Phytogeographic patterns and cryptic diversity in an aposematic toad from NW 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

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) predominantly 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
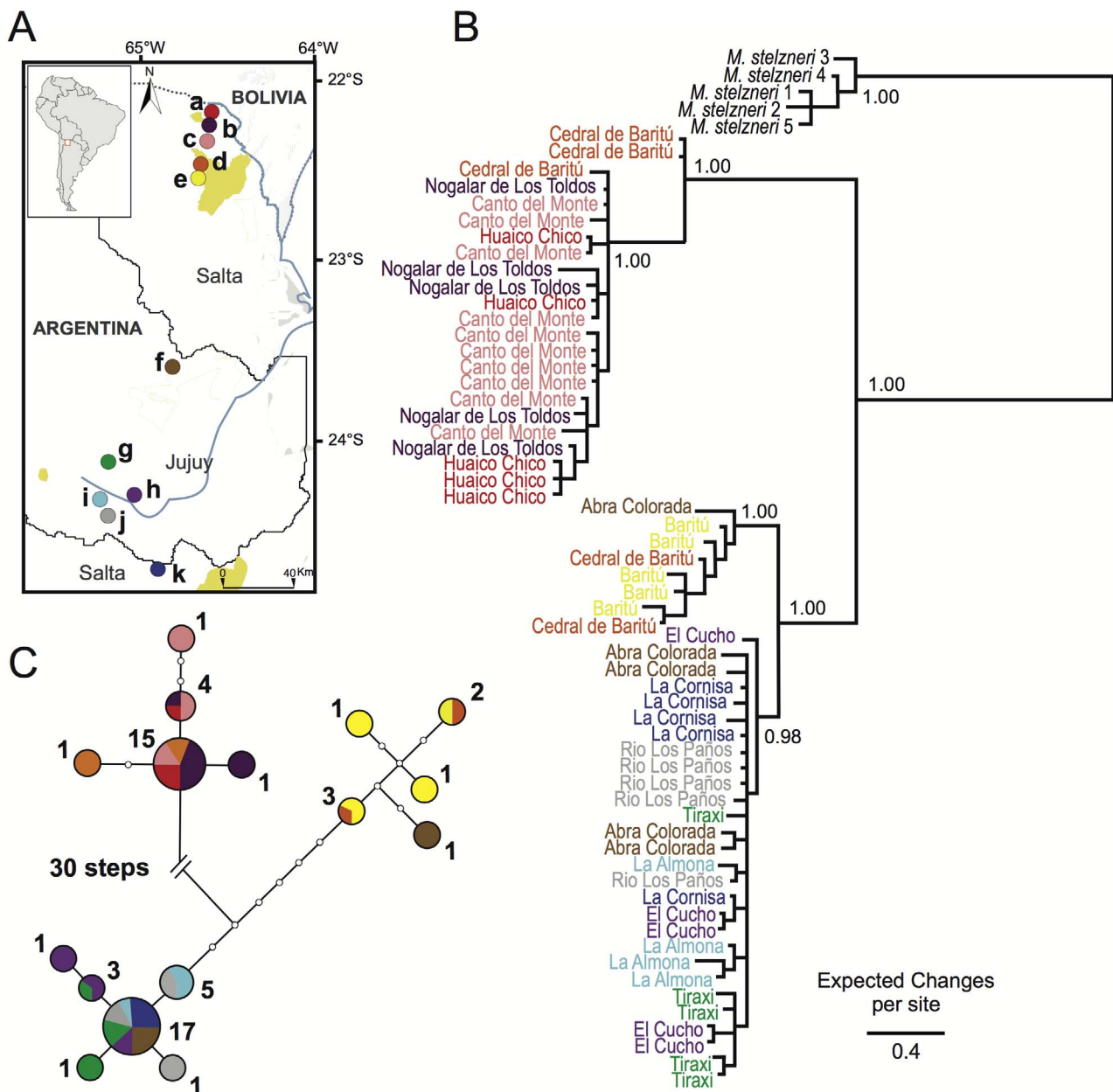
Fig. 1. (A) Map of Northwestern Argentina showing sampling localities for *Melanophryniscus rubriventris*. Amber patches on the map delimit protected areas, including national parks. Locality names: a = Huaico Chico, b = Reserva Nacional El Nogalar de Los Toldos, c = Canto del Monte, d = El Cedral de Baritú, e = Baritú, f = Abra Colorada, Parque Nacional Calilegua, g = Tiraxi, h = El Cucho, i = La Almona, j = Rio Los Paños, and k = La Cornisa. (B) Bayesian phylogeny generated from a concatenated dataset comprising DNA sequences from 3 mitochondrial and 3 nuclear genes (details provided in the text). At relevant nodes, posterior probabilities are indicated. The outgroup is *Melanophryniscus stelzneri*. Individuals are color-coded according to site of origin in panel A. (C) Maximum parsimony network of cytochrome *b* haplotypes. Haplotypes are indicated by colored circles, scaled to number of individuals that each possesses; number of individuals is also indicated beside each haplotype. Pie charts indicate relative proportions within locales for all diagnosed haplotypes. Small open circles indicate missing haplotypes or those not found within our survey. The longest link between bottom and top comprising 30 steps is not drawn to scale.

