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On the distribution and conservation of two "Lost World" tepui summit endemic frogs, *Stefania ginesi* Rivero, 1968 and *S. satelles* Señaris, Ayarzagüena, and Gorzula, 1997

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***Abstract.*—It has been suggested that the inability to migrate in response to climate change is a key threat to tepui summit biota. Tepui summit organisms might thus seriously be threatened by global warming, and there is an urgent need to accurately evaluate their taxonomic status and distributions. We investigated phylogenetic relationships among several populations of *Stefania ginesi* and *S. satelles*, two endemic species reported from some isolated tepui summits, and we examined their IUCN conservation status. Molecular phylogenetic analysis and preliminary morphological assessment indicate that both species are actually restricted to single tepui summits and that five candidate species are involved under these names. We advocate upgrading the conservation status of *S. ginesi* from Least Concern to Endangered, and that of *S. satelles* from Near Threatened to Endangered.**

Key words. Endangered species, Hemiphractidae, IUCN, molecular phylogenetics, molecular taxonomy, Venezuela

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Introduction

The frog genus *Stefania* (Hemiphractidae) is endemic to an iconic South American biogeographical region named "Pantepui" (Mayr and Phelps 1967; McDiarmid and Donnelly 2005) (Fig. 1). Pantepui, often referred to as the "Lost World" because of Arthur Conan Doyle's famous novel (1912), lies in the western Guiana Shield. The region harbors numerous isolated Precambrian sandstone tabletop mountains more formally known as "tepuis" (Fig. 2). Although Pantepui was initially restricted to tepui slopes and summits above 1,500 m elevation (Mayr and Phelps 1967; Rull and Nogué 2007), Steyermark (1982), followed by Kok et al. (2012) and Kok (2013a), expanded the original definition of Pantepui to include the intervening Pantepui lowlands (200-400 m asl) and uplands (400-ca. 1,200 m asl) in order to better reflect the biogeography and biotic interactions in the area (Kok 2013a). The genus *Stefania* currently includes 19 species, 15 of which are restricted to tepui slopes or summits (Duellman 2015; Frost 2015). *Stefania* species are direct-developers (eggs and juveniles carried on the back of the mother) and occupy various types of habitats from lowland rainforest to tepui bogs (Kok 2013a; Schmid et al. 2013; Duellman 2015). The genus *Stefania* was erected by Rivero (1968) to accommodate *Cryptobatrachus evansi* and a few related new species all morphologically divergent from other *Cryptobatrachus*. Shortly later, 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. Kok et al. (2012), followed by Castroviejo et al. (2015), showed that, based on molecular data, these groups are actually not reciprocally monophyletic. A complete molecular phylogenetic analysis of the genus *Stefania* is still lacking, and

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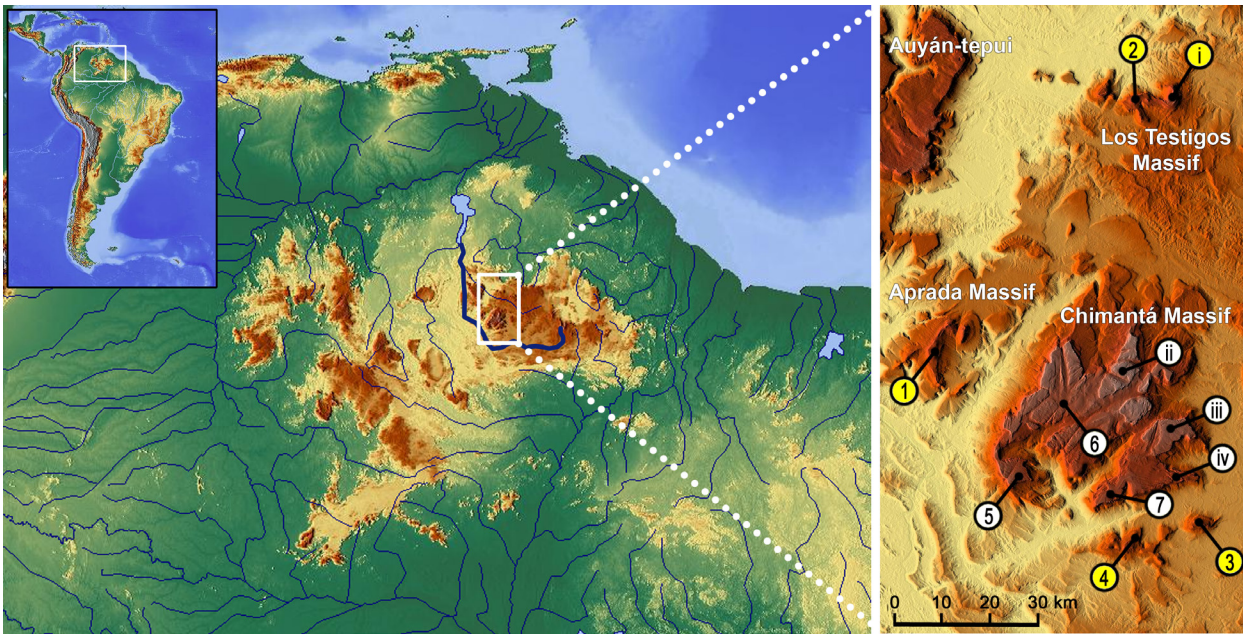

