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**Molecular phylogenetic relationships among *Pristimantis*
summit populations in the eastern tepui chain:
insights from *P. aureoventris* (Anura: Craugastoridae)**

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No less than 500 nominal species are currently recognized in the frog genus *Pristimantis*, the most speciose vertebrate genus in the world (FROST 2016). Only twenty of these species are distributed in Pantepui (area sensu KOK 2013b, see also KOK & BARRIO-AMORÓS 2013, ROJAS-RUNJAIC et al. 2013), nine of them being restricted to tepui summits and tepui upper slopes (KOK 2013a, ROJAS-RUNJAIC et al. 2013). According to MCDIARMID & DONNELLY (2005) and KOK (2013a, b), several undescribed *Pristimantis* species could still be expected in this poorly explored region. *Pristimantis* has a complex taxonomy and biogeographical history (PADIAL et al. 2014), and since most tepui summits and slopes have been inadequately sampled (KOK 2013b) any additional information about tepuian *Pristimantis* populations is crucial. Furthermore, any data about the distribution of tepuian species are important in terms of conservation because tepui summit organisms might seriously be threatened by global warming (e.g., RÖDDER et al. 2010, KOK et al. 2016a).

Pristimantis aureoventris KOK, MEANS & BOSSUYT, 2011 is currently only known from the type locality (the summit of Wei-Assipu-tepui at ca. 2,210 m elevation along the border between Guyana and Brazil), and from the upper slopes of the northern tip of Mount Roraima in Guyana at ca. 2,305 m elevation (KOK et al. 2011). No other records are known from neighboring tepuis or intervening upland/highland areas, but a putative new *Pristimantis* species from the summit of Mount Roraima has been reported to possibly be conspecific with *P. aureoventris* (KOK et al. 2011, see below). The geographically closest tepui summit species to *P. aureoventris* is *P. yuruaniensis*, which occurs on the summit of Yuruaní-tepui, about 19 km (airline) NW of

Wei-Assipu-tepui. Both taxa inhabit similar environments, and although phenotypically similar, these two species diverge in a number of morphological characters (e.g., size, skin texture), colour pattern (including sexual dichromatism), and advertisement call (KOK et al. 2011). In addition, *P. aureoventris* exhibits a high degree of pattern polymorphism, while *P. yuruaniensis* is barely variable. Molecular phylogenetic analyses indicated that populations of these two species are reciprocally monophyletic (KOK et al. 2012). MÄGDEFRAU & MÄGDEFRAU (1994) suggested the presence of *P. yuruaniensis* on the summit of Kukenán-tepui (located 10 km SE of Yuruaní-tepui, airline), mostly based on overall similarities of specimens and similar calls to the human ear (but apparently none of the Kukenán specimens was collected, nor the call of that population recorded). RÖDDER & JUNGFER (2008) mentioned the population from the summit of Kukenán-tepui as *P. cf. yuruaniensis* pending additional data and analyses, thus not ruling out that the specimen from Kukenán-tepui illustrated in RÖDDER & JUNGFER (2008: 64) could belong to an undescribed species. Tissue samples from two *Pristimantis* individuals from the summit of Kukenán-tepui were recently available and we conducted molecular analyses to elucidate their affinities to either *P. aureoventris* or *P. yuruaniensis*.

Kukenán-tepui lies in the Estado Bolívar in Venezuela and reaches a maximum elevation of 2,650 m above sea level (a.s.l.). Its summit area is estimated to be 20.63 km² and is covered by “pioneer vegetation on sandstone summits” according to MCDIARMID & DONNELLY (2005). To date, only two amphibian species have been recorded from the summit of Kukenán-tepui: *Oreophrynella nigra* and *Pristimantis cf. yuruaniensis*, with no addition since

Table 1. List of taxa used in this study, with localities and GenBank accession numbers.

