

Of peaks and valleys: testing the roles of orogeny and habitat heterogeneity in driving allopatry in mid-elevation frogs (Aromobatidae: *Rheobates*) of the northern Andes

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ABSTRACT

Aim The frog genus *Rheobates* (Anura: Aromobatidae) is endemic to mid-elevations in the Colombian Andes. Our aim was to evaluate the role of the northern Andean high peaks and the low Magdalena Valley in creating barriers to dispersal and promoting vicariance within *Rheobates*.

Location Three disjunct mid-elevation flanks of the northern Colombian Andes: the eastern and western flanks of the Eastern Cordillera, separated by high mountain peaks, and the eastern flank of the Central Cordillera, separated from the Eastern Cordillera by the arid Magdalena Valley.

Methods We analysed mitochondrial and nuclear DNA sequences from 37 individuals of *Rheobates* to infer their phylogenetic relationships, divergence times and ancestral areas. We used species distribution models to test the role of climatic variables in determining the present geographical boundaries of the species.

Results The phylogeny of *Rheobates* is largely predicted by geography, with one population from the eastern flank of the Eastern Cordillera sharing a most recent common ancestor with all other *Rheobates* 21 million years ago (Ma; 95% credible interval 31–10 Ma). Populations in the Central Cordillera were monophyletic, isolated across the Magdalena Valley with a divergence time estimated at 13 Ma (19–7 Ma). We also detected one recent crossing of the Eastern Cordillera, along with unanticipated latitudinal phylogeographical structure within the western flank of the Eastern Cordillera. Environmental niche tests indicated that the Magdalena Valley and the high peaks of the Eastern Cordillera are significant environmental barriers for *Rheobates* populations.

Main conclusions Diversification in *Rheobates* coincided with the early Miocene uplift of Colombia's Eastern Cordillera and the hypothesized onset of aridity in the Magdalena Valley during the middle Miocene. Our study offers broad support for the classic mountain orogeny model of vicariant divergence, but suggests that lowland habitat heterogeneity also played a long-term role in promoting vicariance, despite a long history of palaeoclimatic fluctuations.

Keywords

Andean uplift, Colombia, divergence times, Miocene, molecular phylogenetics, niche conservatism, phylogeography, *Rheobates*, species tree, vicariance.

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INTRODUCTION

How geological and climatic factors determine the current geographical distributions of species is a fundamental

question in biogeography (Wallace, 1852; Wiens, 2004). Physical barriers such as mountains and rivers, as well as ecological gradients such as deserts and elevational transitions, may influence the distributional range of species

(Sexton *et al.*, 2009). The classic allopatric divergence scenario invokes physical barriers that divide the distributional range of a species, after which point the populations may accumulate genetic differences via mutation and genetic drift or local adaptation, potentially leading to the formation of distinct species (Wiley, 1988). Hypotheses concerning historical factors such as orogeny and climatic gradients may be evaluated by analysing spatial patterns of standing genetic variation, thereby revealing the evolutionary processes that have shaped the contemporary diversity and distributions of organisms (Bermingham & Moritz, 1998).

In studies of allopatric speciation, the dichotomy between physical and ecological barriers is of course false. Physical geographical features such as mountains act as barriers to lowland organisms and valleys act as barriers to highland organisms because a change in elevation engenders a change in ecological variables such as temperature and precipitation (Janzen, 1967). Thus, the intrinsic inability of organisms to adapt to the novel conditions (niche conservatism) found within an intervening geographical barrier may be a key factor in promoting allopatric speciation (Wiens, 2004; Wiens & Graham, 2005). A physical mountainous barrier may therefore also be regarded as an elevational thermal gradient (Janzen, 1967), highlighting the commonality of ecological and physical barriers in geographical models of divergence (Wang *et al.*, 2013). Recent evidence suggests that the environmental niches in some tropical taxa may be more conserved than those of temperate species (Hua & Wiens, 2010; Cadena *et al.*, 2012). Thus, tropical populations may experience greater opportunities for isolation and allopatric speciation across elevational gradients.

We investigate here the role of Andean peaks and valleys as long-term barriers to gene flow in a mid-elevation frog. The dynamic geological history and extensive environmental heterogeneity of the northern Andes (Gregory-Wodzicki, 2000) are often invoked to explain why this region is a recognized global hotspot of biodiversity and endemism for animals and plants (Hernández Camacho *et al.*, 1992; Kattan *et al.*, 2004; Cadena *et al.*, 2012), and provide an ideal context for testing hypotheses about the relative contributions of geological and environmental forces in shaping the current patterns of genetic diversity and species' range limits (Brumfield & Edwards, 2007). Two main historical mechanisms have been proposed to explain the diversification and high endemism in the Andes: orogenic processes that created novel highland environments while dividing lowland habitats (Bates & Zink, 1994), and Pleistocene climate fluctuations that may have episodically divided and reconnected populations along existing elevational gradients (Lynch *et al.*, 1997; Kattan *et al.*, 2004; Ramírez-Barahona & Eguarte, 2013). Relatively few studies, however, have looked at both processes simultaneously (Ribas *et al.*, 2007).

If orogeny is the main process that promoted diversification in the Andes, then major cladogenic events should coincide temporally with major episodes of Andean uplift. Alternatively, if Pleistocene climatic fluctuations were the main factor promoting diversification, then speciation events

should date to less than 2 Ma (Madrián *et al.*, 2013). Although Pleistocene glaciation left a clear phylogeographical imprint on the fauna and flora at high northern latitudes (Hewitt, 2000), the effects of these climatic fluctuations on tropical species are less clear. One hypothesis posits that as elevational zonation in vegetation types was displaced perhaps 800 metres downwards during the Last Glacial Maximum (LGM; Hooghiemstra & van der Hammen, 2004), mid- to high-elevation populations moved down and increased their connectivity (Ramírez-Barahona & Eguarte, 2013). During interglacials, these populations would have moved upwards again, thus creating cyclical opportunities for vicariant montane diversification (Rossetto *et al.*, 2012). This Andean 'species pump' model predicts that diversification should date to the Pleistocene.

