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ORIGINAL ARTICLE

Evolution in the South American 'Lost World': insights from multilocus phylogeography of stefanias (Anura, Hemiphractidae, *Stefania*)

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ABSTRACT

Aim To investigate the influence of tepuian geomorphology on species diversification in the Pantepui biogeographical region based on the phylogenetic relationships and divergence times of tepui-endemic clades of stefania frogs (*Stefania*, Hemiphractidae).

Location The 'tepuis' and uplands/lowlands of the Pantepui biogeographical region of northern South America, one of the least accessible and least studied areas in the world.

Methods Two mitochondrial and two nuclear DNA sequences from 60 individuals of *Stefania* from 24 localities in Pantepui were employed to infer phylogenetic affinities and estimate divergence times within the genus using both concatenation and species tree analyses. Ancestral areas were inferred using multiple models in a common likelihood framework.

Results Phylogenetic analyses revealed high diversity in the genus *Stefania* with 10 candidate species in the Eastern Pantepui District. Four strongly supported clades are recovered in the area, one being exclusively composed of microendemics on isolated tepui summits. Biogeographical analyses suggest episodes of fragmentation of widespread tepuian ancestors from the onset of diversification of the genus, estimated in the Oligocene (*c*. 26 Ma), therefore suggesting a neglected vicariant model of Pantepui evolution, the Plateau Theory.

Main conclusions Although our results suggest that vicariance played an important role in the diversification of *Stefania*, speciation in Pantepui followed an intricate pattern implying multiple nonexclusive processes. Vicariance and dispersal likely influenced diversification patterns of the Pantepui fauna, possibly according to the following sequence: (1) Cenozoic vicariance; (2) reorganization of species diversity due to periods of climatic instability; (3) recent invasions (Pleistocene) of widespread upland taxa.

Keywords

ancestral areas, anuran, dispersal, divergence times, frog, molecular phylogenetics, phylogeography, tepui, Venezuela, vicariance

INTRODUCTION

Pantepui (Fig. 1a) embraces one of the most fascinating and inaccessible landscapes on Earth. This northern South American bioregion (Mayr & Phelps, 1967) harbours numerous isolated Precambrian sandstone tabletop mountains ('tepuis', Fig. 1b), and is renowned for its floral and faunal endemism (Berry *et al.*, 1995; McDiarmid & Donnelly, 2005). Yet, using a broad sampling of amphibian and reptile taxa, Kok *et al.* (2012) demonstrated that genetic diversity among most tepui summit species and populations is substantially lower than expected. This finding suggests that throughout their history tepuis were not impermeable barriers to local gene flow, and that single-tepui endemism could be the exception rather

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than the rule. Current hypotheses for the diversification of Pantepui endemic organisms can be classified into two major speciation processes, 'ancient' vicariance versus 'recent' dispersals (Mayr & Phelps, 1967). Recent studies (e.g. Salerno *et al.*, 2012) adduce the latter mainly because molecular data indicate that radiations of tepui summit organisms post-date the hypothetical age of physical isolation of tepui summits. However, although little scientific disagreement exists about the Precambrian history of Pantepui and most of the erosional processes that produced the present-day landscape of tepuis, the age, rate, and magnitude of uplift(s), as well as the rate of erosion of the sediment strata, and general lowering of the planation surface are more controversial or at least highly speculative (i.e. Schubert *et al.*, 1986; Briceño & Schubert, 1990; Yanes & Briceño, 1993; Piccini, 1995). Although some authors considered the fragmentation and physical isolation of tepuis to have taken place in the Cretaceous (e.g. Salerno *et al.*, 2012), this has never been demonstrated (see Kok, 2013), and tepui summit isolation is not necessarily concomitant with the Cretaceous uplift of the Guiana Highlands postulated by Gansser (1954). In fact, mechanisms of diversification of tepui summit organisms remain largely unexplained, as exemplified in Leite *et al.* (2015), whose results, based on the phylogenetic relationships and divergence dates of the rare tepui-endemic rodent *Podoxymys roraimae*, do not conform to the predictions of the recent dispersal model of Pantepui faunal evolution, but rather suggest a pre-Pleistocene vicariant scenario.

The frog genus *Stefania* is endemic to Pantepui and includes 19 described species, 15 of which are found only on tepui slopes or summits (Kok, 2013). *Stefania* species are direct-developers, with eggs and juveniles carried on the back of the mother; they occupy various types of habitats ranging in elevation from lowland rain forest to cloud forest and tepui summit low vegetation (Duellman, 2015). Rivero (1970) recognized two species groups within *Stefania*: the *evansi* group including species having the head longer than broad and found in the lowlands and uplands of Pantepui, and the *goini* group including species having the head broader than long and found in the highlands of Pantepui. Both groups were still recognized until recently (e.g. MacCulloch *et al.*, 2006) when Kok *et al.* (2012), followed by Castroviejo-Fisher *et al.* (2015), showed that, based on molecular data, these groups were not reciprocally monophyletic. Rivero (1970) extensively discussed the possible origin, endemism, and distribution of the genus. Duellman & Hoogmoed (1984) revised the taxonomy of *Stefania*, which was later discussed by Señaris *et al.* (1997/1996). Rivero (1968/1966, 1970) suggested that *Stefania goini* Rivero, 1968/1966 and *S. ginesi* Rivero, 1968/1966 (at the time the only known representatives of the *goini* group) represented remnants of an ancestor that once occupied an extensive plateau subsequently dissected into the present-day tepuis. Rivero (1970) doubted that species of the *goini* group could have originated independently from the '*evansi* stock' and acquired similar morphological characteristics by convergence. By contrast, Señaris *et al.* (1997/1996), who still recognized both species groups, assumed that species from the *goini* group (at that time six in number) evolved from the *evansi* group and that their common characteristics were due to convergence, instead of a common phylogenetic origin. Castroviejo-Fisher *et al.* (2015) found the genus as sister to a clade containing *Fritziana*, *Hemiphractus* and *Gastrotheca*. The genus *Stefania* itself has never been the subject of rigorous molecular phylogenetic analyses, mainly because of a lack of comprehensive sampling. Kok *et al.* (2012) highlighted that genetic divergences among summit populations of *Stefania* were relatively high, different from the low genetic diversity found in other anuran Pantepui lineages (e.g. *Oreophrynella*, *Tepuihyla*). Kok *et al.* (2016) recently demonstrated that several populations of *S. ginesi* and *S. satelles* Señaris *et al.* (1997/1996) represented undescribed taxa.

