

# A molecular phylogeny for sub-Saharan amphisbaenians

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**Abstract.**—Amphisbaenians are enigmatic members of the subterranean herpetofauna with the majority of their diversity concentrated in South America and Africa. The largest family, Amphisbaenidae, occurs in South America and Africa, but the phylogenetic relationships among the genera are not clearly understood, especially for African taxa. We present a phylogeny based on two mitochondrial (16S and ND2) and two nuclear genes (CMOS and RAG1) with representatives of six of the nine African genera of Amphisbaenidae. Three African genera with keel and shovel head shapes occur in a single well-supported clade which is sister to all South American members of the Amphisbaenidae. The remaining three African genera (*Chirindia*, *Cynisca* and *Zygaspis*) all with round heads fall outside this clade, although their positions are not well supported. Future challenges rest with sampling species not recorded from the field for decades.

**Key words.**—Homoplasy, worm lizards, amphisbaenians, lacertids, Gondwana

## INTRODUCTION

For subterranean animals, the environment produces such intense constraints on morphology that instances of homoplasy in body plan are likely to be pervasive (Wake *et al.* 2011). Indeed, many subterranean vertebrates are superficially similar, having undergone body elongation, limb reduction or loss, cranial consolidation and changes to sensory systems (Caldwell 2003). Up to 28% of extant squamates have been suggested to be living in the soil (Measey 2006), with limb reduction or loss occurring an estimated 25 times (Wiens *et al.* 2006). Within limbless subterranean squamate clades, taxonomists work with relatively few morphological traits, and this has often led to difficulties in understanding the evolutionary relationships within and among these groups. In recent decades, molecular phylogenies have aided greatly in untangling these relationships, and have directed the subsequent re-examination of morphological characters which provide a backbone upon which morphological descriptions can be placed (e.g. Vidal *et al.* 2010). In contrast, where obvious similarities in morphological traits exist, these may be homoplastic leading to erroneous assumptions regarding evolutionary relationships. One such group that

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has proved difficult in this regard is the poorly known squamate sub-order Amphisbaenia or worm lizards.

Our understanding of relationships between amphisbaenians and other clades of squamates (Gans 1978; Lee 1998; Kearney 2003) underwent a radical shift following relatively recent molecular investigations (Townsend *et al.* 2004; Vidal & Hedges 2004). These studies showed that amphisbaenians are the sister clade to lacertiforms (family Lacertidae), rather than being their own order of squamates, and sister to snakes as previously hypothesised (Lee 1998; Kearney 2003; Townsend *et al.* 2004; Pyron *et al.* 2013). However, new techniques to study morphological traits continue to provide alternative viewpoints (Gauthier *et al.* 2012), and ultimately evolutionary relationships will rely on comprehension of both morphology and molecular tools.

Of particular interest in worm lizards has been the convergent evolution of pectoral scales and distinct cranial shapes: round, keeled, shovel and spade (see Kearney 2003 for definitions of shapes), as well as the repeated loss of limbs (Kearney & Stuart 2004). In a detailed study of six of the seven South American genera, Mott and Vieites (2009) found that three genera defined by head shape were polyphyletic, revealing further instances of convergent evolution. This raises the question as to whether a phylogeny may reveal polyphyly in sub-Saharan taxa, which currently have species placed in genera with round, shovel and keeled heads.

Within the Amphisbaenia there are six currently recognised families (Uetz 2013) and, to date, all molecular phylogenies place the North African and Middle Eastern family Trogonophidae as the sister clade to Amphisbaenidae (Kearney & Stuart 2004; Vidal *et al.* 2008; Mott & Vieites 2009; Fig. 1). However, generic-level relationships within the sub-Saharan African Amphisbaenidae are not well resolved. Kearney and Stuart (2004) found that the African genera *Chirindia* and *Cynisca* are sister taxa with moderate support and good morphological characters (both genera having fused tail vertebrae; see Kearney 2003), and that this grouping is sister to a clade with all derived amphisbaeniids. In other phylogenies, African amphisbaenids are only represented by *Geocalamus acutus*, which is recovered as the sister clade to all South American species with good support (Vidal *et al.* 2008; Mott & Vieites 2009). However, resolution between the genera *Geocalamus* and *Monopeltis* in the only phylogeny that contains them both is not well supported (Kearney & Stuart 2004).

