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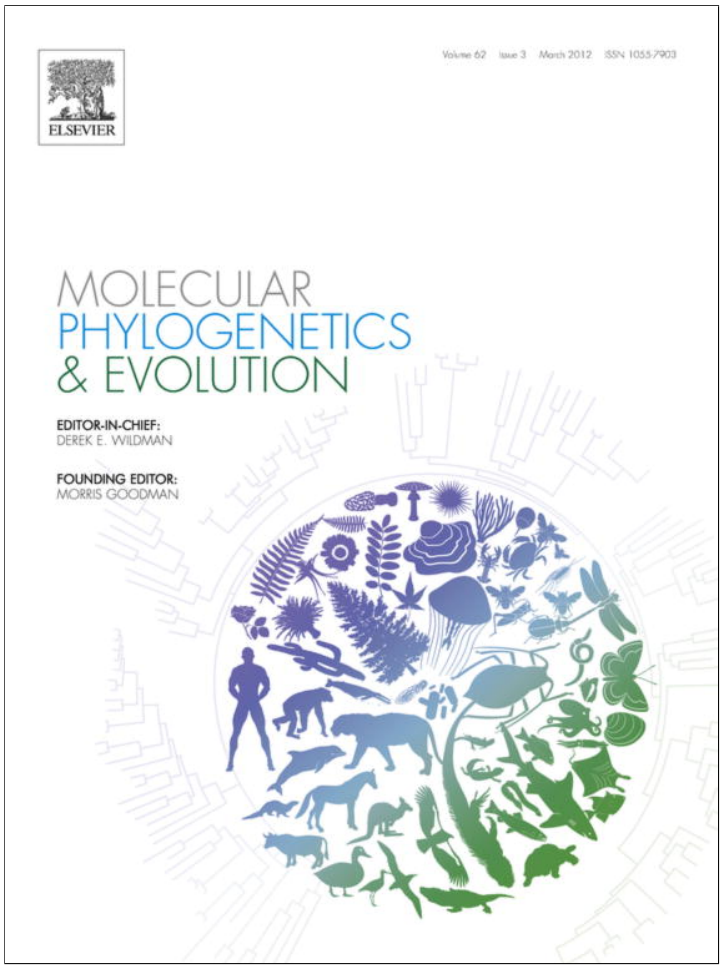

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Phylogeny and cryptic diversity in geckos (*Phyllopezus*; Phyllodactylidae; Gekkota) from South America's open biomes

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ABSTRACT

The gecko genus *Phyllopezus* occurs across South America's open biomes: Cerrado, Seasonally Dry Tropical Forests (SDTF, including Caatinga), and Chaco. We generated a multi-gene dataset and estimated phylogenetic relationships among described *Phyllopezus* taxa and related species. We included exemplars from both described *Phyllopezus pollicaris* subspecies, *P. p. pollicaris* and *P. p. przewalskii*. Phylogenies from the concatenated data as well as species trees constructed from individual gene trees were largely congruent. All phylogeny reconstruction methods showed *Bogertia lutzae* as the sister species of *Phyllopezus maranjonensis*, rendering *Phyllopezus* paraphyletic. We synonymized the monotypic genus *Bogertia* with *Phyllopezus* to maintain a taxonomy that is isomorphic with phylogenetic history. We recovered multiple, deeply divergent, cryptic lineages within *P. pollicaris*. These cryptic lineages possessed mtDNA distances equivalent to distances among other gekkotan sister taxa. Described *P. pollicaris* subspecies are not reciprocally monophyletic and current subspecific taxonomy does not accurately reflect evolutionary relationships among cryptic lineages. We highlight the conservation significance of these results in light of the ongoing habitat loss in South America's open biomes.

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1. Introduction

Molecular phylogenies and DNA sequence data are routinely used to identify species limits and describe biological diversity (Hebert et al., 2004a; Knowles and Carstens, 2007; Sites and Marshall, 2004; Wiens and Penkrot, 2002). Previous research makes it increasingly apparent that large numbers of vertebrate species have yet to be described and molecular phylogenetic analyses that include multiple intraspecific samples often discover deeply divergent lineages in what was thought to be a single, widespread species (Fouquet et al., 2007; Funk and Omland, 2003; Geurgas and Rodrigues, 2010; Hebert et al., 2004b; Oliver et al., 2009; Pfenninger and Schwenk, 2007). These lineages typically represent cryptic species and such studies highlight the inadequate taxonomy of many taxa, including many well-known vertebrate clades. The resulting species descriptions and taxonomic changes can have important conservation implications. Splitting a widespread species into multiple, cryptic species increases the perceived biological diversity of a region. In addition, the distributions of the newly detected species are smaller than the former widespread "taxon", requiring a reevaluation of threats and species viability.

The discovery and description of new species takes on a certain urgency when viewed in light of the ongoing biodiversity crisis. A basic knowledge of the species that occur in an area is crucial to quantifying biological diversity and identifying conservation priorities (Bickford et al., 2007; Bini et al., 2006; Bininda-Emonds et al., 2000; Goldstein et al., 2005; Mace, 2004). Nowhere is this more evident than South America's cis-Andean open biomes: Cerrado, Seasonally Dry Tropical Forests (SDTF, including Caatinga), and Chaco (Werneck, 2011). New vertebrate species continue to be described from these areas every year (Cacciali et al., 2007; Cassimiro and Rodrigues, 2009; Colli et al., 2009; Diniz-Filho et al., 2005;

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Patterson, 2000; Rodrigues and Dos Santos, 2008). South America's open biomes are among the most critically endangered habitats in the world, having been largely transformed by industrial agriculture and cattle production (Altieri, 2009; Klink and Machado, 2005; Leal et al., 2005; Zak et al., 2004). Indeed, Cerrado is considered a global biodiversity hotspot, an area with a remarkably high concentration of endemic species, and experiencing increased habitat loss (Myers et al., 2000). Compounding these problems, very little of the land is formally protected by parks and reserves. For example, only 2.2% of Brazilian Cerrado and less than 1% of Caatinga is protected (Klink and Machado, 2005; Leal et al., 2005). Rapid habitat destruction makes the description and subsequent protection of biological diversity in the region a top priority (Bernard et al., 2011; Cavalcanti and Joly, 2002; Colli et al., 2002; Pimm et al., 2010).

The gecko genus *Phyllopezus* occurs across South America's open biomes and is an ideal model to determine if widespread taxa in these threatened regions are composed of multiple cryptic lineages. *Phyllopezus* is a genus of large-bodied, saxicolous and arboreal geckos consisting of three species. *Phyllopezus periosus* Rodrigues, 1986 occurs in Caatinga habitats in northeastern Brazil. *Phyllopezus maranjonensis* Koch et al., 2006 occurs in SDTF of the Marañon Valley in northern Peru. *Phyllopezus pollicaris* (Spix, 1825) is composed of two subspecies: *P. p. pollicaris* is widespread in Cerrado, Caatinga and other SDTFs habitats in central and eastern Brazil, while *P. p. przewalskii* occurs in Cerrado habitats in southwestern Brazil and the Chaco of Paraguay, southern Bolivia and northern Argentina. *Phyllopezus* has a long and confusing taxonomic history. *P. pollicaris* was originally assigned to the Neotropical gecko genus *Thecadactylus*. Cuvier (1829) synonymized *Thecadactylus pollicaris* with *Hemidactylus mabouia* based on the description and Spiźs (1825) figure but without examining the type specimens. The genus *Phyllopezus* was established for the species *P. goyazensis*, which was later synonymized with *P. pollicaris* (Müller and Brongersma, 1933; Peters, 1877). A second species, *Phyllopezus przewalskii*, was described from western Mato Grosso, Brazil (Koslowsky, 1895). *P. przewalskii* was quickly synonymized with *P. goyazensis* (Boulenger, 1897) until it was afforded subspecific status within *P. pollicaris* in the only taxonomic revision of the genus (Vanzolini, 1953). *Phyllopezus* taxonomy appeared to stabilize after Vanzolini's (1953) treatise and the genus remained monotypic with just two subspecies until the recent descriptions of *P. periosus* and *P. maranjonensis* (Koch et al., 2006; Peters et al., 1986; Rodrigues, 1986).

