

Systematics and conservation of *Colophon* Gray (Coleoptera: Lucanidae)

By

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*To my parents who with love, acceptance and eternal belief in me have motivated,
encouraged and fuelled my education*

“What a wonderful bug is *Colophon*

Each peak has a different kind of one.

On one he is caught with nippers quite short

The next he has claws like a scorpion.”

~Ditty recounted by Frank (Punky) Berrisford Junior

Declaration

I, Angelika Katrin Switala declare that the thesis/dissertation, which I hereby submit for the degree Master of Science (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: _____

Systematics and conservation of *Colophon* Gray (Coleoptera: Lucanidae)

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Summary

The flightless Cape High-mountain stag beetle genus *Colophon* (Coleoptera: Lucanidae) is studied. Represented by 17 species, which are restricted to the highest mountain peaks of the Cape Floristic Region in the Western Cape, South Africa, and show a strict association with the fynbos biome. The study aimed to determine the specific and phylogenetic status of the described species of *Colophon* and to determine the main factors driving their evolution by testing hypotheses of relationship and of a lowland origin. This was achieved by analysing DNA sequence data from three gene regions, the mitochondrial COI and 16S rRNA and the nuclear CAD, using a Maximum Likelihood (ML) and Bayesian approach. Timing of key biogeographical events in the diversification of *Colophon* was estimated in BEAST. The study also undertook to determine diagnostic larval characters for *Colophon* species and also to determine their phylogenetic implications for the sub-familial placement of the genus. Lastly, the study aimed to collate biological information on *Colophon* species so as to make suggestions for their revised conservation status in terms of IUCN and ToPS criteria.

Most species of *Colophon* showed an allopatric distribution, although contact zones between geographically adjacent species are likely. Climate seems to be the main driving factor behind *Colophon* evolution and the hypothesis of a lowland origin appears to be supported. Larvae are soil-living and feed on humus, a habit unique to the family. There are only small inter-specific differences between larvae, with larval characters contributed little equivocal information from which phylogenetic support for family placement of *Colophon* could be deduced. The main threats to *Colophon* survival include overexploitation by commercial collectors, decline in habitat quality and habitat loss due to projected changes in climate. It is suggested that out of the 17 currently described species, eight should be listed as Critically Endangered, seven as Endangered, two as Data Deficient.

Lastly, in light of this project's findings, it is suggested that future considerations in terms of *Colophon* research should focus on obtaining more information on their biology, behaviour and population size and in so doing contribute knowledge for the effective conservation management of each species. A taxonomic revision of the species, focusing on the *C. stokei* varieties and *C. eastmani* subspecies, should be done and a complete taxonomic key of all described species compiled. Future fieldwork should focus on sampling the five species that remained elusive during the project, to eventually be included in phylogenetic analyses.

Keywords: Biogeography; Cape Floristic Region; *Colophon*; Conservation; Divergence time; Larval description; Lucanidae; Phylogeny.

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This thesis would not have been possible without the guidance and help of several individuals, who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

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Chapter I:

General Introduction

Cape Floristic Region

The south-western tip of Africa, the Cape Floristic Region (CFR), with its exceptionally high floral diversity and high levels of biotic endemism, has been recognised as one of six Floral Kingdoms, representing two of the 34 diversity hotspots on the planet, and two of the 200 ecoregions of the world (Cowling, 1992; Myers *et al.*, 2000; Olson & Dinerstein, 2002; Linder *et al.*, 2010). The region is unique from the rest of Africa with its mediterranean type climate and its remarkable geographical structuring. The Cape Fold Mountains, with peaks reaching altitudes higher than 2000m above sea level (asl.), consist of erosion resistant sandstone and form a double range running parallel to the Indian Ocean coast with a single western mountain range running parallel to the Atlantic Ocean coast (Linder, 2003). These mountain ranges act as barriers to moisture moving inland and have a profound effect on the local climate, with moist upward slopes and arid inland plains lying in their rainshadow. During winter, the occurrence of cyclonic fronts and north-westerly winds bring rain and snow storms which often leave the highest mountain peaks covered in a blanket of snow. Dry, hot summers are associated with a high frequency of south-easterly trade winds. These generate updrafts of cool moist air against the mountain slopes which condenses and regularly covers the peaks in a dense cold mist (Cowling, 1992; Mucina & Rutherford, 2006; Linder, 2005; Cowling *et al.*, 2009).

The Cape biota has been the focus of numerous studies, with efforts mainly focused on the area's flowering plant diversity. A unique sclerophyllous, fire-prone shrubland, known as fynbos dominates the Cape flora. Found on the nutrient poor soils of the mountains and mountain plateaux, fynbos contains most of the species diversity in the Cape (Linder *et al.*,

2010). Endemic plants of the CFR make up 1.9% of all plant species worldwide (Myers *et al.*, 2000). Although the overall proportion of animal endemics (mostly terrestrial vertebrates) is lower compared to plants (Myers *et al.*, 2000; Procheş & Cowling, 2006), the CFR faunal diversity is still remarkable. The Cape rates amongst the areas with the highest percentage of ichthyofauna endemism in Africa (Thieme *et al.*, 2005) and is characterised by the highest level of species richness of South African endemic mammals (Gelderblom & Bronner, 1995; Linder *et al.*, 2010). It has been argued that insect diversity in the CFR is comparatively low, considering the unusually high plant diversity (Johnson, 1992; Giliomee, 2003), however, Procheş and Cowling (2006) disagree with this generalisation, arguing that the region is not as insect-poor as originally thought but that the mechanisms linking plant and insect diversity in the Cape are poorly understood (Procheş *et al.*, 2009).

