

Molecular assessment of the systematics of African Scarabaeinae (Coleoptera: Scarabaeidae)

By

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Dedication

For Manelisi and Khanya..... unknowingly, you gave me strength and determination to walk this journey.

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Declaration

I, Sukoluhle Mlambo declare that the thesis, which I hereby submit for the degree Doctor of Philosophy (Entomology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:.....

DATE:.....

Summary

Molecular assessment of the systematics of African Scarabaeinae

(Coleoptera: Scarabaeidae)

Dung beetles of the subfamily Scarabaeinae have a worldwide distribution with the Afrotropical region, the putative origin of the diversification of Scarabaeinae, having the richest diversity. Partial sequences from two ribosomal (16S, 28S) and two protein coding genes (COI, CAD) are used to construct a molecular phylogeny of 55 genera sampled to maximize representation of dung beetle morphological and ecological diversity in all nine tribes that occur in Africa. The ages of the tribes are estimated for comparison with those of other biogeographical regions. The phylogenetic hypothesis was found to largely concur with a recent molecular study done at a global scale. Thus the recent calls for a new classification for the subfamily are validated. Possible changes to the classification are discussed and the likely African origin of the subfamily is corroborated. In addition, recent phylogenetic analyses having pointed to the canthonine dung beetle genus *Epirinus* as the putative African ancestral roller group. Consequently, the roller status of species in the genus is tested with observational studies and a molecular phylogeny based on the above genes is constructed for 16 of 29 species. Tested species were confirmed to be dung rollers. Monophyly of the genus was confirmed, lending support to the synonymisation 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.

Chapter 1

General Introduction

Systematics

Systematics is the field of biology that traces the evolutionary history or phylogeny of organisms and reveals the pattern of events that led to their distribution and diversity (Lipscomb 1998). Phylogenetic systematics seeks to explore and clearly communicate the phylogenetic relationship among species through the use of shared derived characters (Hennig 1965). This branch of biology is important as it facilitates the classification of organisms into natural or monophyletic groups, that is, groups that reflect evolutionary relatedness. Classification systems change with time for a number of reasons which include the addition of new taxa and new sources of character information.

The subfamily Scarabaeinae

The subfamily Scarabaeinae Latreille shares the ability to exploit vertebrate excrements with another Scarabaeidae Latreille subfamily, Aphodiinae Leach, and the family Geotrupidae Latreille (Halffter and Edmonds 1982 sighted in Cabrero-Sañudo and Lobo 2009). Aphodiinae larvae eat and live in dung immediately beneath the soil surface while Geotrupidae pack larval food into the end of a burrow. Scarabaeinae on the other hand, exhibit a range of feeding and nesting strategies for the exploitation of dung that have contributed to their evolutionary success. It is these behaviours and the variety of morphological characteristics that have kept evolutionary biologists interested in the group since the time of Fabre (1918) (Halffter and Matthews 1966).

Dung is such a rich resource, however, that it supports a diverse and abundant insect community. In addition to the Coleoptera, many among the Diptera also feed on dung throughout their larval stages (Hanski 1991).

Geographic Distribution

The subfamily Scarabaeinae has a worldwide distribution with approximately 6000 described species (Scholtz *et al.* 2009) currently grouped into twelve tribes (Cambefort 1991). More than half of the world's scarabaeine genera belong to the tribes Canthonini van Lansberge and Dichotomiini Pereira (Davis *et al.* 2002) and these occur in the southern continents – the Afrotropical, Madagascar, Neotropical and Australian regions. Tribes Canthonini and Dichotomiini have recently been renamed Deltochilini Lacordaire and Ateuchini Perty respectively (Smith, 2006; Bouchard *et al.* 2011), however, the former names will be used for the present work. The tribes Phanaeini Hope, Eucraniini Burmeister and Eurysternini Vulcano, Martínez and Pereira are endemic to the Americas while the Afro-Eurasian endemics are Gymnopleurini Lacordaire, Onitini Laporte and Scarabaeini Latreille. The remaining tribes Onthophagini Burmeister, Coprini Leach, Sisyphini Mulsant and Oniticellini Kolbe are centred in the Afro-Eurasian region but have limited representation in other biogeographical regions.

Habitat

Members of the subfamily occur in a wide range of habitats from forests, grasslands, montane (Cambefort 1991) to deserts (Doube 1991). The Afrotropical region represents the richest in dung beetle diversity with more than 2000 species of which *ca* 780 are found in southern Africa (Doube 1991). Habitat preferences of dung beetles are determined mainly by temperature, soil

type and humidity (Barbero *et al.* 1999; Davis and Scholtz 2001). The dung, upon which these beetles depend, is ephemeral and patchy. It changes as it decomposes progressively, losing moisture and as such, determines among other factors, the composition of the beetle assemblage (Lobo 1991). Habitat and climatic characteristics determine the rate and pattern of decomposition (Sowig and Wassmer 1994). Dung beetles locate their food mainly by its odour (Cambefort and Hanski 1991). The efficiency of transmission of dung odours, as well as the radiant energy level at the soil surface, which affects flight activity, is also influenced by the habitat (Romero-Alcaraz and Avila 2000).

Food and feeding strategies

In most Scarabaeoid dung beetle species, the adults feed on the fresh dung of herbivorous mammals. The dung consists of small particles including microorganisms and dead epithelial cells from the herbivore's gut, large plant fragments, mostly made up of lingo-cellulose cell walls and a high percentage of water (Holter and Scholtz 2005). The high water content makes the dung malleable so that the telecoprids (rollers/ball-rollers), one of the four functional groups of the family Scarabaeinae (Doubé, 1990), can form dung balls that are rolled away from the dung pat.

Ball making and rolling is exhibited by both male and female adults. It is a complex behaviour accompanied by distinct behavioural properties and morphological characteristics of the front legs, hind legs and overall body shape (Monaghan *et al.*, 2007). The behaviour of ball rolling was observed by Matthews (1962) in *Canthon pilularis* (Harold). He described it as involving cutting, using the head and forelegs; shaping using the fore tibiae (with the head being

used for trimming lumps or fibres off the ball surface) and rolling using the forelegs to push backward against the ground while steadying the ball with the middle and hind legs. In their studies, Tomkins *et al.* (1999) observed that the fore and hind legs are critical in the propulsion and direction of the ball for *Kheper nigroaeneus* (Boheman) (Scarabaeini).

The majority of African species are paracoprids which pack dung into pre-excavated tunnels directly beneath the dung. Endocoprids feed and nest within the dung while kleptocoprids utilise dung sequestered by either paracoprids or telecoprids.

Reproduction

Dung is not only patchy and ephemeral, but there is also intense competition for it. A major behavioural trait that has contributed to the success of dung beetles is their ability to construct nests that provide food and shelter for their offspring. Brood masses or balls, in which eggs are laid and develop into larvae, are formed inside the dung or in burrows. The female may abandon or tend the brood until the adult beetle emerges. Nesting behaviour ranges from simple to complex within the subfamily. Nesting patterns are not well-defined for the different functional groups (Cambefort and Hanski 1991) but rather determined by the environment and specific ecological requirements (Scholtz *et al.* 2009). In all cases however, the offspring are protected, thus increasing their chances of survival.

Subfamily classification

Although the global Scarabaeinae dung beetle fauna is divided into 12 tribes (Hanski and Cambefort 1991), these divisions lack phylogenetic support (Medina *et al.* 2003; Philips *et al.*

2004; Monaghan *et al.* 2007; Davis *et al.* 2008). The current classification of the Scarabaeinae is considered to be based on two ‘monophyletic’ groups (Cambefort 1991): 1) the Coprinae containing six tribes of “tunnellers” and 2) Scarabaeinae consisting of six tribes of “rollers”. However, the phylogenetic relationships within and among them are poorly resolved. Consequently, the present study is directed at elucidating phylogenetic relationships within and among the southern African tribes based mainly on a range of molecular analyses.

Aim of study

The major purpose of this study was to trace the evolutionary history of the genera of the African Scarabaeinae and those among species of the canthonine genus, *Epirinus* Reiche, using molecular genetic evidence. In addition the relationship between body size and the ability to roll dung balls was examined.

Research questions

The following research questions were addressed in this study. Chapter 2 focuses on questions 1-3 and Chapter 3 deals with questions 4-7.

1. What are the relationships among the genera and tribes comprising the African Scarabaeinae based on a combined analysis of mitochondrial COI and 16S, and nuclear 28S and CAD genes?
2. What are the divergence dates of different Scarabaeinae lineages?
3. What are the main factors that are likely to have driven the evolution of dung beetles in Africa?
4. Which species of *Epirinus* are ball-rollers and which are not?

5. Is size a constraint to ball-rolling?
6. What are the relationships among *Epirinus* species based on a combined analysis of mitochondrial COI and 16S, and nuclear 28S and CAD genes?
7. Is there congruence between the molecular data set and a published morphological one?

Relevance/Justification of study

The Afrotropical region represents the richest in dung beetle diversity and is the putative origin of the modern dung beetle fauna (Philips *et al.* 2004; Monaghan *et al.* 2007; Sole and Scholtz 2010). Furthermore the major world biogeographical regions (Cox 2001) have been shown to have phylogenetically distinct assemblages (Monaghan *et al.* 2007). It is therefore important to trace the evolutionary history of the African taxa to gain an appreciation of the age of the subfamily as well as divergence times of the different tribes. Relationships among the genera must be ascertained if new tribal delimitations are to be suggested.

Approach

In recent years molecular systematics has become an important tool in studying evolutionary history. According to San Mauro and Agorreta (2010) molecular systematics can be defined as the use of information contained in molecular data to reconstruct phylogenetic relationships. Among the different techniques available, DNA analysis has been used in most analyses. DNA sequences provide different levels of variability for specific evolutionary questions because of the high degree of variation in substitution rates among genes and gene regions (San Mauro and

Agorreta 2010). Four gene regions were used in the present study. Sequence data were generated from two mitochondrial genes - cytochrome oxidase I (COI) and 16S ribosomal RNA (16S rRNA) – and two nuclear genes - a portion of the 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 insect 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; Regier *et al.* 2008; Sole and Scholtz 2010). Mitochondrial genes are mostly used to resolve species level relations while nuclear genes, being more conserved, provide resolution at higher taxonomic levels.

Domain 2 of the 28S rRNA molecule was chosen as within insects they have highly variable expansion segments that have a large suite of phylogenetically informative characters at higher taxonomic levels especially among taxa having diverged over a large evolutionary time scale (Gillespie *et al.* 2005). Carbamoyl-phosphate synthetase-aspartate transcarbamoylase-dihydroorotase (CAD), a relatively recent addition to insect systematic studies (Moulton & Wiegmann 2004; Winterton and de Freitas 2006; Winterton *et al.* 2007; Wahlberg *et al.* 2008), has been shown to be phylogenetically informative, giving results comparable to morphological studies and other gene phylogenies. Consequently, the combined analysis of multiple molecular markers and wide taxon sampling is expected to resolve previously uncertain relationships within and among the Scarabaeinae phylogeny.

While DNA is now regularly used for phylogenetic inference, many studies have also used a combined analysis of molecular and morphological data (Forgie *et al.* 2006; Sole *et al.*

2011). According to the total evidence approach (Kluge 1998; 2004) all available data types should be analysed simultaneously for the most plausible phylogenetic hypotheses. Some studies further evaluate the relative contributions of the different datasets to the phylogenetic hypothesis. For phylogenetic inference the evolutionary model-based Maximum likelihood and Bayesian methods (Felsenstein 2004; Swofford *et al.* 1996) together with Maximum parsimony (Farris 1983) were used.

With dung relocation behaviour having been central in the classification of Scarabaeinae, observational studies were undertaken to determine whether all the species in the “roller” genus, *Epirinus*, actually roll dung balls.

Thesis outline

Chapters 2 and 3 were written as manuscripts for publication in peer-reviewed journals. Each chapter contains its own reference list. Due to this format, there may be instances of duplication across chapters. Both the general introduction and conclusion are tailored from the respective chapters, giving a general overview of the thesis and the overall concluding remarks.

Chapter 2: A molecular phylogeny of the African Scarabaeinae (Coleoptera: Scarabaeidae)

This chapter represents the main focus of the project which was to investigate the relationships amongst the genera of the African Scarabaeinae and estimate divergence times of the tribes. Molecular markers, COI, 16S, 28S and CAD are used to reconstruct the phylogeny of the

subfamily. The monophyly of the tribes is discussed, formation of two new tribes is suggested and the likely African origin of the Scarabaeinae is substantiated.

Chapter 3: **Phylogeny of the African ball-rolling dung beetle genus *Epirinus* Reiche (Coleoptera: Scarabaeidae: Scarabaeinae)**

This chapter has been accepted for publication in *Invertebrate Systematics* (in press). It investigates the relationships among 16 species of the canthonine genus *Epirinus*, one of the earliest diverging roller groups. Four molecular markers, COI, 16S, 28S and CAD are used to reconstruct the phylogeny of the genus. The molecular dataset is combined with a published morphological one to test for congruence. The loss of flight in some species and estimated divergence dates within *Epirinus* are discussed. In addition, small species of *Epirinus*, less than 7mm in length, are used in observational studies to establish whether or not body size is a constraint on the ability to roll dung balls.

Chapter 4: **General conclusions**

This chapter summarises the main findings of this study and draws out their implications for dung beetle classification. These will add to our understanding of the evolution of this group of insects.

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Chapter 2

A Molecular phylogeny of the African Scarabaeinae

(Coleoptera: Scarabaeidae)

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Abstract

Dung beetles of the subfamily Scarabaeinae have a worldwide distribution with the Afrotropical region, the putative origin of the diversification of Scarabaeinae, having the richest diversity. We use partial sequences from two ribosomal (16S, 28S) and two protein coding genes (COI, CAD) to construct a molecular phylogeny of 55 genera sampled to maximise representation of dung beetle morphological and ecological diversity in all nine tribes that occur in Africa. We estimate the ages of the tribes for comparison with those of other biogeographical regions. The phylogenetic hypothesis was found to largely concur with a recent molecular study done at a global scale. Thus the recent calls for a new classification for the subfamily are validated. We discuss possible changes to the classification and corroborate the likely African origin of the subfamily.

