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RESEARCH PAPER

A test of the integrated evolutionary speed hypothesis in a Neotropical amphibian radiation

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ABSTRACT

Aim The evolutionary speed hypothesis is a mechanistic explanation for the latitudinal biodiversity gradient. The recently extended integrated evolutionary speed hypothesis (IESH) proposes that temperature, water availability, population size and spatial heterogeneity influence the rate of molecular evolution which, in turn, affects diversification. However, the evidence for some of the associations predicted by the IESH is not conclusive, and in some cases is contradictory.

Location The Neotropics.

Methods Using a comparative Bayesian method we tested the following predictions of the IESH: the association between the rate of molecular evolution and temperature (and elevation and latitude, as proxies), water availability (using precipitation and relative humidity as proxies), productivity and rate of diversification. We also accounted for the potential confounding effects of body size and UVB radiation. We tested these predictions separately in mitochondrial and nuclear genes.

Results Substitution rates of mitochondrial and nuclear genes were positively associated with temperature and negatively with elevation, while only the mitochondrial coding gene rate was associated with UVB radiation. However, when controlling for temperature, the association between substitution rate and elevation and UVB radiation disappeared, while a negative association with precipitation emerged. Moreover, diversification events were positively correlated with the rate of molecular evolution but only in mitochondrial genes.

Main conclusions Our results support two key predictions of the IESH. They highlight the important association between rate of molecular evolution and temperature within a recently diverged group and also confirm the positive association between molecular evolution and diversification rate, although only in mitochondrial genes. However, the lack of association between diversification and temperature and the low effect size of the relationship between substitution rates and diversification in mitochondrial genes emphasize the important role other factors, such as time, spatial heterogeneity and population size might have in the origin and maintenance of the latitudinal biodiversity gradient.

Keywords

Amphibians, diversification, evolutionary speed hypothesis, latitudinal biodiversity gradient, molecular evolution, substitution rate, temperature.

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INTRODUCTION

The striking difference in biodiversity between tropical and temperate regions is probably the oldest pattern described in ecology (Hawkins, 2001). Despite previous attempts to explain the latitudinal biodiversity gradient, the underlying drivers remain elusive (Hillebrand, 2004; Jablonski *et al.*, 2006; Mittelbach *et al.*, 2007). There is nonetheless a certain consensus that this pattern must be the result of latitudinal differences in the rates of speciation, extinction and migration, or any combination thereof (Dowle *et al.*, 2013). Many studies have attempted to explain the latitudinal biodiversity gradient by focusing on the factors that influence the rate of molecular evolution and its effect on the rate of diversification (reviewed in Dowle *et al.*, 2013). Among them, the explanation that has attracted most attention is the 'evolutionary speed hypothesis'. Rensch (1959) suggested that organisms in warmer environments have shorter generation times, which increases the pace of selection and therefore evolutionary speed. The idea was extended by Rohde (1992), who proposed that organisms in warmer environments have a higher rate of molecular evolution via shorter generation times and a higher mutation rate that lead to faster adaptation and differentiation. Recently, Gillman & Wright (2014) proposed an integrative version of the hypothesis recognizing that the relationship between temperature and rate of molecular evolution is not monotonic, and that in hot climates limited water availability may in fact lead to reduced rates of molecular evolution either directly (e.g. Goldie *et al.*, 2010) or indirectly through its effect on primary productivity. The latter is also proposed to influence the rate of molecular evolution based on the fact that it is a good predictor of species richness (Gillman & Wright, 2014). In addition, the effects of population size and spatial heterogeneity, which can favour isolation of populations as well as the potential for new mutations to be fixed, were also incorporated into the hypothesis. These additions to the original evolutionary speed hypothesis resulted in the 'integrated evolutionary speed hypothesis' (hereafter the IESH; Gillman & Wright, 2014). The predicted end result of the faster rate of molecular evolution is an increased rate of speciation, and therefore the IESH is proposed as a mechanistic explanation of the higher diversity observed in tropical latitudes (Gillman & Wright, 2014).

A diversity of taxa, including plants, invertebrates, fishes, birds, mammals, reptiles and amphibians, show a higher rate of molecular evolution in warmer environments, apparently supporting the IESH (e.g. Davies *et al.*, 2004; Gillooly *et al.*, 2005; Gillman *et al.*, 2009, 2012; Wright *et al.*, 2010; Lourenço *et al.*, 2013). Nonetheless, a limitation of these studies is that in most of them elevation or latitude were used as proxies for temperature (but see Davies *et al.*, 2004; Gillooly *et al.*, 2005), and while elevation and latitude can reflect differences in temperature they are also associated with other factors that may influence the rate of molecular evolution (e.g. UV radiation, oxygen stress, population size, seasonality). Hence, although available evidence is consistent with the predicted positive association between temperature and rate of molecular evolution, it is not conclusive (Gillman & Wright, 2014). Finally, and perhaps most importantly, only a single study has simultaneously tested two of the key predictions of the IESH (Davies *et al.*, 2004): the increase in rate of molecular evolution in warmer environments and the association between the rate of molecular evolution and diversification. This study did not find support for the association between the rate of molecular evolution and diversification, casting doubts on the generality of the IESH. Furthermore, to our knowledge, only one study to date has found the predicted positive relationship between water availability and rate of molecular evolution (Goldie *et al.*, 2010), while the predicted positive association between the rate of molecular evolution and productivity remains untested.