Frogs of the genus *Rheobates* (Anura: Aromobatidae) are endemic to the Colombian Andes, occurring in cloud forests and disturbed habitats near streams and small ponds. Their distribution includes the eastern and western flanks of the Eastern Cordillera (eEC and wEC, respectively) and the eastern flank of the Central Cordillera (CC) at 350–2200 m a.s.l. (Rivero & Serna, 1988). The Central and Eastern cordilleras are separated by the valley of the Magdalena River, and the eEC and wEC are separated longitudinally by a continuous chain of high mountain peaks above 2500 metres (Fig. 1a). The disjunct distribution of *Rheobates* suggests that populations living in different cordilleras and on opposite flanks of the Eastern Cordillera are genetically isolated (Rivero & Serna, 1988; Bernal *et al.*, 2005).

The potential barriers dividing the distribution of *Rheobates* may have arisen in two historical periods: (1) during the Miocene, 26–6 Ma, when the Eastern Cordillera began its uplift and the Magdalena Valley formed as a lowland corridor of arid habitat (Egbue & Kellogg, 2012); and (2) during the Plio-Pleistocene events (5–1 Ma), characterized by dramatic cyclical climate change and rapid final uplift of the Eastern Cordillera (Hooghiemstra & van der Hammen, 2004). Linking the phylogeographical structure and divergence times within *Rheobates* with these two historical periods should elucidate the historical events that promoted the current patterns of genetic divergence (Fig. 1).

We evaluate the following non-mutually-exclusive hypotheses and their corresponding predictions concerning the orogenic and palaeoclimatic processes that may have influenced the diversification and distribution of *Rheobates*.

1. The high elevations of the Eastern Cordillera and low elevation of the Magdalena Valley acted as vicariant barriers in the early Miocene (Fig. 1b), splitting populations of *Rheobates* into the eEC, the wEC and the CC. This scenario predicts old, reciprocally monophyletic clades within each flank (Fig. 1c), with deep genetic discontinuities corresponding to the Magdalena Valley and the high ridges of the Eastern Cordillera. These predictions assume that the fluctuations in vegetational zonation caused by Pleistocene climate changes were insufficient to allow the gene flow between flanks that could have erased the predicted phylogeographical pattern.