Fig. 1. Left: Map of Pantepui and its location within South America (inset); the thick blue line indicates the Río Caroní. Right: Map of the area under study showing localities mentioned in the text (yellow dots represent known localities of occurrence of *Stefania satelles*, white dots represent known localities of occurrence of *Stefania ginesi*). Numbers indicate sampled localities and Roman numerals indicate unsampled localities, as follows: (1) Aprada-tepui, Venezuela; (2) Murisipán-tepui, Venezuela; (3) Upuigma-tepui, Venezuela; (4) Angasima-tepui, Venezuela; (5) Abakapá-tepui, Venezuela; (6) Chimantá-tepui, Venezuela; (7) Amurí-tepui, Venezuela; (i) Kamarkawarai-tepui, Venezuela; (ii) Murei-tepui, Venezuela; (iii) Churí-tepui, Venezuela; (iv) Akopán-tepui, Venezuela.

relationships between many species or populations are unknown. Likewise, the exact distribution of some tepui summit species is uncertain (e.g., Gorzula and Señaris 1999). Among these, two tepui summit endemic *Stefania* species are known from several isolated tepui summits: *Stefania ginesi* Rivero, 1968, which is reported from six tepuis in the Chimantá massif (Chimantá-tepui, Amurí-tepui, Abakapá-tepui, Churí-tepui, Akopán-tepui, and Murei-tepui; Señaris et al. 1997; Gorzula and Señaris 1999; Barrio-Amorós and Fuentes 2012; Fig. 1), and *Stefania satelles* Señaris, Ayarzagüena, and Gorzula, 1997, which has a highly disjunct distribution, being reported from Aprada-tepui (in the Aprada Massif), Angasima-tepui, and Upuigma-tepui (two southern outliers of the Chimantá massif), and from Murisipán-tepui and Kamarkawarai-tepui (in the Los Testigos Massif, north of the Chimantá massif) (Señaris et al. 1997; Gorzula and Señaris 1999; Fig. 1). *Stefania ginesi* is listed as Least Concern (LC) by the International Union for Conservation of Nature (IUCN) (Stuart et al. 2008) and S. *satelles* is listed as Near Threatened (NT) (Stuart et al. 2008). However, preliminary data suggest that their respective distributions could be more restricted than initially thought because more than two species could be involved under these names (the authors, unpublished; see also Señaris et al. 2014 regarding the distribution of *S. ginesi*). Herein we used molecular phylogenetics to investigate the relationships among three populations of *S. ginesi* and four populations of *S. satelles*. We also aim at providing a more precise distribution of these two taxa in order to refine their conservation status. Indeed, tepui ecosystems are reported as particularly sensitive to global warming (Nogué et al. 2009), and tepui summit organisms might be seriously threatened by habitat loss due to upward displacement (Rull and Vegas-Vilarrúbia 2006; see also below). Likewise, climate envelope distribution models of tepui ecosystems based on future scenarios show that potential distributions become drastically smaller under global warming (Rödder et al. 2010). Species restricted to tepui summits are thus clearly at risk of extinction, and there is an urgent need to evaluate their exact taxonomic status and precise distribution.