Here, using the Neotropical amphibian family Centrolenidae as our model system, we tested four of the key predictions of the IESH and also analysed the effects of commonly used proxies and potential confounding factors. More specifically we tested the following predicted positive associations between the rate of molecular evolution and: (1) temperature, (2) water availability (measured as precipitation and relative humidity), (3) primary productivity, and (4) rate of diversification. In addition we tested the effect of variables that are either commonly used as proxies for temperature, i.e. latitude and elevation, or that could possibly have confounding effects on the predicted relationships, i.e. UVB radiation and body size. We used three proxies for the rate of molecular evolution: the synonymous and non-synonymous substitution rates (henceforth d_S and d_N, respectively), and the ratio of non-synonymous to synonymous substitutions (d_N/d_S, hereafter ω). We tested all associations in both nuclear and mitochondrial genes using a whole-tree method (Lartillot & Poujol, 2011).

Centrolenidae, commonly known as glass frogs because of their transparent or semi-transparent venter, is a diverse group of arboreal frogs comprising more than 140 species included in 12 genera, originating from a relatively recent radiation (23.4 Ma; 95% highest posterior density 19.6–28.82 Ma) (Guayasamin *et al.*, 2009; Castroviejo-Fisher *et al.*, 2014). The family presents a wide Neotropical distribution, from southern Mexico to Bolivia with an isolated group in south-eastern Brazil and north-eastern Argentina (Fig. 1) (Guayasamin *et al.*, 2009; Castroviejo-Fisher *et al.*, 2014). Centrolenids are also ecologically diverse as they are found in very distinct habitats, from sea level to high mountain ranges (3300 m a.s.l.) (Guayasamin *et al.*, 2009). Furthermore, centrolenids are highly dependent on water as females lay their eggs on leaves over-hanging streams and when the eggs hatch, tadpoles fall into the water where they complete their development. Recently, a well-resolved molecular phylogeny of Centrolenidae combining mitochondrial and nuclear genes and with high species coverage has become available (Castroviejo-Fisher *et al.*, 2014). Hence, Centrolenidae offers an excellent model system to analyse the influence of environmental energy on the rate of molecular evolution in a diverse but recently diverged group of species.

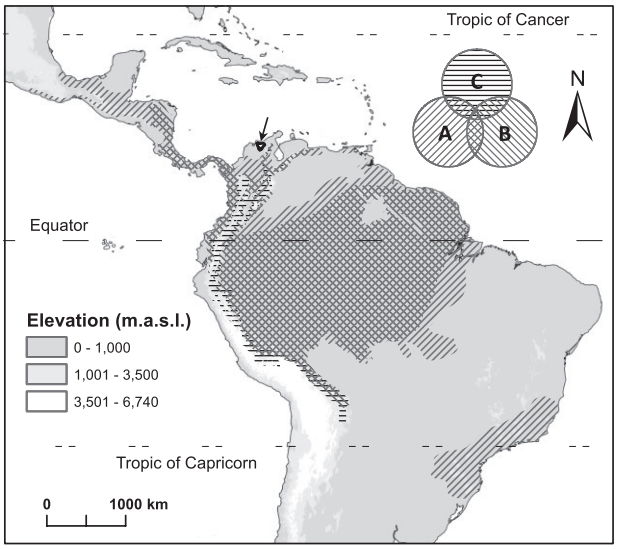

Figure 1 Geographic distribution of Centrolenidae. Clade A (right-angled lines; *Teratohyla* + *Sachatamia* + *Rulyrana* + *Cochranella* + *Espadarana* + *Chimerella* + *Vitreorana*) and clade B (left-angled lines; *Hyalinobatrachium* + *Celsiella*) are basically distributed in lowlands, while clade C (horizontal lines; *Nymphargus* + *Centrolene*) is mainly distributed in the Andes range. The arrow and the black area indicate the distribution of *Ikakogi tayrona*, a centrolenid species outside the three major clades, which also inhabits a mountain range. These three main clades and *Ikakogi tayrona* are represented on the phylogeny shown in Fig. 2, which graphically represents the association between temperature and synonymous substitution rate in the nuclear genes.

