A preliminary molecular phylogeny of the Namib Desert darkling beetles (Tenebrionidae)

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Received 7 August 2009. Accepted 1 March 2010

A systematic classification of Namib Desert darkling beetles (Coleoptera: Tenebrionidae) based on morphological characters is complicated as strong selection pressures exerted by desert conditions have led to a suite of convergent morphological characteristics. Here we present a first and preliminary insight into the relationships within the tribes Zophosini, Eurychorini and Adesmiini using molecular methods. We analysed partial sequences of the mitochondrial genes cytochrome oxidase II and cytochrome b of 16 individuals comprising 12 species. Minimum Evolution, Maximum Parsimony, Maximum Likelihood and Bayesian inference were applied for analysing sequence data. The genus *Pimelia* was used as outgroup and for calibrating divergence time estimates. Overall, results supported phylogenies constructed on morphological characters. The proposed monophyly of the artificially defined tribe Zophosini did receive sufficient support. Speciation events in Namibian darkling beetles likely occurred during periods of aridification about 35 Mya, 16 Mya and 5–10 Mya. Those periods could be related to geological events and climate change due to the glaciation of Antarctica and the development of the Benguela current.

Key words: *Zophosis,* **relaxed molecular clock, time of divergence, Africa, Benguela current.**

INTRODUCTION

The family Tenebrionidae (Insecta: Coleoptera), also known as 'darkling beetles' is one of the most numerous and diverse Coleopteran families recorded in the Namib Desert (Louw 1983). Darkling beetles play an extraordinarily important ecological role in this ecosystem as they are the dominant detritivores (Seely 2004). The Zophosini (Solier 1834), which are in the focus of this study, are an Old World tribe of apterous (flightless) Tenebrionidae. Their centre of origin is assumed to be the northern part of southwestern Africa (Namibia) and the border region of southwestern Angola. Here the highest number of subgenera and species occur and all three of the supposedly earliest divergent lineages are present (Penrith 1986). The Namib Desert was characterized by arid to semi-arid conditions for at least 80 Mya (Goudie & Eckardt 1999; Seely 2004). Climate fluctuations, especially periods of increasing aridification, could have forced the beetles to retreat into isolated patches or to establish psammophilous or petrophilous behaviour. Isolation ('pocket speciation', Endrödy-Younga 1978), adaptation and the availability of abundant ecological niches might have contributed to the radiation of the Zophosini (Penrith 1986).

Speciation events in Namib darkling beetles were obscured by convergent adaptations due to strong selection pressure as a consequence of xeric conditions. Consequently, classification of the Zophosini using morphological characters is a challenge.

Today, the genus *Zophosis* comprises all those species that lack unique character states and could therefore not be assigned to another genus. Revising the whole tribe of the Zophosini, Penrith (1980) recognized an overlap of morphological characters used for previous classification of taxa. Based on fundamental, shared characters, Penrith considered the Zophosini to be monophyletic. In order to satisfy the definition of genera, to avoid further splitting and to achieve stability in the nomenclature, Penrith (1980) fused all genera to one – *Zophosis* – the only genus in the tribe Zophosini that is united by a single synapomorphy: the oblique, more or less approximated metacoxae. The definition of a single genus for the tribe Zophosini, including over 300 species, illustrates the limitations of morphological characters for systematics in this case.

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We present the first, although preliminary, molecular approach to the systematic relationships among flightless species from the tribes Zophosini, Adesmiini and Eurychorini. This study will address three questions: Do molecular data support phylogenetic relationships within the Zophosini, constructed from morphological characters? Is the genus *Zophosis* monophyletic? Are estimates on divergence times of lineages, based on the characters studied, within the Namib Desert darkling beetles related to past geological events and climate changes?