Introduction

The subfamily Scarabaeinae constitutes a group of dung beetles encompassing approximately 5700 species (Scholtz *et al.* 2009). These beetles have a world wide distribution with most being associated with moist herbivore dung. Some, however, utilise a variety of other dung types and even non-dung food sources. Dung is a highly nutritious but patchy and ephemeral resource, characteristics that, together with intense competition at the dung pat, have led to the evolution of an impressive range of morphological (such as horns, mandibles) and behavioural (such as feeding, nesting) attributes in dung beetles (Davis and Scholtz, 2001). Scarabaeines are of biological interest for these attributes and for their important role in ecosystem functioning (Nichols *et al.* 2008; Brown *et al.*, 2010).

The subfamily Scarabaeinae is a monophyletic group morphologically defined by twelve shared apomorphic character states of hindwing articulation (Browne and Scholtz 1998). There is also strong evidence for the monophyly of the group (Philips *et al.* 2004; Ocampo and Hawks 2006; Monaghan *et al.* 2007). It is generally assumed that the sister group of the Scarabaeinae is the Aphodiinae (Browne and Scholtz 1999; Philips *et al.* 2004; Monaghan *et al.* 2007). The 227 genera in Scarabaeinae have been grouped in a number of different ways with earlier classifications having been based on personal intuition or morphology from limited data sets (see Zunino 1983; 1985). Traditionally, the subfamily has been divided into two groups based on either their rolling or tunnelling behaviour (Balthasar 1963; Cambefort 1991). Rollers construct balls of dung and bury them at a distance from the source for feeding and breeding while tunnellers bury dung beneath or near the dung pat. Janssens (1949) grouped the scarabaeines into six tribes: rolling Eurysternini and Scarabaeini, with subtribes Eucraniina, Canthonina,

Gymnopleurina, Scarabaeina and Sisyphina; and tunnelling Onthophagini, Onitini, Oniticellini and Coprini. Coprini was further subdivided into subtribes Dichotomiina, Phanaeina and Ennearabdina. Balthasar's (1963) division of Scarabaeinae was into two subfamilies with six tribes each as follows: Coprinae with tribes Coprini, Dichotomiini, Phanaeni, Oniticellini, Onitini and Onthophagini; and Scarabaeinae with tribes Eucraniini, Eurysternini, Canthonini, Gymnopleurini, Scarabaeini and Sisyphini. The grouping by Balthasar (1963) has been followed by most workers on the Scarabaeinae. These include Hanski and Cambefort (1991) who further envisioned the Canthonini and Dichotomiini as the primitive or "old" tribes that gave rise to the rest of the rollers and tunnellers, respectively. The tribes Scarabaeini, Gymnopleurini, Eucraniini, Eurysternini, Onitini and Phanaeni are considered to be "intermediate" in age (Cambefort 1991a). Sisyphini are considered the most modern/derived of the rollers and Coprini, Oniticellini and Onthophagini the most recently evolved tunnellers (Cambefort 1991a). It was however, an intuitive classification, lacking in phylogenetic support (Medina *et al.* 2003; Philips *et al.* 2004; Monaghan *et al.* 2007).

The earliest phylogenetic studies used morphological data for cladistic analyses with both Zunino (1983) and Luzzatto (1994) (sighted in Philips *et al.* 2004) having used male and female genitalic characters. Although these two studies used limited data sets, their results did indicate some problems with the roller/tunneller division; there was evidence of tunneller groups nested within the rollers such as Phanaeini and Onitini shown in the cladograms in Figures 1a and 1b. In addition, the tribes Canthonini and Dichotomiini did not appear basal in either analysis. Montreuil (1998) used 42 adult morphological characters for his study of the Dichotomiini and Coprini and found the two tribes lacking monophyly. He suggested major changes to scarabaeine classification. The tribe previously known as Dichotomiini was renamed Ateuchini after

reassigning some genera to Coprini. (Dichotomiini has since been renamed Ateuchini (Bouchard *et al.* 2011), however, for this study, the name Dichotomiini will be used).

The first phylogenetic analysis to raise serious doubts about the accepted dung beetle classification was that by Philips *et al.* (2004). Their study was based on 200 morphological characters and 50 taxa covering all 12 currently recognised tribes sampled from six biogeographical regions. The tribes comprise Canthonini and Dichotomiini which are wide spread with their main generic diversity in the southern continents (Neotropical, Afrotropical and Australia), Eucraniini, Phanaeini and Eurysternini restricted to the Americas, Scarabaeini, Gymnopleurini and Onitini showing an Afro-Eurasian distribution and the cosmopolitan Onthophagini, Sisyphini, Oniticellini and Coprini (Davis and Scholtz 2001). Philips *et al.*'s (2004) phylogeny deviated widely from the two-clade scenario with the relationships within the tree differing from the earlier mentioned studies. Rollers and tunnellers were intermixed within the phylogenetic tree and although some Dichotomiini genera were basal within the tree others were not (Figure 2). There was generally poor support for most nodes at the base of their tree with little evidence for the monophyly of the dichotomiines, coprines and canthonines. The remaining nine tribes, however, were monophyletic. From this study it was concluded that the ancestral Scarabaeinae was a tunneller with all other clades having evolved directly or indirectly from the Dichotomiini, and rolling having evolved independently a number of times. Further, according to biogeographical evidence, Scarabaeinae originated and diversified in the Mesozoic, consistent with fossil evidence from a study by Krell (2000). Scholtz and Chown (1995) on the other hand had proposed a Cenozoic origin for the group.

Recently, Vaz de Mello (2007) carried out a taxonomic revision and phylogenetic analysis of the tribe Ateuchini using 87 taxa and 297 morphological characters. With the tribe as defined by Montreuil (1998) lacking synapomorphies, Vaz de Mello (2007) redefined Ateuchini and identified the phylogenetic positions of those genera that previously could not be placed in any tribe with certainty. His work is however, unpublished. Even though the present work follows Balthasar's (1963) 12 tribe scheme, Vaz de Mello's (2007) analysis is relevant and useful as his genera fall into or are related to members of the tribes Canthonini, Dichotomiini and Coprini, whose monophyly is doubtful. Sister relationships obtained were consistent with those in a molecular phylogenetic study by Monaghan *et al.* (2007) for taxa common to both studies. A high proportion of homoplasies were reported by Vaz de Mello (2007).

Generally, many morphological characters are not independent of function as was found to be the case in the study by Philips *et al.* (2004). In addition there are difficulties associated with scoring morphological characters for such a highly modified group as the Scarabaeinae. In such cases the use of other data sets such as molecular markers becomes necessary for robust and stable phylogenetic inferences. Molecular data has its own disadvantages such as difficult amplification of some nuclear genes, alignment problems for ribosomal genes and the linkage of mitochondrial genes on the same chromosome (Simon *et al.* 2006). In addition, the high A/T bias in third basepair positions of mitochondrial genes of insects leads to high levels of homoplasy (Fрати *et al.* 1997). In spite of these setbacks, molecular data are useful in identifying areas in traditional morphological hypotheses that need revisiting.

The Scarabaeinae study by Villalba *et al.* (2002) was the first to use molecular data on the group. It was based on the DNA sequences of the mitochondrial cytochrome oxidase I and II

genes. Thirty-three species representing the seven tribes and all the genera occurring in the Iberian Peninsula were used in the analysis. This study, however, relied solely on mitochondrial genes whereas the current trend is to use both mitochondrial and nuclear genes to produce a more reliable phylogenetic tree. Mitochondrial genes are fast evolving and useful for comparison of closely related taxa while nuclear genes are slower evolving and suitable for comparisons of distantly related taxa (Simon *et al.* 1994). Furthermore, it is desirable to use several markers of independent evolutionary history (Wahlberg and Wheat 2008).

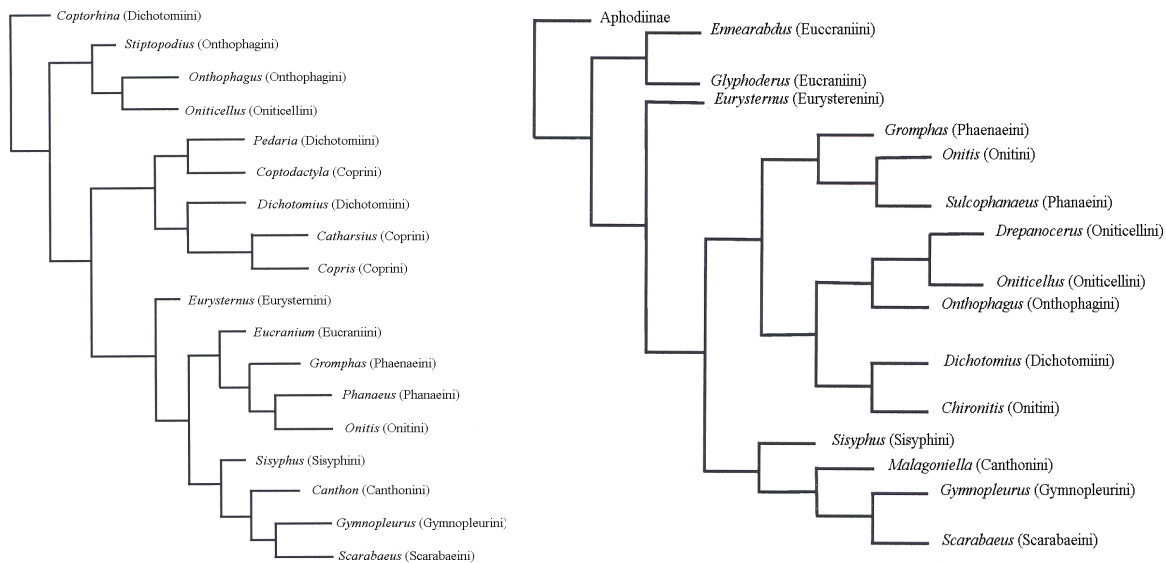


Figure 1a and 1b. Cladograms taken from Zunino's (1983) analysis using male and female genitalia from all the 12 tribes currently recognised in Scarabaeinae, and Luzzatto's (1994) analysis using male and female genitalia from 11 tribes of Scarabaeinae. Coprini was not included.

Villalba *et al.* (2002) showed a contradiction to the accepted classification with Coprini, a tunneller, placed among the rolling tribes in their phylogeny, even though the relationship was poorly supported. Ocampo and Hawks (2006) used two ribosomal nuclear genes for their reconstruction of the scarabaeine phylogeny. Their taxa included genera from all tribes except Sisyphini and Gymnopleurini. Again inconsistencies were shown among sister relationships of the tribes. To date, Monaghan *et al.* (2007) have performed the most extensive molecular phylogenetic study on the Scarabaeinae using 214 species, sampled from across the world. They used three gene regions, two mitochondrial (COI and 16S) and one nuclear (28S) (Figure 3). This study can be compared to that of Philips *et al.* (2004) in that it showed that the relationships within the group are not as simple and clear-cut as previously postulated. They found that rolling has not evolved on a single occasion within the group and some of the most basal “tribes” or lineages are not monophyletic. Although the phylogenetic analysis of Monaghan *et al.* (2007) used a large number of taxa from all the continents, there was little to no support for most of the deeper relationships between taxa which did little to resolve the phylogenetic inconsistencies within the Scarabaeinae.

One of the major findings in the study by Monaghan *et al.* (2007) was the biogeographical distinction in the dung beetle lineages. Each region has endemic clades, which suggests biogeographical isolation over a long time period. The evolutionary history of dung beetles can therefore best be understood by studying regional evolutionary patterns in isolation, as they have evolved independently. To this end, Sole and Scholtz (2010) used sequences from two mitochondrial and three nuclear genes to construct a phylogeny of the basal tribes Canthonini and Dichotomiini. They teased out the previously poorly resolved relationships among the African genera of these ancestral tribes using eight of the nine genera of Dichotomiini

and 17 of 23 canthonine genera. With evidence from recent phylogenetic studies suggesting Africa as the origin of the diversification of Scarabaeinae (Philips *et al.* 2004; Monaghan *et al.*, 2007) they also estimated divergence times for the subfamily. Neither tribe was recovered as monophyletic but three well supported lineages were obtained. Estimates of divergence times were consistent with fossil evidence.

It is clear that although a number of phylogenetic analyses have been undertaken using both morphological and molecular characters, different and often contradictory conclusions have been reached. The predictive power of phylogenetic systematics cannot therefore be applied to the practical and theoretical aspects of Scarabaeinae classification and biology. In essence, the major contentious systematic issues in the Scarabaeinae can be summarised as follows: 1) the traditional classification of the Scarabaeinae into rollers and tunnellers appears to be unfounded and has little phylogenetic support (Zunino 1983; Philips *et al.* 2004; Monaghan *et al.* 2007, Sole and Scholtz 2010); 2) non-monophyly of some of the tribes Dichotomiini and Coprini (Montreuil 1998), Coprini, Canthonini and Dichotomiini (Philips *et al.* 2004), and Onthophagus, Canthonini and Dichotomiini (Monaghan *et al.*, 2007) are a source of confusion leading to 3) uncertain sister-group relationships within the Scarabaeinae and 4) lack of consensus on the time of divergence of the group among morphological and molecular (see Forgie *et al.* 2006; Ocampo and Hawks 2006; Sole and Scholtz 2010) data sets.