MATERIAL AND METHODS

Taxa and molecular data

The study included 97 Centrolenidae species, some of which are yet to be named and described (Castroviejo-Fisher *et al.*, 2014). The sample represents more than 65% of the estimated species richness of the clade and includes representatives from all recognized genera and habitats in which these species are present. We used the most recent and most complete phylogenetic reconstruction based on maximum likelihood inference combining mitochondrial and nuclear genes (Castroviejo-Fisher *et al.*, 2014). Prior to each analysis the tree was pruned to include only those species for which we had accurate phenotypic, environmental and genetic data.

We used three mitochondrial (total 2.8 kb) and seven single-copy nuclear gene fragments (total 3.5 kb). These genes [length, nucleotide diversity (π) (Nei & Li, 1979) and Watterson estimator (θ) (Watterson, 1975) considering only one sequence per species] are: the mitochondrial rRNA genes *12S* (975 bp, $\pi = 0.071$, $\theta = 70.14$) and *16S* (892 bp, $\pi = 0.096$, $\theta = 76.94$); the mitochondrial protein-coding NADH dehydrogenase subunit 1 (*ND1*; 979 bp, $\pi = 0.167$, $\theta = 94.83$); and the nuclear protein-coding brain-derived neurotrophic factor (*BDNF*; 696 bp, $\pi = 0.009$, $\theta = 13.86$), proto-oncogene cellular myelocytomatosis (*C-MYC*; 423 bp, $\pi = 0.044$, $\theta = 24.50$), chemokine receptor 4 (*CXCR4*; 354 bp, $\pi = 0.029$, $\theta = 18.67$), pro-opiomelanocortin A (*POMC*; 615 bp, $\pi = 0.032$, $\theta = 30.32$), recombination activating gene-1 (*RAG-1*; 456 bp, $\pi = 0.031$, $\theta = 22.47$) and solute-carrier family 8 members 1 and 3 (*SLC8A1*; 540 bp, $\pi = 0.024$, $\theta = 21.42$; *SLC8A3*; 465 bp, $\pi = 0.011$, $\theta = 13.23$). Not all gene sequences were available for all species. A complete list of genes used per species with their GenBank accession number is available in Appendix S1 in Supporting Information.

Prior to analysing protein-coding genes, we translated them in EMBOSS Transeq (Rice *et al.*, 2000) to identify the correct open reading frame and compared the sequences with the same gene in the most closely related species using BLASTn (Altschul *et al.*, 1990). For analyses, sequences were concatenated as follows to minimize confounding effects due to potential different effects of traits on the mitochondrial and nuclear genomes: mitochondrial non-coding genes (*12S* and *16S*; 1867 bp, $n = 97$ species) and nuclear protein-coding genes (*BDNF*, *C-MYC*, *CXCR4*, *POMC*, *RAG-1*, *SLC8A1* and *SLC8A3*; 3549 bp, $n = 83$ species). The single mitochondrial protein-coding gene *ND1* (960 bp, $n = 91$ species) was analysed separately.

Traits

We used midpoint elevation, midpoint latitude, mean annual temperature, mean annual precipitation, mean annual relative humidity, mean net primary productivity and mean annual UVB radiation across each species' distribution to characterize correlations between environmental traits and the rate of molecular evolution. Known centrolenid distributions were adjusted based on recent sampling and corrected misidentifications (details in Appendix S2) and analysed with ArcGIS 10 (Environmental Systems Research Institute, 2011) to obtain the values of all these traits, except midpoint elevation. Midpoint elevation values were obtained from the IUCN database (2012), published literature and our own unpublished data (details in Appendix S3). Midpoint latitude was estimated using the addXY command with the geospatial modelling environment (GME) extension (Spatial Ecology, 2012). We used the absolute value of the latitude midpoint because our interest lay in the distance to the equator regardless of the location in the Northern or Southern Hemisphere. We obtained temperature and precipitation data (30 arcsec resolution; *c.* 1 km) from the WorldClim climatic maps (Hijmans *et al.*, 2005), relative humidity (30 arcmin; *c.* 60 km) from the Atlas of the Biosphere (New *et al.*, 1999), net primary productivity data (15 arcmin; *c.* 28 km) from the HANNP datasets (Imhoff *et al.*, 2004; Imhoff & Bounoua, 2006) and UVB radiation data (15 arcmin resolution; *c.* 28 km) from the glUV dataset (Beckmann *et al.*, 2014). We obtained the mean value for each trait for each species' distribution including only distribution points within the known elevational range. One might wonder about the relevance of studying the influence of UVB on nocturnal animals such as centrolenids. However, Cisneros-Heredia & McDiarmid (2007) reported that absence of

pigments from ventral skin (i.e. a transparent parietal peritoneum) was associated with the presence of pigments (i.e. iridophores) covering viscera (e.g. liver, digestive track). These authors hypothesized that pigments covering viscera protect enzymatic activity against the potentially detrimental effects of light and temperature, which could be linked to the rate of molecular evolution via mutagenesis. Finally, we obtained lineage-specific cladogenetic events (a proxy for diversification events) by counting the number of nodes between the root and each tip of the phylogeny, excluding the root node (*sensu* Freckleton *et al.*, 2008) (Appendix S4). We note that although the latter is a nonparametric method, which assumes that rate of extinction is equal across all clades and time, a recent study suggests that extinction in Centrolenidae plays a minor role in explaining current richness patterns (Castroviejo-Fisher *et al.*, 2014). All data are shown in Appendix S3.