Materials and Methods

Tissue sampling and molecular data

We combined available GenBank sequences of *Stefania ginesi* and *S. satelles* for fragments of the mitochondrial 16SrRNA gene (16S) and the protein-coding mitochondrial gene NADH hydrogenase subunit 1 (ND1) with 40 novel DNA sequences of *Stefania ginesi* and *S. satelles*: nine of fragments of 16S, five of ND1, 13 of the nuclear recombination activating gene 1 (RAG1), and 13 of the nuclear CXC chemokine receptor type 4 gene (CXCR4). We combined this dataset with DNA sequences of four additional members of the genus *Stefania* from outside the studied area (three species from east of the Río Caroní: *S. scalae*, an upland species, *S. riveroi* and *S. schuberti*, two highland species; and one highland spe-

Fig. 2. Typical Pantepui landscape. Photograph taken on 8th June 2012 from the summit of Upuigma-tepui, showing Angasima-tepui on the left and Akopán-tepui and Amurí-tepui on the right. Note stretches of savannah mainly caused by anthropogenic fires. *Photo PJRK.*

cies from west of the Río Caroní: *S. riae*; in total 16 novel sequences), and with *Fritziana ohausi*, member of the clade sister to *Stefania* (Castroviejo et al. 2015), which was selected as outgroup (see Table 1). Novel sequences have been catalogued in GenBank under the accession numbers KU958582-958637.

Total genomic DNA was extracted and purified using the Qiagen DNeasy® Tissue Kit following manufacturer's instructions. Fragments of 16S (ca. 550 base pairs [bp]), of ND1 (ca. 650 bp), and of RAG1 (ca. 550 bp) and CXCR4 (ca. 625 bp) were amplified and sequenced using the primers listed in Kok et al. (2012) and Biju and Bossuyt (2003) under previously described PCR conditions (Biju and Bossuyt 2003; Roelants et al. 2007; Van Bocxlaer et al. 2010). PCR products were checked on a 1% agarose gel and were sent to BaseClear (Leiden, The Netherlands) for purification and sequencing. Chromatograms were read using CodonCode Aligner 5.0.2

Table 1. List of *Stefania* taxa and outgroup used in this study, with localities and GenBank accession numbers. Sequences newly generated are in boldface. IRSNB = Institut Royal des Sciences Naturelles de Belgique, Belgium; MZUSP = Museu de Zoologia, Universidade de São Paulo, Brazil.

