

# Population genetics and distribution data reveal conservation concerns to the sky island endemic *Pithecopus megacephalus* (Anura, Phyllomedusidae)

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**Abstract** *Pithecopus megacephalus* is a reticulated monkey–frog species endemic to the highlands of the Espinhaço Mountain Range in southeastern Brazil, an important centre of endemism in South America. This species has a discontinuous distribution and is considered “data-deficient” by the IUCN Red List, raising concerns about its conservation. Understanding the historical dynamics and connectivity of *P. megacephalus* populations can provide guidelines for preservation of this species in the wild. To investigate the population dynamics of *P. megacephalus*, we obtained multilocus DNA data for 55 individuals from different locations along

the species’ known distribution. Spatial population structure, genetic diversity and demographic parameters were evaluated using population genetic and phylogeographical tools. We also evaluated its extent of occurrence and area of occupancy to investigate extinction risk of this species. We found genetic structure along *P. megacephalus*’ spatial distribution in the South Espinhaço Mountain Range corresponding to three population groups: northern, central and southern. Our results could provide important data on geographic distribution and population dynamics for a Data Deficient species. Therefore, we suggest these population data, together with the species’ limited occurrence in sky island environments could be used for a more accurate classification of *P. megacephalus* in the IUCN list, and conservation strategies for the species should be planned accordingly.

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**Keywords** Genetic structure · Data deficient species · Espinhaço Mountain Range · IUCN Red List · Protected areas

## Introduction

Continental montane regions harbour many endemic species of fauna and flora, a fact likely related to their complex topologies and uniqueness of climate and habitat in relation to surrounding low altitude landscapes (Chaves et al. 2015). One important landscape feature that leads to high levels of species endemism is the discontinuous distribution of mountaintops isolated by valleys with different habitats and climate, known globally as ‘sky islands’ (Warshall 1994). This geographical discontinuity, along with specific soil and climate characteristics, frequently results in high levels of genetic structure between populations of endemic biota found on different mountaintops; this in turn

requires appropriate conservation efforts (Freitas et al. 2012; Knowles 2000; Salerno et al. 2015; Silveira et al. 2016). Researchers have expressed concern that the flora and fauna of sky islands, which are typically adapted to cooler temperatures, and especially threatened by global climate change (Galbreath et al. 2009). This is also the case of the endemic biota from the Espinhaço Mountain Range of Brazil (Fernandes et al. 2014; Silveira et al. 2016). Therefore, specific conservation strategies are necessary to assure the preservation of species restricted to those mountains (Silveira et al. 2016).

The Espinhaço Mountain Range is a UNESCO Biosphere Reserve located in the Brazilian Shield (Warshall 1994; Chemale et al. 2011), and it is considered one of the most important areas of endemism in South America (Chaves et al. 2015; Silveira et al. 2016). It extends 1200 km from north to south (or 10.8 latitudinal degrees) along the Brazilian states of Minas Gerais and Bahia. The Espinhaço Mountain Range comprises heterogeneous plant physiognomies associated to high altitudes, and dominated by an ecosystem of grasslands with shrubs and rocky outcrops, known as *campos rupestres* (Silveira et al. 2016). The southern portion of this mountain range divides the Cerrado and Atlantic Forest domains, both considered hotspots for conservation (Myers et al. 2000), while the northern portion is found between the Caatinga and Cerrado domains. Phylogeographic and population genetic studies of different *campos rupestres* endemic plants, such as *Lychnophora ericoides*, *Pilosocereus* spp. and *Vellozia* spp., identified various levels of differentiation among populations of different sky islands, which were attributed to habitat isolation (Collevatti et al. 2009; Lousada et al. 2011, 2013; Bonatelli et al. 2014). This pattern was also found for the leaf frog *Pithecopus ayeaye* (Magalhães et al. in press), and raises the possibility that populations of organisms with limited dispersal abilities, such as other endemic amphibians, may be genetically differentiated among Espinhaço Mountain Range sky islands (Beebee 2005; Leite et al. 2008; Zeisset and Beebee 2008).

The herpetofauna associated with the Espinhaço Mountain Range is one of the most conspicuous examples of endemic Brazilian sky island biota (Leite et al. 2008). Among these endemics, *Pithecopus megacephalus* (Miranda-Ribeiro, 1926) (Anura, Phyllomedusidae) (Fig. 1a), formerly known as *Phyllomedusa megacephala* (see Duellman et al. 2016), is characterized by a green dorsum and colourful, reticulated pattern on its flanks and limbs. The species shows a restricted reproductive period associated with the availability of temporary static water habitats, such as crystal-clear water puddles formed after heavy rains (Eterovick and Barros 2003; Oliveira et al. 2012). It typically occurs at elevations greater than 800 m above sea level (m.a.s.l.) and it is restricted to *campos rupestres* of the South Espinhaço Range (SER) (Oliveira et al.

2012). The SER, mainly located in the state of Minas Gerais, is separated into three mountain regions with four distinct sky islands: the Setentrional (with two sky islands SE1 and SE2), Serra do Cabral (SC), and Meridional (ME) mountain regions (Fig. 1b) (Saadi 1995; Chemale et al. 2011). The presence of *P. megacephalus* in this naturally fragmented habitat results in small populations that are susceptible to the negative effects of genetic drift and other stochastic events, including climate change or anthropogenic impacts, which can increase the probability of local extinction (Diniz-Filho et al. 2008; Chaves et al. 2015). To evaluate these threats, we need to understand the evolutionary dynamics between sky islands populations (Morais et al. 2013).

