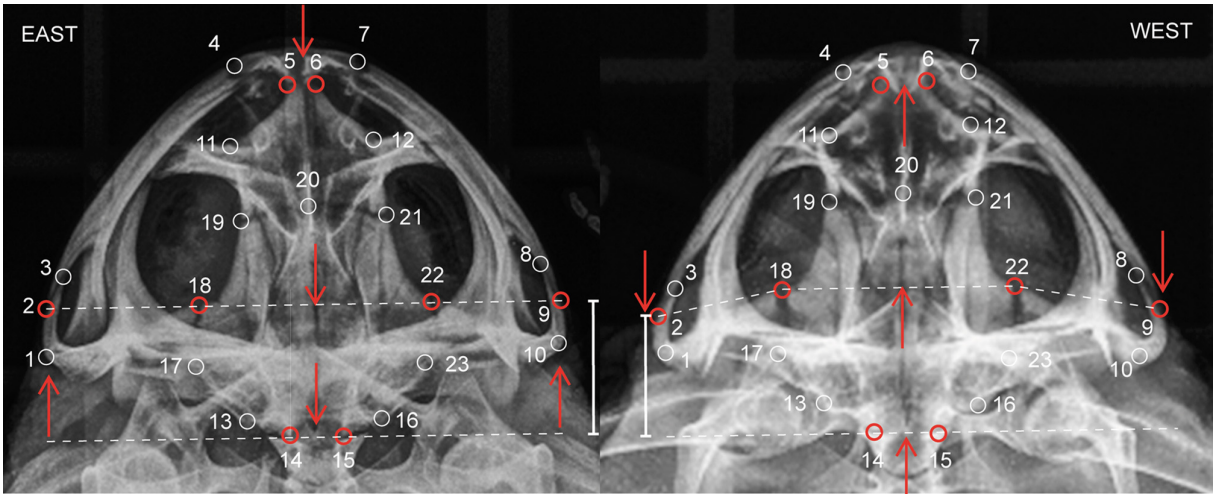

FIGURE 2. Dorsal x-ray images of *Rhinella marina* skulls from one western and one eastern population. Circles show the anatomical landmarks described in Table 2. The red circles correspond to landmarks showing the greatest contributions to the shape differences between eastern and western populations. The red arrows indicate the direction of displacement of landmarks. The white vertical bars indicate differences in measurements in externally visible landmarks between eastern and western populations.

