Recovering the evolutionary history of Africa's most diverse viper genus: morphological and molecular phylogeny of Bitis *(Reptilia: Squamata: Viperidae)*

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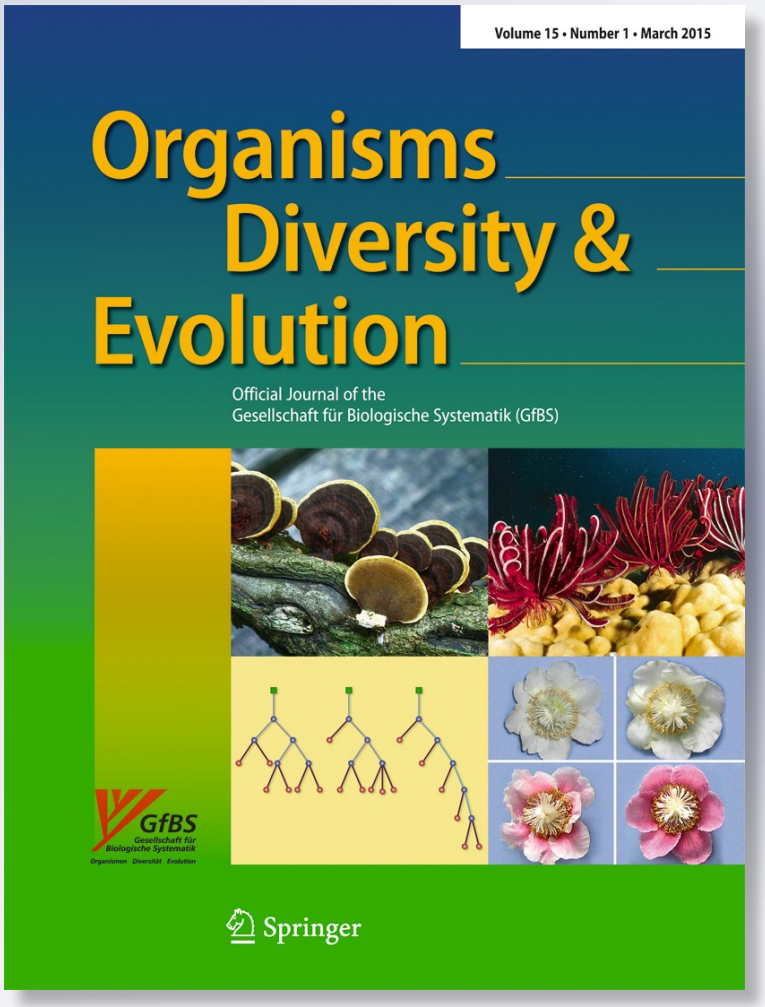

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ORIGINAL ARTICLE

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Recovering the evolutionary history of Africa's most diverse viper genus: morphological and molecular phylogeny of *Bitis* (Reptilia: Squamata: Viperidae)

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Abstract Assessing evolutionary relationships among wide-ranging species can be particularly beneficial to our understanding of speciation patterns and biogeography of taxa, with broad implications for conservation and applications for human health. Integrative phylogenetic analyses that incorporate multiple independent datasets (e.g., DNA, protein, phenotype) can resolve many problematic issues in systematics such as cryptic diversity and incongruence between datasets. Vipers in the genus *Bitis* are widely distributed throughout much of sub-Saharan Africa, filling a variety of ecological niches and presenting an important public health problem. However, evolutionary relationships among this medically and ecologically important genus have not been fully resolved due to inadequate taxon sampling and lack of informative characters. Here, we conduct the first phylogenetic study incorporating complete sampling of known species within the genus *Bitis*. Using morphological, molecular, and combined approaches under multiple criteria, we recovered many of the species groups detected by previous investigators, further validating four currently recognized subgenera. *Bitis arietans* and *Bitis worthingtoni* appear to be early-diverging, monotypic lineages, while the "big *Bitis*" group and the small southern African species form distinct clades. Although our study provides additional information regarding the interspecific relationships within *Bitis*, the placement of *Bitis albanica*, *Bitis heraldica*, and *Bitis inornata* remains problematic. This study enhances our understanding of the evolutionary history of species within the genus *Bitis* incorporating a combined evidence approach to phylogenetics.

Keywords Bayesian · Osteology · Parsimony · Snake · Systematics · Viperinae

Rod D. Wittenberg and Robert C. Jadin are joint first authors.

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Introduction

Resolving evolutionary relationships among organisms provides an essential framework for comparative studies such as those relating to life history, functional anatomy, and behavior (Felsenstein 1985a; Miles and Dunham 1993) and improves our understanding of biogeography, patterns of speciation, conservation need, and biomedical applications. Evolutionary relationships within a specific ingroup are more accurately resolved by sampling all members, and downstream uses of phylogenies benefit from comprehensive sampling; therefore, recent reconstructions have combined multiple sources of evidence and included species represented in some datasets and not others (e.g., Eernisse and Kluge 1993; Littlewood and Smith 1995; Wahlberg et al. 2005; Fenwick et al. 2009; Wiens et al. 2010; Pyron 2011). However, these taxon-dense

phylogenies may include species with very low amounts of data, which can compromise the accurate placement of otherwise well-resolved lineages (Wiens 1998). Ongoing work is needed to find a balance between taxon sampling and missing data.

Recent research aimed at reconstructing phylogenetic relationships of vipers has advanced our understanding of the relationships primarily among pitvipers in the subfamily Crotalinae (Gutberlet and Harvey 2002; Murphy et al. 2002; Wüster et al. 2002; Castoe and Parkinson 2006; Fenwick et al. 2009; Jadin et al. 2011, 2012; Carrasco et al. 2012; but see Fenwick et al. 2012). As noted by Wüster et al. (2008), relationships among true vipers in the subfamily Viperinae have remained problematic and the relationships of many groups are still unresolved. To date, our understanding of the intrageneric relationships within *Bitis*, Africa's most morphologically and ecologically diverse viper genus, has been limited by incomplete taxon sampling. *Bitis* species are considered medically important as they contribute to the estimated 500,000 envenomations that occur on the African continent each year (Chippaux 2006). Unlike endothermic predators, the low energy requirements of vipers may allow them to maintain relatively high population densities even when prey densities are low (Nowak et al. 2008). During such periods of low prey density, vipers may be especially effective at preventing increases in the prey population (Nowak et al. 2008). A phylogeny that includes all species of *Bitis* is needed to provide a comparative framework for studies of these medically and ecologically important vipers.