Several studies show fynbos as the most species rich biome for gall-forming insects (Price *et al.*, 1998; Wright & Samways, 1998) and the Cape has also been recognised as one of the six globally important hotspots for bee diversity (Kuhlmann, 2009). Thus contrary to previous assumptions (e.g., Johnson, 1992) the CFR, and specifically the Cape Fold Mountains, provide habitat to a diverse group of insect species (Cowling, 1992; Linder, 2003; Giliomee, 2003; Galley & Linder, 2006; Cowling *et al.*, 2009; Linder *et al.*, 2010). One such insect species group is the enigmatic, Cape High-mountain stag beetle genus *Colophon* (Coleoptera: Lucanidae).

Enigmatic Beetles of the Mountains

The genus name, *Colophon*, is of Greek origin meaning ‘summit’ or ‘finishing touch’, referring to a publisher’s emblem or trademark usually placed on the title page of a book. Endemic to the CFR and geographically restricted to the summits of the high mountains of the Western Cape, this primitive, flightless beetle genus forms part of a taxonomically poor,

but nonetheless interesting, fauna of southern African Lucanidae. *Colophon* is represented by 17 flightless species and two subspecies (Fig. 1), each geographically restricted to a mountain peak, separated by deep gorges or low lying areas (Gray, 1832; Westwood, 1855; Barnard, 1929; Barnard, 1932; Endrödy-Younga, 1988; Bartolozzi, 1995; Mizukami, 1996). Adults of all species are strongly sexually dimorphic, with males boasting large mandibles and females small, non-diagnostic mandibles, as is typical for virtually all members of Lucanidae. Females cannot easily be differentiated into the various species as they closely resemble each other in appearance; species identification is thus based only on adult male characters.

Barnard (1929; 1932) described ten of the *Colophon* species, designating names for each in honour of their collectors, or as he put it ‘colophonologists’, all fellow mountaineers of the Mountain Club of South Africa (Stiller, 2000). Endrödy-Younga (1988) later honoured this tradition when he described two additional species in a taxonomic revision of *Colophon*. This review represents the most recent comprehensive work on of the genus since the study by Barnard. Endrödy-Younga (1988) further separated *Colophon* into two distinct species groups (Apomorphic and Plesiomorphic) based on the structure of their aedeagus. He also hypothesised a lowland origin for *Colophon*, suggesting the genus is a relict of a past temperate climate regime that prevailed at lower elevations. With the gradual increase in aridification and temperatures across the continent, the low-lying country would have become uninhabitable, forcing *Colophon* to higher altitudes and leading to their isolation and to the localised distribution of distinct species on more hospitable mountain tops. Several years later, Bartolozzi (1995; 2005) added two new species to the genus. Other publications on the genus, mostly written in Japanese, include the travel accounts of Mizukami and Kawai (1997) in which they report on aspects of the biology of *Colophon* and describe a new species and subspecies (Mizukami, 1996).

