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Phylogenetic relationships within *Bothrops neuwiedi* group (Serpentes, Squamata): Geographically highly-structured lineages, evidence of introgressive hybridization and Neogene/Quaternary diversification

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ABSTRACT

Eight current species of snakes of the *Bothrops neuwiedi* group are widespread in South American open biomes from northeastern Brazil to southeastern Argentina. In this paper, 140 samples from 93 different localities were used to investigate species boundaries and to provide a hypothesis of phylogenetic relationships among the members of this group based on 1122 bp of *cyt b* and *ND4* from mitochondrial DNA and also investigate the patterns and processes occurring in the evolutionary history of the group. Combined data recovered the *B. neuwiedi* group as a highly supported monophyletic group in maximum parsimony, maximum likelihood and Bayesian analyses, as well as four major clades (Northeast I, Northeast II, East–West, West-South) highly-structured geographically. Monophyly was recovered only for *B. pubescens*. By contrast, *B. diporus*, *B. lutzi*, *B. erythromelas*, *B. mattogrossensis*, *B. neuwiedi*, *B. marmoratus*, and *B. pauloensis*, as currently defined on the basis of morphology, were polyphyletic. Sympatry, phenotypic intergrades and shared mtDNA haplotypes, mainly between *B. marmoratus* and *B. pauloensis* suggest recent introgressive hybridization and the possible occurrence of a narrow hybrid zone in Central Brazil. Our data suggest at least three candidate species: *B. neuwiedi* from Espinhaço Range, *B. mattogrossensis* (TM173) from Serra da Borda (MT) and *B. diporus* (PT3404) from Castro Barros, Argentina. Divergence estimates highlight the importance of Neogene events in the origin of *B. neuwiedi* group, and the origin of species and diversification of populations of the Neotropical fauna from open biomes during the Quaternary climate fluctuations. Data reported here represent a remarkable increase of the *B. neuwiedi* group sampling size, since representatives of all the current recognized species from a wide geographic range are included in this study, providing basic information for understanding the evolution and conservation of Neotropical biodiversity.

1. Introduction

1.1. Taxonomic status

The *Bothrops neuwiedi* group currently comprises eight species: *B. diporus*, *B. erythromelas*, *B. lutzi*, *B. mattogrossensis*, *B. marmoratus*, *B. neuwiedi*, *B. pauloensis*, and *B. pubescens* (Castoe and Parkinson, 2006; Fenwick et al., 2009; Silva, 2004; Silva and Rodrigues, 2008; Werman, 1992; Wüster et al., 2002). Historically, the variability observed in *Bothrops neuwiedi* was associated with geographic distribution, and the group was considered to comprise a complex of 12 subspecies (Table 1) (see review in Campbell and Lamar, 1989).

Silva and Rodrigues (2008) proposed a new taxonomic arrangement for the complex mainly based on qualitative features such as coloration and blotch patterns. As a result of this work, six subspecies were elevated to the species level, others synonymized, and one new species was described, setting the present formation of the *B. neuwiedi* group (Silva, 2004; Silva and Rodrigues, 2008) (Table 1).

Representatives of the *B. neuwiedi* group are widespread in open areas from northeastern Brazil to southeastern Argentina (Werman, 2005). *Bothrops erythromelas* is restricted to Caatinga areas; *B. lutzi*, *B. pauloensis* and *B. marmoratus* occur mainly in Cerrado; *B. mattogrossensis* and *B. diporus* are distributed mostly in Chaco; *B. pubescens* is limited to the Pampas in southernmost Brazil and Uruguay, and *B. neuwiedi* occurs throughout the mountains in the southeast Brazilian coast (Silva, 2004; Silva and Rodrigues, 2008). A wide range of overlapping species from the *B. neuwiedi* group was observed; however, only 25 sympatry records were reported. Most overlaps (12) were between *B. neuwiedi* and *B. pauloensis*, and nine cases involved *B. pauloensis* and *B. mattogrossensis* (Silva, 2004; Silva and Rodrigues, 2008).

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Table 1
Compilation of the *Bothrops neuwiedi* group, with valid taxa and synonyms from Campbell and Lamar (1989), Silva (2004), Silva and Rodrigues (2008).

Valid taxa	Synonyms
B. neuwiedi Wagler (1824)	*B. n. neuwiedi* Amaral (1925)
	B. n. goyazensis Amaral (1925)
	B. n. paranaensis Amaral (1925)
	B. urutu Lacerda (1884), *B. n. minasensis* Amaral (1925), *B. n. urutu* Amaral (1937), Hoge (1966)
	B. atrox meridionalis Cope (1885), *B. n. fluminensis* Amaral (1933), *B. n. meridionalis* Hoge (1966)
B. mattogrossensis Amaral (1925)	*B. n. mattogrossensis* Amaral (1925)
	B. n. boliviana Amaral (1927), *B. n. bolivianus* Hoge (1966)
B. pubescens Cope (1870)	*Trigonocephalus pubescens* Cope (1870), *B. n. riograndensis* Amaral (1925), *B. n. pubescens* Hoge (1959, 1966)
B. diporus Cope (1862)	*B. n. meridionalis* Amaral (1930), *B. n. diporus* Cochran (1961), Hoge (1966)
B. pauloensis Amaral (1925)	*B. n. pauloensis* Amaral (1925)
B. lutzi Miranda-Ribeiro (1915)	*Lachesis lutzi* Miranda-Ribeiro (1915), *B. n. bahiensis* Amaral (1925), *B. n. lutzi* Amaral (1930), Hoge (1966)
	B. n. piauhyensis Amaral (1925)
	B. iglesiasi Amaral (1923)
B. erythromelas Amaral (1923)	*Lachesis neuwiedi itapetiningae* Ihering (1911)
B. marmoratus Silva and Rodrigues (2008)	–

1.2. Phylogenetic relationships

Burger (1971) first proposed the *B. neuwiedi* group based on morphological traits grouping *B. neuwiedi* with *B. itapetiningae* and *B. iglesiasi*. Werman (1992), based on morphology and protein electrophoresis, expanded the *B. neuwiedi* group to include a wider assemblage composed of the species with a divided lacunolabial scale, i.e. *B. alternatus, B. erythromelas, B. itapetiningae* and *B. neuwiedi*.

Wüster et al. (2002), on the basis of mitochondrial *cyt b* and *ND4* sequences, first hypothesized *B. neuwiedi* as the sister group of *B. erythromelas;* and *B. itapetiningae* as the sister group of *B. alternatus*. Castoe and Parkinson (2006) recovered similar results adding sequences of 12S and 16S rRNA genes for *B. diporus, B. erythromelas* and *B. alternatus*. Both analyses recovered *B. jararaca* as the sister group of the *B. neuwiedi* group. These results are in accordance with the *B. erythromelas* as the sister taxon to a clade comprising the rest of the *B. neuwiedi* group. Another point of agreement, contrary to previous morphological results (Burger, 1971; Werman, 1992), is that the *B. neuwiedi* group is more closely related to the *B. jararaca* group than to any other *Bothrops*, regardless of the condition of the lacunolabial scale.

Fenwick et al. (2009) performed the most complete analysis for the *B. neuwiedi* group, including molecular and morphological data of four out of eight species of the group: *B. diporus, B. erythromelas, B. neuwiedi* and *B. pauloensis*. The analyses for *B. mattogrossensis* were obtained exclusively using morphological traits, and *B. lutzi, B. pubescens* and *B. marmoratus* were not included in the study. The *Bothrops neuwiedi* group was monophyletic in the analysis performed using exclusively molecular information (*B. erythromelas* (*B. neuwiedi* (*B. pauloensis*, *B. diporus*))), and *B. jararaca* was the sister group. Morphological data grouped *B. mattogrossensis* with *B. alternatus,* and placed *B. sanctaecrucis* as the sister taxa of the remaining B. *neuwiedi* group. However, the authors allocated *B. mattogrossensis* to the *B. neuwiedi* group, based on the morphological description proposed by Silva and Rodrigues (2008).

Additionally, Fenwick et al. (2009) suggested the maintenance of the genera *Bothrocophias* and *Bothriopsis* and split *Bothrops* in three genera: *Bothropoides* (*B. neuwiedi* group and *B. jararaca* group), *Rhinocerophis* (*B. alternatus* group) and *Bothrops sensu strict* (*B. atrox* group).

Recently Carrasco et al. (2012) highlighted the incongruence between their analyses with combined evidence and the one presented by Fenwick et al. (2009), excluding taxa that have only morphological information. The authors also observed that although splitting *Bothrops* in different genera as suggested by Fenwick et al. (2009), *Bothrops* still remains paraphyletic.

Carrasco et al. (2012) performed analyses using morphological, ecological and molecular information. Species groups were recovered similar to those groups obtained in Wüster et al. (2002), although incongruences between morphological and molecular characters lead to alternative phylogenetic hypotheses. Total evidence recovered *B. neuwiedi* as the sister group of *B. jararaca*. However when *cyt b* and *ND4* were excluded, the analysis recovered *B. neuwiedi* as the sister group of *B. alternatus*. Then, the authors suggested an arrangement that rectifies the paraphyly of *Bothrops* by assigning *Bothrops andianus* to *Bothrocophias* and recognizing the sister clade as *Bothrops,* synonymizing *Bothropoides, Bothriopsis* and *Rhinocerophis*.