A recent molecular phylogeny of New World geckos called into question current *Phyllopezus* taxonomy (Gamble et al., 2011a). That study found deep divergences among the three *Phyllopezus* specimens sampled. In addition, *Phyllopezus* was paraphyletic with regard to *Bogertia lutzae* Loveridge, 1941, a small gecko from Restinga habitats on Brazil's Atlantic coast. *Bogertia* was nested within *Phyllopezus* and the sister taxon of *P. maranjonensis*. These findings challenge *Phyllopezus* monophyly and prompted us to look more closely at the phylogenetic relationships among members of the genus. Here we use a multi-gene dataset to estimate phylogenetic relationships among all described *Phyllopezus* taxa to determine if *Bogertia* and *Phyllopezus* represent reciprocally monophyletic lineages. We also use these data to determine if the widespread *P. pollicaris* is composed of multiple cryptic lineages and test whether current subspecific taxonomy adequately reflects phylogenetic diversity within the species.

2. Materials and methods

2.1. Taxon sampling and molecular methods

We included samples from 40 *Phyllopezus* specimens, including exemplars from all described species: *P. periosus*, *P. pollicaris*, and *P. maranjonensis*. We included specimens of *P. pollicaris przewalskii* from Argentina, Paraguay and Bolivia, and *P. pollicaris pollicaris* from multiple sites in central and eastern Brazil (Fig. 1). Specimens of the closely related phyllodactylid taxa *B. lutzae*, *Gymnodactylus amarali* and *Phyllodactylus xanti* were included as outgroups. A complete list of sampled specimens and sampling localities can be found in Table 1.

Genomic DNA was extracted from muscle, liver and tail clips using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). We used PCR to amplify fragments of the mitochondrial ribosomal

Fig. 1. Map of South America showing major open biomes: Chaco, Cerrado, and Caatinga. Collecting localities for *Phyllopezus* and *Bogertia* specimens used in this study are shown. Letters refer to the localities of *P. pollicaris* clades identified by the GMYC analysis.

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Table 1
Details of material examined. Haplotype number refers to duplicate haplotypes collapsed for the GMYC analysis. Brazilian states: TO = Tocantins, GO = Goiás, BA = Bahia, PB = Paraíba, AL = Alagoas, PI = Piauí.

Haplotype number	Species	ID	Locality	16S	RAG1	RAG2	ACM4	C-MOS
1	*Phyllodactylus xanti*	ROM38490	Baja California Sur, Mexico	AY763284	EF534807	EF534975	EF534890	EF534933
2	*Gymnodactylus amarali*	CHUNB11179	Palmas, TO, Brazil	JN935544	JN935427	JN935398	JN935508	JN935468
3	*Gymnodactylus amarali*	CHUNB37128	Paranã, TO, Brazil	JN935545	JN935428	JN935399	JN935509	JN935469
4	*Gymnodactylus amarali*	CHUNB37161	São Domingos, GO, Brazil	JN935546	JN935429	JN935400	JN935510	JN935470
5	*Gymnodactylus amarali*	CHUNB38646	Cocalzinho, GO, Brazil	JN935547	HQ426288	HQ426457	HQ426364	HQ426539
6	*Bogertia lutzae*	CHUNB50461	Mata de São João, BA, Brazil	JN935548	JN935430	JN935401	JN935511	JN935471
6	*Bogertia lutzae*	CHUNB50462	Mata de São João, BA, Brazil	JN935549	HQ426265	HQ426438	HQ426345	HQ426522
6	*Bogertia lutzae*	CHUNB50463	Mata de São João, BA, Brazil	JN935550	JN935431	JN935402	JN935512	JN935472
7	*Phyllopezus periosus*	MTR 887022	Cabaceiras, PB, Brazil	JN935552	JN935439	JN935405	JN935519	JN935480
8	*Phyllopezus maranjonensis*	ZFMK84995	Balsas, Amazonas, Peru	JN935555	EU293633	EU293723	EU293655	EU293678
8	*Phyllopezus maranjonensis*	ZFMK84997	Balsas, Amazonas, Peru	JN935557	JN935438	–	–	JN935479
9	*Phyllopezus maranjonensis*	ZFMK84996	Balsas, Amazonas, Peru	JN935556	JN935437	–	JN935518	JN935478
10	*Phyllopezus p. pollicaris* Clade A	(MTR) JC 1185	Mucugê, BA, Brazil	JN935553	JN935435	JN935406	JN935516	JN935476
10	*Phyllopezus p. pollicaris* Clade A	(MTR) JC 1219	Mucugê, BA, Brazil	JN935554	JN935436	JN935407	JN935517	JN935477
11	*Phyllopezus p. pollicaris* Clade B	LG1309	Serra da Mesa, GO, Brazil	JN935569	JN935449	JN935413	JN935528	JN935489
12	*Phyllopezus p. pollicaris* Clade C	LG1792	Palmas, TO, Brazil	JN935572	JN935452	–	JN935530	JN935492
13	*Phyllopezus p. pollicaris* Clade C	LG1845	UHE Lajeado, TO, Brazil	JN935574	JN935454	JN935417	–	JN935494
14	*Phyllopezus p. przewalskii* Clade D	TG00105	unknown, Paraguay	JN935565	JN935445	EU293724	EU293656	EU293679
14	*Phyllopezus p. przewalskii* Clade D	LG1093	Fuerte Esperanza, Chaco, Argentina	JN935567	JN935447	–	JN935526	JN935487
14	*Phyllopezus p. przewalskii* Clade D	MTD43490	Fortin Toledo, Boqueron, Paraguay	JN935578	–	–	–	–
14	*Phyllopezus p. przewalskii* Clade D	MTD43492	Fortin Toledo, Boqueron, Paraguay	JN935579	–	JN935421	–	–
15	*Phyllopezus p. przewalskii* Clade D	MNCN5903	Serranía Aguarague, Tarija, Bolivia	JN935577	JN935457	JN935420	JN935533	JN935497
16	*Phyllopezus p. pollicaris* Clade E	MTR3074	Santo Inácio, BA, Brazil	JN935584	JN935462	JN935423	JN935538	JN935502
16	*Phyllopezus p. pollicaris* Clade E	MTR3287	Santo Inácio, BA, Brazil	JN935586	JN935464	JN935424	JN935540	JN935504
17	*Phyllopezus p. pollicaris* Clade E	MTR3263	Gentio do Ouro, BA, Brazil	JN935585	JN935463	–	JN935539	JN935503
18	*Phyllopezus p. pollicaris* Clade F	LG1310	Niquelândia GO, Brazil	JN935568	JN935448	JN935412	JN935527	JN935488
19	*Phyllopezus p. pollicaris* Clade G	CHUNB36991	Paranã, TO, Brazil	JN935559	JN935440	JN935408	JN935520	JN935481
19	*Phyllopezus p. pollicaris* Clade G	CHUNB36992	Paranã, TO, Brazil	JN935560	JN935441	JN935409	JN935521	JN935482
19	*Phyllopezus p. pollicaris* Clade G	CHUNB37001	Paranã, TO, Brazil	JN935561	JN935442	–	JN935522	JN935483
20	*Phyllopezus p. pollicaris* Clade H	CHUNB43849	São Domingos, GO, Brazil	JN935562	JN935443	–	JN935523	JN935484
21	*Phyllopezus p. pollicaris* Clade H	CHUNB43850	São Domingos, GO, Brazil	JN935563	HQ426313	HQ426487	HQ426396	HQ426569
22	*Phyllopezus p. pollicaris* Clade H	CHUNB43852	São Domingos, GO, Brazil	JN935564	JN935444	JN935410	JN935524	JN935485
23	*Phyllopezus p. pollicaris* Clade I	MTR 916557	Rio de Contas, BA, Brazil	JN935551	JN935433	JN935404	JN935514	JN935474
–	*Phyllopezus p. pollicaris* Clade I	MTR 916556	Rio de Contas, BA, Brazil	–	JN935432	JN935403	JN935513	JN935473
24	*Phyllopezus p. pollicaris* Clade J	MTR 887020	Cabaceiras, PB, Brazil	JN935558	JN935434	–	JN935515	JN935475
25	*Phyllopezus p. pollicaris* Clade J	LG1011	Porto Seguro, BA, Brazil	JN935566	JN935446	–	JN935525	JN935486
25	*Phyllopezus p. pollicaris* Clade J	LG807	Xingó, AL, Brazil	JN935575	JN935455	JN935418	JN935532	JN935495
25	*Phyllopezus p. pollicaris* Clade J	LG808	Xingó, AL, Brazil	JN935576	JN935456	JN935419	–	JN935496
26	*Phyllopezus p. pollicaris* Clade J	LG1342	Campo Formoso, BA, Brazil	JN935570	JN935450	JN935414	JN935529	JN935490
27	*Phyllopezus p. pollicaris* Clade J	LG1343	Campo Formoso, BA, Brazil	JN935571	JN935451	JN935415	–	JN935491
28	*Phyllopezus p. pollicaris* Clade J	MTR2346	Uruçuí-Una, PI, Brazil	JN935580	JN935458	–	JN935534	JN935498
28	*Phyllopezus p. pollicaris* Clade J	MTR2807	Uruçuí-Una, PI, Brazil	JN935581	JN935459	–	JN935535	JN935499
29	*Phyllopezus p. pollicaris* Clade J	MTR2958	Uruçuí-Una, PI, Brazil	JN935582	JN935460	–	JN935536	JN935500
29	*Phyllopezus p. pollicaris* Clade J	MTR2959	Uruçuí-Una, PI, Brazil	JN935583	JN935461	JN935422	JN935537	JN935501
30	*Phyllopezus p. pollicaris* Clade J	MTR3681	Ilha do Gado Bravo, BA, Brazil	JN935587	JN935465	–	JN935541	JN935505
31	*Phyllopezus p. pollicaris* Clade J	MTR3748	Alagoado, BA, Brazil	JN935588	JN935466	JN935425	JN935542	JN935506
32	*Phyllopezus p. pollicaris* Clade J	MTR4960	Serra das Confusões, PI, Brazil	JN935589	JN935467	JN935426	JN935543	JN935507
33	*Phyllopezus p. pollicaris* Clade J	MZUSP92491	Serra das Confusões, PI, Brazil	JN935590	EU293635	EU293725	EU293657	EU293680