(Darriba et al., 2012). For the analyses of the concatenated data, all partitions were unlinked, allowing parameter values to vary independently. All Bayesian analyses included two simultaneous runs each with random starting trees, four incrementally heated Markov chains, and default priors for all parameters. Analyses were run until the standard deviation of split frequencies was < 0.01, implying convergence (see Table S1 from model details and number of generations run for each partition). We sampled trees every 100 generations, and discarded the first 20% as burn-in. From the remaining trees for both runs combined, we constructed a 50% majority-rule consensus tree. The potential scale reduction factor (Gelman and Rubin, 1992) was close to one for all parameters for all runs, indicating that we had sufficient effective sample size to have adequately sampled the posterior

distributions. Finally, we used the 'cumulative' and 'compare' functions implemented in the software AWTY (Nylander et al., 2008) to confirm that runs had reached stationarity.

2.4. Estimating TMRCA and Extended Bayesian Skyline Plots

We used BEAST v1.6.1 (Drummond and Rambaut, 2007) to estimate TMRCA of major clades using the only protein-coding mitochondrial gene, *cytochrome b*. As we do not have fossils or defining geological events to provide independent calibration for a *Melanophryniscus* specific clock, we use Crawford's (2003) *ND2* calibration of approximately 1% change per lineage per million years. We used an HKY + I model, assuming a relaxed uncorrelated lognormal molecular clock, and a

Table 1

Sampling details for *Melanophryniscus rubriventris* including locality name and province, approximate geographical coordinates, and number of individuals sequenced and photographed for the present study. Localities are arranged from northernmost to southernmost distribution in Argentina.

Locality name	Latitude	Longitude	Individuals sequenced	Individuals with photo
Arroyo Huaico Chico, Los Toldos, Departamento Santa Victoria, Provincia de Salta	22 °16′23.82″S	64 °42′46.72″W	5	44
Reserva Nacional El Nogalar de Los Toldos, Departamento Santa Victoria, Provincia de Salta	22 °16′41.06″S	64 °42′42.62″W	5	16
Canto del Monte, Departamento Santa Victoria, Provincia de Salta	22 °22′13.74″S	64 °43′16.69″W	10	248
El Cedral de Baritú, Parque Nacional Baritú, Departamento Santa Victoria, Provincia de Salta	22 °27′35.76″S	64 °44′33.54″W	4–5	184
Baritú, Departamento Santa Victoria, Provincia de Salta	22 °29′55.59″S	64 °45′56.65″W	5	18
Abra Colorada, Parque Nacional Calilegua, Departamento Valle Grande, Provincia de Jujuy	23 °40′52.36″S	64 °53′53.00″W	5	202
Tiraxi, Departamento Dr. Manuel Belgrano, Provincia de Jujuy	24 °01′23.92″S	65 °23′24.78″W	4–5	296
El Cucho, Departamento Palpalá, Provincia de Jujuy	24 °14′38.41″S	65 °07′03.35″W	5	14
La Almona, Departamento Dr. Manuel Belgrano, Provincia de Jujuy	24 °16′29.64″S	65 °24′29.21″W	4	34
Rio Los Paños, Departamento Dr. Manuel Belgrano, Provincia de Jujuy	24 °18′12.34″S	65 °24′58.10″W	5	–
Abra de la Sierra (Camino de La Cornisa), Departamento La Caldera, Provincia de Salta	24 °29′55.59″S	65 °18′29.47″W	5	20

random starting clock, and ran the analysis for 50 million steps sampling every 1000, and used a burn-in of 5 million steps. We examined the output in Tracer v1.5 (Rambaut and Drummond, 2007) to evaluate effective sample sizes for all parameter estimates (all > 2000), and examine traces to ensure convergence. We conducted two separate analyses using different tree priors: 1. Constant population size. 2. Exponential growth (reflecting probable population growth since the last glacial maximum – see below).