Seventeen currently recognized species of *Bitis* are widely distributed throughout sub-Saharan Africa including portions of Morocco and the Arabian Peninsula (Spawls and Branch 1995). The genus contains the world's most massive viperid as well as its smallest. *Bitis rhinoceros* may exceed 2.0 m in total length and weigh as much as 8.5 kg, thereby allowing it to prey on large mammals including hares, mongooses, and monkeys (Spawls and Branch 1995). By contrast, *Bitis schneideri* only attains 27.6 cm in total length and feeds primarily on small lizards (Spawls and Branch 1995). Additional morphological diversity includes species with prominent internasal "horns" (*B.* nasicornis, *B. rhinoceros*), supraocular ornamentation (*B. albanica*, *B. armata*, *B. caudalis*, *B. cornuta*, *B. srubida*, *B. worthingtoni*, and some *B. schneideri*), keeled subcaudal scales (*B. caudalis*, *B. peringueyi*, *B. schneideri*, and some *B. arietans* and *B. cornuta*), and dorsally situated eyes (*B. peringueyi*) (Wittenberg 2001). *Bitis* species inhabit tropical forests (*B. gabonica*, *B. nasicornis*, *B. parviocula*, and *B. rhinoceros*), boulder piles and rocky slopes (*B. albanica*, *B. armata*, *B. cornuta*, *B. heraldica*, *B. rubida*, and *B. xeropaga*), deserts (*B. caudalis*, *B. peringueyi*, and *B. schneideri*), montane grassland (*B. atropos* and *B. inornata*), and grassland scrub (*B. arietans* and *B. worthingtoni*) (FitzSimons 1962; Pitman 1974; Broadley and Cock 1975; Spawls and Branch 1995; Branch 1999).

Despite such diversity in body form and ecological habits, both morphological (Marx and Rabb 1965; Groombridge 1980, 1986; Ashe and Marx 1988) and molecular (Herrmann and Joger 1997; Herrmann et al. 1999; Lenk et al. 2001; Wüster et al. 2008) studies of the Viperinae have yielded strong support for *Bitis* as a monophyletic group. In particular, the genus *Bitis* is strongly supported by a suite of morphological synapomorphies that include the following: (1) large flange on the ectopterygoid (Marx and Rabb 1965; Groombridge 1980; Ashe and Marx 1988), (2) broad postorbital (Marx and Rabb 1965; Ashe and Marx 1988), (3) unique position of the parietal relative to the postorbital with medial contact (Ashe and Marx 1988), (4) two or three scales in nasal shield (Ashe and Marx 1988), (5) extreme relative length of the longest maxillary tooth (Ashe and Marx 1988), (6) spike-like laterodorsal process of septomaxilla (Groombridge 1980), (7) scale surface microornamentation with plate-like projecting laminae (Groombridge 1980), and (8) semicircular supranasal scale that overlaps the nasal forming a well-developed supranasal sac (Marx and Rabb 1965; Groombridge 1980). Interestingly, the supranasal sac is innervated by the ophthalmic division of the trigeminal nerve (York et al. 1998), but despite the findings of Breidenbach (1990), recent studies indicate that this structure is not used to detect thermal cues (Safer and Grace 2004; Roelke and Childress 2007).

Beginning in the 1980s, several researchers have attempted to resolve the interspecific relationships within *Bitis* using both morphological and molecular data. Groombridge (1980) used cranial osteology and myology, visceral anatomy, hemipenial morphology, and scalation to recover four subgroups including a monotypic "early-diverging" species (*B. worthingtoni*), a small southern African group (*B. atropos*, *B. cornuta*, *B. heraldica*, and *B. xeropaga*), the "*caudalis*" group (*B. caudalis*, *B. peringueyi*, and *B. schneideri*) and the "big *Bitis*" species (*B. arietans*, *B. gabonica*, and *B. nasicornis*). Although *B. parviocula* was not examined, Groombridge used the description of Böhme (1977) and color slides to infer that this species belonged to the "big *Bitis*" subgroup. Herrmann and Joger (1997) reanalyzed characters from Groombridge (1980) and used their own immunological distance data to produce both morphological and molecular trees containing 11 and eight *Bitis* species, respectively. Later, Herrmann et al. (1999) used 29 morphological characters from Groombridge (1980) and 33 amino acid characters to construct a consensus phylogeny of the Viperinae that included a clustering of eight *Bitis* species. The findings of Herrmann and Joger (1997) and Herrmann et al. (1999) were consistent with Groombridge's subgroup arrangement. Lenk et al. (1999) studied the relationships within *Bitis* and presented separate phylogenies based on immunological distance and DNA

sequence data. Based on congruence (i.e., identical grouping of species) between their molecular data and the morphology-based hypothesis of Groombridge (1980), the authors erected four *Bitis* subgenera (Table 1). Additionally, *B. gabonica rhinoceros* was elevated to a full species as genetic divergences supported equivalent taxonomic status for *B. gabonica*, *B. gabonica rhinoceros*, and *B. nasicornis* (Lenk et al. 1999). Wüster et al. (2008) recovered a grouping of *Bitis* species within their mitochondrial DNA phylogeny of Viperidae that strongly supported the subgeneric grouping of Lenk et al. (1999). Although the authors felt that the subgeneric designations of Lenk et al. (1999) were effective in highlighting phylogenetic structure, they argued that elevating these subgenera to full genera would not enhance our understanding of the evolution within this monophyletic group. Most recently, Fenwick et al. (2012) used viperine datasets from Lenk et al. (1999) and Wüster et al. (2008) in a mitochondrial phylogeny of Viperidae that included 11 species of *Bitis* (Fenwick et al. 2012, Figs. S1 and S3), and unsurprisingly, their analyses supported the subgeneric classification of Lenk et al. (1999). Although these studies have greatly advanced our knowledge, the most taxonomically well-sampled phylogenies to date have only included 11 of the 17 known *Bitis* species (Groombridge 1980; Wüster et al. 2008; Fenwick et al. 2012). Unfortunately, incomplete sampling has hindered our understanding of the evolutionary history of these medically and ecologically important vipers. Furthermore, past analyses have omitted species with restricted ranges that may provide unique information about the relationship between speciation patterns and regional endemism.