With nine genera and 62 species (Uetz 2013), the Amphisbaenidae in sub-Saharan Africa remain relatively under-sampled but are clearly in need of a complete molecular phylogeny. The subcontinent contains genera encompassing three principle head morphologies: keeled (*Ancylocranium*, *Baikia*, *Geocalamus*), round (*Chirindia*, *Cynisca*, *Loveridgea*, *Zygaspis*) and shovel (*Dalophia*, *Monopeltis*; see Kearney 2003). In particular, there is a need to investigate traditional genus-level taxonomy based on morphology, and to re-examine areas of poor support in amphisbaenian phylogenies that have previously included African amphisbaenians. In this study, we construct a phylogeny for sub-Saharan African amphisbaenians including six of the nine genera, covering three head morphologies: round, shovel and keeled.

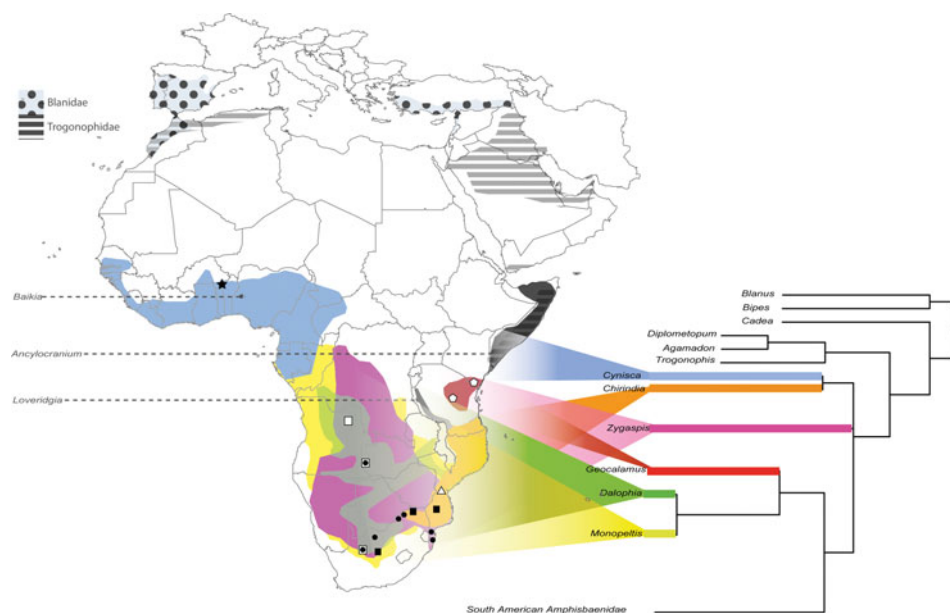


Figure 1. The distribution of sub-Saharan African genera of amphisbaenians, and a generic level tree (see Fig. 2). Each African genus of Amphisbaenidae is mapped with a minimum convex hull polygon approach and data from the literature (Broadley 1997; Gans 2005). Those genera included in the study are shown on the generic level tree (right), while those not included due to lack of available tissues are indicated to the left. The families Trogonophidae and Blaniidae are shaded with symbols. Symbols represent sampling localities for animals from which tissue samples were taken for this study (*Chirindia*: white triangle; *Cynisca*: black star; *Dalophia*: white square; *Geocalamus*: white pentagon; *Monopeltis*: black square; *Zygaspis*: black circle).

## METHODS AND MATERIALS

### DNA isolation and sequencing

Tissue samples (liver or muscle tissue) of amphisbaenians were used to extract DNA, with a standard salt extraction protocol, and amplify two mitochondrial (16S and ND2) and two nuclear genes (RAG1 and CMOS), following Mott and Vieites (2009). We used published primers for a selected fragment of the 16S ribosomal rRNA gene (Palumbi 1996), and for NADH dehydrogenase subunit 2 we used L4349 (Measey & Tolley 2011) and H5934 (Macey *et al.* 1997) (40 cycles 57 C). For the nuclear genes, we amplified a fragment of the recombination activation factor 1 gene (RAG1) using primers R1067 (Matthee *et al.* 2004) or R80 and R75 (Townsend *et al.* 2009), and for the oocyte maturation factor gene (CMOS) using the primers CO8 and CO9 (Han *et al.* 2004).