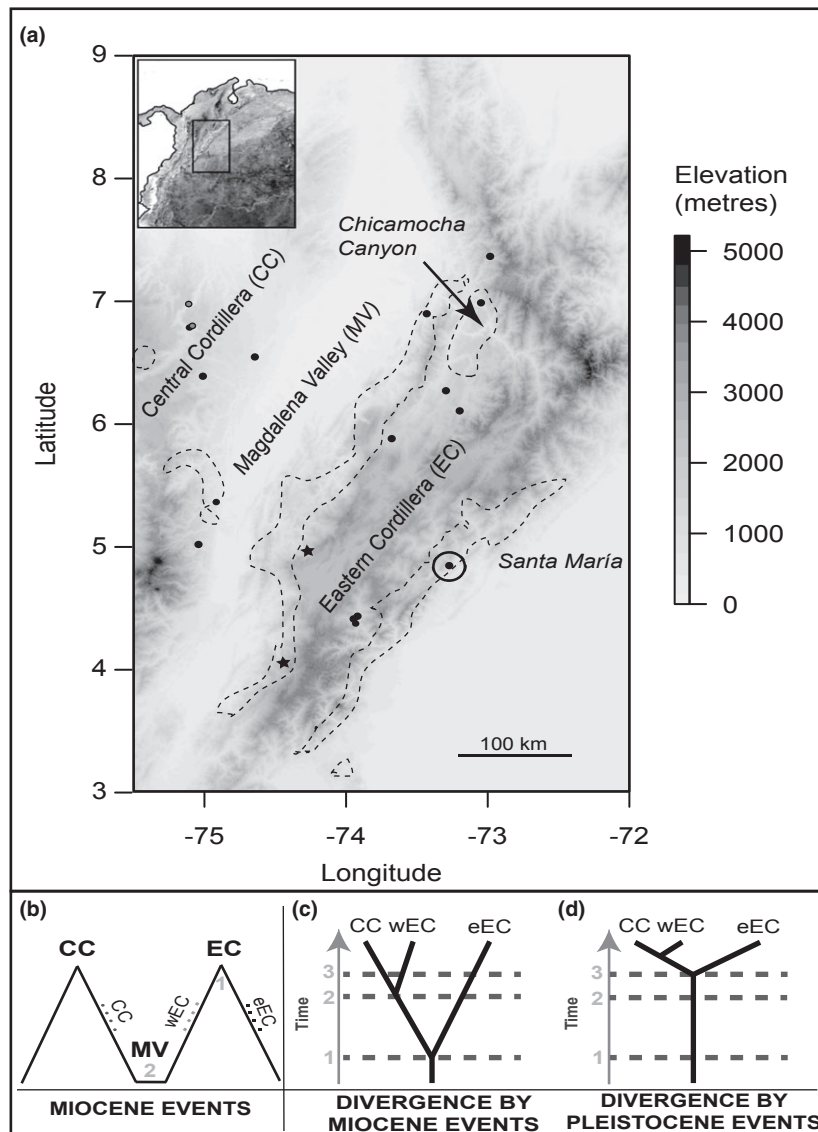


Figure 1 Localities for genetic samples of *Rheobates* in the Colombian Andes, along with graphical illustrations of temporal phylogenetic predictions made by *a priori* hypotheses addressed in this study. Abbreviations: CC, Central Cordillera; EC, Eastern Cordillera; wEC, western flank of the Eastern Cordillera; eEC, eastern flank of the Eastern Cordillera; MV, Magdalena Valley. Numbers correspond to potential vicariance events: 1, EC Miocene uplift; 2, MV aridification in the Miocene; and 3, Pleistocene glaciation events. (a) The 19 symbols (points and stars) indicate localities represented by genetic samples (see Appendix S1 for details). Stars indicate two wEC localities (San Francisco (AAV 167) and Icononzo (MAR 2175)) whose samples are phylogenetically placed close to the eEC clade, whereas the circle highlights Santa María (smEC), the phylogenetic sister to all other samples of *Rheobates*. Points in black and grey represent *R. palmatus* and *R. pseudopalmatus* localities, respectively. The thin dotted line delineates the disjunct distribution of the genus *Rheobates* according to the Global Amphibian Assessment (GAA) as provided by the International Union for Conservation of Nature Red List portal (Ramírez Pinilla *et al.*, 2010), including *R. pseudopalmatus* (recorded only at the type locality). Note that several of our sampling localities lie outside the estimated distribution. (b) Diagrammatic cross-sectional view running east-west through both cordilleras and the MV, where 1 and 2 indicate position of Miocene events potentially associated with divergence of *Rheobates* populations and 3 indicates Pleistocene glaciation events. (c) Hypothesis 1: genetic diversification of *Rheobates* is associated with Miocene events. (d) Hypothesis 2: diversification within *Rheobates* is associated with glaciation Pleistocene events.

2. Pleistocene climate fluctuations led to cycles of gene flow between adjacent flanks of the cordilleras, i.e. across the highlands of the Eastern Cordillera during interglacial periods, and across the Magdalena Valley during glacial maxima. This scenario predicts that divergences between cordilleran flanks should be younger than the onset of the Pleistocene

(Fig. 1d), and would be supported by the non-monophyly of populations on opposite sides of the present-day barriers, caused by recent dispersal.

3. The disjunct distribution and allopatric diversification in *Rheobates* has been promoted by niche conservatism (not niche divergence). This hypothesis predicts that the

environmental niche is similar amongst disjunct populations of *Rheobates*, and that the habitat characteristics of the intervening geographical barriers are outside the potential niche of *Rheobates*. In contrast, if disjunct populations have diverged within their ecological niches, such a result would instead support the possibility that local adaptation may have promoted or reinforced isolation, potentially in the absence of permanent barriers.

MATERIALS AND METHODS

Study system

The genus *Rheobates* (Anura: Aromobatidae) is apparently the only mid-elevation dendrobatoid present in two Andean cordilleras in Colombia, making it an excellent model to simultaneously test the role of Andean uplift and the formation of the Magdalena Valley on diversification. *Rheobates* contains two named species: *R. palmatus* (Werner, 1899) and *R. pseudopalmatus* (Rivero & Serna, 1995). *Rheobates palmatus* inhabits the eEC, wEC and CC, whereas *R. pseudopalmatus* is known only from the region of the type locality (Anorí, Antioquia) in the CC (Frost, 2013). The validity of *R. pseudopalmatus* has been questioned (Grant *et al.*, 2006) and we therefore offer a molecular perspective on its taxonomic status.

Tissue sampling and molecular methods

Thirty-seven tissue samples of *Rheobates* were obtained through field collections and donations from museums (see Table S1 in Appendix S1 of the Supporting Information), representing 19 localities that covered most of the geographical distribution of the genus (Fig. 1a). For phylogenetic analyses, we included as outgroups seven samples from three aromobatid species closely related to *Rheobates*, as demonstrated by previous family-level molecular phylogenetic analyses (Grant *et al.*, 2006; Santos *et al.*, 2009; Table S1).

Genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Fragments of the mitochondrial genes cytochrome oxidase I (COI; Hebert *et al.*, 2003) and the 16S ribosomal RNA gene (16S) were amplified by polymerase chain reaction (PCR) from 33 and 30 samples, respectively (Appendix S1). To complement the phylogenetic inference based on mitochondrial DNA (mtDNA), we amplified a fragment of the nuclear gene pro-opiomelanocortin (POMC) for 20 specimens from 15 locations. (See Table S2 in Appendix S1 for PCR primers and cycling conditions.)

Molecular phylogenetic analyses

Chromatograms were assembled and edited, and protein-coding genes were translated, with SEQUENCHER 4.7 (GeneCodes, Ann Arbor, MI, USA). Preliminary phylogenetic analyses (see below) were performed on each gene fragment

individually to check for any significant conflicting phylogenetic signal among genes (Appendix S2: Fig. S1).

Concatenated phylogenetic inference was conducted using maximum likelihood (ML) and Bayesian inference (BI). ML trees were constructed by stepwise addition with the tree bisection–reconnection (TBR) branch-swapping algorithm in RAxML 7.2.8 (Stamatakis *et al.*, 2008). ML support was assessed using the nonparametric bootstrap with 1000 pseudoreplicates (Felsenstein, 1985). For the Bayesian Markov chain Monte Carlo (MCMC) phylogenetic analyses, we used MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). To corroborate the concatenated gene tree results, we also estimated a species tree using coalescent analyses (Edwards, 2009) as implemented in *BEAST (Heled & Drummond, 2010). Major mtDNA clades within *R. palmatus* corresponding to disjunct geographical regions were treated as terminal taxa. (See Appendix S2 for an extended description of phylogenetic methods.)