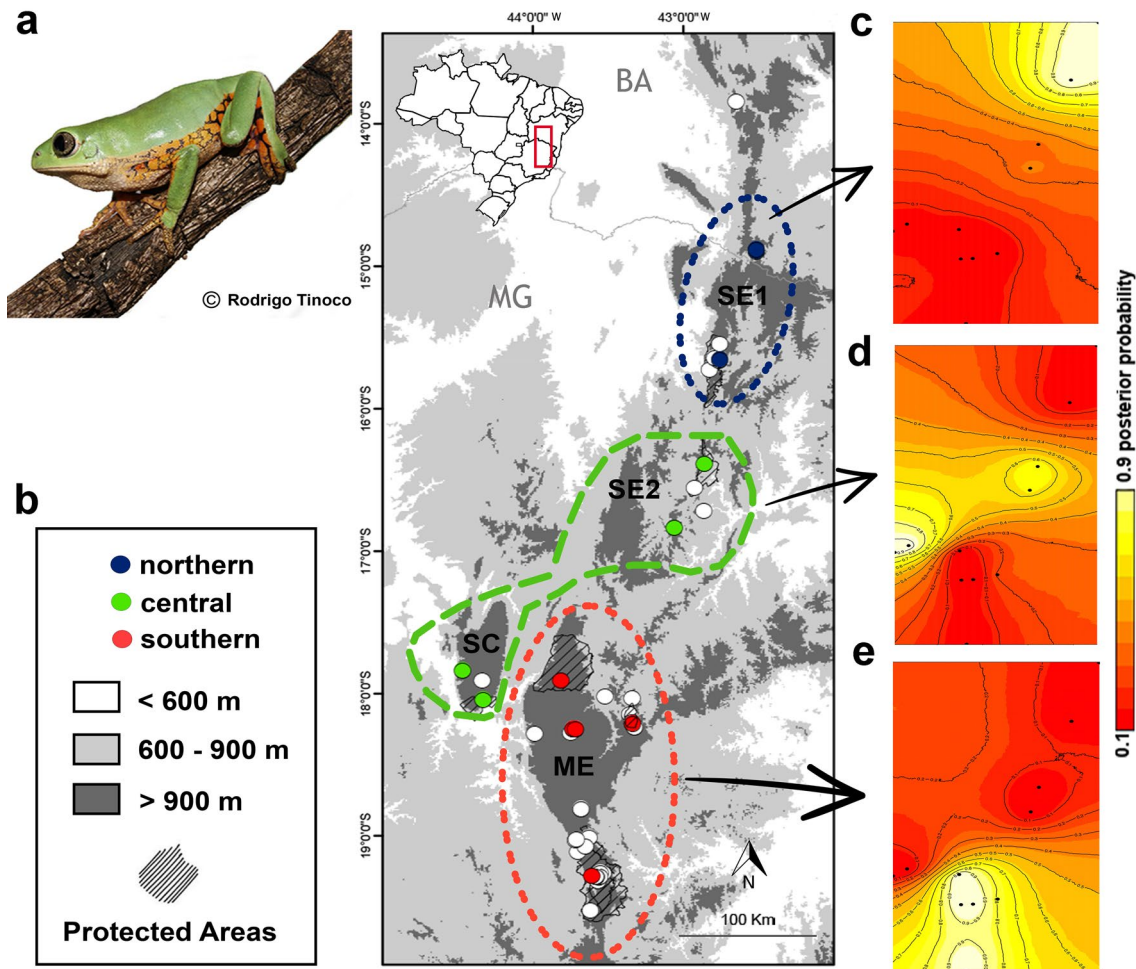
*Pithecopus megacephalus* is classified by the International Union for Conservation of Nature and Natural Resources (IUCN) as ‘Data Deficient’ (Angulo 2008). This is likely because, despite it is relatively well-known ecology and distribution (Brandão et al. 2012; Oliveira et al. 2012), there is a lack of information regarding its population dynamics and population connectivity. Some characteristics of this species should be considered for an assessment of its degree of threat: (1) it is endemic to isolated high altitude environments with great importance for conservation; (2) its reproduction is restricted to transient pools and temporary streams produced by rainfall (Brandão et al. 2012; Oliveira et al. 2012; Silveira et al. 2016). Indeed, previous studies of *P. megacephalus* have suggested a change to the IUCN status from Data Deficient to Endangered, based on the limited extent of their occurrence (Morais et al. 2013; Barata et al. 2016). However, these studies were not considered for the current IUCN classification.

In this work, we used phylogeographic analyses to understand the historical dynamics and connectivity of *P. megacephalus* populations, providing useful information in a ‘data deficient’ species by analysing their genetic diversity across its spatial range and suggesting an accurate evaluation of the conservation status of *P. megacephalus* populations.

## Materials and methods

### Sampling and laboratory methods

We sampled 55 individuals of *P. megacephalus* from 10 localities along the species’ known distribution (Fig. 1b; Table 1). Tissue samples of liver and/or tail muscle were obtained from specimens deposited at the following herpetological collections: ‘Coleção de Anfíbios Adultos e Girinos do Centro de Coleções Taxonômicas da Universidade Federal de Minas Gerais’, Belo Horizonte, Minas Gerais, Brazil (UFMG-AMP/GIR); ‘Coleção do Laboratório de Zoologia de Vertebrados da Universidade Federal de Ouro Preto (LZV-UFOP)’; ‘Coleção de Herpetologia do Museu



**Fig. 1** **a** Individual of *P. megacephalus* from the Serra do Cabral sky island. **b** Distribution of *P. megacephalus* sampling sites (circles) in South Espinhaço Mountain Range sky islands (medium grey). Coloured circles are localities with samples used in the genetic analyses, while white circles are other registered occurrences of the species. Sky islands (>900 m.a.s.l.) numbered according to Chemale

et al. 2011: Setentrional sky island (SE1 and SE2), Serra do Cabral sky island (SC), and Meridional sky island (ME). Areas hatched with diagonal lines are Protected Areas (EPA, NP, and SP in Table 2). BA Bahia state, MG Minas Gerais state. **c** Northern, **d** central and **e** southern spatial probabilities of *P. megacephalus* populations in the Espinhaço Mountain Range. (Color figure online)

