



Phytogeographic patterns and cryptic diversity in an aposematic toad from NW Argentina



Rute B. Clemente-Carvalho^a, Marcos Vaira^b, Laura E. King^c, Daria Koscinski^d, Maria I. Bonansea^e, Stephen C. Loughheed^{a,*}

^a Department of Biology, Queen's University, Kingston, Ontario K7L 3N6, Canada

^b Centro de Investigaciones y Transferencia de Jujuy, Conicet – Universidad Nacional de Jujuy, Gorriti 237, 4600 S.S. de Jujuy, Argentina

^c Wildlife Preservation Canada, 5420 Highway 6 North Guelph, Ontario, N1H 6J2, Canada

^d Department of Biology, University of Western Ontario, London, Ontario, N6A 5B7, Canada

^e Facultad de Ciencias Agrarias, Universidad Nacional de Jujuy, Alberdi 47, 4600 S. S. de Jujuy, Argentina

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ABSTRACT

The Yungas Redbelly Toad, *Melanophryniscus rubriventris*, is patchily distributed in Argentina, confined to the upland portion (1000–2000 m above sea level) of the montane forests of northern and central regions of Salta, and in central-eastern and south-eastern Jujuy. This species is known for its striking aposematic color variation across its geographic distribution, and was once treated as a complex of three subspecies based on distinctive color patterns. Here we assess the geographical genetic variation within *M. rubriventris* and quantify divergence in color and pattern among individuals sampled from Northwestern Argentina. We compare multi-gene phylogeography of *M. rubriventris* to patterns of dorsal and ventral coloration to test whether evolutionary affinities predict variation in warning color. Our results reveal two well-supported species lineages: one confined to the extreme northern portion of our sampling area, and the other extending over most of the Argentine portion of the species' range, within which there are two populations. However, these well-supported evolutionary relationships do not mirror the marked variation in warning coloration. This discordance between DNA genealogy and warning color variation may reflect selection brought about by differences in local predation pressures, potentially coupled with effects of sexual selection and thermoregulation.

1. Introduction

As phylogeographic studies accumulate on neotropical taxa, it becomes increasingly clear that cryptic species are pervasive (e.g. insects – Hebert et al., 2004; Smith et al., 2008; Bernasconi et al., 2010; fishes – Keck and Near, 2010; Piggott et al., 2011; amphibians – Elmer et al., 2007; Fouquet et al., 2007; Townsend et al. 2013a; bats – Clare et al., 2011; plants – Govindarajulu et al., 2011; birds – Welch et al., 2011; reptiles – Devitt et al., 2008; Leaché et al., 2009; Jadin et al., 2011; Townsend et al. 2013b), potentially more so in tropical versus higher-latitude taxa. Moreover, detailed phylogeographic studies reveal deep genealogical divisions within many long-recognized neotropical species (e.g. Chek et al., 2003; Kerr et al., 2009; Gehara et al., 2014). Such observations together imply that we have profoundly underestimated the number of species at lower latitudes, presenting greater challenges for conservation and implying a much steeper latitudinal gradient in species richness than traditionally thought. Moreover, it implies that

lineages may diverge for millions of years without concomitant divergence in the phenotypic characters that are typically used to classify species.

Amphibians have featured prominently in discussions of cryptic species, potentially because of their conserved morphology and divergence in less appreciated aspects of phenotype including mate recognition system (Cherry et al., 1977; Emerson, 1988). For example, Fouquet et al. (2007) deployed a variety of analyses of 16S rDNA from 60 Amazonia-Guianan frog species and identified 129 new candidate species. Elmer et al. (2007) found deep divergences between lineages within what had been considered a single species of upper Ecuadorean Amazon frog, *Pristimantis ockendeni* (previously *Eleutherodactylus ockendeni*).

Warning coloration can markedly impact patterns of diversification and speciation (Mallet and Joron, 1999; Stevens and Ruxton, 2012; Santos et al., 2014), but also can mask the presence of deep divergences (Vences et al., 2003). For example, the striking New World radiation of

* Corresponding author at: Department of Biology, Biosciences Complex, 116 Barrie Street, Queen's University, Kingston, Ontario K7L 3N6, Canada.