Gene fragments were amplified in 25  $\mu$ L reactions with 2  $\mu$ L of template DNA from extractions, and a standard recipe containing 0.2  $\mu$ L of each primer, 0.2 mM dNTPs, 2.5 mM MgCl<sub>2</sub>, 10 $\cdot$ thermophilic buffer (50 mM KCl, 10 mM Tris-HCl, pH 9) and 0.25 U Super-Therm Taq DNA polymerase. The profile of the polymerase chain reaction (PCR) was 95°C for 60 s, followed by 35–40 cycles of 60 s at 95°C, 30 s at 48–57°C (primer dependent) and 1 min at 72°C, with a final extension at 72°C for

Table 1. Specimens of amphisbaenians newly sequenced in this study including species localities, museum accession numbers. NMZB (National Museum of Zimbabwe), NMBO (National Museum, Bloemfontein), TM (Ditsong Museum of South Africa – formerly Transvaal Museum), NA (tissue but no specimen was collected). Data for additional sequences used in Fig. 2 are available online (see Online Supplementary Material) and are published in Mott and Vieites (2009).

Genus	Species	Specimen Number	Lat	Long	Locality	Country
<i>Chirindia</i>	<i>swynnertoni</i>	NA	–19.85	34.03	Buzi	Mozambique
<i>Cynisca</i>	<i>kraussi</i>	ZMB 79093	11.05	1.52	Batia savanna	Benin
<i>Dalophia</i>	<i>ellenbergeri</i>	NMZB 1643	–15.50	22.50	Barotseland	Zambia
<i>Dalophia</i>	<i>pistillum</i>	NMBO 9126	–28.52	22.12	Upington	South Africa
<i>Dalophia</i>	sp.	NA	–9.39	20.39	Saurimo	Angola
<i>Geocalamus</i>	<i>acutus</i>	NA	–3.85	38.65	Bunguli, Nr Voi	Kenya
<i>Monopeltis</i>	<i>capensis</i>	NMBO R8702	–28.68	24.92	Kimberley	South Africa
<i>Monopeltis</i>	<i>capensis</i>	NMBO R8701	–28.76	24.74	Kimberley	South Africa
<i>Monopeltis</i>	<i>sphenorhynchus</i>	TM 85591	–22.67	33.25	Banhine Park	Mozambique
<i>Zygaspis</i>	<i>quadrifrons</i>	PEM R20393	–23.23	28.83	Makgabeng	South Africa
<i>Zygaspis</i>	<i>quadrifrons</i>	PEM R20392	–27.12	23.93	Vryburg	South Africa
<i>Zygaspis</i>	<i>nigra</i>	NMZB 1640	–15.50	22.50	Barotseland	Zambia
<i>Zygaspis</i>	<i>vandami</i>	TM 85592	–26.90	32.88	Kosi Bay	South Africa
<i>Zygaspis</i>	<i>vandami</i>	NMBO R8703	–26.28	32.35	Porto Henrique	Mozambique

30 s. Electrophoresis of the PCR product was made on a 1% agarose gel containing Sybr Green (Life Technologies), and trans-illuminated using ultraviolet light. PCR products were cleaned up and sequenced by Macrogen (Seoul, Korea). Sequences were checked and aligned in GeneiousPro v. 4.8 (Drummond *et al.* 2007). All new sequences generated have been deposited in the EMBL Nucleotide Sequence Database ([Online Supp. Mat.](#)).

### Phylogenetic analysis

The dataset included 45 amphisbaenian taxa representing all families except Rhineuridae (after Wiens *et al.* 2012), of which 29 were available on GenBank. In addition, two outgroup taxa (*Blanus strauchi* and *Bipes canaliculatus*) from GenBank were included ([Online Supp. Mat.](#)). We chose these outgroup taxa because of their close sister relationship with the ingroup taxa (e.g. Townsend *et al.* 2004; Pyron *et al.* 2013). The partition homogeneity test was run to examine whether the two genomes had different phylogenetic signals, but this test indicated no conflict ( $p = 0.90$ ), so the combined dataset was used to produce a single phylogeny. jModelTest 0.0.1 (Posada 2008) was run for each marker to investigate the evolutionary model that best fits the dataset using the Akaike information criterion (AIC) (GTR+I+G for 16S, ND2 partitions; GTR+G for CMOS and RAG). The analysis was run with separate partitions for each marker, but with partitioned codons (1+2, 3) for the ND2 marker as the third codon was saturated. A Bayesian inference (BI) was run using MrBayes