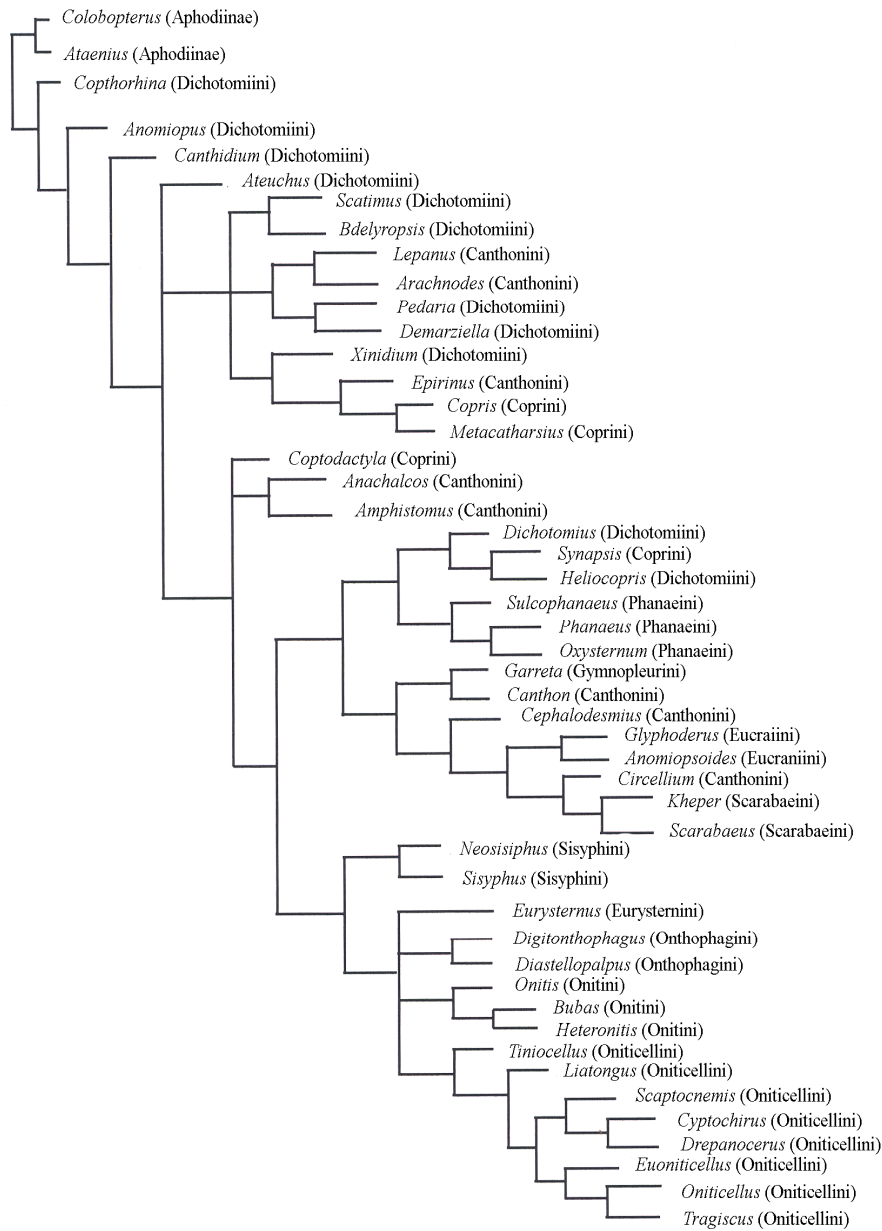


Figure 2. Cladogram taken from the Philips *et al.* (2004) analysis based on 200 morphological characters from members of all 12 Scarabaeinae tribes.

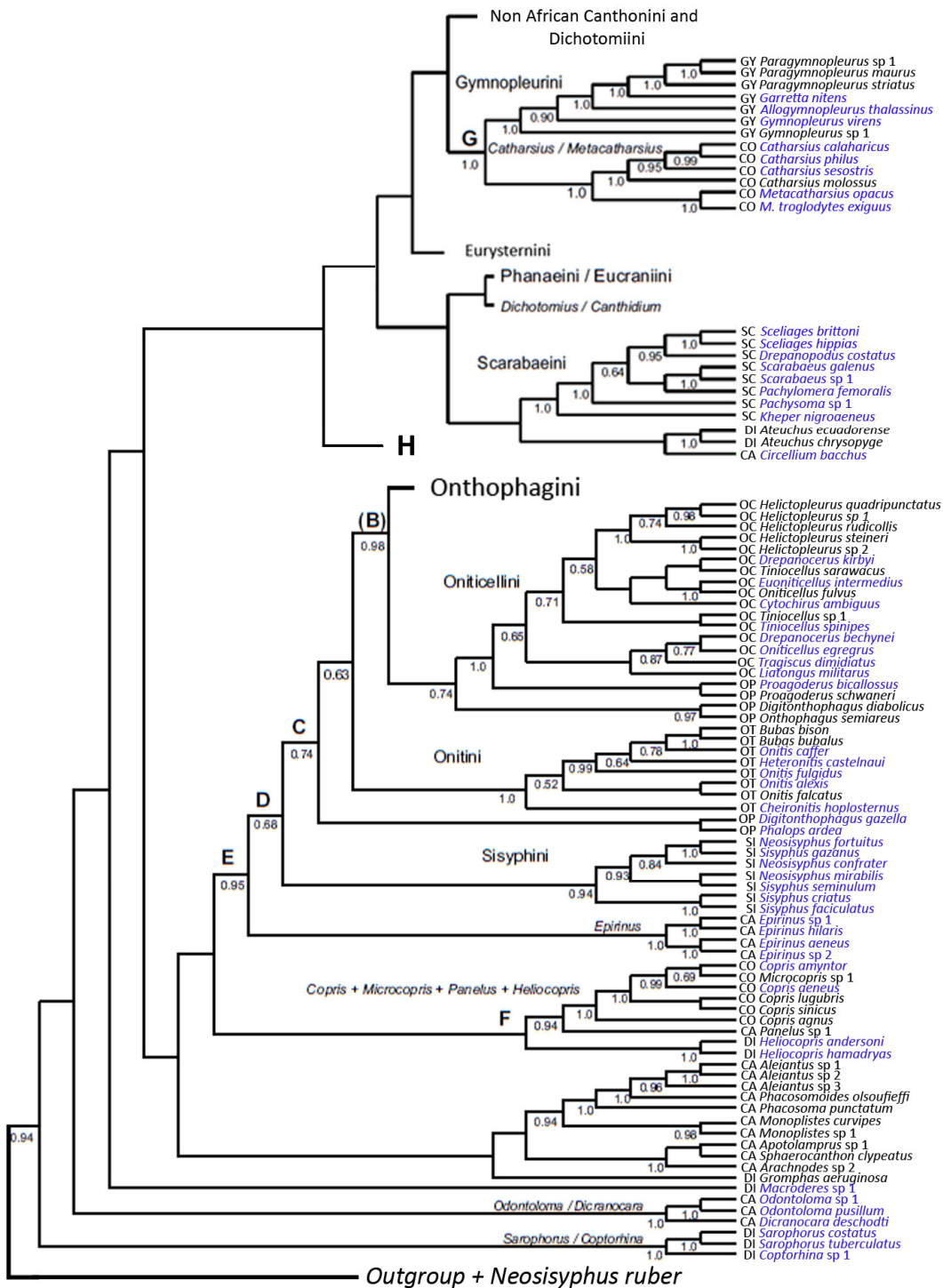


Figure 3. Phylogeny of Scarabaeinae adapted from Monaghan *et al.* (2007). African taxa are shown in blue.

The present study is aimed at reconstructing the genealogical ties between the tribes of the subfamily in the African contingent as well as estimating the time of divergence since they last shared a common ancestor. To the two tribes in the Sole and Scholtz (2010) study, we added genera from the remaining seven African tribes and, for continuity, used four (16S, CO1, D2 and CAD) of the five gene regions they utilised in their study, to construct a phylogeny of Scarabaeinae in an effort to untangle the history of the subfamily on the continent. The Afrotropical region represents the richest in dung beetle diversity, with more than 2000 species (Scholtz *et al.* 2009) of which about 780 are found in southern Africa. Here we use a large sample of more than half of the known African genera. We compared our phylogeny to that of the African taxa in the study of Monaghan *et al.* (2007) and to other recently proposed phylogenies. Additionally, we examine evolutionary trends among the African scarabs.

Materials and Methods

Taxon sampling

We obtained samples of species belonging to all the nine tribes of Scarabaeinae that occur in Africa. An attempt was made to include as many as possible of the African genera. In-group taxa include 56 genera. Two species of *Aphodius* were used as out-groups (Monaghan *et al.* 2007, Sole and Scholtz 2010). The choice of this taxon as a valid out-group was based on the phylogenetic study of Scarabaeoidea (Brown and Scholtz 1999) (See table 1 for details)

Gene sampling

As our focus lay in resolving both the relationships between the genera as well as the deeper branches of the tree we used two genes from the fast evolving mitochondrion: cytochrome oxidase subunit I (COI) and the large ribosomal subunit (16S), as well as two from the slower evolving nucleus: a portion of the rRNA large subunit – 28S (28S rRNA) domain 2 – and the CPSase region of Carbamoyl-phosphate synthetase-aspartate transcarbamoylase-dihydroorotase (CAD). The DNA sequences generated in this study have been submitted to GenBank; see Table 1 for accession numbers. For details on genera and sequences used for the tribes Dichotomiini and the majority of the Canthonini see Sole and Scholtz (2010). Sequences were obtained from three specimens per species whenever possible. The specimens were field collected in dung or dung/carrion baited traps and then preserved in absolute ethanol. All the specimens in this study were collected in Southern Africa.

Table 1. Taxa studied and their Genbank numbers.

Taxon	ID GenBank accession numbers				
		COI	16S	28S	CAD
Tribe Coprini					
<i>Copris cornitrans</i>	CCU1	JN804613	JN804686	JN804759	-
<i>Catharsius jacchoides</i>	CJU1	JN804618	-	-	-
<i>Catharsius fidius</i>	CFB1	-	JN804687	JN804760	JN804572
<i>Catharsius fidius</i>	CFB2	-	JN804688	JN804761	-
<i>Catharsius fidius</i>	CFB3	-	JN804689	JN804762	-
<i>Litocopriss muticus</i>	LTC1	JN804639	JN804711	JN804781	JN804583
<i>Litocopriss muticus</i>	LTC2	JN804640	JN804712	JN804782	JN804584
<i>Litocopriss muticus</i>	LTC3	JN804639	-	JN982316	JN804585

<i>Metacatharsius exiguiformis</i>	MEG1	JN804647	JN804718	JN804788	JN804589
<i>Metacatharsius exiguiformis</i>	MEG2	JN804648	JN804719	JN804789	JN804590
<i>Metacatharsius exiguiformis</i>	MEG3	JN804649	JN804720	JN804790	-
<i>Metacatharsius marani</i>	MMG1	JN804650	JN804721	JN804791	JN804591
<i>Metacatharsius marani</i>	MMG2	-	-	JN804792	JN804592
Tribe Gymnopleurini					
<i>Allogymnopleurus thalassinus</i>	ALG1	JN804607	JN804678	JN804751	JN804566
<i>Allogymnopleurus thalassinus</i>	ALG2	JN804608	JN804679	JN804752	JN804567
<i>Allogymnopleurus thalassinus</i>	ALG3	JN804609	JN804680	JN804753	-
<i>Garreta nitens</i>	GRA1	JN804630	JN804703	JN804773	JN804579
<i>Garreta nitens</i>	GRA2	JN804631	JN804704	JN804774	JN804580
<i>Garreta nitens</i>	GRA3	JN804632	JN804705	JN804775	JN804581
<i>Gymnopleurus humanus</i>	GMP1	JN804627	JN804700	-	-
<i>Gymnopleurus humanus</i>	GMP2	JN804628	JN804701	JN804772	-
<i>Gymnopleurus humanus</i>	GMP3	JN804629	JN804702	-	-
Tribe Onitini					
<i>Anonychonitis freyi</i>	ANY1	JN804610	JN804681	JN804754	JN804568
<i>Anonychonitis freyi</i>	ANY2	JN804611	JN804682	JN804755	JN804569
<i>Anonychonitis freyi</i>	ANY3	JN804612	JN804683	JN804756	-
<i>Cheironitis scabrosus</i>	CHR1	JN804616	JN804690	JN804763	JN804573
<i>Cheironitis scabrosus</i>	CHR2	JN804617	JN804691	JN804764	-
<i>Heteronitis castelnaui</i>	HTN1	JN804636	JN804708	JN804778	
<i>Heteronitis castelnaui</i>	HTN2	JN804637	JN804709	JN804779	JN804582
<i>Heteronitis castelnaui</i>	HTN3	JN804638	JN804710	JN804780	-
<i>Onitis caffre</i>	OTC1	-	-	JN804798	-
<i>Onitis sp.</i>	OTS1	JN804656	JN982323	-	-
<i>Onitis sp.</i>	OTS2	JN804657	JN804729	-	-
Tribe Oniticellini					
<i>Cyptochirus sp.</i>	CYP1	JN804620	JN804693	JN804766	JN804574