E-mail addresses: rute_beatriz@hotmail.com (R.B. Clemente-Carvalho), marcos.vaira@conicet.gov.ar (M. Vaira), king.laura.elizabeth@gmail.com (L.E. King), Daria.Koscinski@uwo.ca (D. Koscinski), inesbonan@gmail.com (M.I. Bonansea), steve.loughheed@queensu.ca (S.C. Loughheed).

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Heliconius butterflies (Bates, 1862) is a classic example of Müllerian mimicry, where subspecies may exhibit markedly divergent phenotypes across a species range (Jiggins et al., 2001), while sympatric subspecies of distantly related species can evolve similar color patterns independently in response to common predators (Turner, 1976; Mallet and Gilbert, 1995). Importantly warning coloration plays a dual role in many taxa, serving both as a signal to potential predators and in mate choice (e.g. dart-poison frogs: Summers et al., 1999; Maan and Cummings, 2008; *Heliconius* spp. Melo et al., 2009). Mate selection based on warning coloration has been implicated in reinforcing reproductive isolation (e.g. Brusa et al., 2013), and in driving the evolution of new species (Wang and Summers, 2010) including via homoploid speciation (Brower, 2013). Some aposematic species are highly polymorphic, varying both within and among populations across their distributions (e.g. Summers et al., 2003; Wollenberg et al., 2008; Bonansea and Vaira, 2012; Rojas and Endler, 2013), raising the possibility that multiple independent evolutionary lineages are found within single diagnosed taxa. In sum, taxa with warning coloration provide compelling albeit complicated systems for studying factors implicated in speciation, divergence, and the evolution of cryptic species (Przeczek et al., 2008).

Here we examine phylogeographic patterns in an anuran taxon from the montane forests of the Andes of Northwestern Argentina, the Yungas Redbelly Toad (*Melanophryniscus rubriventris*), combining DNA sequence data from three nuclear and three mitochondrial genes. This aposematic toad is mostly diurnal, and reproduces throughout a prolonged spring-summer breeding season (November to February). Breeding activities involve large aggregations that vary markedly in numbers among years, with short and explosive reproductive events where toads use shallow temporary ponds in marshy areas to lay eggs (Vaira, 2005; Goldberg et al., 2006). The species is patchily distributed in Argentina, confined to areas within 1000–2000 m above sea level within the montane forests (Yungas) of northern and central Salta, and central-eastern and south-eastern portions of Jujuy (Bonansea and Vaira, 2012).

The species is polymorphic throughout much of its geographic range, with four different dorsal patterns and three ventral patterns varying within and among populations (Figs. S1 and S2). Warning coloration of northern and central populations (Salta and central-east region of Jujuy) is characterized by bright dorsa, differing mainly in the extent of black patches. The bright dorsal coloration shows differences in intensity, with bright patches ranging from vivid red, and orange to pale pink. Individuals of southern populations (south-eastern Jujuy and southern Salta) predominately exhibit a more cryptic olive or black dorsal pattern, with brighter dorsal coloration limited to two small patches of muted yellow. Concomitantly, individuals from northern and central populations have a mostly uniform orange-to-red belly, whereas toads from southern populations have well-demarcated yellow, red and black speckled bellies (Bonansea and Vaira, 2012). The species was once treated as a complex of three subspecies by Laurent (1973) based on these color patterns; however, studies by Vaira (2000, 2002) showed that *M. rubriventris* was one morphologically, osteologically, and acoustically variable species with striking variability in color pattern, and that color characters used by Laurent (1973) were not diagnostic of subspecies.