On the basis of this scenario, the present study aims to reconstruct phylogenetic relationships and investigate historical biogeography and diversification of the *B. neuwiedi* group. The analyses were conducted including all species of the group, using a broad sample representing the wide range of the group, and employing partial mitochondrial gene sequences (*cyt b* and *ND4*).

2. Material and methods

2.1. Sampling

A total of 140 samples representing the eight species recognized for *B. neuwiedi* group were obtained from 93 localities throughout the distribution area (Fig. 1 and Table 2). Samples from *Bothrops* and *Bothrocophias* (24 taxa) were used as outgroups for phylogenetic analysis (Appendix S1). Sequences from GenBank for seven specimens of the *B. neuwiedi* group were included in the analysis as well (Appendix S1). All haplotype sequences used in this study were deposited in GenBank under the accession numbers (KF801106 - KF801356) presented in Table 2.

All specimens used in this study came from known localities and were morphologically identified according to Silva and Rodrigues (2008). The sample includes: 14 *Bothrops diporus*, seven *B. erythromelas*, seven *B. lutzi*, 13 *B. mattogrossensis*, 10 *B. marmoratus*, 46 *B. neuwiedi*, 24 *B. pauloensis,* 14 *B. pubescens* and five specimens showing intermediate morphology between species *B. neuwiedi* and *B. pauloensis*; *B. marmoratus* and *B. mattogrossensis;* and *B. pauloensis* and *B. marmoratus* (Table 2).

2.2. Laboratory methods

Total genomic DNA was extracted from liver and scales preserved in ethanol and dry shed skin, following Fetzner (1999).

The mitochondrial data set comprises a total of 1122 bp. Partial cytochrome b gene (378 bp) was amplified with the primers LGL 765 (Bickham et al., 1995) and H15149 (Kocher et al., 1989), NADH dehydrogenase subunit 4 gene (640 bp) plus 104 bases from two

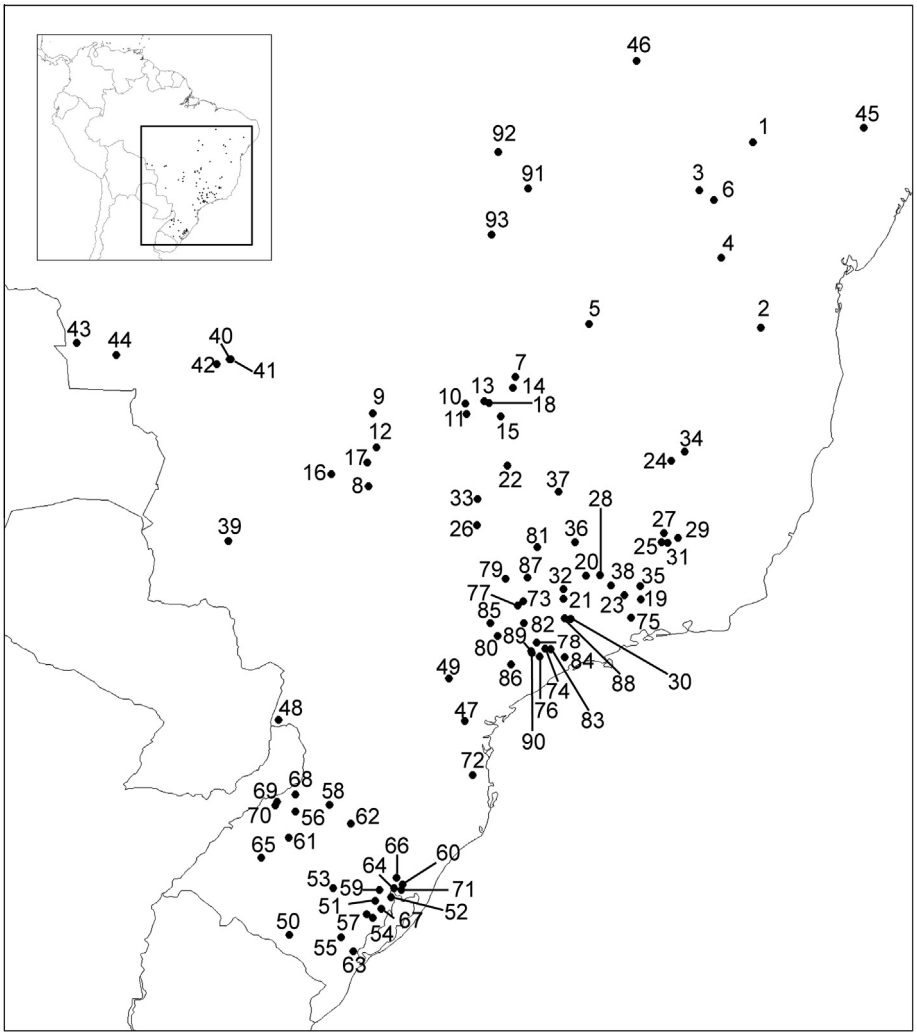

Fig. 1. Geographic distribution of *B. neuwiedi* group sampled in this study from 93 localities in Brazil.

transfer RNA (tRNA) genes flanking the 3′ end of the *ND4* gene ($tRNA^{HIS}$, $tRNA^{SER}$) with ND4F and Leu (Arèvalo et al., 1994).

Polymerase chain reactions were performed in 25 μL (2.5 μL PCR buffer, 2.5 μL 2 mM dNTPs, 1.5 μL 25 mM $MgCl_2$, 2.0 μL of each 5 μM primers, 0.25 μL 5 U/μL Taq DNA polymerase, 11 μL H2O and 3.25 μL 20 ng/μL DNA). Reaction conditions were as follows: an initial denaturation of 94 °C for 5 min, followed by 35 cycles (*cyt b*) or 40 cycles (*ND4*) of denaturation at 94 °C (40 s), annealing at 48 °C (45 s), extension at 72 °C (40 s), and a final extension of 72 °C (10 min).

2.3. Phylogenetic analysis

Sequences were edited using CodonCode Aligner 3.0 (Li-COR Inc) and aligned using Muscle (Edgar, 2009) available in this program. Uncorrected genetic distances were calculated based on *cyt b* in PAUP 4.0b10 (Swofford, 2001).

Four strategies for data partitioning were tested using Treefinder (Jobb et al., 2004): a single partition (all data), two partitions (per gene), three partitions (per codon position) and six partitions (per gene and codon position). For each data partition, the most appropriate model of sequence evolution was determined in Modeltest 3.7 (Posada and Crandall, 1998), based on the Akaike Information Criterion (AIC) corrected for small sample size (Posada and Buckley, 2004). A single partition was selected as the best partitioning, and the evolutionary model used was GTR + I + G.

Phylogenetic analyses under maximum parsimony (MP) were performed using TNT 1.1 (Goloboff et al., 2008) with 1000 replicates of Jackknife. Maximum likelihood (ML) was performed using PhyML 3.0 (Guindon et al., 2010), with aLRT SH-like supports. Data were analyzed under Bayesian inference (BI) with MrBayes v.3.0b4 (Ronquist and Huelsenbeck, 2003), with two independent runs of four Markov chains run for 10 million generations and sampled every 1000 generations. The first 25,000 trees were discarded and a 70% consensus topology was obtained from the last 75,001 trees in the chain. Significant support for phylogenetic hypothesis Bayesian posterior probability values >95% were considered and for MP and ML supports >70%.

2.4. Molecular dating

Using the concatenated *cytb* and *ND4* sequences, we estimated the time to the most recent common ancestor (TMRCA) for each clade using a relaxed Bayesian molecular clock with uncorrelated lognormal rates (BEAST 1.7.4 – Drummond and Rambaut, 2007; Drummond et al., 2012). Sequences with missing data were discarded and haplotypes were combined to represent only one terminal when possible. Four analyses were run with 10 million generations, sampled every 1000 generations. A Birth and Death branching process with lognormal priors, UPGMA generated starting tree, and a GTR + I + G model were implemented. The resulting BEAST log files were viewed in Tracer 1.5 (Rambaut and

Table 2
Taxa used in this study: species, field number/voucher, Institution, locality, Brazilian state (ST), locality number (N°) in the map (Figure. 1), tissue (S = scale, L = liver, SS = shed skin) and GenBank accession number for cyt *b* and *ND4*.