Divergence time and ancestral area analyses

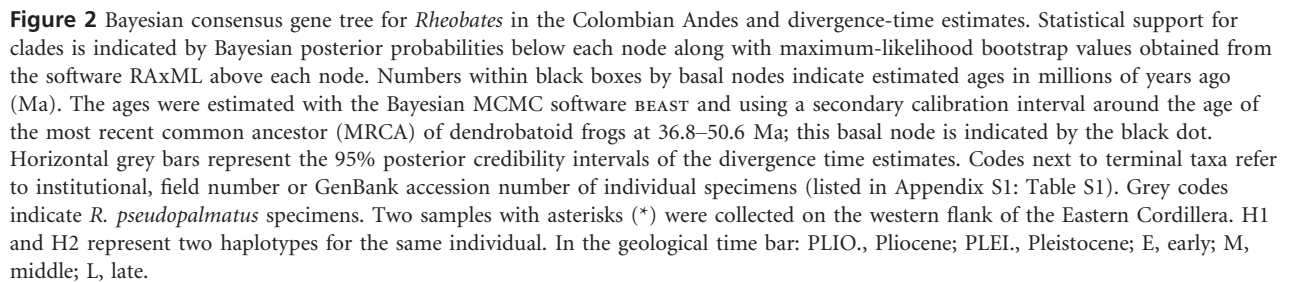
Divergence times were estimated using a Bayesian MCMC approach, as implemented in BEAST 1.7.4 (Drummond & Rambaut, 2007), under concatenated and species-tree approaches. Temporal information was incorporated by placing a secondary calibration interval around the age of the most recent common ancestor (MRCA) of dendrobatoid frogs (Aromobatidae plus Dendrobatidae, or dendrobatid frogs *sensu lato*), which was estimated in a previous large-scale analysis of anurans to be 43.7 Ma with a standard deviation (SD) of 6.9 Myr (Table S7 in Santos *et al.*, 2009; calibration details are given in Appendix S2).

To estimate historical patterns of dispersal and cladogenesis within *Rheobates*, we performed a likelihood analysis of the dispersal–extinction–cladogenesis (DEC) model of geographical range evolution as implemented in LAGRANGE 2013-05-26 (Ree & Smith, 2008). We estimated ancestral areas for relevant nodes in the concatenated Bayesian consensus phylogeny (Fig. 2) and defined three disjunct distributional areas *a priori*: CC, wEC and eEC (Fig. 1).

Niche modelling analyses

Environmental data and distribution modelling

To evaluate the potential strength of environmental barriers and the possible role of abiotic niche divergence in structuring populations of *Rheobates*, we used MAXENT 3.3.3e (Phillips *et al.*, 2010). Environmental niche models (ENM; Warren, 2012) were estimated using 97 independent localities (see Table S3 in Appendix S3) and 19 spatial bioclimatic variables from the WorldClim database with 30-arc-second resolution (Hijmans *et al.*, 2005). These 19 variables have been shown to be associated with long-term biogeographical barriers in other studies of Neotropical frogs (Wiens *et al.*, 2006). We assessed the accuracy of the distribution



niches, we performed a background similarity test between these disjunct areas using the R package `PHYLOCLIM` (Heibl & Calenge, 2009). The background similarity test asks whether differences in the ENMs of pairs of predefined regions are more similar than expected by chance, by comparing a focal region to pseudoreplicate models generated by randomly subsampling the background of the other region (Warren *et al.*, 2008). We used two metrics to quantify the similarity of niches, Schoener's *D* statistic for niche overlap (Schoener, 1968) and the similarity statistic *I* (Warren *et al.*, 2008). These statistics quantify predicted niche similarity and range from 0 (no overlap) to 1 (identical niche models).

Geographical barriers test

To test whether the geographical position of genetic discontinuities corresponds to potential geographical barriers we used the software BARRIER 2.2 (Manni *et al.*, 2004). This program generates a geometric network connecting all sampled haplotypes in order to identify boundaries where the differences between pairs of haplotypes are largest, using Monmonier's maximum-difference algorithm on a genetic distance matrix (Manni *et al.*, 2004). We estimated the robustness of each barrier by bootstrapping the DNA alignment (100 replicates) using SEQBOOT and DNADIST in the PHYLIP package (Felsenstein, 1989).

RESULTS

Phylogeny of *Rheobates*

Our three methods of phylogenetic inference recovered nearly identical topologies, with only minor differences. For simplicity, we present here the time-calibrated Bayesian consensus phylogeny obtained from the concatenated analysis (Fig. 2; individual gene trees and the species tree are presented in Figs S1 & S2 in Appendix S2). The monophyly of the genus was strongly supported in all analyses (ML bootstrap, 100%; posterior probability in gene tree analysis, $PP_{GT} = 1$; posterior probability in species tree analysis, $PP_{SPT} = 0.99$; Fig. 2, Fig. S2). Within *Rheobates*, we found three well-supported clades that were consistent with the geographical distribution of the samples, with the notable exception of two lineages (AAV 167 and MAR 2175) occurring in eEC. Samples from Santa María (Fig. 1) comprised the sister lineage to all other *Rheobates* (Fig. 2). A well-defined clade of CC samples, including the nominal *R. pseudopalmaris*, formed the sister clade to all other EC

samples, excluding Santa María (ML bootstrap, 100%; $PP_{GT} = 1$; $PP_{SPT} = 0.99$). The third clade contained all remaining samples from the EC (ML bootstrap, 94%; $PP_{GT} = 0.99$; $PP_{SPT} = 0.92$; Fig. 2). Within this EC clade, we recovered three principal lineages consisting of the northern (ML bootstrap 100%; $PP_{GT} = 1$) and southern (ML bootstrap, 95%; $PP_{GT} = 1$) ends of the wEC, plus a clade made up of the remaining samples of the eEC (ML bootstrap, 92%; $PP_{GT} = 1$). The eEC was recovered as more closely related to the southern than the northern clade of the wEC, but with low support (ML bootstrap, 48%; $PP_{GT} = 0.82$; Fig. 2). Relationships among the three EC lineages were also poorly supported in the species tree analysis ($PP_{SPT} = 0.55$; Fig. S2).

Divergence times and ancestral-area estimation

The estimated divergence times at basal nodes were similar in the concatenated gene tree and species tree analyses (Table 1, and Fig. 2 cf. Appendix S2: Fig. S2). For brevity, we will only mention a few gene-tree results here. The MRCA of *Rheobates* and its sister lineage *Anomaloglossus* + *Aromobates* were dated to the late Eocene (35 Ma), with the 95% posterior credibility interval (CI) stretching from the early Eocene to the early Miocene (48–22 Ma; Fig. 2). The crown age of *Rheobates*, separating Santa María (smEC) from the rest of *Rheobates* was estimated at 21 Ma, with the 95% CI ranging from the late Eocene to the middle Miocene (35–12 Ma). The MRCA of the Central Cordillera clade and its sister clade in the Eastern Cordillera was estimated at 13 Ma, with the 95% CI ranging from the early to late Miocene (20–7 Ma; Fig. 2), a period congruent with the formation of the modern Magdalena Valley and presumably its aridification (Kay & Madden, 1997; Horton *et al.*, 2010). Divergences among the remaining lineages of the Eastern Cordillera took place between 9 and 3 Ma (Table 1, Fig. 2).