Herein, using a comprehensive molecular sampling of the genus (DNA sequence data from two mitochondrial and two nuclear markers from 60 individuals from 24 localities in Pantepui), we estimated the phylogenetic relationships and divergence times in *Stefania* to investigate its historical biogeography and the role that tepuian geomorphology may have played in Pantepui species diversification. Specifically, we evaluated the two following hypotheses about the origin of tepui summit *Stefania* populations.

1. Tepui summit populations originated from neighbouring lowland/upland populations by upward altitudinal dispersal. Given the peculiar geomorphology of tepuis, and the highly specialized habitat found on their summits, speciation promoted by dispersal would likely have been triggered by temperature fluctuations, local changes in precipitation or seasonality, and habitat heterogeneity, that is, a scenario similar to the 'Disturbance-Vicariance Theory' (Colinvaux, 1993). In this case, most divergences between tepui summits are expected to have occurred during the Pleistocene climatic oscillations (< 2.6 Ma). Altitudinal dispersal to tepui summits in the absence of any climatic constraint cannot be ruled out, but considering the extreme physiographical and ecological isolation of tepui summits this scenario requires that ancestors shifted their habitat preference, or had sufficient ecological plasticity, that is, a scenario similar to the 'Habitat Shift Theory' (Mayr & Phelps, 1967). This scenario assumes the presence of closely related taxa in intervening areas.

2. Tepui summit populations originated from vicariance, that is, extant tepui summit species would have derived from highland ancestors that occupied a large, more or less continuous plateau that was gradually eroded and dissected into several isolated tepuis. This scenario conforms to the 'Plateau Theory' (Mayr & Phelps, 1967). In this case, most divergences between tepui summits are expected to coincide with major uplifts and erosional processes in the area, and therefore to predate the Pleistocene climatic oscillations. In a vicariant scenario, a high level of relatively old microendemism is expected on isolated tepui summits, and the species phylogeny is supposed to mirror the geographical phylogeny (Avise, 1994).