MATERIALS & METHODS

Samples were provided by the Gobabeb Training and Research Centre (GTRC, 23°24′S, 15°03′E) in Namibia or were sampled during field-trips in July 2006 and April 2007 (permit numbers 1075/2006 and 1129/2007; Table 1). Further samples from Orupembe (northwestern Namibia, 18°18′S, 12°52′E, Table 1) were provided by the Museum of Natural History (Stuttgart, Germany). Specimens collected from the field were identified to species level using the reference collection available at GTRC by J. Meyer and subsequently confirmed by J. Steckel and M.L. Penrith. Specimens collected during field-trips were immediately stored in 70% ethanol, while samples provided from the Museum in Stuttgart were dry. The specimens provided by the GTRC were collected within the framework of the BIOTA-South Africa project. Those specimens were captured with Barber traps filled with mono-ethylene glycol and preserved in methyl alcohol. Unfortunately, these chemical compounds strongly interfered with DNA-extraction procedures and special treatments were required. DNA quality was substantially improved by passing extracts through semi-permeable filters (0.025 μm pore size; Millipore GmbH, Schwalbach, Germany) on distilled water. A further pre-treatment was the two-fold washing of the sample material in phosphate-buffered saline (PBS). From most beetles, one complete hind leg with coxa and one middle leg as well as the pronotum were ground. DNA was extracted using the 'DNeasy Tissue Kit' (Qiagen, Hilden, Germany). Whenever possible, DNA was extracted from several individuals per species and analysed for each specimen separately.

Partial sequences of the mitochondrial cytochrome oxidase II (*COII*) gene and the cytochrome b (*CytB*) gene were amplified by polymerase chain reaction (PCR). We chose these gene fragments due to their proven utility in insect phylogenetic studies (e.g. Contreras-Diaz *et al.* 2003; Vogler & Welsh 1997; Friedrich & Muquim 2003). Total reaction volume for PCR was 20 μl yielding 10x reaction buffer (Thermopol, NEB), 2.5 mM $MgCl_2$, one unit Taq-polymerase (Thermopol, NEB) and 13 to 450 ng template. Final primer concentration was 0.5 μM each for *COII* and 1 μM each for *CytB*, respectively. The dNTP concentration was 100 μM for CO II fragments and 200 μM for Cyt B, respectively. Primers for *COII* (TL-J-3037; 5′-TAATATG GCAGATTAGTGCATTGGA-3′ and TK-N-3785; 5′-GAGACCATTACTTGCTTTCAGTCATCT-3′) have been previously used by Contreras-Díaz *et al.* (2003) in a study on the darkling beetle genus *Pimelia* on the Canary Islands. PCR started with an initial cycle of 4 min at 95°C followed by 35 cycles of: 30 sec denaturation at 95°C, 1 min annealing at 50°C and 2 min extension at 72°C. Final extension lasted for 10 min at 72°C. *CytB* sequences were amplified using primers CB-J-10933 (5′-TATG TACTACCATGAGGACAAATATC-3′) and CB-N-11367 (5′-ATTACACCTCCTAATTTAT TAGGAAT-3′; Simon *et al.* 1994). DNA was amplified with an initial step of 5 min at 95°C followed by 40 cycles of 1 min denaturation at 95°C, 1 min annealing at 45°C and 90 sec extension at 72°C, with a final extension at 72°C for 10 min. The quality of PCR-products was assessed by gel-electrophoresis (2% agarose). Gels were stained with ethidium bromide (2 mg/l) and visualized under UV. PCR products were purified using the MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced by an external laboratory (Seqlab GmbH, Göttingen, Germany).

Trace files of sequences were edited and aligned manually using BIOEDIT (version 7.0.5.3; Hall 1999). Codon positions were assigned according to the mitochondrial genome of *Tribolium castanaeum* (accession no. AJ312413). Both genes were translated into amino acid sequences using MEGA version 3.1 (Kumar *et al.* 2004) to check for reading frame errors and stop-codons. Prior to the phylogenetic analyses, MODELTEST 3.7 (Posada & Crandall 1998) was used for selecting the substitution model which best described the data. Models were evaluated by three methods: the hierarchical Likelihood Ratio Test (Frati *et al.* 1997; Huelsenbeck & Crandall 1997; Posada & Crandall 1998), the corrected Akaike Information Criterion (Akaike 1973, 1974) and the Bayesian Information Criterion (Schwarz 1978).