Table 1 Subgenus arrangement of the monophyletic viper genus *Bitis* as proposed by Lenk et al. (1999)

Subgenus *Bitis* (Gray 1842)
Bitis arietans (Merrem 1820)
Subgenus *Calechidna* (Tschudi 1845)
Bitis albanica (Hewitt 1937)
Bitis armata (A. Smith 1826)
Bitis atropos (Linnaeus 1758)
Bitis caudalis (A. Smith 1849)
Bitis cornuta (Daudin 1803)
Bitis heraldica (Bocage 1889)
Bitis inornata (A. Smith 1838)
Bitis peringueyi (Boulenger 1888)
Bitis rubida (Branch 1997)
Bitis schneideri (Boettger 1886)
Bitis xeropaga (Haacke 1975)
Subgenus *Keniabitis* (Lenk et al. 1999)
Bitis worthingtoni (Parker 1932)
Subgenus *Macrocerastes* (Reuss 1939)
Bitis gabonica (Duméril, Bibron and Duméril 1854)
Bitis nasicornis (Shaw 1802)
Bitis parviocula (Böhme 1977)
Bitis rhinoceros (Schlegel 1855)

In this study, we reanalyzed 29 morphological characters from the unpublished thesis of Wittenberg (2001) that included data from all 17 species of *Bitis* and combined this dataset with mitochondrial and nuclear sequence data available for subsets of 14 of these species. We used maximum parsimony and Bayesian optimality criteria to evaluate the subgeneric taxonomy of Lenk et al. (1999). Finally, we place the evolution of Africa's most diverse viper genus within a biogeographical context and discuss why some species relationships within our phylogeny remain poorly supported, an important area for further study.

Materials and methods

Morphological data collection and phylogenetic analysis

We collected morphological data from 207 alcohol-preserved museum specimens and 29 osteological preparations (Appendix S1). We included morphological data from Groombridge (1980) and Branch (1999) for some characters that were missing data. We attempted to obtain at least 20 specimens of each species. For wide-ranging species such as *Bitis arietans*, we increased the sample size and selected specimens from throughout their known range to assess and account for within-species variation (Wiens and Servedio 1997).

Our morphology dataset for phylogenetic analysis comprised 29 characters of scalation, osteological, visceral, and hemipenial morphology (Appendix S2). Descriptions for characters are derived from Dowling (1951), Groombridge (1980), Spawls and Branch (1995), and Branch (1999). Characters 1–13, 20, and 21 are meristic; characters 14–17 and 22–25 are qualitative and multistate. In parsimony analyses using PAUP* v4.0b10 (Swofford 2002), multistate characters were treated as ordered, and polymorphic characters (1–17, 20, 21, 23–25) were coded using generalized frequency coding (Smith and Gutberlet 2001) with unequal subcharacter weighting. All other characters were nonpolymorphic and were simply coded with the state displayed by all individuals. Morphological data were entered into the software program FastMorphologyGFC (Chang and Smith 2003) to convert raw data into a nexus file that could be used in PAUP*. Analyses used heuristic searches under a parsimony criterion with 10,000 random-taxon-addition sequences and tree bisection reconnection (TBR) branch swapping. To assess confidence in the relationships depicted by the shortest tree, nonparametric bootstrapping (Felsenstein 1985b) was applied using 2000 full heuristic pseudoreplicates and two random-taxon-addition sequence replicates per pseudoreplicate. In each of the three analyses, all 17

species of *Bitis* were included as the ingroup while another Old World viper, *Vipera berus*, served as the outgroup.

In parsimony analyses using Tree analysis using New Technology (TNT) (Goloboff et al. 2008, provided via sponsorship of the Willi Hennig Society), morphological data were treated as continuous, and polymorphic characters (1–17, 20, 21, 23–25) for each species were input as ranges of one standard deviation around the mean. We selected the heuristic search option under TNT's Traditional Search criteria, with 200 random-taxon-addition sequences and TBR branch swapping. We conducted resampling with standard bootstrapping, searching 1000 pseudoreplicates and two random-taxon-addition sequence replicates per pseudoreplicate.

For Bayesian Markov chain Monte Carlo (BMCMC) analyses conducted using MrBayes v.3.0b4 (Ronquist and Huelsenbeck 2003), limitations of the program required fewer frequency bins than parsimony-based analyses (six compared to 26 in PAUP*). We therefore coded meristic characters under gap weighting (1–13, 20, 21; Thiele 1993), polymorphic characters with three or fewer states under unscaled coding (14, 15, 17, 23–25; Campbell and Frost 1993), and polymorphic characters with more states under majority coding (16; Johnson et al. 1988). We used the standard Markov model for phenotypic data (Lewis 2001); preliminary analyses also supported using gamma-distributed rate variation across this dataset. We conducted two simultaneous BMCMC runs (with the default MCMC settings) for a total of 4.0×10^6 generations, sampling trees and parameters every 400 generations. We used TRACER v. 1.6 (Rambaut and Drummond 2009) to confirm stationarity in the Markov chain within the burn-in period and discarded the first 4×10^5 generations as burn-in.