Species	Field N°/Voucher	Institution	Locality	ST	N°	Tissue	cyt *b*	ND4
B. diporus	BNF9302-06	IB	Itaipu	PR	48	S	KF801115	-
	BNF9302-10	IB	Itaipu	PR	48	S	KF801116	KF801248
	NOPA3748	FZB	Chiapeta	RS	56	S	KF801113	KF801246
	UPF503	UPF	Constantina	RS	58	S	KF801114	KF801247
	UPF561	UPF	Jóia	RS	61	S	KF801117	-
	NOPA3034	FZB	Jóia	RS	61	S	-	KF801249
	NOPA3209 – E	FZB	Passo Fundo	RS	62	S	KF801122	-
	MCN16672	FZB	Santiago	RS	65	S	KF801118	KF801250
	NOPA3161H	FZB	Três Passos	RS	68	S	KF801121	KF801251
	MCN16783	FZB	Tucunduva	RS	69	S	-	KF801252
	MCN16990	FZB	Tucunduva	RS	69	S	KF801120	KF801253
	NOPA2277	FZB	Tuparendi	RS	70	S	KF801119	KF801254
	IBSP60323	IB	Blumenau	SC	72	L	KF801111	KF801245
	IBSP60327	IB	Blumenau	SC	72	L	KF801112	-
B. erythromelas	MRT3742	IBUSP	Alagoado	BA	1	L	KF801123	KF801255
	UFBA3699	UFBA	Anagé	BA	2	SS	KF801124	-
	UFBA4485	UFBA	Ibiraba	BA	3	SS	KF801125	KF801256
	7030681	IB	Ibitira	BA	4	S	KF801126	KF801257
	MTR11185	IBUSP	Santo Inácio	BA	6	L	KF801129	KF801260
	MRT3497	IBUSP	Santo Inácio	BA	6	L	KF801128	KF801259
	LAPT467	UFPE	Petrolândia	PE	45	S	KF801127	KF801258
B. lutzi	PHV1479		Jaborandi	BA	5	L	KF801133	KF801262
	PHV1994		Jaborandi	BA	5	L	KF801134	KF801263
	MZUSP12536	IBUSP	Uruçuí-Una	PI	46	L	KF801136	KF801264
	IBSP73209	IB	Piracicaba	SP	82	L	KF801135	-
	MTR14196	IBUSP	EESGT (Estação Ecológica Serra Geral do Tocantins)	TO	91	L	KF801131	KF801261
	MTR14488	IBUSP	EESGT	TO	91	L	KF801132	-
	MTR14800	IBUSP	EESGT	TO	91	L	KF801130	-
B. marmoratus	CHUNB19281	UnB	Reserva Ecológica do IBGE (RECOR)	DF	7	L	KF801145	KF801273
	CEPB8171	UCG	Goiânia	GO	10	S	KF801137	KF801265
	CEPB6511	UCG	Hidrolândia	GO	11	S	KF801138	KF801266
	CEPB13422	UCG	Jataí	GO	12	S	KF801139	KF801267
	CEPB12742	UCG	Leopoldo de Bulhões	GO	13	S	KF801142	KF801270
	CEPB14067	UCG	Luziânia	GO	14	S	KF801143	KF801271
	CEPB6816	UCG	Orizona	GO	15	S	KF801144	KF801272
	MZUSP15006	MZUSP	Serra da Canastra - São Roque de Minas	MG	36	L	KF801146	KF801274
	IBSP73286	IB	Serra do Salitre	MG	37	L	KF801147	KF801275
	BN0202	IB	Lajeado	TO	92	S	KF801140	KF801268
	BN0203	IB	Lajeado	TO	92	S	KF801141	KF801269
B. marmoratus/mattogrossensis	CEPB12734	UCG	Caiapônia	GO	9	S	KF801108	KF801240
B. marmoratus/pauloensis	CEPB13237	UCG	Aporé	GO	8	S	KF801110	KF801241
	CEPB13417	UCG	Hidrolândia	GO	11	S	KF801109	KF801242
B. mattogrossensis	CBGM833	PUCRS	Aquidauana	MS	39	SS	KF801148	KF801276
	NORMAT95	UFMT	Chapada dos Guimarães	MT	41	S	KF801150	KF801278
	NORMAT113	UFMT	Chapada dos Guimarães	MT	41	S	KF801149	KF801277
	NORMAT126	UFMT	Cuiabá	MT	42	S	KF801152	KF801280
	NORMAT239	UFMT	Cuiabá	MT	42	S	KF801154	KF801282
	NORMAT292	UFMT	Cuiabá	MT	42	S	KF801155	KF801284
	NORMAT280	UFMT	Cuiabá	MT	42	S	-	KF801283
	NORMAT109	UFMT	Cuiabá	MT	42	S	KF801151	KF801279
	NORMAT97	UFMT	Cuiabá	MT	42	S	KF801156	KF801285
	NORMAT213	UFMT	Cuiabá	MT	42	S	-	KF801281
	NORMAT199	UFMT	Cuiabá	MT	42	S	KF801153	-
	TM173	UFMT	Serra da Borda, V. Bela da Santíssima Trindade	MT	43	L	KF801158	-
	NORMAT134	UFMT	Jauru, Vale de São Domingos	MT	44	S	KF801157	KF801286
B. neuwiedi	IBSP71063	IB	Aiuruoca	MG	19	L	KF801159	-
	BN0101	IB	Aiuruoca	MG	19	S	KF801160	KF801287
	BN0318	IB	Aiuruoca	MG	19	S	KF801161	KF801288
	-	UNIFAL	Alfenas	MG	19	SS	KF801162	KF801289
	IBSP73007	IB	Andradas	MG	21	S	KF801164	KF801290
	BN0406	IB	Andradas	MG	21	S	KF801163	-
	IBSP74565	IB	Baependi	MG	23	L	KF801169	KF801294
	IBSP74563	IB	Baependi	MG	23	L	KF801167	KF801293
	IBSP74564	IB	Baependi	MG	23	L	KF801168	-
	IBSP74566	IB	Baependi	MG	23	L	KF801170	-
	BN9926	IB	Congonhas	MG	25	S	KF801171	KF801295
	MTR9979	IBUSP	Diamantina, Comarca de Biribiri	MG	24	L	KF801173	KF801296
	-	UFV	Itabirito	MG	27	SS	KF801175	KF801298
	IBSP74040	IB	Machado	MG	28	L	-	KF801302
	BN0309	IB	Machado	MG	28	S	KF801181	KF801301
	BN0413	IB	Machado	MG	28	S	KF801182	-
	JC870	IBUSP	Mariana	MG	29	L	KF801183	KF801303
	MZUSP15724	IBUSP	Mariana	MG	29	S	-	KF801304
	IBSP72925	IB	Munhoz	MG	30	L	KF801185	KF801305

(*continued on next page*)

Table 2 (*continued*)

Species	Field N°/Voucher	Institution	Locality	ST	N°	Tissue	cyt *b*	ND4
	IBSP73477	IB	Munhoz	MG	30	L	KF801186	-
	BN0203-02	IB	Munhoz	MG	30	S	KF801184	-
	UFOP6315	UFOP	Ouro Branco	MG	31	L	KF801187	KF801306
	UFOP6645	UFOP	Ouro Branco	MG	31	L	KF801188	-
	BN0410	IB	Poços de Caldas	MG	32	S	KF801189	KF801307
	IBSP72736	IB	São Gonçalo do Rio Preto	MG	34	L	KF801192	KF801310
	IBSP74088	IB	São Gonçalo do Rio Preto	MG	34	L	KF801193	KF801311
	IBSP73004	IB	São Vicente de Minas	MG	35	L	KF801198	KF801314
	IBSP73005	IB	São Vicente de Minas	MG	35	L	KF801199	KF801315
	IBSP73006	IB	São Vicente de Minas	MG	35	L	KF801200	KF801316
	IBSP71102	IB	Três Corações	MG	38	L	-	KF801319
	BN0319	IB	Curitiba (CPPI)	PR	47	S	KF801172	-
	BN9735	IB	Jaguariaiva	PR	49	S	KF801180	KF801300
	BN0608	IB	Araçariguama	SP	74	S	KF801165	KF801291
	IBSP74218	IB	Areias	SP	75	L	KF801166	KF801292
	IBSP72734	IB	Ibiúna	SP	76	L	KF801174	KF801297
	IBSP70337	IB	Itu	SP	78	L	KF801178	-
	IBSP70723	IB	Itu	SP	78	L	KF801179	-
	BN0102	IB	Itu	SP	78	S	KF801176	-
	BN0108	IB	Itu	SP	78	S	KF801177	KF801299
	20007010726	IB	Santana de Parnaíba	SP	83	S	KF801191	KF801308
	IBSP73292	IB	Santo André	SP	84	L	KF801197	KF801309
	BN0412	IB	São Miguel Arcanjo	SP	86	S	KF801194	KF801312
	BN9809-05	IB	São Simão	SP	87	S	KF801190	KF801313
	BN0301	IB	Socorro	SP	88	S	KF801195	KF801317
	IBSP71611	IB	Sorocaba	SP	89	L	KF801196	KF801318
	IBSP73637	IB	Votorantim	SP	90	L	KF801201	-
B. pauloensis	CEPB12528	UCG	Aporé	GO	8	S	KF801203	KF801321
	CEPB12094	UCG	Goiânia	GO	10	S	KF801210	KF801326
	CEPB13598	UCG	Goiânia	GO	10	S	KF801211	KF801327
	CEPB11832	UCG	Jataí	GO	12	S	KF801214	KF801330
	CEPB15162	UCG	Jataí	GO	12	S	KF801215	KF801331
	E134	IBUSP	Parque Nacional das Emas	GO	16	L	KF801206	KF801336
	E129	IBUSP	Parque Nacional das Emas	GO	16	L	KF801205	KF801335
	CEPB12070	UCG	Serranópolis	GO	17	S	KF801223	KF801340
	CEPB12976	UCG	Silvânia	GO	18	S	KF801224	KF803141
	BP0661	IB	Araguari	MG	22	S	KF801204	KF801322
	IBSP71110	IB	Frutal	MG	26	L	KF801208	KF801324
	IBSP71111	IB	Frutal	MG	26	L	KF801209	KF801325
	BN0321	IB	Frutal	MG	26	S	KF801207	KF801323
	IBSP71473	IB	Prata	MG	33	L	KF801221	KF801337
	IBSP71474	IB	Prata	MG	33	L	KF801222	KF801338
	MZUSP11849	IBUSP	APM Manso, Chapada dos Guimarães	MT	40	L	KF801216	KF801332
	MZUSP11851	IBUSP	APM Manso, Chapada dos Guimarães	MT	40	L	KF801217	KF801333
	IBSP71785	IB	Analândia	SP	73	L	KF801202	KF801320
	ITS357	IB	Itirapina	SP	77	L	KF801213	KF801329
	BN0501	IB	Itirapina	SP	77	S	KF801212	KF801328
	IBSP72943	IB	Motuca	SP	79	L	KF801218	KF801334
	IBSP70827	IB	São Manuel	SP	85	L	KF801225	KF801339
	MZUSP15681	IBUSP	UHE-Peixe Angical	TO	93	L	KF801220	-
	MZUSP15546	IBUSP	UHE-Peixe Angical	TO	93	L	KF801219	KF801342
B. neuwiedi/pauloensis	BN0605	IB	Pardinho	SP	80	S	KF801106	KF801243
	BN0310	IB	Patrocínio Paulista	SP	81	S	KF801107	KF801244
B. pubescens	NOPA2934G	FZB	Bagé	RS	50	S	KF801226	KF801343
	NOPA3860	FZB	Barão do Triunfo	RS	51	S	KF801227	KF801344
	MCN16832	FZB	Barra do Ribeiro	RS	52	S	KF801228	KF801345
	NOPA3314B	FZB	Cachoeira do Sul	RS	53	S	KF801229	KF801347
	MCN16986	FZB	Camaquã	RS	54	S	KF801230	KF801348
	NOPA3681	FZB	Canguçu	RS	55	S	KF801231	KF801349
	NOPA3253	FZB	Chuvisca	RS	57	S	KF801232	KF801350
	MCN16853	FZB	Eldorado do Sul	RS	59	S	KF801233	KF801351
	NOPA3842	FZB	Gravataí	RS	60	S	KF801234	KF801352
	NOPA2762	FZB	Pelotas	RS	63	S	KF801235	KF801353
	NOPA3218	FZB	Porto Alegre	RS	64	S	KF801236	KF801354
	MCN17137	FZB	São Leopoldo	RS	66	S	KF801238	-
	NOPA3259	FZB	Sentinela do Sul	RS	67	S	KF801237	KF801355
	NOPA3295B	FZB	Viamão	RS	71	S	KF801239	KF801356