We conducted Minimum Evolution (ME), Maximum Parsimony (MP), Maximum Likelihood

Table 1. Overview of the darkling beetle specimens included in our analyses. The specimens were provided by the Gobabeb Training and Research Centre (GTRC), the Natural Museum at Stuttgart (MNHS) or collected near Gobabeb (FT). Sequences of the outgroup *Pimelia* were obtained from Genbank (NCBI). The available partial sequences and their accession numbers (GenBank) are given.

Tribe/Genus/(Subgenus)/Species	Source	mtDNA-fragment	Accession No. (NCBI)
Zophosini			
Zophosis Solier, 1834			
Zophosis Latreille			
dorsata Péringuey, 1892	GTRC	CO II	EU342408
dorsata Péringuey, 1892	GTRC	CO II, Cyt b	EU342409, EU342394
Calosis Deyrolle stat. nov.			
amabilis Deyrolle, 1867	GTRC	CO II, Cyt b	EU342405, EU342397
amabilis Deyrolle, 1867	GTRC	CO II	EU342399
Gyrosis Gebien			
moralesi Koch, 1962	FT	CO II, Cyt b	EU342407, EU342398
moralesi Koch, 1962	GTRC	CO II	EU342406
orbicularis Deyrolle, 1867	FT	CO II, Cyt b	EU342404, EU342389
Occidentophosis Penrith stat. nov.			
parentalis Péringuey, 1908	FT	CO II, Cyt b	EU342401, EU342392
parentalis Péringuey, 1908	FT	CO II, Cyt b	EU342402, EU342391
parentalis Péringuey, 1908	MNHS	CO II, Cyt b	EU342400, EU342395
parentalis Péringuey, 1908	MNHS	CO II, Cyt b	EU342399, EU342396
damarina Péringuey, 1908	GTRC	CO II, Cyt b	EU342403, EU342393
Adesmiini			
Onymacris			
rugatipennis rugatipennis Haag, 1875	FT	CO II, Cyt b	EU342416, EU342384
rugatipennis rugatipennis Haag, 1875	FT	CO II, Cyt b	EU342413, EU342385
plana Péringuey, 1886	FT	CO II, Cyt b	EU342415, EU342386
laeviceps Gebien, 1938	FT	CO II, Cyt b	EU342414, EU342387
Stenocara			
gracilipes Solier, 1835			
Physadesmia			
globosa Haag-Rutenberg, 1875			
Eurychorini			
Stips			
stali Haag, 1875	FT	CO II, Cyt b	EU342412, EU342390
Pimeliini			
Pimelia Fabricius, 1775			
sparsa sparsa Brullé, 1838	NCBI	CO II, Cyt b	AJ536507, AJ565980
sparsa serrimargo Wollaston, 1864	NCBI	CO II, Cyt b	AJ536512, AJ565982
sparsa albohumeralis Lindberg, 1950	NCBI	CO II, Cyt b	AJ536495, AJ565981
granulicollis Wollaston, 1864	NCBI	CO II, Cyt b	AJ536538, AJ565983
estevezi Oromí, 1990	NCBI	CO II, Cyt b	AJ536527, AJ565984
fernandezlopezi Machado, 1979	NCBI	CO II, Cyt b	AJ536516, AJ565985

(ML) analyses and Bayesian inferences (BI) for constructing phylogenies. ME-analyses were conducted by MEGA (Kumar *et al.* 2004), while MP and ML-trees were constructed using PAUP (version 4.0 b 10, Swofford 1998). Analyses on BI were performed by MRBAYES (version 3.1.2, Ronquist & Huelsenbeck 2003) and BEAST (version 1.4.7; Drummond & Rambaut 2002). Results from the ME-approach derived from a heuristic tree search in MEGA on an initial neighbour-joining-tree. Gaps in the alignment were treated as complete deletion. Number of substitutions per site was calculated using the Tamura-Nei substitution model (Tamura & Nei 1993). For evaluation of nodes, 1000 bootstrap replicates were performed for each. The MP-analyses were