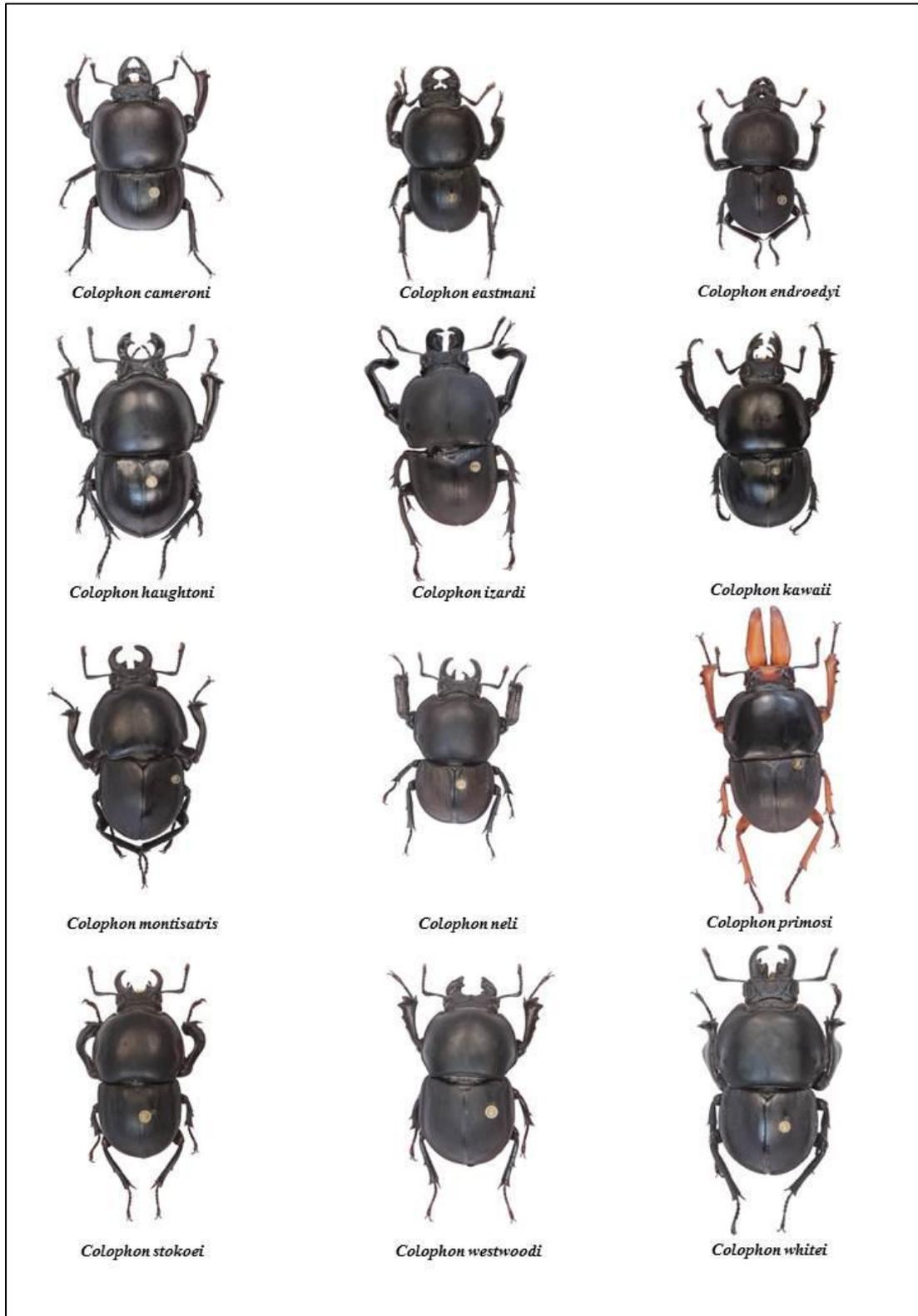


Figure 1. Males of twelve of the seventeen described *Colophon* species (*C. berrisfordi*, *C. barnardi*, *C. cassoni*, *C. oweni* and *C. thunbergi* were not available for study)

Colophon habitat is restricted to the highest peaks of the Cape Fold Mountains, with beetles usually found amongst typical montane fynbos vegetation, interspersed with rocks (Fig. 2). Some differences in terms of habitat diversity have been observed between the different mountain ranges occupied by *Colophon* (Endrödy-Younga, 1988; personal observation). Habitat of the Swartberg species is thought to be generally more restricted to a tiny area (400m horizontally and 20m vertically for *C. montisatris* as suggested by Endrödy-Younga (1988)) when compared to the wider distribution of the Langeberg species (*C. izardi* found at sites several kilometres apart). Another difference in habitat is observed for *Colophon westwoodi* which occupies the lowest altitude within the *Colophon* distribution. Their habitat is characterised by taller restioid vegetation on dark, poorly drained soil on the marshy flatlands of Table Mountain (Geertsema & Owen, 2007; Roets *et al.*, 2012). These differences between habitats are notably due to the general climatic differences between the mountains. The Swartberg are much drier and warmer, with less frequent cloud cover, compared to Table Mountain and Langeberg. *Colophon* activity is also shown to be greatly influenced by the prevailing climate, with optimal weather conditions for surface activity defined as cool and misty during late afternoons and early mornings (Barnard, 1929; Endrödy-Younga, 1988). However, specimens of several species have been collected in the heat of day following a misty evening/morning (Barnard, 1929; personal observation). A study by Roets *et al.* (2012), on the abiotic variables influencing adult *Colophon westwoodi* activity, showed that their activity was significantly influenced by illuminance (suggesting they might be nocturnal) as well as by the presence of mist (moisture) during the day.

Virtually nothing is known of *Colophon* biology. It is not known whether adults feed, and if so, on what. Endrödy-Younga (1988) observed several adult specimens in captivity over a period ranging from 15 to 84 days. None was observed to feed and they did not accept any food provided for them, including plants from their habitat. *Colophon* larvae are known from

only two specimens of *C. neli*. These were found in humus-rich soil and were maintained for several months in the laboratory, appearing to feed on a sample of the soil in which they were collected (Scholtz & Endrödy-Younga, 1994). This soil feeding behaviour is unique as all other Lucanidae are associated with decaying wood on which the larvae feed (Scholtz & Holm, 2008). Details about the lifecycle of *Colophon* are also vague. Larvae seem to be long-lived, with 3rd-instar larvae of *C. neli* showing no signs of growth over a six-month period in the laboratory (Scholtz & Endrödy-Younga, 1994). Another large, possibly 3rd-instar larva, originally thought to be that of a fruit-chafer (Scarabaeidae: Cetoniinae) and kept in humus-rich soil indoors, yielded a female specimen of *Colophon cameroni* approximately one year later (Tony Brinkman, personal communication).