Table 1 Estimated divergence times in millions of years ago (Ma) for the principal clades of *Rheobates* in the Colombian Andes along with climatic and geological events temporally and potentially causally associated with these dates. Divergence time analyses were based on three gene sequence fragments: *COI* ($n = 33$), *16S* ($n = 33$) and *POMC* ($n = 20$). Clades are defined by the most recent common ancestor (MRCA) of pairs of lineages, and time estimates represent crown age of said clade. A cross in the age column indicates the clade was not recovered in that analysis (Fig. 2, Appendix S2: Fig. S2). Abbreviations of clades: smEC, Santa María; CC, Central Cordillera; EC, Eastern Cordillera; eEC, eastern flank of the Eastern Cordillera; wEC, western flank of the Eastern Cordillera.

Clade	Crown age (Ma) based on gene tree	Crown age (Ma) based on species tree	Event in Earth history	Time (Ma)	Source
MRCA of <i>Rheobates</i>	31.07–10.83	35.81–12.10	Eastern Cordillera uplift – Usme formation (reaching 25–40% of its current height)	38–33	Ochoa <i>et al.</i> (2012)
MRCA of CC + EC (i.e. sister to smEC)	19.55–7.43	21.14–6.16	Magdalena Valley aridification	15–10	Gregory-Wodzicki (2000)
north-wEC + (eEC + south-wEC)	12.85–4.65	×	Eastern Cordillera uplift (40% of current elevation)	10–2	Gregory-Wodzicki (2000)
eEC + south-wEC	10.74–4.10	×			
eEC + wEC	×	13.5–4.14			
north-wEC + south-wEC	×	9.85–1.17			

At highly nested nodes, some discrepancy between concatenated and species-tree approaches was observed, with the latter yielding more recent dates (Fig. S2).

Ancestral-area reconstruction based on a DEC model for geographical range evolution failed to provide statistical support for the area of the MRCA of *Rheobates*, but did resolve with high support other important nodes such as smEC, CC and wEC (Appendix S2: Fig. S3). More precisely, DEC suggested that the most likely ancestral area for the wEC + eEC clade was on the western flank (Fig. S3).

Test of niche conservatism

ENM analyses for complete locality data for *Rheobates*, as well as subsets thereof, were characterized by high AUC values ($AUC > 0.8$; Appendix S3: Fig. S4). Among the 19 WorldClim variables, the mean temperature of the warmest quarter (BIO10) made the largest contribution to ENMs for three clades (all *Rheobates*, 40.1%; all EC, 46.1%; wEC alone, 29.5%), whereas temperature seasonality (BIO4) and the precipitation of the coldest quarter (BIO19) explained substantial variation in CC (36.4%) and eEC (34.2%), respectively (Table 2).

The background similarity test revealed a statistically significant similarity of abiotic niches between the CC clade and the complete EC clade, with values of Schoener's *D* and

the similarity statistic *I* significantly higher than the null distributions obtained from 100 randomizations ($P < 0.01$; Table 3, Appendix S3: Fig. S5). Tests between the CC clade and the wEC clade were marginally significant, but no other pairwise comparisons between clades were significant (Table 3, Fig. S5). Thus, the prediction of the niche conservatism hypothesis was supported specifically at broader geographical and phylogenetic scales.

Geographical barrier test

We obtained congruent BARRIER results from each gene (*COI*, *16S* and *POMC*) separately and using the concatenated alignment (Fig. 3, Appendix S3: Fig. S6). Here, we show the results of the more informative mtDNA dataset. We found three significant genetic discontinuities (bootstrap 100%; Fig. 3), the first separating the Santa María population (smEC) from all other *Rheobates*. A second barrier isolated CC from EC, geographically corresponding to the low elevations of the Magdalena Valley. The third barrier separated the northern and southern clades along the wEC, and is geographically close to the arid Chicamocha Canyon, which reaches elevations below 400 m (Fig. 1). We found no robust genetic discontinuity that separated the eastern and western flanks of the EC.

Table 2 Percentage contribution of WorldClim variables to predicting the distribution of clades of *Rheobates* in the Colombian Andes. Within each subset of the locality data (column), the sum of the bold values explained at least 80% of the environmental variation amongst localities.

Code	WorldClim variable	Geographical range of clade being modelled (number of georeferenced localities)				
		All <i>Rheobates</i> localities (<i>n</i> = 97)	Central Cordillera (<i>n</i> = 20)	Eastern Cordillera (<i>n</i> = 77)	Western flank of Eastern Cordillera (<i>n</i> = 54)	Eastern flank of Eastern Cordillera (<i>n</i> = 23)
BIO1	Annual mean temperature	0.0	0.0	0.1	0.0	16.8
BIO2	Mean diurnal temperature range: mean of monthly (max. temp. – min. temp)	0.2	11.2	0.5	0.0	1.2
BIO3	Isothermality (BIO2 / BIO7) ($\times 100$)	5.4	16.3	1.9	1.3	1.6
BIO4	Temperature seasonality (standard deviation $\times 100$)	2.7	36.4	3.6	16.1	4.6
BIO5	Maximum temperature of the warmest month	0.0	0.0	0.0	0.5	1.3
BIO6	Minimum temperature of the coldest month	1.9	2.4	5.1	0.0	24.5
BIO7	Annual temperature range (BIO5 – BIO6)	0.1	0.0	1.0	0.4	3.2
BIO8	Mean temperature of the wettest quarter	1.8	0.0	0.5	0.4	0.0
BIO9	Mean temperature of the driest quarter	0.0	0.0	0.0	0.0	0.0
BIO10	Mean temperature of the warmest quarter	40.1	0.0	46.1	29.5	0.0
BIO11	Mean temperature of the coldest quarter	0.0	0.0	0.0	0.0	0.0
BIO12	Annual precipitation	0.1	0.3	0.0	0.0	0.0
BIO13	Precipitation of the wettest month	1.3	14.3	2.8	0.3	1.7
BIO14	Precipitation of the driest month	10.4	14.6	6.0	13.9	0.8
BIO15	Precipitation seasonality (coefficient of variation)	22.8	3.8	16.1	16.0	9.8
BIO16	Precipitation of the wettest quarter	3.5	0.0	4.2	13.8	0.3
BIO17	Precipitation of the driest quarter	3.3	0.0	3.7	5.2	0.1
BIO18	Precipitation of the warmest quarter	1.0	0.7	1.3	2.1	0.0
BIO19	Precipitation of the coldest quarter	5.5	0.0	7.0	0.4	34.2

