Phylogeny and Taxonomic Subdivision of *Bitis* (Reptilia: Viperidae) Based on Molecular Evidence

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Zusammenfassung

Abstract
The genus *Bitis* is a well-defined monophylum within the African vipers. An immunologically derived phylogenetic hypothesis based on albumin is now corroborated by mitochondrial DNA sequences. A 597 bp portion of cytochrome b was sequenced and used to reconstruct phylogenetic trees by Neighbor Joining, Maximum Parsimony and Maximum Likelihood. The resulting topologies are similar to the albumin tree, thus confirming the following partition of *Bitis* into different clades: (1) *Bitis arietans*, (2) *B. gabonica*, *B. nasicornis*, (3) *B. caudalis*, *B. peringueyi*, *B. schneideri*, (4) *B. atopus*, *B. cornuta*, *B. xenopaga*, *B. inornata*, (5) *B. worthingtoni*. The South African small *Bitis* (groups 3 and 4) form a monophyletic group. Therefore four groups are recognised as subgenera. Of these, *Keniabitis*, monotypic with *Bitis* (*Keniabitis*) *worthingtoni*, is described as a new subgenus. *Keniabitis* appears as sister group of the remaining subgenera.

Introduction
Within the subfamily Viperinae, *Bitis* is the most widespread and diverse African genus. *Bitis* occurs widely in the sub-Saharan region but also in south-western Morocco and a restricted area of the Arabian Peninsula (Swains & Branch 1995). All members are predominantly terrestrial snakes that can be found in a variety of biotopes such as sand dunes, rocky hillsides, open grasslands or tropical rainforests.

The first substantial attempt [minor attempts include: Boulenger (1896), Werner (1922), Parker (1932), Mertens (1958), Haacke 1975, Böhme (1977), Branch (1989)] dedicated to the intrageneric relationships of *Bitis* was undertaken by Groombridge (1980). His hypothesis of cladistic relationships within *Bitis* entails four monophyletic lineages. *Bitis worthingtoni* from the East African Rift Valley appears to be the most divergent species, as indicated by the most basal branch within the genus (Fig. 1). A trichotomy comprises two "dwarf adder" groups from southern Africa and the large *Bitis* group with *B. arietans* and the equatorial African forest species *B. gabonica* and *B. nasicornis*.

The basal position of *B. worthingtoni* is supported by four character states that appear plesiomorphic within *Bitis*: (1) postorbital-prootic contact, (2) sculation of snout region, (3) anterior ridge of septomaxilla, and (4) form of maxilla. Although biogeography implies that the remaining dwarf adders form a monophyletic group, it has not been possible to find morphological characters suggesting either that these southern African 'small *Bitis* reveal a closer relationship or that any one is more related to the large *Bitis* group. Ashe & Marx (1988) found many synapomorphic characters to characterise *Bitis* as a genus, but none that separates groups within *Bitis*.

In this communication we present additional data on the phylogenetic relationships between members of the genus. We focus on the question to which extent molecular data corroborate or refine the morphological hypothesis and whether
alternatives can be figured out. To this means we employ immunological comparisons of blood serum albumins and sequence analyses of mitochondrial DNA.

Material and methods

Material
Species selection was dictated by the testing of previous hypotheses based on morphological data. Our goal was to include species from the four different groups suggested by GROOMBRIDGE (1980). In the DNA analysis we met the requirements fully, as at least one species of each group (see above) could be sequenced. For the immunological part, however, no representative of B. worthingtoni was available. Thus we were forced to examine slightly different sets of species in both analyses.

Blood samples were collected from live specimens of the taxa listed in Tab. 1. Serum and blood cells were separated after a short centrifugation step.

Immunological comparison
Immunological methods were applied as described by JOGER (1984). Albumins were separated on vertical PAGE, purified and injected into rabbits for antisera production. Antisera were produced against the following taxa: Bitis arietans (Togo), B. gabonica (Togo), B. nasicornis (Congo), B. atropos (South Africa), B. peringueyi (Namibia), B. cornuta (South Africa), B. caudalis (South Africa). Except for B. peringueyi, at least two rabbits were used per species. Antisera obtained were pooled in quantities reciprocal to their reactivity.

Quantitative precipitin tests were carried out and immunological distances (IDS) were calculated as percentage of homologous reaction. Corrections for ‘non-random elements’ were completed according to SARICH & CRONIN (1976).