Total: 140 samples

IB – Instituto Butantan, MZUSP - Museu de Zoologia da Universidade de São Paulo, CHUNB - Coleção Herpetológica – Universidade de Brasília, LAPT-UFPE -Laboratório de Animais Peçonhentos e Toxinas - Universidade Federal de Pernambuco, UPF - Universidade de Passo Fundo, NOPA-FZB - Núcleo Ofiológico de Porto Alegre - Fundação Parque Zoobotânico, UFBA - Universidade Federal da Bahia, CEPB-UCG - Centro de Estudos e Pesquisas Biológicas – Universidade Católica de Goiás, CBMG-PUCRS - Centro de Biologia Molecular e Genômica – Pontifícia Universidade Católica do Rio Grande do Sul, NORMAT-UFMT - Núcleo de Ofiologia Regional de Mato Grosso – Universidade Federal de Mato Grosso, UFV - Universidade Federal de Viçosa, UFOP - Universidade Federal de Ouro Preto, UNIFAL – Universidade Federal de Alfenas, IBUSP – Instituto de Biociências da Universidade de São Paulo.

Drummond, 2007) to estimate the point of convergence. Effective sample sizes were >200 for all parameters and the time estimates were similar among runs.

2.5. Calibration points

The choice of calibration points is a critical part of any molecular dating analysis, because of the use of fossil evidence and constraints based on geological scenarios require careful evaluation (Wüster et al., 2008). Fossil evidence can provide an approximate minimum age for the existence of a clade, and geological events can provide maximum age constraints (Weir and Schluter, 2008). Earlier analyses are subjected to methodological advances, such as the most accurate methods of dating. As an example Gibbard et al. (2010) dated the Pleistocene upper bound to 2.58 Ma, which affects the interpretation of the molecular dating results. Additionally, analyses were subjected to methodological nuances, such as the incorporation of minimum- and maximum-age bounds, which can lead to very different prior times and be responsible for large differences in posterior time estimates (Inoue et al., 2010).

Four calibration points were used as minimum constraints: two fossils and two biogeographic points.

2.5.1. Sistrurus/Crotalus

Parmley and Holman (2007) cite a fossil of *Sistrurus* reported by them as being 9 Ma, but a revised date of the associated faunal stage (Clarendonian NALMA) locates it at a minimum of 10.3 Ma (Paleobiology Database on 06 May, 2013) and we used this latter information. For the maximum age, we used a conservative soft upper bound of 25 Ma, based on the oldest fossils known for Viperinae and Crotalinae (Ivanov, 1999) and for the Viperidae as a whole (Szyndlar and Rage, 2002). To include such a range (25–10.3 Ma) in BEAST, we used a lognormal prior with an offset of 10.3 Ma (hard lower bound), with mean = 1.865 and SD = 0.5 (both in log scale), which altogether places 25 Ma as a 95% soft upper bound.

2.5.2. Agkistrodon piscivorus/A. contortrix

Holman (2000) identifies the origin of *Agkistrodon piscivorus* at the Hemiphillian NALMA, having a minimum age of occurrence at 4.9 Ma (Paleobiology Database on 06 May, 2013). To include such range (25–4.9 Ma), a lognormal prior was used with offset = 4.9 Ma (hard lower bound), with mean = 1.36 and SD = 1.0 (both in log scale), which altogether places 25 Ma as a 95% soft upper bound.

2.5.3. Porthidium dunni/P. arcosae

Porthidium dunni is distributed in Mexico, while *P. arcosae* is endemic to Ecuador. We assume here that the uplift of the Isthmus of Panama can be regarded as a biogeographic maximum for this split (Wüster et al., 2002, 2008). Until recently, the isthmus was considered to have originated around 3.95–3 Ma (Coates and Obando, 1996; Ibaraki, 2002), but recent evidence indicates its closure seems to have initiated earlier, during the late Oligocene and early Miocene, 25–23 Ma (Farris et al., 2011; Montes et al., 2012), therefore permitting dispersion since then. An exponential prior was used with mean = 8.35, providing a 95% confidence interval of 25–0 Ma (zero being a conservative minimum estimate, and 25 Ma a soft upper bound).

2.5.4. Lachesis muta/L. stenophrys

We used sequences of *Lachesis muta* from Peru and *L. stenophrys* from Costa Rica, which represent separated evolutionary lineages according to Zamudio and Greene (1997) and Parkinson et al. (2002), and used the same rationale as above (formation of Isthmus of Panama as a soft upper bound of 25 Ma, and lower bound of zero; parameter settings as in "*Porthidium dunni*/*P. arcosae*").

3. Results

This study reports the first phylogeny that includes all 8 species of the *Bothrops neuwiedi* group. The analyses were conducted using partial sequences of mitochondrial genes *cyt b* and *ND4*, with a total of 1122 bases from a remarkable sample of 140 specimens.

3.1. Monophyly and relationships within B. neuwiedi group

Monophyly of the *B. neuwiedi* group was highly supported in MP, ML and BI, and the *B. jararaca* group was recovered as its sister group (Fig. 2).

The consensus of MP, ML and BI recovered the same tree topology for the *B. neuwiedi* group (Fig. 2). Nine lineages (clades A to I) with high supports and genetic distances were recovered; however, monophyly was recovered only for *B. pubescens;* the other species, *B. diporus*, *B. erythromelas*, *B. lutzi, B. mattogrossensis*, *B. neuwiedi*, *B. marmoratus* and *B. pauloensis,* contained paraphyletic or polyphyletic groupings of mitochondrial haplotypes (Figs. 2 and 3).

Analyses revealed a clear split into four clades geographically highly-structured in Brazilian territory: Northeast I (clade A), strongly supported by all analyses (MP = 99, ML = 100, BA = 100), Northeast II (clade B) strongly supported by maximum likelihood and Bayesian analyses (ML = 99, BA = 100); and moderately supported by parsimony (MP = 85); East–West (clades C, D and E) strongly supported by all analyses (MP = 100, ML = 100, BA = 100), and West-South (clades F, G, H and I) strongly supported by Maximum likelihood and Bayesian analyses (ML = 99, BA = 100) and moderately supported by parsimony (MP = 92) (Figs. 2 and 3). Moreover the East–West clade makes geographic contact with the West-South clade in the state of Goiás, in Central Brazil (Fig. 3).