conducted on a equally weighted heuristic search with 1000 random stepwise sequence additions and 'tree bisection reconnection' (TBR) as branch swapping algorithm. A 50% majority-rule consensus tree was created. Nonparametric bootstrap analysis (Felsenstein 1985) with 1000 pseudoreplicates and ten random sequence additions were conducted through heuristic search. For the ML-analysis a heuristic search was performed via random stepwise sequence additions for ten replicates and subsequent TBR as branch swapping algorithm. Analyses on BI were run using a gamma distribution (eight rate categories). Model parameters were estimated within analyses runs, starting with random trees, running for 10^7 generations while sampling the Markov chains every 1000 generations. Running the analysis twice ensured that it was not trapped at local optima. Enhancement of the tree-climbing capability of the Markov chains was achieved by using one cold and three heated Markov chain Monte Carlo chains. The first 250 trees were discarded as burn-in (Huelsenbeck & Ronquist 2001; Knoop & Müller 2006).

Phylogenetic relationships between the taxa were inferred for the sequence data of *COII* and *CytB* singly as well as for the combined sequences of both partial genes.

Times of divergence among lineages were estimated using a Bayesian approach implemented in the package BEAST (Drummond & Rambaut 2002). Defining a calibration point for estimating divergence among Namib Desert darkling beetles lineages is a challenge, as no fossil records of darkling beetles from the Namib Desert are available (J. Henschel and G. Schneider, pers. comm.; see also Sole *et* al. 2005 and references therein). To overcome this problem we used sequence data of a group of six *Pimelia* (Tenebrionidae) species aged approximately 10 (9.3–10.7) Mya (Contreras-Díaz *et al.* 2003), which are endemic to the 15 My old island Gran Canaria (Canary Islands; Table 1). Likelihood of the Bayesian tree (MRBAYES) for the combined data set was –log 7004.9 without, and –log 7020.9 with enforcement of a molecular clock, respectively. The difference was not significant (LRT; $P = 0.13$), indicating a consistent molecular clock. Ages of nodes were drawn from 10^7 Markov-Chain-permutations, defining the first 1000 trees as burn-in and sampling every 1000th tree ($n = 9999$ trees). A relaxed log-normal molecular clock was enforced, using the GTR+G+I model and a Yule speciation process for setting priors.

RESULTS & DISCUSSION

We successfully amplified partial gene fragments of 604 bp of *COII* from 19 and 367 bp of *CytB* from 16 samples, representing 12 species (Table 1). Trees derived from analyses of individual gene regions were congruent regarding numbers of shared nodes and measures of support. Consequently, sequence data of all those individuals, for which fragments of both genes have been successfully amplified ($n = 16$) were combined, resulting in a data set comprising 16 samples with a total length of 971 bp each.

Saturation plots (not shown) of both gene fragments indicated multiple hits at the third codon position. However, results from several scenarios of weighting codon positions and allowing for different substitution rates always resulted in almost identical tree topologies. The combined sequence data for the ingroup taxa revealed 365 variable sites (37.6%) of which 20% occur at the first, 6% at the second and 74% at the third codon position. Of the 971 sites, 300 (30.9%) were parsimony-informative. The model of general time reversal with a gamma-distribution of rate heterogeneity (alpha = 0.76) and a proportion of invariable sites (46.8%; GTR+G+I) was, according to the hierarchical Likelihood Ratio Test, the corrected Akaike Information Criterion as well as the Bayesian Information Criterion, the most likely model of sequence evolution of our data set.

Phylogenetic trees constructed using the methods ME, MP and ML revealed almost identical topologies (consensus tree shown, Fig. 1). Combining data sets of both gene fragments always led to improved topologies in terms of higher support values. Those species belonging to the tribe of the Adesmiini have been grouped into one clade by all phylogenetic approaches. Within this clade, the genus *Onymacris* always received highest supporting values. Overall, phylogenies of Namib Desert darkling beetles suggested by molecular approaches were in agreement with previous phylogenies drawn from morphological characters.