ver. 3.1.2 (Ronquist & Huelsenbeck 2003) via remote upload at the CBSU cluster (cbsuapps.tc.cornell.edu). The phylogeny was estimated using uniform priors for all parameters, with each partition allowed to run independently. For each partitioning scheme, the Markov chain Monte Carlo (MCMC) was run twice in parallel for 10 million generations, sampling trees every 1 000 generations. Burn-in was determined by examining stationarity of log likelihood tree scores, standard deviation of split frequencies, and that effective sample size (ESS)  $> 200$  for all parameters, using Tracer v1.4.1 (Rambaut & Drummond 2007). For all runs, the first one million generations (1 000 trees) were removed as burn-in before constructing a 50% majority rule tree. Nodes with posterior probability  $\geq 0.95$  were considered supported.

A maximum likelihood (ML) search was run in RAxML 7.2.7 (Stamatakis 2006; Stamatakis *et al.* 2008) via CIPRES Science Gateway v. 3.1 ([http://www.phylo.org/sub\\_sections/portal/](http://www.phylo.org/sub_sections/portal/)), with a search for the best scoring ML tree, plus rapid bootstrapping. The same data partitions were used as in the Bayesian analysis, using the GTR+I+G model of evolution. The dataset was run both with and without invariant sites for the two nuclear genes, for which GTR+G was the most appropriate model. The ML analysis was run three times to ensure that independent ML searches produced the same topologies. Nodes with a bootstrap value of  $\geq 70\%$  were considered supported in this analysis.

## RESULTS AND DISCUSSION

The Bayesian and likelihood searches produced trees with the same basic topology and node support (Fig. 2). At the family level, our analysis revealed the same topology previously reported for families, with Cadeidae, Bipedidae and Trogonophidae as sister groups to the largest family with African and South American distribution: Amphisbaenidae (Kearney & Stuart 2004; Vidal *et al.* 2008). This also supports the single origin for South American members of this family, and hence the scenario of overseas dispersal suggested by Vidal *et al.* (2008). Furthermore, there was good support for a sister relationship between the South American clade (clade A) and the African clade containing *Geocalamus*, *Dalophia* and *Monopeltis* (clade B). There is Bayesian support (0.97) for the relationship between *Geocalamus* and the genera *Dalophia* and *Monopeltis* (clade B). *Chirindia* and *Cynisca* (clade D) and *Zygaspis* (clade C) form a polytomy within Amphisbaenidae, although the node defining the family Amphisbaenidae did receive 0.88 posterior probability support, which could potentially be improved with better taxon sampling.

Although not well supported, the clade containing genera *Cynisca* (West Africa) and *Chirindia* (East Africa; clade D) is sister to all other amphisbaeniids as reported by Kearney and Stuart (2004). Living members of these geographically disparate genera (see Fig. 1) are all small, with narrow round heads. Their current distributions and deep divergence between the two genera are reminiscent of an ancient East–West African division shown by some subterranean amphibians (Loader *et al.* 2007).

South American amphisbaenians, including round, keeled and spade headed species (clade A), form a well-supported clade together with keel and shovel headed amphisbaenians from the African genera *Geocalamus*, *Monopeltis* and *Dalophia* (clade B), a topography in accordance with Mott and Vieites (2009). However, this