Three internal relationships were recovered with low support values: *Bothrops erythromelas* (clade A) was recovered as the sister group of the remaining *B. neuwiedi* group, *B. lutzi* (clade B) was recovered as the sister group of the East–West clade (B, C and D) and West-South clade (F, G, H and I). Two other phylogenetic relationships nested in West-South clade were recovered with low supports: the haplotype PT3404 from Castro Barros, Argentina, was recovered as sister group of the clade G and the haplotype (TM173) from Serra da Borda, Mato Grosso State, was recovered as the sister group of the clades H and I.

3.2. Genetic distance

The uncorrected genetic distances within and among clades of the *B. neuwiedi* group are presented in Table 3; genetic distances between subclades and some specific haplotypes are reported below (Item 3.4). Distances within clades were lower than distances among clades in almost all of the cases, except for clades H and I, whose internal distances overlapped distances between clades. Another exception observed was related to elevated distances within clades A and F.

3.3. Monophyly of B. pubescens

The only monophyletic species of the group is *B. pubescens* (clade I, Fig. 2), which was strongly supported by Bayesian analysis (BA = 95), moderately supported by maximum likelihood (ML = 73) and weakly supported by parsimony (MP = 20). The sample showed North–South genetic structure – the only exception was

the specimen from Bagé, state of Rio Grande do Sul (NOPA2934-G), which was recovered in the Southern clade.

3.4. Polyphyletic taxa

Seven species of the *B. neuwiedi* group were recovered as paraphyletic and polyphyletic (Fig. 2).

B. erythromelas (clade A) was strongly supported (MP = 99, ML = 100, BA = 100) except for the exclusion of one haplotype (UFBA4485 – Ibiraba, State of Bahia). Haplotypes were recovered in two subclades, with divergence ranging from 2.0% to 3.5%. The haplotype of *B. erythromelas* from Ibiraba with genetic distance from 4.1% to 6.2% of the remaining haplotypes was recovered in *B. lutzi* clade.

Clade B (*B. lutzi* and *B. erythromelas*) was strongly supported by likelihood and Bayesian analyses (ML = 99, BA = 100) and moderately supported by parsimony (MP = 85). Haplotypes from the same locality were recovered in two subclades. One specimen of *B. lutzi* (IBSP7320927 - Piracicaba) had a geographically disjunct distribution, occurring in the Brazilian southeast, although it was recovered in the Northeast II clade.

B. neuwiedi was recovered in three clades (C, D and E), comprising the more inclusive East–West clade. These specimens are collected from throughout the mountain ranges on the east coast of Brazil. Clade C is strongly supported (MP = 98, ML = 97, BA = 100)

Fig. 2. Strict consensus topology obtained in MP, ML and BI based on mitochondrial genes *cyt b* and *ND4*. Numbers in the nodes indicate MP Jackknife support, ML aLRT SH-like support and BI posterior probability. Species of *B. neuwiedi* group are classified morphologically according to Silva (2004) and Silva and Rodrigues (2008).

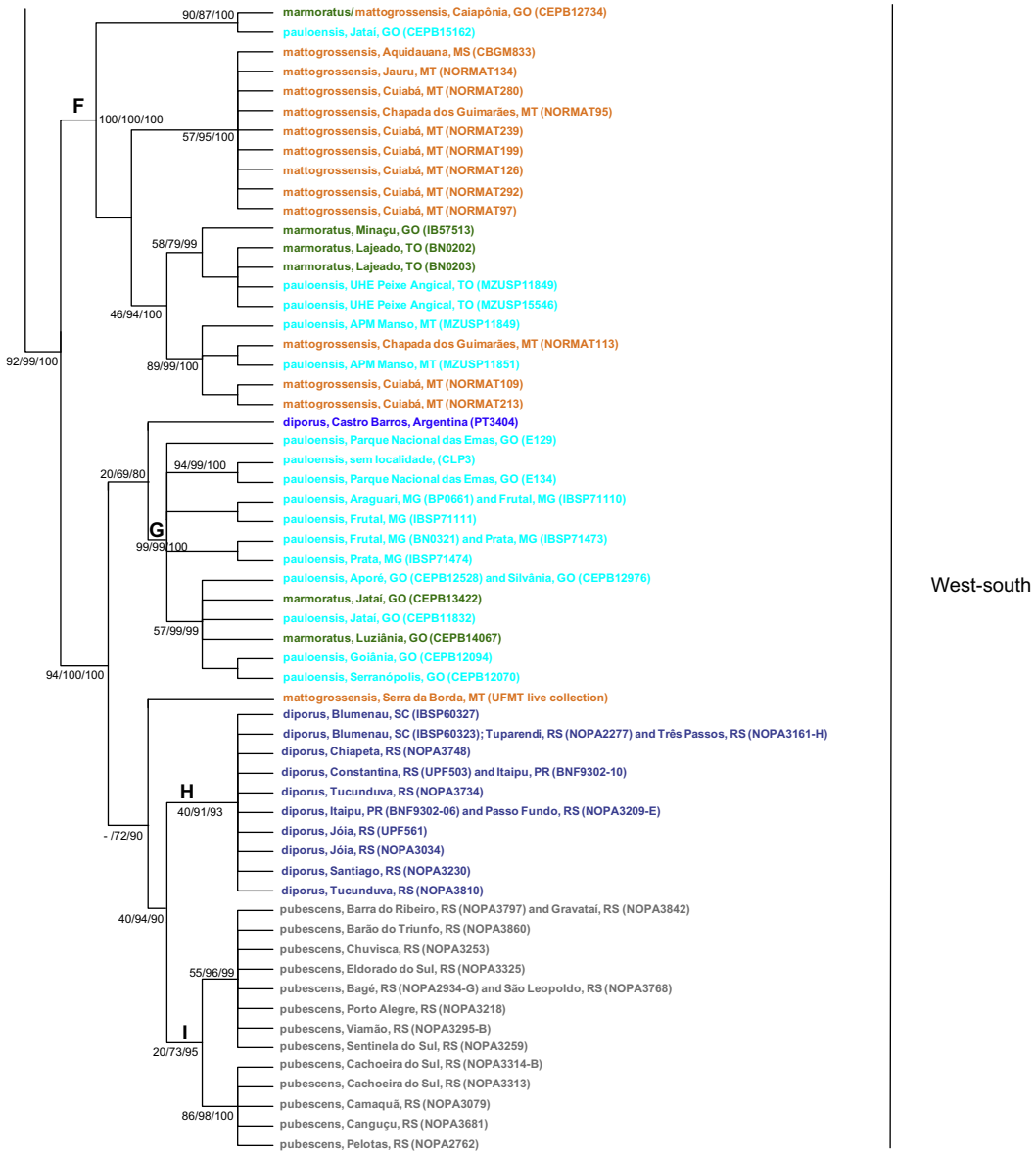

Fig. 2 (*continued*)

and it was composed exclusively of *B. neuwiedi* haplotypes. Haplotypes from Ouro Branco, Minas Gerais State, were recovered in Clade C and D, and they have 1.6% genetic distance; this is the only occurrence of *B. neuwiedi* in clade D. Clade E was supported by all analyses (MP = 80, ML = 93, BA = 100) and it was mostly composed of haplotypes sampled from populations morphologically recognized as *B. neuwiedi.* Haplotypes from São Gonçalo do Rio Preto, Minas Gerais State, were recovered in Clade C and E, and they have 3.8% genetic distance.

B. pauloensis haplotypes were recovered in four clades (D, E, F and G). Clade G was strongly supported (MP = 99, ML = 99, BA = 100) and composed of almost all haplotypes of *B. pauloensis* and two haplotypes of *B. marmoratus.* Haplotypes from Jataí (CEPB11832 and CEPB15162) occur in clades G and F, respectively; they had 6.1% genetic distance. Similarly, *B. pauloensis* from Goiânia (CEPB12094 and CEPB13598) occurs in clades G and D, and these haplotypes had 6.7% of distance. In the other clades, *B. pauloensis* specimens are a minority, occurring with *B. neuwiedi*, *B. marmoratus* and *B. mattogrossensis*, respectively in clades E, D and F.

B. marmoratus was recovered in three clades (D, F and G). Clade D was strongly supported by likelihood and Bayesian analysis (ML = 100, BA = 100), and moderately supported by parsimony (MP = 86) and mostly composed of haplotypes sampled from populations of *B. marmoratus.* Phylogenetically associated with these haplotypes there is one haplotype from *B. neuwiedi* - UFOP6645. Additionally, in clade D, one haplotype of an intergrade between *B. marmoratus* and *B. pauloensis* was recovered from Hidrolândia (CEPB13417) and another from Aporé (CEPB13237); and a single haplotype shared by three specimens: *B. marmoratus,* CEPB6816 from Orizona; *B. pauloensis*, CEPB13598 from Goiânia, and *B. marmoratus*/*pauloensis,* CEPB13273 from Aporé, all localities from Goiás State.

The Brazilian sample of *B. diporus* was recovered as a monophyletic group (clade H) in all analyses (MP = 40, ML = 91, BA = 93), without genetic substructure. The haplotype PT3404 from Castro Barros, Argentina, was not recovered in this clade or other major clades, and it shows genetic distance between 2.3% to 4.1% from clade H.