Molecular data and phylogenetic analysis

We used published sequence data of five mitochondrial genes [NADH dehydrogenase subunits four and two (ND4 and ND2), cytochrome *b* (cyt *b*), 16S ribosomal RNA (rRNA), and 12S rRNA] and two nuclear loci [prolactin receptor (PRLR) and anonymous locus Ba34] from subsets of 14 species of *Bitis* as well as from the outgroup *Vipera berus* (e.g., Lenk et al. 2001; Wüster et al. 2008; Barlow et al. 2012; Table S1). Sequences for each gene fragment were aligned separately using MUSCLE (Edgar 2004) in MEGA 5.0 (Tamura et al. 2011).

Bayesian inference and maximum parsimony were implemented to reconstruct phylogenies for the 14 ingroup species with sequence data. TNT was used to evaluate relationships via parsimony, using the same settings described above for morphological data. For Bayesian analysis, model likelihoods for each gene fragment were calculated, models were chosen, and partitioning strategies were evaluated using the Akaike information criterion with sample size being the number of sites (AICc4) in Kakusan4 (Tanabe 2011; Table 2). Kakusan4 determined that a partitioning strategy treating codon positions separately and assuming branch lengths are proportional across partitions (CodonProportional) was optimal for mitochondrial protein coding genes, and nonpartitioning of codon positions was optimal for nuclear loci. Stems and loops of rRNA genes were not partitioned separately due to a lack of informative characters. Analysis used MrBayes, with the same settings described above for morophological data.

Combined morphology and molecular phylogenetic analyses

We analyzed the morphological and molecular datasets together in a combined evidence approach, with both parsimony and BMCMC methods. Parsimony analyses were conducted with TNT, and BMCMC analyses were conducted with MrBayes, using the settings described above.

Results

Our morphological, molecular, and combined phylogenetic analyses are generally congruent with respect to several previously identified relationships within *Bitis*. One notable difference is that several of our analyses recovered *B. worthingtoni* as the earliest diverging member of the genus: BMCMC with morphology only (Fig. 1, 0.91 posterior probability (*Pp*) for a clade of *Bitis* species excluding *B. worthingtoni*), MP with morphology only [Fig. 1, 51–100

Table 2 Partitioned models for mitochondrial gene fragments and codon positions selected by Kakusan (Tanabe 2011) under AICc4. Note the positions given below are nucleotide alignment positions and may not correspond to codon positions

Bitis phylogeny	Total characters	AICc4 model
ND4, 1st pos	193	GTR+Γ
ND4, 2nd pos	193	GTR+Γ
ND4, 3rd pos	193	HKY85+Γ
Cyt *b*, 1st pos	199	SYM+Γ
Cyt *b*, 2nd pos	199	HKY85+Γ
Cyt *b*, 3rd pos	199	GTR+Γ
16S	381	GTR+Γ
12S	373	GTR+Γ
ND2, 1st pos	338	GTR+Γ
ND2, 2nd pos	338	GTR+Γ
ND2, 3rd pos	338	GTR+Γ
PRLR	525	HKY85+Γ
Ba34	523	K80+Γ

ND4 NADH dehydrogenase subunit 4, *Cyt b* cytochrome *b*, *16S* and *12S* small ribosomal RNA fragments, *ND2* NADH dehydrogenase subunit 2, *PRLR* prolactin receptor, *Ba34* anonymous nuclear locus from Barlow et al. (2012)

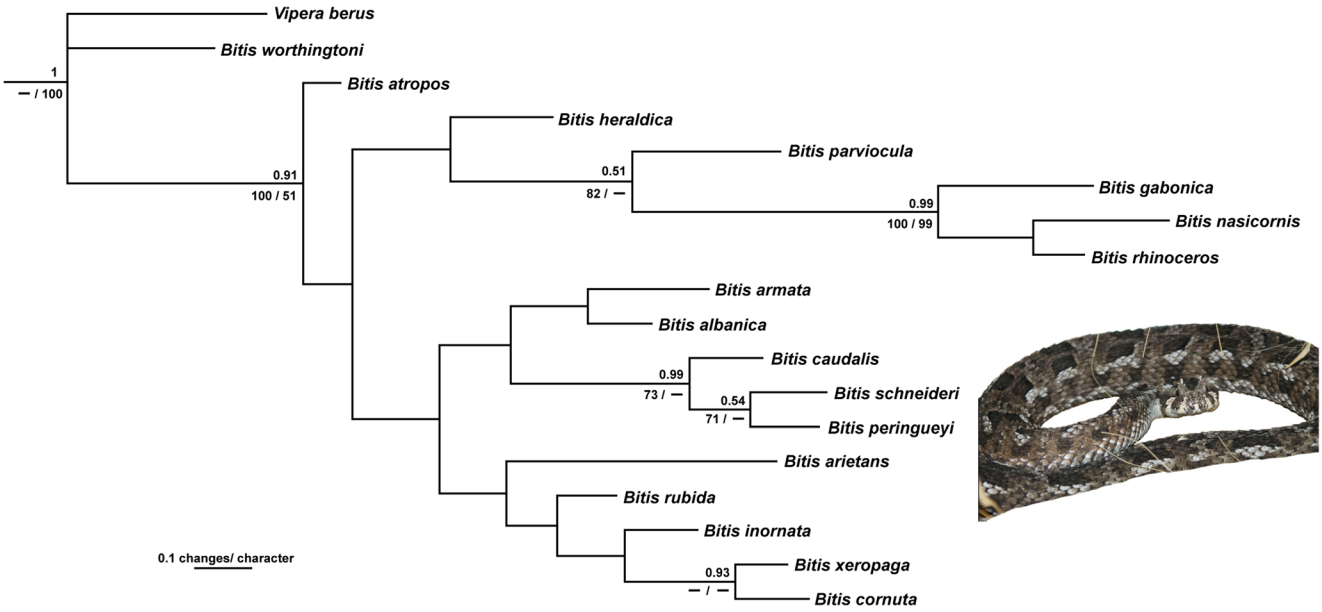

Fig. 1 Bayesian 50 % majority-rule consensus phylogram of *Bitis* species based on 29 morphological characters (Appendix S2). Support values include posterior probabilities (Bayesian, *above*) and bootstrap values (maximum parsimony with PAUP*/TNT, *below*). Photograph of *B. cornuta* (*insert*) by A. Saunders

bootstrap (*Bs*)], and MP including DNA (not shown, 94 *Bs* with DNA and 97 *Bs* with combined evidence). In contrast, BMCMC analyses including DNA recovered *B. arietans* (Fig. 2 insert) as the first-diverging member of the genus with moderate to high support (Figs. 2 and 3, 0.91–0.99 *Pp* for a clade of *Bitis* species excluding *B. arietans*).