We evaluated the historical demography of *M. rubriventris* overall, and for identified major clades separately using Extended Bayesian Skyline Plots (EBSP) in BEAST v1.6.1. Results were visualized in Tracer v1.5 (Rambaut and Drummond, 2007) and using Python scripts provided by Joseph Heled. The EBSP analysis employed all six loci (see Heled and Drummond, 2008), and used relaxed uncorrelated lognormal clock priors. Each analysis was run for 50 million generations with every 5000 generations retained and the first 5 million generations (10%) discarded as burn-in. We set the mtDNA *cytochrome b* mutation rate to be consistent with a divergence rate of 1% per lineage per million years, and estimated the rates for other data partitions relative to this rate for each of the remaining loci under a lognormal prior. Site models were those used in the TMRCA analysis (Table S1).

We used the program TCS v1.2.1 (Clement et al., 2000) to construct maximum parsimony networks for our *cytochrome b* data as they formed the single largest data partition. We systematically lowered the parsimony limit until we found the value that allowed for connection between the two most divergent clusters of haplotypes (30 steps).

2.5. Analysis of color patterns

Between 2007 and 2009 we scored a suite of dorsal and ventral color-pattern characters from 1076 individuals sampled from ten of the 11 previously sampled localities (excluding Rio Los Paños) to allow us to quantify color pattern morph frequencies. Pattern morphs were described from adult toads to obtain a reasonably comprehensive characterization using standardized high-resolution digital color photographs of the dorsum and ventral parts of toads. Individuals of each population were hand-captured on a single day and lightly anesthetized with a diluted dose of Ethyl-p-Aminobenzoate (Benzocaine). Each toad was placed on a blue board on flat ground and photographed *in situ*. We used a tripod-mounted digital camera with macro lens, set 20 cm above the board. The digital camera was set to 2 megapixel resolution, and we manually controlled white balance, focus, and exposure. We maintained the consistency of ambient light conditions by taking pictures only during cloudless days. Toads were released at the point of capture. One of us (MIB) scored every individual based on our categorization scheme for dorsal and ventral patterns, separately. Dorsal patterns of toads were scored on a four-point scale with (1) comprising almost completely black or drab morphs to (4) signifying an equal proportion of black and bright colors. Ventral patterns were scored on a three-point

scale: (a) brightly uniform coloration; (b) bright belly with a few dark patches; (c) completely speckled belly (see Figs. S1 and S2).

To allow comparison to relatedness of populations deduced using DNA sequences, we summarized among-population differences in dorsal and ventral color patterns using multidimensional scaling (MDS) based on Morisita's index distances. The significance of groupings was tested with a nonparametric one-way analysis of ranked similarities (ANOSIM) (Clarke, 1993), and the global R calculated based on a color-pattern similarity matrix. Significance for global R was assessed by creating a null distribution using 9999 random permutactions of population membership. We used PAST v2.17 statistics software (Hammer et al., 2001) to run MDS and ANOSIM analyses.

3. Results

In total we sequenced 3644 base pairs for 64 individuals, 59 ingroup and 5 outgroup individuals. The Bayesian analysis of our concatenated dataset revealed two well-supported clades (pp = 1.0), which we interpret as diagnosing two species lineages: one confined to the extreme northern portion of our sampling area near the border between Bolivia and Argentina and centred on Baritú (hereafter the "Northern Lineage"), and the other extending over most of the Argentine species range from our Cedral de Baritú site to the border between the provinces of Jujuy and Salta (hereafter the "Southern Lineage", Fig. 1B). Within the broadly distributed Southern Lineage, there is further phylogenetic structure with one subpopulation including individuals from Parque Nacional Calilegua (hereafter Calilegua), Cedral de Baritú, and Baritú, while the other sublineage comprises individuals from Calilegua and from all southernmost sites (Fig. 1B). The Cedral de Baritú site shows admixture of haplotypes from the Southern and Northern Lineages, while all individuals from the geographically proximate Baritú site belong to the Southern Lineage. A maximum parsimony network of *cytochrome b* haplotypes (Fig. 1C) reveals similar phylogeographic structure with a northern cluster, separated by more than 30 mutational steps from a southern cluster within which we found evidence of two separate subclusters with high (≥95%) parsimony support. Indeed most of the phylogenetic signal is contained within the mitochondrial data, although no single gene tree contradicts our conclusions regarding the overarching phylogeographic patterns that we describe above (Fig. S3). Two *cytochrome b* haplotypes were common and presumably ancestral. In the northern cluster, the common haplotype was found in 15 individuals (68% of all individuals within that cluster), while in the southern cluster the common haplotype was present in 17 individuals (46% of all surveyed individuals).