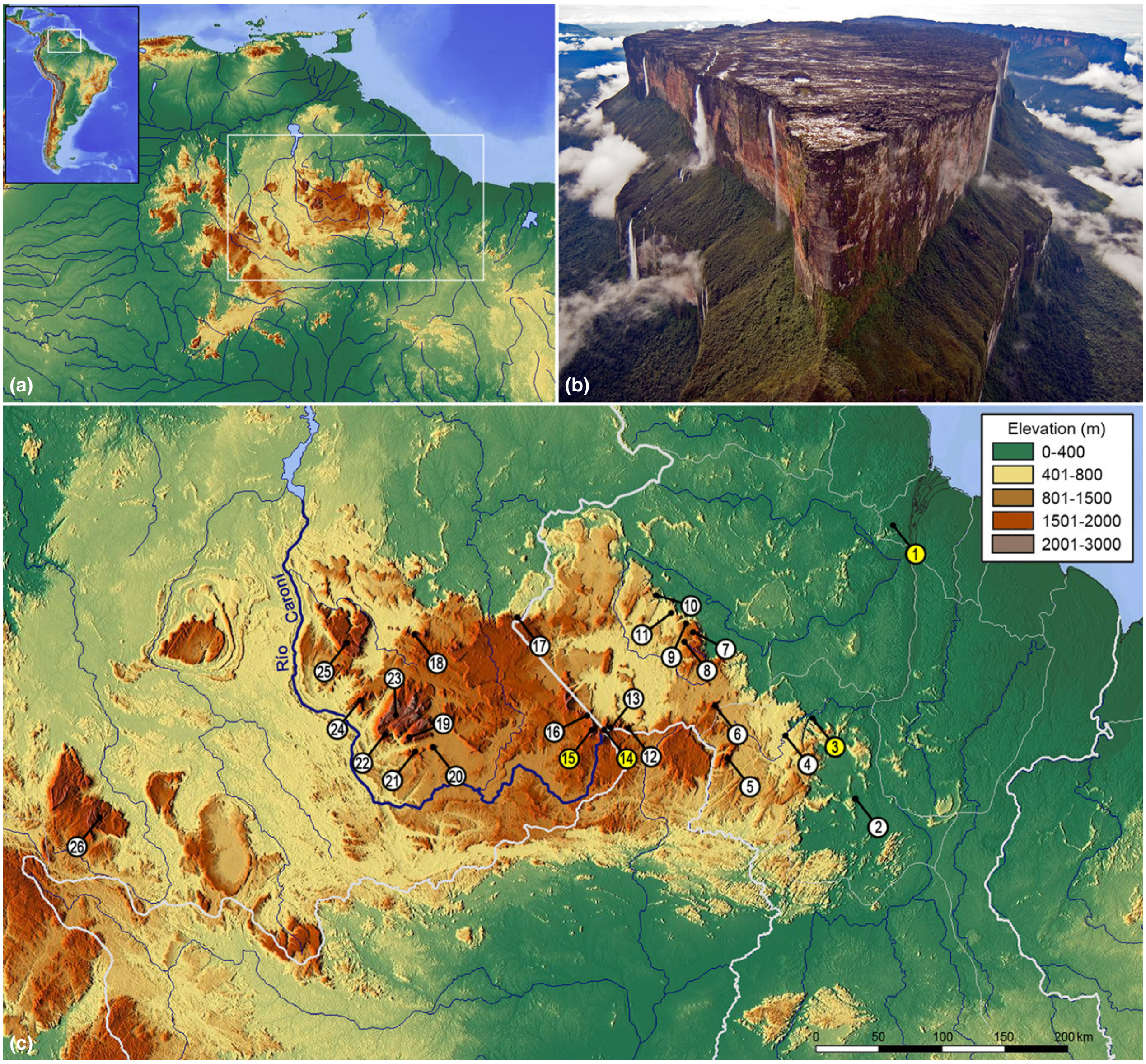


Figure 1 (a) Map of Pantepui and its location within South America (inset); white rectangle enlarged in (c). (b) Aerial view of the northern part of Mount Roraima (the Prow), Guyana, showing typical tepuian sheer cliffs and lower forested slopes. Photo: DBM. (c) Map of eastern Pantepui showing localities mentioned in the text (yellow dots represent localities for which no *Stefania* genetic sample was available): 1. Groete Creek, Guyana; 2. Pakatau Creek, Guyana; 3. Mount Kanaima, Guyana; 4. Kaieteur National Park (including Tukeit), Guyana; 5. Wokomung Massif, Guyana; 6. Mount Ayanganna, Guyana (including Ayanganna airstrip); 7. Apakai, Guyana; 8. Partang, Guyana; 9. Merume Mountain, Guyana; 10. Seroun, Guyana; 11. Kurupung, Guyana; 12. Maringma-tepui (including Wayalayeng), Guyana; 13. Wei-Assipu-tepui, Guyana; 14. Mount Roraima, Guyana/Venezuela/Brazil; 15. Kukenán-tepui, Venezuela; 16. Yuruaní-tepui, Venezuela; 17. Salto El Danto, Venezuela; 18. Murisipán-tepui, Venezuela; 19. Amurí-tepui, Venezuela; 20. Upuigma-tepui, Venezuela; 21. Angasima-tepui, Venezuela; 22. Abakapá-tepui, Venezuela; 23. Chimantá-tepui, Venezuela; 24. Aprada-tepui, Venezuela; 25. Auyán-tepui, Venezuela; 26. Sarisariñama-tepui, Venezuela.

MATERIALS AND METHODS

Study area

This study mainly focuses on the eastern Pantepui region in Venezuela and Guyana (Fig. 1c), the Eastern Pantepui District (EPD) of McDiarmid & Donnelly (2005). We consider the EPD as including the Pantepui lowlands, uplands and highlands east of the Río Caroní, a major drainage that flows north into the lower Río Orinoco. Most tepuis of the EPD are in the Caroní river basin, but tepuis in western Guyana are drained by tributaries of the Essequibo river (McDiarmid & Donnelly, 2005).

Definitions of the main Pantepui landscape types mostly follow Kok (2013) and are as follows: the 'lowlands' (L) are the surface covered by rain forest or savanna below 400 m elevation; the 'uplands' (U) are the surface covered by savanna, rain forest or montane forest below tepui slopes, between *c.* 400–1200 m elevation; the 'highlands' (H) start from *c.* 1200 m elevation, and usually correspond to the mostly inclined talus slopes covered by cloud forest and separating tepui summits/cliffs from the uplands or the lowlands; the 'tepui summits' (TS), which are relatively flat, correspond to areas that (1) are usually higher than *c.* 1200 m elevation and isolated from tepui slopes by vertical walls, or (2) have an elevation of more than *c.* 1900 m, which usually favours different climate and vegetation (e.g. dwarf tepui forest, tepui scrub, tepui meadows) from that on the slopes even if there is no substantial demarcation between slopes and summit. Tepui summits as understood here are isolated topographically and/or ecologically, usually both. Poorly differentiated summits of low elevation tepuis are considered similar to slopes.

Tissue sampling and molecular data

Available targeted sequences of *Stefania* for the mitochondrial 16SrRNA gene (16S), the protein-coding mitochondrial gene NADH hydrogenase subunit 1 (ND1), the nuclear Recombination activating gene 1 (RAG1), and the nuclear CXC chemokine receptor type gene (CXCR4) were retrieved from GenBank, and combined with 86 novel DNA sequences of *Stefania*. Novel sequences have been deposited in GenBank under the accession numbers KX399765–KX399850. We combined this data set with GenBank data of additional members of the family Hemiphractidae, and some members of the families Bufonidae, Hylidae and Eleutherodactylidae (Hyloidea), as well as one species belonging to the family Limnodynastidae (member of the clade sister to Hyloidea) to root our tree. Our final data set comprised 75 taxa (see Tables S1 & S2 in Appendix S1 in Supporting Information). See Appendix S1 for technical details about DNA extraction, PCR, sequencing and sequence alignment.

Molecular phylogenetic analyses

The combined 16S+ND1+RAG1+CXCR4 data set (totalling 2301 bp, after exclusion of regions of ambiguous alignment) was subjected to phylogenetic inference using maximum likelihood and Bayesian analyses (Yang & Rannala, 2012). The limnodynastid frog *Limnodynastes salmini* was selected as outgroup taxon as previous studies recovered Limnodynastidae as the sister group of Hyloidea (Roelants *et al.*, 2007). Optimal partitioning schemes were estimated with PartitionFinder 1.1.1 (Lanfear *et al.*, 2012) using the 'greedy' algorithm, either the 'mrbayes' or 'raxml' set of models and the Bayesian information criterion to compare the fit of different models. Maximum likelihood (ML) analyses were conducted in RAxML-HPC2 8.1.11 (Stamatakis, 2006) on the CIPRES Science Gateway V 3.3 (https://www.phylo.org/, Miller *et al.*, 2010); nodal bootstrap values (Felsenstein, 1985) were estimated using 500 pseudoreplicates under the best substitution model inferred by PartitionFinder 1.1.1, and in paup* 4.0a134 for Mac OS X (Swofford, 2002) with similar settings. Bayesian posterior probabilities (PP) were used to estimate clade credibility in MrBayes 3.2.2 (Ronquist *et al.*, 2012) on the CIPRES Science Gateway V 3.3. The Bayesian analyses implemented the best substitution models inferred by PartitionFinder 1.1.1 partitioned over the different gene fragments, flat Dirichlet priors for base frequencies and substitution rate matrices and uniform priors for among-site rate parameters. Four parallel Markov chain Monte Carlo (MCMC) runs of four incrementally heated (temperature parameter = 0.2) chains were performed, with a length of 2.10^6 generations, a sampling frequency of 1 per 1000 generations, and a burn-in value corresponding to 10% of the saved trees. Convergence of the parallel runs was confirmed by split frequency SDs (< 0.01) and potential scale reduction factors (*c.* 1.0) for all model parameters, as reported by MrBayes. All analyses were checked for convergence by plotting the log-likelihood values against generation time for each run, using Tracer 1.5 (Rambaut & Drummond, 2009). All parameters had effective sample sizes (ESS) largely over 200. Results were visualized and edited in FigTree 1.4.1 (Rambaut, 2014). We additionally conducted species tree analyses using a Bayesian multispecies coalescent approach implemented in *beast (beast 1.8.0 package; Drummond *et al.*, 2012). Our data set consisted of two mitochondrial and two nuclear genes (see above). We used a Birth-Death prior for the species tree and applied the best fit model identified by PartitionFinder 1.1.1 (Lanfear *et al.*, 2012) for each locus. Other parameters were left at their default values. *beast analysis was run with a chain length of 10^7, and a 10% cut-off was used for the burn-in. Resulting trees were summarized in TreeAnnotator 1.8.0 (part of the beast package). Results were visualized and edited in FigTree 1.4.1 (Rambaut, 2014).