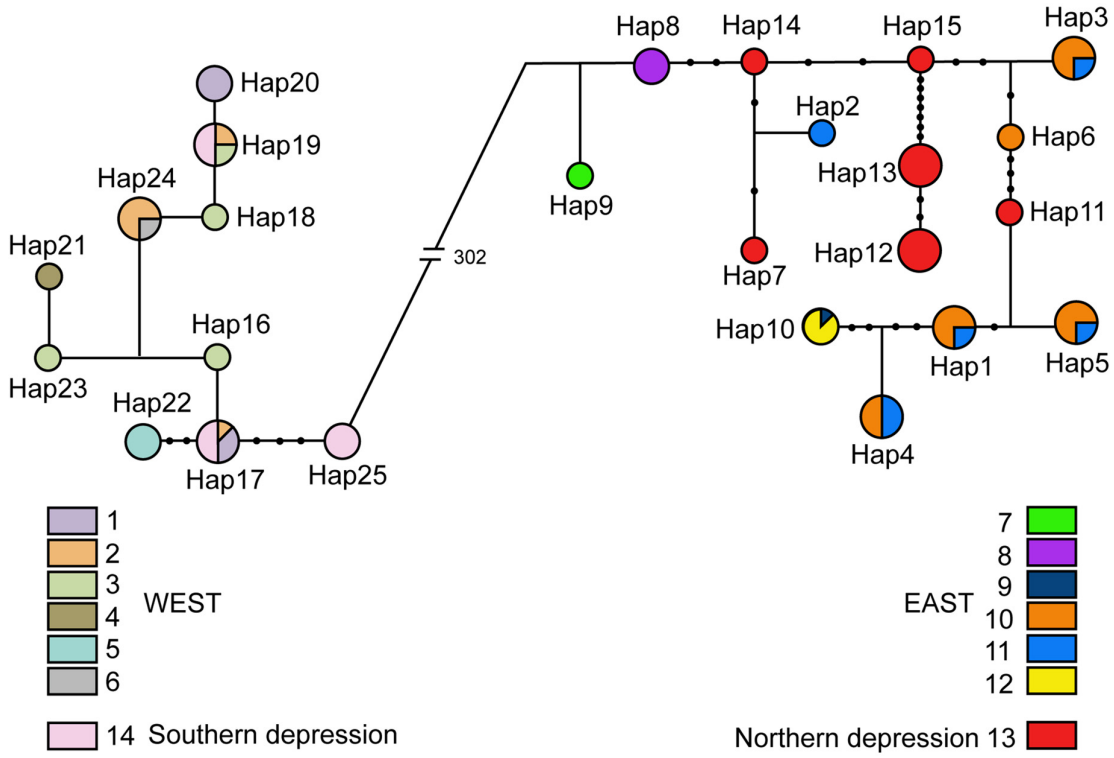

FIGURE 3. Statistical parsimony network for the *Rhinella marina* species complex in northern Venezuela based on concatenated ND3+CR mitochondrial haplotypes. The left branches correspond to western populations and the right branches to eastern populations. Connections between haplotypes correspond to one mutational step and small dots indicate additional mutational steps.

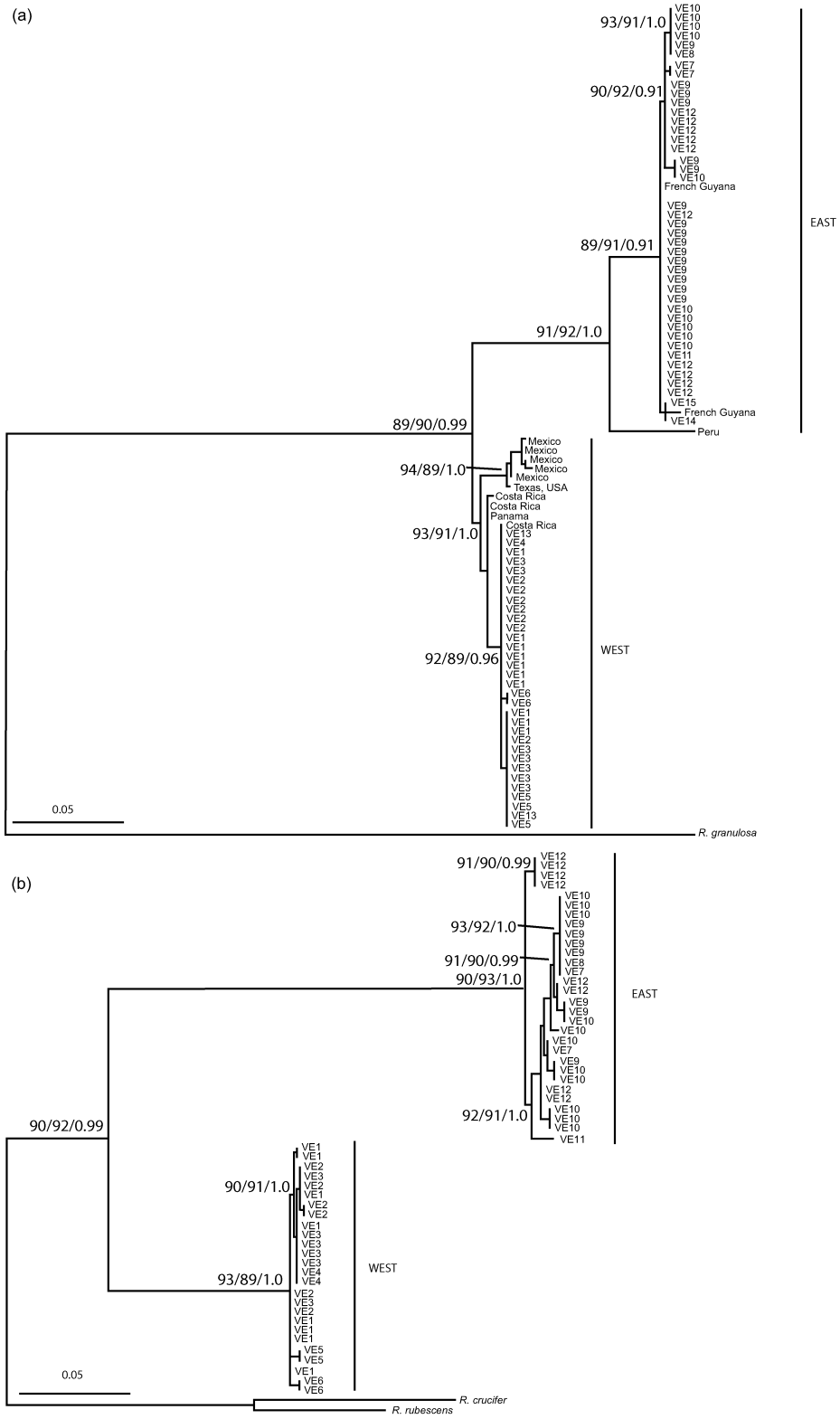

FIGURE 4. Trees showing phylogenetic relationships for the *Rhinella marina* species complex in northern Venezuela. Phylogenetic relationships are inferred from 429bp fragment of the ND3 (a) and 737bp for CR (b). The names refer to the location where the haplotype was collected and east and west to their location in relation the Andean Cordillera, as in Figure 1. The numbers at the nodes indicate maximum likelihood, maximum parsimony and Bayesian inference bootstrap values (ML/MP/BI).

