

# Phylogeny of the African ball-rolling dung beetle genus *Epirinus* Reiche (Coleoptera: Scarabaeidae: Scarabaeinae)

S. Mlambo,<sup>A,B</sup> C. L. Sole<sup>A</sup> and C. H. Scholtz<sup>A</sup>

<sup>A</sup>Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa.

<sup>B</sup>Corresponding author. Email: smlambo@zoology.up.ac.za

**Abstract.** Recent phylogenetic analyses have pointed to the dung beetle genus *Epirinus* as the putative African ancestral roller group. Consequently, we tested the roller status of species in the genus with observational studies and constructed a molecular phylogeny based on partial sequences of two mitochondrial and two nuclear genes for 16 of 29 species. Tested species were confirmed to be dung rollers. Monophyly of the genus was confirmed, lending support to the synonymy of the wingless genus *Endroedyantus* with *Epirinus*. Moreover, the phylogenetic hypothesis was found to have a similar topology with a previously published one based on morphological data. A combined molecular/morphology analysis showed congruence between the molecular and morphological datasets. The loss of flight in some species and estimated divergence dates within *Epirinus* are discussed.

## Introduction

The subfamily Scarabaeinae is the largest and most important group of dung insects. They have a worldwide distribution and the approximately 6000 described species are grouped into 334 genera (Scholtz *et al.* 2009). Scarabs are well-studied because evolutionary biologists have long been fascinated by their complex behaviours (Fabre 1918), unusual morphology and interesting biology (Halffter and Matthews 1966), but also because of their role in maintaining ecosystem functionality and as biodiversity indicators (Davis *et al.* 2004; Nichols *et al.* 2008). Members of the subfamily occur across different biogeographic regions (Scholtz *et al.* 2009) in a wide range of habitats ranging from forests, grasslands and mountains to deserts. The Afrotropical region

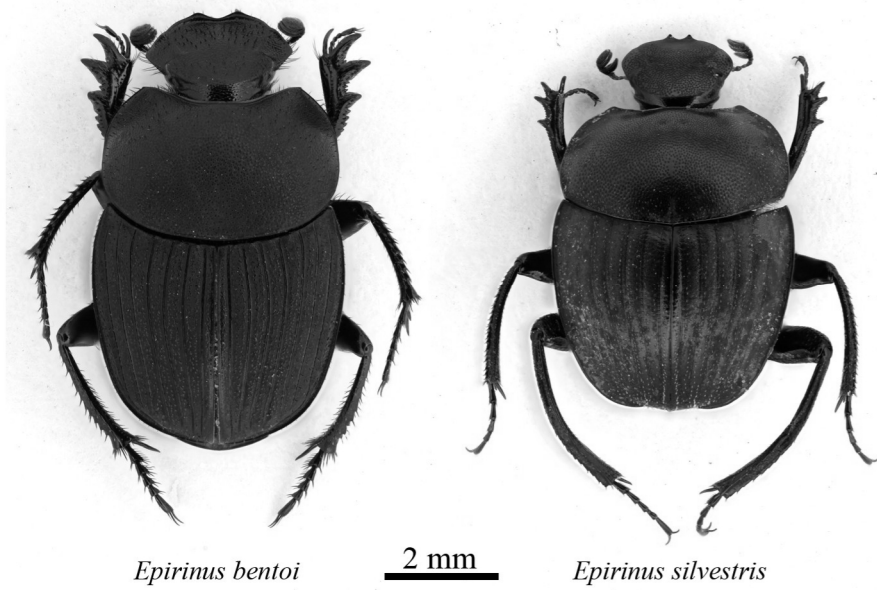
represents the richest in dung beetle diversity with more than 2000 species (Scholtz *et al.* 2009) of which *ca* 780 are found in southern Africa (Davis *et al.* 2008).

Food relocation behaviour has traditionally been used to classify dung beetles. “Tunnellers” bury dung beneath the dung source while “rollers” form a piece of the dung into a ball, which they roll away and bury at some distance from the dung source. Although the global Scarabaeinae dung beetle fauna is divided into 12 tribes (Hanski and Cambefort 1991), some of these divisions lack phylogenetic support (Medina *et al.* 2003; Philips *et al.* 2004; Monaghan *et al.* 2007; Davis *et al.* 2008; Sole and Scholtz 2010). The current classification is considered to be based on two large ‘monophyletic’ groups (Cambefort 1991): 1), one containing six tribes of “tunnellers” with Dichotomiini being basal; and 2), the other consisting of six tribes of “rollers” with Canthonini as the basal tribe. The two basal tribes are thought to be of Gondwana origin and that the distribution of the modern representatives reflects this ancient ancestry. However, the phylogenetic relationships within and among them are poorly resolved.

The differentiation between dung beetles that roll and those that tunnel has been the focus of several recent phylogenetic studies in an attempt to explain their evolutionary history, and some have revealed that rollers and tunnellers are phylogenetically mixed and that the relationships within the groups are not as simple and clear-cut as previously thought. Philips *et al.* (2004) used 200 morphological characters in their analysis which included representative genera from all 12 tribes and concluded that the ancestral Scarabaeinae was a tunneller and that rolling has evolved independently a number of times. Monaghan *et al.* (2007) performed an extensive molecular phylogenetic analysis and came to several of the same general conclusions as those of Philips *et al.* (2004). One of these was that the basal extant dung beetle groups are African genera currently placed in the Canthonini and Dichotomiini. Sole and Scholtz (2010) analysed several gene regions of most of the African genera in these two tribes and confirmed that they are, indeed, polyphyletic and that none of the most basal groups actually rolls. The Sole and Scholtz (2010) study left the canthonine genus *Epirinus*, which was considered to be a genus of “rollers” in previous studies, although this was untested, as the most basal rolling dung beetle group of the African genera. In view of the fact that all of the basal “rollers” have now been shown to not roll (Sole and Scholtz 2010) and that *Epirinus* is apparently the most basal African roller, it warranted further study.

Average body length of *Epirinus* species ranges from approximately 2.5 mm to 12.0 mm. The larger species are known to roll balls from the herbivore dung with which they are associated. Some of the smallest species had only been collected on carrion and in leaf litter and were therefore thought not to exhibit this behaviour (Scholtz and Howden 1987). For “rollers” to be able to form and roll dung balls away from the dung pat, the dung must be malleable. This is facilitated by the high percentage of water in fresh herbivore dung (Holter and Scholtz, 2005). In their studies of the canthonines and some of the food choice restrictions faced by rollers, Scholtz *et al.* (2009) suggested that in addition to malleability of the food, body size could also be a constraint on the ability to form and roll balls, with beetles smaller than about 7.0 – 8.0 mm possibly incapable of rolling. To date most species of *Epirinus* recorded as exhibiting ball rolling behaviour have a body length longer than about 8.0 mm. With species both smaller and larger than this within the genus, representatives of *Epirinus* are suitable candidates for testing the hypothesis that size may be a constraint to making and rolling dung balls. This is of particular interest because of the genus’ position as the basal roller among the African genera of the subfamily Scarabaeinae.