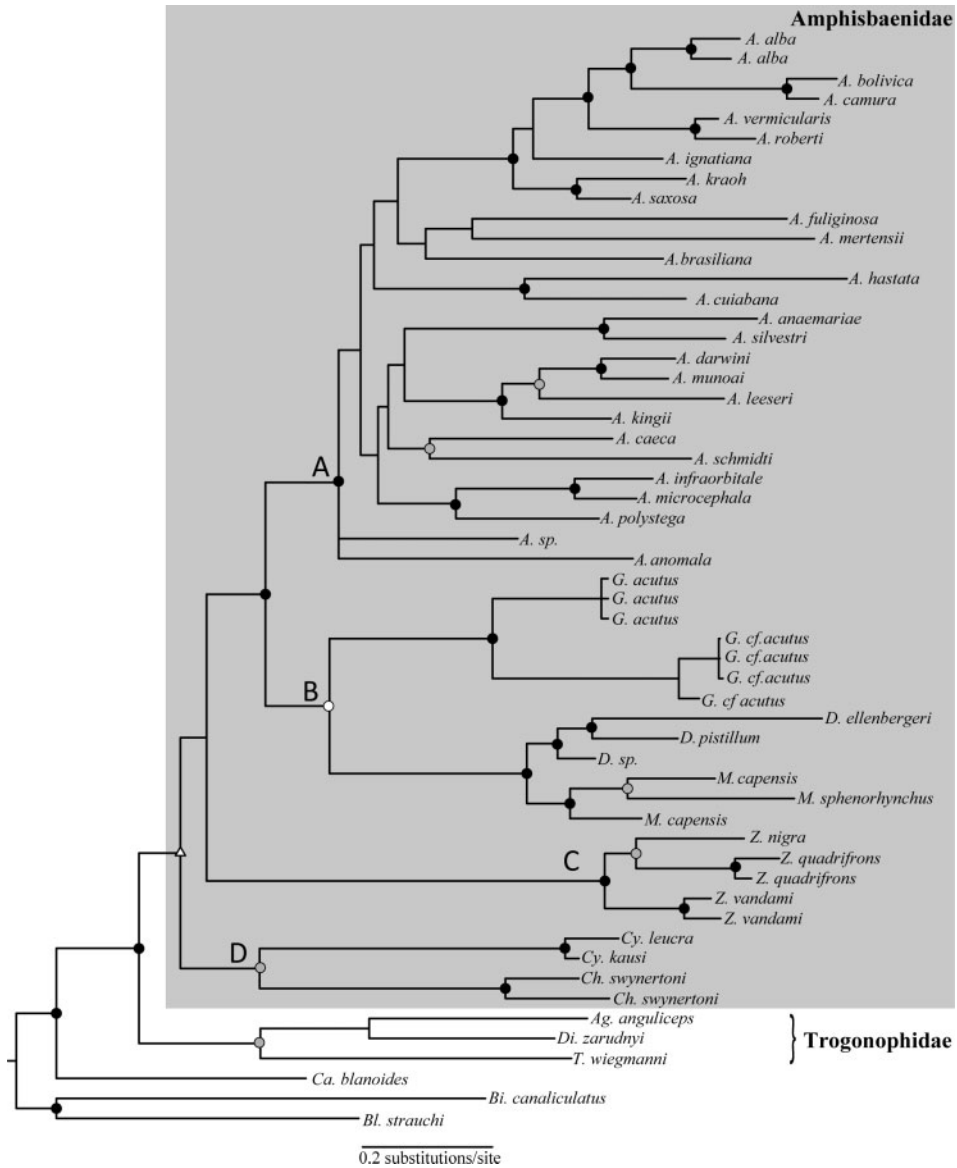


Figure 2. Bayesian topology for amphisbaenians included in this study. Supported nodes are indicated by circles (black:  $\geq 70\%$  likelihood bootstrap and  $\geq 0.95$  Bayesian posterior probability; grey:  $\geq 0.95$  Bayesian posterior probability and  $\geq 60\%$  likelihood bootstrap; white  $\geq 0.95$  Bayesian posterior probability but not supported by ML). Triangle indicates the node that defines Amphisbaenidae (shown within the grey box), which was supported with 0.88 Bayesian posterior probability. (Generic assignments: *A* *Amphisbaena*, *Ag* *Agamodon*, *Bi* *Bipes*, *Bl* *Blanus*, *Ca* *Cadea*, *Ch* *Chirindia*, *Cy* *Cynisca*, *D* *Dalophia*, *Di* *Diplometopon*, *G* *Geocalamus*, *M* *Monopeltis*, *T* *Trogonophis*, *Z* *Zygaspis*.)

larger clade (A+B) does not include the other African round headed genera (*Chirindia*, *Cynisca* and *Zygaspis*). This suggests the independent origins of both keeled and shovel headed African genera, and the homoplastic evolution of shovel headed amphisbaenians proposed by Kearney and Stuart (2004). Within clade B, the keel and shovel headed genera are divided into two separate clades (keel: *Geocalamus*; shovel: *Dalophia*, *Monopeltis*) although this relationship was only supported in the Bayesian analysis. It is also noteworthy that new samples of *Geocalamus acutus* collected from near the type locality in Voi, Kenya are substantially divergent from individuals of this taxon (from GenBank) from Dodoma, Tanzania (here referred to as *G. cf. acutus*). It is possible that Dodoma specimens were of *G. modestus* as this is in close geographic proximity to the type locality (Mpwawa, 80 km SSE of Dodoma; see Gans & Kochva 1966).