DNA sequencing
Total DNA was extracted from blood cells after Proteinase K digestion and phenol chloroform extraction (SAMBROOK et al. 1989). Polymerase chain reaction was performed using the primer pair L-14983 5'-CGGCTGAAATYATACACAAA-3' and H-15149 5'-AAACTGAGCCCCCTCAGATGA-TATTGTCCCTCA-3'. The nucleotide sequence of a 597 bp portion of the cytochrome b gene was determined and aligned.

Phylogenetic analysis
To establish evolutionary hypotheses on the basis of immunological and DNA data, the following methods were applied.

Immunological distances were used to reconstruct a phylogeny with the Fitch-Margoliash subroutine of the PHYLP program (FELSENSTEIN 1995). This method is additive and does not require the assumption of a similar evolutionary rate. The outgroup CAUSUS rhombeatus was used to root the tree.

DNA-sequences were analysed using the programs MEGA 1.01 (KUMAR et al. 1993) and PAUP 4d 60 test version for DOS (SWOFFORD 1997). Pairwise genetic distances were calculated with MEGA 1.01. In addition, the Neighbour Joining algorithm of MEGA was used to reconstruct phylogeny based on distance matrices.

For Maximum Parsimony and Maximum Likelihood heuristic searches with PAUP 4d, 60 replications were conducted using default settings with the following parameters specified: TBR branch swapping, MULPARS option in effect, steepest descent option not in effect.

Bootstrap consensus trees were computed with MEGA (Neighbour Joining) and PAUP (Maximum Parsimony).

Abbreviations:
NJ Neighbour Joining
MP Maximum Parsimony
ML Maximum Likelihood
Fig. 2: Fitch-Margoliash dendrogram computed from albumin immunological distances. Numbers indicate branch lengths in % of ID.

Table 1: Species used in immunological and DNA analyses and their geographical origins.

<table>
<thead>
<tr>
<th>Species</th>
<th>Immunology</th>
<th>DNA</th>
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</thead>
<tbody>
<tr>
<td><em>Bitis gabonica</em></td>
<td>Congo: Kivu, Irangi</td>
<td>Congo: Kivu, Irangi</td>
</tr>
<tr>
<td><em>Bitis gabonica rhinoceros</em></td>
<td>Togo</td>
<td>Togo</td>
</tr>
<tr>
<td><em>Bitis nasicornis</em></td>
<td>Congo: Kivu, Irangi</td>
<td>Congo: Kivu, Irangi</td>
</tr>
<tr>
<td><em>Bitis peringueyi</em></td>
<td>Namibia: Swakopmund</td>
<td>Namibia: Swakopmund</td>
</tr>
<tr>
<td><em>Bitis caudalis</em></td>
<td>South Africa: Transvaal, Messina</td>
<td>South Africa: Cape, Swartberg</td>
</tr>
<tr>
<td><em>Bitis atropos</em></td>
<td>South Africa: Drakensberg, Barberton</td>
<td>South Africa: Saldanha Bay</td>
</tr>
<tr>
<td><em>Bitis cornuta</em></td>
<td>South Africa: Cape, Saldanha Bay</td>
<td>South Africa: Saldanha Bay</td>
</tr>
<tr>
<td><em>Bitis xeropaga</em></td>
<td>South Africa: Cape, Aggenys</td>
<td>South Africa: Port Elizabeth</td>
</tr>
<tr>
<td><em>Bitis schneideri</em></td>
<td>South Africa: Cape, Kleinsee</td>
<td>Rwanda</td>
</tr>
<tr>
<td><em>Bitis arietans</em></td>
<td>Togo</td>
<td>Kenya</td>
</tr>
<tr>
<td><em>Bitis arietans</em></td>
<td></td>
<td></td>
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<tr>
<td><em>Bitis worthingtoni</em></td>
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</tr>
</tbody>
</table>

Results

Immunological tree
Figure 2 provides a phylogenetic hypothesis based on the immunological distances between the species listed in Tab. 1. It minimises the 'sum of squares' of up to 486 alternative dendrograms. An identical topology was generated by ten trials with a different sequence of taxa. *Bitis schneideri* and *B. xeropaga*, against which no antisera were available, were added to the tree topology according to the method of BEVERLY & WILSON (1982). The tree distinguishes three well-separated clades: *Bitis gabonica* / *nasicornis*, *B. arietans* (monotypic) and the dwarf adders, which divide into two groups. The latter are identical with the morphologically defined groups (Fig. 1) of GROOMBRIDGE (1980).

Sequence analysis
For the complete dataset 226 sites were variable and 155 were parsimony-informative. Typically for mtDNA, a highly biased nucleotide content is observed with the frequencies A = 0.28, C = 0.32, G = 0.13, and T = 0.28. The skewness of 1000 randomly sampled trees generated with PAUP indicates the presence of significant phylogenetic signals (HILLIS & HUelsenbeck 1992) (g 1 = -0.961, p<0.01). As no other viperine taxon has been unambiguously identified as a closer relative to *Bitis*, we have chosen another African viperine species, *Atheris squamiger*, as an appropriate outgroup.