In addition to considerable size variation members of the genus *Epirinus* vary in the presence or absence of wings and in their perceived ability to roll since very small species have been considered physically incapable of forming and rolling balls (Scholtz *et al.* 2009). In view of some of these differences *Epirinus* was previously separated into two genera, *Endroedyantus* Cambefort and *Epirinus* Reiche, based on the morphological differences associated with the presence or absence of wings. *Endroedyantus*, representing the flightless group/genus, was synonymised with *Epirinus* by Scholtz and Howden (1987) in their revision of the genus. The flightless species have also subsequently been shown to comprise two distantly related lineages within the group (Medina and Scholtz 2005) indicating at least two flight loss events. To date *Epirinus* comprises 29 described and some undescribed species making it by far the largest group of relictual “canthonines” in Africa. All *Epirinus* species are restricted to southern Africa (Scholtz and Howden 1987; Medina and Scholtz 2005). Of the described species, 10 are wingless. Winged and wingless species, *E. bentoi* and *E. silvestris* respectively, are shown in Figure 1. The wingless species are distributed mainly in isolated forest patches along the southeast or northeast coastal regions of southern Africa while the winged species are more widespread in habitats which include inland open grassland and the Nama-Karoo biome.



**Figure 1.** Photograph showing some superficial morphological differences between winged (*Epirinus bentoii*) and wingless (*E. silvestris*) species.

Consequently, *Epirinus* is a good model group to test for relatedness among winged and flightless species and to answer questions relating to the effects of flight loss on speciation; and whether small size is a constraint on the ability to roll. In this study we used partial gene sequences from two nuclear and two mitochondrial genes for a molecular analysis of relationships amongst sixteen *Epirinus* species as well as an analysis combining molecular and morphological data. From these we aimed to compare the result of the combined analysis with the morphology-based hypothesis of *Epirinus* phylogenetics by Medina and Scholtz (2005). This would provide for a better understanding of the evolutionary trends relating to flightlessness within the genus and together with the observations of food relocation behaviour of the smaller species within the genus, confirm them as true rollers. Lastly speciation and biogeography of *Epirinus* in southern Africa are discussed.

## **Materials and Methods**

### *Sampling for observation of food relocation behaviour*

Dung beetles were trapped in localities where the target beetles (body length less than 7.0 mm) are known to occur. Live beetles were trapped in standard baited pitfall traps. Cow and pig dung were used for bait. Species collected were *E. scrobiculatus* (6.8 mm), *E. aeneus* (5.9 mm), *E. comosus* (5.2 mm), *E. convexus* (4.0 mm), *E. ngomae* (3.7 mm) and *E. hluhluwensis* (3.3 mm). Body length was calculated as the average of the maximum length listed in the literature (Scholtz and Howden 1987; Medina and Scholtz 2005). The first three species are found mostly in the Fynbos, a habitat of mixed scrub with broken canopy, whereas the remaining three species occur in dense wet forest (Scholtz and Howden 1987; Medina and Scholtz 2005). Little is known about the dung preference among these species.

At least five individuals per species were placed in each of at least two observation chambers and provided with cow dung to determine food relocation behaviour. Observations of the behaviour of the dung beetles were made during the day as it is thought that *Epirinus* species have diurnal activity patterns (Davis *et al.* 2008).

### *Taxa for phylogenetic study*

Sixteen of the 29 described species of *Epirinus* were included in this phylogenetic study including representatives from each of the four major groupings obtained in the morphological phylogeny (Medina and Scholtz 2005). Three members of the Canthonini, *Circellium bacchus*, *Gyronotus pumilis* and *Anacalchos convexus* were used as out-group representatives as used by Medina and Scholtz (2005) for direct comparison between the molecular and morphological data. However, the phylogenetic study of the Canthonini and Dichotomiini by Sole and Scholtz (2010) places them in derived positions relative to *Epirinus*, therefore *Namakwanus davisi* and *Dicranocara deschodti* were added as out-groups as they appeared to be sister and basal to the genus. The new out-group species were used in all analyses that were not used for direct comparison with the morphological data. Details of the individuals and species used are presented in Table 1.

### *DNA extraction and sequencing*

Four gene regions were used for the phylogenetic reconstruction of *Epirinus*. Sequence data were generated from two mitochondrial genes - cytochrome oxidase I (COI) and 16S ribosomal RNA (16S rRNA) - a portion of the nuclear rRNA large subunit – 28S (28S rRNA) domain 2 – and the CPSase region of Carbamoyl-phosphate synthetase-aspartate transcarbamoylase-dihydroorotase (CAD). These gene regions were chosen as they have been shown in previous studies to be phylogenetically informative in the Coleoptera and other insect groups (Sole *et al.* 2005; Forgie *et al.* 2006; Orsini *et al.* 2007; Monaghan *et al.* 2007; Wahlberg and Wheat 2008; Wild and Maddison 2008; Sole and Scholtz 2010). Muscle tissue from the metathoracic femur was used for extraction from at least three individuals per species wherever possible, using the Roche High Pure PCR Template Preparation Kit (Roche, Penzberg, Germany). Primers used for the gene regions are shown in Table 2. For COI, 16S and 28S domain 2, polymerase chain reactions (PCR) were performed in a final volume of 50µl containing 1x buffer, 25mM MgCl<sub>2</sub>, 10mM DNTP's, 20pmol of each primer and 1U of Super-Therm® DNA polymerase (Southern Cross Biotechnology). Thermal cycling conditions were as follows: COI- denaturing at 94°C for 90 seconds, followed by 35 cycles of 94°C for 22 seconds, primer annealing at 48-52°C for 30 seconds and elongation at 72°C for 90 seconds, followed by a final elongation at 72°C for 60 seconds; 16S - initial denaturation at 94°C for 90 seconds, followed by 35 cycles of 94°C for 60

**Table 1.** *Epirinus* in-group and the out-group (OG) species sequenced for the study. – indicates PCR amplification failed, \* indicates specimens used in the combined data analysis.