<i>Euoniticellus africanus</i>	ETA1	JN804626	JN804699	JN804771	JN804578
<i>Liatongus militaris</i>	LTG1	-	JN804713	JN804783	JN804586
<i>Liatongus militaris</i>	LTG2	JN804642	JN804714	JN804784	JN804587
<i>Liatongus militaris</i>	LTG3	JN804643	JN804715	JN804785	JN804588
<i>Oniticellus egregius</i>	ONT1	JN804652	JN804723	JN804794	JN804593
<i>Oniticellus egregius</i>	ONT2	JN804653	JN804724	JN804795	JN804594
<i>Oniticellus egregius</i>	ONT3	JN804654	JN804725	JN804796	JN804595
<i>Tiniocellus spinipes</i>	TCL1	JN804674	-	JN804819	JN804605
<i>Tiniocellus spinipes</i>	TCL2	JN804675	JN804746	JN804820	-
<i>Tiniocellus spinipes</i>	TCL3	JN804676	JN804747	JN804821	-
Tribe Onthophagini					
<i>Caccobius sp.</i>	CCB1	JN804614	JN804684	JN804757	JN804571
<i>Caccobius sp.</i>	CCB2	JN804615	JN804685	JN804758	-
<i>Digitonthophagus gazella</i>	DTP1	JN804621	JN804694	-	-
<i>Digitonthophagus gazella</i>	DTP2	JN804622	JN804695	JN804767	-
<i>Onthophagus interstitialis</i>	ENT1	JN804623	JN804696	JN804768	JN804575
<i>Onthophagus interstitialis</i>	ENT2	JN804624	JN804697	JN804769	JN804576
<i>Onthophagus interstitialis</i>	ENT3	JN804625	JN804698	JN804770	JN804577
<i>Hylonthophagus alcyonides</i>	HTA2	JN804634	JN804706	JN804776	-
<i>Hylonthophagus alcyonides</i>	HTA3	JN804635	JN804707	JN804777	-
<i>Milichus apicalis</i>	MCH1	JN804645	JN804716	JN804786	-
<i>Milichus apicalis</i>	MCH2	JN804646	JN804717	JN804787	-
<i>Onthophagus sp.</i>	OTH1	JN804655	JN804728	JN804799	JN804596
<i>Onthophagus variegatus</i>	OPV1	-	-	JN804797	-
<i>Phalops rufosignatus</i>	PLP1	JN804660	JN804732	JN804800	JN804599
<i>Phalops rufosignatus</i>	PLP2	JN804661	JN804733	JN804801	JN804600
<i>Phalops rufosignatus</i>	PLP3	JN804662	JN804734	JN804802	JN804601
<i>Proagoderus aciculatus</i>	PRS1	JN804663	JN982324	-	JN804602
<i>Proagoderus aciculatus</i>	PRS2	-	JN804735	JN804803	JN804603

<i>Proagoderus aciculatus</i>	PRS3	JN804664	JN804736	JN804804	JN804604
Tribe Scarabaeini					
<i>Scarabaeus Scarabaeus caffer</i>	SBF1	-	JN804737	JN804805	JN819270
<i>Scarabaeus Scarabaeus caffer</i>	SBF2	-	JN804738	JN804806	
<i>Scarabaeus proboscideus</i>	SBP1	-	JN804739	JN804807	JN819269
<i>Scarabaeus zambezianus</i>	SBZ1	-	-	JN804808	JN819268
<i>Scarabaeus Kheper bonelli</i>	SKB1	-	JN804740	JQ088080	JN819271
<i>Scarabaeus Kheper bonelli</i>	SKB2	JN804665	-	JN804809	-
<i>Scarabaeus Kheper bonelli</i>	SKB3	JN804666	-	JN982317	-
<i>Scarabaeus Scarabaeolus sp.</i>	SSB1	JN804671	JN804743	JN804814	-
<i>Scarabaeus Scarabaeolus sp.</i>	SSB2	JN804672	JN804744	JN804815	-
<i>Scarabaeus Sceliages sp.</i>	SSS1	-	JQ088081	JN804816	-
<i>Scarabaeus Sceliages sp.</i>	SSS2	JN804673	JN804745	JN804817	-
<i>Scarabaeus Sceliages sp.</i>	SSS3	JN982320	-	JN804818	-
<i>Pachylomerus femoralis</i>	PLF1	JN804658	JN804730	JN982318	JN819267
<i>Pachylomerus femoralis</i>	PLF2	-	JN804731	JN982319	-
<i>Pachylomerus femoralis</i>	PLF3	JN804659	JN982322	-	-
<i>Pachysoma hippocrates</i>	LA08	AY965170	JN804749	JN804823	JN804597
<i>Pachysoma hippocrates</i>	SK04	AY965190	JN804750	JN804824	JN804598
<i>Pachysoma hippocrates</i>	KK04	AY965161	JN982321	JN804825	-
Tribe Sisyphini					
<i>Sisyphus fasciculatus</i>	SPF1	JN804668	-	JN804811	-
<i>Sisyphus fasciculatus</i>	SPF2	JN804669	JN804742	JN804812	JN819266
<i>Sisyphus fasciculatus</i>	SPF3	JN804670	-	JN804813	-
<i>Neosisyphus sp.</i>	NSP1	JN804651	JN804722	JN804793	JN819265
Tribe Canthonini (Deltachilini)					
<i>Aliuscanthoniola similaris</i>	SMA1	JN804667	JN804741	JN804810	-
<i>Silvaphilus oubosiensis</i>	TIN1	JN804677	JN804748	JN804822	JN804606

Molecular protocol

Muscle tissue from the meta-thoracic femur was used for extraction 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 1 X buffer, 25mM MgCl₂, 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 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 MgCl₂ 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. In the second amplification step 1µl of the resulting amplified product was used in the same reaction mixture except at a 1.5mM MgCl₂ 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). Additional primers CD439F/CD688R and CD667F (Wild and Maddison 2008), for nested PCR were used for taxa that were difficult to amplify. 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 (Katoh and Toh 2008). Thereafter the sequences were adjusted manually. The aligned data consisted of 2748 bp (≈368 bp from 16S, 748 bp from COI, ≈699 bp from 28S D2 and 921 bp from CAD). The phylogenetic relationships among Scarabaeinae 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. 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). Ten 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.

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 was 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 model (Table 3). The analyses were run for 150 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.

The use of these “standard” substitution rates is controversial as researchers use different combinations of mitochondrial genes and different time scales. This is despite the fact that, for example, the arthropod substitution rate of 1.15% substitutions per million years is based on

short sequences and a small sample size of closely related species (Brower 1994). Pons *et al.* (2010) point out that for deep level phylogenies age estimations using COI may be erroneous, suggesting the use of NAD2, NAD4 and NAD5 which exhibit more homogenous rates among codon positions compared to COI. Without reliable fossil evidence, however, researchers continue to use these rates. Moreover COI has become a standard gene region to sequence as it is used for bar-coding (Song *et al.* 2008) and costs for sequencing additional gene regions may be prohibitive. For this study the gene regions COI, 16S, 28S domain2 and CAD were all used.

Table 2. Primers used for PCR amplification

Locus	Primer name and sequence (5'–3')	Reference
Cytochrome oxidase 1	C1-J-2183 CAACATTTATTTTGATTTTTTGG	Simon <i>et al.</i> (1994)
	TL2-N-3014 TCCAATGCACTAATCTGCCATATTA	Simon <i>et al.</i> (1994)
16S rRNA	16Sb2 TTTAATCCAACATCGAGG	Vogler, <i>et al.</i> (1993)
	LR-N-13398 CGCCTGTTTAACAAAAACAT	Simon <i>et al.</i> (1994)
28S rRNA domain 2	D2-3551 CGTGTTGCTTGATAGTGCAGC	Gillespie <i>et al.</i> (2005)
	D2-4057 TCAAGACGGGTCCTGAAAGT	Gillespie <i>et al.</i> (2005)
CAD	54F GTNGTNTTYCARACNGGNATGGT	Moulton and Wiegmann (2004)
	680R AANGCRTCNCGNACMACYTCRTAYTC	Moulton and Wiegmann (2004)
	338F ATGAARTAYGGYAATCGTGGHCA YAA	Winterton <i>et al.</i> (2010)
	654R TCYTTCCANCCYTTYARSGATTTRTC	Winterton <i>et al.</i> (2010)
	CD439F TTCAGTGTACARTTYCAYCCHGARCAYAC	Wild and Maddison (2008)
	CD668R ACGACTTCATAYTCNACYTCYTTCCA	Wild and Maddison (2008)

	CD688R TGTATACCTAGAGGATCDACRTTYTCCATRTTRCA	Wild and Maddison (2008)
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Table 3. Best model and estimated substitution parameter values estimated using AIC with MrModeltest

Gene	COI	16S	28S	CAD
Length (bp)	748	≈368	≈699	921
Best model	GTR+I+G	GTR+I+G	GTR+I+G	GTR+I+G
FreqA	0.3064	0.3907	0.1957	0.4470
FreqC	0.1948	0.1250	0.2832	0.0979
FreqG	0.1699	0.0861	0.3083	0.0318
FreqT	0.3289	0.3983	0.2128	0.4233
rA-C	1.12739	0.4211	1.4131	0.6856
rA-G	5.1234	5.3172	2.1257	8.8656
rA-T	0.6673	1.5486	2.2605	0.4700

rC-G	1.5038	0.4825	0.5878	2.1632
rC-T	5.5907	1.0305	3.6351	8.2627
rG-T	1.0000	1.0000	1.0000	1.0000
P_{inv}	0.2422	0.1884	0.2822	0.2936
alph	0.6625	0.5663	0.6576	0.2862

Table 4. Selected characteristics of the African taxa used in this study

Taxon (number of known species in brackets)	Habitat	Size range (mm)	Food type /relocation	Diel activity
Tribe Canthonini				
<i>Aliuscanthoniola</i> (1)	Leaf litter in dense forest	2.5-2.6	Unknown , trapped with pig dung	unknown
<i>Anachalcos</i> (9)	Forest to savanna	14.5-38.0	Moist dung	nocturnal
<i>Aphengoecus</i> (2)	Open scrubland	4.1-5.9	Dung, decomposing material	unknown
<i>Bohepilissus</i> (2)	forest	1.7-3.1	Probably dung and decomposing material	unknown
<i>Byrrhidium</i> (3)	Succulent karoo to desert	10.5-13.0	Herbivore and omnivore dung, hyrax pellets	unknown
<i>Canthodimorpha</i> (1)	Coastal dune forest	14.6-15.1	unknown	unknown
<i>Circellium</i> (1)	Dense shrubland	22.0-50.0	Rollers / moist dung	diurnal
<i>Dicranocara</i> (3)	desert	9.0-12.0	Hyrax pellets, pick up with front legs and walk backwards	nocturnal
<i>Dwesasilvasedis</i> (1)	forest	4.2-4.8	Dung, decomposing material	unknown
<i>Endroedyolus</i> (1)	forest	3.0-4.0	Probably dung and decomposing material	unknown

<i>Epirinus</i> (29)	Forest/savanna/grassland	3.0-13.5	Carrion, herbivore and omnivore dung, probably decomposing material/rollers	diurnal
<i>Gyronotus</i> (6)	forest	11.0-18.0	Moist dung	likely diurnal
<i>Hammondantus</i> (1)	desert	5.0-6.0	Kleptoparasites of <i>Pachysoma</i> nest	diurnal
<i>Namakwanus</i> (3)	desert	7.0-9.0	Hyrax pellets	unknown
<i>Odontoloma</i> (20)	Forest/savanna/grassland	1.9-3.9	Carrion, herbivore and omnivore dung	diurnal
<i>Outenikwanus</i> (1)	forest	1.6-2.1	Probably dung and decomposing material	unknown
<i>Peckolus</i> (2)	forest	1.8-2.3	Probably dung and decomposing material	unknown
<i>Pycnpanelus</i> (3)	savanna	3.0-4.5	dung	diurnal
<i>Silvaphilus</i> (1)	forest	4.2	Herbivore dung pellets	unknown
Tribe Dichotomiini				
<i>Coptorhina</i> . (15)	Savanna to grassland	8.2-20.7	mushrooms	diurnal
<i>Delopleurus</i> (3)	Savanna to grassland	5.4	mushrooms	unknown
<i>Frankenbergerius</i> (7)	Savanna to forest	4.5-16.0	Mushroom and dung	diurnal
<i>Heliocopriss</i> (49)	Savanna to grassland	26.5-65.3	Dung, tunnellers	nocturnal

<i>Macroderes</i> (14)	Succulent karoo	7.5-13.0	Dung, carry dung pieces with front legs and move backwards to burrows	nocturnal
<i>Pedaria</i> (56)	Savanna/forest/grassland	5.2-10.0	Kleptoparasites of <i>Helicopris</i> nest,/ dung	nocturnal
<i>Sarophorus</i> (10)	shrubland	6.0-10.0	Very old dung/ carrion remains	diurnal
<i>Xinidium</i> (3)	Grassland to forest	8.0-13.0	Dung, tunnellers	nocturnal
Tribe Coprini				
<i>Copris</i> (105)	Forest, savanna, grasslands	8.5-27.3	Omnivore /herbivore dung, tunnellers	diurnal
<i>Catharsius</i> (80)	Forest, savanna, grasslands	13.1-44.6	Dung /tunnellers	nocturnal
<i>Metacatharsius</i> (62)	Arid and moist savannas	4.8-15.2	Herbivore /omnivore dung	nocturnal
<i>Litocopris</i> (4)	savanna, grasslands	10.3-13.6	Old dung	unknown
Tribe Onitini				
<i>Onitis</i> (130)	Forest,savanna, grasslands	12.0-24.9	Dung, tunnellers	Diurnal/nocturnal
<i>Heteronitis</i> (5)	savanna	24.9-38.8	Elephant dung, tunnellers	diurnal
<i>Cheironitis</i> (10)	savanna, grasslands	10.2-16.5	Coarse-fibred dung, tunnellers	diurnal
<i>Anonychonitis</i> (1)	savanna	14.7-17.0	Coarse-fibred dung, tunnellers	unknown