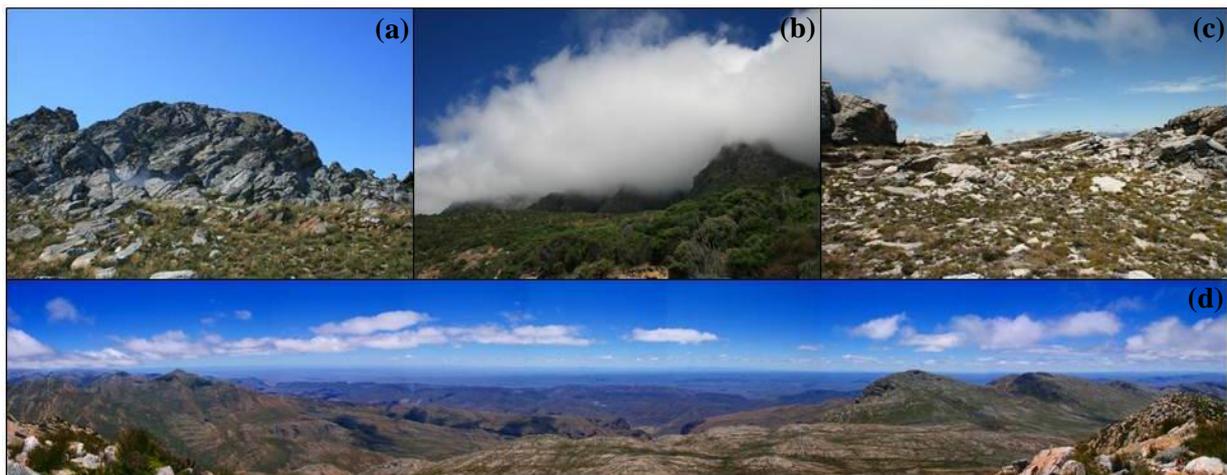


Figure 2. Typical *Colophon* habitat of montane fynbos vegetation, interspersed with rocks. (a) Langeberg; (b) cloud cover over Table Mountain; (c) Hex River Mountains; (d) Swartberg Mountains.

Seasonality for adult activity ranges mainly from October to March (Barnard, 1929; Endrödy-Younga, 1988; Geertsema & Owen, 2007) and probably peaks during early summer (December to early January) (Roets *et al.*, 2012). The adult sex ratio seems to be very low, with fieldwork observations yielding many more males than females (Endrödy-Younga,

1988; Geertsema & Owen, 2007; Roets *et al.*, 2012; personal observation). This might, however, be due to sampling bias as males could be more active than females and thus easier to find amongst vegetation. Another variable unknown for *Colophon* is their population size. Roets *et al.* (2012) attempted a mark-recapture study in hopes of calculating the population size for *C. westwoodi*. None of the marked individuals were recaptured during their study and they were thus unable to predict total population size; this suggests that the *C. westwoodi* population is larger than originally thought. Nothing is, however, known for any of the other *Colophon* species.

Threats and Conservation concerns

Colophon beetles are highly prized by insect collectors with specimens trading for astronomical amounts (up to US \$15 000 per specimen) on the black market. Due to their perceived rarity and the assumed threats of over collecting imposed on their survival, *Colophon* have been given provincial legal protection (Cape Nature and Environmental Conservation Ordinance #24) in 1992 and are currently listed by CITES (Convention on International Trade in Endangered Species) in Appendix III. Fourteen of the 17 species have also been included on the IUCN (International Union for Conservation of Nature) Red List, and the genus as a whole was placed on the South African ToPS (Threatened or Protected Species) list in 2007. However, since so little is known about *Colophon* biology it is exceedingly difficult to do a threat assessment of the genus and the need for their protection has largely been emotionally driven. Thus, for the effective implementation of a conservation management programme for *Colophon*, a comprehensive study confirming species' identity, and distribution, phylogenetic relationships and phylogeography, will be required.

Aim of this study

Given the background above, the objectives and key questions of the present study were:

- i. To determine the specific and phylogenetic status of the various described species of *Colophon*. (Chapter 2)
- ii. To test the theory of a lowland origin of *Colophon* as proposed by Endrödy-Younga (1988) and thereby determining the main factors driving their evolution. (Chapter 2)
- iii. To determine distinct larval characters for *Colophon* species identification and their phylogenetic implications. (Chapter 3)
- iv. To make suggestions for the revised conservation status of *Colophon*. (Chapter 4)

To achieve these aims, DNA sequence data from multiple gene regions were analysed. These provide a powerful basis for testing hypotheses and estimating evolutionary processes, including the estimation of divergence times (San Mauro & Agorreta, 2010) and the designation of species boundaries (i & ii). Several *Colophon* larvae were examined and the morphological characters used to distinguish between species determined (iii). Combining this information with field observations and what is known of their morphology, aided in developing recommendations for the sound conservation management of *Colophon* (iv).

Thesis outline:

Chapter 2 and 3 were written as manuscripts for publication and are formatted accordingly. Chapter 2 has been submitted to the Journal of Biogeography and Chapter 3 to Insect Systematics and Evolution. Due to this, there may be instances of duplication across chapters and differences in formatting between chapters. Chapter 4 is written as a postscript as it incorporates knowledge gained from the previous two chapters to make recommendations regarding *Colophon* conservation status. Each chapter contains its own set of references and all appendices are found at the end of the thesis. The General Introduction and Conclusion are tailored from the respective chapters.

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Chapter II:

Phylogeny, historical biogeography and divergence time estimates of the genus *Colophon* Gray (Coleoptera, Lucanidae)

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Abstract:

Aim: To determine the specific and phylogenetic status of the described species of *Colophon* and to determine the main factors driving their evolution by testing the hypothesis of a lowland origin.

Location: Cape Floristic Region, Western Cape, South Africa.

Methods: The status and phylogeny of *Colophon* species was analysed from the mitochondrial COI, 16S rRNA and nuclear CAD genes, using a Maximum Likelihood (ML) and Bayesian approach. Timing of key biogeographical events in the diversification of *Colophon* was estimated in BEAST.