Our coalescent analysis of *cytochrome b* to estimate TMRCA indicates that the deepest divergence within *M. rubriventris* probably dates to the early Pleistocene or late Pliocene. Assuming a lineage-specific rate of divergence of 1% per million years yields estimates of 2,069,400 years before present (95% HPD: 704,470–3,615.100) and
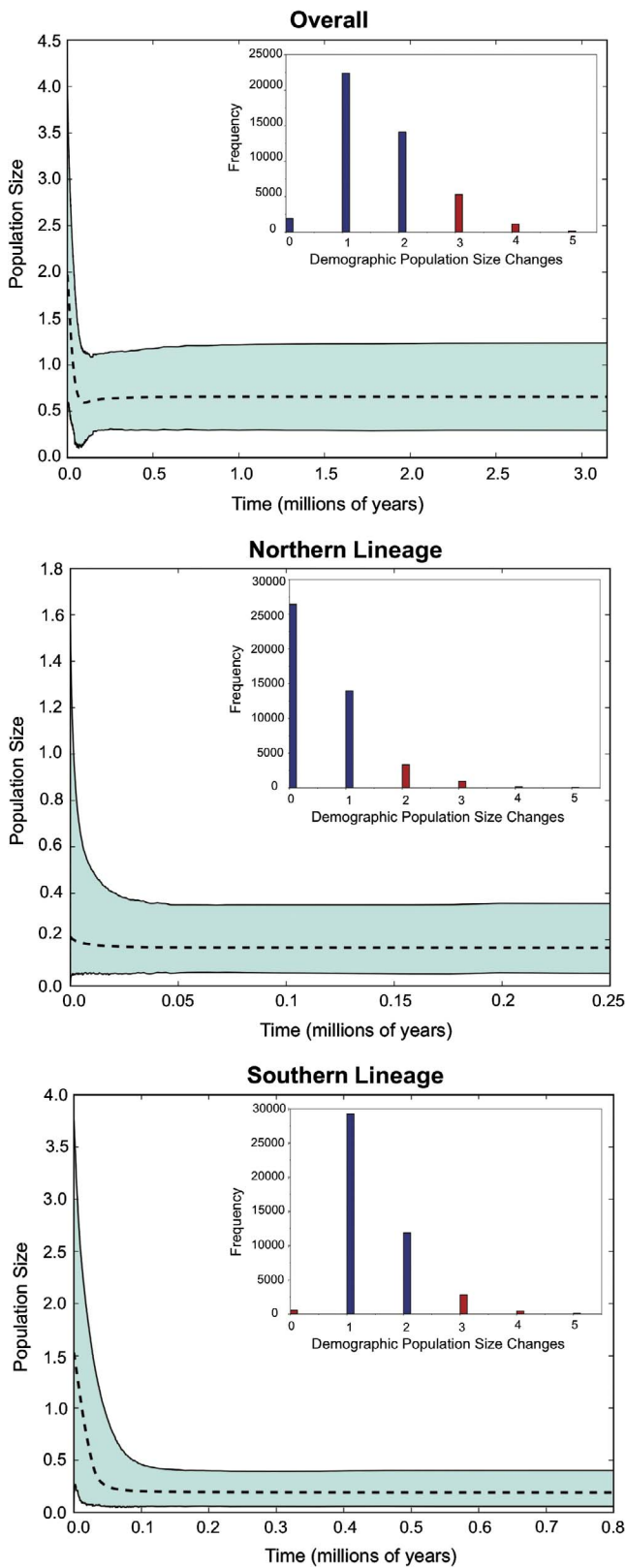
Overall