Divergence time estimation

We employed a Bayesian multi-gene relaxed-clock approach (Battistuzzi *et al.*, 2010) using the beast 1.8.0 package (Drummond *et al.*, 2012) for the estimation of divergence times in *Stefania*. The beast input file was initially created using BEAUTi 1.8.0 bundled with beast 1.8.0 applying the best fit model identified by PartitionFinder v1.1.1 for each locus; minor editing was performed on the xml file (such as the inclusion of a starting tree). We used the MCMC sampling algorithm as implemented in beast 1.8.0, and employed an uncorrelated lognormal relaxed-clock model that allows for evolutionary rates to vary along the tree branches (Drummond *et al.*, 2006), with the 'Birth-death process with incomplete sampling' as tree prior. This model was preferred to the 'Yule' tree prior because it allows for extinction events (while the Yule prior does not). We calibrated the root of Hyloidea by setting a normally distributed prior on the node corresponding to the most recent common ancestor of Hyloidea genera (mean = 65.5 Ma, standard deviation = 10 Ma) following Roelants *et al.* (2007). We also incorporated information from two fossils using a uniform prior to calibrate two shallower nodes on the phylogeny: divergence time between *Eleutherodactylus* and *Diasporus* (minimum 15 Ma, Heinicke *et al.*, 2009) and divergence time between *Acris* and *Hyla* (minimum 16 Ma, Heinicke *et al.*, 2009). Other priors were left at the default values. We performed four independent runs of 5.10^6 generations each, sampling for trees and parameters every 1000 generations; results from all runs were combined for final results. All analyses were checked for convergence using TRACER 1.5 (Rambaut & Drummond, 2009), and the first 5.10^5 generations were discarded as burn-in; posterior parameter estimates had ESS largely above 200, confirming stationarity and adequate sample sizes. All dating analyses were performed on the CIPRES Science Gateway V 3.3 (Miller *et al.*, 2010). Resulting trees were summarized in TREEANNOTATOR 1.8.0 (part of the BEAST package). Results were visualized and edited in FIGTREE 1.4.1 (Rambaut, 2014).