The approaches of ME, MP and ML did not fully support monophyly in the tribe Zophosini (Fig. 1) as a polytomy occurred within the *Zophosis* species. The proposed subgenera *Occidentophosis* and *Gyrosis* were identified by those phylogenetic approaches. In contrast, results from BI-analyses showed a grouping of all Zophosini into one clade. This clade is sub-divided into two major lineages: the *Occidentophosis* on one hand and all the remaining species on the other

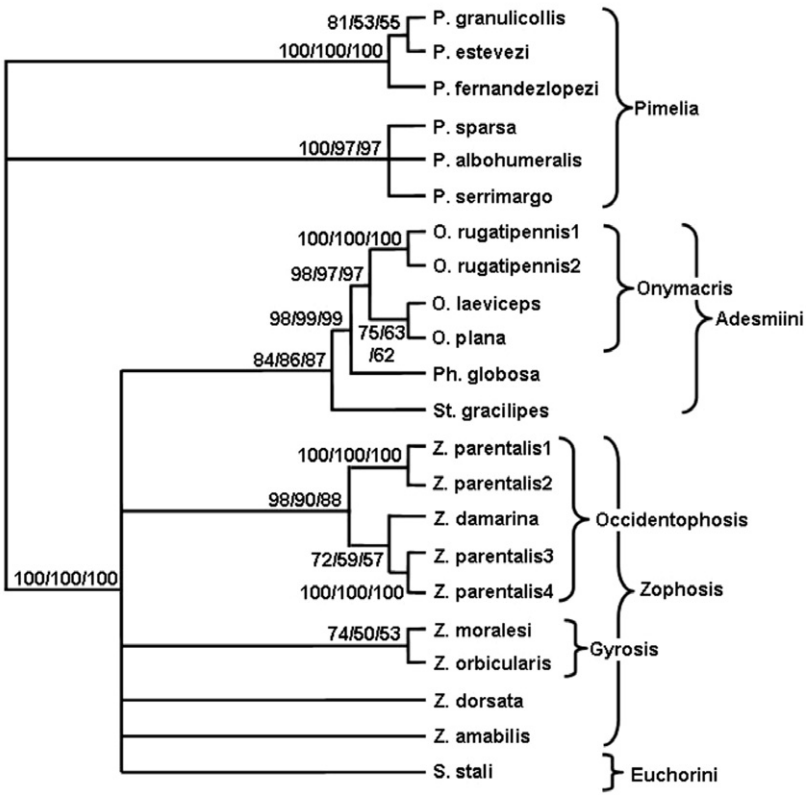

Fig. 1. Consensus tree on the phylogenetic relationships of darkling beetles from the Namib combining results from Minimum Evolution, Maximum Parsimony and Maximum Likelihood analyses. Supporting values (bootstrap) are shown at nodes. The genus *Pimelia* was used as outgroup.

hand with a 0.83 BI posterior probability.

No conclusions can be drawn on the phylogeny of the Euchorini, here represented by *Stips stali.* According to the consensus tree (Fig. 1), they were included in the polytomy along with the *Zophosis,* while they were arranged together with the *Pimelia* group by BI. However, the node separating the clades of the *Pimelia* group and *S. stali* had a posterior probability support value of less than 50% (Fig. 2). Thus, the alternative configuration of *S. stali* being a sister group to the Namibian and/or the Canary Island darkling beetles is possible.

Note the clear separation among specimens of *Z. parentalis* collected at Orupembe (labels 1 & 2 in Figs 1 & 2) and in the Namib Desert (labels 3 & 4).

This finding suggests the presence of either cryptic species or may represent phylogeographic variation at the two locations, which are about 600 km apart.

The subgenus *Gyrosis* showed low bootstrap support but had high posterior probability. The tree drawn from BI showed a clear separation of *Occidentophosis* and *Zophosis s. str.,* which corresponds to the lineages described by Penrith (1986, Fig. 11, lineage 4 and 7 therein). It reflects the different habitat preferences of both groups: the ancestor of the *Occidentophosis* was supposedly *Hologenosis*-like, a basic genus frequently occurring on hard substrates or under stones. Referring to four morphological categories that correspond with habitat preferences (Fig. 253 in Penrith 1977), *Occidentophosis* could indeed be assigned to a group harbouring species that hide under objects, like stones or debris, and emerge preferably during cooler parts of the day. The clade of the *Zophosis,* uniting all the remaining species, might also have derived from one common ancestor. All of them are diurnal, fast-running species living on the surface (category A of Penrith 1977).