Northern Lineage

Southern Lineage

Fig. 2. Extended Bayesian Skyline Plots (EBSP) showing historical demographic patterns for *M. rubriventris* overall, and for the Northern Lineage and Southern Lineage separately. Extended Bayesian skyline plots provide a means for graphically visualizing trends in effective population sizes through time estimated using multi-locus DNA sequence data. The dashed line shows the estimated median value for population size (millions of individuals), with the light blue shading within solid lines indicating the 95% highest posterior density (HPD) or credible interval, analogous to a 95% confidence interval in traditional frequentist statistics. The inset shows a bar chart of frequencies for each number of inferred demographic changes, with blue bars indicating those changes that are within the credible set, and red bars those that are outside. If zero is not included within the credible set (i.e. colored red), then this implies that there has been at least one detected shift in population size. Overall demographic population size changes occur only at change points inferred for any single gene. These are generated through an approximately coalescent process, and thus not evenly distributed through time. Our sampling of only 3644 base pairs for six genes also constrains our ability to quantify deeper historical population changes, and thus the apparent constancy over longer durations simply may be a consequence of recent demographic changes having overwritten the signatures of deeper events.

Northern Lineage has been more or less demographically stable over the last 2–3 million years, while the signature of population expansion is evident in the Southern Lineage (one demographic change is the most frequently identified outcome within the credible set – 95% HPD; see Fig. 2).

Melanophryniscus rubriventris shows substantial variation in warning coloration across the sampled range (Fig. 3). Our MDS analyses of dorsal and ventral color patterns revealed three different groups of populations (Fig. 4). ANOSIM for the MDS-based analysis yielded a highly significant global R = 1 (p < 0.001). Populations from Huaico
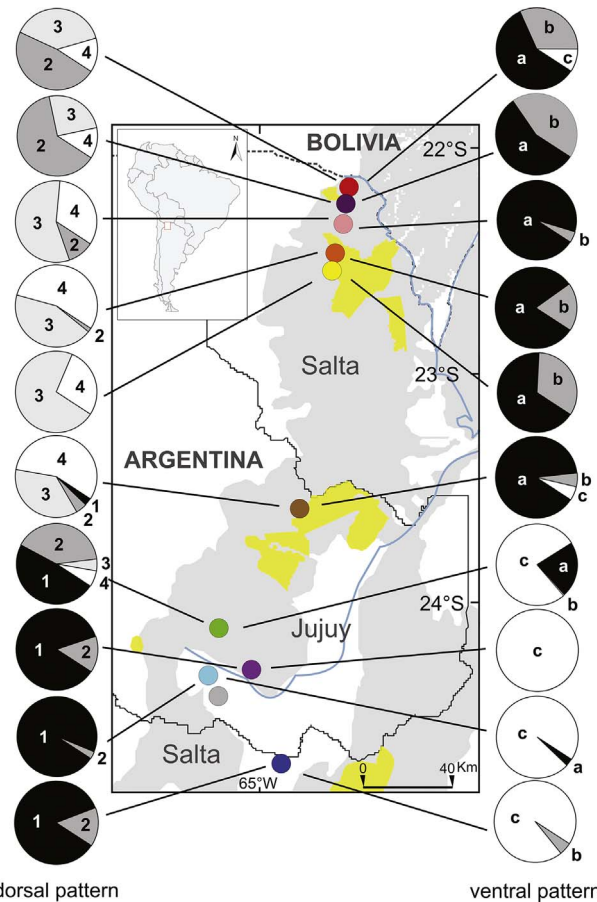

Fig. 3. Variation in dorsal and ventral pattern coloration of *M. rubriventris* across its Northwestern Argentine geographical distribution. Numbers and letters in each pie chart reflect the classification scheme used (four dorsal patterns and three ventral patterns respectively, see text and Figs. S1 and S2 for details). Populations are color-coded according to Fig. 1A.

2,231,700 years before present (95% HPD: 809,970–3,816,900) for the constant and population growth tree priors, respectively. TMRCA estimates for all other diagnosed subpopulations within this species fall into the second half of the Pleistocene (Table S2).