The objectives of our study were two-fold. First, we wished to assess the phylogeographic relationships within *M. rubriventris* and to examine divergence patterns in terms of the dynamic history of Northwestern Argentina and phylogeographic patterns of other sympatric taxa. Contemporary Northwestern Argentina exhibits great topographic, climatic, and phytogeographic complexity (Handford, 1988; Strecker et al., 2007), and it is clear that climate and vegetation patterns have changed dramatically over the Pliocene and Pleistocene coincident with Milankovitch cycling (e.g. Hinojosa and Villagran, 1997; Hinojosa, 2005; Morrone, 2014). The few geographically intensive molecular surveys of taxa from this region show striking north-to-south

phylogeographic structure, interpreted to result from episodic isolation in historical refugia, followed by post-refugial expansion: sigmodontine rodents, *Akodon* sp. (Braun et al., 2008), *Podocarpus parlatorei* (Quiroga and Premoli, 2007); *Polylepis australis* (Hensen et al., 2011), and two frog species (*Hypsiboas andinus* – Koscinski et al., 2008; *Pleurodema borellii* – Koscinski et al., unpubl. data). Our second goal was to compare phylogeographic patterns in *M. rubriventris* to patterns of dorsal and ventral warning coloration described by Bonansea and Vaira (2012) to test whether there is correlation between evolutionary history and warning color patterns that might imply a role for range fragmentation and shifting in the evolution of color patterns evident today.

2. Materials and methods

2.1. Sampling

Our study includes tissue samples from 59 individuals of *M. rubriventris* from 11 sites throughout all the mountain systems in Northwestern Argentina spanning the known Argentine distribution along the northern range of the ecoregion of Southern Andean Yungas, provinces of Salta and Jujuy. The sampled populations ranged in elevation from 1300 to 1700 m above sea level (Fig. 1A, Table 1). Samples from locales reported by De la Riva (1995) and De la Riva et al. (2000) from the inter-Andean valleys of the departments of Tarija, Chuquisaca, and Cochabamba in southern and central Bolivia are not included in this study. Liver tissues were obtained during multiple fieldworks conducted from May 1998 through January 2008 (during the rainy season). We took samples of toads along short transects in marshy areas using diurnal encounter surveys (Crump and Scott Jr., 1994). Sampling began at 0900 h, and ceased at 1900 h, by which time most of the toads had ceased their breeding activities. Toads were killed by submersion in an anaesthetic (MS 222, Syndel Laboratories, Qualicum BC) as reference specimens and deposited in the amphibian collection of the Instituto de Bio y Geociencias del NOA (IBIGEO-A; Table S1). Between 100 and 200 mg of liver tissue was excised from each specimen, and stored in 70% ethanol at -20°C until DNA extraction. We also obtained tissue samples from 5 individuals for a single outgroup taxon, *Melanophryniscus stelzneri* from the Smithsonian Institution – National Museum of Natural History (USNM 253718 – 253722). DNA was extracted using a QIAGEN DNeasy Tissue kit (QIAGEN, Mississauga, ON, Canada) following the manufacturer's protocol.

2.2. PCR and sequencing

Multi-marker phylogeographic studies can provide great insight into evolutionary relationships of closely-related species (Dupuis et al., 2012) but are lacking in most phylogeographic surveys of neotropical taxa (Turchetto-Zolet et al., 2013). Thus we assayed DNA sequence variation from portions of 6 different genes: three mitochondrial – 12S rDNA (838 bp), 16S rDNA (495 bp), and *cytochrome b* (858 bp), and three nuclear – rhodopsin (*RHOD*, 360 bp), recombination activating gene-1 (*RAG1*, 495 bp), chemokine receptor 4 (*CXCR-4*, 599 bp). Details of amplification are provided in the Supplementary Methods.

2.3. Phylogenetic and phylogeographic analysis

Sequences were aligned using CLUSTALX v2.0 (Larkin et al., 2007). Protein-coding genes were visually inspected to confirm lack of indels and translated into amino acids to verify absence of stop codons. We first conducted individual-based Bayesian phylogenetic analysis using MRBAYES v3.1.3 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) on each dataset separately, and on a concatenated dataset comprising DNA sequences from all six genes. For this analysis, individuals heterozygous for polymorphic sites for the nuclear genes were encoded as ambiguous using IUPAC codes. For each gene, we selected the model of nucleotide evolution using JMODELTEST v2.1.2

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