To control for the potentially confounding effects of generation time, longevity and metabolic rate (for which no data are available) we used body size as a proxy. Body size is positively associated with generation time (Galtier *et al.*, 2009) and longevity (Nabholz *et al.*, 2008), and negatively associated with metabolic rate (Martin & Palumbi, 1993), all of which might influence the rate of molecular evolution (i.e. Gillooly *et al.*, 2005; Nabholz *et al.*, 2008; Lartillot & Poujol, 2011; Santos, 2012). We used body size, measured as the midpoint of snout–vent length (SVL; distance from the tip of the snout to the anterior margin of the cloaca). Midpoint SVL was obtained from the IUCN database (2012), published literature, and our own unpublished data (details in Appendix S3). Estimates of midpoint SVL included SVL of both males and females.

Analyses

An essential first step, prior to testing whether there is any association between phenotypic traits or environmental variables and rate of substitution, is to discard any potential confounding effects of the node-density artefact, an underestimation of the branch lengths in areas of the tree with few taxa (Venditti *et al.*, 2006). We tested for the presence of the node-density artefact using the 'Test for punctuational evolution and the node-density artifact' available online (http://www.evolution.reading.ac.uk/pe/index.html) (Webster *et al.*, 2003; Venditti *et al.*, 2006). Presence of a node-density effect was discarded ($\delta < 1$).

To analyse the association between the rate of molecular evolution and the different traits we used Coevol, v.1.4 (Lartillot & Poujol, 2011). We analysed correlations between traits and d_S, d_N and ω. Note that only an overall substitution rate can be calculated for non-coding genes (*12S* and *16S* mitochondrial rRNA). Coevol estimates the correlations jointly modelled as a multivariate Brownian diffusion process along all branches of the tree (whole-tree method). 'Whole-tree' methods now enable analyses including all the information obtained from a phylogenetic reconstruction of the clade of interest taking into account the phylogenetic dependence (Lanfear *et al.*, 2010). The input was the multiple sequence alignment, a matrix of log-transformed continuous traits for the same taxa, and the phylogenetic tree. A covariance matrix is estimated using a Bayesian Markov chain Monte Carlo (MCMC) method. We used a geodesic averaging method for computing branch-specific mean values of the molecular evolution parameters, as it is suggested to be more precise than an arithmetic averaging method (Lartillot & Poujol, 2011). We ran each analysis twice until stabilization of all estimated parameters, which was visually verified by plotting the trace file of each trait. We ensured convergence by comparing the two independent runs of each analysis using the tracecomp module of Coevol. Burnin was set after stabilization (checked visually). To compute posterior estimates of the covariance matrix, we used the component readcoevol. We tested the key association between temperature and diversification predicted by the IESH using phylogenetic generalized least squares (Martins & Hansen, 1997) in the R package 'caper' (Orme *et al.*, 2012) to control for phylogenetic non-independence. Analyses were run under a Brownian motion model of evolution with the lambda parameter, which estimates the necessary correction for phylogenetic non-independence of the residuals (Freckleton *et al.*, 2002; Revell, 2010). Data were log-transformed prior to analyses to meet assumptions of the model of evolution. Figure 2, depicting the main result of these analyses, was constructed using the phytools package (Revell, 2012) in R (R Development Core Team, 2012).

RESULTS

We confirmed the convergence of the two independent runs of each analysis. In all cases effective sample sizes of parameters and the discrepancy between independent runs were well within the range of values for the runs to be considered as good, with a single exception, a run for which some values were in the range for it to be considered as acceptable (see Appendix S5 for details). The number of cycles, burn-in and number of sampled iterations in the posterior distribution for all analyses are shown in Appendix S6.