We found strong support for a *B. gabonica*–*B. nasicornis*–*B. rhinoceros* clade in all but one analysis (0.99–1.0 *Pp*, 98–100 *Bs*, but see Fig. 2 and <50 *Bs*) and also recovered a sister relationship between *B. gabonica* and *B. rhinoceros* based on combined evidence (0.89 *Pp*, 91 *Bs*). This three-species clade is supported by averaging a greater number of circumorbitals (except *B. worthingtoni* which averages more), interoculabials, interrictals, middorsal scale rows, supralabials, dentary teeth, scales between the nasal and first supralabial, and infralabials contacting each chin

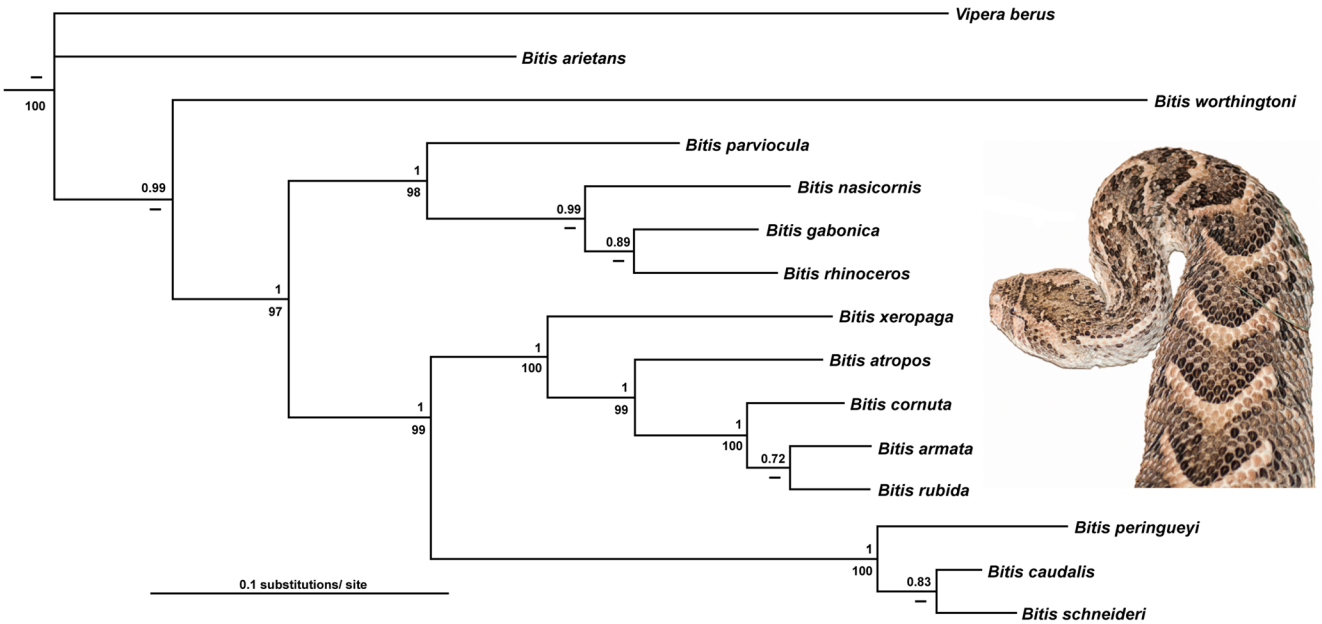

Fig. 2 Bayesian 50 % majority-rule consensus phylogram of *Bitis* species from partitioned analysis of four mitochondrial gene fragments (ND4, cyt *b*, 16S, and 12S; total of 1930 bp). Support values include posterior probabilities (Bayesian, *above*) and bootstrap (maximum parsimony, *below*). Photograph of *B. arietans* (*insert*) by A. Saunders