Tribe Oniticellini				
<i>Oniticellus</i> (5)	Forest,savanna	6.5-14.7	Tunnellers /endocoprid; older, drier dung	diurnal
<i>Cyptochirus</i> (4)	savanna, grasslands	8.3-10.4	Herbivore dung, tunnellers	diurnal
<i>Tiniocellus</i> (4)	savanna	4.9-7.6	tunnellers	diurnal
<i>Euoniticellus</i> (15)	savanna, grasslands	3.0-14.0	Tunnellers / very fresh dung	diurnal
<i>Liatongus</i> (17)	Forest, grassland	7.4-10.9	Tunnellers /older, drier dung	diurnal
Tribe Onthophagini				
<i>Onthophagus</i> (970)	Forest,savanna, grasslands	2.5-11.0	Carrion,dung, fruit, fungi /tunnellers, some kleptocoprids	Diurnal/nocturnal
<i>Caccobius</i> (55)	Forest,savanna, grasslands	2.5-8.5	Endocoprid/ kleptocoprids	Diurnal/nocturnal
<i>Hylonthophagus</i> (10)	Savanna	6.8-10.3	Endocoprid/ kleptocoprids	diurnal
<i>Euonthophagus</i> (16)	savanna, grasslands	5.7-9.6	Herbivore dung /tunnellers	dusk
<i>Milichus</i> (15)	Forest,savanna	5.9-9.5	Herbivore dung	nocturnal
<i>Digitonthophagus</i> (1)	arid/moist savannas	12.1-14.4	Herbivore dung, tunnellers	nocturnal
<i>Proagoderus</i> (107)	Savanna/grassland	10.0-22.0	Omnivore dung /tunnellers	diurnal

<i>Phalops</i> (31)	arid/moist savannas	6.6-13.0	Herbivore dung / tunnellers	diurnal
Tribe Scarabaeini				
<i>Scarabaeus</i> (110)	Arid savanna/grassland/forest/desert	7.3-48.5	Dung/ millipedes/ rollers	Diurnal/nocturnal
<i>Pachylomera</i> (2)	Arid to moist savanna	23.4-44.7	Herbivore dung/rollers	diurnal
<i>Pachysoma</i> (13)	desert	15.0-36.3	Dry dung pellets/ plant detritus, held by hind legs and dragged forward	diurnal
Tribe Sisyphini				
<i>Sisyphus</i> (20)	Forest/grassland/savanna	4.0-11.6	Omnivore/ruminant herbivore/rollers	diurnal
<i>Neosisyphus</i> (22)	savanna	6.7-14.2	Omnivore/ruminant, elephant/rollers	diurnal
Tribe Gymnopleurini				
<i>Gymnopleurus</i> (33)	savanna	7.8-14.0	Dung/rollers	diurnal
<i>Allogymnopleurus</i> (20)	grassland/savanna	8.1-18.2	Dung/rollers	diurnal
<i>Garreta</i> (11)	Forest/grassland/savanna	10.7-24.0	Dung/rollers	diurnal

Results

Phylogenetic analysis

The subfamily is monophyletic based on all three tree-drawing methods (posterior probability = 1.00; ML bootstrap value = 100%; MP bootstrap value = 100%). The Bayesian tree for the combined dataset is shown in Figure 4. The three analyses yielded trees that exhibit essentially congruent topologies with high posterior probability values while bootstrap support appeared mixed. As expected from the results of previous analyses on the group, the five tribes Scarabaeini (SCA), Gymnopleurini, (GYM) Sisyphini (SIS), Oniticellini (OTC) and Onitini (ONI) are all monophyletic in all analyses.

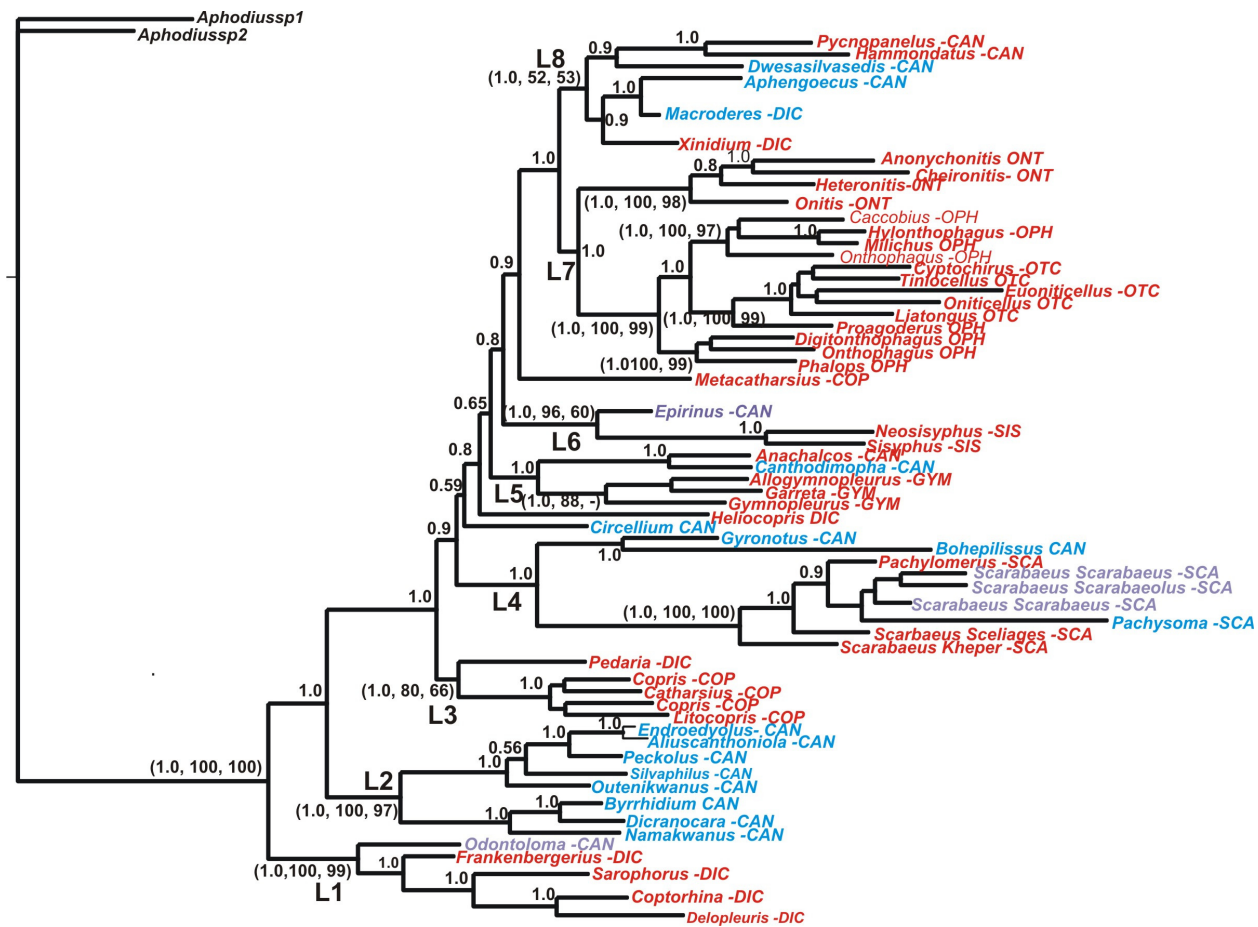


Figure 1. Bayesian phylogram with posterior probabilities for the nine African Scarabaeinae tribes. Bootstrap support for maximum likelihood and parsimony, respectively, added in brackets where applicable). Genera in blue consist of flightless species, those in red, winged species and those in purple have both flying and flightless species. CAN- Canthonini; DIC- Dichotomiini; COP- Coprini; SCA- Scarabaeini; GYM- Gymnopleurini; SYS- Sysiphini; OPH- Onthophagini; OTC- Oniticellini; ONT- Onitini

Addition of taxa did not alter the composition of Lineages 1 and 2 in Figure 1 as these remain the same as those obtained by Sole and Scholtz (2010) and have strong support (posterior probability = 1.0, MP bootstrap = 99% and pp = 1.0, MP bootstrap = 97% respectively. Lineage 1 (L1) comprises *Odontoloma*, *Frankenbergerius*, *Sarophorous*, *Coptorhina*, *Delopleuris*, all dichotomiines, except *Odontomoloma*. These are sister to lineage 2, which contains *Namakwanus*, *Dicranocara* and *Byrrhidium*, *Outenikwanus*, *Silvaphilus*, *Peckolus*, *Aliuscanthoniola* and *Endroedyolus*, all of which are canthonines. Lineage 2 (L2) has two well-supported lineages within it, one of which is made up of *Namakwanus*, *Dicranocara* and *Byrrhidium* while the other consist of *Outenikwanus*, *Silvaphilus* *Peckolus*, *Aliuscanthoniola* and *Endroedyolus*. Lineage 3 (L3) consists of all of the genera from the tribe Coprini (except *Metacatharsius*) as well as the genus *Pedaria* traditionally classified as a dichotomiine (pp = 1.0, ML bootstrap = 80%, MP bootstrap = 66%). Lineage 4 (L4) includes a monophyletic Scarabaeini and the canthonine genera *Gyronotus* and *Bohepilissus* (pp = 1.0, ML bootstrap = 100%, MP bootstrap = 100%). The genera *Circellium*, a canthonine, and *Heliocopris*, a dichotomine form two distinct separate lineages. In Lineage 5 (L5) a monophyletic Gymnopleurini is sister to two canthonine genera *Anachalcos* and *Canthodimorpha* (pp = 1.0). Sisyphini genera *Sisyphus* and *Neosisyphus* sister to the canthonine genus *Epirinus* (pp = 1.0, ML bootstrap = 96%, MP bootstrap = 66%) form Lineage 6 (L6). *Metacatharsius*, a distinct lineage, is sister to all the genera in Lineages 7 (L7) and 8 (L8).

Within Lineage 7 a monophyletic Onitini (pp = 1.0, ML bootstrap = 100%, MP bootstrap = 96%) is sister to Onthophagini (pp = 1.0, MP bootstrap = 99%) with Oniticellini (pp = 1.0, ML bootstrap = 100%, MP bootstrap = 99%) nested within Onthophagini. Lineage 8 (pp = 1.0, ML bootstrap = 80%, MP bootstrap = 53%) has two sister lineages within it, one containing the

canthonine genera *Pynopanelus*, *Hammondatus* and *Dwesasilvasedis*. In the sister lineage are *Aphengoeus*, a canthonine, and two dichotomiines genera namely, *Macroderes* and *Xinidium*.

Estimation of divergence times

Our analysis places the earliest split in the subfamily in Africa between 82 (63/103) and 50 (39/64) MYA for the rates of 0.0075 and 0.012 substitutions /site/MYA respectively (upper and lower credibility limits in brackets). Confidence intervals are wide for the slower mutation rate (0.0075). The posterior distributions of a number of parameters were poor (low ESS values) therefore there is doubt on the validity of these age estimates. For this reason discussion will be based on the faster evolutionary rate. Below are the estimates of selected lineage ages based on a BEAST analysis of COI, 16S, 28S domain 2 and CAD sequence data.

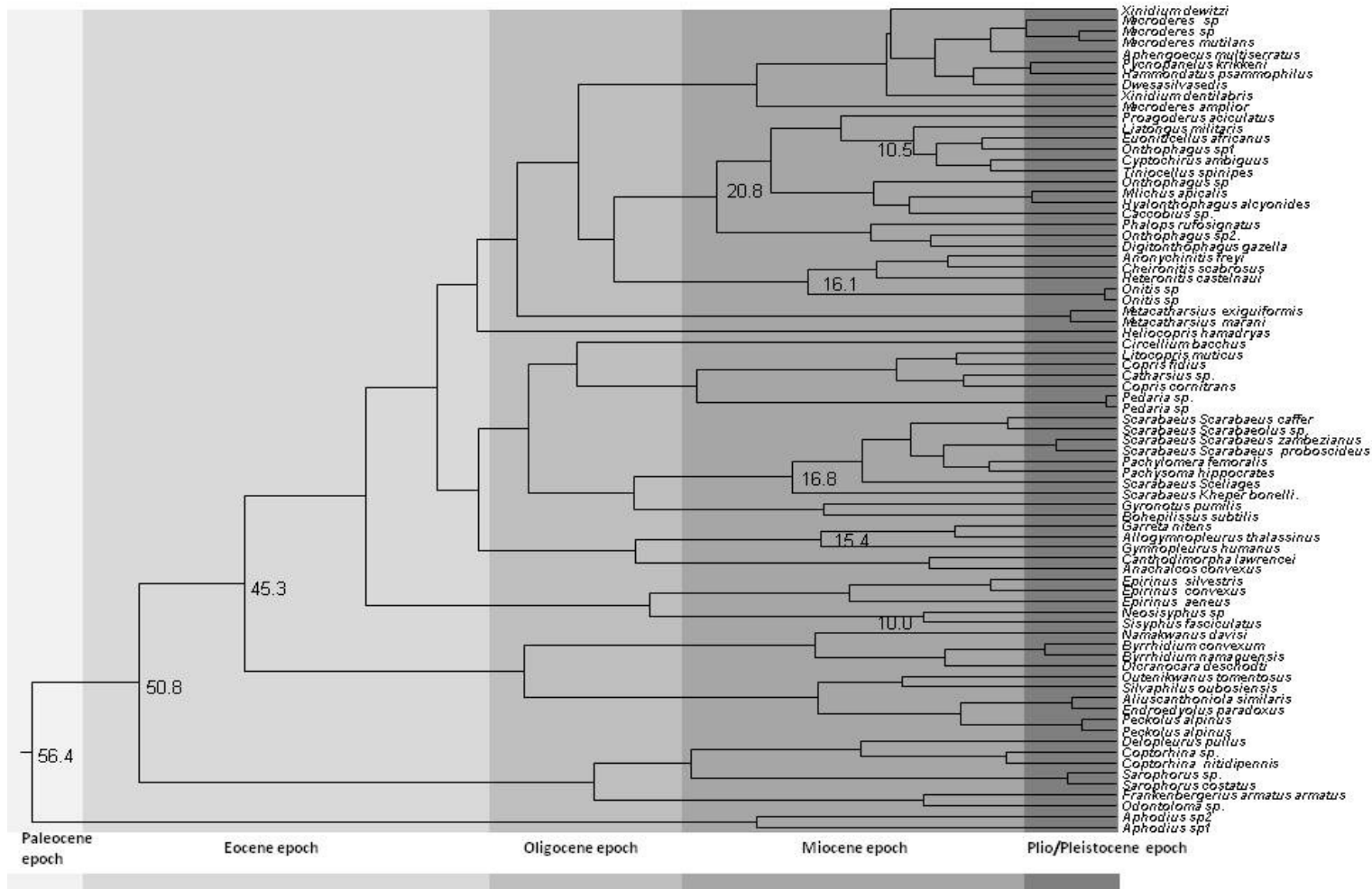


Figure 2. Divergence times for the African Scarabaeinae.