Name	Size (mm)		Locality	Specimen ID	GenBank accession numbers			
					COI	16S	28S	CAD
<i>Epirinus aeneus</i>	5.6	winged	Darling	EADAR01*	-	GQ289727	GQ289866	GQ289935
				EADAR03	GQ290002	GQ289728	GQ289867	GQ289936
				EADAR04	GQ290003	GQ289729	GQ289868	GQ289937
<i>E. aquilus</i>	3.4	wingless	Alexandria Forest	EAQA01	-	HQ289896	HQ289945	-
				EAQA02	HQ289974	HQ289897	HQ289944	-
				EAQA03*	HQ289975	HQ289898	HQ289943	HQ289923
<i>E. comosus</i>	5.2	winged	Koeggelberg	ECOK01	HQ289991	-	-	HQ289924
				ECOK02	HQ289993	-	HQ289946	HQ289925
				ECOK03*	HQ289992	HQ289899	HQ289947	HQ289926
<i>E. convexus</i>	4.0	winged	Dwesa Forest	ECDFT02*	GQ289984	GQ289704	GQ289841	-
				ECDFT05	GQ289985	GQ289705	GQ289842	GQ289924
				ECDFT07	GQ289986	GQ289706	GQ289843	GQ289925
				ECDFT14	-	GQ289707	GQ289844	-
<i>E. flagellatus</i>	8.8	winged	Koeggelberg	EFLK01*	HQ289983	HQ289903	HQ289951	HQ289929
				EFLK02	HQ290000	HQ289904	HQ289952	-
				EFLK03	HQ290001	HQ289905	HQ289953	HQ289928
<i>E. hluhluwensis</i>	3.3	wingless	Hluhluwe	EHLH01	HQ289995	HQ289906	HQ289954	-

				EHLH02*	HQ289996	HQ289907	HQ289955	HQ289930
				EHLH03	HQ289997	HQ289908	HQ289956	HQ289931
<i>E. minimus</i>	2.3	wingless	Alexandria Forest	EMIA02*	HQ289978	-	-	-
<i>E. ngomae</i>	3.7	Wingless	Ngome forest	ENGN01*	HQ289999	HQ289909	HQ289957	HQ289932
				ENGN02	HQ289987	HQ289910	HQ289958	HQ289933
				ENGN03	HQ289998	HQ289911	HQ289959	HQ289934
<i>E. obtusus</i>	8.0	Winged	Cradock	EOBC01	HQ289982	HQ289912	HQ289960	HQ289935
				EOBC02	HQ290004	HQ289913	HQ289961	-
				EOBC03*	-	HQ289914	HQ289962	HQ289936
<i>E. pygidialis</i>	6.9	Winged	Utrecht, Lentz Farm	EPYU01*	HQ289994	-	HQ289963	-
<i>E. relictus</i>	7.8	winged	Karkloof NR	EREK01*	HQ290003	HQ289915	HQ289964	HQ289937
<i>E. scrobiculatus</i>	6.8	winged	Vanrhynsdorp	ECRV01*	HQ289979	HQ289900	HQ289948	HQ289927
				ECRV02	HQ289980	HQ289901	HQ289949	-
				ECRV03	HQ289981	HQ289902	HQ289950	-
<i>E. sebastiani</i>	3.4	wingless	Alexandria Forest	ESEA01	HQ289976	-	HQ289965	HQ289938
				ESEA02*	HQ290002	HQ289916	HQ289966	HQ289939
				ESEA03	HQ289977	HQ289917	HQ289967	HQ289940
<i>E. silvestris</i>	5.0	wingless	Outenikwa Pass	EH01*	GQ290019	GQ289750	GQ289800	GQ289946
				EH02	GQ290020	GQ289751	GQ289801	GQ289947



				ESUL01	HQ289989	-	HQ289968	-
<i>E. sulcipennis</i>	10.3	winged	Lesotho	ESUL02*	HQ289990	HQ289918	HQ289969	HQ289941
				ESUL03	HQ289988	HQ289919	HQ289970	HQ289942
<i>E. validus</i>	11.8	winged	Grahamstown	EVDG01*	HQ289985	HQ289920	HQ289971	-
				EVDG02	HQ289986	HQ289921	HQ289972	-
				EVDG03	HQ289984	HQ289922	HQ289973	-
<i>Anachalcos</i>	23.8	winged	Island Blue	ACIB01*	GQ289996	GQ289717	GQ289768	GQ289929
<i>convexus</i> (OG)				ACIB02	GQ289997	GQ289718	GQ289769	-
<i>Circellium</i>	37.3	wingless	Addo	C1*	GQ290011	GQ289737	-	-
<i>bacchus</i> (OG)			Elephant Park	C3	GQ290012	GQ289738	GQ289876	GQ289942
<i>Dicranocara</i>	10.5	wingless	Boom River	DC01*	GQ290014	GQ289740	GQ289793	GQ289944
<i>deschodti</i> (OG)				DC02	GQ290015	GQ289741	GQ289794	GQ289945
<i>Gyronotus</i>	12.6	wingless	Bosbok	BSGP01*	GQ289993	GQ289714	GQ289765	GQ289950
<i>pumilis</i> (OG)			Strand	BSGP02	GQ289994	GQ289715	GQ289766	GQ289951
<i>Namakwanus</i>	8.0	wingless	Hardap Dam	NHD01*	GQ290021	GQ289745	GQ289802	GQ289955
<i>davisi</i> (OG)				NHD02	GQ290022	GQ289746	GQ289803	GQ289956

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**Table 2.** Primers used for PCR amplification

Locus	Primer name and sequence	Reference
Cytochrome oxidase 1	C1-J-2183 (5'CAACATTTATTTTGATTTTTTGG 3')	Simon <i>et al.</i> (1994)
	TL2-N-3014 (TCCAATGCACTAATCTGCCATATTA 3')	Simon <i>et al.</i> (1994)
16S rRNA	16Sb2 (5' TTTAATCCAACATCGAGG 3')	Vogler, <i>et al.</i> (1993)
	LR-N-13398 (5' CGCCTGTTTAACAAAAACAT 3')	Simon <i>et al.</i> (1994)
28S rRNA domain 2	D2-3551 (5' CGTGTTGCTTGATAGTGCAGC 3')	Gillespie <i>et al.</i> (2005)
	D2-4057 (5' TCAAGACGGGTCCTGAAAGT 3')	Gillespie <i>et al.</i> (2005)
CAD	54F (5' GTNGTNTTYCARACNNGGNATGGT 3')	Moulton and Wiegmann (2004)
	680R (5' AANGCRTCNCGNACMACYTTCRTAYTC 3')	Moulton and Wiegmann (2004)
	338F (5' ATGAARTAYGGYAATCGTGGHCAAYAA 3')	Winterton <i>et al.</i> (2010)
	654R (5' TCYTTCCANCCYTTYARSGATTTTRTC 3')	Winterton <i>et al.</i> (2010)

seconds, annealing 48-50°C for 60 seconds and 72°C for 90 seconds with a final elongation at 72°C for 3 minutes; 28S domain 2 a three cycle touchdown PCR program was followed: initial denaturation at 96°C for 20 seconds, followed by 3 cycles of (15s at 96°C, 20s at 54-55°C, 1 min at 72°C) then 7 cycles (12s at 96°C, 18s at 53-54°C, 55s at 72°C) and 30 cycles (12s at 96°C, 15s at 52-53°C, 50s at 72°C) with a final extension of 1 minute at 72°C.