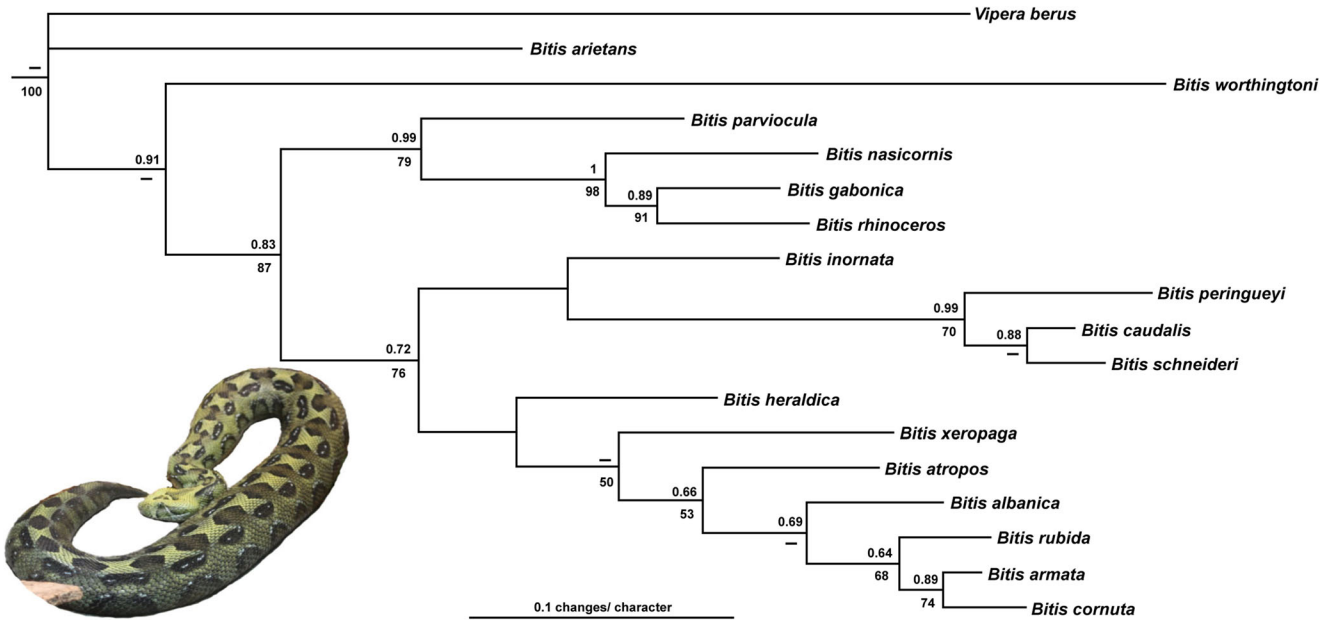

Fig. 3 Bayesian 50 % majority-rule consensus phylogram of *Bitis* species from combined evidence. Bayesian and parsimony analyses were conducted from a 29 morphological character dataset (Appendix S2) and a partitioned dataset of sequences from four mitochondrial gene fragments (ND4, cyt *b*, 16S, and 12S; total of 1930 bp). Support values include posterior probabilities (Bayesian, *above*) and bootstrap (maximum parsimony, *below*). Photograph of *B. parviocula* (*insert*) by RDW

shield (Table 3). Additionally, only members of this clade have (1) tracheal cartilage that terminates either at or before the anterior portion of the heart, (2) a lateral process of the ectopterygoid that is distinctively concaved outwards, (3) flounces present distal to the sulcus fork on the hemipenes (other species in the genus have spines instead of flounces), and (4) internasal horns, a trait only shared with *B. arietans* (however, in *B. arietans*, this trait is manifested as a slight ridge that never forms a true "horn"). A sister relationship between *B. parviocula* and the *B. gabonica* clade had generally low support from morphology (0.51 *Pp*, <50–82 *Bs*) but strong support in the analyses including DNA (0.99–1 *Pp*, 79–98 *Bs*). This relationship is supported by *B. parviocula* sharing a similarly large number of middorsal scale rows, interocunasals, scales between the nasal and first supralabial, and infralabials contacting each chin shield with the *B. gabonica* clade than other *Bitis* taxa.

A strongly supported *B. caudalis–B. peringueyi–B. schneideri* clade was recovered in all but one analysis (0.99–1.0 *Pp*, 70–100 *Bs*, but see Fig. 1 and <50 *Bs* from TNT). These three species possess the fewest middorsal scale rows and interrictals. Interestingly, MP analysis of combined evidence recovered a clade of *B. albanica–B. caudalis–B. peringueyi–B. schneideri* with strong support (not shown, 70 *Bs*), with low support for a clade of *B. albanica* and *B. schneideri* (57 *Bs*). *Bitis albanica* is represented by morphological data only, and in our analyses using only morphology, it does not have support for group membership.

Our molecular phylogeny shows strong support for a clade consisting of *B. armata*, *B. cornuta* (Fig. 1 insert) and *B. rubida* (1.0 *Pp*, 100 *Bs*) that is sister to *B. atropos* (1.0 *Pp*, 99 *Bs*), which together are sister to *B. xeropaga* (1.0 *Pp*, 100 *Bs*). Combined evidence recovers similar relationships with low support (<0.5–0.89 *Pp*, <50–74 *Bs*) and with *B. albanica* sister to the *B. armata–B. cornuta–B. rubida* clade with low or no support (0.69 *Pp*, <50 *Bs*). Morphological data only found support for a sister relationship between *B. xeropaga* and *B. cornuta* and only from BMCMC analysis (0.93 *Pp*, <50 *Bs*).

Our molecular and combined evidence phylogenies support a sister relationship between the *B. caudalis–B. peringueyi–B. schneideri* clade and the *B. armata–B. atropos–B. cornuta–B. rubida–B. xeropaga* clade (0.72–1.0 *Pp*, 76–99 *Bs*) and a sister relationship between these groups and the *B. gabonica–B. nasicornis–B. parviocula–B. rhinoceros* clade (0.83–1.0 *Pp*, 87–97 *Bs*). Support for these relationships decreases with the addition of morphology, and the morphological phylogeny does not recover the same relationships. The morphological dataset appears to lack distinct synapomorphies for higher level relationships within *Bitis*.

Discussion

Enigmatic history of *B. parviocula* and relationships within the "big *Bitis*" clade

Bitis parviocula (Fig. 3 insert) is one of the most poorly known vipers in the world and is considered to be a montane endemic of southwest Ethiopia, occurring on either side of the