An overview of the main results is presented in Table 1 (see Appendix S7 for a comprehensive description). Following the prediction of the IESH, substitution rate and d_S were positively correlated with temperature in mitochondrial non-coding genes and mitochondrial and nuclear coding genes (Fig. 2). Temperature was only positively correlated with d_N in the mitochondrial coding gene and negatively correlated with ω in the nuclear genes. However, contrary to the predictions of the IESH, substitution rates were not correlated with mean annual precipitation, mean annual relative humidity or mean annual net primary productivity for the mitochondrial or nuclear genes. Finally, in agreement with the IESH, the substitution rate in the mitochondrial non-coding genes as well as d_S in the mitochondrial coding gene were positively correlated with diversification rate. However, d_N and ω in the mitochondrial coding gene and substitution rates (d_N or d_S) and ω in the nuclear genes were not correlated with diversification rate.

With regard to potential confounding factors and commonly used proxies for temperature we found that, firstly, body size was not correlated with substitution rate (d_S or d_N) or ω in the

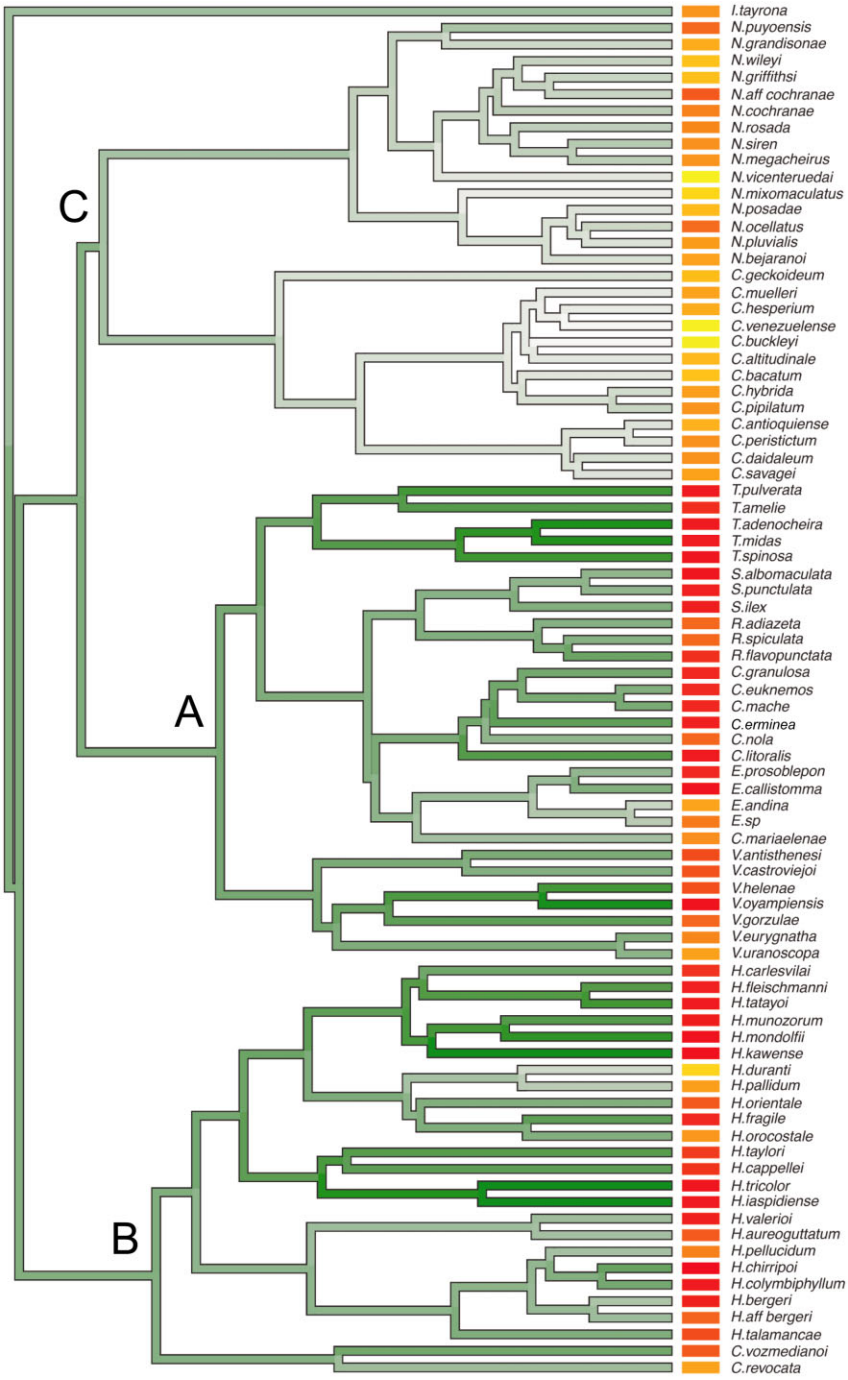

Figure 2 Phylogenetic tree of Centrolenidae. Colours of branches represent the synonymous substitution rate (d_S) of the nuclear genes: where the rate of substitution increases from light grey to dark green. Rectangles at the tips of the phylogeny represent the mean annual temperature of the species' distribution range (mean annual temperature increases from yellow to red). This figure highlights the faster synonymous substitution rate in species inhabiting warmer habitats (clades A and B versus C). Letters indicate the three major clades whose distribution is shown in Fig. 1.