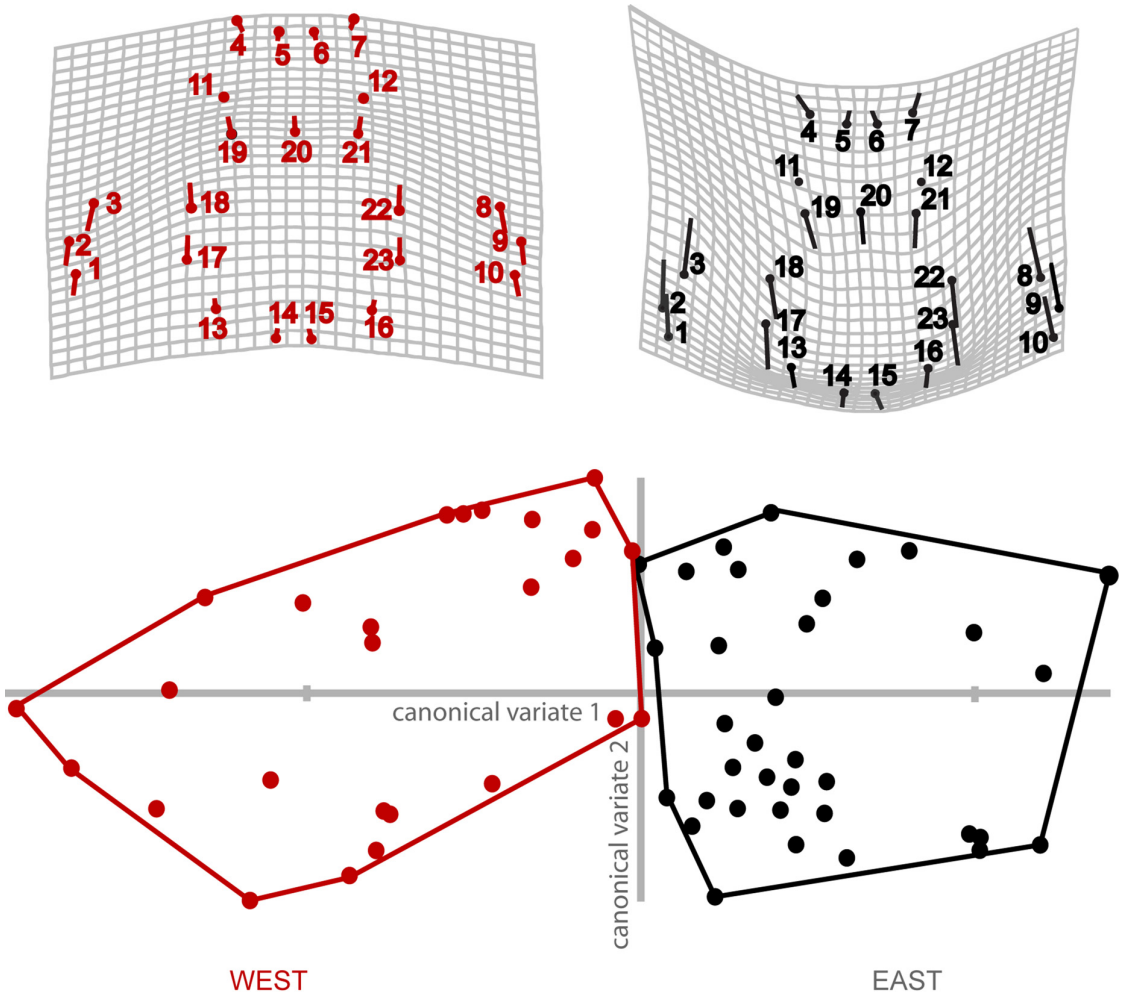

FIGURE 5. Morphological differences in skull shape between east and west populations of *Rhinella marina*. Changes with respect to the average on the deformation grids (a) and morphospaces in the canonical variates (b) resulting from comparisons between eastern and western populations.