The missing keel headed species from the genus *Ancylocranium* are all distributed in East Africa (Gans 2005; Fig. 1), and we suggest that they are likely to fall within clade B due to their geographic proximity and morphological similarity to species of the genus *Geocalamus*. More intriguing will be the phylogenetic position of the monotypic species *Baikia africana*, known from only nine specimens most recently collected in central Nigeria in the 1960s (see Dunger 1964). The distant geographic proximity to all species of *Ancylocranium* in Tanzania and Somalia gives rise to the question of whether *B. africana* occurs within this clade B, or could be an independent homoplastic lineage of the keeled head shape. Interestingly, there has already been a suggestion that members of the genus *Ancylocranium* may belong to the genus *Baikia* due to their shared skull morphology (Gans & Kochva 1966). These three keel headed genera (*Ancylocranium*, *Baikia* and *Geocalamus*) together have the smallest distributions for sub-Saharan amphisbaenian taxa (Gans & Kochva 1966), likely influenced by the specialist soil types required for this type of burrowing.

The two shovel headed genera, *Monopeltis* and *Dalophia*, have very similar head morphologies with the six species of *Dalophia* sharing a derived terminal pad of the tail which has been speculated to be an anti-predator mechanism against *Xenocalamus* snakes (W.R. Branch pers. comm.). The two genera have a largely sympatric distribution in central and southern Africa (Fig. 1). Some species of *Monopeltis* have been found to have particularly high morphological plasticity, leading to many taxonomic problems (Broadley 1997; Boudzoumou *et al.* 2013) which may be resolved with more phylogenetic investigations. Distributions of some species are large, and we likely do not know the true extent of many (dos Santos 2013).

In summary, our phylogeny shows that the sub-Saharan genera *Geocalamus*, *Dalophia* and *Monopeltis* (all keel and shovel headed species) are a sister clade to the South American genera of the family Amphisbaenidae. The remaining African genera form a polytomy in the family although this may be improved with additional taxon sampling and a more complete dataset. The two round headed genera, *Chirindia* and *Cynisca*, are monophyletic with Bayesian support and low ML support, and the division within this clade corresponds to their East and West African distribution, respectively. The phylogenetic position of the round headed genus *Zygaspis* is not resolved, with respect to the other African genera.

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## ONLINE SUPPLEMENTARY MATERIAL

Specimens of amphisbaenians newly sequenced in this study including species localities, museum accession numbers, GenBank numbers. NA indicates no corresponding information. (New accession numbers will be supplied upon publication.)