**Table 1** Sampling localities of *Pithecopus megacephalus*, number of individuals per locality (N) and respective sky islands and population assignments

Locality	N	Sky island	Population
Augusto de Lima—MG	2	Serra do Cabral	Central
Botumirim—MG	3	Setentrional 2	Central
Buenópolis—MG	1	Meridional	Southern
Diamantina—MG	5	Meridional	Southern
Grão Mogol—MG	2	Setentrional 2	Central
Jacaraci—BA	2	Setentrional 1	Northern
Lassance—MG	1	Serra do Cabral	Central
Rio Pardo de Minas—MG	21	Setentrional 1	Northern
Santana do Riacho—MG	16	Meridional	Southern
São Gonçalo do Rio Preto—MG	2	Meridional	Southern

BA Bahia state, MG Minas Gerais state

de Ciências Naturais da Pontifícia Universidade Católica de Minas Gerais’, Belo Horizonte, Minas Gerais, Brazil (MCNAM); ‘Coleção Herpetológica da Universidade de Brasília’, Brasília, Distrito Federal, Brazil (CHUNB) and ‘Coleção Célio F. B. Haddad, Universidade Estadual Paulista—Campus de Rio Claro’, Rio Claro, São Paulo, Brazil (CFBH). Specimen localities and voucher numbers are given in Table S1 (Supporting Information). We extracted genomic DNA from the tissue samples with a modified phenol–chloroform protocol (Sambrook and Russel 2001). We extracted DNA from some small tissue samples using a DNeasy kit (Qiagen) following the manufacturer’s protocol.

For 55 sampled specimens, we amplified the Cytochrome b (*Cyt-b*) gene of the mitochondrial DNA (mtDNA), and four nuclear DNA (nDNA) markers: intron 5 of ribosomal protein L3 (L3i5), intron 7 of  $\beta$ -Fibrinogen ( $\beta$ Fib7), and

partial exons of the Proopiomelanocortin (POMC) and Tensin 3 (TNS3) genes. PCR reactions of 15  $\mu$ L included 20 ng of genomic DNA, 1X *Taq* buffer (Thermo Fisher Scientific), 2.5 mM  $MgCl_2$ , 1.25  $\mu$ M of each primer, 3 mM dNTPs, 0.72  $\mu$ g bovine serum albumin, and 0.625 units of Platinum *Taq* DNA polymerase (Thermo Fisher Scientific). Amplifications were performed with an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of denaturation (95 °C for 30 s), variable annealing temperatures and times (see Table S2), extension at 72 °C for 1 min/1000 bp, and a final extension at 72 °C for 7 min. For samples with poor amplification, we adapted the PCR conditions by reducing the annealing temperature, increasing the annealing time and/or increasing the  $MgCl_2$  concentration up to 3.5 mM. Primers, PCR conditions, and the amplicon sizes for each locus are provided in Table S2.

All PCR products were cleaned up by precipitation using 20% polyethylene glycol with 2.5 M NaCl. Sequences were obtained in an automatized ABI 3130xl DNA sequencer (Applied Biosystems) using the same primers used in the PCR reactions. We used the software SEQSCAPE 2.6 (Applied Biosystems) to assemble sequence contigs, interpret chromatograms, and edit sequences. Edited sequences were aligned with the CLUSTALW module of the software MEGA 7 (Larkin et al. 2007; Kumar et al. 2016). Gametic phases for all diploid nuclear markers were resolved with the program PHASE 2.1.1, under default conditions (Stephens et al. 2001), using the SeqPHASE input/output interconversion tool (Flot et al. 2010). Heterozygous indels were found in some sequences of  $\beta$ Fib7 and L3i5 markers, which were previously phased using the Indelligent web tool (Dmitriev and Rakitov 2008), and next were aligned and edited by eye. When more than one haplotype pair was reconstructed for one individual, the most probable pair was selected (when at least one pair already existed in the population). Some  $\beta$ Fib7 and L3i5 sequences remained unresolved (Posterior probability; PP < 0.9), and ambiguous nucleotide positions were maintained as such only for analyses based in genealogical reconstructions (BEAST software); for the other analyses the phased sequences were used, as described above. We selected the best-fit model of molecular evolution among 88 substitution schemes for each marker using the BIC criterion in the software JModelTest 2.1.10 (Darriba et al. 2012).

### Genetic diversity and population structure

Summary statistics, including the number of segregation sites (S), number of haplotypes (h), and haplotype diversity (Hd) were calculated for mitochondrial and nuclear data using the software DNAsp v.5.10.01 (Librado and Rozas 2009).

The optimal number of populations (K) was estimated using the total dataset with the R package Geneland 4.0.3

(Guillot et al. 2005; R Core Team 2016). Geographical coordinates of samples are shown in Table S1. For the genetic analysis, the coordinates of another sample from the same locality were used for samples without geographical coordinates. Samples with and without precise coordinate information are also shown in Table S1. We performed ten independent runs with the number of populations (K) ranging from one to ten, assuming an uncorrelated allele frequencies model. Each run consisted of 10,000,000 iterations, a thinning interval of 1000 and a burn-in phase of 250,000. After the discovery of the optimal number of populations, we performed a new round of runs restricting the number of potential populations to the most likely number of groups. This approach was used to generate resulting figures because more accurate probabilities are obtained with a fixed K value in the Geneland package (Guillot et al. 2005). To assess the level of genetic structure among populations we performed an 2-level analysis of molecular variance (AMOVA) for *Cyt-b* using Arlequin 3.5 to infer the  $\phi_{ST}$  values between the three groups (populations) indicated by the Geneland analysis (Excoffier and Lischer 2010). We selected only *Cyt-b* for this analysis because mtDNA displays higher nucleotide substitution rates and smaller effective population size ( $N_e$ ) when compared to the nuclear genome. This is important for intraspecific evolutionary studies, because mtDNA will experience a more rapid divergence between isolated lineages due to genetic drift (Hare 2001; Nabholz et al. 2007; Avise 2009).