Taxon	Country	Locality	GenBank voucher	GenBank accession number	Reference
<i>Pristimantis abakapa</i>	Venezuela	Abakapá-tepui	VUB3749	JQ742162	KOK et al. 2012
<i>P. aureoventris</i>	Venezuela	Kukenán-tepui	3513	KY495891	This study
<i>P. aureoventris</i>	Venezuela	Kukenán-tepui	3514	KY495892	This study
<i>P. aureoventris</i>	Guyana	Mt. Roraima	VUB3741	JQ742151	KOK et al. 2012
<i>P. aureoventris</i>	Guyana	Wei-Assipu-tepui	VUB3748	JQ742152	KOK et al. 2012
<i>P. aureoventris</i>	Guyana	Wei-Assipu-tepui	VUB3742	JQ742153	KOK et al. 2012
<i>P. aureoventris</i>	Guyana	Wei-Assipu-tepui	VUB3747	JQ742154	KOK et al. 2012
<i>P. aureoventris</i>	Guyana	Wei-Assipu-tepui	VUB3743	JQ742158	KOK et al. 2012
<i>P. sp. 'Ayanganna'</i>	Guyana	Mt. Ayanganna	ROM40164	EU186676	HEDGES et al. 2008
<i>P. jamescameroni</i>	Venezuela	Aprada-tepui	SBH268110	EU186721	HEDGES et al. 2008
<i>P. jester</i>	Guyana	Maringma-tepui	VUB3493	JQ742169	KOK et al. 2012
<i>P. pulvinatus</i>	Guyana	Iwokrama Forest Reserve	VUB3751	JQ742164	KOK et al. 2012
<i>P. pulvinatus</i>	Venezuela	La Escalera	VUB3674	JQ742165	KOK et al. 2012
<i>P. saltissimus</i>	Guyana	Mt. Ayanganna	ROM43913	EU186692	HEDGES et al. 2008
<i>P. saltissimus</i>	Guyana	Mt. Wokomung	ROM43310	EU186693	HEDGES et al. 2008
<i>P. saltissimus</i>	Guyana	Maringma-tepui	VUB3490	JQ742168	KOK et al. 2012
<i>P. sp. 'Angasima'</i>	Venezuela	Angasima-tepui	VUB3750	JQ742163	KOK et al. 2012
<i>P. yuruanensis</i>	Venezuela	Yuruani-tepui	VUB3717	JQ742160	KOK et al. 2012

the first herpetological exploration on its summit in 1977 (MÄGDEFRAU & MÄGDEFRAU 1994, RÖDDER & JUNGFER 2008, McDIARMID & DONNELLY 2005).

Two *Pristimantis* individuals were caught by hand by DG on 27th November 2015 on the summit of Kukenán-tepui (5.18873° N, -60.81715° E, 2,249 m a.s.l.; coordinates and elevations acquired using a Garmin 60CSx Global Positioning System unit and referenced to map datum WGS84). Both individuals were collected during a wet, foggy and rainy night, ca. five meters from each other. Habitat at the sampled locality consisted of steep sandstone cliffs along rocky outcrops, with dense fern ground cover at their base (Figs 1E, F). Vicinity of the locality consisted of classic “tepuian vegetation”, with large areas of coarse herbs mixed with woody subshrubs on peat soils, some quaking bogs and extensive patches of dwarf forests dominated by *Bonnetia roraimae* (Theaceae). The first individual (unsexed adult, Figs 1A–B) was found 1.3 m above the ground on a rocky cliff, the second one (calling male, Figs 1C–D) was caught ca. 5 meters above the ground in a shrub growing along the cliff. Temperature during the night was approximately 13°C at the site of capture. Tissue samples (a phalange of each specimen) were collected and preserved in 96% ethanol and deposited in the tissue collection of DJ in the Department of Zoology, at the Comenius University in Bratislava under numbers 3513 (unsexed individual) and 3514 (male) and duplicated in the tissue collection of the Amphibian Evolution Lab, Vrije Universiteit Brussel, Belgium. Colour pattern in life was estimated using colour digital photographs. No measurements were taken, and both specimens were immediately released at the site of capture.