gene *16S* and portions of 4 nuclear protein-coding genes: recombination-activating gene 1 (*RAG1*), recombination-activating gene 2 (*RAG2*); oocyte maturation factor MOS (*C-MOS*), and acetylcholinergic receptor M4 (*ACM4* or *CHRM4*). Primers used for PCR and sequencing are listed in Table 2. We purified PCR products using Exonuclease I and Shrimp Alkaline Phosphatase (Hanke and Wink, 1994). Sequencing was performed using Big Dye terminator cycle sequencing with an ABI 3730xl at the Advanced Genetic Analysis Center, University of Minnesota. Sequences were edited and assembled with Sequencher 4.8 (Gene Codes Corporation). Nuclear gene sequences were aligned using T-Coffee (Notredame et al., 2000) and translated to amino acids using MacClade 4.08 (Maddison and Maddison, 1992) to confirm alignment and gap placement. *16S* sequences were aligned using ClustalW (Thompson et al., 1994) using a gap open penalty of 10 and a gap extension penalty of 0.1 with subsequent minor adjustments by eye.

2.2. Phylogenetic analyses

We analyzed the concatenated dataset with partitioned Maximum Likelihood (ML) using RAxML 7.2.6 (Stamatakis, 2006). Nuclear gene data were partitioned by codon with a separate partition for *16S* for a total of four partitions. Each partition utilized the GTR + Gamma model of sequence evolution, the model handled by RAxML. Nodal support was assessed using 1000 nonparametric bootstrap replicates (Felsenstein, 1985). We also analyzed the concatenated data using Bayesian analysis with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The partitioning strategy was the same as the ML analysis using models of sequence evolution selected with the Akaike information criterion (AIC) as implemented in jModelTest (Posada, 2008) and model parameters estimated independently using the unlink option. We conducted two independent runs, each consisting of six

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parallel Markov chain Monte Carlo (MCMC) chains for 5 million generations and sampled every 1000 generations. Finally, we estimated phylogenies for each locus independently in MrBayes as above. Heterozygous genotypes in the nuclear gene data were resolved using PHASE 2.1.1 (Stephens and Donnelly, 2003), implementing the default options of 100 main iterations, 1 thinning interval, 100 burn-in iterations, and confidence probability thresholds of 0.90. We assessed convergence and stationarity in all Bayesian analyses by plotting likelihood values in Tracer 1.5 (Rambaut and Drummond, 2007) and plotting split frequencies between independent runs using AWTY (Nylander et al., 2008).

Inconsistencies between gene trees and species trees, especially those due to incomplete lineage sorting, can cause errors in phylogenetic reconstruction (Degnan and Rosenberg, 2009; Maddison, 1997; Slowinski et al., 1997). This prompted us to estimate species trees in a coalescent framework incorporating individual gene genealogies. Most species tree estimation methods require *a priori* assignment of individuals to a species before estimating the phylogenetic relationships among those species. Large uncorrected *16S* distances among clades (see Section 3) suggested the presence of multiple cryptic species in *P. pollicaris* and this required that we delimit presumptive taxa within *P. pollicaris* if we were to incorporate this newly uncovered diversity in our phylogenetic analyses. The lack of an independent hypothesis to partition taxa within *P. pollicaris*, such as morphology, prompted us to use the mtDNA data to provisionally assign individuals to "species". Simply picking mtDNA haploclades to represent species was considered an arbitrary exercise given the hierarchical nature of a phylogenetic tree. We therefore implemented an objective means of species delimitation using the general mixed Yule coalescent (GMYC) model implemented in the software SPLITS (Ezard et al., 2009; Pons et al., 2006). The GMYC model estimated the number of phylogenetic clusters or "species" by identifying the transition between intra- and interspecific branching patterns on an ultrametric phylogeny (Pons et al., 2006). A likelihood-ratio test was used to determine if the model with a shift in the branching processes provided a better fit to the data than the null model lacking a shift in branching processes. The ultrametric phylogeny of unique *16S* haplotypes was constructed with BEAST 1.6.1 (Drummond and Rambaut, 2007) under a strict molecular clock with the substitution rate set to 1.0 and a substitution model determined using the AIC in jModeltest (Posada, 2008). We used a coalescent tree prior with a constant population size. The analyses were run for 10,000,000 generations sampling every 1000th generation and used a UPGMA starting tree. We conducted 3 replicate runs and pooled samples using LogCombiner 1.6.1 (Drummond and Rambaut, 2007) excluding the first 1,000,000 generations from each run.

We used *BEAST (Heled and Drummond, 2010), as implemented in BEAST 1.6.1, to estimate a species tree from the multilocus data. We resolved heterozygous genotypes in the nuclear gene data using PHASE 2.1 as described above. We assigned individuals to "species" based on clusters identified by the GMYC analysis. The mtDNA substitution rate was set to 1.0 and mutation rates for other loci were co-estimated relative to the mtDNA under a strict molecular clock with substitution models as determined using the AIC in jModeltest (Posada, 2008). We ran the analyses for 50,000,000 generations sampling every 1,000th generation. A UPGMA starting tree was used for each locus. We conducted 3 replicate runs and pooled samples using LogCombiner 1.6.1 (Drummond and Rambaut, 2007) excluding the first 5000,000 generations from each run.