### Genealogies and demographic expansion

We reconstructed a haplotype network for *Cyt-b* using the median-joining algorithm (Bandelt et al. 1999) in POPART 1.7 (Leigh and Bryant 2015). Concerning nuclear data, we used the genpofad algorithm to convert pairwise haplotype distance matrixes of all nDNA phased loci into a distance matrix among individuals with the software POFAD 1.07 (Joly et al. 2015). Then, a neighbor-net distance method (Bryant and Moulton 2003) was used to build a nuclear network from this matrix, using the software SplitsTree 4 (Huson and Bryant 2006).

A population tree was reconstructed using StarBEAST2 package, implemented in BEAST v2.4.3 (Bouckaert et al. 2014), with all markers and a priori population information identified by GENELAND. We included *Pithecopus rohdei*, sister species of *P. megacephalus* (Faivovich et al. 2010) to test the monophyly of the ingroup. We used a coalescent constant population tree model (Drummond and Bouckaert 2014) and a relaxed lognormal clock for all markers. Two independent runs of 100,000,000 generations were conducted, sampled every 2000th generation. We assessed convergence by examining the likelihood plots through time and accepting only ESS values >200 using TRACER v. 1.6.

Then we used TreeAnnotator v2.4.2 to find the maximum credibility tree within all generated trees.

To characterize past population demography, we performed neutrality tests using all markers (nuclear and mitochondrial) with the software Arlequin 3.5 (Excoffier et al. 2005). For these tests, we analysed our entire dataset and each of the three populations separately ( $K=3$ ; see “Results” section).

To evaluate the isolation by distance or the effects of geographical distances on population differentiation, we performed a Mantel test through IBDWS 3.23 (Jensen et al. 2005) with 30,000 randomizations. We calculated a genetic distance matrix from all loci (now including mtDNA) with the algorithm genpofad in POFAD 1.07, and the matrix of geographical distances among sampling sites was estimated using the Geographic Distance Matrix Generator 1.2.3 (GEODIS) platform of the American Museum of Natural History (Posada et al. 2000). In IBDWS we used the same four datasets used for the neutrality tests: one comparing the ten localities altogether and other three sets comparing only localities within each of the three populations detected by Geneland ( $K=3$ ; see “Results” section).

### Geospatial conservation assessment

The extinction risk evaluation of *P. megacephalus* followed the criteria and guidelines of the IUCN Red List Categories (IUCN 2012, 2016). We calculated the extent of occurrence (EOO; i.e., the area contained within the shortest continuous imaginary boundary that includes all known distribution points of a species), and the area of occupancy (AOO; i.e., the area within its EOO that is really occupied by a taxon) (IUCN 2001). The EOO and the AOO were evaluated using geographical data from the collections of the following institutions: UFMG-AMP/GIR; MCNAM; CFBH, CHUNB, LZV-UFOP, and ‘Coleção de Girinos do Departamento de Zoologia da Universidade Paulista’, São José do Rio Preto, SP, Brazil (DZSJRP-Amphibia-tadpoles). The institutional databases were accessed directly via open-access internet databases (SiBBR ‘Sistema de Informação sobre a Biodiversidade Brasileira’; Species Link Network-CRIA, ‘Centro de Referência em Informação Ambiental’; PortalBio—ICMBio ‘Portal da Biodiversidade, Instituto Chico Mendes de Conservação da Biodiversidade’) and the literature (Eterovick et al. 2005; Caramaschi 2006; Brandão et al. 2012; Oliveira et al. 2012). The compiled dataset was composed of 54 distinct occurrence sites (see Table S1). These data were analysed using the open source program GeoCAT (Geospatial Conservation Assessment Tool, available at <http://geocat.kew.org/>) (Bachman et al. 2011). We analyzed EOO data separately, for the three mountain regions in the South Espinhaço Mountain Range: Setentrional, Serra do Cabral, and Meridional. Then EOO value of each mountain region

was added together to produce a single value. Therefore, because the species is not found below 800 m.a.s.l., the extent of the valleys was not accounted for EOO calculation (Eterovick et al. 2005; Caramaschi 2006; Brandão et al. 2012; Oliveira et al. 2012). As recommended by IUCN for AOO analyses, we used a 2-km grid (cell area of 4 km<sup>2</sup>) (IUCN 2012, 2016). We also calculated the total area of all protected areas (PAs, in km<sup>2</sup>) (see Table S1), where the species has ever been recorded. The area values of each protected unit were taken from Instituto Chico Mendes de Biodiversidade, Ministério do Meio Ambiente (ICMBio) web site (<http://www.icmbio.gov.br/portal/unidadesdeconservacao/biomas-brasileiros>); and from Instituto Estadual de Florestas de Minas Gerais (IEF/MG) website (<http://www.ief.mg.gov.br/areas-protegidas>). We subtracted that area from the area of the species’ extent of occurrence (EOO) to estimate the percentage of the species’ distribution that is found within PAs.