Discussion

Here we present a phylogenetic analysis of the nine tribes that make up the African Scarabaeinae. Taxon sampling was comprehensive and included a number of genera that have not been used in recent analyses of the subfamily (Philips *et al.* 2004; Monaghan *et al.* 2007). Earlier analyses made little provision for many in the African group, a number of which may have been unknown or poorly studied at the time. These include recently described genera such as *Silvaphilus*, and *Aliuscanthoniola* and others, for example *Namakwanus* and *Outenikwanus*, which are amongst some of the earliest diverging lineages.

Phylogeny

Below we compare our results (Figure 1) with the African taxa in the phylogeny of Monaghan *et al.* (2007), and to some extent, other recent molecular phylogenies and the morphological one of Philips *et al.* (2004). Table 4 provides information on the number of species, feeding characteristics, habitats and size of taxa included in this study. Generally there is congruence between our tree and that of Monaghan *et al.* (2007) providing a reliable basis for the evolutionary analysis of different traits. Both phylogenies support the monophyly of the tribes Scarabaeini, Sisyphini, Gymnopleurini, Onitini and Oniticellini. Monaghan *et al.* (2007) found Coprini, Dichotomiini Canthonini and Onthophagini to be polyphyletic with three, four, four and three lineages, respectively. In the present study the same tribes form two, five, eight and three lineages respectively.

Gymnopleurini

There are three representatives of the tribe in Africa, namely *Gymnopleurus*, *Allogymnopleurus* and *Garreta*. In the studies of Villalba *et al.* (2002) *Gymnopleurini* is sister to Scarabaeini. Our analysis returns a sister relationship with the canthonine genera *Anachalcos* and *Canthodimorpha*, similar to Monaghan *et al.*'s (2007) phylogeny which places *Gymnopleurini* in a clade that is sister to *Anachalcos*, showing congruence between the analyses. The genera in this lineage (5) are all rollers except *Canthodimorpha* whose food exploitation activities and diet are unknown. *Canthodimorpha* is a monotypic and flightless forest genus whereas *Anachalcos* is one of the few canthonines that is fairly widely distributed. It is nocturnal and food association varies with species ranging from carrion to herbivore and omnivore dung.

There are no phylogenetic studies of *Gymnopleurini* in the literature; however, Monaghan *et al.*'s (2007) phylogeny suggests divergence in the order *Gymnopleurus*, *Garreta* and *Allogymnopleurus*. Our dating analysis also suggests that *Gymnopleurus* is the earliest diverging genus among the three.

Sisyphini

This tribe consists of three genera, two of which occur in Africa. All the species in *Sisyphini* are rollers. In our phylogeny *Sisyphini* is sister to the rolling canthonine genus *Epirinus*. The two are in turn sister to a clade comprising the genus *Metacatharsius* and all the genera from the tribes Onthophagini, Onitini, Oniticellini and a dichotomine/canthonine group (labeled lineage 8). A similar relationship is shown in Monaghan *et al.*'s study (2007) where *Epirinus* is sister to a

clade comprising Sisypkini, Onitini, Onthophagini and Oniticellini. Philips *et al.* (2004) found similar patterns. We found Sisypkini to be the most recently diverged tribe with an age of 10 Mya (3.3/18.3 Mya), which is in line with Cambefort's (1991a) categorisation of the tribe as a "modern" roller.

Scarabaeini

A number of recent phylogenetic analyses have included this tribe (Forgie *et al.* 2005; 2006; Philips 2004, Monaghan *et al.* 2007, Sole *et al.* 2011) and none has questioned its monophyly. In both Monaghan *et al.* (2007) and Philips *et al.*'s (2004) analyses Scarabaeini was sister to a clade that included the monotypic genus *Circellium*. In our analysis the tribe formed a sister relationship with canthonine genera *Gyronotus* and *Bohepilissus*.

The genus *Pachysoma* has had a variable taxonomic history. It has been considered a genus (Ferreira 1953), synonymised with *Scarabaeus* (Mostert and Holm 1982), considered a subgenus of *Scarabaeus* (Harrison and Philips 2003; Forgie *et al.* 2005) and currently has full generic status (Forgie *et al.* 2006). Sole *et al.* (2007) also supported the idea of *Pachysoma* as a genus). Interestingly, as with *Khepher* and *Sceliages*, it groups as sister to *Scarabaeus* species in our study confirming its placement as a genus.

It was originally postulated that the Scarabaeini was an old group with Madagascan representatives as old as the Mesozoic separation of the island (Davis *et al.* 2008). A recent study has estimated the African and Madagascan scarabaeine taxa have a shared origin of between 24 and 15 my (Sole *et al.* 2011). Forgie *et al.* (2005) estimated the origin of the Scarabaeini at

between 23 and 9.8 mya. Both studies used 16S and COI sequence data but different analytical methods. Our estimate of their divergence is approximately 17 Mya (10.9/22.6Mya falling within the estimates of both Forgie et al (2005) and Sole et al (2011).

Onitini

The genera of Onitini form a monophyletic lineage which is sister to the Onthophagini and Oniticellini. Onitini has 17 genera that occur in Africa. All the species in this tribe are tunnellers and coprophagous (Scholtz *et al.* 2009). *Onitis* and *Cheironitis* are species-rich whereas the remaining genera have no more than five species each. The monophyly of Onitini has not been questioned with recent analyses, both molecular (Villalba *et al.* 2002; Monaghan *et al.* 2007) and morphological (Philips *et al.* 2004) data, having determined the tribe to be sister to Onthophagini and Oniticellini. Suggestions by Cambefort (1995), sighted in Scholtz *et al.* (2009), that the genera associated with non-ruminant dung diverged earlier than those associated with the dung of ruminants (in tandem with the respective herbivores) may be true since *Anonychonitis*, in our dating analysis (based on COI), is basal to the remaining three genera. The genera here are too few however, to draw any concrete conclusions.

Oniticellini

Oniticellini is divided into three subtribes. The Helictocpleurina are restricted to Madagascar whereas Drepanocerina and Oniticellina are represented in Africa. There has been much work recently to stabilise the systematics of the Afrotropical fauna in this tribe (Barbero *et al.* 2009a,

b; Barbero *et al.* 2011). In the present study one drepanocerine genus, *Cyptochirus* and four oniticellines *Tiniocellus*, *Euoniticellus*, *Oniticellus* and *Liatongus* form a monophyletic group that is nested within Onthophagini. This group is sister to the genus *Proagoderus*, an onthophagine.

In our Bayesian analysis and that of Ocampo and Hawks (2006) the tribe Oniticellini was found to be a monophyletic group nested within Onthophagini whereas Monaghan *et al.* (2007) found both Onitini and Oniticellini to form monophyletic clades within Onthophagini. Our MP analysis returns some sister relationships between Onthophagini and Oniticellini genera. In their study Wirta *et al.* (2008) found both Onthophagini and Oniticellini to be paraphyletic with an Onitini species branching off within the two tribes. This instability points to a need for further examination of these relationships.

Onthophagini

Onthophagini is the most species-rich tribe in the subfamily Scarabaeinae with more than 2500 species. The genera are widely distributed in Africa. Onthophagini is made up of mainly tunneling genera, although, some small-bodied species are kleptocoprids, relying on dung buried by other dung beetles for breeding. Examples include some *Caccobius* and *Hylonthophagus* species. Within the tribe there is also ant or termite-association in some genera such as *Haroldius* (not included in the present study).

The Onthophagini in our analysis are paraphyletic. *Phalops*, *Digitonthophagus* and *Onthophagus* form a clade that is basal to the rest of the genera, with *Proagoderus* and

Oniticellini genera next in succession and finally *Euonthophagus*, *Milichus*, *Hylonthophagus* and *Caccobius*. This order of derivation is consistent with the findings of Monaghan *et al.* (2007).

Coprini

Coprini is a tunneling tribe, which according to its traditional constitution comprised 10 genera of which seven occur in Africa. Three of the genera in our analysis form a natural group that is sister to *Pedaria*, with *Metacatharsius*, the fourth genus, forming a distant distinct lineage. The separation of these genera was unexpected since the African *Metacatharsius* and *Catharsius* in Monaghan's (2007) analysis grouped together. The divergence of this tribe is estimated to have occurred between 50 and 21 mya. However, currently, composition of the tribe and the phylogenetic validity of the characters that define and separate Coprini and Dichotomiini are questionable (Davis *et al.* 2008).

Dichotomiini and Canthonini

For a long time there has been doubt concerning the monophyly of Canthonini (Scholtz and Howden 1987; Frolov and Scholtz 2003a; Philips *et al.* 2004). In addition, many of the African canthonines are either monotypic or have only a few species and are therefore likely to be relicts of otherwise extinct lineages (Frolov and Scholtz 2003a). There has been similar instability within Dichotomiini. The Afrotropical region has nine Dichotomiini genera with 126 species. Previous phylogenetic studies have confirmed its polyphyletic nature since the first detailed study of Coprini and Dichotomiini by Montreuil (1998).

There is congruence between our findings and those of Monaghan *et al.* (2007) concerning the position of *Coptorhina* + *Sarophorus* being in the most basally derived lineages. *Sarophorus* shares a number of apparently synapomorphic characters with *Coptorhina* and *Delopleurus* (Frolov and Scholtz 2003b). Our analysis and that of Sole and Scholtz (2010) concur with the relatedness of these genera; however, there is need for morphological studies to determine the relationship between this group of four genera and *Odontoloma*, which is currently placed among the canthonines. *Odontoloma*, a genus with an African distribution range is not a typical canthonine. Therefore its placement in the tribe was uncertain (Howden and Scholtz 1987), with some species being thought to be more closely related to the onthophagines.

The three genera in the earliest diverging lineage, *Coptorhina*, *Delopleurus*, and *Frankenbergerius*, feed and breed on fungi while *Sarophorus* is a coprophagous genus. Cambefort (1991b) and Scholtz and Chown (1995) hypothesised that fungus feeding was the ancestral feeding pattern of dung beetles with a change later, to feeding on dung. With the beetles depending nutritionally on the microbes within the substrate, the change would have been easy (Cambefort 1991b; Scholtz and Chown 1995).

The flightless genera *Byrrhidium*, *Namakwanus* and *Dicranocara* form a distinct sub-lineage in our analysis. The close relationship among these genera alluded to by Frolov and Scholtz (2003a; b) is confirmed in our molecular study. *Byrrhidium*, *Namakwanus* and *Dicranocara* each have three species and all but one species of *Byrrhidium* are only known from deposits of dung pellets in rock hyrax middens (*Procavia capensis*) (Deschodt *et al.* 2007). These genera, together with many other canthonines are thought to be relicts of a fauna that was once widespread but have been forced into restricted environments and trophic specialisations

through increasing aridity. In addition, the feeding habits of this group fall in line with what Cambefort (1991a) postulated, that the 'taxa possibly turned to the dung of primitive small mammals that were forest dwellers' giving the example of the Vietnamese genus *Panelus* that feeds on rat faeces.

Peckolus, *Endroedyolus*, *Aliuscanthoniola*, *Outenikwanus* and the newly described monotypic *Silvaphilus* (Roets and Oberlander 2010), are in a clade that is sister to *Byrrhidium*, *Namakwanus* and *Dicranocara*. Our molecular analysis confirms the close relationship among these taxa that is suggested by general morphology (Roets and Oberlander 2010). These are all small (less than 5mm) flightless forest endemics (Deschodt and Scholtz 2008; Davis *et al.* 2008; Roets and Oberlander 2010). Our analysis and that of Sole and Scholtz (2010) validate the need for a new classification that will re-categorise these two monophyletic groups.

Pycnopanelus and *Hammondatus*, positioned in lineage 8, doubtfully belong to the tribe Canthonini, and have been closely associated with Onthophagini. However, there is no implication of such a relationship in our analysis. Whereas the two genera are found in southwestern arid areas, they appear closely related to *Dwesasilvasedis* a flightless forest endemic. This is an example of the diversity that occurs among closely related genera expressed by Monaghan *et al.* (2007). Taxa diversify to fill different ecological niches. The other canthonine in this lineage is *Aphengoecus*, a coastal sandveld genus. It comprises two flightless species whose habitat in deep coastal sands and mountains has been greatly reduced due human infrastructural developments. *Aphengoecus* appears closely related to *Macroderes*, a Coprini-associated dichotomine. The genus *Xinidium* appears as a monophyletic genus also in the same lineage. It is also a dichotomine that is associated with Coprini.

Food and its relocation

There is intense competition at a dung pat among dung beetles as with other insects groups. One of the reasons for their success has been the evolution in dung beetles of specialised ways of using this resource. The two most well known methods involve either forming and rolling dung balls away, or burying the dung beneath the source, thus facilitating its quick removal from competition (Hanski and Cambefort 1991). Some dung beetles may be dwellers and others kleptocoprids (Doubé 1990). Previously the two main relocation methods had been used for classification in Scarabaeinae but it has become clear that the character of food relocation cannot be used for this purpose in the subfamily. There is much evidence for the plasticity of this trait alluded to in previous studies and dung beetles appear to be true opportunists that use whatever dung type is available with a suitable relocation method associated with the habitat. Examples are the forward dragging of detritus and dry dung pellets on sand in *Pachysoma* (Scholtz 1989), and carrying of pieces of dung by “tunneller” genus *Macroderes* (Davis *et al.* 2008). This trait varies widely within tribes showing no traceable pattern.