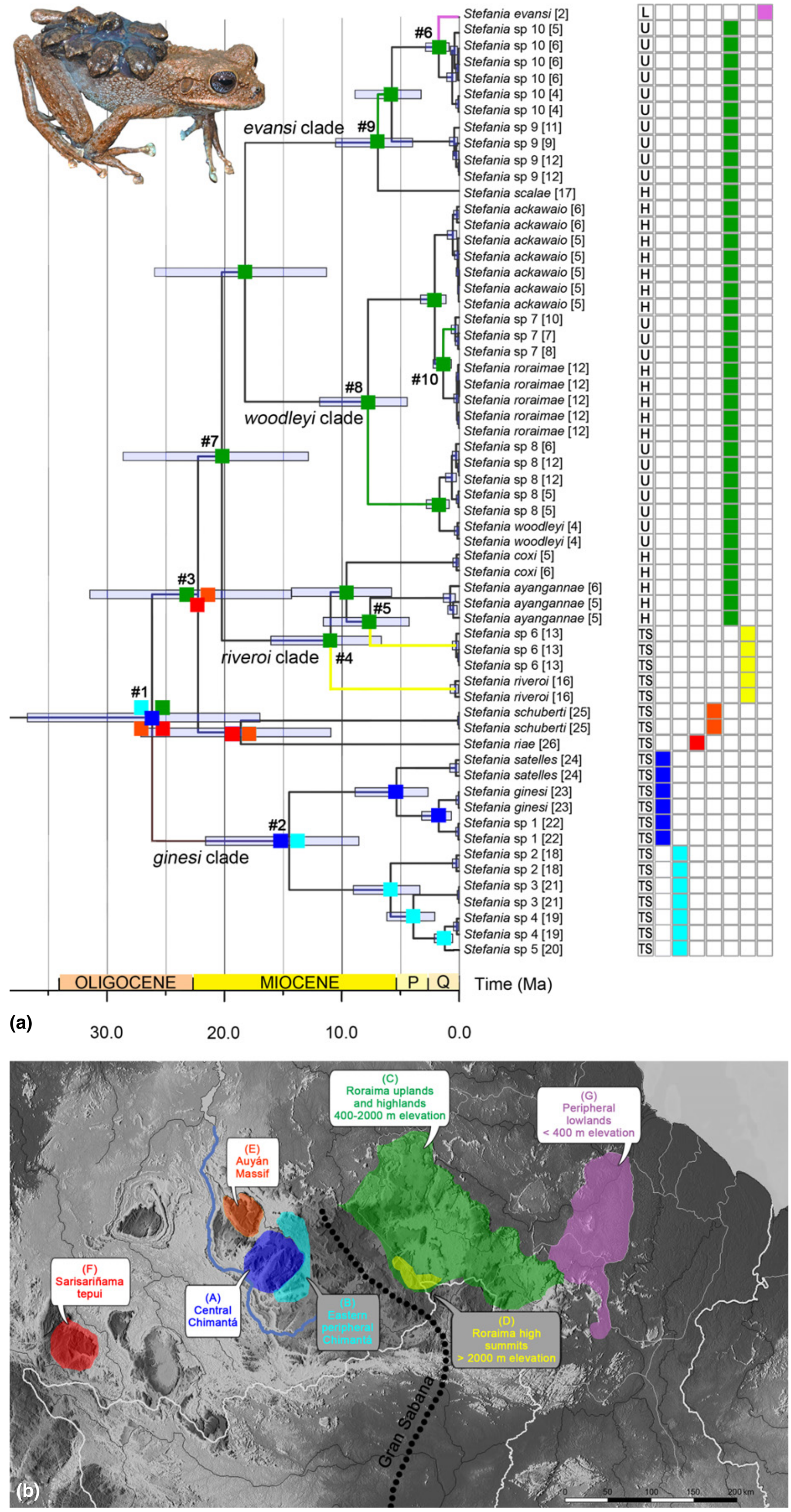
Ancestral range estimation

We used the BIOGEOBEARS package [Biogeography with Bayesian (and Likelihood) Evolutionary Analysis in R Scripts] (Matzke, 2013) in R (R Core Team, 2014) to perform inference of biogeographical history on the dated phylogeny of *Stefania* (i.e. ancestral range estimation). BIOGEOBEARS implements several models of ancestral range estimation in a common likelihood framework. We compared six biogeographical models implemented in BIOGEOBEARS to determine their fit to our data (see Matzke, 2013 for details): (1) the Lagrange's model of dispersal-extinction-cladogenesis (DEC; Ree *et al.*, 2005); (2) DEC+J (DEC including founder-event speciation); (3) DIVALIKE, a likelihood version of the parsimony-based dispersal-vicariance analyses (DIVA; Ronquist, 1997); (4) DIVALIKE+J (DIVALIKE including founder-event speciation); (5) BAYAREALIKE, a likelihood version of the Bayesian inference of historical biogeography for discrete areas (BayArea; Landis *et al.*, 2013); and (6) BAYAREALIKE+J (BAYAREALIKE including founder-event speciation). Likelihood values of the models were compared using Likelihood Ratio Test (LRT), and model-fit was assessed in BIOGEOBEARS by comparing weighted Akaike's information criterion scores (Matzke, 2013).

To perform ancestral range estimation we excluded non-*Stefania* taxa, pruned our dated tree to include only one terminal per population, and left the maximum number of areas per node unconstrained because the distribution of the taxa in the recent past was unknown and previous studies suggested possible recent faunal exchange among tepuis (Kok *et al.*, 2012). Likewise, because of the speculative nature of the tepuian geomorphological history, no constraint could be made regarding potential ancestral distributions and other values were left at default values. Implementing a large number of areas in the biogeographical analysis may alter the results and render them statistically non-significant (e.g. Upchurch *et al.*, 2015). Therefore, and for computational convenience, we defined seven major geographical areas for the BIOGEOBEARS analysis based on the current distribution of *Stefania*. These areas did not totally conform to the subdivisions proposed by McDiarmid & Donnelly (2005), and were as follows (sampled localities are in parentheses, see also Fig. 2b): (A) 'Central Chimantá subdistrict', which encompassed the Aprada Massif and tepuis of the Chimantá Massif north of the Río Toronó (Aprada-tepui, Chimantá-tepui, Abakapá-tepui); (B) 'Eastern peripheral Chimantá subdistrict', which encompassed tepuis of the Chimantá Massif south of the Río Toronó, Angasima-tepui, Upuigma-tepui, and tepuis of the Ptari Massif and the Los Testigos Massif (Angasima-tepui, Upuigma-tepui, Amurí-tepui, Murisipán-tepui); (C) 'Roraima subdistrict uplands and highlands 400–2000 m elevation' (Kaieteur, Kurupung, Merume, Wayalayeng, El Danto, Seroun, Apakai, Partang, Maringma-tepui, Tukeit, Mount Ayanganna, Mount Wokomung); (D) 'Roraima subdistrict high summits > 2000 m elevation' (Yuruaní-tepui, Wei-Assipu-tepui); (E) 'Auyán Massif' (Auyán-tepui); (F) 'Western Pantepui District' (Sarisariñama-tepui); and (G) 'Pantepui eastern peripheral lowlands < 400 m elevation' (Pakatau).

The results obtained prompted us to investigate each of the two major clades separately, defining more discrete geographical units for each of them: EPD Highlands (> *c.* 1200 m elevation), EPD Uplands (*c.* 400–1200 m elevation), EPD Lowlands (*c.* < 400 m elevation), the summit of Wei-Assipu-tepui, and the summit of Yuruaní-tepui for the large clade containing the *riveroi*+*woodleyi*+*evansi* clades (see below), and all tepui summits (Aprada-tepui, Abakapá-tepui, Chimantá-tepui, Amurí-tepui, Angasima-tepui, Upuigma-tepui, and Murisipán-tepui) as single areas for the *ginesi*

Figure 2 Graphical representation of the historical biogeography of *Stefania* in eastern Pantepui. (a) Bayesian timetree obtained from the concatenated data set (2301 bp, *Stefania* only, outgroup not shown). The 95% confidence intervals for selected nodes are highlighted by horizontal light blue bars. Numbers between brackets correspond to sampling localities as illustrated in Fig. 1c. Numbers at nodes refer to speciation events described in the Results. The distribution of each taxon within one of the seven major geographical areas used in the BIOGEOBEARS analysis is given in a geographical matrix; colours refer to areas in the geographical map illustrated below in (b). L = lowlands; U = uplands; H = highlands; TS = tepui summits. Colour squares at nodes indicate the most probable ancestral distributions inferred from BIOGEOBEARS. Coloured branches indicate dispersals (pink = U to L; green = H to U; yellow = H to TS). (b) Geographical map (same as illustrated in Fig 1c, but in black and white) showing the seven major geographical areas used for the BIOGEOBEARS analysis.