The amplification of the desired CAD fragment was a two-step process. Thermal cycling was performed in a final volume of 25µl containing 1x buffer, 25mM MgCl<sub>2</sub> 10mM dNTP's, 10pmol of each primer and 0.15 U of SuperTherm® polymerase. Dimethyl sulphoxide (DMSO) was added to both amplification steps to improve the sensitivity of the reaction. For the first amplification step primers 54F and 680R were used in a three-cycle touchdown PCR: initial denaturation at 94°C for 4 minutes followed by 4 cycles (30 s at 94°C, 30 s at 51°C, 2 min at 72°C), 6 cycles (30 s at 94°C, 1 min at 47°C, 1 min at 72°C and 36 cycles (30 s at 94°C, 20 s at 42°C, 2½ min at 72°C) and a final extension of 3 min at 72°C. Touchdown PCR is useful for improving the specificity, sensitivity and yield of PCR amplification (Korbie and Mattick 2008) particularly for templates that are difficult to amplify such as CAD. The gradual lowering of temperature to the calculated annealing temperature during cycling favours amplification of the desired product. In the second amplification step 1µl of the resulting amplified product was used in the same reaction mixture except at a 1.5mM MgCl<sub>2</sub> concentration using internal primers 338F and 654R under the following conditions: initial denaturation for 4min at 94°C followed by 4 cycles (30 s at 94°C, 30 s at 51°C, 80 s at 72°C), then 36 cycles (30 s at 94°C, 30 s at 45°C, 80s at 72°C) and a final extension of 3 min at 72°C in a Geneamp® PCR System 9700 (Applied Biosystems). Forward and reverse strands were sequenced using the amplification primers and BigDye v.3.1 (ABI).

#### *Phylogenetic analysis and dating*

The sequences were viewed in Chromas version 2.0, assembled and edited in CLC Bio Main Workbench 5 (<http://www.clcbio.com>), and subsequently aligned in MAFFT version 6 (Kato and Toh 2008). Thereafter the sequences were adjusted manually. The phylogenetic relationships among *Epirinus* sequences were inferred using maximum parsimony (MP) and maximum likelihood (ML) analyses implemented in PAUP\*4.010b (Swofford 2003) while MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) was used to infer a Bayesian phylogram.

Phylogenetic trees were generated for individual and combined gene regions. For the Parsimony analysis all characters were equally weighted and unordered and tree statistics calculated excluding uninformative sites. Heuristic searches with 10 random addition sequences were used for each of 1000 bootstrap replicates. Partitioned Bremer Support (PBS) (Baker *et al.* 1998) and Partition Congruence Indices (PCI) (Brower 2006) implemented in PAUP\* were applied using TreeRot version 3 (Sorenson and Franzosa 2007) to test whether these DNA sequences are congruent with the morphological data set analysed elsewhere (Medina and Scholtz 2005). Conflict between the molecular and morphological data was identified and the relative contributions of the different data sets to the phylogenetic tree were determined. When there is no conflict between partitions PCI and PBS values are equal and as conflict increases, PCI values decrease until they become negative (Brower 2006).

Maximum likelihood analyses employed MrModeltest version 3.7 (Nylander, 2004) (Table 3) to determine the model of sequence evolution which best fit the DNA data. Parameters from MrModelTest were used in a heuristic search and nodal support was estimated using 1000 bootstrap replications. Bayesian analysis allows the data to be partitioned and optimal models of nucleotide substitution applied to each partition. The model of nucleotide substitution for each gene region was assessed using the Akaike Information Criteria (AIC) in MrModeltest version 3.7 (Nylander 2004) (Table 3). In both molecular and combined molecular and morphological data analyses 5 million generations were run with 3 cold and one heated chain with trees sampled every 100 generations. Posterior probabilities were calculated after discarding the initial 20% as burn-in. Two independent runs were performed for each analysis.

There are many approaches to molecular dating. To estimate divergence times of the different lineages within *Epirinus* we chose a method that corrects for evolutionary rate heterogeneity among gene regions (Rutschmann 2006). The program Beast version 1.4.8 (Drummond and Rambaut 2007), which uses MCMC estimation, was used to estimate lineage ages. Cytochrome Oxidase I data for all the individuals successfully sequenced were used. The published rates of evolution of 0.0075 and 0.012 mutation changes per million years were used to cover the range of rates reported (Brower 1994; Juan *et al.* 1995; Farrell 2001; Smith and Farrell 2005; Wirta *et al.* 2008; Sole and Scholtz 2010). Lineage age was estimated under the log normal uncorrected model assuming the Yule speciation for all estimates using the GTR+I+G

**Table 3.** Estimated model parameters and data characteristics for 16S, COI, 28S domain 2, CAD and combined dataset for *Epirinus*.

	<b>16S</b>	<b>COI</b>	<b>D2</b>	<b>CAD</b>	<b>Combined</b>
Number of samples	36	38	40	27	16
Aligned positions	396	748	591	899	2639
Parsimony informative sites	206	179	142	230	826
Tree length (MP)	417	528	248	477	2030
Number of trees (MP)	100	100	27	100	1
CI/RI	0.729/0.858	0.489/0.486	0.802/0.867	0.669/0.682	0.586/0.656
Best-fit model	GTR+G	GTR+I+G	GTR+I	GTR+G	GTR+I+G
A frequency	0.3522	0.3386	0.19	0.2929	0.2863
C frequency	0.1313	0.123	0.3063	0.216	0.2004
G frequency	0.1508	0.1268	0.3313	0.2346	0.2128
T frequency	0.3656	0.4116	0.1724	0.2565	0.3006
Gamma	0.9625	1.1077	equal	0.291	1.2154
Invariable sites	0	0.4652	0.4985	0	0.3929

model (Table 3). The analyses were run for 170 million generations starting from a random tree and the results were analysed with the programme Tracer version 1.3 (Drummond and Rambaut 2007). The analyses were run twice to validate consistency of the time estimates between runs.