We estimated the Bayesian concordance analysis (BCA) tree using BUCKy 1.4.0 (Ané et al., 2007) using the posterior distributions of trees from the analyses of individual loci performed in MrBayes. Nodal support, the concordance factor, was measured as the proportion of loci that shared a specific clade across the majority of sampled loci. We conducted three separate analyses, each with a different *a priori* level of discordance among loci, which was controlled by the Dirichlet process prior α (Ané et al., 2007). An interactive web tool (<http://bigfork.botany.wisc.edu/concordance/>) was used to calculate α values for our data: $\alpha = 0.01$ placed a high prior on 1 tree; $\alpha = 1$ placed a high prior on 1–3 trees; and $\alpha = 10$ placed a high prior on 4–5 trees. Analyses were run for 1100,000 generations with the first 100,000 generations discarded as burn-in.

2.3. Genetic distances

Net among-group distances (Nei and Li, 1979) between *Phyllopezus* lineages for the *16S* data were calculated using MEGA 4 (Kumar et al., 2008). We calculated both uncorrected *p*-distances and ML corrected distances using the GTR + I + G model. Standard error estimates were calculated using 500 bootstrap replicates.

2.4. Testing phylogenetic hypotheses

Two taxonomic hypotheses were tested with our data: (1) monophyly of *Phyllopezus*, exclusive of *Bogertia*; and (2) reciprocal monophyly of *P. p. pollicaris* and *P. p. przewalskii* to determine if current subspecific nomenclature adequately reflects evolutionary history and phylogenetic diversity. We tested both hypotheses in a ML framework using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) and the approximately unbiased (AU) test (Shimodaira, 2002). We estimated constrained phylogenies using the concatenated data in RAxML as above. Per-site likelihood values were calculated in RAxML and *p*-values estimated using the software CONSEL (Shimodaira and Hasegawa, 2001). We also tested each hypothesis in a Bayesian framework. Bayesian posterior probabilities of the alternative hypotheses were calculated using the filter option in PAUP* (Swofford, 2002). Briefly, we filtered the post burn-in posterior distribution of trees from the Bayesian analyses with a constrained tree representing the alternative hypothesis to be tested. The proportion of trees consistent with the constrained tree approximates the posterior probability of the alternative hypothesis. We tested alternative hypotheses using results from the concatenated Bayesian analysis, the Bayesian analyses of each individual locus, and the Bayesian species trees from the *BEAST analyses.

3. Results

3.1. Molecular data

We sequenced fragments from one mitochondrial gene and four nuclear protein-coding loci for a total of 2758 aligned base pairs of sequence data. A deletion of six continuous base pairs, or two codons, was found in the coding region of *C-MOS* in the three *P. pollicaris* specimens in Clade E, comprising individuals from the sand dunes of the middle Rio São Francisco region (Gentio do Ouro and Santo Inácio, Bahia). No other insertions or deletions were observed among protein-coding loci.

3.2. Phylogenetic relationships within Phyllopezus

Maximum likelihood and Bayesian phylogenies of the concatenated data were largely congruent and most nodes well supported (Fig. 2). Both methods recovered *P. periosus* as the sister taxon of the remaining *Phyllopezus* and *Bogertia* samples; *P. maranjonensis* and *B. lutzae* were strongly supported sister taxa; and we recovered multiple, well supported, deeply divergent clades within *P. pollicaris*. Bayesian consensus trees of each of the individual loci were generally poorly resolved and had posterior probabilities <0.95 at most nodes (Fig. 3).

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Table 2
Primers used for PCR and sequencing.

Primer name	Primer sequence (5′–3′)	Source
RAG1		
R13	TCTGAATGGAAATTCAAGCTGTT	Groth and Barrowclough (1999)
R18	GATGCTGCCTCGGTCGGCCACCTTT	Groth and Barrowclough (1999)
F700	GGAGACATGGACACAATCCATCCTAC	Bauer et al. (2007)
R700	TTTGTACTGAGATGGATCTTTTTGCA	Bauer et al. (2007)
693R	TGRATCTTTTTGCAGTTGGTAAT	Gamble et al. (2011b)
R1tgR	CTCCACCTTCTTCTTTCTCAGCA	Gamble et al. (2011b)
RAG2		
EM1-F	TGGAACAGAGTGATYGACTGCAT	Gamble et al. (2008a)
EM1-R	ATTTCCCATATCAYTCCCAAACC	Gamble et al. (2008a)
PY1-F	CCCTGAGTTTGGATGCTGTACTT	Gamble et al. (2008a)
PY1-R	AACTGCCTRTTGTCCCCTGGTAT	Gamble et al. (2008a)
C-MOS		
G73	GCGGTAAAGCAGGTGAAGAAA	(Saint et al., 1998)
G74	TGAGCATCCAAAGTCTCCAATC	(Saint et al., 1998)
FU-F	TTTGGTTCKGTCTACAAGGCTAC	Gamble et al. (2008a)
FU-R	AGGGAACATCCAAAGTCTCCAAT	Gamble et al. (2008a)
ACM4		
tg-F	CAAGCCTGAGAGCAARAAGG	Gamble et al. (2008a)
tg-R	ACYTGACTCCTGGCAATGCT	Gamble et al. (2008a)
16S		
16S-F	CTAACCGTGCAAAGGTAGCGTAATCAC	Gamble et al. (2008b)
16d	CTCCGGTCTGAACTCAGATCACGTAG	(Reeder, 1995)