Results: The combined molecular dataset supports the described species analysed. High genetic divergence was found between the described varieties *C. stokoei* and the two subspecies of *C. eastmani*. The mean estimated divergence of the genus was mid-Cretaceous, with a split into two lineages during the early Paleocene to mid Eocene. Species divergence was shown to have occurred during mid to late Miocene.

Main Conclusions: Most species of *Colophon* showed an allopatric distribution, although contact zones between geographically adjacent species are likely as is the case between *C. kawaii* and *C. haughtoni*. Climate seems to be the main driving factor behind *Colophon* evolution and the hypothesis of a lowland origin appears to be supported. Addition of the

outstanding species and analyses of a slower evolving gene-region could help improve the resolution of the deeper phylogenetic nodes. We suggest the taxonomic revision of the *C. stokoei* varieties and the *C. eastmani* subspecies.

Keywords: Biogeography; Cape Floristic Region; *Colophon*; Divergence time; Endangered; Phylogeny.

Introduction

The south-western tip of southern Africa has been recognised as one of the six Floral Kingdoms on earth, the Cape Floristic Region (CFR) (Good, 1953; Takhtajan, 1986; Cowling, 1992). Its uniqueness is owed to its remarkable floral diversity and high levels of biotic endemism. A contributing factor to the high diversity in the region is the variable topography, including the series of folded mountains, which reach altitudes higher than 2000m above sea level (asl). These Cape Fold Mountains consist of erosion-resistant sandstone and form a double range of eastern mountains parallel to the Indian Ocean coast with a single western mountain range running parallel to the Atlantic Ocean coast (Linder, 2003). The mountain ranges act as barriers to moisture moving inland and have a profound effect on the local climate, with moist upward slopes and arid inland plains lying in their rainshadow. During the dry summer months the mountains are frequently under cloud cover from moisture driven in by strong south-easterly winds. In winter, the occurrence of cyclonic fronts and north-westerly winds bring rain and snow storms which often leave the high mountain peaks covered in a blanket of snow. This characteristic mediterranean type climate of the CFR, makes it distinct from the rest of Africa and provides the perfect habitat for a unique sclerophyllous, fire-prone shrubland known as fynbos (Goldblatt & Manning, 2000; Linder, 2003; Linder, 2005; Galley & Linder, 2006; Mucina & Rutherford, 2006; Procheş & Cowling, 2006; Cowling *et al.*, 2009) and its attendant fauna.

The CFR, and the Cape Fold Mountains specifically, also provide habitat to a diversity of insect species (Cowling, 1992; Linder, 2003; Giliomee, 2003; Galley & Linder, 2006; Cowling *et al.*, 2009). One such insect species group is the enigmatic, Cape High-mountain stag beetle genus *Colophon* (Coleoptera: Lucanidae). Endemic to the CFR and geographically restricted to the high mountains of the Western Cape, this primitive apterous genus forms part of a taxonomically poor, but nonetheless interesting, fauna of southern African Lucanidae. The taxonomic placement of *Colophon* has been riddled with ambiguity. Originally placed in the subfamily Lucaninae (Lacordaire, 1856), the genus was later transferred by Perry (1864) to the subfamily Chiasognathinae (which included Lampriminae at this stage). Didier and Séguy (1953) considered the subfamilies Chiasognathinae and Lampriminae as separate, placing *Colophon* in the latter. A later examination of the structure of the male genitalia by Holloway (1960) resulted in *Colophon* being transferred back to Lucaninae, with which Endrödy-Younga (1988) tentatively agreed. However, a unique suite of larval characters was considered by Scholtz and Endrödy-Younga (1994) to exclude *Colophon* from any of the currently recognised subfamilies. An on-going study by David Hawks (University of California, Riverside) and Matt Paulsen (University of Nebraska, Nebraska), on the phylogeny of the world Lucanidae will give more insight into the placement of *Colophon*.

The genus was described by Gray in 1832, and more generic details were added two years later by Westwood (Barnard, 1929). It is currently represented by 17 flightless species and two subspecies (Gray, 1832; Westwood, 1855; Barnard, 1929; Barnard, 1932; Endrödy-Younga, 1988; Bartolozzi, 1995; Mizukami, 1996). A review of the genus by Endrödy-Younga (1988) represents the most recent comprehensive work on *Colophon*. He separated *Colophon* into two distinct species groups (Apomorphic and Plesiomorphic) based on the symmetry of their aedeagus, with further species delineation based on specific morphological characters of the male mandibles and fore tibiae. Endrödy-Younga (1988) suggested that

Colophon originated in the Cape lowlands, where conditions were similar to those they now live in. With the gradual increase in aridification and temperatures across the continent, the low-lying country would have become uninhabitable, forcing *Colophon* species to higher altitudes and leading to isolation of populations and speciation and the current localised distribution of distinct species on more hospitable mountain tops.