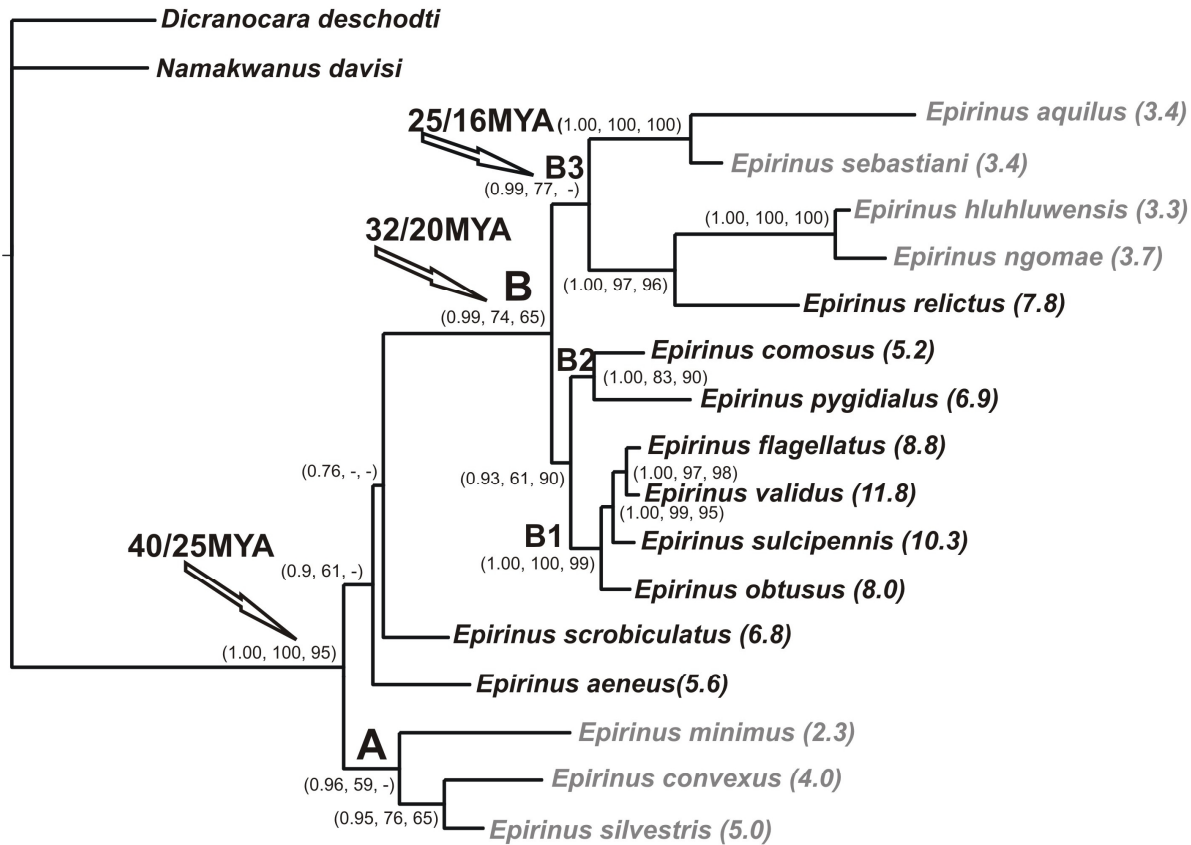
## Results

### *Food relocation behaviour*

All of the six small *Epirinus* species collected to test for rolling behavior were observed to roll dung balls in typical scarabaeine fashion for the first time (except *E. aeneus* reported by Davis *et al.* (2008)). Both round (food) and pear-shaped (brood) balls were observed for *E. ngomae* and *E. hluluwensis* while *Epirinus scrobiculatus* rolled irregularly shaped balls.

### *Molecular phylogenetic analysis*

The complete assembled matrix across four gene regions consisted of 2639 aligned base pairs (bp) from 16 of 29 species. The matrix was made up as follows: COI mtDNA  $\approx$  748 bp/179 parsimony informative sites (PI); 16S rRNA  $\approx$  414 bp/206 PI, 28S D2 rRNA  $\approx$  628 bp/142 PI and CAD  $\approx$  899 bp/230 PI. Table 3 shows the other statistics associated with the parsimony analysis. The genus is monophyletic based on all three tree drawing methods (posterior probability = 1.00; ML bootstrap value = 100%; MP bootstrap value = 95 %). The three trees were similar in topology and only the Bayesian phylogram is presented (Figure 2) with posterior probabilities, ML and MP bootstrap support. Relationships in the Bayesian and ML analyses were the same, however, overall the Bayesian phylogram was better resolved with moderate to strong support. In the parsimony analysis *E. aquilus* + *E. sebastiani*, are sister to the rest of the species in lineage B and *E. scrobiculatus*, *E. aeneus*, *E. minimus* and *E.convexus* + *E. silvestris* form a polytomy at the base of the tree. Figure 2 shows lineage A consisting of three species, *E. silvestris*, *E. convexus* and *E. minimus* with strong support (0.96 posterior probability) as sister to the rest of the species in the genus. The species *E. aeneus* and *E. scrobiculatus* branch off in succession next. Lineage B consists of three groups. B1 includes *E. obtusus*, *E. sulcipennis*, *E. validus* and *E. flagellatus* (1.00 posterior probability and 99 % MP bootstrap value) and sister to that are *E. comosus* and *E. pygidialis* in lineage B2, (1.00 posterior probability and 90 % MP



**Figure 2.** Molecular (16S, COI, D2 and CAD) Bayesian inference phylogram. Posterior probabilities, maximum likelihood and parsimony bootstrap values indicated where 0.5/50% or higher. Divergence times for the flightless lineages are indicated at nodes A, B and B3. Flightless taxa in grey font. Size in brackets in mm in front of species name.

bootstrap value). Lineage B<sub>3</sub> with posterior probability 0.99, includes *E. relictus* sister to the flightless pair *E. hluluwensis* and *E. ngomae*, which all share the same ancestor with the flightless pair *E. sebastiani* and *E. aquilus*.