Voucher	16S	ND1	RAG1	CXCR4	Genus	Species	Locality	Country	Coordinates	Elevation (m)
IRSNB16724	JQ742191	JQ742362	**KU958600**	**KU958619**	*Stefania*	*scalae*	Salto El Danto	Venezuela	N 5°57'52" W 61°23'31"	1208
Uncatalogued	JQ742172	JQ742343	**KU958601**	**KU958620**	*Stefania*	*riae*	Sarisariñama-tepui	Venezuela	N 4°41' W 64°13'	ca. 1100
IRSNB15703	JQ742177	JQ742348	**KU958602**	**KU958621**	*Stefania*	*riveroi*	Yuruaní-tepui	Venezuela	N 5°18'50" W 60°51'50"	2303
IRSNB15716	JQ742178	JQ742349	**KU958603**	**KU958622**	*Stefania*	*riveroi*	Yuruaní-tepui	Venezuela	N 5°18'50" W 60°51'50"	2303
IRSNB16725	JQ742173	JQ742344	**KU958604**	**KU958623**	*Stefania*	*"ginesi"*	Abakapá-tepui	Venezuela	N 5°11'23" W 62°17'52"	2137
IRSNB16726	JQ742174	JQ742345	**KU958605**	**KU958624**	*"ginesi"*	*"ginesi"*	Abakapá-tepui	Venezuela	N 5°11'07" W 62°17'21"	2209
IRSNB15839	JQ742175	JQ742346	**KU958606**	**KU958625**	*Stefania*	*"satelles"*	Angasima-tepui	Venezuela	N 5°02'36" W 62°04'51"	2122
IRSNB15844	JQ742176	JQ742347	**KU958607**	**KU958626**	*Stefania*	*"satelles"*	Angasima-tepui	Venezuela	N 5°02'36" W 62°04'51"	2122
IRSNB16727	**KU958582**	**KU958593**	**KU958608**	**KU958627**	*Stefania*	*"satelles"*	Upuigma-tepui	Venezuela	N 5°05'10" W 61°57'32"	2134
IRSNB16728	**KU958583**	—	**KU958609**	**KU958628**	*Stefania*	*satelles*	Aprada-tepui	Venezuela	N 5°24'39" W 62°27'00"	2551
IRSNB16729	**KU958584**	—	**KU958610**	**KU958629**	*Stefania*	*satelles*	Aprada-tepui	Venezuela	N 5°24'43" W 62°27'03"	2576
IRSNB16730	**KU958585**	**KU958594**	**KU958611**	**KU958630**	*Stefania*	*"ginesi"*	Amurí-tepui	Venezuela	N 5°08'34" W 62°07'08"	2215
IRSNB16731	**KU958586**	**KU958595**	**KU958612**	**KU958631**	*Stefania*	*"ginesi"*	Amurí-tepui	Venezuela	N 5°08'35" W 62°07'08"	2213
IRSNB16732	**KU958587**	**KU958596**	**KU958613**	**KU958632**	*Stefania*	*schuberti*	Auyán-tepui	Venezuela	N 5°45'56" W 62°32'25"	2279
IRSNB16733	**KU958588**	**KU958597**	**KU958614**	**KU958633**	*Stefania*	*schuberti*	Auyán-tepui	Venezuela	N 5°45'56" W 62°32'25"	2279
IRSNB16734	**KU958589**	**KU958598**	**KU958615**	**KU958634**	*Stefania*	*"satelles"*	Murisipán-tepui	Venezuela	N 5°52'03" W 62°04'30"	2419
IRSNB16735	**KU958590**	**KU958599**	**KU958616**	**KU958635**	*Stefania*	*"satelles"*	Murisipán-tepui	Venezuela	N 5°52'03" W 62°04'30"	2419
IRSNB16736	**KU958591**	—	**KU958617**	**KU958636**	*Stefania*	*ginesi*	Chimantá-tepui	Venezuela	N 5°19'12" W 62°12'07"	2180
IRSNB16737	**KU958592**	—	**KU958618**	**KU958637**	*Stefania*	*ginesi*	Chimantá-tepui	Venezuela	N 5°19'12" W 62°12'07"	2180
MZUSP139225	JN157635	KC844945	KC844991	—	*Fritziana*	*ohausi*	n/a	Brazil	n/a	n/a