Stefania evansi [2]
Stefania sp 10 [5]
Stefania sp 10 [6]
Stefania sp 10 [6]
Stefania sp 10 [6]
Stefania sp 10 [4]
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Stefania sp 9 [11]
Stefania sp 9 [9]
Stefania sp 9 [12]
Stefania sp 9 [12]
Stefania scalae [17]
Stefania ackawaio [6]
Stefania ackawaio [6]
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Stefania sp 7 [10]
Stefania sp 7 [7]
Stefania sp 7 [8]
Stefania roraimae [12]
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Stefania sp 8 [6]
Stefania sp 8 [12]
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Stefania sp 8 [5]
Stefania sp 8 [5]
Stefania woodleyi [4]
Stefania woodleyi [4]
Stefania coxi [5]
Stefania coxi [6]
Stefania ayangannae [6]
Stefania ayangannae [5]
Stefania ayangannae [5]
Stefania sp 6 [13]
Stefania sp 6 [13]
Stefania sp 6 [13]
Stefania riveroi [16]
Stefania riveroi [16]
Stefania schuberti [25]
Stefania schuberti [25]
Stefania riae [26]
Stefania satelles [24]
Stefania satelles [24]
Stefania ginesi [23]
Stefania ginesi [23]
Stefania sp 1 [22]
Stefania sp 1 [22]
Stefania sp 2 [18]
Stefania sp 2 [18]
Stefania sp 3 [21]
Stefania sp 3 [21]
Stefania sp 4 [19]
Stefania sp 4 [19]
Stefania sp 5 [20]
L
U
H
TS
evansi clade
woodleyi clade
riveroi clade
ginesi clade
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
OLIGOCENE
MIOCENE
P
Q
Time (Ma)
30.0
20.0
10.0
0.0
(a)
(C) Roraima uplands and highlands 400-2000 m elevation
(G) Peripheral lowlands < 400 m elevation
(E) Auyán Massif
(F) Sarisariñama tepui
(A) Central Chimantá
(B) Eastern peripheral Chimantá
(D) Roraima high summits > 2000 m elevation
Gran Sabana
0 50 100 150 200 km
(b)

clade (see below). The ancestral area probability was computed for each node and subsequently plotted on the majority-rule chronogram using R scripts.

RESULTS

Phylogenetic relationships of *Stefania*

For brevity and clarity purposes, and because the three methods we used for phylogenetic inference resulted in similar tree topologies with no major conflict (i.e. no highly supported alternatives), we only discuss the MrBayes tree topology (see Fig. S1 in Appendix S2). The *beast tree topology is shown in Fig. S2 (see Appendix S2).

The monophyly of *Stefania* is strongly supported (PP = 1), and we recovered four main clades in the EPD, all strongly supported: one clade was exclusively composed of tepui summit microendemics from the Chimantá Massif and peripheral tepuis (herein called the *ginesi* clade, distributed in areas A + B in Fig. 2b); one clade was composed of highland species and tepui summit microendemics from the Roraima subdistrict (herein called the *riveroi* clade, distributed in areas C + D in Fig. 2b); one clade was composed of upland and highland species from the Roraima subdistrict (herein called the *woodleyi* clade, distributed in area C in Fig. 2b); and one clade was composed of lowland and upland species from the Roraima subdistrict (herein called the *evansi* clade, distributed in areas C + G in Fig. 2b). The position of two tepui summit species received poor support in our tree and deserves further investigation: *Stefania schuberti* Señaris *et al.* (1997/1996), which is restricted to Auyán-tepui, and *Stefania riae* Duellman & Hoogmoed, 1984; which is restricted to Sarisariñama-tepui (Western Pantepui District). Phylogeography, molecular divergences and/or congruent differences in morphological characters were used to detect candidate species (*sensu* Padial *et al.*, 2010). Our preliminary results indicated the occurrence of 10 (confirmed or unconfirmed) candidate species in the EPD. This revision corresponds to an increase of 100% of the number of *Stefania* species previously reported in the area, and highlights a surprisingly high number of microendemics (see also Kok *et al.*, 2016).

The *ginesi* clade (Fig. 2) consists of two well-defined subclades, each strongly supported and containing three and four species respectively. Samples of *Stefania ginesi* and *S. satelles*, as currently understood, are not reciprocally monophyletic, challenging their taxonomic classification. In the first subclade, which contains samples from the Aprada and Chimantá Massifs, *Stefania satelles* from the type locality (Aprada-tepui) is recovered sister to a clade containing *S. ginesi* from the type locality (Chimantá-tepui) and a candidate species from Abakapá-tepui (hereafter *Stefania* sp. 1) previously misidentified as *S. ginesi* (e.g. Gorzula & Señaris, 1999/1998). Sister to that clade, our phylogenetic results reveal a previously unknown subclade of four candidate species (hereafter *Stefania* sp. 2-5), from Amurí-tepui and peripheral tepuis east of the Aprada and Chimantá Massifs. These four candidate species have been previously confused either with *S. ginesi* or with *S. satelles* (e.g. Gorzula & Señaris, 1999/1998; Señaris *et al.*, 2014). All seven species sampled in the *ginesi* clade seem to have their distribution restricted to single tepui summits, and thus qualify as microendemics.

The *riveroi* clade (Fig. 2) contains three recognized and one undescribed species as already suggested in Kok *et al.* (2012). *Stefania riveroi* Señaris *et al.* (1997/1996) from the type locality (summit of Yuruaní-tepui) is recovered sister to the clade [*S. coxi* MacCulloch & Lathrop, 2002 (*S. ayangannae* MacCulloch & Lathrop, 2002 + *S.* sp. 6)] with strong support. Two species, *S. riveroi* and *S.* sp. 6, are restricted to single tepui summits above 2000 m elevation (Yuruaní-tepui and Wei-Assipu-tepui respectively).