### *Congruence of molecular and morphological data sets*

#### Phylogenetic trees

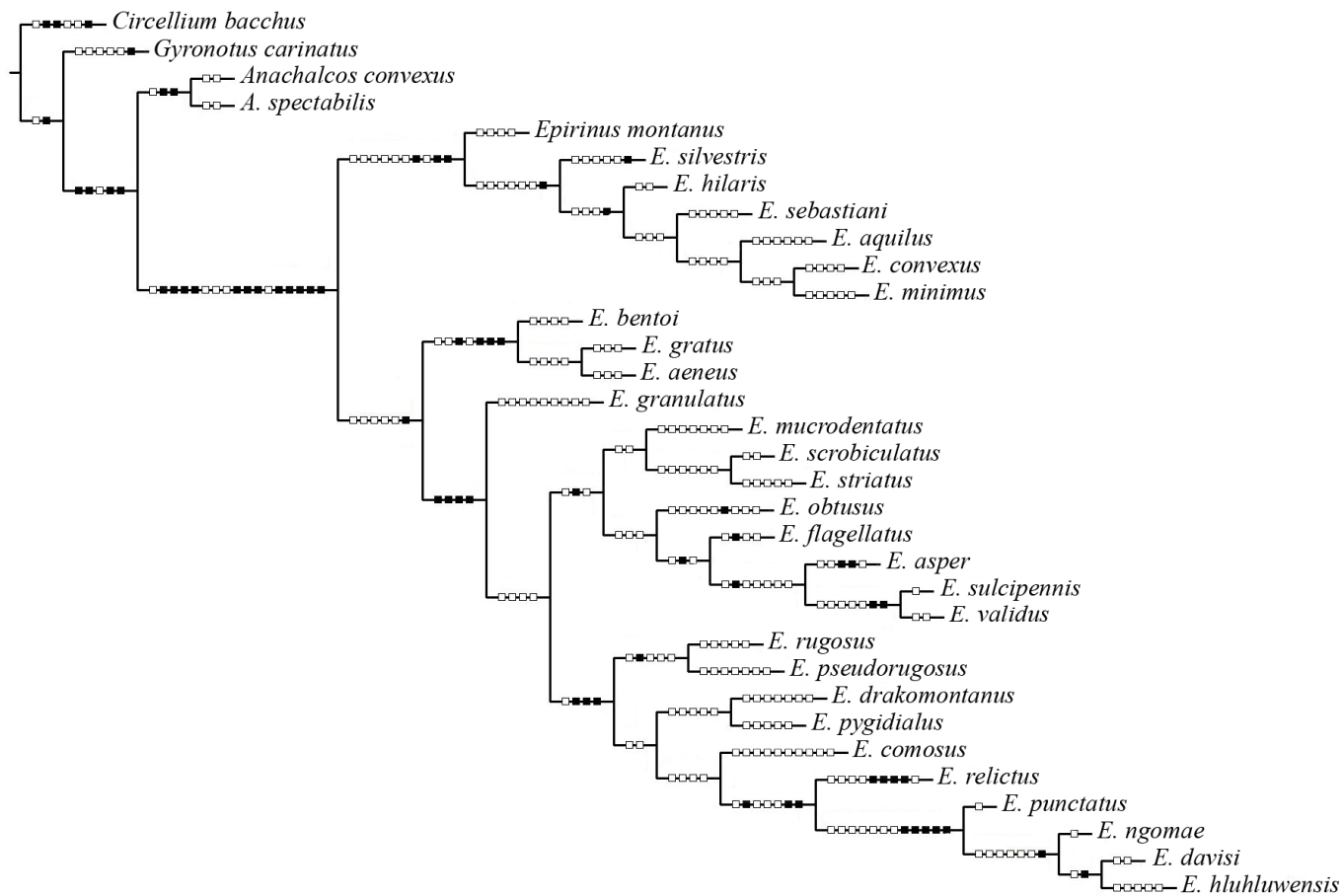
Phylogenetic relationships within *Epirinus* were investigated by Medina and Scholtz (2005), shown in Figure 3. Their analysis was based on 79 morphological characters including male and female genitalia. Monophyly of the genus was supported. However, as in other scarabaeines (Philips *et al.* 2004), there was a high degree of homoplasy in the morphological characters that were used.

The combined molecular and morphological phylogeny Bayesian phylogram (Figure 4) is largely congruent with the morphological species groupings of Medina and Scholtz (2005) (Figure 3) according to both Bayesian and Parsimony analyses. Essentially the species are grouped in the same way except that in the molecular phylogeny *E. aquilus* and *E. sebastiani* (Figure 2) are placed among the more derived group of flightless species whereas the morphological data places them in the basal clade of flightless *Epirinus*. The molecular tree is more reliable for this clade (B<sub>3</sub>) as the morphological *E. sebastiani* (*E. aquilus* (*E. convexus* + *E. minimus*)) clade is poorly supported without a single unique character supporting it.