Table 3 Background similarity tests of niches occupied by *Rheobates* clades in the Colombian Andes. Asterisks (*) indicate statistically significantly ($\alpha = 0.05$) similar niches as determined by randomization test (see Materials and Methods and Appendix S3: Fig. S5 for details). Niche similarity is predicted by the hypothesis of niche conservatism. 'Null' indicates the $1 - (1 - \alpha / 2)$ and $(1 - \alpha / 2)$ quantiles of the null distribution obtained by 100 randomizations and used to evaluate significance of observed D and I statistics. The numbers of georeferenced localities within each region was: Central Cordillera, 18; Eastern Cordillera, 88; Eastern Cordillera western flank, 63; and Eastern Cordillera eastern flank, 25.

Pairwise comparison		Background similarity test					
		Schoener's D statistic			Similarity statistic I		
Model X	Model Y	D	X vs. random Y (null)	Y vs. random X (null)	I	X vs. random Y (null)	Y vs. random X (null)
Central Cordillera	Eastern Cordillera	0.52*	0.30, 0.40	0.250, 0.48	0.79*	0.60, 0.71	0.53, 0.71
Eastern Cordillera western flank	Eastern Cordillera eastern flank	0.31	0.299, 0.373	0.207, 0.311	0.57	0.603, 0.683	0.468, 0.60
Central Cordillera	Eastern Cordillera western flank	0.40*	0.335, 0.40	0.174, 0.323	0.70*	0.639, 0.711	0.418, 0.611
Central Cordillera	Eastern Cordillera eastern flank	0.39	0.234, 0.397	0.311, 0.414	0.69	0.519, 0.712	0.616, 0.731

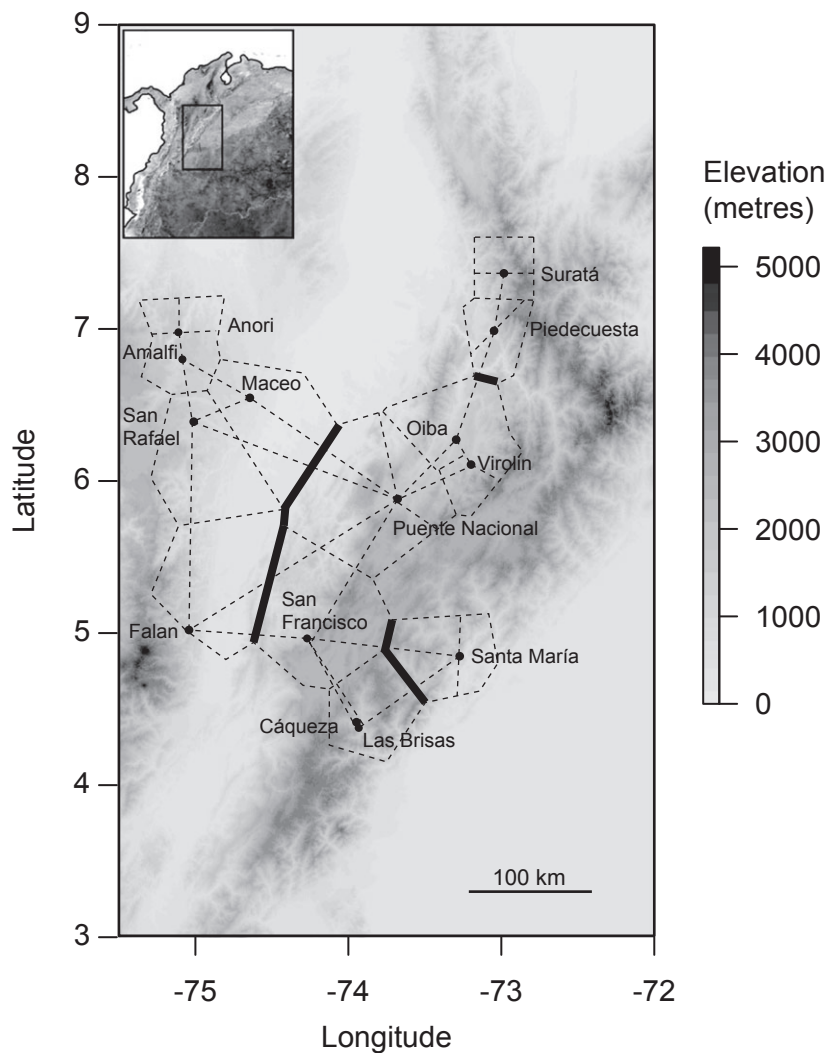


Figure 3 Geographical barrier test for *Rheobates* in the Colombian Andes. The results of the more informative mtDNA dataset (*COI* localities) are shown. Solid black lines show the three main genetic discontinuities (bootstrap = 100%). Dotted lines, Delaunay triangulation and Voronoi tessellation.