Total genomic DNA was extracted from the two samples using the Qiagen DNeasy® Blood and Tissue Kit following manufacturer's protocols. DNA was amplified for a fragment of the widely used 16S ribosomal RNA standard phylogenetic marker (16S) with primers 16Sar-L and 16Sbr-H (PALUMBI et al. 1991). PCR products were sent to Macrogen Inc. (Amsterdam, the Netherlands) for purification and sequencing. The two novel sequences were deposited in GenBank under accession numbers KY495891 and KY495892. The new sequences were combined with (1) available 16S GenBank sequences for *P. aureoventris* and *P. yuruanensis*; and (2) selected 16S sequences of closely related Pantepui *Pristimantis* species (Table 1). For intraspecific relationships within *P. aureoventris* we only used the distinct haplotypes published in KOK et al. (2012), not the all-individual dataset. The 16S fragments (563 bp) were aligned using Clustal W algorithm (THOMPSON et al. 1994) as is implemented in BioEdit (HALL 1999). Alignments were checked by eye and low quality ends trimmed. Ambiguously aligned region/gaps were ignored for the subsequent analysis. We used a network approach (POSADA & CRANDALL 2001) to infer inter-individual relationships. First, we generated a phylogenetic network of closely related species of Pantepui *Pristimantis* (see above) using the Neighbor-Net algorithm (BRYANT & MOULTON 2004) implemented in the software SplitsTree 4.10 (HUSON & BRYANT 2006). To assess the support for the observed structure, bootstrap analysis was performed with 1000 replicates. Nodes were considered strongly supported if they received bootstrap values > 70%. This type of analysis is a powerful tool for inferring and visualizing conflicting and consistent evidence



Figure 1. Individuals of *Pristimantis aureoventris* from Kukenán-tepui and their habitat. (A) Dorsolateral view of an unsexed individual; (B) Ventral view of the same individual; (C) Dorsolateral view of a male; (D) Ventral view of the same individual; (E) Locality of capture (arrow in F) on the summit of Kukenán-tepui; and (F) Macrohabitat on Kukenán-tepui. All photos by DG except 1F (P. FENŐA).

in the dataset (HUSON & BRYANT 2006). Second, we constructed a haplotype network for *P. aureoventris* and *P. yuruaniensis* using the 95% limit of parsimony as implemented in TCS 1.21 (CLEMENT et al. 2000).

Both methods supported the identification of the two individuals from Kukenán-tepui as *Pristimantis aureoventris*, which is the first evidence of the presence of *P. aureoventris* in Venezuela. As indicated in the network, the sequences obtained from the new specimens clearly cluster with sequences of *P. aureoventris* (JQ74251–54, 58), all of them being very close to *P. yuruaniensis* (JQ742160), the most closely related species according to previous phylogenetic analyses (KOK et al. 2012). Both new sequences from Kukenán-tepui are distinct haplotypes separated from haplotypes from Mount Roraima by one and/or two mutation steps, and by three and four mutation steps from haplotypes from Wei-Assipu-tepui.

The newly discovered population of *Pristimantis aureoventris* is located (airline distances) 11 km from the single known locality of *P. aureoventris* on Mount Roraima (northern upper slopes), 13 km from the type locality of *P. aureoventris* (Wei-Assipu-tepui), and 14 km from Yuruaní-tepui (type locality of *P. yuruaniensis*). Coloration

and pattern of the Kukenán individuals (Figs 1A–D) are consistent with the colour pattern variation observed in *P. aureoventris* as illustrated in KOK et al. (2011).

The mechanisms underlying the species/populations structure on a single tepui and among isolated tepui summits are still not fully understood. KOK (2013b) and KOK et al. (2016b) hypothesized an intricate pattern of multiple nonexclusive processes to explain species diversification in the area, and suggested that different lineages may have responded differently to the same historical events. It seems indeed difficult to find a generalized evolution pattern applicable to all tepui summit amphibian species/populations. For example, although the current distribution of *Oreophrynella quelchii* (Wei-Assipu-tepui and Mount Roraima) and *O. nigra* (Kukenán-tepui and Yuruaní-tepui) would suggest closer affinities within each of these two pairs of tepuis, our results indicate that this is not relevant to *Pristimantis*. Our observations also suggest that *Pristimantis* sp. “J” from the summit of Mount Roraima in Venezuela (see McDIARMID & DONNELLY 2005) could be *P. aureoventris*, but tissue samples and specimens from that population are necessary to corroborate this assumption.