mitochondrial or the nuclear genes, thus we can rule out confounding effects of body size. Secondly, midpoint latitude was not correlated with substitution rates or ω in the mitochondrial or nuclear genes. On the other hand, midpoint elevation was negatively correlated with the rate of substitution in mitochondrial non-coding genes, and also with the rate of synonymous (d_S) and non-synonymous (d_N) substitution in the mitochondrial coding gene, but it was only correlated with the rate of synonymous substitution (d_S) in the nuclear coding gene. Midpoint elevation was not correlated with the ratio of non-synonymous to synonymous substitutions (ω) in any gene. Finally, contrary to what we expected, UVB radiation was negatively correlated with d_S in the mitochondrial coding gene while all other relationships were non-significant.

Interestingly, some of the significant correlations described above disappeared and others became significant when tested by controlling for other factors with which they were correlated. The partial correlations we analysed suggest that elevation and UVB radiation are correlated with the rate of molecular evolution via temperature, since their association with the rate of molecular evolution disappeared when controlling for the effect of temperature. Surprisingly, when controlling for temperature

Table 1 Covariance matrix between substitution rate (Sub. rate in mitochondrial non-coding genes or synonymous (d_S) and non-synonymous (d_N) substitution rates, and the ratio of non-synonymous to synonymous substitutions (ω) in mitochondrial and nuclear coding genes) and body size, diversification events (Div. events), elevation (Elev.), latitude (Lat.), temperature (Temp.), relative humidity (RH), net primary productivity (NPP), precipitation (Precip.) and UVB radiation (UVB). We show covariances (cov), which indicate the direction of the correlation, the effect size (r^2) and the posterior probabilities (pp); values ≤ 0.05 or ≥ 0.95 can be taken as indicating that the relationships are extremely unlikely to occur by chance and are shown in bold.

		Mitochondrial genes				Nuclear genes		
		Non-coding	Coding			Coding		
		Sub. rate	d_S	d_N	ω	d_S	d_N	ω
Body size	cov	−0.03	−0.02	0.00	0.00	−0.01	−0.01	−0.01
	r^2	0.04	0.04	0.00	0.01	0.02	0.01	0.00
	pp	0.08	0.06	0.51	0.62	0.25	0.30	0.40
Div. events	cov	**0.06**	**0.03**	0.03	−0.00	0.01	0.01	0.01
	r^2	**0.10**	**0.06**	0.03	0.00	0.00	0.00	0.00
	pp	**1.00**	**0.97**	0.86	0.51	0.60	0.56	0.56
Elev.	cov	**−0.31***	**−0.16***	**-0.20***	−0.00	**−0.21***	0.02	0.20
	r^2	**0.14**	**0.08**	**0.10**	0.00	**0.24**	0.00	0.12
	pp	**0.00**	**0.03**	**0.05**	0.52	**0.01**	0.55	0.92
Lat.	cov	−0.12	−0.11	0.11	0.02	−0.11	−0.09	−0.01
	r^2	0.01	0.01	0.01	0.00	0.02	0.00	0.00
	pp	0.28	0.25	0.65	0.54	0.25	0.41	0.48
Temp.	cov	**0.07**	**0.05**	**0.05**	−0.00	**0.04**	−0.00	**−0.04**
	r^2	**0.31**	**0.34**	**0.25**	0.00	**0.35**	0.00	**0.18**
	pp	**1.00**	**1.00**	**0.99**	0.48	**1.00**	0.40	**0.04**
RH	cov	−0.00	0.00	0.01	0.00	0.01	−0.00	−0.01
	r^2	0.00	0.01	0.04	0.00	0.07	0.01	0.08
	pp	0.49	0.73	0.81	0.54	0.87	0.35	0.14
NPP	cov	0.06	−0.03	0.04	0.00	0.06	0.01	−0.05
	r^2	0.02	0.01	0.01	0.00	0.10	0.00	0.04
	pp	0.82	0.32	0.69	0.48	0.92	0.49	0.18
Precip.	cov	−0.01†	−0.01†	0.04	0.00	0.06	−0.06	−0.13
	r^2	0.00	0.00	0.01	0.00	0.07	0.04	0.17
	pp	0.41	0.46	0.70	0.53	0.86	0.23	0.06
UVB	cov	0.00	**−0.02***	−0.01	−0.00	−0.01	0.01	0.01
	r^2	0.00	**0.07**	0.03	0.00	0.04	0.00	0.03
	pp	0.53	**0.04**	0.22	0.51	0.19	0.59	0.76

When controlling for the effect of temperature: correlations indicated by *are no longer significant, while those indicated by †become significant (see details in Results and Appendix S7).

we found a significantly negative correlation between precipitation and substitution rate and d_S in mitochondrial non-coding genes and the mitochondrial coding gene, respectively.