DISCUSSION

Our concatenated and species-tree phylogenies manifested a close spatial and temporal concordance between the history

of lineages within *Rheobates*, their geographical distribution, and the geological and environmental history of the northern Andes. With two exceptions (see below), the geographically disjunct populations corresponded to well-supported clades

that were geographically separated during the Miocene by the highest elevations of the Andes and the dry Magdalena Valley. This result supports the topological and temporal predictions of our first *a priori* hypothesis – that the high peaks of the Eastern Andes and the depth of the Magdalena Valley acted as vicariant barriers dividing *Rheobates* into three clades: eEC, wEC and CC (Fig. 1a,b). We found two notable exceptions to this prediction, however. First, we uncovered a second, cryptic and highly divergent population: the smEC clade, which formed the sister-group to the rest of *Rheobates* (Fig. 2). Second, some samples from eEC were closely related to populations from wEC, suggesting that at least one lineage may have recrossed the Eastern Cordillera following the primary vicariant separation of ancestral populations on the two sides of the Cordillera.

Cryptic diversity within *Rheobates*

The genus *Rheobates* is currently composed of two species: *R. palmatus* and *R. pseudopalmatus*. Our mitochondrial and nuclear DNA data indicate that the individuals at or near the type locality of *R. pseudopalmatus* (Fig. 2, Appendix S2) form part of a relatively low-diversity CC clade along with samples of *R. palmatus*. Previous authors have suggested that *R. pseudopalmatus* may be conspecific with *R. palmatus* (Grant *et al.*, 2006) and our data support this hypothesis and also offer an alternative solution to this taxonomic problem. Given that the CC lineage last shared a common ancestor with its sister clade in the EC an estimated 13 Ma (95% CI, 21–6 Ma; Table 1), a synapomorphy may exist for the CC lineage, such that *R. pseudopalmatus* could be redescribed to encompass the complete CC lineage, restricting *R. palmatus* to the Eastern Cordillera.

Our phylogenetic results also clearly show that the Eastern Cordillera is not monophyletic, thus providing evidence for the existence of a second cryptic species within *Rheobates*. Frogs from Santa María, Boyacá Province (smEC; Fig. 2, Appendix S2), form the sister lineage to all other *Rheobates* populations, having diverged approximately 22 Ma (11–36 Ma). The smEC lineage shows a Kimura two-parameter (K2P) distance much higher than the 10–12% threshold suggested for candidate species in frogs based on *COI* (Crawford *et al.*, 2010). Thus, although we do not formally propose any taxonomic changes here, we suspect that future work will reveal three species of *Rheobates*.

The role of geographical barriers within *Rheobates*

Physical landscape features such as rivers may directly divide populations by physically preventing dispersal (Ribas *et al.*, 2012). More commonly, perhaps, geographical barriers split populations by creating environmental conditions within the barrier that lie outside the species' ecophysiological tolerances (Janzen, 1967; Wiens & Graham, 2005; Crawford *et al.*, 2007). The most striking environmental barrier uncovered here corresponds to the low elevations of the relatively arid

Magdalena Valley. Arid river systems are important geographical barriers in several Andean bird species (Weir, 2009), but their long-term effects on amphibian phylogeography are largely unknown. *Rheobates* is normally found along small streams and ponds in montane humid areas, although a few lowland records exist for the Magdalena Valley (Fig. 1a). Given the history of Pleistocene climatic fluctuations (Hooghiemstra & van der Hammen, 2004), we find it surprising that this lowland environmental barrier has apparently shut off dispersal by *Rheobates* since the Miocene (21–6 Ma; Table 1). This divergence time coincides with the aridification of the Magdalena Valley, and may therefore have been caused indirectly by the rise of the EC (Gregory-Wodzicki, 2000; Horton *et al.*, 2010). Thus, despite a long history of environmental fluctuations, and despite the fact that *Rheobates* can be found at elevations as low as 350 m, the Magdalena Valley has been a continuous barrier since around 13 Ma (minimum 6 Ma). One important implication of this finding may be that, although the compression of habitat types along elevation gradients during Pleistocene maxima may have pushed high-elevation species downwards (Hoorn *et al.*, 2010), mid-elevation species are unlikely to have entered the lowlands, because the Pleistocene maxima also increased aridity (Ramírez-Barahona & Eguiarte, 2013).

Although we argue that the long-term barrier effect of the Magdalena Valley is likely to be due to its harsh, arid environment, it could also be simply a riverine barrier (Wallace, 1852; Ribas *et al.*, 2012) caused by the Magdalena River. We therefore used ENM analyses to ask whether aridity is in fact the most likely environmental variable that prevented colonization by *Rheobates*. The bioclimatic variable with highest weight in the ENM of all *Rheobates* and in the subsets EC and wEC was BIO10, the mean temperature of the warmest quarter. For the CC localities, BIO4 (temperature seasonality) was the strongest predictor (Table 2; cf. Wiens *et al.*, 2006). Thus, whereas aridity may be the primary environmental challenge for lowland rain-forest frogs (Navas *et al.*, 2004; Prates & Navas, 2009), montane species may be more limited by high temperature than low precipitation (see below).

A second, unexpected yet highly supported genetic discontinuity within *Rheobates* corresponds roughly to a north–south split centred on another lowland arid region, the Chicamocha Canyon (Fig. 1). This landscape feature apparently divided the EC into northern and southern clades around 8 Ma in the gene tree (12–5 Ma; Figs 2 & 3) and 5 Ma in the species tree (9.85–1.17 Ma; Fig. S2). This barrier could have been caused by the aridity and high temperatures associated with the low canyon floor, as suggested for the Magdalena Valley barrier.