The *woodleyi* clade (Fig. 2) consists of two well-defined subclades, each strongly supported and containing two and three species respectively. In the first subclade, comprising upland and highland species, *Stefania ackawaio* MacCulloch & Lathrop, 2002 from the type locality (Mount Ayanganna) and from Mount Wokomung (all these samples previously confused with *S. roraimae* Duellman & Hoogmoed, 1984, e.g. MacCulloch & Lathrop, 2002; MacCulloch *et al.*, 2006) is recovered sister to a clade containing *S. roraimae* from the eastern highlands of the eastern tepui chain and a candidate species distributed in the uplands east of the upper Mazaruni river (hereafter *Stefania* sp. 7). In the second subclade, comprising two upland species, *S. woodleyi* Rivero, 1968/1966 [samples from Kaieteur National Park and Tukeit, close to the type locality (Mount Kanaima)] is sister to a candidate species (hereafter *Stefania* sp. 8) distributed westwards and at higher elevations than *S. woodleyi sensu stricto*. That candidate species was previously misidentified either as *S. woodleyi* or as *S. ackawaio* (e.g. MacCulloch & Lathrop, 2002; MacCulloch *et al.*, 2006).

The *evansi* clade (Fig. 2) contains four species from the lowlands and uplands of the EPD and its eastern periphery, of which two are still undescribed. *Stefania scalae* Rivero, 1970 from the type locality (La Escalera, Venezuela) is found sister to the clade [*S.* sp. 9 (*S. evansi* Boulenger, 1904 + *S.* sp. 10)] with strong support. *Stefania* sp. 9 and sp. 10 have been previously misidentified as *S. evansi* (e.g. MacCulloch & Lathrop, 2002; MacCulloch *et al.*, 2006; Kok & Kalamandeen, 2008). The three species of that clade are currently restricted to Guyana.

Divergence times within *Stefania*

The age of the most recent common ancestor (MRCA) of *Stefania* and its sister lineage (*Fritziana*) is estimated at *c.* 40 Ma (95% HPD: *c.* 26–55 Ma, see Fig. S2 in Appendix S2) in the Eocene, but that node is poorly supported in all analyses.

Although the beast analysis indicates a prevalence of pre-Pleistocene divergence events (see Fig. 2, Table S3 in Appendix S2), divergences between extant taxa are almost equally distributed before and after the onset of the Pleistocene. Estimated divergence times among and within *Stefania* clades are presented in Table S3 in Appendix S2.

Historical biogeography of *Stefania*

The results of the six biogeographical models used in BioGeoBEARS are presented in Fig. 2, Table S4 and Fig. S3 in Appendix S3; additional results for each of the two major clades investigated separately (see Materials and Methods) are provided in Tables S5 & S6 and Figs S4 & S5 in Appendix S3.

In analysing the full data set (Fig. 2, see Fig. S3 in Appendix S3), the DIVALIKE model including founder-event speciation (DIVALIKE+J) provided the best fit of all models to our data (see Table S1 in Appendix S1). Results indicate that the MRCA of extant *Stefania* (*c.* 26 Ma) had a widespread distribution [we infer a joint origin of areas ABCEF, probability of distribution for prior speciation (P) = 0.58; node #1 in Fig. 2], suggesting gene flow across these areas (and occurrence between them), consistent with an ancestor living on a highland/upland plateau. Two subsequent speciation events may be indicative of vicariant events in the eastern Pantepui. First, the *ginesi* clade diversified (P = 0.92; node #2 in Fig. 2) *c.* 14.5 Ma into two main lineages today roughly separated by the broad valley between the Los Testigos and the Chimantá Massifs to the north, and the large valley formed by the Río Toronó to the southeast. Second, the large clade sister to the *ginesi* clade diversified (P = 0.62; node #3 in Fig. 2) *c.* 22.3 Ma into two clades today separated by the Gran Sabana. These two clades are represented by a large highland/upland radiation to the east, and an Auyán Massif + Western Pantepui District clade to the west. Three distinct dispersal events occurred from the large eastern highland/upland radiation. Initial dispersals occurred from the Miocene onwards, to the summit of Yuruaní-tepuí first (from *c.* 11.0 Ma onwards), and subsequently to the summit of Wei-Assipu-tepui (from *c.* 7.6 Ma onwards) [P = 0.81 (node #4 in Fig. 2) and P = 0.89 (node #5 in Fig. 2) respectively]. The third dispersal, to the eastern peripheral lowlands (P = 0.99; node #6 in Fig. 2), likely occurred in the Quaternary, *c.* 1.8 Ma,

When analysed separately the large eastern highland/upland radiation (see Fig. S4 in Appendix S3) yielded the highest likelihood for the DEC+J model (see Table S4 in Appendix S3). Results suggest a highland origin for that clade (P = 0.51; node #7 in Fig. 2), which mostly expanded downward by dispersal, first from the Miocene onwards [two events, P = 0.69 (after node #8 in Fig. 2) and P = 0.72 (after node #9 in Fig. 2)], then in the Quaternary [two events, P = 0.84 (after node #10 in Fig. 2) and P = 0.95 (after node #6 in Fig. 2)]. Two upward dispersal events appear to have taken place from the Miocene onwards, to the summit of Yuruaní-tepuí (P = 0.67; node #4 in Fig. 2), then to the summit of Wei-Assipu-tepui (P = 0.92; node #5 in Fig. 2) (see above and Discussion).

Analysis of the *ginesi* clade taken separately revealed equal likelihoods from both the DIVALIKE and DIVALIKE+J models, in which all changes of character states are explained by vicariance (see Fig. S5 and Table S5 in Appendix S3). These results were predictable as each terminal corresponded to a different area, and we could not provide any prior knowledge about possible dispersal routes and dispersal abilities.

DISCUSSION

Our Bayesian relaxed-clock analysis suggests the onset of diversification of *Stefania* starting in the Oligocene, around 26 Ma with credibility intervals ranging from Eocene to Miocene. Our findings refine the prior estimate of 30.8 Ma (Schmid *et al.*, 2013/2012), and *c.* 18 Ma (Duellman, 2015) as these two studies included only five and four *Stefania* species respectively.