## Results

### Descriptive data and population structure

A total of 3104 bp were sequenced from five DNA regions (see Table S2). The resulting sequenced loci had 0.4% of missing data across the entire dataset. The *Cyt-b* locus and two of the nuclear introns ( $\beta$ Fib7 and L3i5) of *P. megacephalus* showed higher genetic diversity than the other nuclear non-intronic markers (Table 2). We found indels only in sequences of the two introns. Sequence data is available in GenBank under accession numbers:  $\beta$ Fib7 (MF171732–MF171764), *Cyt-b* (MF171765–MF171789), L3i5 (MF171790–MF171822), POMC (MF171823–MF171838) and TNS3 (MF171839–MF171845) (see Table S3 for individual haplotype assignment).

The number of estimated groups or populations ( $K$ ) based on genetic and geographical information was calculated as three ( $K=3$ ) in all Geneland runs, with an average frequency of 41% among MCMC iterations (after burn-in). *Pithecopus megacephalus* range was partitioned into: (i) a northern population, corresponding to the Setentrional 1 (SE1) sky island (Fig. 1b, c); (ii) a central population, distributed across the Setentrional 2 (SE2) and the Serra do Cabral (SC) sky islands (Fig. 1b, d); and (iii) a southern population, corresponding to the Meridional (ME) sky island (Fig. 1b, e; Table 1). The AMOVA based on *Cyt-b* revealed substantial differentiation among these groups ( $F_{ct}=0.15893$ ;  $p=0.03842$ ; Table 3). Furthermore, the northern and central populations had higher genetic diversities ( $\pi$  and  $H_d$ ) than the southern population for mtDNA and nDNA markers (Table 2).

**Table 2** Population summary statistics for sampled loci

	Model	N	S	h	H	Hd	$\pi$	Tajima's D	Fu's Fs
<i>Cyt-b</i>	HKY+I								
Northern		23	13	8	6	0.858	0.00513	1.07300	0.89193
Central		8	12	6	5	0.929	0.00586	0.67610	-0.38278
Southern		24	17	12	11	0.848	0.00264	-1.72196*	-5.54816**
Total		55	34	25	22	0.941	0.00546	-1.13474	-10.02776**
$\beta$ Fib7	JC+I+G								
Northern		23	32	11	9	0.730	0.02008	2.19771	6.67115
Central		8	23	5	3	0.650	0.01676	1.81476	6.63041
Southern		24	25	21	18	0.876	0.01310	1.28551	-2.79372
Total		55	36	33	30	0.865	0.01841	1.87810	-2.39707
L3i5	K80+I								
Northern		23	31	27	20	0.958	0.01962	1.26251	-6.58223*
Central		8	27	9	3	0.858	0.01510	-0.34278	0.34851
Southern		24	25	6	2	0.560	0.00814	-0.94508	4.29138
Total		55	40	32	25	0.836	0.01556	0.03477	-5.36839
POMC	HKY+I								
Northern		23	9	5	2	0.733	0.00528	1.56089	4.06989
Central		8	17	9	5	0.908	0.00937	0.39488	-0.48577
Southern		24	8	8	5	0.533	0.00357	0.49459	-0.30592
Total		55	19	16	12	0.828	0.00665	0.30041	-0.92195
TNS3	F81+I								
Northern		23	3	4	1	0.650	0.00243	1.60885	1.32722
Central		8	4	5	2	0.750	0.00276	0.41837	-0.64345
Southern		24	2	3	1	0.228	0.00047	-0.87325	-1.11780
Total		55	4	7	4	0.605	0.00224	0.90368	-0.75299

*N* shown are the number of individuals, *S* number of polymorphic sites, *h* number of haplotype copies, *H* number of unique haplotypes, *Hd* haplotype diversity,  $\pi$  nucleotide diversity per site

Significant values are shown for  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*)

**Table 3** AMOVA calculated using mtDNA (*Cyt-b*)

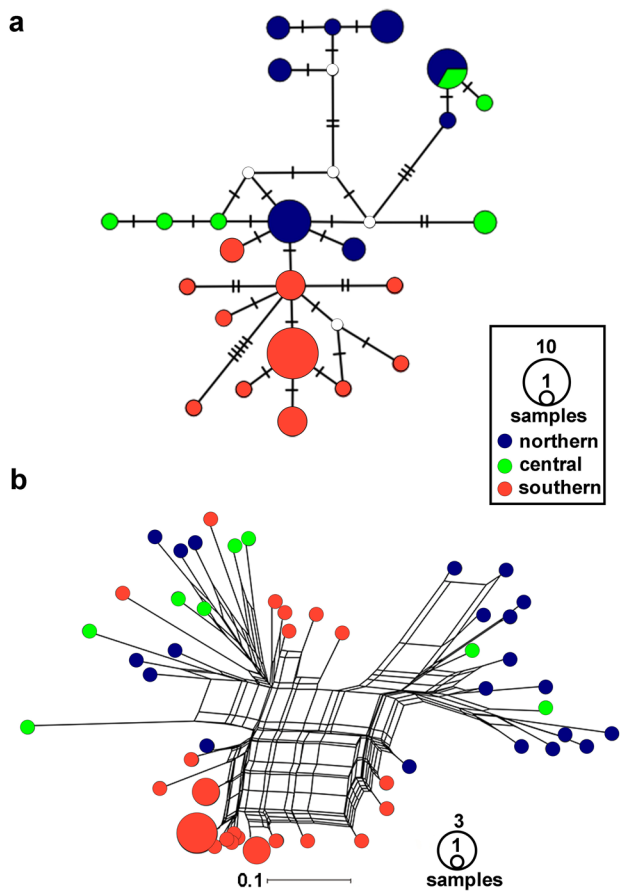
Source of variation	d.f	Sum of squares	Variance components	Variation (%)	FSC	FST	FCT
Among groups	2	35.826	0.44781 Va	25.61	0.36788**	0.46835**	0.15893*
Among localities within groups	6	27.284	0.87182 Vb	22.01			
Within localities	46	68.908	1.49801 Vc	52.38			