Our third barrier is the EC itself, which may be both the oldest landscape feature discussed here and the most recently penetrated barrier. We argue that the rise of the EC (Gregory-Wodzicki, 2000; Hoorn *et al.*, 2010; Horton *et al.*, 2010) separated the smEC lineage from the rest of *Rheobates* some 22 Ma (11–36 Ma; Table 1), as predicted by our

orogeny hypothesis (Fig. 1c). The maximum elevation for *Rheobates* is around 2200 m, just below the uninterrupted Cocuy ridge at ≥ 2500 m. This ridge starts near Ocaña (in the department of Norte de Santander) and extends 600 km south to the depression of Paso de Andalucía in the southern portion of the department of Meta (Rivero & Serna, 1988). The wEC and eEC clades are separated by 10% K2P genetic divergence at *COI*, and diverged an estimated 7 Ma (14–4 Ma).

The EC barrier is, however, likely to have been breached recently. We note that the members of the eEC clade from the western slope are the two most basal samples: they are paraphyletic with respect to a nested, monophyletic group of five samples on the eastern slope (ANDES-A 1475, ANDES-T 2356, ANDES-T 2354, ANDES-A 1474 and ANDES-T 2355). The likelihood analysis of geographical range evolution suggests that the ancestor of the wEC and eEC clades inhabited the western slope, with the ancestor of the five eastern slope samples having crossed over to the eastern slope only very recently, perhaps through the Paso de Andalucía depression (Fig. S3). This scenario was predicted by the hypothesis that Pleistocene climate fluctuations permitted frogs to cross what currently appear to be barriers (Fig. 1d), and the gene-tree analysis suggests a Pleistocene time frame for this event (Fig. 2).

Alternatively, the eEC clade could have been split vicariantly around 3 Ma (Fig. 2) and, given large effective population sizes relative to the age of splitting, stochastic sorting of mtDNA lineages may not yet have been completed (Moore, 1995). Paraphyly of the western samples would be unlikely after roughly three coalescent time units (Rosenberg, 2003), so the effective population sizes would need to be greater than 1 million, or 4 million at nuclear loci. We find these parameter values rather high (Crawford, 2003), and thus suggest that the EC barrier was indeed penetrated in the Pleistocene, perhaps facilitated by climate fluctuations.

Niche conservatism versus niche divergence

Vicariant speciation requires environmental heterogeneity that isolates populations, coupled with an inability of populations to adapt to novel environmental conditions, or niche conservatism (Wiens, 2004; Wiens & Graham, 2005), our third hypothesis (see Introduction). Alternatively, lineages may diversify through local adaptation into distinct, potentially adjoining environments, such as along a gradient (Doebeli & Dieckmann, 2003). We used ENM to test for a pattern of niche conservatism in populations along each flank of the CC and EC. The background similarity test indicated that the CC and EC clades occupy similar niches, as do the wEC and CC clades, as predicted by niche conservatism (Table 3, Fig. S5), supporting the idea that the Magdalena Valley is an environmental barrier to which the CC and EC populations have not been able to adapt. Niche conservatism is not uncommon in frogs, and may even be responsible for driving biogeographical patterns over long stretches of evolutionary time (Wiens *et al.*, 2006).

CONCLUSIONS

We found that *Rheobates* is a highly genetically structured and deeply divergent genus, and that two types of geographical features may explain such divergence: high peaks and low valleys. Weir (2009) suggested that high-elevation barriers are less frequent than low-elevation and riverine barriers in Andean birds, because the major uplift activity of the northern Andes was too recent for strong genetic divergences to accrue across such barriers, whereas low-elevation barriers have had more time to generate divergence. We observed a similar pattern in *Rheobates*, where the low-elevation geographical barriers, the Magdalena Valley and perhaps the Chicamocha Canyon, seem to have produced deeper genetic divergences than across the high-elevation Andean peaks. The asynchronous uplift of the EC produced seemingly contradictory patterns: the isolation of the smEC lineage in the Miocene as well as the apparent Pleistocene crossing by a wEC lineage to the eastern flank. Although much vertebrate diversification in the northern Andes seems to have occurred during the late Pliocene–early Pleistocene, *Rheobates* appears far older. The inference that the Magdalena Valley has been a continuous ecological barrier since 13 Ma supports the hypothesis that valleys are stronger barriers than peaks for montane taxa (Weir, 2009), and may provide a historical explanation for the almost complete biogeographical turnover in amphibian species between the CC and the wEC (Kattan *et al.*, 2004).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Description of genetic samples (Table S1) and primers (Table S2) used to generate DNA sequence data used in phylogenetic analyses.

Appendix S2 Phylogenetic inference based on single genes, extended phylogenetic methods, results obtained from species tree inference, and results of ancestral area reconstruction obtained by likelihood analysis of a dispersal–extinction–colonization (DEC) model of geographical range evolution (includes Figs S1–S3).

Appendix S3 Description of geographical data, extended methods and results of the niche modelling analyses (includes Table S3 and Figs S4–S6).

DATA ACCESSIBILITY

DNA sequence data from the 37 tissues of *Rheobates* and two outgroups (*Allobates femoralis* and *A. ranooides*) are available in the BoLD Systems page under Project *Rheobates* phylogeography and in the GenBank database under accession numbers KJ130660–KJ130747 and KJ871616–KJ871619.

BIOSKETCH

The **Crawford Lab** was formed in 2009 at the Universidad de Los Andes, focusing on evolutionary genetic studies to elucidate processes promoting or reducing biodiversity in the Neotropics. Current research is focused on the interaction between organismal biology and the environment and its influence on the spatial and historical patterns of biodiversity. Students integrate multiple approaches including phylogeography, niche modelling, experimental ecophysiology and transcriptomics. Astrid Muñoz-Ortiz obtained her PhD at Los Andes studying the evolutionary genetics of *Heliconius* butterflies, and recently joined the faculty at the Universidad de La Salle. Her primary interest is in speciation genetics. This work was submitted to the Universidad de los Andes by Álvaro Velásquez-Álvarez in partial fulfilment of the requirements of the master's degree.

Author contributions: A.A.V.-A. and A.J.C. conceived the ideas; A.M.-O. and A.A.V.-A. collected the data; A.M.-O., A.A.V.-A. and C.E.G. analysed the data; and A.M.-O., C.E.G. and A.J.C. led the writing.

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