Considering the pairwise p distances (proportional distances) of all sequences shown in Tab. 3, we found that divergence between taxa is fairly high. This concurs with the finding that more than one quarter of all pairwise transition/transversion ratios of our dataset lay beyond 2.0.

BROWN et al. (1979) and MINDELL & THACKER (1996) indicated that saturation effects on mitochondrial DNA appear to be frequent if divergences exceed 15-20%. In a case like this there is no reason to
equal the evolution rates of the three codon positions or different substitution types (transversions and transitions) during phylogeny reconstruction.

Hence, we applied evolution models that consider different rates of substitutions to reduce noise generated by multiple changes per site. For the NJ approach we used the TrN model (Tamura & Nei 1993) considering different nucleotide frequencies and different rates of two types of transitions and one type of transversions. In the MP analysis, we gave transversions a 10-fold higher weight than transitions. In ML analysis, we allowed for six substitution types that correspond to the GTR (general time-reversible) model of nucleotide changes (Yang 1994a).

In protein coding genes the assumption of equal substitution rates between all sites is usually violated because of particular constraints on the different triplet positions or on functional domains of the enzyme. Statistical analyses of the distribution of the number of substitutions at different sites have suggested that the rate varies approximately according to the Gamma distribution (Uzzell & Corbin 1971, Kocher & Wilson 1991, Tamura & Nei 1993). Hence, in the distance approach we considered that rates for variable sites follow the Gamma distribution and used the parameter 1 for describing the shape of substitution rates. To reduce the effects of homoplasy in the MP analysis, we allowed for unequal substitution rates at the three codon positions by weighting pos. 1 = 0.5, pos. 2 = 1, and pos. 3 = 0.1 based on Tab. 2. In the ML analysis, the discrete four-parameter Gamma model of Yang (1994b) was employed with the Gamma shape parameters to be estimated.

In congruence, all tree constructing methods (Figs. 3a-c) readily identified four assemblages, which can be comprised to the following four clades: gabonica group (B. gabonica, B. nasicornis), arietans group, worthingtoni group (monotypic), and the dwarf adders (B. atropos, B. cornuta, B. caudalis, B. peringueyi). The latter were subdivided by all three procedures into two clades (B. atropos and B. cornuta; and B. caudalis and B. peringueyi). Only in the NJ tree, the gabonica clade and B. arietans, comprising the large Bitis species, represent a monophyletic group. No close relationship between Bitis worthingtoni and the dwarf adders as well as the remaining species can be found. Each tree supports a rather distinct state for B. worthingtoni, which takes the most basal position in the NJ tree, whereas in the other procedures it is replaced by B. arietans. In a strict sense, Bitis worthingtoni and B. arietans are the only variable elements within a rather stable phylogenetic framework.

Considering the more terminal nodes, only minor variations can be found. The monophyly of the atropos group, the caudalis group and the gabonica group is unequivocal. However, within the latter lineage the position of B. gabonica rhinoceros appears to be unresolved. In the NJ tree, it clusters with B. g. gabonica while MP and ML suggest a closer relationship to B. nasicornis.

Bootstrap values (Felsenstein 1985) reflect this general pattern. As shown in Figs. 3a and 3b, the highest values were achieved for the monophyly of the gabonica clade, the caudalis clade, and the atropos clade, whereas the other fucrations revealed lower confidence limits. Phyletic groups concurring in both bootstrap analyses revealed 60% or more confidence limits. The basal position of B. worthingtoni, as suggested in the NJ analysis, was found in 28% of the MP replicates.

The monophyly of the arietans-gabonica-clade, supported with 58% in the NJ tree, is rejected by the MP analysis as recovered in only 4.5% of resampled trees.

Table 2: Variability of the partial cytochrome b gene analysed and of particular subsets of the data, based on pairwise comparisons using the p-distance of MEGA.