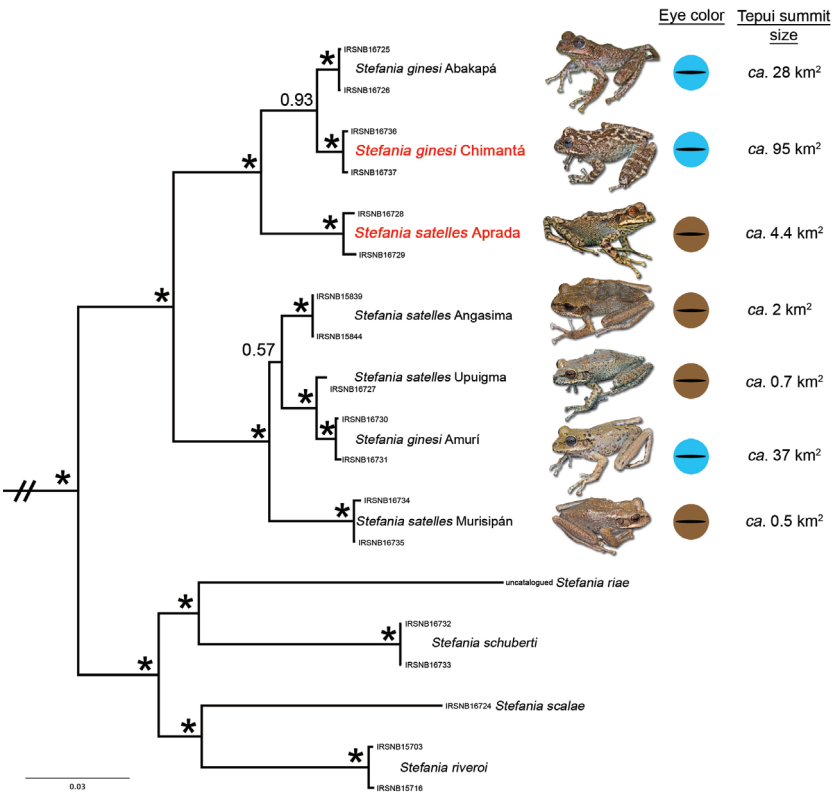

Fig. 3. Phylogenetic relationships as recovered in the MrBayes analysis (concatenated dataset, 2359 bp), outgroup not shown. Values at each node represent Bayesian posterior probabilities; asterisks indicate values > 95%. *Stefania ginesi* sensu stricto, and *S. satelles* sensu stricto are highlighted in red. Relation between eye color and tepui summit surface is indicated on the right side of the figure. *Photos PJRK.*

(http://www.codoncode.com/index.htm) and a consensus sequence was assembled from the forward and reverse primer sequences. MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) was used to perform preliminary alignment using G-INS-i and default parameters. Minor alignment corrections were made using MacClade 4.08 (Maddison and Maddison 2005). Protein-coding sequences were translated into amino-acid sequences to check for unexpected stop codons. Alignment-ambiguous regions of 16S were excluded from subsequent analyses.

Molecular phylogenetic analyses

The combined 16S + ND1 + RAG1 + CXCR4 dataset (totalling 2,359 bp after exclusion) was subjected to phylogenetic inference using Bayesian analyses. Optimal partitioning schemes were estimated with PartitionFinder v1.1.1 (Lanfear et al. 2012) using the "greedy" algorithm, the "mrbayes" set of models, and the Bayesian Information Criterion (BIC) to compare the fit of different models. 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 (https://www.phylo.org/, Miller et al. 2010). The Bayesian analyses implemented the best substitution models inferred by PartitionFinder v1.1.1 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 20,000,000 generations, a sampling frequency of 1 per 1,000 generations, and a burn-in corresponding to the first 1,000,000 generations. Convergence of the parallel runs was confirmed by split frequency SDs (<0.01) and potential scale reduction factors (~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 and Drummond 2009). Effective sample sizes (ESS) largely over 200 were obtained for every parameter. Results were visualized and edited in FigTree 1.4.1 (Rambaut 2014).

Results

Stefania ginesi and *S. satelles* as currently recognized are recovered non-reciprocally monophyletic (Fig. 3). Our molecular phylogeny also reveals the occurrence of five candidate species (sensu Padial et al. 2010) that have been misidentified for more than a decade as *S. ginesi* (two candidate species) or *S. satelles* (three candidate species) (e.g., Señaris et al. 1997; Gorzula and Señaris 1999). Preliminary morphological analyses (in progress) indicate a few, sometimes subtle, morphological characters allowing discrimination among these candidate spe-

cies and *S. ginesi* and *S. satelles*. Our combined results indicate that *S. ginesi* sensu stricto is likely restricted to its type locality, Chimantá-tepui, as we suspect that populations from other tepuis in the Chimantá Massif that were not sampled in this study will prove to be distinct as well. As for *Stefania satelles*, the species is restricted to its type locality, Aprada-tepui.