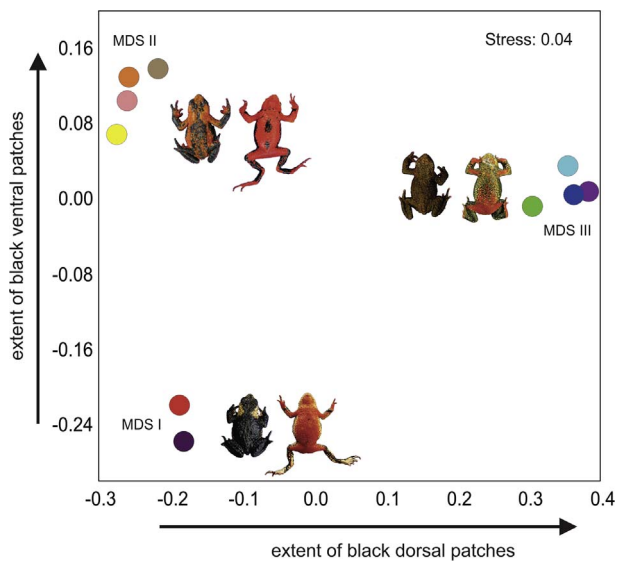Our extended Bayesian skyline analysis (Fig. 2) implies that the

Fig. 4. Two-dimensional non-metric scaling (nMDS) plot based on a matrix of pairwise Morisita's index distances of color patterns for north-western populations of *M. rubriventris*. MDS is an ordination method that visualizes the relationship among objects (here locales or populations) seeking to preserve the between object-distances. Here populations were grouped into three clusters, MDS-I through –III according to the extent of dorsal (Dimension 1) and ventral black patches (Dimension 2) of individuals. Color circles represent population origin and are coded as in Fig. 1A. Frequencies of color patterns vary regionally (Fig. 3), but specific ventral and dorsal patterns tend to predominate regionally. Exemplars of these are shown for each nMDS cluster of populations.

Chico and Nogalar de Los Toldos (MDS I in Fig. 4) exhibit pale pink to orange dorsal colors with varying extents of black and bright dorsal color patches (dorsal patterns 2–4; see Fig. S1), and vivid red to orange bright ventral coloration with a few dark patches but almost no speckled patterns (mostly ventral patterns a and b; see Fig. S2). Toads from Canto del Monte, Cedral de Baritú, Baritú, and Calilegua (MDS II in Fig. 4) had larger extents of bright dorsal colors or equal extents of black and bright dorsal colors (primarily dorsal patterns 3 and 4), and mostly vivid red to orange bright ventral colors with fewer individuals with dark patches, but almost no individuals with speckled patterns (ventral patterns a and b). Populations from Tiraxi, El Cucho, La Almona and La Cornisa (MDS III in Fig. 4) show no bright coloration (dorsal pattern 1) or a small extent of dull yellow coloration (dorsal pattern 2), and red or yellow/red coloration with predominantly speckled venters (ventral pattern c).

4. Discussion

Our analysis revealed marked phylogeographic structure with two well-supported lineages in Northwestern Argentina, the Northern Lineage confined to the extreme north centered on Parque Nacional Baritú, and the Southern Lineage extending from Baritú to the southern extent of the species distribution on the border between the Argentine provinces of Salta and Jujuy. One site, Cedral de Baritú, was a zone of admixture between the Northern and Southern Lineages, although even within the Northern Lineage individuals from this locale exhibited substantial diversity (Fig. 1). The estimated time of divergence implies early Pleistocene or late Pliocene origins to this split, and we find some evidence of population expansion within the last 100,000 years, consistent with historical range fragmentation and subsequent expansion, and similar to patterns that have been documented for other broadly-sympatric taxa. While there exists substantial variation in color and pattern among populations (Bonansea and Vaira, 2012), we found no compelling evidence to suggest that aposematic coloration is related to phylogeographic history of this species. We discuss these results in turn in the ensuing text.

4.1. Phylogeographic patterns and Pleistocene history of Northwestern Argentina

While the evolutionary history of the biota of many temperate and polar regions has been directly influenced by glaciation and post-glacial range expansion (e.g. Patagonia – Zemlak et al., 2008; northern North America – Weir and Schluter, 2004; Europe and Asia – Hewitt, 1996), global climate change and shifting vegetation have also been posited to have influenced phylogeographic patterns of taxa in tropical and subtropical regions around the world (e.g. Bell et al., 2012; Fjeldså et al., 2012; Fouquet et al., 2012; Shirley et al., 2014).

Paleoclimatic data are relatively scarce for Northwestern Argentina, but those that do exist suggest a dynamic local climate with highly variable precipitation patterns both spatially and temporally during the Holocene (e.g. Trauth et al., 2003; Tchilinguirian and Morales, 2013), but extending throughout the Quaternary (e.g. May et al., 2011). Such climatic shifts have influenced the distribution and extent of montane habitats across the Andes (e.g. Cárdenas et al., 2011; González-Carranza et al., 2012), including the humid forests of the eastern flanks of the Central Andes from Bolivia south to Argentina (Williams et al., 2011).