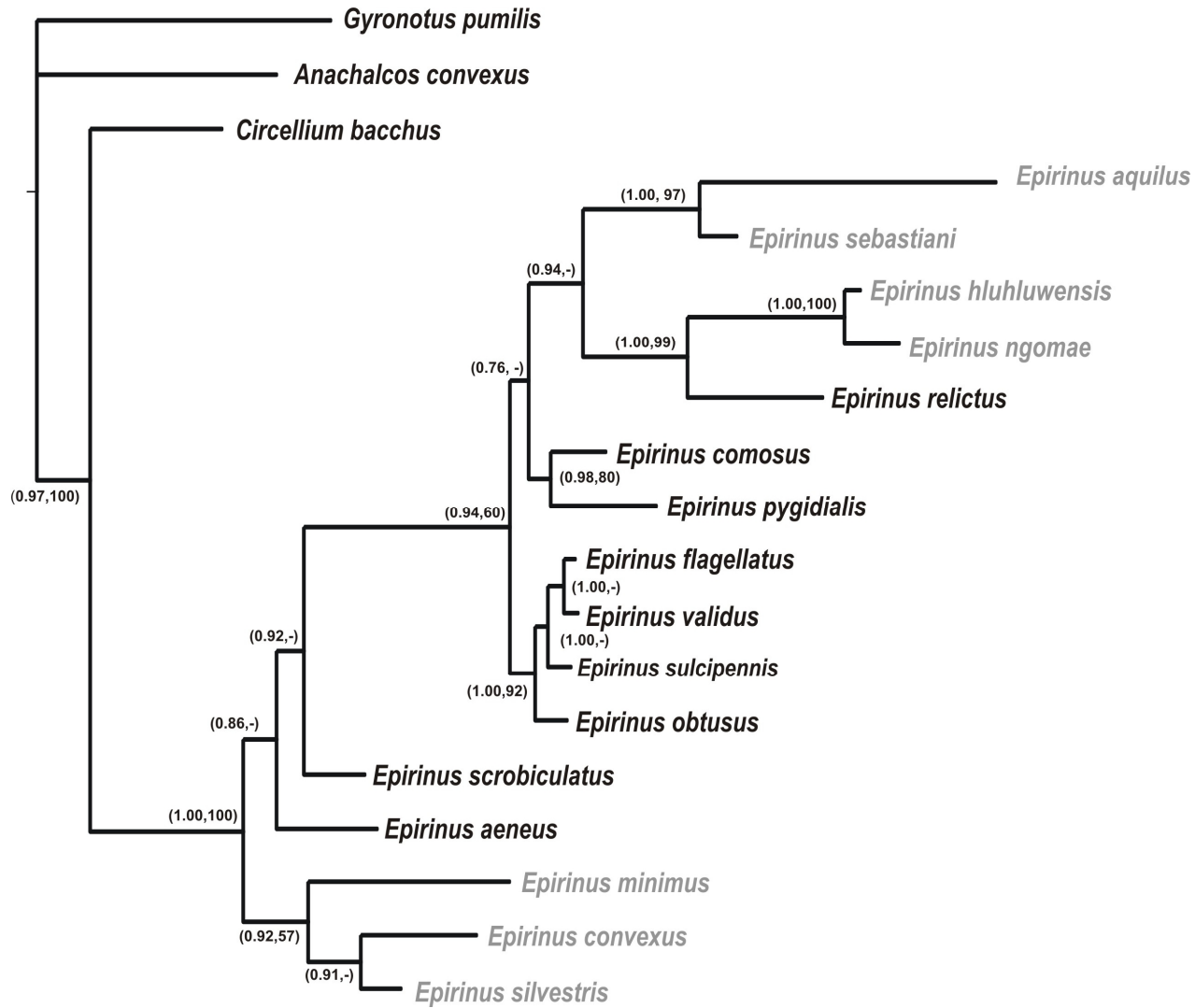
#### Bremer Support

An assessment of the relative contribution of each data partition of the combined tree indicated that most conflicts were from the nuclear gene partitions CAD and D2. Three of the 15 nodes in the parsimony phylogram had negative PCI values indicating strong incongruence between the data sets (Table 4; Figure 5). For nodes 2 and 4, D2 contained the most nodal conflict while for node 8 it was COI. Lending the most support to the nodes was COI (14) dataset followed by 16S (10) and thereafter the morphological data contributed to eight nodes.





**Figure 3.** Strict consensus tree from 3 cladograms (331 steps, CI = 40, RI = 67) based on morphological characters. Black hatch marks indicate changes without homoplasy and white hatchmarks indicate changes with homoplasy. Reproduced from Medina and Scholtz (2005).



**Figure 4.** Combined morphological and molecular Bayesian inference phylogram with posterior probabilities and parsimony bootstrap values respectively where 0.5/50% or higher. Flightless taxa – grey font.

**Table 4.** Bremer support indices calculated from the parsimony analysis. Node numbers correspond to those on the parsimony phylogram (Fig. 5)

Node	Bremer	PCI	PBS values				
			D2	COI	16S	CAD	morphology
1	16	19.5	-2.5	1	4	-1	14.5
2	0.3	-21.3	-2.5	0	4	-1	-0.5
3	0	0	2.5	0	4	-1	-0.5
4	2	-1.5	-3.5	0	7	-4	2.5
5	0	0	-2.5	0	4	-1	-0.5
6	103.5	107.5	43	21	9	27	3.5
7	11	14.3	-2.5	4	4	-1	6.5
8	2	-3	0	-7	-2	0	11
9	8	11.8	0	0	-1	3	6
10	7	9.7	3.5	7	-4	1	-0.5
11	4	4.8	2.5	7	-4	1	-2.5
12	6	6.3	-3.5	2	14	-8	1.5
13	0	0	-2.5	0	4	-1	-0.5
14	20	22.9	4.5	22	-12	1	4.5
15	0	0	-2.5	0	4	-1	-0.5

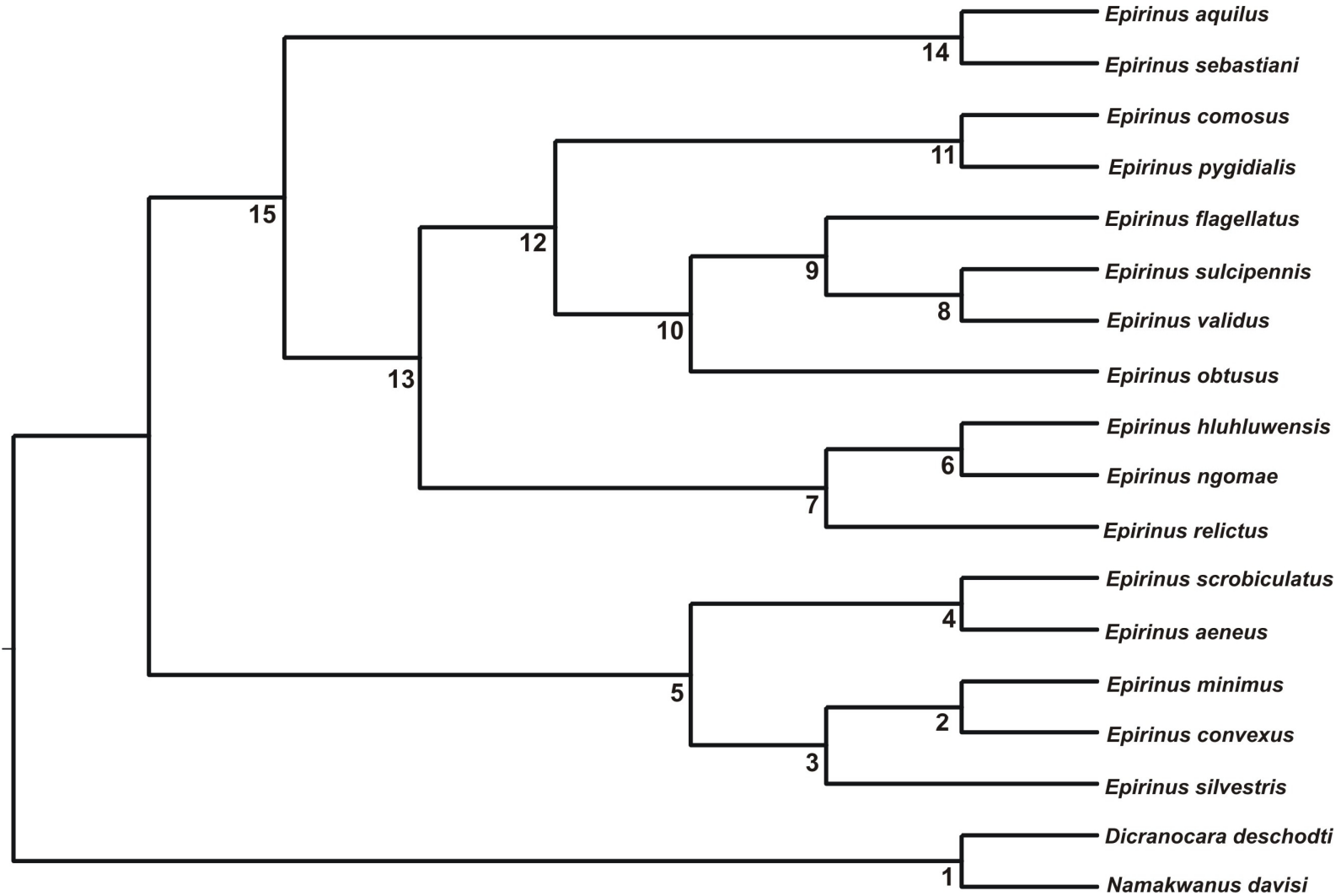


Figure 5. Shortest tree constrained to include nodes obtained in the Bayesian analysis. Node numbers indicated below branches.