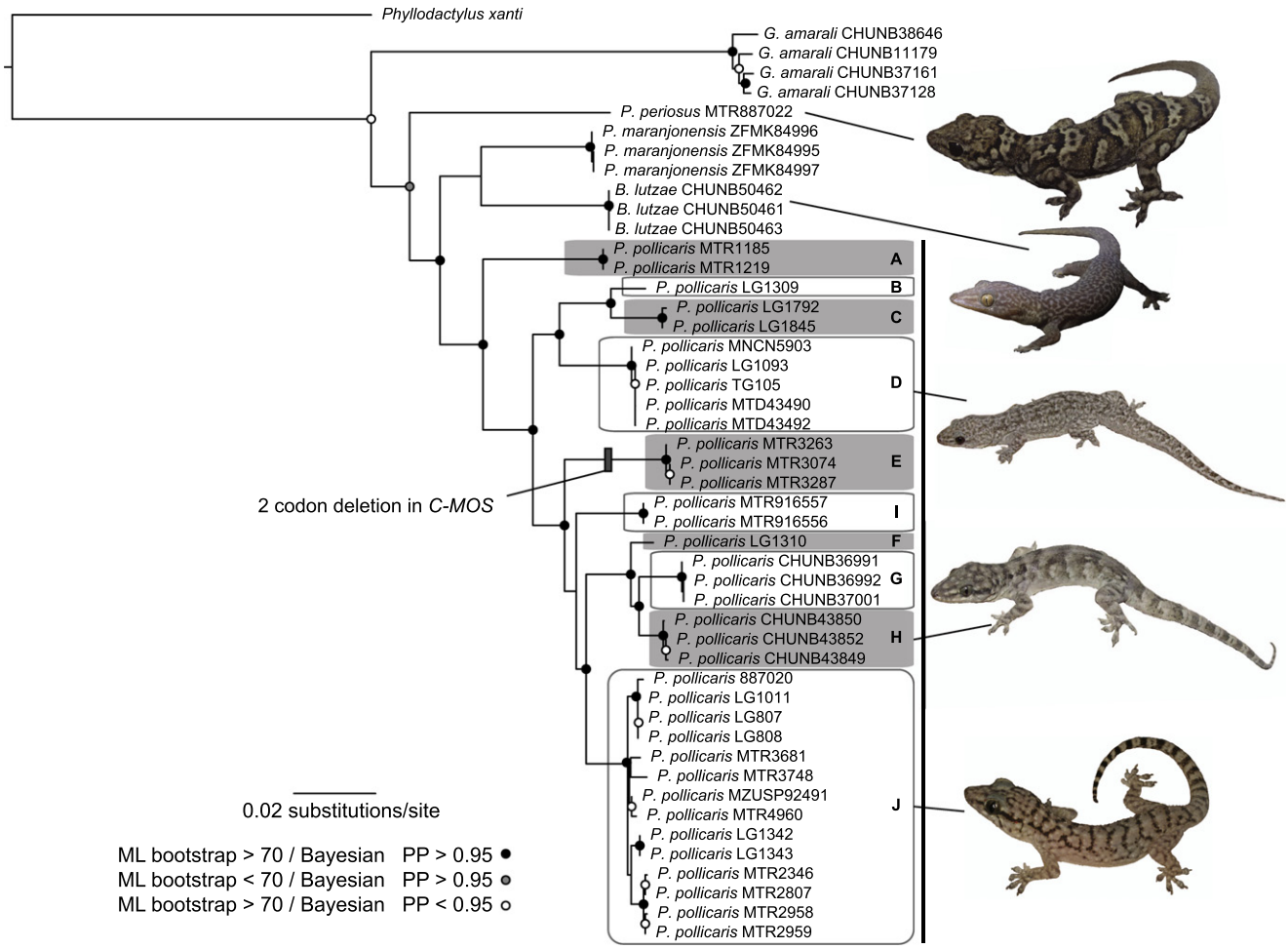

Fig. 2. Maximum likelihood phylogeny of the concatenated dataset. Taxa enclosed by lettered boxes indicate clades identified by the GMYC analysis. Circles indicate levels of clade support from the maximum likelihood bootstrap analysis and Bayesian posterior probabilities from the Bayesian analyses. Photos by MTR and TG.

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The GMYC analysis recovered 15 maximum likelihood entities with a confidence interval of six to 25 (Fig. 4). The likelihood-ratio test to determine if there was a shift from interspecific to intraspecific processes was not significant ($P = 0.116$). Specimens of *P. pollicaris* were separated into 10 clusters, which, for convenience, were labeled A though J (Table 1, Fig. 4).

Relationships among the major clades identified using the GMYC analysis using both species tree methods, *BEAST analyses and BCA (Fig. 5), were identical to each other and to the concatenated phylogenies. Nodal support was high (>0.95) for most nodes of the *BEAST consensus tree (Fig. 5). BCA concordance trees with varying α levels were identical to each other and had identical concordance factors. Several clades had concordance factors >0.50 although it can be difficult to determine what represents a significant concordance factor (Baum, 2007).

3.3. Genetic distances

Uncorrected net among-group distances for *16S* between *Phyllopezus* clades ranged from 4.7% to 18.0% (Table 3). Maximum likelihood corrected distances ranged from 5.7% to 36.8%. Uncorrected net among-group distances among *P. pollicaris* clades A–J ranged from 4.7% to 15.6%, while ML corrected distances ranged from 5.7% to 28.8% (Table 3).

3.4. Phylogenetic hypothesis testing

We tested the monophyly of *Phyllopezus* and reciprocal monophyly of *P. p. przewalskii* (Clade D) and *P. p. pollicaris* subspecies in a likelihood framework by comparing the concatenated ML tree (Fig. 2) to trees constrained to reflect the alternative hypotheses (Table 4). Both constrained trees were significantly different from the ML tree using the AU test, but only the tree that constrained reciprocal monophyly of the two *P. pollicaris* subspecies was significantly different from the ML tree using the SH test. Bayesian posterior probabilities of the alternative hypotheses were either zero or very low for all analyses of individual loci and the concatenated data as well as the species trees from the *BEAST analyses (Table 5).

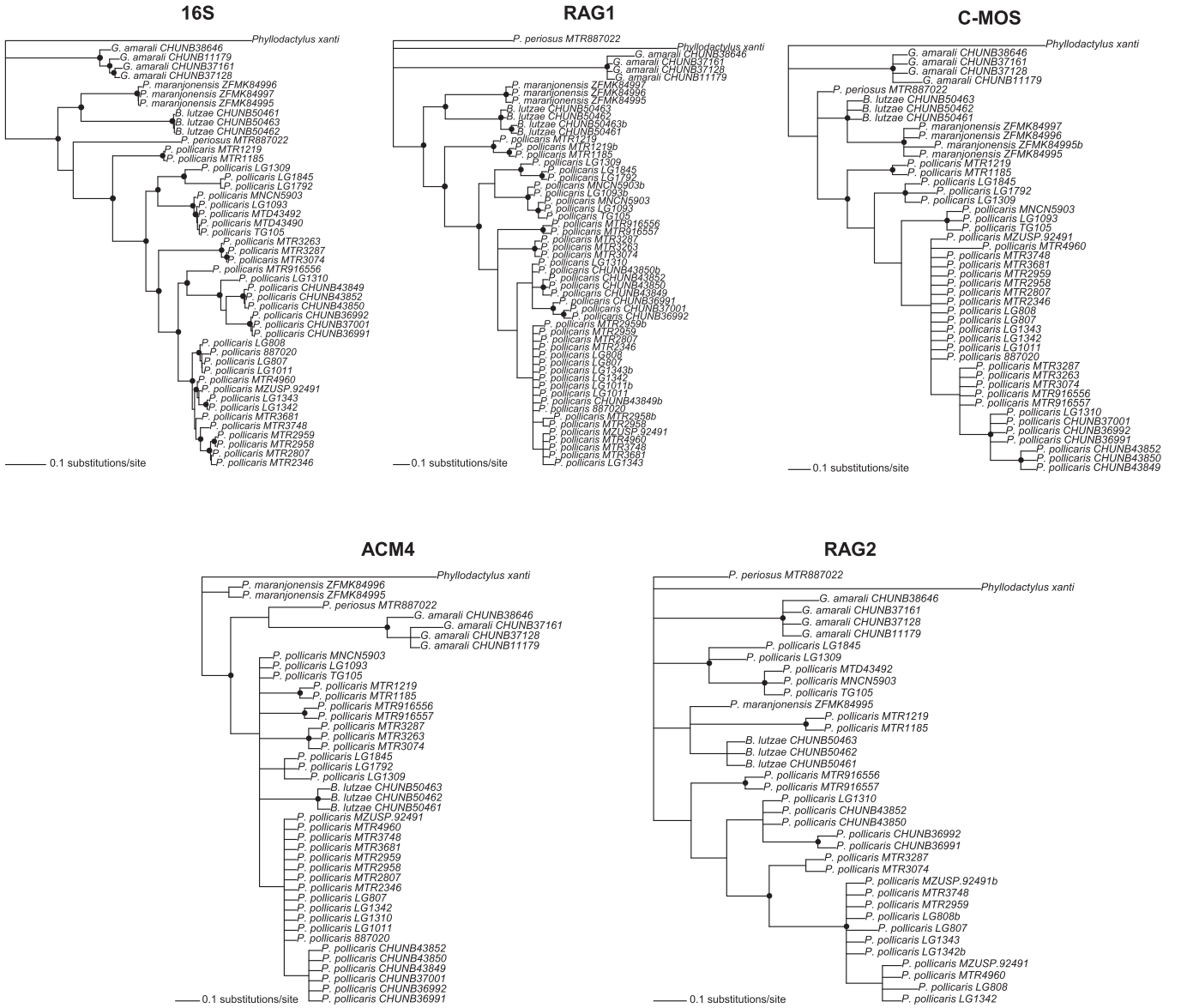

Fig. 3. Bayesian phylogenies for each locus. Circles at nodes indicate clades with posterior probabilities >0.95. Taxon names appended with a "b" represent the alternative phased allele for heterozygous individuals.