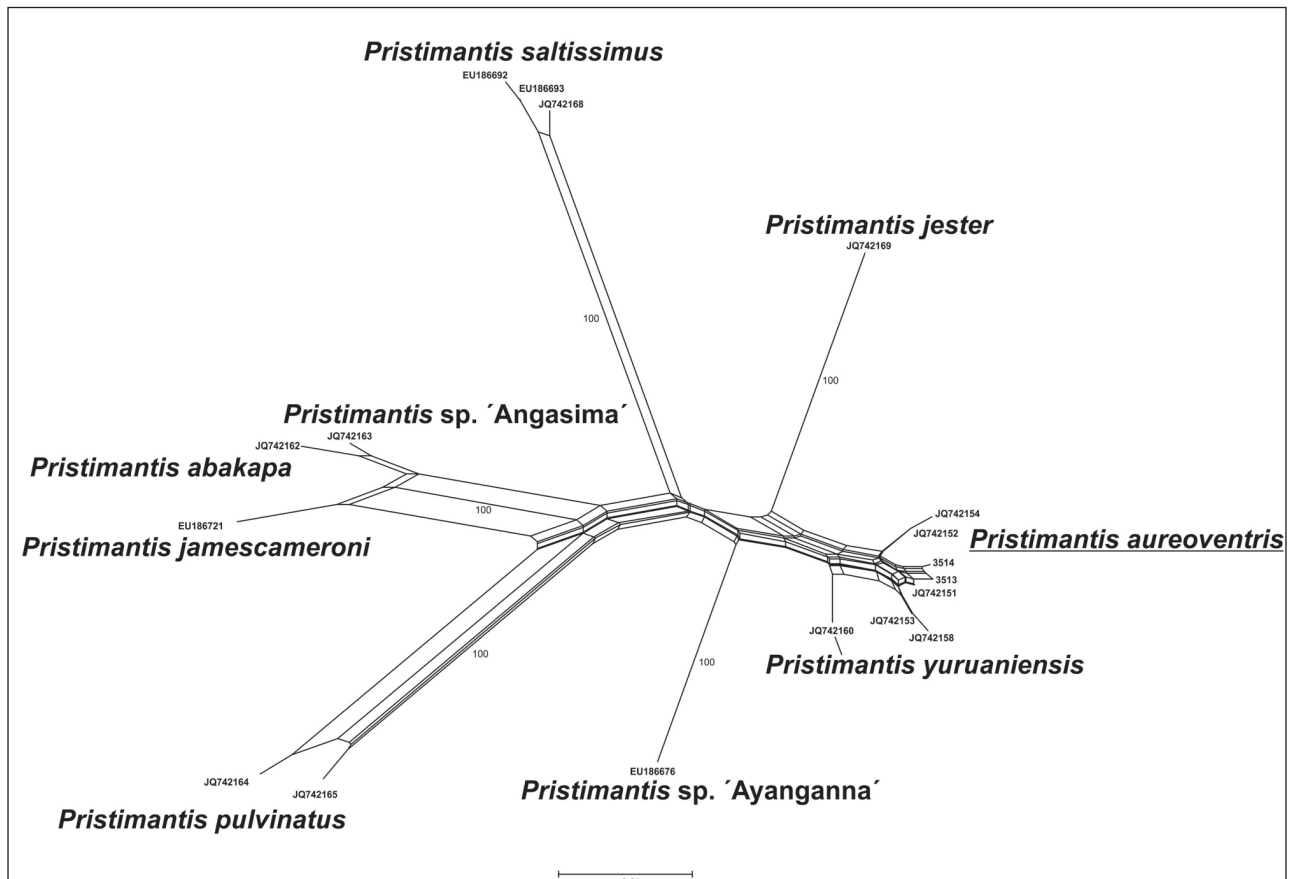


Figure 2. Mitochondrial 16S rRNA neighbor-net networking of selected *Pristimantis* species from Pantepui using SplitsTree. Scale bar represents 1% sequence divergence; numbers correspond to bootstrap value.