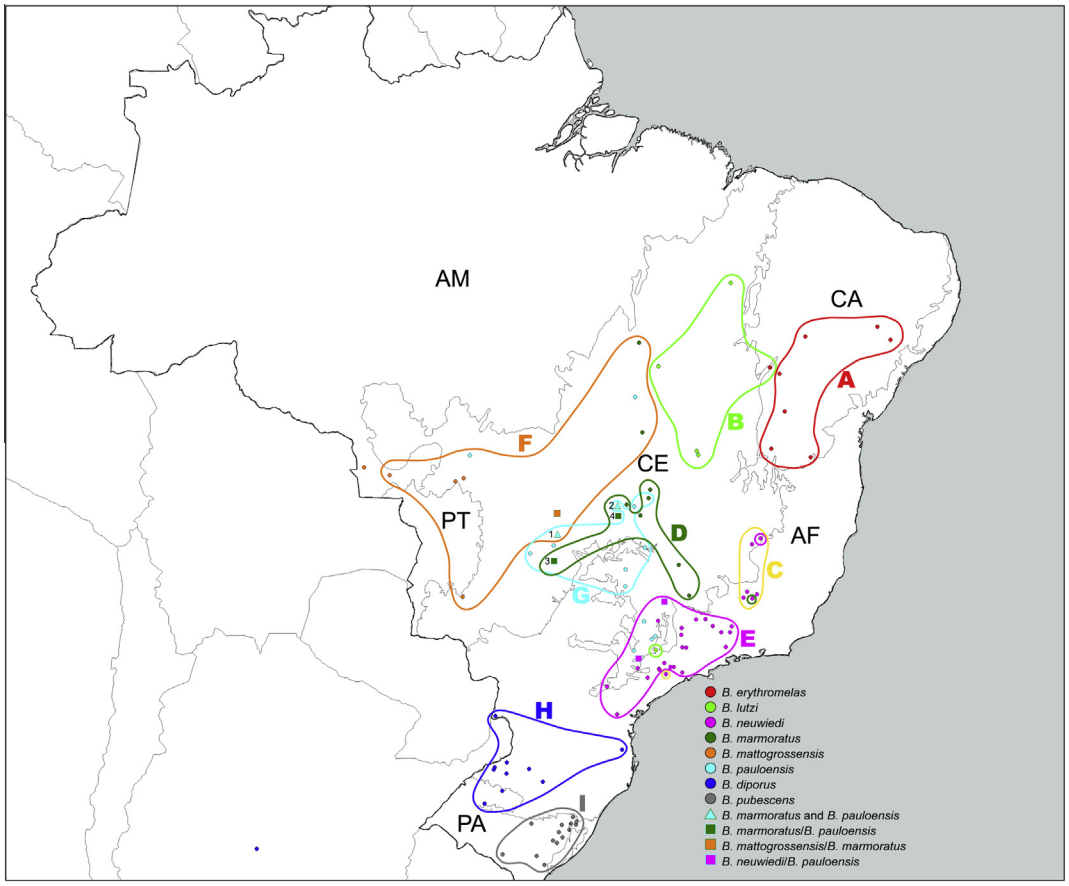

Fig. 3. Geographic distribution of clades obtained from molecular analyses (A to I). Brazilian territory division by biomes: AM = Amazon Forest, CE = Cerrado, PT = Pantanal, CA = Caatinga, AF = Atlantic Forest and PA = Pampas. Occurrence of sympatry in four localities: 1 (Jataí, GO) between *B. marmoratus* and *B. pauloensis*; 2 (Goiânia, GO) between *B. marmoratus* and *B. pauloensis*; 3 (Aporé, GO) between *B. marmoratus*/*pauloensis and B. pauloensis*; 4 (Hidrolândia, GO) between *B. marmoratus*/*pauloensis* and *B. marmoratus*.

Table 3
Uncorrected genetic distances based on cytochrome *b*, minimum and maximum values among *Bothrops neuwiedi* clades and other species. Genetic distances within *Bothrops neuwiedi* clades are in bold.

	Clade A	Clade B	Clade C	Clade D	Clade G	Clade E	Clade F	Clade H	Clade I
Bothrocophias hyoprora	0.136–0.153	0.126–0.131	0.127–0.135	0.131–0.135	0.130–0.142	0.119–0.139	0.123–0.140	0.122–0.135	0.126–0.127
Bothrops alternatus	0.115–0.131	0.098–0.106	0.116–0.121	0.108–0.112	0.110–0.122	0.104–0.116	0.099–0.122	0.102–0.114	0.106–0.110
Bothrops atrox	0.134–0.138	0.114–0.134	0.157–0.161	0.143–0.147	0.127–0.137	0.143–0.157	0.124–0.144	0.111–0.130	0.117–0.127
Bothrops bilineatus	0.104–0.112	0.081–0.103	0.110–0.114	0.094–0.094	0.100–0.112	0.076–0.096	0.100–0.124	0.092–0.104	0.096–0.103
Bothrops jararaca	0.089–0.097	0.081–0.089	0.111–0.115	0.099–0.103	0.089–0.109	0.080–0.099	0.090–0.117	0.074–0.085	0.078–0.085
Bothrops insularis	0.089–0.096	0.072–0.080	0.094–0.098	0.090–0.094	0.080–0.092	0.072–0.090	0.081–0.100	0.065–0.077	0.069–0.077
Clade A	**0–0.035**	0.049–0.067	0.085–0.095	0.079–0.092	0.067–0.077	0.067–0.086	0.067–0.083	0.063–0.085	0.070–0.079
Clade B		**0–0.024**	0.061–0.076	0.058–0.070	0.044–0.061	0.049–0.078	0.052–0.073	0.044–0.064	0.049–0.061
Clade C			**0–0.01**	0.016–0.018	0.067–0.094	0.038–0.053	0.071–0.101	0.068–0.091	0.073–0.079
Clade D				**0–0.005**	0.061–0.073	0.032–0.049	0.068–0.091	0.064–0.088	0.067–0.076
Clade G					**0–0.013**	0.055–0.072	0.050–0.069	0.021–0.043	0.021–0.032
Clade E						**0–0.018**	0.072–0.107	0.047–0.085	0.055–0.072
Clade F							**0–0.024**	0.049–0.073	0.052–0.079
Clade H								**0–0.024**	0.009–0.027
Clade I									**0–0.010**

B. mattogrossensis was recovered in Clade F and strongly supported by all analyses (MP = 100, ML = 100, BA = 100). Nested in this clade there are three haplotypes of *B. marmoratus*, five of *B. pauloensis* and one intergrade of *B. mattogrossensis*/*marmoratus*. Only the *B. mattogrossensis* haplotype (TM173) from Serra da Borda, Mato Grosso State, did not cluster in clade F or in the other major lineages, and it has genetic distances between 5.9% to 7.0% from other *B. mattogrossensis* haplotypes.

3.5. Divergence times

Our estimates of divergence place the most recent common ancestor (MRCA) of the New World pit vipers in the early Miocene 20.91 Ma (95% Credibility Interval – $CI_{95\%}$ – 23.66–18.26 Ma) and MRCA of the bothropoids in the middle Miocene 14.07 Ma ($CI_{95\%}$ 16.37–11.75 Ma) (Appendix S2).

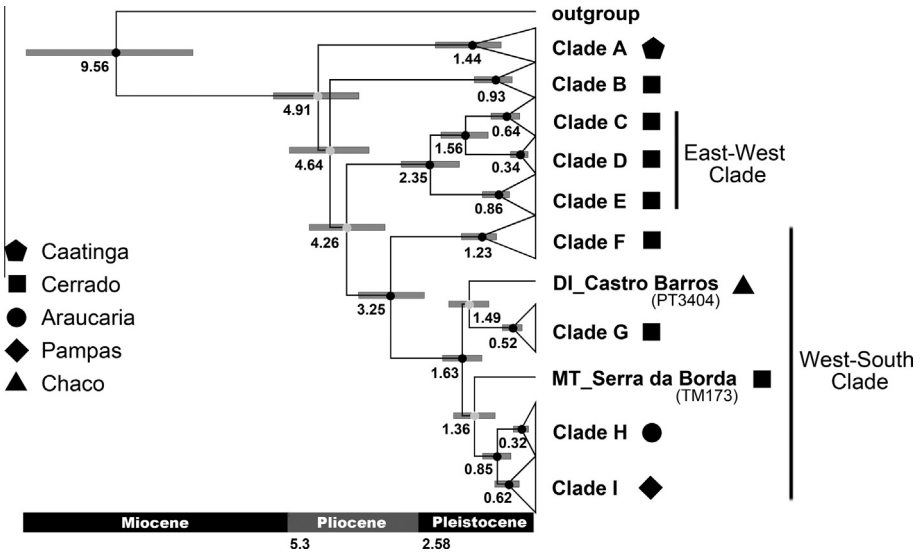

Fig. 4. Bayesian estimates of divergence times (Ma) for *B. neuwiedi* group. Numbers on nodes indicate mean and bars represent 95% credibility intervals for divergence estimates. Geometry symbols represent biomes in which clades occur. Circles represent node support, MP Jackknife support, ML aLRT SH-like support and BI posterior probability. Black circle for JK/aLRT SH-like > 70 PP > 95; and grey circle for JK/aLRT SH-like < 70 PP < 95. Period boundaries according to International Chronostratigraphic Chart v 2013/01 available in www.stratigraphy.org.