Our analyses showed that (1) the MRCA had a widespread distribution and probably originated in the highlands, (2) most divergence events predated the Pleistocene, and (3) the presence of *Stefania* in Pantepui uplands and peripheral lowlands is probably the result of dispersal events from highland areas having taken place in the Miocene and in the Pleistocene. Alternative scenarios retrieved from the biogeographical analyses do not strongly affect these conclusions. The next best alternative biogeographical scenarios also suggested early vicariant events (see Table S7 in Appendix S3) and a highland origin for tepui summit species/populations (see Table S8 in Appendix S3). The most likely ancestral range of the large eastern highland/upland radiation was in the highlands (P = 71 when results are combined) (see Table S8 in Appendix S3). It is possible that the ancestors of this clade had distributions that included some uplands (P = 30 when results are combined). Such a scenario, however, would not significantly affect our interpretation of the results, which indicate that vicariance among tepuis influenced diversification in *Stefania,* favouring the Plateau Theory (hypothesis 2). Our findings, suggesting a period of high tepui fragmentation and vertical isolation from surrounding areas extending from the Eocene to the Oligocene, do not disagree with time estimates for appropriate geological events suggested by Gansser (1954), Simpson (1979), Gómez *et al.* (2003) and Orme (2007). Although two dispersal episodes from highlands to tepui summits occurred, the hypothesis of dispersal from uplands/lowlands for the origin of tepui summit species is unlikely (hypothesis 1 rejected). Dispersal events from highland to upland areas are evident in the *woodleyi* and in the *evansi* clades. The earliest dispersal events in both clades could relate to a period of substantial Andean uplift causing major geographical changes, such as the Amazon river reaching the Atlantic and the Orinoco changing course (Ollier & Pain, 2000); local changes in precipitation and seasonality are also reported during these periods (Bradshaw *et al.*, 2012). The more recent downward dispersals in the Pleistocene may correspond to glacial-interglacial cycles and environmental instability (Gibbard & van Kolfschoten, 2004).

The historical biogeography of *Stefania* contrasts with that of other Pantepui amphibian genera, in which extant summit populations apparently diverged more recently (Kok *et al.*, 2012; Salerno *et al.*, 2012). Extinctions, bottlenecks, and repeated vertical migrations caused by climatic instability (induced by the climax of the Andean orogeny, the emergence of the Central American Isthmus, and Quaternary climate oscillations) have been postulated as the main factors for the recent origin of most extant amphibian tepui summit species (Kok, 2013). Botanical studies suggest that tepui summits had no or very limited organic soils during the Last Glacial Maximum, and were therefore too inhospitable for the maintenance of viable floral and faunal populations (e.g. Schubert *et al.*, 1986). Our results suggest that climate instability had a lower impact on *Stefania* species than on other Pantepui lineages, with *Stefania* persisting where other amphibian lineages went extinct. Kok (2013) hypothesized that the breeding ecology of the genus induced a different response to environmental challenges. Unlike other tepui summit amphibians, which are either direct developers, or have a biphasic life cycle, *Stefania* females carry eggs and metamorphs on their back until juveniles are fully formed and independent (Jungfer & Boehme, 1991). This adaptation makes *Stefania* species independent of the permanent or semi-permanent water bodies necessary for tadpoles, and permanent moist places necessary for eggs of direct developers. *Stefania* species are the only tepui summit amphibians able to actively procure the right conditions for their eggs to successfully develop. This peculiar breeding ecology could have helped the genus to endure drier climatic conditions by using *in situ* microrefugia like deep fractures and canyons present on many tepui summits, or on upper tepui slopes. This hypothesis implies that the absence of such microrefugia at the time of climate changes would have caused extinction of the species, which would explain the apparent absence of *Stefania* on some tepui summits, such as Mount Roraima and Kukenán-tepui (which seem to lack such appropriate microrefugia). Why these tepuis were not reinvaded from the surrounding highlands and uplands remains a mystery as other amphibian genera (*Oreophrynella*, *Pristimantis*) were able to do it (Kok *et al.*, 2012).

CONCLUSION

Our analyses point to a Pantepui highland origin of *Stefania*, and indicate that vicariance played an important role in the diversification of the genus. Our results also highlight a complex pattern implying several dispersal events, downward into the surrounding uplands and lowlands as well as upward onto currently isolated tepui summits. The postulate that the current distribution of the tepui summit fauna is only the result of dispersal from the surrounding lowlands (e.g. Salerno *et al.*, 2012) is oversimplified and not supported by our data, or recent molecular studies based on smaller data sets [e.g. Capurucho *et al.*, 2013 (birds); Leite *et al.*, 2015 (rodents)]. Rather, our data are consistent with a period of accelerated uplift and high fragmentation during the Eocene/Oligocene (25–45 Ma), and a more recent period for complete isolation of individual tepui summits (late Miocene to Holocene?). A pattern of recent isolation in many extant tepui summit species (Kok *et al.*, 2012) does not necessarily refute the Plateau Theory, and several elements seem to support that hypothesis for the early origin of the Pantepui fauna. For example, known divergences between amphibian genera endemic to Pantepui (or that have multiple representatives on tepui summits) and their known sister group usually pre-date the Neogene and are estimated in the Eocene/Oligocene, *c.* 25–45 Ma (e.g. this work; Heinicke *et al.*, 2009; Santos *et al.*, 2009; Van Bocxlaer *et al.*, 2010), and up to the Palaeocene for *Ceuthomantis* (Heinicke *et al.*, 2009). Likewise, many lineages of amphibians and reptiles, but also in other zoological groups such as birds (e.g. Mayr & Phelps, 1967), invertebrates (e.g. Breure, 2009), and in plants (e.g. Steyermark, 1986) are currently endemic to the remnant of that assumed ancient plateau.

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SUPPORTING INFORMATION

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

Appendix S1 Sampling and source of DNA.

Appendix S2 Phylogenetic inference and dating.
Appendix S3 Results of the BioGeoBEARS analyses.

BIOSKETCH

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