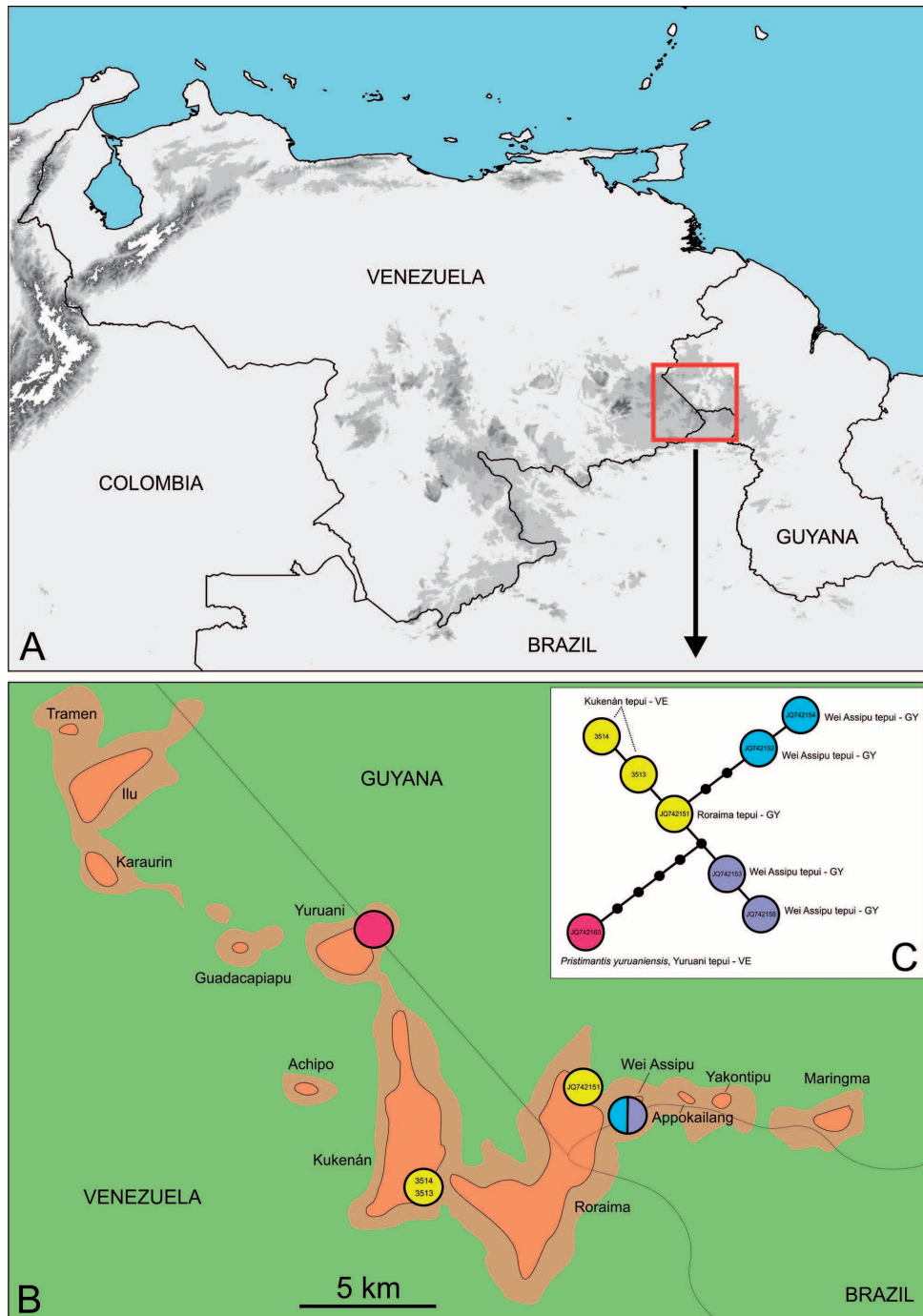


Figure 3. (A) Location of the eastern tepui chain (enlarged red rectangle) in northern South America; (B) Map of the eastern tepui chain (modified from KOK et al. 2011) showing the distribution of known haplogroups of *Pristimantis aureoventris* on Kukenán-tepui, Mount Roraima and Wei-Assipu-tepui, and of *P. yuruaniensis* on Yuruani-tepui; and (C) Haplotype network inferred for 16S rDNA dataset in TCS under the 95% parsimony threshold of *P. aureoventris* and the closely related *P. yuruaniensis*. Colours are concordant with those used in Fig. 3B.

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