Genus	Species	16S	ND2	RAG1	CMOS
<i>Agamadon</i>	<i>anguliceps</i>	NA		AY444040	AY444013
<i>Amphisbaena</i>	<i>alba</i>	FJ441700	FJ441943	FJ441820	FJ441760
<i>Amphisbaena</i>	<i>alba</i>	FJ441702	FJ441945	FJ441822	FJ441762
<i>Amphisbaena</i>	<i>anaemariae</i>	FJ441668	FJ441911	FJ441788	FJ441728
<i>Amphisbaena</i>	<i>bolivica</i>	FJ441670	FJ441913	NA	FJ441730
<i>Amphisbaena</i>	<i>caeca</i>	EU203647	FJ441914	FJ441791	FJ441731
<i>Amphisbaena</i>	<i>camura</i>	FJ441672	FJ441915	NA	FJ441732
<i>Amphisbaena</i>	<i>darwini</i>	FJ441693	FJ441936	FJ441813	FJ441753
<i>Amphisbaena</i>	<i>fuliginosa</i>	FJ441682	FJ441925	FJ441802	FJ441742
<i>Amphisbaena</i>	<i>hastata</i>	FJ441678	FJ441920	FJ441798	FJ441737
<i>Amphisbaena</i>	<i>ignatiana</i>	FJ441679	FJ441922	FJ441799	FJ441739
<i>Amphisbaena</i>	<i>leeseri</i>	FJ441694	FJ441937	FJ441814	FJ441754
<i>Amphisbaena</i>	<i>mertensii</i>	FJ441674	FJ441917	FJ441794	FJ441734
<i>Amphisbaena</i>	<i>munoi</i>	FJ441687	FJ441930	FJ441807	FJ441747
<i>Amphisbaena</i>	<i>schmidti</i>	EU203655	FJ441924	FJ441801	FJ441741
<i>Amphisbaena</i>	<i>silvestri</i>	FJ441688	FJ441931	FJ441808	FJ441748
<i>Amphisbaena</i>	<i>sp.</i>	FJ441690	FJ441933	FJ441810	FJ441750
<i>Amphisbaena</i>	<i>vermicularis</i>	FJ441685	FJ441928	FJ441805	FJ441745
<i>Amphisbaena</i>	<i>kingii</i>	FJ441726	FJ441969	FJ441845	FJ441786
<i>Amphisbaena</i>	<i>anomala</i>	FJ441712	FJ441955	FJ441832	FJ441772
<i>Bipes</i>	<i>canaliculatus</i>	NC_006288	NC_006288	FJ518701	FJ518700
<i>Blanus</i>	<i>strauchi</i>	FJ518702	FJ518703	AY444050	AY444024
<i>Bronia</i>	<i>brasiliana</i>	FJ441708	FJ441951	FJ441828	FJ441768
<i>Bronia</i>	<i>kraoh</i>	FJ441692	FJ441935	FJ441812	FJ441752
<i>Bronia</i>	<i>saxosa</i>	FJ441709	FJ441952	FJ441829	FJ441769
<i>Cadea</i>	<i>blanoides</i>	EU203661	NA	EU203662	EU203613
<i>Amphisbaena</i>	<i>cuabana</i>	FJ441695	FJ441938	FJ441815	FJ441755
<i>Amphisbaena</i>	<i>roberti</i>	FJ441711	FJ441954	FJ441831	FJ441771
<i>Chirindia</i>	<i>swynertoni</i>	HG425323	HG425312	HG425278	HG425300
<i>Cynisca</i>	<i>kausi</i>	HG425322	NA	HG425277	HG425295
<i>Dalophia</i>	<i>ellenbergeri</i>	HG425315	HG425304	HG425283	HG425299
<i>Dalophia</i>	<i>pistillum</i>	HG425320	HG425313	HG425282	HG425294
<i>Dalophia</i>	<i>sp.</i>	HG425321	NA	HG425279	NA
<i>Diplometopon</i>	<i>zarudnyi</i>	NC_006283	NA	NA	AY444023
<i>Geocalamus</i>	<i>acutus</i>	FJ441724	FJ441967	FJ441844	FJ441784
<i>Geocalamus</i>	<i>acutus</i>	HG425319	HG425306	NA	HG425301
<i>Amphisbaena</i>	<i>infraorbitale</i>	FJ441723	FJ441966	FJ441843	FJ441783
<i>Amphisbaena</i>	<i>microcephala</i>	FJ441716	FJ441959	FJ441836	FJ441776
<i>L. Amphisbaena</i>	<i>polystega</i>	FJ441719	FJ441962	FJ441839	FJ441779
<i>Monopeltis</i>	<i>capensis</i>	HG425318	HG425305	HG425280	HG425297
<i>Monopeltis</i>	<i>capensis</i>	HG425324	NA	HG425281	HG425298
<i>Monopeltis</i>	<i>sphenorhynchus</i>	NA	HG425309	HG425276	HG425296
<i>Trogonophis</i>	<i>wiegmanni</i>	FJ441667	AY662542	FJ441787	AY444025
<i>Zygaspis</i>	<i>nigra</i>	HG425317	HG425310	HG425288	HG425293
<i>Zygaspis</i>	<i>quadrijfrons</i>	HG425316	HG425308	HG425286	HG425291
<i>Zygaspis</i>	<i>quadrijfrons</i>	NA	HG425307	HG425287	HG425292
<i>Zygaspis</i>	<i>vandami</i>	HG425314	HG425302	HG425284	HG425289
<i>Zygaspis</i>	<i>vandami</i>	TBA	HG425303	HG425285	HG425290