Because of their localised distribution patterns and their perceived rarity, *Colophon* species were declared a “protected species” by South African Cape Provincial law in 1992 and listed by CITES (Convention on International Trade in Endangered Species) in Appendix III in 2000. Fourteen of the 17 *Colophon* species are also included on the IUCN Red List (<http://www.iucnredlist.org>) and the genus as a whole was placed on the South African ToPS (Threatened or Protected Species) list in 2007. However, these assessments of *Colophon* are largely outdated and in need of revision (Samways *et al.*, 2012). Initially listed by CITES (Anonymous, 1994) to protect them from commercial exploitation, this listing has only succeeded in drawing more attention to the beetles and possibly increasing their black market value (Gess & Gess, 1993; Melisch & Schütz, 2000; Geertsema & Owen, 2007). Apart from the on-going collection pressures, their survival has already been under threat by warming conditions induced by gradual changes in climate and continental movement since the Miocene when *Colophon* likely evolved (David Hawks, personal communication). Projected global warming scenarios (various models e.g., Lutjeharms *et al.*, 2001; Meadows, 2006), and predicted change in climate, also loom as imminent danger to the survival of lineages such as *Colophon*.

For the implementation of effective conservation management programmes, sound knowledge of the species’ habitat requirements and biology is needed (New, 2009; McGeoch *et al.*, 2011; New, 2012). This is often lacking for invertebrate species and is particularly true for *Colophon*, thus a comprehensive study confirming the species’ identity and, their

distribution, phylogenetic relationships and phylogeography is desirable. A recent study by Roets *et al.* (2012) on the abiotic variables influencing adult *Colophon westwoodi* activity is the start to obtaining the needed information. The current study aims to contribute to the growing knowledge base by:

1. Determining the specific and phylogenetic status of the various described species of *Colophon*
2. Testing the theory of a lowland origin of *Colophon* as proposed by Endrödy-Younga (1988) and thereby determining the main factors driving their evolution.

To achieve these aims, DNA sequence data from multiple gene regions were analysed. These provide a powerful basis for testing hypotheses and estimating evolutionary processes, including the estimation of divergence times, due in large part to the high degree of variation in substitution rates among genes and gene regions (San Mauro & Agorreta, 2010).

Methodology

Taxon sampling

Using locality and activity data from museum collections as well as published records from Endrödy-Younga (1988), several mountain peaks in the Western Cape fynbos biome were searched for *Colophon* activity during three successive summer periods, starting November 2007. Twelve of the 17 described species were observed, photographed and their locality data recorded (see Appendix I: Table S1). A few of the specimens were collected (CapeNature Permit AAA004-00106-0035, South African National Parks Permit for Table Mountain National Park) and preserved in absolute ethanol for subsequent genetic analyses. Several leg, mandible and thorax fragments from dead specimens were also collected for possible genetic and morphological analyses. Samples were identified to species level using the dichotomous

key provided by Endrödy-Younga (1988) as well as several publications of subsequently described species (Bartolozzi, 1995; Mizukami, 1996).

Several scarabaeiform larvae were also collected from under rocks and amongst the roots and tussocks of the Restionaceae. These were identified as Lucanidae based on the shape of their anal opening (Ritcher, 1966) and ascribed to species based on their locality and the associated adults collected. Larvae were preserved in absolute ethanol for inclusion in the genetic analyses and a subsequent description of the species was done based on larval characters (not presented in this paper).

Out-group

No suitable out-group taxon for *Colophon* was known when Endrödy-Younga conducted his cladistic analysis of the species. For the current study four specimens from the family Bolboceratidae (Coleoptera: Scarabaeoidea) were chosen based on the higher classifications of Scarabaeoidea (Browne & Scholtz, 1999; Smith *et al.*, 2006) that indicate that Lucanidae Latreille, 1804 and Bolboceratidae Mulsant, 1842 are members of closely related clades.

DNA extractions and sequencing

Where possible, at least three adult individuals per species and per locality were selected for genetic characterisation. DNA was extracted from one hind leg using the Roche extraction kit (Roche Diagnostic System, Inc.) according to the manufacturers' specifications. DNA was also extracted from the head muscle of larval specimens, allowing for the retention of the head capsule which contains several diagnostic structures for subsequent larval description.

Three gene regions were used for the phylogenetic analyses of *Colophon*, these include the two mitochondrial genes Cytochrome oxidase I (COI) and 16S rRNA (16S) and the nuclear CPSase region of carbamoyl-phosphate synthetase-aspartate transcarbamoylase-

dihydroorotase (CAD). These gene regions were chosen for their ease of amplification and proven phylogenetically informative properties in Coleoptera and other insect groups (Simon *et al.*, 1994; Moulton & Wiegman, 2004; Wahlberg & Wheat, 2008; Wild & Maddison, 2008; Sole & Scholtz, 2010; Winterton *et al.*, 2010; Mlambo *et al.*, 2011). Several other gene regions (28S domain 2, Elongation factor-1 α , Wingless and ITS2) were screened for their suitability to delineate *Colophon* species but were either not variable enough or gave inconsistent amplification results and are therefore not included in this study.

Primers used for the gene regions are shown in Table 1. A 764-bp fragment of COI and a 353-bp fragment of 16SrRNA was amplified by PCR, using an ABI 2720 thermocycler (Applied Biosystems, Foster City, CA, USA), in a final volume of 25 μ l containing 2.5mM MgCl₂, 10pmol of each primer, 10mM dNTP's and 1X Buffer in the presence of 1 unit Super-Therm *Taq* (JMR Holdings, USA) DNA polymerase. Thermal cycling conditions were as follows: COI - initial denaturation for 2 min at 94°C, followed by 35 cycles of 94°C for 22s, primer annealing at 45°C for 30s and elongation at 72°C for 90s, followed by a final elongation at 72°C for 60s; 16S – denaturing at 94°C for 90s, followed by 35 cycles of 94°C for 60s, annealing at 48°C for 90s and 72°C for 90s with a final elongation of 60s at 72°C.