DISCUSSION

Temperature is the key factor related to the rate of molecular evolution

The bivariate correlations show that the rate of molecular evolution is significantly correlated with temperature, elevation and UVB radiation (Table 1). The IESH predicts that these factors, with the exception of UVB radiation, are associated directly or indirectly with the rate of molecular evolution, and all were included within the concept of biologically available energy (Wright *et al.*, 2006). According to the IESH, ectothermic species living in environments with higher biologically available energy have a higher body temperature and therefore a higher metabolic rate (Allen *et al.*, 2006; Wright *et al.*, 2010), increasing the production of reactive oxygen species that may lead to a higher mutation rate. A recent study with poison frogs empirically tested the relationship between metabolic rate and rate of molecular evolution, and showed that a higher active metabolic rate, but not resting metabolic rate, is indeed associated with a higher rate of molecular evolution in both mitochondrial and nuclear genes (Santos, 2012). We propose that the observed correlations between temperature, elevation, UVB radiation and the rate of molecular evolution are the result of an increased rate of mutation in warmer environments, in support of the IESH. Indeed, the fact that the correlations between rate of molecular evolution and elevation or UVB radiation disappear when we control for temperature supports our interpretation and emphasizes the crucial role of temperature as the principal factor behind these associations. The absence of an association between latitude and the rate of molecular evolution can be explained by the lack of correlation between latitude and temperature (Pearson's $r = 0.07$). Centrolenid species present a mainly tropical distribution, and this limited variation in the latitudinal range (Fig. 1) is a potential explanation for why variation in temperature is linked to elevational rather than latitudinal differences. Our results also stress the importance of testing the IESH using the environmental variable proposed to

play the key role, i.e. temperature, rather than proxies as these may not always accurately reflect differences in environmental temperature.

According to the IESH, reduced water availability is proposed to limit the rate of molecular evolution, as was observed in Australian plants (Goldie *et al.*, 2010). Somewhat contrary to this prediction, we found that precipitation is negatively correlated with substitution rate and d_S in mitochondrial non-coding genes and the mitochondrial coding gene, respectively, but only after controlling for the effect of temperature. It is certainly possible that, since centrolenids require a relatively wet microhabitat, our data measured over the entire distribution range does not properly reflect water stress. Relative humidity, however, was not associated with the rate of molecular evolution. We propose two non-mutually exclusive explanations for why, for a given temperature, in drier conditions centrolenids present higher rate of molecular evolution in mitochondrial genes. On the one hand, under hydric stress mitochondria might produce higher amounts of reactive oxygen species, which could in turn lead to a higher mutation rate. Alternatively, it is possible that in adverse conditions the population size of centrolenids, which depend on liquid water for reproduction, presents more pronounced demographic fluctuations resulting in a higher rate of molecular evolution (Balloux & Lehmann, 2011). Our results do not allow us to distinguish between these two potential explanations.

Contrary to the prediction of the IESH, we did not find any correlation between net primary production and the rate of molecular evolution (Table 1) (Gillman & Wright, 2014). We cannot rule out that there is insufficient variation in net primary productivity in this mainly tropical clade for a significant association to be detected. Nonetheless, we note that the relationship is based on the fact that productivity is a good predictor of species richness. But, as discussed below, the rate of molecular evolution may not be directly associated with species richness, as other factors may also play important roles therefore mitigating the correlation between molecular evolution and primary productivity. The absence of a correlation between productivity and the rate of molecular evolution suggests that the relationship between temperature and/or water availability and the rate of molecular evolution might not be via productivity.

In sum, our results are in accord with previous studies in ectotherms (Wright *et al.*, 2010; Lourenço *et al.*, 2013) and endotherms (Gillman *et al.*, 2009, 2012) that found faster substitution rates for species living in warmer environments. The observed discrepancies between mitochondrial and nuclear data might be due to differences in the statistical power to detect associations or natural differences between both kinds of genomes, such as the lower effective population size (Wright, 1931) and the higher mutation rate of the mitochondrial genome (Martin & Palumbi, 1993).