Loss of flight

In the African Scarabaeinae we see the loss of flight on a number of occasions within the subfamily. In some genera there are both flightless and flight capable species. Examples are the canthonine genera *Epirinus* (Scholtz and Howden 1987; Medina and Scholtz 2005; Mlambo *et al.* 2011) and *Odontoloma* (Davis *et al.* 2008) as well as the scarabaeine genus *Scarabaeus* (Forgie *et al.* 2005; 2006). Changes in a habitat or habitat isolation are generally thought to be the main factors that lead to flightlessness. Scenarios such as deserts and forests proposed by

Roff (1990) and Scholtz (2000) as leading to flightlessness are seen in the subfamily. The earliest diverging flightless lineage comprising *Byrrhidium*, *Namakwanus* and *Dicranocara* is an example. Their desert habitat likely drove the loss of flight but we also propose that a change in feeding habit from saprophytic to coprophagous also contributed, as in Ikeda *et al.*'s (2008) suggestion that other evolutionary processes independent of change in habitat or isolation may result in flight loss.

Biogeography and Origin of dung beetles

The dates for the origin of dung beetles from this study are consistent with those of Sole and Scholtz (2010) placing the events in the early Cenozoic era. This also compares well with the dates calibrated by Scholtz *et al.* (2009) for the phylogeny of Monaghan *et al.* (2007). Dung beetle distribution is influenced by two ecological factors, namely climate and the number of dung types (Davis and Scholtz, 2002). Sole and Scholtz (2010) discuss the conditions that have led to the current distribution of dung beetles on the continent.

While it was believed that the Scarabaeinae had a Gondwanaland origin (Cambefort 1991a; Davis *et al.* 2002). Monaghan *et al.* (2007) in their analysis of the global fauna suggested that it was the African group that was ancestral to lineages in all other regions. Moreover, they asserted that most clades in their phylogeny were derived from African clades. The proportion of global dung beetles on the African continent alone counts for much in support of this assertion. There are approximately 100 genera in the Afrotropical region compared to 66 in the Neotropical region, the next in dung beetle diversity. This greater morphological diversity in the African lineages is likely due to greater lineage age in this biogeographical region.

For the tribe Scarabaeini Monaghan *et al.* (2007) only had African species whereas the phylogenies of Forgie *et al.* (2005) and Sole *et al.* (2011) included Oriental and Malagasy taxa. In these latter studies the African genera appear ancestral to the rest. For the tribe Gymnopleurini, *Gymnopleurus* appears to be the ancestral genus (Monaghan *et al.* 2007). This genus has an Afrotropical and Palearctic distribution pattern. A similar situation is true for earliest diverging genera in Oniticellini. They are most diverse in Africa, and have some representation in other biogeographic regions. Helictopleurina, a subtribe of Oniticellini that is endemic to Madagascar, is thought to have colonised Madagascar from an African origin (Wirta *et al.* 2008). Monaghan *et al.* (2007) found Helictopleurini most closely related to species from the Afrotropical-Oriental and Palearctic regions. Barbero *et al.* (2009b) hypothesise that the ancestor of *Eodrepanus*, a new genus in the subtribe Drepanocerina, was from the Afrotropical-Oriental region.

Among the onthophagine species sampled by Monaghan *et al.* (2007) the South African species *Phalops ardea* and *Digitonthophagus gazella* appear ancestral while Tarakov and Solodovnikov (2011) proposed an Afrotropical-Oriental ancestor for the Onthophagini they analysed morphologically. The largest and most widespread genus among the Scarabaeinae, *Onthophagus*, and (Davis *et al.* 2008) was shown by Emlem *et al.* (2005) to be of African origin.

All this evidence suggests Africa as the major diversification region for Scarabaeinae with subsequent dispersal to other biogeographical regions.

Conclusion

Some of the relative ages for the different tribes that were proposed by Cambefort (1991a) have proven to be inaccurate. Among the tunnellers for example, the divergence of “intermediate” Onitini is more recent than that of Coprini, Onthophagini and Oniticellini. Moreover although some of the genera in Dichotomiini and Canthonini are indeed “old”, the divergence estimates indicate that the terminal taxa in these tribes have diverged as recently as the tribes Onthophagini and Oniticellini. In addition, these taxa are very distantly related to the rest of the genera in their current tribes. We suggest morphological re-examination of these taxa with the use of novel characters and the assignment to a new tribe(s). Scotland *et al.* (2003) suggest rigorous examination of few morphological characters in the framework of molecular phylogenies as the best way to integrate the strengths of molecular and morphological data. We recommend the same treatment for apparently oddly placed taxa such as *Pedaria* which is superficially similar to *Sarophorus* (Frolov and Scholtz 2003b) and *Circellium* and *Metacatharsius* with possible tribal reassignment. The enigmatic genus *Circellium* has no close relatives and has been placed in Scarabaeini in the past but is currently placed in the tribe Canthonini (Davis *et al.* 2008). The placement of this genus in our analysis has low support and therefore remains uncertain.

Another challenge also remains to combine the results from this molecular study with morphological data to consolidate African dung beetle systematics. The findings, together with those from other biogeographical regions can then be used to gain a better understanding of worldwide dung beetle relationships.

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Chapter 3

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

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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 synonymisation 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 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 1991a): 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.

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. hlulhluwensis* (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 davisii* 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 MgCl₂, 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 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 MgCl₂ 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 MgCl₂ 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 (Katoh 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 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 bootstrap value). Lineage B₃ with posterior probability 0.99, includes *E. relictus* sister to the flightless pair *E. hluhluwensis* 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 (B3) 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.

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 synonymisation 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 1991a). 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 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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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	Vanhynsdorp	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	EH01*	GQ290019	GQ289750	GQ289800	GQ289946
			Pass	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

Table 2. Primers used for PCR amplification

Locus	Primer name and sequence	Reference
Cytochrome oxidase 1	C1-J-2183 (5'CAACATTTATTTTGATTTTGG 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' GTNGTNTTYCARACNGGNATGGT 3')	Moulton and Wiegmann (2004)
	680R (5' AANGCRTCNCGNACMACYTCRTAYTC 3')	Moulton and Wiegmann (2004)
	338F (5' ATGAARTAYGGYAATCGTGGHCAYAA 3')	Winterton <i>et al.</i> (2010)
	654R (5' TCYTTCCANCCYTTYARSGATTTRTC 3')	Winterton <i>et al.</i> (2010)

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

	16S	COI	D2	CAD	Combined
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

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

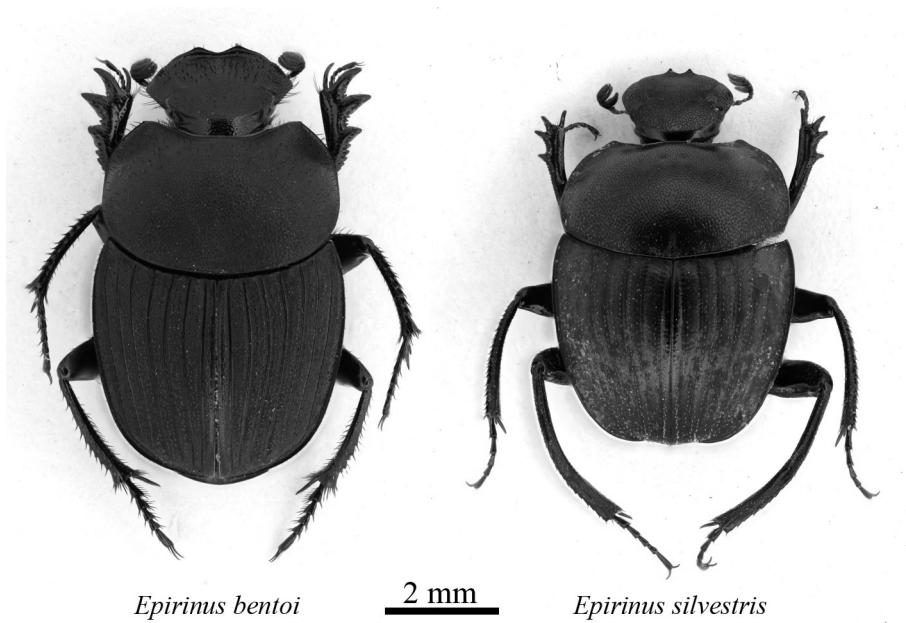


Figure 1. Photograph showing some superficial morphological differences between winged (*Epirinus bentoii*) and wingless (*E. silvestris*) species.

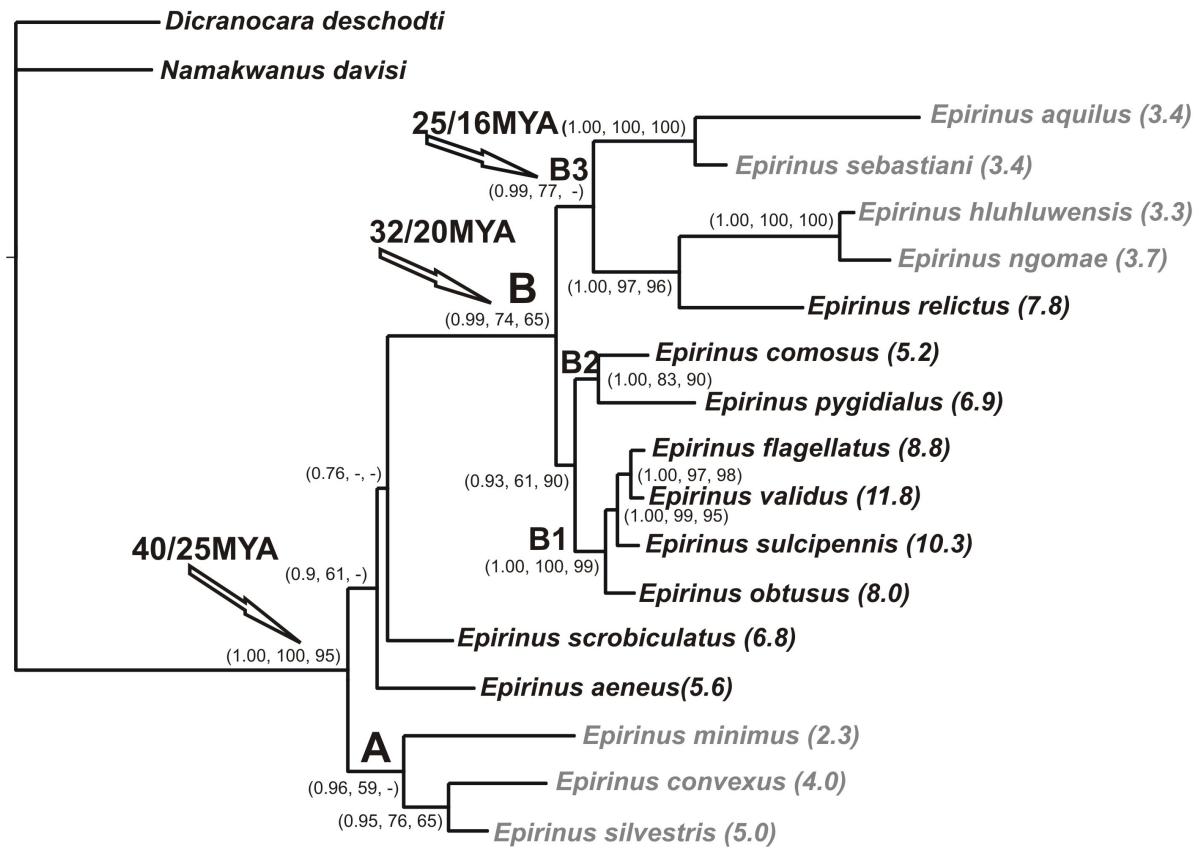


Figure 2. Molecular (16S, COI, D2 and CAD) Bayesian inference phylogram. Posterior probabilities, maximum likelihood and parsimony bootstrap values indicated where 0.5/50% or higher. Divergence times for the flightless lineages are indicated at nodes A, B and B3. Flightless taxa in grey font. Size in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

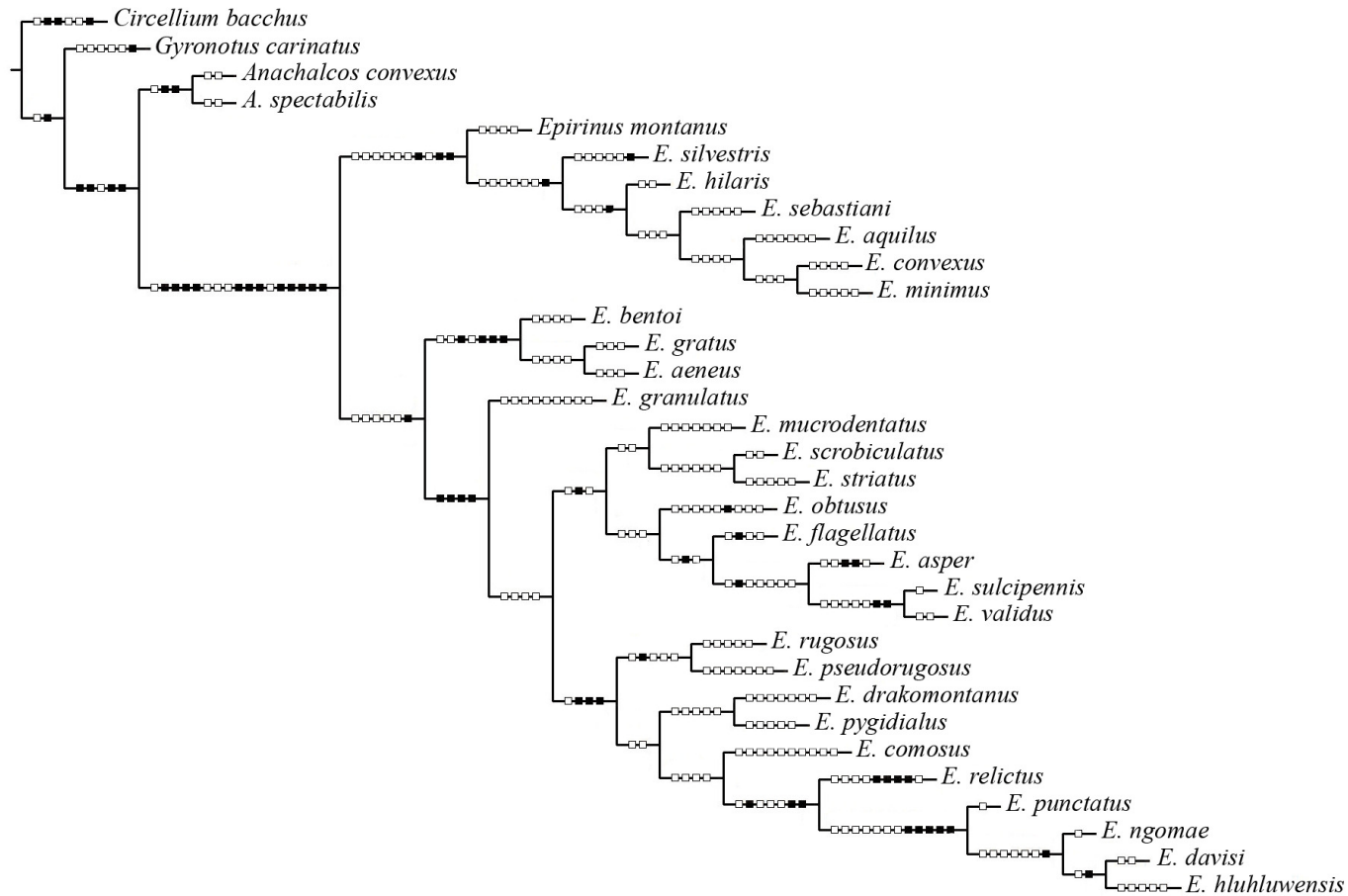


Figure 3. Strict consensus tree from 3 cladograms (331 steps, CI = 40, RI = 67) based on morphological characters. Black hatch marks indicate changes without homoplasy and white hatchmarks indicate changes with homoplasy. Reproduced from Medina and Scholtz (2005).