Groups are defined according to the Geneland results

Significant values are shown for  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*)

The patterns represented in the mtDNA network (Fig. 2a) and in the nDNA neighbor-net (Fig. 2b) are somewhat consistent with the results found by the Geneland analyses, showing one group with samples mainly from the southern population, one with samples mainly from the northern population, and a third consisting of samples from all three populations. However, this group shows only one haplotype shared with northern population and only exclusive nuclear genotypes (Fig. 2). IBDWS tests did not support a significant

correlation between genetic and geographical distances for *P. megacephalus*, neither along the whole species distribution nor when considering each of the three populations delimited by Geneland separately (Fig. S1). Northern and central populations had higher values for the correlation coefficient between genetic and geographical distances, but the non-significance of these IBD could possibly be related to the low power in the analyses, due to the small number of sampling locations.



**Fig. 2** **a** Haplotype (MJ) network based on *Cyt-b*, and **b** multilocus nuclear neighbor-net of *Pithecopus megacephalus*. The bar indicates standardized distances between genotypes. White points in network represent unobserved haplotypes and potential intermediates. (Color figure online)

The results of the population tree analysis strongly support that northern and central populations inferred by Geneland are more related, with strong support (Fig. 3), indicating greater historical connectivity between them. This result is consistent with the mtDNA and nDNA networks (Fig. 2).

**Demographic fluctuation and gene flow**

The neutrality tests did not indicate significant changes in population size for the *P. megacephalus* nuclear markers, but recovered a significantly negative value only in the Fu and Li’s  $F_s$  statistic ( $F_s = -10.03$ ,  $p < 0.01$ ) obtained with *Cyt-b* data (Table 2). Furthermore, the southern population presented a significant value of Tajima’s  $D$  ( $D = -1.72196$ ,  $p < 0.05$ ) and Fu and Li’s  $F_s$  statistic ( $F_s = -5.548$ ,  $p < 0.01$ ) obtained with *Cyt-b* data (Table 2).

The neighbor-net result (Fig. 2b) shows the presence of a group with closely related individuals belonging to different populations found in the Geneland analysis. This group

is composed of similar numbers of individuals from each population. It is important to note that 75% of the samples from the central population were assigned to this group (two from Grão Mogol, two from Augusto de Lima and two from Botumirim). The second group is composed mainly by samples from the southern population. Finally, the third group consists mostly of individuals from the northern population, with exception of two samples, one from Augusto de Lima and another from Botumirim, which were attributed to the central population by the Geneland analysis. One possible interpretation of this result is that the populations of *P. megacephalus* consist of a metapopulation with stepping-stone pattern, and the sky islands where the central population lives must have been the area of greatest connectivity among all populations in the past.

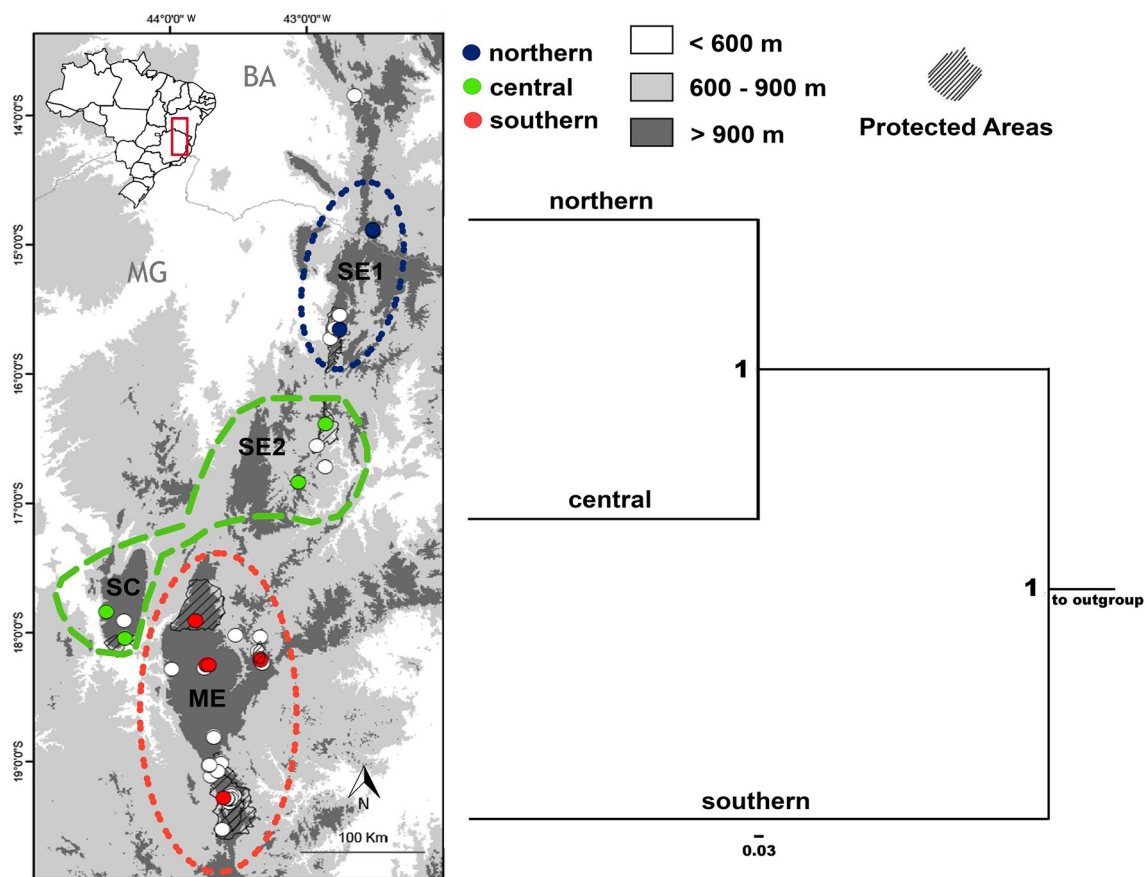
**Conservation Assessment of species distribution and protected areas**