<table>
<thead>
<tr>
<th>Sequence divergence</th>
<th></th>
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<tr>
<td>Overall</td>
<td>5.2–19.6</td>
</tr>
<tr>
<td>Only transitions</td>
<td>4.4–15.0</td>
</tr>
<tr>
<td>Only transversions</td>
<td>0.8–6.0</td>
</tr>
<tr>
<td>Only first position</td>
<td>2.0–10.0</td>
</tr>
<tr>
<td>Only second position</td>
<td>0.0–5.0</td>
</tr>
<tr>
<td>Only third position</td>
<td>12.0–47.7</td>
</tr>
</tbody>
</table>

Discussion

Our sequence data showed an essential robustness with respect to different phylogeny reconstruction methods applied, and revealed only minor variations. Typically ambiguous positions revealed by different methods were weakly supported by permutation tests and vice versa. However, there is strong evidence for the existence of five major monophyletic clades, of which only the two groups of small Bitis exhibit an apparently closer relationship towards each other. While Groombridge found only poor support for the monophyly of the south African dwarf adders, DNA and especially immunological data reveal a significant association. Each reconstruction showed a dichotomy leading to the caudalis group (B. caudalis, B. peringueyi, B. schneideri) and to the atropos group (B. atropos, B. cornuta, B. xeropaga). The other major clades are confirmed by all three data sets, comprising morphological, DNA and immunological data.

In conclusion, Bitis represents a reasonably divergent genus of viperine snakes. All data agree in that they suggest a gabonica clade, an arietans clade, and a "small Bitis" clade comprising the Karoo –
Kalaharian – South African species. The latter can be divided into two subgroups: the desert-dwelling caudalis group and the more rock-dwelling atropos group. Another small Bitis from East Africa, B. worthingtoni, appears to be a fifth basal phylogenetic entity.

We were unable to resolve an unambiguous cladogenetic pattern that could seriously reflect the relationships within the genus. Probably this pattern indicates a sudden radiation founded by an ancient precursor of the genus Bitis.

It turns out that the large Bitis species are not as closely related as suggested (GROOMBRIDGE 1980) and accepted (SPAWLS & BRANCH 1995) in morphological studies. Conversely, they represent two independent lineages consisting of an arietans and a gabonica clade, both are probably not sister taxa. If GROOMBRIDGE’s data are re-analysed with HENNIG 86 and enriched with apomorphic cytochrome b amino acids (HERRMANN et al. this volume: Fig. 4), a non-additive tree joins arietans with gabonica/nasicornis, but an additive tree does not find any synapomorphy of the two clades. We like to point out that GROOMBRIDGE (1980) found seven autapomorphies characterising the gabonica clade, whereas – except for the large body size – only two shared characters were found between arietans and gabonica groups. One of them is also observed in B. worthingtoni.

Another striking feature revealed by our analysis is the position of B. gabonica rhinoceros, which appears not closely related to B. g. gabonica but claims a rather independent position within the gabonica group. Genetic divergences would support equivalent taxonomic ranks for the three taxa B. gabonica, B. nasicornis, and B. g. rhinoceros.

Two specimens of the most widely ranging African species, Bitis arietans, from Rwanda and from South Africa, exhibit a considerable extent of sequence divergence. A third specimen, from Morocco, appeared closer to the animal from Rwanda (3.85% sequence divergence versus 5.03% towards the South African snake). DNA analysis takes into consideration that the continuous distribution is obviously accompanied by a substantial spatial structure of mitochondrial haplotypes at least between southern and north-central African populations.

Congruence between morphology, immunological and DNA data is obtained in the remaining species studied. Bitis worthingtoni is apparently a monotypic basal lineage within the genus with no closer affinity to any other Bitis.

Taxonomical consequences
The good congruence between the three data sets and their identical grouping of species justifies taxonomic consequences, i.e. the definition of subgenera within Bitis. Type species of Bitis GRAY 1842 is B. arietans. This was confirmed by the International Commission of Zoological Nomenclature together with the suppression of the older name Cobra in 1945 (Opinion 188).

The following names are available for other subgenera:
<table>
<thead>
<tr>
<th>OTUs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atheris</td>
<td>1.642</td>
<td>1.792</td>
<td>1.893</td>
<td>2.027</td>
<td>1.826</td>
<td>1.859</td>
<td>1.725</td>
<td>1.843</td>
<td>1.893</td>
<td>1.742</td>
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</tr>
<tr>
<td>B. arietans (South Africa)</td>
<td>0.019</td>
<td>1.524</td>
<td>1.675</td>
<td>1.575</td>
<td>1.558</td>
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<td>1.457</td>
<td>1.441</td>
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<tr>
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<td>1.960</td>
<td>1.608</td>
<td>1.558</td>
<td>1.390</td>
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<td>1.508</td>
<td>1.474</td>
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<td>1.508</td>
<td>1.173</td>
<td>1.524</td>
<td>1.441</td>
<td>1.642</td>
<td>1.390</td>
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<td>1.843</td>
<td>1.776</td>
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<td>B. caudalis</td>
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<td>1.407</td>
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<tr>
<td>B. rhinoceros</td>
<td>0.972</td>
<td>1.390</td>
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<tr>
<td>B. nasicornis</td>
<td>1.608</td>
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<tr>
<td>B. peringueyi</td>
<td>1.608</td>
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</table>

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Macrocrastes Reuss 1939, type species: B. nasicornis,
Hallowellius Reuss 1939, type species: B. gabonica,
Calechidna Tschudi 1845, type species: B. atropos.