The distributions of the Northern and Southern Lineages (Fig. 1) and the evidence that we found for recent population expansion (Fig. 2) in *M. rubriventris* are consistent with patterns reported for other Northwestern Argentine taxa across different habitats and altitudes. The authors of these studies have invoked a dynamic regional history of vicariance and range expansion driven by changing climate in the late Pliocene and Quaternary to explain contemporary phylogeographic and genetic patterns. For example, Koscinski et al. (2008) found well-supported mitochondrial DNA lineages distributed along a north-south axis in the treefrog, *Hypsiboas riojanus*, asserted to be evidence of Pleistocene range fragmentation and subsequent range expansion. D'Elía et al. (2008) reported that the grassland sigmodontine rodent, *Necromys lactens*, also has well-supported northern and southern mtDNA *cytochrome b* clades, which they interpreted as the signature of the range fragmentations driven by shifting distributions of high-altitude grasslands in the central Andes. In a mitochondrial DNA phylogenetic survey of a different sigmodontine taxon (*Akodon varius* group) from Paraguay, Bolivia and Argentina, Braun et al. (2008) diagnosed four Yungas forest lineages/species distributed in a latitudinal series from Bolivia to Catamarca Province, Argentina. The timing of divergence was suggested to span the Pliocene and Pleistocene, consistent with a vicariant regional history. *Podocarpus parlatorei* (Podocarpaceae) is a tree of the Yungas forests distributed from 1000 to 2000 m elevation. Using allozyme data, Quiroga and Premoli (2007) described genetically distinct northern, central, and southern populations for this species, with a diminution in genetic diversity from south to north and from low to high elevations, interpreted as evidence of northern expansion during episodes of cooling, and range contraction towards the highlands during periods of warming. Finally, Hensen et al. (2011) investigated the genetic structure of *Polylepis australis*, a tree endemic to mountainous regions of central and western Argentina, using AFLPs. For the Northwestern Argentine populations they concluded that patterns of genetic diversity reflected waxing and waning habitat connectivity and historical climate-driven range shifts.

Within the Southern Lineage there appears to be additional phylogeographic structure with a north-south split (Fig. 1B and C) that may correspond to contact zones that have been diagnosed for other anuran taxa in the region (e.g. *Hypsiboas riojanus* – Koscinski et al., 2008). Ultimately the contemporary phylogeographic patterns evident in *M. rubriventris* potentially reflect the interaction of complex orogenic and paleoclimatic factors in this region, with diversification potentially initiated during the Tertiary but evolutionary trajectories shaped by changing climate and vegetation throughout the Pleistocene (see Turchetto-Zolet et al., 2013).

4.2. Warning coloration and divergence among lineages

We see striking among-population variation in both dorsal and ventral color patterns (Fig. 3) and three clusters that emerge from our MDS analysis (Fig. 4). While these MDS clusters do make sense geographically, they do not mirror the deepest phylogeographic divisions that we found in our Bayesian analyses. Indeed our northernmost two populations (Huaico Chico and Nogalar de Los Toldos) are as phenotypically divergent from their geographically proximate counterparts (Canto del Monte, Baritú) within the northern clade, and from the admixed population (Cedral de Baritú), as they are from the southernmost populations (Tiraxi, El Cucho, La Almona and La Cornisa – see Fig. 4), differing clearly from the northern populations in the extent of dorsal black coloration and the speckled venter. That warning color patterns in *M. rubriventris* do not exhibit a strong relation to phylogeographic history is consistent with at least one other study that shows aposematic coloration to be evolutionarily malleable in anurans. Wang and Shaffer (2008) used ancestral character-state reconstruction on a mtDNA tree to document very frequent changes in color and pattern in the Central American populations of *Oophaga pumilio*, with multiple independent shifts to neutral dorsal coloration (see also Galindo-Uribe et al., 2014).