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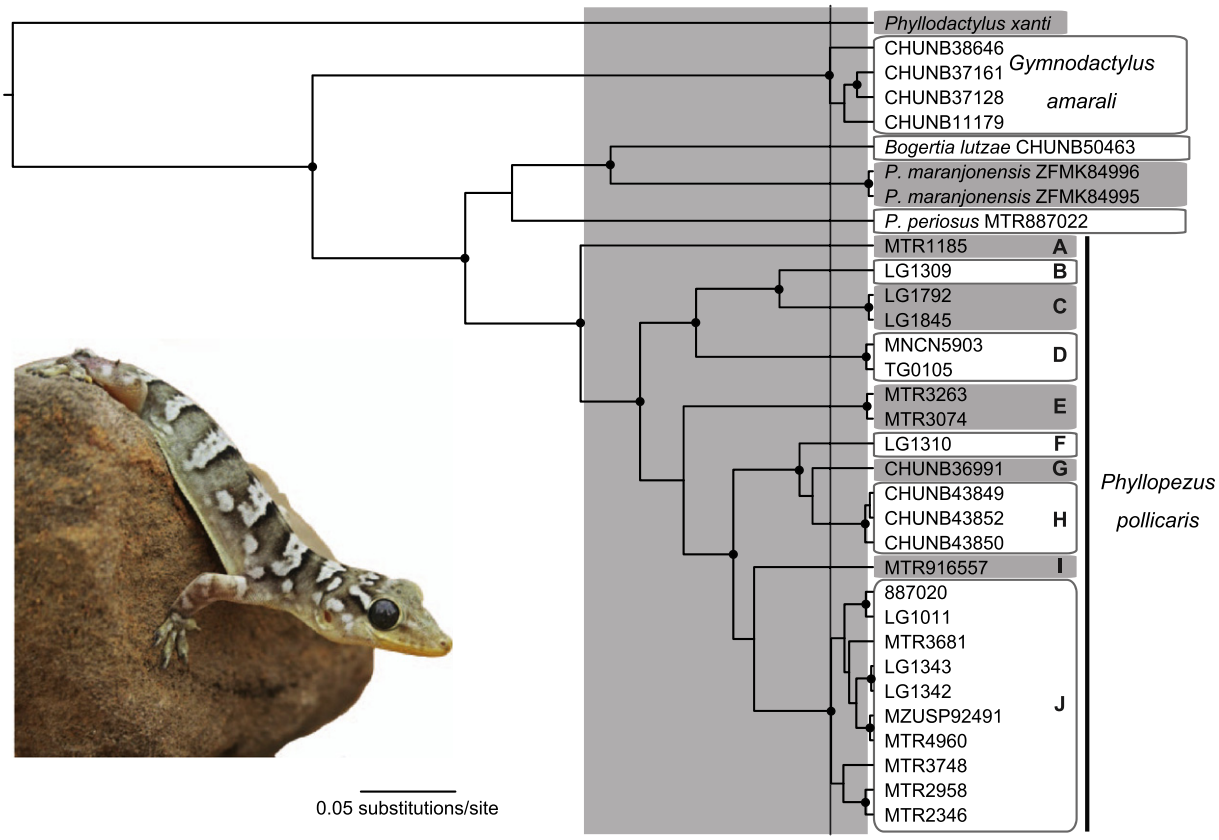

Fig. 4. Ultrametric phylogeny of unique *16S* haplotypes. The vertical line represents the cutoff for species-level clusters identified by the GMYC analysis. Taxon names/IDs of species-level clusters are enclosed by boxes. The large gray box shows the confidence interval of species-level clusters (6–25 clades). Circles indicate nodes with posterior probabilities >0.95. Photo of *Phyllopezus periosus* by MTR.

4. Discussion

4.1. Phylogenetic relationships

We inferred phylogenetic relationships among *Phyllopezus* species from a multi-gene dataset using several methods including Maximum Likelihood and Bayesian analyses of the concatenated data, and species trees constructed from individual gene trees using both BCA and the coalescent-based *BEAST. The same topology was recovered, with varying levels of nodal support, from all of these techniques. All multi-gene analyses recovered *P. periosus* as the sister species to the remaining *Phyllopezus* species plus *Bogertia*. We also recovered a *Bogertia* + *P. maranjonensis* clade that rendered *Phyllopezus* paraphyletic, consistent with a previous phylogenetic analysis (Gamble et al., 2011a). Phylogenetic relationships estimated from mtDNA were largely congruent with the multi-locus results, with the exception of the placement of *P. periosus*. The Bayesian analyses using MrBayes recovered *P. perio-*

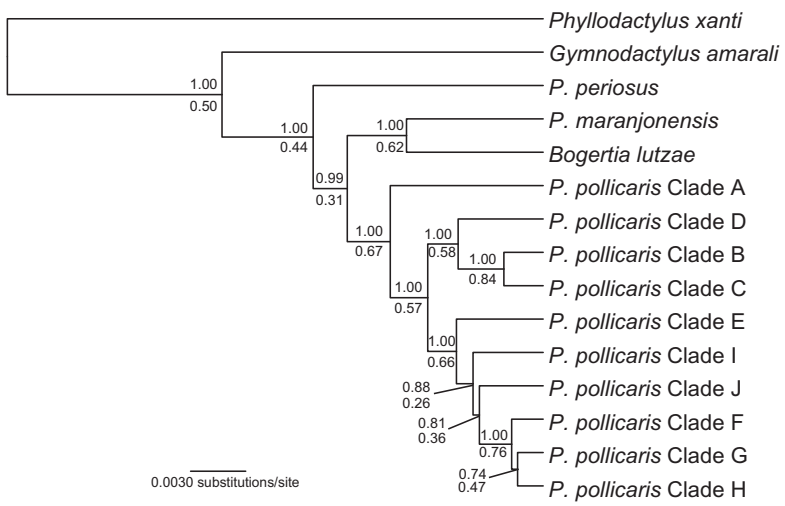

Fig. 5. Phylogenetic relationships among *Phyllopezus* species. Topology estimated using Bayesian coalescent analyses with *BEAST, which is identical to the topology from the Bayesian concordance analysis (BCA) using BUCKy. Node values indicate Bayesian posterior probabilities (top) and posterior mean concordance factors from the BCA analysis (bottom). Lettered *P. pollicaris* clades refer to clusters identified by the GMYC analysis.

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Table 3
Net among group distances between sampled clades for *16S* data. Values below the diagonal are uncorrected *p*-distances. Values above the diagonal are ML corrected distances using the GTR + I + G model. Standard error values, calculated using 500 bootstrap replicates, are in parentheses.