Discussion and conservation recommendations

We assumed that misidentifications were likely due to a rather conserved external morphology (e.g., head broader than long, skin strongly granular, absence of prominent cranial crests) of all tepui summit species previously identified as *Stefania ginesi* or *S. satelles*. This conserved morphology appears to be symplesiomorphic, and probably the result of an allopatric non-adaptive radiation (lineage diversification with minimal ecological diversification, see Rundell and Price 2009). It is, however, intriguing that two slightly divergent phenotypes (a "*satelles* phenotype" with brown eyes and a "*ginesi* phenotype" with blue eyes) evolved independently in each subclade (see Fig. 3). Interestingly, selection towards one of these two phenotypes seems closely associated with the size of the summit surface on which the species occur (see Fig. 3). The "*ginesi* phenotype" is found on large tepui summits (surface > 25 km^2) in the central Chimantá Massif, whereas the "*satelles* phenotype" is found on much smaller tepui summits (surface < 5 km^2) in the periphery of the core Chimantá Massif. Disentangling this phenomenon and the nature of the ecological constraints possibly involved and their influence on phenotypic trajectories is beyond the scope of this paper and will be treated in a separate study.

Most importantly, our results have direct implications on the conservation status of the populations under study. A complete taxonomic revision of the genus is in progress, but meanwhile we wish to emphasize the restricted distributions of all the populations previously known as *Stefania ginesi* or *S. satelles*. Our results argue for the upgrading of the conservation status of *S. ginesi* from LC to Endangered (EN), and that of *S. satelles* from NT to EN, based on the same argument recently developed for other species restricted to the summit of one or two tepuis, e.g., *Pristimantis imthurni* and P. *jamescameroni* (Kok 2013b), or *P. aureoventris* (IUCN SSC Amphibian Specialist Group 2014), thus in accordance with criteria B1 a-b (iii) and B2 a-b (iii) of the IUCN Red List of Threatened Species (IUCN 2014). We indeed argue that (1) extents of occurrence of *S. ginesi* and *S. satelles* are much less than 5,000 km^2 (less than 100 km^2 and five km^2, respectively); (2) areas of occupancy of *S. ginesi* and *S. satelles* are much less than 500 km^2 (less than 100 km^2 and five km^2, respectively); (3) there is an inferred and projected decline in the quality of habitat due to the effects of global warming upon tepui ecosystems, with an expected 2–4 °C increase in temperature in the region through the next century (IPCC 2007). As stressed by Nogué et al. (2009) and Rödder et al. (2010), this rise in temperature will likely cause a decrease in habitat suitability for tepui biota. In addition, numerous anthropogenic fires in the region (Means 1995; Rull et al. 2013, 2016), coupled with a global rise of temperature, may cause an up to 10% decrease in precipitation (IPCC 2007) instigating an increase in fire range and intensity (Rull et al. 2013, 2016); and (4) the altitudinal range of *Stefania ginesi* and *S. satelles* allows no vertical migration in order to avoid these threats. As mentioned by Rull and Vegas-Vilarrúbia (2006), the inability to migrate to compensate for the climate change is a key threat to tepui summit biota.

There is an urgent need to gain a greater understanding of species boundaries and distributions in Pantepui, especially in Venezuela where the threats are the highest due to ongoing uncontrolled anthropogenic fires (Rull et al. 2013, 2016). However, it is assumed that an even greater threat to Pantepui biota is global climate change. Local actions (such as stopping fires), even if necessary, might only have a limited impact on the long-term survival of Pantepui organisms. Conservation awareness is critically important in the area, notably due to the inaccessibility of tepui ecosystems where an out of sight, out of mind effect may have taken place.

This study adds to the many studies now available demonstrating that estimates of amphibian diversity based on morphology alone are often misleading. Molecular data have indeed been shown to be of great help in detecting cryptic species (e.g., Hebert et al. 2004; Vences et al. 2005; Fouquet et al. 2007; Burns et al. 2008; Fouquet et al. 2016). Unfortunately, while everyone seems to agree that gaining a greater understanding of the world biodiversity is needed in order to prioritize biodiversity conservation (e.g., Wilson 2016), the task turns more and more often into a bureaucratic obstacle course, if not an impossible mission for scientists working with molecular data.

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