Table 3 Mean values for morphological characters evaluated in this study. These data represent counts (1–13, 20, and 21) and states (14–19 and 22–29) for characters found in Appendix S2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
B. albanica	27	119	22	4	13.5	12	27	9	15	4	1	1	3	0	0	2	2	0	0	?	?	?	?	?	?	?	?	?	?
B. arietans	32.5	133	24.5	5.1	13.8	14	27.1	10.7	8	4.2	1.1	1	3.8	1	1	0	1	1	0	13.5	17.1	1	0	0/b	2	0	1	1	0
B. armata	27.3	123	29.7	3.3	13.8	12.5	27	8.8	12	4.3	1	1	3	1	0	3	?	0	0	?	?	?	?	?	?	?	?	?	?
B. atropos	29.4	127.2	24.1	3.9	13.4	11.3	29.3	9	11	3.3	0.9	0.9	3.6	0	0	0	3	0	0	13.3	14	0	0	1/c	1	0	1	1	1
B. caudalis	25.5	134	26.2	4.5	13.8	11.5	25.3	9.1	11.3	4.1	1.2	1.1	2.8	3	0	1	?	1	0	12.5	15.4	0	2	1	1	0	1	1	1
B. cornuta	27.8	139.6	29.2	4.7	14.5	13.9	28.3	9.8	13	4.2	1.5	1.1	3	1	0	3	2	0	0	14.3	15.8	0	2	1	1	0	1	1	1
B. gabonica	39.8	131.1	20.3	6.1	16.8	14.9	36.1	12.2	11.3	5.1	3.8	3.5	4.7	0	2	0	1	0	1	14	21	1	1	0	2	1	1	0	0
B. heraldica	27	129	24	4	12.5	13	31	8.7	11	3.5	1.5	1.5	3	0	0	0	3	0	0	12	13	0	1	0	2	0	1	1	1
B. inornata	28.3	134.3	24.7	5.3	14	12.9	30.7	8.6	14.8	4.4	1.3	1	2.8	1	0	0	?	0	0	?	?	?	?	?	?	?	?	?	?
B. nasicornis	36.5	125.1	24.6	3.8	17.8	16.6	31.3	9.5	12.1	5.6	3.3	4.6	4.8	0	3	0	1	0	1	16.3	20.5	1	1	0	2	1	1	0	0
B. parviocula	36	144	19	4	14	14	29	9	9	5	2	2.5	4.5	0	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?
B. peringueyi	24.7	133.3	22.6	3.6	11.7	11.2	22.8	9	6.2	4.4	1.2	1	2.7	2	0	0	2	1	0	12.5	14.8	0	1	1	1	0	1	1	1
B. rhinoceros	36.8	126.5	23.7	6	16.9	14.3	34.2	11.6	11.7	4.8	3.3	3.3	4.6	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
B. rubida	29.3	135	25.7	4.3	13.2	13.5	27.7	8.7	14.3	4.5	1	1	3.2	0	0	2	2	0	0	?	?	?	?	?	?	?	?	?	?
B. schneideri	25	110.3	20.3	3.3	11.2	11.3	23.3	8.7	10.3	4.2	1.3	1	3	3	0	0.6	2	1	0	?	?	?	?	?	1	?	1	1	1
B. worthingtoni	28	138	27.8	3.4	17.9	11.9	27.5	8.1	11.8	3.3	1	0	4.9	0	?	1	0	1	0	?	?	?	?	?	0	?	0	1	0
B. xeropaga	27	148.7	29.3	4.7	15.5	14.2	29.7	9.7	13.7	4	2	1.7	3	0	0	0	2	0	0	?	?	?	?	?	1	?	1	1	1

Rift Valley in forests and rolling grasslands (Largen and Rasmussen 1993; Spawls and Branch 1995). When Böhme (1977) examined the damaged holotype, he erroneously described the eye and nasal morphology of *B. parviocula* as reduced and therefore concluded that *B. parviocula* might be semifossorial. Few specimens of *B. parviocula* existed in 2000 when authors RDW and RLG, assisted by Stephen Spawls, searched unsuccessfully for this species in Ethiopia's southwestern forests. In 2001, *B. parviocula* first appeared for sale in the USA as part of the live reptile trade and the species is becoming increasingly common in private collections (Sánchez et al. 2011). Although *B. parviocula* remains unstudied in its vanishing habitat, genetic material recently available on GenBank has allowed us to confirm the phylogenetic position of this species previously assessed in morphological phylogenies conducted by Wittenberg (2001). Our phylogenies resolve *B. parviocula* sister to the *B. gabonica–B. nasicornis–B. rhinoceros* clade with strong support from the inclusion of molecular data (Figs. 2 and 3, 0.99–1 *Pp*, 79–98 *Bs*; Fig. 1, 0.51 *Pp*, <50–82 *Bs* with morphology only). These analyses place *B. parviocula* within the "big *Bitis*" clade and support the validity of the subgenus *Macrocerastes* for these species. This relationship was proposed by Groombridge (1980) based only on photographs, owing to the paucity of specimens available for character analysis. Within the "big *Bitis*" clade, we find moderate to strong support in our molecular and combined evidence analyses for a *B. gabonica* and *B. rhinoceros* clade that is sister to *B. nasicornis*.

One of the major differences among phylogenetic analyses of *Bitis* has been the phylogenetic placement of *B. arietans*. Phylogenies generated by Groombridge (1980, 1986) and Herrmann et al. (1999) show *B. arietans* as a sister lineage to the *B. gabonica–B. nasicornis–B. rhinoceros* clade, forming part of the "big *Bitis*" clade. Although Lenk et al. (1999) detected this relationship in one of their analyses, the majority of molecular analyses show *B. arietans* to be an early-diverging lineage, along with *B. worthingtoni* (Herrmann and Joger 1995; Lenk et al. 1999; Wüster et al. 2008). We find strong support from the molecular dataset (Fig. 2, 1.0 *Pp* and 97 *Bs*) and moderate support from the combined dataset (Fig. 3, 0.83 *Pp* and 87 *Bs*) for clades of all *Bitis* excluding *B. arietans* and *B. worthingtoni*. This finding supports the suggestion of Lenk et al. (1999) to place *B. arietans* in the monotypic subgenus *Bitis*. Although Lenk et al. (1999) treated *B. arietans* from Rwanda and South Africa as distinct taxonomic units and reported considerable sequence divergence between them, a recent phylogeographic study by Barlow et al. (2013) suggests that *B. arietans* is a monophyletic species.

Phylogenetic position of the morphologically primitive *B. worthingtoni*

Our finding of *B. worthingtoni* as one of two earliest diverging lineages in all analyses, with generally strong support, has been corroborated by several studies starting with Groombridge (1980), who considered the species to be the most primitive. We agree with these earlier studies and identify several distinct and possibly ancestral characters represented in *B. worthingtoni* that differ from other *Bitis* species. This relationship suggests that a hemipenial naked zone and terminal awn, an anterior ridge of the septomaxilla, dorsal ridge of the maxilla, scales between the nasal and first supralabial, and a greater number of rictals are derived characters in the other *Bitis* taxa. This finding supports the subgeneric recognition of *Keniabitis* for *B. worthingtoni* by Lenk et al. (1999), while our morphological data suggest that elevation of this lineage to a distinct monotypic genus may be warranted.