Elevated diversification is correlated with increased rate of molecular evolution in mitochondrial genes

The IESH also predicts that the faster rate of molecular evolution in warmer environments favours diversification because of the more rapid build-up of incompatibilities between populations (Dowle *et al.*, 2013). Hence, a positive correlation between diversification and the rate of molecular evolution would be expected. In agreement with this prediction, we found a correlation between diversification events and the rate of molecular evolution in mitochondrial genes (Table 1). However, the effect size of the correlation between substitution rates and diversification was relatively low, ranging from 6 to 10% (Table 1), indicating that other factors possibly play an important role in the association with rate of molecular evolution. The absence of a correlation between diversification events and substitution rates in nuclear genes (Table 1) is surprising and counter to results of theoretical and empirical studies (Ohta, 1992; Balloux & Lehmann, 2011; Buschiazzo *et al.*, 2012; Popadin *et al.*, 2013). As mentioned above, it is possible that our sample of nuclear genes is not sufficient to allow us to detect the association, in contrast with previous studies (e.g. Barraclough & Savolainen, 2001; Lanfear *et al.*, 2010). Compared with the mitochondrial genome, the nuclear genome has a higher effective population size that can dilute the effect of demographic fluctuations associated with diversification events (Popadin *et al.*, 2013).

Body size is not correlated with the rate of molecular evolution

We did not find any correlation between body size and substitution rates or ω in mitochondrial or nuclear genes (Table 1). This result contrasts with previous findings in mantellid and poison frogs (Wollenberg *et al.*, 2011; Santos, 2012). The absence of a relationship between body size and the rate of molecular evolution in Centrolenidae may be a result of the reduced variation in body size in this clade. Excluding the exceptionally large *Centrolene geckoideum* (SVL 71.3 mm), the largest species is only twice as large as the smallest Centrolenidae (SVL range 18.5–36.4 mm). On the other hand, the difference between the smallest and largest species is one order of magnitude or more in mantellids (SVL range 10–110 mm SVL) and poison frogs (range 0.2–6 g). The lack of correlation between body size and molecular evolution rate places the focus on the influence of environmental variables ruling out potential confounding effects of allometry.

Do our results support the IESH?

The IESH is proposed as an explanation for the latitudinal species gradient (Gillman & Wright, 2014). Our results for a recently diverged clade of Neotropical amphibians support two key predictions of the IESH. First, we found that the rate of molecular evolution is positively correlated with temperature. Second, we found that the rate of molecular evolution is positively associated with diversification, although only in mitochondrial genes and with a small effect size ($r^2 = 6$–10%). Hence, at first sight our results appear to support the IESH. However, our results do not support the additional tested predictions of the IESH, namely the positive associations between

rate of molecular evolution and productivity or water availability. The absence of a correlation between temperature and diversification events in centrolenids ($\lambda = 1$, $\beta = 0.16187$, SE = 0.1543, t-value = 1.0491, P-value = 0.2968) is not necessarily contrary to the IESH. As indicated by Rohde (1992) and Gillman & Wright (2014), the relationship between temperature and speciation (or species richness) is indirect and other key factors intervene, such as time or the possibility of genetic isolation. A recent study points to the early colonization of mountain ranges, in combination with time allowing for richness to build up, as potential explanations for centrolenid diversity (Hutter *et al*., 2013), and other studies of Neotropical amphibians also suggest that mountain ranges play a key role favouring isolation of populations and therefore diversification (e.g. Santos *et al*., 2009; Gonzalez-Voyer *et al*., 2011). We suggest that while lowland habitats are indeed associated with a higher rate of molecular evolution, as a result of higher temperature, they nonetheless lack the ecological and geographical features favouring isolation of populations, and hence speciation, compared with mountain ranges such as the Andes. A recent study supports this suggestion as it found higher genetic divergences among frog species in mountains than lowlands (Guarnizo & Cannatella, 2013). Spatial heterogeneity therefore appears to overlay the important effect of temperature via the rate of molecular evolution favouring speciation.

In conclusion, we found that species inhabiting warmer environments have a higher rate of molecular evolution, which increases even more in the mitochondrial genes in drier conditions. We also found that a higher rate of molecular evolution is positively correlated with the rate of diversification in mitochondrial genes. As far as we know, this is the first study to show both an association between temperature and a higher rate of molecular evolution as well as a positive correlation between the rate of molecular evolution and the diversification rate. These results support the IESH, emphasizing the important association between the rate of molecular evolution and temperature ($r^2 = 25$–35%) even within a relatively short time-scale and the relationship between the latter and the rate of diversification. However, the absence of a positive correlation between temperature and diversification, together with the small effect size of the relationship between substitution rates and diversification ($r^2 = 6$–10%), highlight the influence of other factors, such as time, spatial heterogeneity or population size, that must be taken into consideration (Gillman & Wright, 2014).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Appendix S1 GenBank accession numbers for each species and gene.
Appendix S2 References used to adjust IUCN distributions, when necessary.
Appendix S3 Matrix of characters used in the analyses.
Appendix S4 Phylogenetic tree with the number of nodes.
Appendix S5 Convergence diagnostics.
Appendix S6 Details for the Coevol analyses.
Appendix S7 Comprehensive results of the Coevol analyses.

BIOSKETCH

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