Both names published by Reuss (1939) in the same article and on the same page are available according to the International Code of Zoological Nomenclature. We chose Macrocrastes, which appears a few lines before Hallowellius, as a new subgenus of Bitis with Hallowellius becoming a younger synonym.

**Subgenus Macrocrastes Reuss 1939: 14**

Type species: Echidna gabonica Duméril, Bibron & Duméril 1854 (= Macrocrastes gabonica).

Species included: Bitis (Macrocrastes) gabonica (Duméril, Bibron & Duméril 1854); Bitis (Macrocrastes) rhinoceros (Schlegel. 1855); Bitis (Macrocrastes) nasicornis (Shaw 1802), ? Bitis (Macrocrastes) parvcula Böhm 1977.

Bitis parvcula is included only tentatively, as its extent of nasal-rostral separation is similar to Macrocrastes spp. Its allocation was not verified osteologically or biochemically.

Definition: Large, stoutly built vipers with triangular head which bears one or several pairs of horn-like scales on the snout tip. Nasal separated from first supralabial by four scales or more, from rostral by 3-5 scales. Lateromedial body scales in oblique rows, with downward-pointing keels. Dorsal scale rows often duplicated. Prootic modified in relation to the origin of the Musculus retractor pterygoideus (see Groombridge 1980). Camouflage body pattern, characterised by rectangular light spots mid-dorsally, and a wave-like pattern laterally. Restricted to wet forest areas of tropical Africa.

Calechidna was originally described from Peru, South America. The accompanying plate, however, can be identified as Bitis atropos. Coluber atropos was a Linnaean species erroneously assumed to be American. We follow Williams & Wallach (1989) in assigning Bitis atropos to Calechidina as type species.

Species included: Bitis (Calechidina) albicana Hewitt 1937; Bitis (Calechidina) atropos (Linnaeus 1758); Bitis (Calechidina) armata (A. Smith 1826); Bitis (Calechidina) caudalis (A. Smith 1849); Bitis (Calechidina) cornuta (Dauidin 1803); Bitis (Calechidina) inornata (A. Smith 1838); Bitis (Calechidina) peringueyi (Boulenger 1888); Bitis (Calechidina) rubida Branch 1997; Bitis (Calechidina) schneideri (Boettger 1886); Bitis (Calechidina) xoropaga Haacke 1975; ? Bitis (Calechidina) heraldica (Bocage 1889) (incertae sedis); Bitis heraldica, a poorly known species of Angola which we could not study, is only tentatively included, on the basis of Groombridge's (1980) study.

Definition: Small to medium-sized vipers (maximum total length 750 mm, usually much smaller). No anterolateral pocket in (right) lung. A gap present between heart and liver. Angular and splenial bones are united into a single, much reduced bone, lacking close approach to the dentary (Groombridge 1980). Geographically restricted to southern Africa.

Comment: An apparent evolutionary diversification into two morphological/genetic groups may justify the separation of two subgenera.

No generic name has been proposed for Bitis worthingtoni which is clearly the most distinctive taxon within Bitis. Therefore we here describe:

**Subgenus Keniabitis new subgenus**

Monotypic; containing only the type species: Bitis (Keniabitis) worthingtoni Parker 1932.

Definition: Small-sized vipers (maximum total length 600 mm), characterised by a single horn-
like scale above each eye, direct contact between nasal and rostral and undivided subcaudals. No anterior ridge on septomaxilla; postorbital does not contact prootic (Groombridge 1980). A very specific cytchrome b containing modified unique amino acids at four positions (Histidine at pos. 42, Isoleucine at pos. 63, Threonine at pos. 89, Valine at pos. 155). Geographically restricted to Kenya.

Comment: If it holds true that Keniabitis occupies a basal position with regard to the other subgenera of Bitis, it may be justified to rank it as a separate genus.

**Subgenus Bitis Gray 1842**

The remaining typical subgenus Bitis contains only one species: B. arietans (Merrrem 1820). It is characterized by the absence of the character states used for the definition of the other subgenera, in particular by the following combination of characters:

Nasal separated from first supralabial by one to three scales, from rostral by one or two scales, no horn-like scales on head, and a different cytchrome b.

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**References**