### *Times of divergence*

Our results indicated that the mean estimate of the earliest split in the genus occurred 40 (26/56) and 25 (16/35) MYA for the rates of 0.0075 and 0.012 substitutions /site/MYA respectively (upper and lower credibility limits in brackets). Lineage B3 which includes possibly the most derived flightless species, appears to have diverged around 26 (16/37) and 16 (10/23) MYA (Fig.3). Our results for *Epirinus* are consistent with those for other African canthonine genera reported by Sole and Scholtz (2010) with the oldest having diverged from its closest relative in the Eocene epoch 56-34 MYA, and many splits subsequently occurring during the Oligocene and Miocene. In their study of five (out of a possible 13) Madagascan canthonine genera, the oldest have an estimated divergence date in the Miocene (14 MYA) (Orsini *et al.* 2007). The “roller” tribes Scarabaeini and the Argentine endemic Eucraniini also have a Miocene origin 23-10 MYA (Forgie *et al.* 2005) and, 16-12 MYA (Ocampo and Hawks, 2006) respectively.

### **Discussion**

Our research has shown that even the smallest species of *Epirinus* are rollers; the monophyly of the genus has been confirmed, and the molecular and combined molecular and morphological phylogenetic hypotheses have been shown to be largely congruent with the existing morphological phylogeny. The poor support of *E. aquilus* and *E. sebastiani* in the morphological dataset of Medina and Scholtz (2005) has meant that their phylogenetic placement and relationships with sister taxa are better resolved with the gene regions used in this study.

### *Food relocation*

Recent phylogenetic studies have postulated that in Scarabaeinae rolling balls of dung evolved independently several times (Philips *et al.* 2004; Monaghan *et al.* 2007). Sole and Scholtz (2010) tried to “tease apart the different taxa of tunnellers and rollers” concluding that within the African Canthonini and Dichotomiini *Epirinus* appeared to be the most basal of all rollers. Our assessment of food relocation behaviour confirms that the genus *Epirinus* appears to be a ‘true roller’. It also appears that these species may exhibit plasticity in their food relocation behaviour as seen in other dung beetle taxa (Philips *et al.* 2004 and references therein). Admittedly, most

dung beetles show selectivity for particular dung based on qualities such as dropping size, texture and fibre content (Rahagalala *et al.* 2009) but they may also be opportunists that exhibit different behaviours to suit their environment.

In addition, most of these smaller species are flightless and occur in forests so that locating dung pellets of small animals could potentially be easier for them than locating large herbivore dung pats (Gill 1991). Competition at a large dung pat would also favour large beetles (Scholtz *et al.* 2009). It must also be pointed out that some species of *Epirinus* are known to be attracted to bait containing a mixture of dung and carrion, a fact that may lend support to the hypothesis of plasticity concerning food and its relocation.

### *Phylogeny*

Overall the phylogenetic hypothesis produced for *Epirinus* is well supported. The genus is monophyletic so there is strong support for the synonymy of the flightless species (*Endroedyantus*) with *Epirinus* (Scholtz and Howden 1987). However, broader taxon sampling would possibly give better support for resolution to nodes in lineage B. Lineage A, which appears basal in the phylogeny, consists of three flightless species. This is corroborated by the morphological phylogeny of Medina and Scholtz (2005). The species in lineage B are all winged while all but one species in lineage B3 are flightless. Flightlessness is generally accepted as a derived condition from a flying ancestor. It has evolved independently many times in different Scarabaeoidea groups (Scholtz 2000). In the Scarabaeinae, examples include aptery evolving independently at least three times within Scarabaeini (Harrison and Philips 2003,) and twice in *Epactoides* (Canthonini) (Wirta and Montreuil 2008).

There appear to be at least two origins of flightlessness in our analysis- a monophyletic group of three species from the basal node and four others in the most derived clade. Both groups are sister to winged species so we can conclude that flightlessness in *Epirinus* is derived from winged ancestors. Its evolution was likely driven by environmental pressures. According to Scholtz (2000) increased environmental homogeneity is one of the conditions that favour secondary loss of wings. All the flightless species are found in forest habitats which provide stable and persistent microhabitats that promote flightlessness.

Flightlessness has been suggested as a factor influencing speciation in other dung beetle groups such as the flightless genera of Scarabaeini (Forgie *et al.* 2005). However, although there is no data to make precise comparisons between speciation rates in winged and flightless lineages, branch lengths of ancestral nodes do not suggest any differences. Although flightlessness appears not to have driven speciation, this adaptation may have promoted endemism in these species. All the flightless species occur in isolated forest patches along the eastern coast of South Africa

### *Historical biogeography*

Although our COI phylogeny does not match the combined phylogeny exactly, the winged and wingless species of *Epirinus* form distinct clades so that we can estimate the divergences of the different groups. Our molecular dating results appear to confirm that dung beetle biogeography mostly reflects evolution since the Miocene to Pleistocene (Cambefort 1991). Many canthonines in Africa appear to be represented by relicts of a once widespread temperate fauna (Deschodt and Scholtz 2008). All the flightless, and a few winged species of *Epirinus*, are forest relicts found in undisturbed disjunct forest habitats (Davis *et al.* 2008). According to Eeley *et al.* (1999) the warmer, wetter conditions characteristic of Pleistocene hyperthermal periods may have caused an expansion of the forest biome whereas cooler drier hypothermal periods would have caused a reduction in the extent of forests. The expansion and contraction would have resulted in fragmentation of the forests and in that way taxa would have been isolated in forest refugia. The rest of the species are found in a range of more open habitats. The winged species are bigger and have probably been able to successfully compete with highly efficient rollers from the tribes Scarabaeini, Gymnopleurini and Sisyphini where they overlap.

### **Conclusion**

In conclusion, we provide evidence that *Epirinus* is a monophyletic group, with support for the synonymisation of *Endroedyantus* with *Epirinus* and demonstrate that even some of the smallest species within the genus are rollers. These beetles seem prone to behaviour shifts to suit different environmental conditions for their survival. The hypothesis that body size is a

constraint on the ability to form and roll balls is thus rejected. Our analysis has also shown that flightlessness evolved twice within *Epirinus* with environmental pressure the likely cause for this condition.

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