The divergence between *B. jararaca* and *B. neuwiedi* groups occurred in the late Miocene 9.56 Ma ($CI_{95\%}$ 11.51–7.76 Ma) (Fig. 4). Three main splits occurred almost simultaneously during the Pliocene: clade A – 4.91 Ma ($CI_{95\%}$ 5.98–3.97 Ma), clade B – 4.64 Ma ($CI_{95\%}$ 5.63–3.74 Ma), and clade East–West/West-South – 4.26 Ma ($CI_{95\%}$ 5.22–3.45 Ma). The West-South clade diversified earlier in the Pliocene – 3.25 Ma ($CI_{95\%}$ 4.03–2.53 Ma) than the East–West clade in the Pleistocene – 2.35 Ma ($CI_{95\%}$ 3.06–1.71 Ma). The origin and diversification of the clades C, D, E, F G, H, I, PT3404 (Castro Barros) and TM173 (Serra da Borda) occurred during the Pleistocene (Appendix S2).

4. Discussion

Phylogenetic relationships and biogeographic patterns obtained in this study underline the spatial and temporal complexity of biological diversification in the South American open biomes. Because of its widespread distribution throughout this region, the *B. neuwiedi* group seems to be an excellent model for understanding historical patterns of diversification in the Neotropics. Our results challenge current taxonomic arrangement and the traditional use of morphology to delimit evolutionary units in the referred group. They also highlight the importance of Neogene events for the origin of the *B. neuwiedi* group and the Quaternary climate fluctuations for the species origin and diversification of populations of the Neotropical fauna.

4.1. Phylogenetic relationships

Similar topologies were recovered by all methods, with nodal supports varying in different levels. The *B. jararaca* group was recovered as the sister group of the *B. neuwiedi* group corroborating and reiterating previous studies (Wüster et al., 2002; Fenwick et al., 2009; Carrasco et al., 2012).

Our molecular dating estimates the divergence between *B. jararaca* and *B. neuwiedi* groups in the late Miocene 9.56 Ma (11.51–7.76 Ma) and refines previous estimation for the *Bothrops neuwiedi* group (12–7 Ma) (Wüster et al., 2008; Fenwick et al., 2011).

MRCA of the New World pit vipers estimated between 23.66 and 18.26 Ma corroborates New World's Crotalinae colonization in the early Miocene, 30–16 Ma (Wüster et al., 2002), 26.9–17.9 Ma (Wüster et al., 2008), and 18.55–14.33 Ma (Castoe et al., 2009).

B. neuwiedi is a highly supported monophyletic group. Nine clades (A to I) were strongly supported. *Bothrops erythromelas* (clade A) was the sister group of the remaining *B. neuwiedi* group, *B. lutzi* (clade B) was the sister group of the East–West (B, C and D) and West-South clades (F, G, H, I, PT3404 and TM173) (Fig. 4). These three cladogenetic events occurred almost simultaneously in the Pliocene, and rapid radiation could explain low supports for these nodes in the phylogenetic analysis. This hypothesis could also be relevant to the less inclusive clades C, D, G, H and I (Fig. 4), although they are strongly supported.

4.1.1. B. erythromelas vs. B. lutzi

B. erythromelas samples used in this study were collected in localities along the São Francisco River. The internal structure of the clade does not correspond to the barrier formed by the riverbanks, as observed for other herpetofaunal genera such as *Calyptommatus* and *Nothobachia* (Siedschlag et al., 2010), and *Eurolophosaurus* (Passoni et al., 2008). Instead of differences between organisms from the left or right side of the river, a differentiation pattern was observed upstream and downstream at the central region of the middle São Francisco River. Similarly, the same pattern upstream and downstream for anurans and rodents was observed along the Juruá River in the Amazon basin (Lougheed et al., 1999; Patton et al., 2000). A stable biogeographic barrier in this region of Juruá River was suggested since the break was found for several taxa of rodents (Patton et al., 2000).

Additionally, one break was verified in the region of the middle São Francisco River between the north–northeast phytogeographic province of the Cerrado and Caatinga (Ratter et al., 1997), which coincides with the subclades within *B. erythromelas*. A temporal agreement is observed, despite the discordances among diversification patterns of Caatinga herpetofauna, and it is probable that the same vicariant events that occurred during the Pleistocene (2.28–0.78 Ma) were responsible for the species diversification. The shared haplotype between *B. lutzi* and *B. erythromelas* is suggested here to be due to introgressive hybridization, and the relictual presence of *B. lutzi* in southeastern Brazil could be hypothesized due to expansion/retraction dynamic of the Caatinga – seasonally dry tropical forest (SDTF) nucleus – and Cerrado bio-

mes during the Pleistocene (Pennington et al., 2000; Prado and Gibbs, 1993; Werneck, 2011). To corroborate such a hypothesis, there are new records for *B. lutzi* associated with transition areas between Caatinga and Cerrado, expanding its previous geographic distribution (Loebmann, 2009), and the molecular dating estimated that the process occurred between 0.63 and 0.11 Ma.

4.1.2. East–West clade

The origin of this lineage occurred in the Plio-Pleistocene, with divergence approximately 2.35 Ma (3.06–1.71) after the final uplift of the Central Brazilian Plateau took place (7–5 Ma) according to Werneck (2011). Such an event probably affected the Espinhaço Range, Serra do Mar and Mantiqueira between the late Pliocene and early Pleistocene (4–2 Ma) (Colli, 2005; Porzecanski and Cracraft, 2005) affecting the diversification of this lineage in the eastern mountains of Brazil. Clade E is the older one (1.2–0.58 Ma), composed mainly of *B. neuwiedi*, occurring in the "campos de altitude" from Serra da Mantiqueira, with recent east–west dispersion (0.47–0.19 Ma) in the southern boundaries of the Cerrado, where specimens of *B. pauloensis* are found. Clades C and D are sister groups with 1.56 Ma (2.12–1.06) MRCA. Clade C diverged earlier 0.64 Ma (0.99–0.34 Ma) and it has a restricted distribution in the meridional region of Espinhaço Range. This exclusive *B. neuwiedi* lineage is morphologically indistinguishable from Clade E. Evidence suggested a species candidate and such results corroborate the Espinhaço region as an endemic area, as suggested for squamate reptiles and anuran fauna (Nogueira et al., 2011; Valdujo, 2011). Clade D has the most recent diversification 0.34 Ma (0.58–0.16 Ma) and occurs in the core of Cerrado with recent sympatry with the clade G (West-South clade). Shared haplotypes among the three clades of East–West clade are better explained by incomplete lineage sorting (Avise, 2000). The basal phylogenetic position of the haplotypes and the lack of geographic proximity of inter-specifically shared haplotypes are characteristics of the retained ancestral polymorphism (Funk and Omland, 2003). *Hypsiboas albopunctatus,* for instance, from Central Cerrado and Southeast clades (Prado et al., 2012) shares the same distribution and time of origin and diversification of the East–West clade; however, these frogs show less structuration than the observed for *B. neuwiedi* East–West clade.

4.1.3. West-South clade

The origin of this lineage was in the Pliocene (4.26 Ma), approximately when the final uplift of the Central Brazilian Plateau took place (Werneck, 2011). Another event influencing this lineage during this period probably was the marine regression of the Paranense Sea, which exposed a continental area in Southern Brazil, northeastern Argentina and western Uruguay (Le Roux, 2012). Clade F, the older clade (1.23 Ma), is mostly composed of *B. mattogrossensis,* and occurs in the Cerrado core, with a wide distribution in the Central Brazilian Plateau along a vast latitudinal range. Each of the four subclades of Clade F had a disjunct geographic origin between 0.49 and 0.29 Ma and different times of diversification during Pleistocene, with their geographic occurrence overlapping only recently and probably this is why the intergrade *B. marmoratus*/*B. mattogrossensis* (CEPB12734) was detected. The uplift of the Brazilian Plateau along the Espinhaço Range, Serra do Mar and Mantiqueira (4–2 Ma), and the subsidence of the Chaco and Pantanal due to the Andean uplift (Colli, 2005; Porzecanski and Cracraft, 2005) are among the possible vicariant events accounting for the Cerrado and Chaco grouping.

Clade G, composed of *B. pauloensis,* occurs in the Cerrado core, is the sister group of the Chaco specimen, from Argentina, and diverged approximately 1.49 Ma. The geographic occurrence of Clade G overlapped with Clade F and D, only recently as well (0.17 Ma). And the most recent clade diverged approximately 1.36 Ma, represented by the specimen TM173 from Serra da Borda, Mato Grosso State, as the sister group of the *B. pubescens* (Clade I) from Pampas and *B. diporus* (clade H) from the Araucaria Forest, which diverged recently (0.85 Ma), justifying the low divergence between them. The West-South clade presents a complex biogeographic pattern; a better sampling of the neighboring regions of Brazil will provide a more complete perspective of this lineage.

4.2. Cryptic diversity

Phylogenetic position, genetic divergence, geographic location and time of divergence suggest a review, with a clear possibility of characterizing new species for at least three candidate species: *B. neuwiedi* (clade C) from the meridional region of Espinhaço Range, *B. diporus* (PT3404) from Castro Barros, Argentina, and *B. mattogrossensis* (TM173) from Serra da Borda, Mato Grosso State. Clade F, mainly composed of *B. mattogrossensis,* requires further investigation.