Taxa	*Phyllodactylus xanti*	*Gymnodactylus amarali*	*Bogertia lutzae*	*P. periosus*	*P. maranjonensis*
Phyllodactylus xanti	–	0.485 (0.111)	0.481 (0.114)	0.480 (0.107)	0.492 (0.116)
Gymnodactylus amarali	0.190 (0.019)	–	0.355 (0.086)	0.484 (0.114)	0.362 (0.083)
Bogertia lutzae	0.198 (0.020)	0.151 (0.016)	–	0.334 (0.071)	0.233 (0.054)
P. periosus	0.206 (0.020)	0.188 (0.018)	0.169 (0.018)	–	0.289 (0.061)
P. maranjonensis	0.199 (0.020)	0.161 (0.017)	0.130 (0.016)	0.153 (0.017)	–
P. pollicaris Clade A	0.210 (0.020)	0.166 (0.017)	0.143 (0.017)	0.152 (0.017)	0.148 (0.017)
P. pollicaris Clade B	0.219 (0.021)	0.172 (0.018)	0.150 (0.018)	0.148 (0.018)	0.141 (0.017)
P. pollicaris Clade C	0.219 (0.020)	0.165 (0.018)	0.172 (0.019)	0.157 (0.018)	0.144 (0.018)
P. pollicaris Clade D	0.214 (0.021)	0.170 (0.018)	0.171 (0.018)	0.159 (0.018)	0.142 (0.017)
P. pollicaris Clade E	0.218 (0.020)	0.188 (0.018)	0.164 (0.018)	0.154 (0.019)	0.148 (0.017)
P. pollicaris Clade F	0.225 (0.020)	0.173 (0.017)	0.162 (0.017)	0.158 (0.017)	0.156 (0.016)
P. pollicaris Clade G	0.227 (0.020)	0.191 (0.018)	0.173 (0.018)	0.180 (0.018)	0.155 (0.017)
P. pollicaris Clade H	0.235 (0.020)	0.182 (0.017)	0.175 (0.018)	0.178 (0.018)	0.156 (0.016)
P. pollicaris Clade I	0.213 (0.020)	0.153 (0.017)	0.144 (0.017)	0.140 (0.017)	0.140 (0.016)
P. pollicaris Clade J	0.214 (0.020)	0.166 (0.017)	0.149 (0.016)	0.158 (0.018)	0.136 (0.016)
Taxa	*P. pollicaris* Clade A	*P. pollicaris* Clade B	*P. pollicaris* Clade C	*P. pollicaris* Clade D	*P. pollicaris* Clade E
Phyllodactylus xanti	0.546 (0.126)	0.562 (0.129)	0.565 (0.131)	0.574 (0.139)	0.587 (0.142)
Gymnodactylus amarali	0.372 (0.086)	0.387 (0.084)	0.373 (0.084)	0.418 (0.097)	0.510 (0.125)
Bogertia lutzae	0.258 (0.054)	0.271 (0.057)	0.336 (0.068)	0.368 (0.080)	0.339 (0.075)
P. periosus	0.291 (0.066)	0.266 (0.059)	0.304 (0.068)	0.311 (0.067)	0.298 (0.066)
P. maranjonensis	0.272 (0.058)	0.254 (0.053)	0.271 (0.059)	0.266 (0.057)	0.278 (0.062)
P. pollicaris Clade A	–	0.241 (0.052)	0.288 (0.063)	0.188 (0.043)	0.258 (0.061)
P. pollicaris Clade B	0.137 (0.017)	–	0.093 (0.020)	0.146 (0.033)	0.188 (0.041)
P. pollicaris Clade C	0.156 (0.018)	0.073 (0.013)	–	0.165 (0.035)	0.227 (0.049)
P. pollicaris Clade D	0.115 (0.015)	0.095 (0.014)	0.107 (0.015)	–	0.153 (0.035)
P. pollicaris Clade E	0.136 (0.017)	0.117 (0.016)	0.131 (0.016)	0.101 (0.014)	–
P. pollicaris Clade F	0.133 (0.016)	0.121 (0.016)	0.133 (0.016)	0.105 (0.014)	0.100 (0.014)
P. pollicaris Clade G	0.128 (0.016)	0.153 (0.017)	0.148 (0.017)	0.125 (0.016)	0.117 (0.016)
P. pollicaris Clade H	0.135 (0.016)	0.138 (0.016)	0.145 (0.016)	0.121 (0.015)	0.114 (0.015)
P. pollicaris Clade I	0.127 (0.016)	0.110 (0.016)	0.115 (0.016)	0.107 (0.015)	0.089 (0.014)
P. pollicaris Clade J	0.120 (0.016)	0.101 (0.015)	0.104 (0.014)	0.085 (0.013)	0.082 (0.013)
Taxa	*P. pollicaris* Clade F	*P. pollicaris* Clade G	*P. pollicaris* Clade H	*P. pollicaris* Clade I	*P. pollicaris* Clade J
Phyllodactylus xanti	0.602 (0.131)	0.612 (0.139)	0.667 (0.150)	0.550 (0.128)	0.593 (0.138)
Gymnodactylus amarali	0.412 (0.089)	0.479 (0.104)	0.455 (0.099)	0.334 (0.075)	0.409 (0.092)
Bogertia lutzae	0.310 (0.059)	0.341 (0.065)	0.363 (0.072)	0.271 (0.057)	0.306 (0.063)
P. periosus	0.306 (0.063)	0.364 (0.075)	0.374 (0.077)	0.248 (0.054)	0.323 (0.070)
P. maranjonensis	0.294 (0.060)	0.291 (0.058)	0.297 (0.061)	0.251 (0.051)	0.259 (0.056)
P. pollicaris Clade A	0.231 (0.047)	0.207 (0.041)	0.234 (0.047)	0.215 (0.046)	0.205 (0.044)
P. pollicaris Clade B	0.187 (0.037)	0.255 (0.050)	0.228 (0.042)	0.166 (0.034)	0.155 (0.031)
P. pollicaris Clade C	0.215 (0.042)	0.254 (0.049)	0.251 (0.048)	0.184 (0.039)	0.164 (0.034)
P. pollicaris Clade D	0.163 (0.035)	0.193 (0.039)	0.198 (0.040)	0.164 (0.036)	0.124 (0.027)
P. pollicaris Clade E	0.161 (0.037)	0.185 (0.040)	0.190 (0.040)	0.131 (0.031)	0.126 (0.030)
P. pollicaris Clade F	–	0.077 (0.019)	0.057 (0.016)	0.092 (0.021)	0.098 (0.022)
P. pollicaris Clade G	0.062 (0.012)	–	0.077 (0.019)	0.122 (0.027)	0.120 (0.027)
P. pollicaris Clade H	0.047 (0.010)	0.061 (0.011)	–	0.110 (0.024)	0.101 (0.022)
P. pollicaris Clade I	0.070 (0.012)	0.088 (0.013)	0.079 (0.012)	–	0.071 (0.017)
P. pollicaris Clade J	0.070 (0.012)	0.084 (0.013)	0.070 (0.011)	0.054 (0.011)	–

Table 4
Results of topology tests for the concatenated data using the likelihood-based Shimodaira–Hasegawa (SH) test and the approximately unbiased (AU) test.

Trees	Lln	Difference in Lln	*P* (SH test)	*P* (AU test)
ML tree	−9009.410766	–	–	–
Monophyly of *Phyllopezus* exclusive of *Bogertia*	−9025.826721	16.415955	0.163	0.019
Reciprocal monophyly of *P. p. pollicaris* and *P. p. przewalskii*	−9046.704113	37.293347	0.013	0.001

sus as the sister taxon to *P. pollicaris* samples, while the BEAST analyses found that *P. periosus* formed a clade with *P. maranjonensis* + *B. lutzae*. These alternative positions of *P. periosus* were not well supported in either analysis. Individual nDNA loci showed conflicting patterns of relationships, both among loci and with the multi-locus analyses, and nodal support was generally low for most nodes.

4.2. Taxonomic status of Bogertia

All of the multi-gene phylogenetic analyses recovered *B. lutzae* samples nested within *Phyllopezus* as the sister species of *P. maranjonensis*. Phylogenetic hypothesis testing, except for the SH test of the concatenated data, also rejected a monophyletic *Phyllopezus* exclusive of *Bogertia*. The SH test is known to be conservative, particularly when multiple trees are examined, as was the case here (Shimodaira, 2002; Strimmer and Rambaut, 2002). Implementing a correction for multiple trees, the weighted SH test (Buckley et al., 2001), resulted in a marginally significant difference between the ML tree and the constraint tree ($P = 0.049$).

Phyllopezus and *Bogertia* share many characteristics. Digits of both genera possess undivided scansors with a prominent claw that extends beyond the toepads. The first digit on both manus and pes is either reduced, e.g. *Phyllopezus*, or rudimentary, e.g. *Bogertia* (Loveridge, 1941; Vanzolini, 1953). Both genera have large paraphalanges, cartilaginous structures associated with the adhesive pads, that take up most of the internal area of the toepads

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Table 5
Posterior probabilities of alternative phylogenetic hypotheses from the Bayesian analyses of each individual locus, the concatenated data and the species tree analyses using *BEAST.