PCR amplification of the 796-bp fragment of CAD was achieved by a two-step process using an ABI 2720 thermocycler in a final volume of 25 μ l containing 25mM MgCl₂, 10pmol of each primer, 10mM dNTP's and 1X Buffer in the presence of 0.15 unit Super-Therm *Taq* (JMR Holdings, USA) polymerase. Dimethyl sulphoxide (DMSO) was added to both amplification steps to improve the sensitivity of the reaction. A three-cycle touchdown PCR, with primers 54F and 680R (Table 1), was used for the first amplification step: initial denaturation of 4 min at 94°C followed by 4 cycles (30s at 94°C, 30s at 52°C, 2 min at 72°C) thereafter 6 cycles (30s at 94°C, 1 min at 47°C, 2 min at 72°C) and 36 cycles (30s at 94°C, 20s at 42°C, 2.5 min at 72°C) and a final extension of 3 min at 72°C. In the second

amplification step, 1 µl of the resulting amplified product was used in the same reaction mixture except at a 1.5mM MgCl₂ concentration with internal primers 338F and 654R (Table 1). Initial denaturation was obtained at 94°C for 4 min followed by 4 cycles of (30s at 94°C, 30s at 53°C, 80s at 72°C), 36 cycles (30s at 94°C, 30s at 47°C, 80s at 72°C) with a final extension of 3 min at 72°C.

Automated DNA sequences in both directions for each specimen were obtained by cycle sequencing with the Big Dye method Ver. 3.1 (Applied Biosystems). These were inspected and assembled in CLC bio[®] Ver 5.0.2 (www.clcbio.com) and subsequently aligned in MAFFT (Kato *et al.*, 2009) using default parameters to deal with 16S gap opening penalties. After verifying the electropherograms for recognisable sequencing artefacts, all ambiguous sites were coded using the appropriate IUB symbols. All sequences were submitted to GenBank under accession numbers KC763191-KC763331 (see Appendix I: Table S1).

Species status and phylogeny

The relationships among *Colophon* sequences were inferred using Maximum Likelihood (ML) analyses as implemented in PAUP*4.0b10 (Swofford, 2003) while a Bayesian phylogram was inferred using the Metropolis-coupled Monte Carlo Markov Chain (MCMC) method as implemented in MrBayes Ver. 3.1.2 (Huelsenbeck & Ronquist, 2001). Bayesian analyses allow for the partitioning of data and the use of optimal models of nucleotide substitution for each partition. Analyses were initiated in MrBayes from random starting trees using one cold and three incrementally heated chains (0.01) run for 30 million iterations with trees being sampled every 100th iteration. Twenty per cent of these were discarded as burn-in and the posterior probabilities calculated from the remaining saved majority rule consensus trees. The best model of nucleotide substitution for each gene region was assessed in MrModeltest Ver. 2.3 (Nylander, 2004) based on the Akaike Information Criteria (AIC)

(Akaike, 1973) (Table 2) and phylogenetic trees were generated for both individual and combined gene regions.

For the ML analysis the starting tree was obtained using the Neighbor-joining method (Saitou & Nei, 1987). We used a heuristic tree search protocol with 10 random addition sequences and tree bisection and reconnection (TBR) while nodal support was estimated using 100 bootstrap (Felsenstein, 1985) replicates. For the ML inference a model as favoured by the AIC for the entire dataset was estimated in MrModeltest. Pairwise genetic distances were calculated between species using MEGA Ver. 5 (Tamura *et al.*, 2011) for all gene regions.

Divergence time estimates

The program BEAST 1.6.2 (Drummond & Rambaut, 2007), a Bayesian coalescent analysis with MCMC procedure, was used to estimate timing of key biogeographical events in the diversification of the genus *Colophon*. Reflecting the phylogenetic analysis, a monophyletic constraint was placed on the genus. The dataset was partitioned by gene region, with respective substitution models applied to each partition. Lineage age was estimated under the uncorrelated lognormal model (relaxed molecular clock) with unconstrained nodes derived from a birth-death tree model. A normal prior was applied to the root using a mid-point of the Jurassic (172 million years ago (mya) with a standard deviation of 11, allowing for soft minimum and maximum bounds) in accordance with the radiation of Lucanidae (Krell, 2007). A fossil record for *Bolboceras inermis* was described by Piton (1940), from the Paleocene (54-65 mya). Thus an exponential prior, with 54 million years as hard minimum age, was used to constrain the out-group. Three independent analyses were run for 10 million iterations each using a random starting tree. Convergence between runs and posterior probabilities of estimates were assessed using the program TRACER 1.6 (Drummond & Rambaut, 2007).

Results

Species status and phylogeny

The combined molecular data set consists of 1913 base pairs (bp): COI = 764 bp; 16S = 353 bp and CAD = 796 bp. A ML tree was obtained assuming the HKY85 model. The phylogram depicted in Fig. 1 is the Bayesian consensus tree with nodal support values above 0.75 Bayesian Posterior Probability (PP) and 50% ML Bootstrap (BS) indicated. Nodal support of 0.75-0.90 PP and 50-70% BS are considered as moderately supported with values above 0.90 PP and 70% BS respectively considered as strongly supported nodes. Individual gene trees show similar phylogenetic groupings but with poor nodal support and are thus not presented.