The total EOO of *P. megacephalus* was estimated as 13,974 km<sup>2</sup> and its AOO as 160 km<sup>2</sup>. The total area of protected regions where *P. megacephalus* is registered was estimated as 4377 km<sup>2</sup> (2248 km<sup>2</sup> are fully protected and 2129 km<sup>2</sup> are partially protected) and the rest of its EOO (9597 km<sup>2</sup>) is found in regions/locations without any kind of private or government protection. Thus, only 31.3% of *P. megacephalus*’ potential distribution is within PAs.

**Discussion**

Amphibians are the most endangered vertebrates on the planet and species are continuously being added to the IUCN list’s various threat categories (Beebee 2005; Stuart 2008; Pimm et al. 2014). Species with limited information regarding their conservation status may be at risk of extinction and could disappear if they are not identified in a timely manner (Morais et al. 2013). This study provides an example of such a species that is not considered in current conservation strategies. We were interested in contributing to the conservation needs of the Brazilian frog *P. megacephalus*, by documenting its population dynamics, distribution and connectivity in the wild. To that end, we assessed the species’ genetic diversity and collected data on its geographical distribution. We detected genetic structure between populations from different sky islands of the Espinhaço Mountain Range and estimated that a small portion of those populations exist in protected areas.

When we associate the genetic data to the geographical distribution information of *P. megacephalus*, we observe the separation among the three populations identified by Geneland and the multilocus population tree: northern (Setentrional 1), central (Setentrional 2 and Serra do Cabral), and



**Fig. 3** Coalescent species tree for *Pithecopus megacephalus* lineages. Nodes labels show posterior probabilities of each clade

southern (Meridional) populations (Fig. 1a). Only the central population includes individuals from two different SER sky islands (Serra do Cabral and Setentrional 2). Conversely, considering only the haplotype networks and nDNA neighbor-net analyses, this species has only two clearly recognizable phylogenetic units: one in the south, with samples from the southern population collected in the Meridional sky island (Fig. 1a), and another with samples from the other islands (SE1, SE2 and Serra do Cabral). However, taking also into account the high number of unique haplotypes found in the localities of Grão Mogol, Botumirim and Serra do Cabral (Fig. 2; Table 2), together with the whole set of phylogeographical, population genetic and spatial distribution analyses, we suggest that *P. megacephalus* be subdivided into three management units (MU), northern, central and southern, but this may be evaluated in a more rigorous way, including more samples and statistical phylogeography methods, to test them as evolutionarily independent lineages (see Magalhães et al. in press).

Differentiation among sky island populations is also found in different species that inhabit the Espinhaço Mountain Range. Species endemic to the Espinhaço Mountain Range present population differentiation associated with

different sky islands because valleys can act as barriers to gene flow, as for example, in many birds of the *campos rupestres* (Chaves et al. 2015). Such population differentiation is also observed in plants. For example, the cactus *Pilosocereus aurisetus* presents different lineages between Meridional and Setentrional sky islands (Bonatelli et al. 2014), and the shrubby plant *Vellozia hirsuta* shows a much more restricted structure, with four highly differentiated populations among four different ER sky islands (Barbosa et al. 2012).

There is evidence of historical gene flow among *P. megacephalus* populations (demonstrated mainly in the nuclear neighbor-net in Fig. 2b), evidenced by individuals assigned to different populations forming a closely related group. This gene flow is probably not associated with changes in mountain conformation, since this range acquired its present main physiography after the Miocene, which was characterized by tectonic stability (Saadi 1995). Therefore, it is expected that these populations were in closer contact in the recent past, when climatic conditions of the Pleistocene promoted the historical expansion of habitats between current sky islands in past glaciation periods (Chaves et al. 2015). The few phylogeographical studies on high altitude tropical areas, as *campos*



*rupestres*, indicate a complex history of diversification when Quaternary climatic oscillations played a central role in shaping the population dynamics (Turchetto-Zolet et al. 2013). The diversification events and population expansions on this ecosystem, mainly evidenced by plant populations and related organisms, date from the late Miocene at 5.8 Mya (Prado et al. 2012) to the middle Pleistocene at 0.8–0.2 Mya (Gibbard and Kolfshoten 2004; Moraes et al. 2009; Collevatti et al. 2012; Bonatelli et al. 2014; Magalhães et al. in press). Population expansions during glacial periods followed by retractions during the interglacial periods culminated in the current distribution of these species, with lineages structured across mountain ranges (Trovó et al. 2013). To test if potential population range of endemic species shifted in the past, population distribution modelling algorithms should be implemented (Alvarado-Serrano and Knowles 2014).

The association of population structure and the sky island landscape can be also investigated by testing for isolation by distance (IBD) (DeChaine and Martin 2004). The lack of IBD may result from a disruption of gene flow, represented by a high genetic divergence found between geographically close areas separated by physical or climatic barriers, or other environmental disruptions like valleys between sky islands (He and Jiang 2014). Therefore, the three MUs occupying separated sky islands should be considered in the threat assessment of the species, given that habitat fragmentation and climate change impacts are among the main threats for this group of organisms (Young et al. 2004; Pounds et al. 2006; Becker et al. 2007). Because *P. megacephalus* has limited dispersal abilities and its habitat is characterized by naturally fragmented areas separated by inhospitable valleys, an environment-specific strategy needs be planned for long-term conservation of its MUs (Leite 2012; Chaves et al. 2015; Magalhães et al. in press).