Dataset	Monophyly of *Phyllopezus* exclusive of *Bogertia*	Reciprocal monophyly of *P. p. pollicaris* and *P. p. przewalskii*
RAG1	0.0005	0.0000
RAG2	0.0018	0.0003
C-MOS	0.0028	0.0040
ACM4	0.0000	0.0088
16S	0.0253	0.0000
Concatenated data	0.0000	0.0000
Species tree	0.0000	0.0000

(Russell and Bauer, 1988). Other internal digital characteristics related to tendons, muscles and osteology are also very similar (Russell and Bauer, 1988). *Phyllopezus* and *Bogertia* share similarities in cranial osteology, including the absence of a stapedial foramen and a short parietal (Abdala, 1996). *B. lutzae* also has a $2n = 40$ karyotype, like specimens of *P. periosus* and *P. pollicaris* from clades E and J (Pellegrino et al., 1997).

The molecular phylogenies presented here, coupled with morphological and karyotypic similarities, indicate a close relationship between *Phyllopezus* and *Bogertia*. These results also have taxonomic implications. To prevent a paraphyletic *Phyllopezus* and maintain a classification that is isomorphic with respect to phylogenetic history, we synonymize *Bogertia* with *Phyllopezus*. *B. lutzae* becomes *Phyllopezus lutzae* comb. nov.

The large distributional gap between *P. lutzae*, which is restricted to Restinga habitat on Brazil's Atlantic coast (Vrcibradic et al., 2000), and *P. maranjonensis* in the Peruvian Andes (Koch et al., 2006) is remarkable and not a commonly encountered pattern. Most Restinga endemics have sister taxa in nearby areas, e.g. *Liolaemus lutzae* and *L. occipitalis* (Schulte et al., 2000), or have close relatives with large distributions, e.g. *Bothrops leucurus* and *B. atrox* (Fenwick et al., 2009). The disjunct distributions of *P. lutzae* and *P. maranjonensis* suggest their ancestor had a widespread distribution across the continent followed by extinction across the intervening areas. Although the original factors responsible for this enormous ancestral distribution and subsequent extinction are presently obscure, extinction likely played an important part in *Phyllopezus'* history.

4.3. P. pollicaris subspecies

Phyllopezus p. przewalskii (Clade D), the subspecies from southwestern Brazil and the Chaco, was monophyletic with both mtDNA and multi-gene analyses. *Phyllopezus p. przewalskii* are also morphologically diagnosable and differ from *P. p. pollicaris s.l.* by ventral scale number (26–29 in *P. p. przewalskii* vs. 28–32 in *P. p. pollicaris*) and the absence of postcloacal tubercles (Peters et al., 1986; Vanzolini, 1953, 1968). The karyotype of *P. p. przewalskii* ($2n = 38$) was also distinct from both *P. periosus* ($2n = 40$) and *P. p. pollicaris* ($2n = 40$) (Pellegrino et al., 1997). The *P. p. pollicaris* specimens examined by Pellegrino et al. (1997) were from Alagoado, Manga and Santo Inácio, BA; Cabaceiras, PB; and Xingó, AL. Specimens from these Caatinga localities, with the exception of Manga, which we did not sample here, correspond to clades E and J in our analyses.

The molecular and morphological evidence demonstrates that *P. p. przewalskii* is a distinct species under a lineage-based species concept (de Queiroz, 2007); raising it to species status would render *P. pollicaris* paraphyletic. This is because, under the current nomenclature, all *P. pollicaris* that are not *P. p. przewalskii* are *P. p. pollicaris*. Phylogenetic topology tests rejected the reciprocal monophyly of the *P. pollicaris* subspecies indicating that current subspecific names do not accurately reflect evolutionary history within the species. Addressing this problem requires the erection of new names for the other *P. pollicaris* clades to ensure that taxonomy is isomorphic with phylogeny. The erection of new names to represent clades within *P. pollicaris*, as mentioned below, is premature based on our data. An accurate assessment of *Phyllopezus* species diversity will require more thorough taxonomic and geographic sampling and an examination of additional characters, such as morphological, karyological, and ecological data.

4.4. Cryptic genetic diversity

We recovered substantial phylogenetic diversity among *P. pollicaris* samples indicating the presence of multiple cryptic species. The GMYC analysis identified 10 species-level clusters within *P. pollicaris*. These clusters were strongly supported by both mtDNA alone and the combined mtDNA and nDNA data and had uncorrected *16S* distances (4.7–15.6%) equivalent to distances among other gekkotan sister species, which typically range from 4% to 10% (Bauer and Lamb, 2002; Hass, 1996; Rocha et al., 2009; Ziegler et al., 2008). This is similar to the 5% uncorrected sequence divergence in *16S* that has been used as a tentative cutoff for identifying cryptic amphibian species (Vences et al., 2005). Although these distances are comparable to genetic distances in other amphibian and reptile species, we are not advocating species delimitation based on some pre-determined amount of sequence divergence. Variation in lineage-specific substitution rates and alterations in coalescent times due to differences in effective population sizes makes the application of a universal threshold to delimit species difficult to implement (Barraclough et al., 2009; Hickerson et al., 2006; Moritz and Cicero, 2004; Pons et al., 2006). That said, examining the amount of sequence divergence among closely related taxa can serve as a heuristic to identify taxa that warrant a closer examination using other taxonomic methods.

The large mitochondrial DNA distances among *P. pollicaris* clades suggest that many may be valid species. Even so, we are reluctant to identify these clades as species in a taxonomic sense at this time. The likelihood-ratio test from the GMYC analysis was not significant calling into question the robustness of the results. There are several explanations for a non-significant outcome; the most likely reason in this case was our sparse sampling within several of the clades. Incomplete taxon sampling can weaken the ability to detect the transition between inter- and intraspecific branching patterns (Pons et al., 2006). Indeed, some of the *P. pollicaris* clades were represented by just single individuals, e.g. clades A, B, F and I. Sparse taxon sampling is also very likely the cause for the wide confidence interval for the GMYC results (six to 25 species). Additionally, a thorough analysis of morphological and ecological data (e.g. niche modeling) should be conducted to confirm our preliminary results and provide diagnostic characters for putative species (Bauer et al., 2011). Morphological data in particular will likely prove informative for this purpose as morphological variation among *P. p. pollicaris* populations has been commented on previously (Borges-Nojosa and Caramaschi, 2005; Rodrigues, 2005b).

The presence of multiple cryptic lineages in *P. pollicaris* provides evidence that current estimates of herpetofaunal diversity in South America's open biomes are underestimated, perhaps substantially so. This stems primarily from the existence of large unsampled expanses (Colli et al., 2002; Rodrigues, 2005b; Souza et al., 2010), the paucity of systematists (Rodrigues, 2005a; Yahner, 2004), the meager financial support for biological collections (Bockmann, 2011), and the still incipient utilization of molecular data for species delimitation and taxonomic studies (Werneck, 2011). For instance, from 2000 to 2009, 32 new species of Cerrado squamates were described, at a rate of 3.56 new species per year, representing approximately 36% of all Cerrado squamate species (92) described during

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the 20th century (Nogueira et al., 2010)! Further, 29 (92%) of these species are endemic and have small ranges (see also Diniz-Filho et al., 2005).

The deeply divergent and geographically structured *Phyllopezus* haploclades recovered here emphasize the region's complex biogeographical history and challenges initial views that South America's open biomes possess a homogenous herpetofauna and shared diversification history (Vanzolini, 1976). On the contrary, we found each of the open biomes possessed one or more unique lineages not shared by the other biomes. *P. pollicaris*, because of its complex phylogenetic history and its occurrence across heterogeneous habitats in the open biomes, represents an ideal group to test several of the recently proposed biogeographic hypotheses to explain species diversification in South American open biomes (Werneck, 2011; Werneck and Colli, 2006; Werneck et al., 2011). Further molecular genetic work with *Phyllopezus*, that attempts to broaden the geographic scope and the number of individuals sampled should be a priority.

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