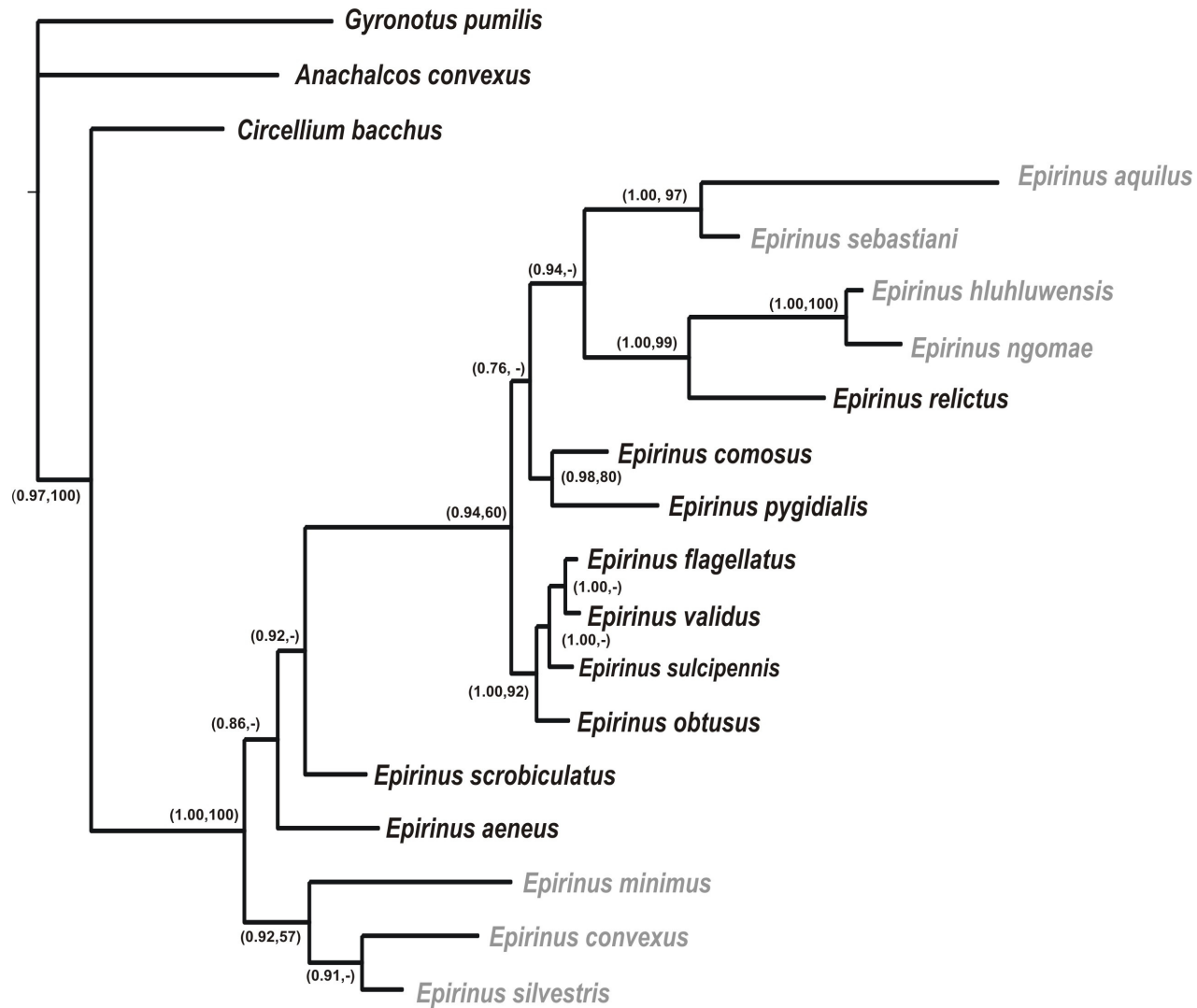


Figure 4. Combined morphological and molecular Bayesian inference phylogram with posterior probabilities and parsimony bootstrap values respectively where 0.5/50% or higher. Flightless taxa – grey font.

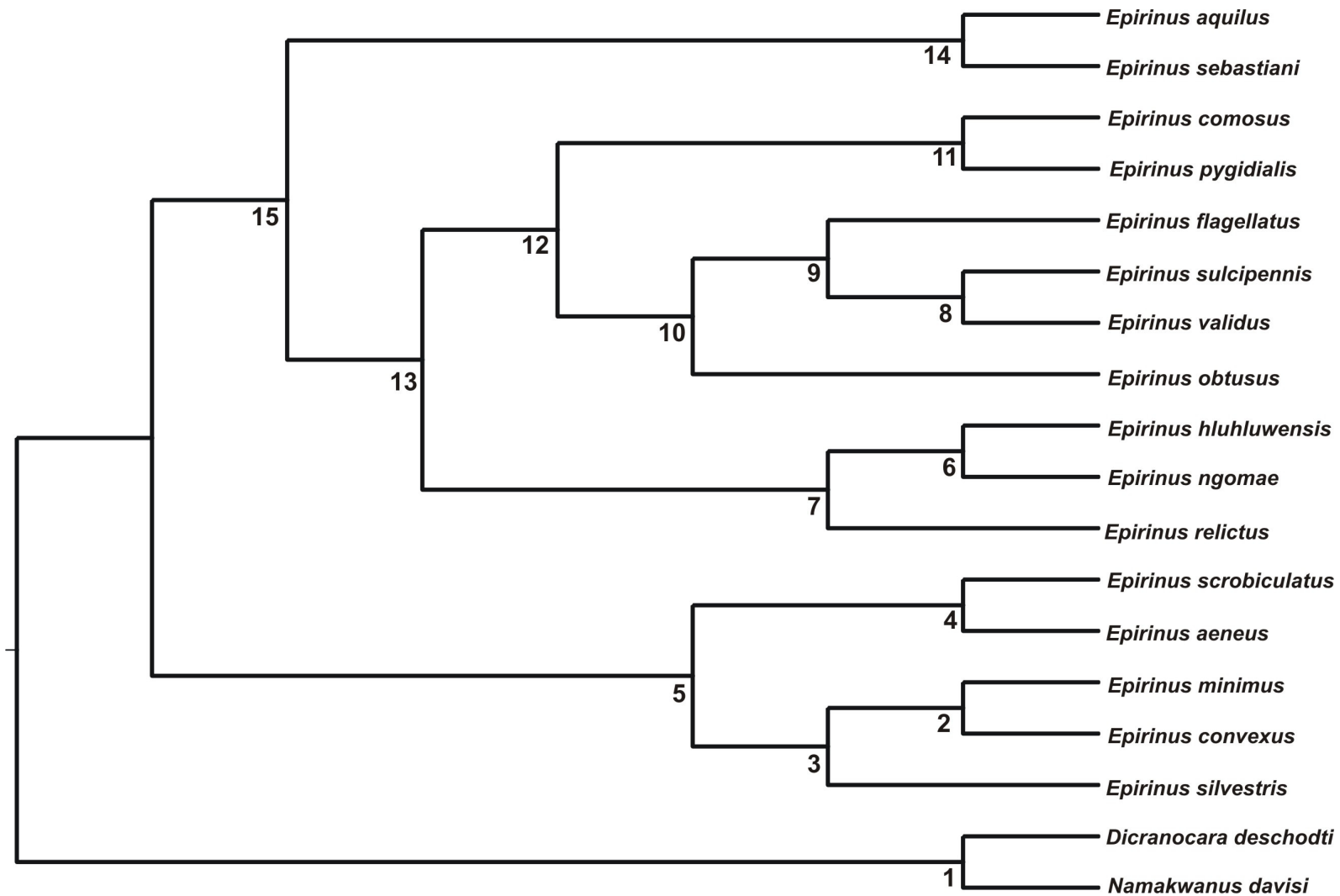


Figure 5. Shortest tree constrained to include nodes obtained in the Bayesian analysis. Node numbers indicated below branches.

Chapter 4: General Conclusions

Presented in this section are the key findings of the project as well as recommendations for avenues of future research.

Phylogeny of the African Scarabaeinae

One of the major aims of this study was to estimate the phylogeny of the African Scarabaeinae for comparison with a global-scale phylogeny. Robust resolution was attained for a majority of the deep nodes with the topology of the phylogeny largely similar to that of the global study. The tribes Canthonini, Dichotomiini, Coprini and Onthophagini were found to be polyphyletic while Sisyphini, Scarabaeini, Gymnopleurini, Onitini and Oniticellini appear to be natural groups. It is recommended that, based on the results of this study, which confirm the findings of Sole and Scholtz (2010), lineages 1 and 2 should be given tribal status. The lineages are based on monophyly and are not disruptive to the current classification of the subfamily.

Another major goal was to determine the approximate ages of the nine tribes that occur on the continent. For the first time the divergence times of the different tribes were estimated and some of the relative ages are not consistent with the conventional “ancient, intermediate and modern” ages that have previously been suggested. Evidence from this study supports the hypothesised African origin of Scarabaeinae.

Further work on this subfamily should include combined molecular and morphological phylogenetic analyses for each biogeographic region after which critical taxa should be identified and their data collated for a better understanding of the evolutionary history of the global Scarabaeinae.

Phylogeny of Epirinus Reiche

In the second part of the project, the hypothesis that size is a constraint on the ability to form and roll dung balls was rejected. Six species which are among the smallest in the genus were observed rolling dung balls. Considering the increasing evidence for the plasticity of feeding behaviour, further investigation could be carried out as a number of questions still remain unanswered. Do these species construct and roll dung balls in their natural environments? If presented with less malleable feeding material such as dung pellets of non-grazing herbivorous mammals or carrion, what behaviour would they exhibit?

The molecular phylogeny for *Epirinus* was found to be congruent with a published one based on morphology. The monophyly of the genus was confirmed with flightlessness having evolved twice.

Appendix 1. Character states as defined by Medina and Scholtz (2005) of the taxa used in the combined analyses for *Epirinus*. (0-5) character state; ? unknown character state; - not applicable character state.

[illegible]

<i>Epirinus striatus</i>	20100001001231000130000111110100101001000112101125114010110140--110413011110000
<i>Epirinus obtusus</i>	20000000001221000110010110100200220001000111101125114010010120-0110413001210000
<i>Epirinus mucrodentatus</i>	21101001001221000121000110110100110000000112101125114011210140--110412011110000
<i>Epirinus pseudorugosus</i>	21100011001232000120010110102200101112000112201124113010010140-111141200110-000
<i>Epirinus silvestris</i>	211001102012420001111011101102001200001112111011241160110100-10-0--51200140-000
<i>Epirinus montanus</i>	210010002012420001101001101101001210001002011211241150112100-10-0--512????????
<i>Epirinus davisi</i>	211001102012420100312001101013111201001102011111????????????????????00150-110
<i>Epirinus convexus</i>	211001102112420100210001101003111100001102112111241150100100-11-0--51200140-001
<i>Epirinus hiliaris</i>	211001102012420000100001111102001110001102111111241150112100-10-0--51200140-001
<i>Epirinus hluluwensis</i>	21100110201242011021100110101311120000110211121123111010010110--10-21100150-110
<i>Epirinus sebastiani</i>	211001102012420000200000111103011210001102111211241150112100-11-0--51200140-001
<i>Epirinus minimus</i>	2110011010124200001100011010031111000011010121112411501100-0-10-0--51200140-001
<i>Epirinus ngomae</i>	2110011010124201102120011010131112010011021111112311101000-110--10-21100150-100
<i>Epirinus aquilus</i>	211001102112420000211011101103111100001112111111241150112110-10-0--51200140-001
<i>Dicranocara deschodti</i>	012011----200322000000000010110 10000000021001300002????????????????????????????
<i>Namakwanus davisi</i>	0 1 1 1 1 1 ---- 100322000000000001011020100000021011300002????????????????????????????