Relationships within the "*B. atropos*" and "*B. caudalis*" groups

Groombridge (1980) placed the remaining species into a "small *Bitis*" clade, further divided into a "*B. atropos* group" and a "*B. caudalis* group." Our phylogenetic analyses also detect these broad clades within *Bitis*, and recent studies also strongly support these findings (Groombridge 1986; Lenk et al. 1999, 2001; Wüster et al. 2008).

Within Groombridge's "small *Bitis*" clade, Herrmann and Joger's (1995) immunological blood serum albumin comparisons found a different phylogenetic arrangement of species. Interestingly, after adding information from three additional "small *Bitis*" species to their dataset (Herrmann and Joger 1997), the phylogeny of "small *Bitis*" resembled that of Groombridge (1980). Within the "small *Bitis*" clade, we generally find strong BMCMC and MP support for a *B. caudalis–B. peringueyi–B. schneideri* clade (0.99–1.0 *Pp*, 70–100 *Bs*, but see Fig. 1 and <50 *Bs* from TNT). Based on morphological data, only the species *B. albanica*, *B. armata*, and *B. inornata* are found sister to this clade, but none have support (<0.5 *Pp* and <50 *Bs*).

Based on molecular data, the second "small" *Bitis* clade includes *B. armata*, *B. atropos*, *B. cornuta*, *B. rubida*, and *B. xeropaga*. Combined evidence finds low support for this group plus *B. albanica*, and morphological evidence does not find support for this group. The placement of *B. albanica*, *B. heraldica*, and *B. inornata* remains problematic; however, the broader monophyletic subgenus *Calechidna* suggested by Lenk et al. (1999) is supported in our analyses.

Biogeographical and evolutionary patterns

An approximate minimum age for the *Bitis* lineage is provided by the early fossil record of *Bitis* sp. from Namibia from the lower Miocene (Szyndlar and Rage 2002). However, molecular dating suggests that *Bitis* diverged from its sister clade, *Atheris*, approximately 35.2 mya near the Eocene–Oligocene

boundary and underwent an extensive species radiation throughout the Miocene (Wüster et al. 2008). While *Atheris* (bush vipers) exploited an arboreal niche, *Bitis* remained a ground-dwelling clade that invaded most terrestrial habitats. As grasslands developed and spread across Africa during the Miocene (Retallack et al. 1990), a viper preferring open habitat could have become widespread before giving rise to other forms. Although our analysis using morphological characters suggests two independent origins of large body size (Fig. 1), the more robustly supported molecular phylogeny and less-supported combined evidence phylogeny suggest that a single, early origin of large body size followed by a trait reversal is as parsimonious as two independent origins of large body size (Figs. 2 and 3). Our combined and molecular analyses also indicate that *B. arietans* is an early-diverging lineage, while the forest-dwelling species of the "big *Bitis*" group (*B. gabonica*, *B. nasicornis*, *B. parviocula*, and *B. rhinoceros*) represent derived forms. Although *B. gabonica* is widespread, populations have been isolated in southeastern Africa due to forest fragmentation. The varied climate and topography of southern Africa supports the greatest diversity of species, including a desert adapted clade (*B. caudalis*, *B. peringueyi*, and *B. schneideri*) and a clade consisting primarily of montane, rock-dwelling species (*B. armata*, *B. atropos*, *B. cornuta*, *B. rubida*, and *B. xeropaga*, potentially also *B. albanica* and *B. heraldica*) (Fig. 3). Species such as *B. albanica*, *B. armata*, and *B. inornata* have restricted distributions and may be threatened by habitat loss (Branch 1999).

Effect of morphological and molecular datasets on tree topology

In comparing analyses using a single dataset to the combined evidence phylogeny, we find several relationships that differ. Most of the differences affect the placement of taxa for which DNA evidence is lacking. We suggest that our dataset of 29 phenotypic characters is below the theoretical threshold of informative characters suggested by Wiens (2003) needed for full resolution of a 17-taxon ingroup. The lack of support for a number of nodes across datasets indicates that additional informative characters are needed to enhance resolution. Although missing DNA data for a number of taxa (over 98 % of the matrix) might not be problematic (Wiens and Morrill 2011), we predict that more phenotypic evidence will need to be collected in order to confidently place the data-sparse species in the phylogeny. Alternatively, the effects of missing data on this dataset may be caused by nuances of the relationships in this clade and/or the phylogenetic signal in the dataset (Dragoo and Honeycutt 1997). Therefore, accurate placement of data-sparse species may not be achieved simply by increasing the amount of data available for each lineage.

Conclusions

A complete phylogeny of *Bitis* allows for comparative studies of anatomy, physiology, behavior, and venom biochemistry to be placed within an evolutionary context (Felsenstein 1985a; Miles and Dunham 1993). The importance of venom studies cannot be overstated, as only South Asia has a higher incidence of annual snakebite mortality than portions of sub-Saharan Africa (Kasturiratne et al. 2008). It is well documented that *B. arietans* is responsible for much of the snakebite morbidity and mortality on the African continent (Visser and Chapman 1978; Spawls and Branch 1995); however, information regarding the frequency of envenomation by other *Bitis* species is sparse. As the human population expands, bites from other members of the genus may become more prevalent. Although a large degree of variation in venom composition has been documented to occur among *Bitis* species (Calvete et al. 2007), a complete and robust phylogeny might allow toxinologists and clinicians to predict these patterns of variation and advance treatment protocols rapidly (Fry et al. 2003). Our study is the first to combine morphological and molecular data and modern phylogenetic analyses to resolve relationships within the genus *Bitis*. Additionally, these morphological data should support future studies aimed at better understanding the evolutionary relationships of these medically and ecologically important vipers.

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