As stated by Werneck (2011), Nogueira et al. (2011), and Prado et al. (2012), an early misleading notion was that Caatinga, Cerrado, and Chaco had low diversity levels, depauperate fauna, and lack unique species (endemic). Recent studies been changed this view, since knowledge of the South American open biomes have shown that levels of endemism are higher than previously thought (Nogueira et al., 2011) and the presence of cryptic lineages underestimated diversity of herpetological fauna in these areas (Werneck et al., 2012; Prado et al., 2012).

4.3. Hybrid zone in Central Brazil

Sympatric occurrence (Jataí, Goiânia, Aporé and Hidrolândia), intergrades (Aporé and Hidrolândia) and shared mtDNA haplotypes (Goiânia, Aporé and Orizona), as well as the two haplotypes of *B. marmoratus* recovered in clade G (composed of *B. pauloensis*), suggest recent hybridization (0.65–0.02 Ma) between *B. marmoratus* and *B. pauloensis* in a narrow zone of secondary contact in the state of Goiás (Central Brazil).

The sympatric sharing of geographically localized mtDNA sequence haplotypes among genetically and morphologically divergent species is considered the clearest signature of introgression (Funk and Omland, 2003). According to Weisrock et al. (2005) these patterns would not be expected under a process of stochastic sorting of ancestral polymorphism; consequently, introgressive hybridization would be a more likely explanation, as observed for salamanders from the genus *Plethodon.* The hypothesis of introgression by secondary contact between clades D and G has in its favor the occurrence of the Cerrado vegetation transition zone in this region (Bridgewater et al., 2004; Ratter et al., 2003). Furthermore, no differences were found in the hemipenis of the *B. neuwiedi* group (Silva and Rodrigues, 2008); and additionally the occurrence of interspecific crosses of *Bothrops* in captivity (Balestrin et al., 2002) support the assumption that crossings can also occur in nature especially if the animals are sympatric.

4.4. Retention of ancestral morphology or morphological parallelism

The most parsimonious explanation for the widespread occurrence of *B. pauloensis* haplotypes is the retention of ancestral morphology. However morphological parallelism could also explain such polyphyly, as observed for the *Leptodeira anullata* and *L. septentrionalis* (Daza et al., 2009), precluding previous taxonomic efforts to accurately identify evolutionary units. All the evidence previously reported for *B. pauloensis* in the literature has favored a single taxonomic entity. The *Bothrops pauloensis* diagnosis is very distinct from other species of the *B. neuwiedi* group except *B. marmoratus*, whose characters overlap (Silva, 2004; Silva and Rodrigues, 2008). Valdujo et al. (2002) detect no geographic variation

in the ecological aspects of *B. pauloensis* from Itirapina (SP), Emas National Park (GO) and the IBGE Ecological Reserve – RECOR (DF), contrasting directly with results presented here.

The name *B. pauloensis* is related to the type locality, Leme, São Paulo State, where specimens were recovered in clade E together with *B. neuwiedi*. Although both species are clearly morphologically distinct, low genetic divergence was observed.

4.5. Polymorphism

Morphological divergence within *B. mattogrossensis* might be explained by phenotypic plasticity. Polymorphisms play an important role in adaptive species evolution, allowing populations to explore distinct sources and occupy new habitats (Losos et al., 2006). Such polymorphisms can be randomly fixed during development, genetically determined or set by environment (Leimar, 2005). They occur as distinctive variants coexisting within a population, as observed in the snake complex *Elaphe obsoleta* and *Elaphe guttata,* traditionally diagnosed by divergent color and blotch patterns, with seven and five subspecies, respectively (Burbrink et al., 2000; Burbrink, 2001, 2002). Based on molecular data, geographic isolation and genetic distance, the authors suggested only three full species within each complex (Burbrink et al., 2000; Burbrink, 2001, 2002).

4.6. Biogeographic pattern

Bothrops neuwiedi group distribution pattern seems to have a southward diversification along the Chacoan subregion of Neotropical region (Morrone, 2000). Such subregion grouped open biomes of South America: Caatinga, Cerrado, Chaco and Pampas. Historically, the relationships among Cerrado, Caatinga and the Chaco have been proposed, due to the intermixture of some species ranges that are widely distributed in these biomes (Cabrera and Willink, 1973). However, only recently, the relationships among them have been investigated by phylogenetic and phylogeographic approaches, searching for spatial and temporal patterns of biological diversification in the South American open biomes.

The basal split of the *Bothrops neuwiedi* group in late Miocene and species diversification during the Plio-Pleistocene is also observed in other examples of Neotropical fauna of open biomes: the rodent *Calomys* (Almeida et al., 2007), lizards *Kentropix paulensis* group (Werneck et al., 2009) and *Phyllopezus pollicaris* complex (Werneck et al., 2012), and anuran *Rhinella marina* group (Maciel et al., 2010). These groups highlight the importance of Neogene events, characterized by significant tectonic and paleogeographic reorganizations, such as the uplift of the Andean Range and the Central Brazilian plateau, and deep diversification of Neotropical fauna as well, which influenced both open and forested biomes (Hoorn et al., 2010; Rull, 2011; Werneck, 2011).

The *Bothrops neuwiedi* group diverged firstly in the Caatinga with *Bothrops erythromelas*, thus in the Cerrado with *B. lutzi*. The other six clades diverged in the Cerrado, and clades underwent diversification at least once in the Chaco, Pampas and Araucaria Forest. Such a pattern agrees with the statement that the Cerrado core seems to be the center stage of speciation dynamics with adjacent ecosystems (Almeida et al., 2007; Werneck et al., 2009, 2012).

Additionally, Pleistocene environmental shifts played an important role in the origin of species and in the genetic diversification of populations of the *B. neuwiedi* group, as well in the species such as *Cerdocyon thous* (Tchaicka et al., 2006), *Calomys* (Almeida et al., 2007), *Hymenea stignocarpa* (Ramos et al., 2007), *Drosophila gouveai* (Moraes et al. 2009), *Kentropix* (Werneck et al., 2009), *Rhinella marina* (Maciel et al., 2010), *Phyllopezus pollicaris* (Werneck et al., 2012), *Hypsiboas albopunctatus* (Prado et al., 2012). Despite the temporal agreement for the deep and shallow divergences among the different examples for the South American open biomes, heretofore, a spatial concordance among the cases has not been found. For *Caenonomada,* endemic bees to the same open biomes considered here, Zanella (2002) used morphological phylogeny to derive an area cladogram suggesting that the Cerrado is more closely related to Chaco, with the Caatinga occupying an ancestral position. The same relationship was proposed for herpetofauna (Colli, 2005; Werneck et al., 2012) and birds (Porzecanski and Cracraft, 2005), although they used different methodological approaches.

5. Conclusions

The current taxonomy of *B. neuwiedi* group is based entirely on morphology. Silva (2004) and Silva and Rodrigues (2008) defined alpha taxonomy mainly based on color and blotch patterns. Consequently, species status should be revisited in light of phylogenetic studies in order to reflect the evolutionary history of the *B. neuwiedi* group. A new taxonomic arrangement is required to accommodate current nominal species in the phylogenetic context presented herein, and also more diagnosable characters are required for the *B. neuwiedi* group, except for *B. erythromelas*, *B. lutzi* and *B. pubescens.*

On the whole, this study revealed the presence of at least three candidate species: *B. neuwiedi* from clade C, *B. mattogrossensis* (TM173) from Serra da Borda (MT) and *B. diporus* (PT3404) from Castro Barros, Argentina.

Evidence for introgressive hybridization between some species mainly at the borders of their geographic distributions was found for *B. lutzi* and *B. erythromelas,* and *B. marmoratus* and *B. pauloensis*.

Historical biogeography supported the relationships among South American open biomes, and delimited a temporal pattern of diversification. While events of Neogene are responsible for the origin of the group, Quaternary climate changes act in the origin of species and intraspecific divergence.

Additionally, the *Bothrops neuwiedi* group distribution pattern seems to have a southward diversification along open biomes of South America and the Cerrado as the center stage of speciation dynamics with adjacent ecosystems. Sympatric regions in the Cerrado are associated with younger subclades with shallower branches and less resolution, representing more recent expansion scenarios.

It is important to understand that the evolutionary history of the *B. neuwiedi* group has two additional implications. One involves interests in public health since these are venomous snakes. *Bothrops neuwiedi* Wagler, 1824 (*sensu lato*) was considered the third commonest species of venomous snakes received by the Butantan Institute from 1900 to 1962 (Belluomini, 1971), responsible for snakebite accidents in Brazil (Oliveira et al., 2010).

The other important aspect is related to conservation. The *Bothrops neuwiedi* group represents an excellent indicator for understanding the South American open formation zoogeography, since the rapid environmental degradation of the Cerrado and Caatinga - because of economic exploitation and the existence of rare units of conservation in these biomes - has complicated studies of species diversification and biodiversity conservation (Espírito-Santo et al., 2009; Gamble et al., 2012; Myers et al., 2000; Nogueira et al., 2009; Ratter et al., 1997; Werneck, 2011).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.10.003.

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