Discussion

The combined morphometric and molecular evidence allowed for the identification of two cryptic species within *Rhinella marina*. The mitochondrial DNA datasets from ND3 and CR confirmed the phylogenetic break between populations located east and west of the Andean cordillera identified earlier (Sequeira *et al.* 2011; Slade & Moritz 1998; Vallinoto *et al.* 2010), with more than 97% of the observed genetic variation attributed to differences between regions. In agreement with the genetic evidence, the morphometric geometry analysis revealed, for the first time, a divergent pattern of skull evolution in *R. marina*. The identification of an eastern and a western morphotype with no overlapping morphospaces suggests independent evolutionary histories of morphological traits across the cis and trans-Andean cordillera. Our fine-scale survey revealed no common haplotypes or morphotypes between regions, despite the proximity between some eastern and western locations, suggesting that the two lineages may be allopatric. This concordance between the observed geographic patterns in morphometric and genic traits calls for the taxonomic revision and the recognition of two species *R. marina*, one east and one west to the Andean cordillera.

Previous molecular studies based on mtDNA suggested that the divergence of *R. marina* across the Andes predated the speciation of some other species of the *R. marina* group in South America (Slade & Moritz 1998; Vallinoto *et al.* 2010). According to molecular clocks using mitochondrial DNA, eastern and western populations

could have split during the uplift of the Andean cordillera (early Pleistocene) (Slade & Moritz 1998) or earlier during marine incursions and the formation of the Pebas system (middle-late Miocene) (Vallinoto *et al.* 2010). The fine-scale geographic pattern of genetic variation of mitochondrial markers observed in this study showed no evidence of recent geographic or demographic expansions in *R. marina* populations around the Andean cordillera (east or west), in contrast to populations at the southern banks of the Amazon river (Sequeira *et al.* 2011). These authors hypothesize a recent southward expansion of populations located south of the Amazon as a result of a southward re-establishment of the Cerrado during the Holocene.

Cranial morphology in bufonids is highly variable and, for the most part, reflects the evolutionary history of major taxonomic lineages within this group (Pramuk 2006). At lower taxonomic levels, however, large phylogenetic breaks are often associated with subtle or indistinguishable changes in morphology, a disparity that results in the frequent occurrence of cryptic species in bufonids. For example, molecular markers have revealed cryptic speciation in *Anaxyrus punctatus* (Jaeger *et al.* 2005), *Rhinella margaritifera* (Fouquet *et al.* 2007) and in the *Dendrophryniscus, Amazophrynella* (Fouquet *et al.* 2012), *Frostius* (Junca *et al.* 2012) and *Peltophryne* genera (Alonso *et al.* 2012). Most of these species, however, remain taxonomically cryptic because the assignment of valid names requires establishing links with voucher type specimens that are frequently unsuitable for molecular studies. Thus, the identification of diagnostic morphological characters is necessary for the taxonomic revaluation of species.

Among South American toads, the species belonging to the *R. marina* group show distinctively large parotoid glands and, internally, the medial ramus of the pterygoid and the parasphenoid alae join forming a jagged suture (Pramuk 2006). Within this group, *R. marina* lacks of tibial glands and has particularly large parotoid glands that protrude from the outline of the lateral surface of the head (Kwet *et al.* 2006). However, a depression of the pre-maxillar region and a posterior elongation of the frontoparietal and occipital regions (*e.g.* condyles and sphenethmoids) of *R. marina* adults from eastern populations result in a slightly rounder head compared to western populations. In western populations, the quadratojugal bones tend to be closer to the transverse line formed by the occipital condyles than in eastern populations (Figure 2). These shape differences can unequivocally separate the eastern from the western lineage and can be captured by the position of diagnostic landmarks that, despite involving non-visible internal structures, can be used for the evaluation of voucher specimens with non-destructive x-ray or ecography techniques.

The type locality for the first description of *Rhinella marina* [*Rana marina* (Linneus 1758)] was probably located in Surinam (Muller & Hellmich 1936), east to the Andean cordillera. Later, at least three synonyms were proposed for *R. marina* specimens collected west of the Andes: *Bufo horribilis* (Wiegmann 1833), *B. angustipes* (Taylor and Smith 1945), *B. pithecodactylus* (Rivero 1961) and *B. marinus horribilis* (Lynch and Fulger 1965). The earliest description from a western location *Bufo horribilis* corresponds to a specimen from Mexico (Veracruz). These suggest that eastern populations should maintain the *Rhinella marina* nomenclature and the name *Rhinella horribilis* should be revalidated for western populations, as suggested earlier (Frost 2015), until further studies integrate a specimens from its complete geographic distribution. Given the possibility of using non-distructive x-ray imaging techniques in voucher specimens, landmark-based geometric morphometry is likely to open new avenues for solving the taxonomy of *R. marina*.

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