Strong divergent natural selection mediated by predation is thought to be one of the primary drivers of diversification among populations in aposematic species. Such effects have been noted in other aposematic frogs (e.g. *Dendrobates tinctorius* – Noonan and Comeault, 2009; *Oophaga pumilio* – Hegna et al., 2012). Predator-mediated selection on warning coloration in *M. rubriventris* may be more intense in northern compared to southern populations, favoring greater signal conspicuousness and less variable pattern (Joron and Mallet, 1998). If population differences in coloration arise because of divergence in local predators' ability to interpret aposematic signals, this implies that there may be differences among suites of predators in their capacity to learn avoidance of different morphs among our surveyed locales; in other words, different suites of local predators would exert differential selection on local morphs (Endler and Mappes, 2004; Mochida, 2011; Willink et al., 2014).

In brightly-colored, diurnal toads like *M. rubriventris*, mate choice may involve both acoustic and visual cues, providing another mechanism for divergence among populations. For example, Crothers and Cummings (2013) suggest that sexual and natural selection work synergistically to cause brighter morphs when toxicity is high in Bocas del Toro, Panama populations of *Oophaga pumilio*. Brusa et al. (2013) found two deeply-diverged, putatively reproductively-isolated lineages of the Costa Rican dart-poison frog, *O. granulifera*, and suggested that warning coloration plays a role in mating-system evolution and ultimately reproductive isolation at a secondary contact zone. However, there is little evidence to suggest that color variation in *M. rubriventris* is implicated in mate selection, or evidence for such synergistic effects. Studies of several populations show that color pattern and intensity do not correlate with toxicity (alkaloid composition), immune response (ectoparasite levels), or mate choice (Bonansea and Vaira, 2012; Bonansea, unpubl. data). Moreover, skin reflectance measurements of males and females of the same populations do not reveal the sexual dichromatism that would be expected under a scenario of sexual selection (Bonansea and Vaira, unpubl. data).

An alternative explanation for the geographical patterns in skin-color variation is selection for thermoregulatory efficiency. Dorsal color is expected to affect the amount of visible light absorbed. Dark-colored toads absorb all wavelengths of visible light, while lighter-colored toads reflect some wavelengths, reducing heat absorption during daylight hours when toads are most active. Sanabria et al. (2014) showed experimentally that dark *M. rubriventris* have consistently warmer body temperatures than light ones after exposure to an infrared lamp. However, it seems unlikely that the remarkable among-population differences in coloration could be attributable to thermoregulation alone – given that, throughout its range the species occupies a diverse range of habitats, including primary or secondary forests, open areas, and even human-modified landscapes (e.g. drainage ditches along dirt roads).

4.3. Conclusions and recommendations for future work

The montane forests of the eastern flanks of the Andes contain a diverse array of species that is under increased threat from climate change (Pacheco et al., 2010). Our study implies that this biota may be even more diverse than previously thought with long-recognized species containing cryptic lineages as we found for *M. rubriventris*, mirroring what has been found in other taxa. The marked phylogeographic patterns that we describe, with deepest divisions dating to the early Quaternary or late Pliocene, are probably the result of historical range fragmentation and subsequent expansion driven by Milankovitch cycles (Bennett, 1990) and shifting distributions of these southern montane forests. We found no apparent similarity between warning coloration and phylogeographic patterns. Patterns in warning coloration may thus reflect the signature of selection from local predation, perhaps coupled with the effects of sexual selection and thermoregulation – matters that should be considered for future research.

Future work would also benefit from increased sampling in the middle part of the Argentine distribution, and from the Bolivian portion of the species' range. Particular attention should be given to the Cedral de Baritú population, where there is admixture of haplotypes of the two lineages and perhaps some interesting consequences of contact between the two. Yungas Redbelly Toads breed in small ephemeral ponds in humid montane forest, and can reach high breeding densities in these wetlands. This implies that dispersal may be low despite suitable habitat being almost continuously distributed within their range. Mountain ridges and major rivers can be effective physical barriers for amphibians (Lougheed et al., 1999; Li et al., 2009; Turchetto-Zolet et al., 2013). While little is known of either juvenile or adult movement patterns in *M. rubriventris*, its distribution within the montane forest encompasses many major rivers and disjunct mountain ridges that might contribute to the observed phylogeographic structure that we document. Clearly further work on dispersal is needed.

We feel it premature to recommend taxonomic revisions for *M. rubriventris* despite the pronounced molecular and phenotypic divergence among sampled Argentine populations, and recommend more detailed quantification of ecological, genetic and morphological variation. Our study does provide some guidelines for conservation prioritization to mitigate loss of genetic diversity in *M. rubriventris*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.07.013.

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