Results from analyses performed within this study agree with the assumption of monophyly of *Zophosis.* It is, however, still open to further analyses and discussion if the *Zophosis* shall be considered as a genus. The similar genetic distances across

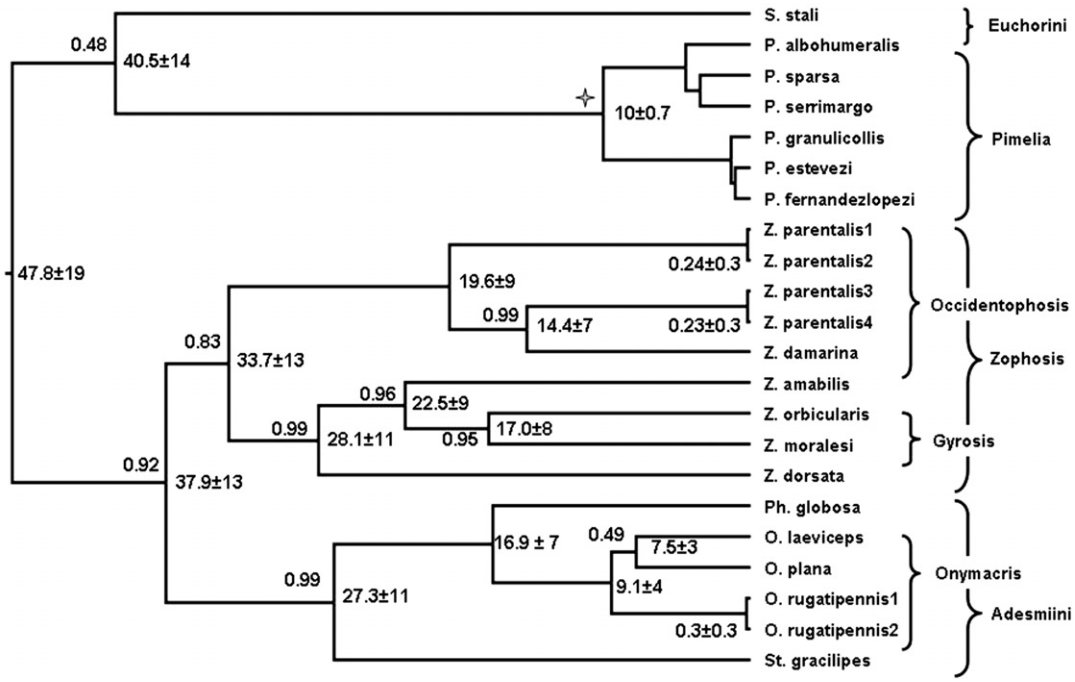

Fig. 2. Phylogenetic relationships among darkling beetle species from the Namib constructed by Bayesian interference. Values at nodes give mean estimated time of divergence and 95% credibility intervals. The node marked with the star was used as the calibration point. Posterior probabilities are indicated at nodes only if they were below '1'.

different proposed levels in both genes (Table 2) give reason for a critical revision of groups and hierarchical levels in the Namibian darkling beetles.

In Penrith's (1986) revision, *Calosis* and *Gyrosis* were described as '(ultimately) derivative subgenera' of *Zophosis s. str.* The latter was claimed to have arisen from an *Oculosis*-like ancestor during drier periods in Central Africa with arid corridors to East Africa (Endrödy-Younga 1978). *Calosis* is supposed to have evolved at an earlier stage, before psammophilous subgenera like *Gyrosis* evolved (Penrith 1986). This chronology is reflected in the position of *Z. dorsata,* followed by *Z. (Calosis) amabilis,* ending up with both *Gyrosis* species. Hence, the grouping of those species is in accordance with the suggested chronology.

Estimations of divergence times suggest two

Table 2. Average pairwise genetic *p*-distances in % within (diagonal) and between groups of Namibian darkling beetles. Uncorrected values are below, corrected values (according to K2P-model) above diagonal (first and second value at the diagonal, respectively).