An excess of rare alleles was found for *P. megacephalus* only for the mitochondrial marker, as indicated by Tajima's D and Fu's FS, which can be explained by the fact that nuclear markers present four times the effective sizes of mtDNA making this marker more sensitive to recent evolutionary history and population size fluctuations than nuclear sequences (Sunnucks 2000). For the neutrality tests, the results for the southern population may be compatible with a scenario of recent demographic expansion. However, this result for *Cyt-b* data should be taken with care, as it could be also a sign of purifying selection as previously shown (Hahn et al. 2002). Therefore, the lack of evidence of population decline found for *P. megacephalus* (Appendix 1, supplementary material) corroborates the past climatic stability of *campo rupestre*. This favours the existence of several endemism areas and refugia on it, which may have maintained the relatively high genetic diversity of populations (Collevatti et al. 2009; Bonatelli et al. 2014).

However, distribution models based on climatic conditions predict that *campos rupestres*, the environment occupied by *P. megacephalus*, will be reduced 95% by the end of this century due to synergistic actions of climate change and anthropogenic factors (Fernandes et al. 2014). Under this scenario, only the Meridional sky island of the SER will likely remain climatically stable (Fernandes et al. 2014). Severe changes in this ecosystem would result in reductions in population sizes mainly in the Setentrional sky islands, where populations may become more vulnerable to genetic drift and negative environmental pressures (Fernandes et al. 2014). These predictions raise questions about the persistence of *P. megacephalus* in the wild, because it suggests that populations where we found the least amount of genetic variability may serve as potential refugia for the species, and much of the genetic variation and distinctiveness of the species could be lost.

In addition, our results show that *P. megacephalus* has an AOO smaller than 500 km<sup>2</sup> and an EOO smaller than 20,000 km<sup>2</sup> with naturally fragmented populations in altitudinal islands that have a continued decline in habitat quality due to chronic threats to the its natural environment (Fernandes et al. 2014; IUCN 2016). Although all lineages of *P. megacephalus* identified in this study are represented in at least one protected area, only 31.3% of the species range occurs in legally PAs. This is considered partially satisfactory according to the IUCN I–IV PAs categories (Barata et al. 2016) (Fig. 1b). In these naturally fragmented landscapes, the use of measures such as creating ecological corridors to connect the populations is hardly feasible because there are no suitable areas of habitat in the valleys between sky islands for some species (Chaves et al. 2015). Thus, populations will need to be managed separately. However, this type of management cannot avoid reduction in genetic diversity and loss of population fitness (Weeks et al. 2016). An alternative, focussed on genetic information and differentiation, would be the application of a management technique based on individual translocation between populations (Weeks et al. 2011, 2016), but this strategy requires a high level of anthropic interference in the evolution of the species that should be avoided.

Considering a scenario of future climate change and the potential displacement of suitable areas for this species, the expansion or creation of new PAs and more thorough inspections of conservation units are among the most effective measures that could be implemented (Fernandes et al. 2014). Populations with higher genetic diversity (i.e., the northern and central populations) could act as pools of variation for recovery of the species, but they are limited to small and restricted distributions, while the population with a more continuous distribution has lower genetic diversity. Consequently, the three different populations are susceptible to reduction of their environments according to future

estimates (Fernandes et al. 2014). To increase efficiency in conservation, *P. megacephalus* MUs should be considered as independent units for conservation, which may display different responses to environmental changes due to differences in their evolutionary potential (Bálint et al. 2011; Pauls et al. 2013).

However, our conclusions must be considered with caution due to limitation of our sampling. Our geographical coverage was limited to the specimens available from museums and collections, representing an incomplete survey of the species distribution. Additionally, the smaller sample size of the central population could have biased the spatial Bayesian analyses performed in Geneland (Corander et al. 2003; Corander and Marttinen 2006). For example, more samples from Serra do Cabral, the most isolated SER sky island, would be necessary to investigate whether individuals from this area could represent an independent management unit. To better explain genetic variation among populations, other multivariate analyses can be performed that include landscape variables (Storfer et al. 2010). In addition, the use of markers with greater capacity to capture more recent demographic processes, such as microsatellites or SNPs, could be used to infer gene flow and demographic dynamics between populations of *P. megacephalus*. Regardless of these limitations, this study provides important data on geographic distribution and population dynamics for this Data Deficient species. Geographic distribution data raise the hypothesis that *P. megacephalus* is a threatened species, but the genetic results were not as clear. Our data points to a historical genetic structure, which must be considered for the conservation of this species together with the importance of its habitat. Therefore, we suggest these results could be used for a more accurate classification of *P. megacephalus* in the IUCN list, considering its fragmentation.

We demonstrated how sky islands of the Espinhaço Mountain Range have shaped the population dynamics of an endemic amphibian, and we used this information to make suggestions regarding future conservation strategies. We encourage future researchers to improve our understanding of phylogeographical patterns of endemic species in the Espinhaço Mountain Range by comparing those phylogeographical patterns with geology, climate, and historical data that may influence ecosystem dynamics. Considering the high number of species of endemic amphibians that co-occur in the different sky islands of the SER (Leite et al. 2008), further investigations must be made to unravel the patterns and processes of the evolutionary history of this region, and to assist in a broader conservation planning to this ecosystem. Such studies will likely prove important for protecting the habitat of organisms in this unique region.

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