	Pimelia genus	*Occidentophosis* subgenus	*Zophosis* genus	Adesmiini tribe
CO II				
Pimelia	6.5 / 6.9	23.2	21.1	22.4
Occidentophosis	19.9	10 / 11.1	16.9	19.1
Zophosis	18.3	15.0	10.4 / 11.5	18.0
Adesmiini	19.2	16.7	15.9	10.8 / 11.9
Cyt b				
Pimelia	8.8 / 9.7	23.3	26.4	24.8
Occidentophosis	19.9	11.4 / 12.9	22.3	22.3
Zophosis	22.1	19.1	16.6 / 18.9	24.1
Adesmiini	20.9	19.1	20.5	11.6 / 13

periods where lineages of the tribes studies diversified: a first around 30 to 40 Mya and a second at around 15 to 22 Mya (Fig. 2). Both periods can be related to historical events that intensified arid conditions in the Namib. About 35 My ago, Antarctica reached its present-day location at the South Pole. In the course of increasing glaciation of Antarctica, cold, upwelling current systems began to develop. The Benguela upwelling system on the west coast of Namibia is one of these. It started to develop in the Middle Miocene about 15–16 Mya and reached its full extension about 5–10 Mya (Van Zinderen Bakker 1975; Siesser 1978; Endrödy- Younga 1982; Goudie & Eckardt 1999; Pickford & Senut 1999; Ségalen *et al.* 2004). Within the genus *Zophosis* four out of six nodes (mean age ±95% CL) span the period of Benguela system establishment. Within the Adesmiini, the genus *Onymacris* probably radiated during that period.

The Benguela did not only intensify arid conditions in the Namib, it also provided an important source of water: fog. Some species like members of the subgenus *Gyrosis* (*Z. moralesi* and *Z. orbicularis*) and the genus *Onymacris* have adapted behaviourally (e.g. fog-basking in *O. unguicularis*) to the water-supply by fog (e.g. Seely 1979).

In a study on flightless Namib Desert dung beetles *Scarabeus (Pachysoma),* Sole *et al.* (2005) concluded a rapid radiation of dung beetles since the establishment of the Benguela current. For estimating divergence times based on sequence data of the *COI* gene, the authors applied a substitution rate (percent nucleotide change per million years) of 2.3%/My (Brower 1984).

Our results, calibrated against the age estimates of darkling beetles from the Canary Islands, suggest lower substitution rates in flightless Namib Desert darkling beetles. Genetic distances (Kimura-2P-distances) among the Namib tenebrionid beetle species of our data set range from 0.10 to 0.25 (mean 0.188). The resulting substitution rates among species were in the range of 0.63 to 1.28%/My and 0.43 to 0.55%/My among the groups of *Zophosis, Occidentophosis* and Adesmiini. Substitution rates of gene fragments studied in Namib Desert darkling beetles are considerably lower than those reported for other insects like Hawaiian *Drosophila* (about 2%/My; DeSalle *et al.* 1987), darkling beetles of the genus *Pimelia* from the Canary Islands (about 2%/My; Juan *et al.* 1995) and the general rate for arthropods of about 2.3%/My as supposed by Brower (1994). However, substitution rates are in good agreement with rates found in Mediterranean carabid species (0.54 to 0.98%/My; Prüser & Mossakowski 1998).

As the study presented here is of preliminary character, estimations on divergence times as well as on phylogenies may, of course, change by including sequences of additional genes, species and even haplotypes into analyses and by selecting other calibration points than in our study. Despite these limitations, our results allow the following observations: results generally support the phylogenies developed from morphological characters. They also provide support for the monophyly of the genus *Zophosis*. Past aridity-intensifying geological and climate change events are likelyto have triggered lineage divergences.

ACKNOWLEDGEMENTS

The study is dedicated to the 50th anniversary of the Gobabeb Training and Research Centre and is also in honour of the 70th birthday of Mary Seely, who encouraged research in the Namib for decades. We are thankful to W. Schawaller (Museum of Natural History, Stuttgart, Germany) for providing us with material. The study was approved by the Namibian Ministry of Environment and Tourism and the GTRC, and was funded by the German Federal Ministry of Education and Research (01LC0024).

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Responsible Editor: M.D. Picker