The genus *Colophon* is strongly supported as a monophyletic group (1.00 PP; 100% BS). Deeper nodes are not well supported in the ML analysis. Two lineages can be identified within the phylogenetic tree labelled I (1.00 PP; 82% BS) and II (0.98 PP; 52% BS), respectively (Fig. 1) with the morphologically spectacular *Colophon primosi* (Fig. 2) as sister taxon (0.92 PP) to all the species. The sister grouping of *C. primosi* is not supported in the ML analysis. The lineages correspond to the original morphological designation of Plesiomorphic (Lineage I) and Apomorphic (Lineage II), as originally defined by Endrödy-Younga (1988). The Bayesian analyses strongly support 11, excluding *C. kawaii*, of the 17 described species, with the ML only strongly supporting eight.

Lineage I contains the species *Colophon stokoei* (1.00 PP; 90% BS), *C. eastmani* (1.00 PP; 79% BS), comprising the two subspecies *C. eastmani eastmani* (1.00 PP; 100% BS), *C. eastmani nagaii* (1.00 PP; 97% BS), *C. cameroni* (1.00 PP; 100% BS) and *C. haughtoni* (1.00 PP; 92% BS). *Colophon kawaii* is not supported, with one specimens (*C. kawaii**) grouping as sister to *C. cameroni* (1.00 PP) and the other falling within the *C. haughtoni* group, respectively. Neither grouping is supported in the ML. *Colophon cameroni*, *C. haughtoni*, *C.*

kawaii and *C. eastmani* are sister species (1.00 PP; 88% BS) to *C. stokoei* which Barnard (1929) originally divided into five varieties based on the morphological form of the mentum. These analyses strongly support three of the varieties: *C. stokoei forma typica* (1.00 PP; 98% BS), *C. stokoei* Var. C (1.00 PP; 99% BS) and *C. stokoei* Var. A (1.00 PP; 98% BS).

Lineage II is not well supported by the ML analysis but very well supported by the Bayesian analysis (0.98 PP; 52 % BS). It contains the species *Colophon westwoodi* (1.00 PP; 97% BS), *C. izardi* (1.00 PP; 100% BS), *C. neli* (1.00 PP; 57% BS), *C. endroedyi* (0.97 PP; 51% BS), *C. montisatris* (1.00 PP; 99% BS) and *C. whitei* (1.00 PP; 100% BS). Two live females collected (indicated as *C. neli* * in Fig. 1) were tentatively identified as *C. neli* due to the male fragments found in close proximity. The mitochondrial COI gene shows pairwise genetic distances between species ranging from 3.0% to 10.7% (Table 3). For comparative reasons, since most studies use genetic distances from only the COI gene, the pairwise distances for CAD and 16S are only added as supplementary information (see Appendix I: Table S2) and all results focus on COI.

Divergence time estimates

Divergence time estimates are presented in Fig. 2, with the blue bars indicating the 95% high posterior density interval of each divergence. The mean estimated divergence of the genus *Colophon* is mid-Cretaceous, *ca.* 85.81 mya (124.52-53.27 mya upper and lower estimates respectively). There is a clear split between Lineage I and II corresponding to the lineages illustrated in Fig. 1. This split appears to have occurred during the early Paleocene (*ca.* 63.94 mya) (94.15-39.97 mya upper and lower estimates) to mid Eocene (*ca.* 46.81 mya) (71.17-27.61 mya upper and lower estimates). The ancestral lineages of *C. westwoodi*, *C. izardi* and *C. primosi* diverged during the early Paleocene (*ca.* 63.94 mya for *C. westwoodi*) to early Eocene (*ca.* 55.4 mya and *ca.* 48.43 mya for *C. izardi* and *C. primosi* respectively). Three of

the species, *C. stokoei*, *C. eastmani* and *C. neli*, diverged from early- to mid Miocene (*ca.* 21-15 mya) with the remaining species showing a more recent divergence during the last *ca.* 8 mya.

Discussion

Species status and phylogeny

This is the first molecular and most comprehensive phylogenetic study conducted on relationships in the genus *Colophon* and is complementary to the morphological phylogeny hypothesised by Endrödy-Younga in 1988. Endrödy-Younga examined 14 species and, based on general morphological structures, suggested that the group is monophyletic and that all species group closely in a single genus. The species were, further, separated into two distinct groups based on the symmetry of their aedeagi and the level of their clypeal margin. These he interpreted as two distinct evolutionary lineages. His results are supported by the Bayesian and Maximum Likelihood analyses reported here, with the genus *Colophon* being monophyletic and the separation of species into a strongly supported lineage (Lineage I) and a second, moderately supported lineage (Lineage II) (Fig. 1).

Lineage I corresponds to the morphological designation of Plesiomorphic as defined by Endrödy-Younga (1988). Within this lineage, *Colophon stokoei* has a large and fragmented distribution from the Palmietriver Mountains in the south to the Wellington Mountains in the north (Fig. 3). The species was originally divided into five varieties by Barnard (1929), marking the types as '*forma typica*, variety A, B, C, and D'. Considering its wide distribution area, Endrödy-Younga (1988) suggested that the marked differences between varieties could indicate subspecifically distinct populations, but due to the inadequacy of the material available and the unreliable locality records at his disposal he refrained from drawing any taxonomic conclusions and kept with Barnard's designations.

The Bayesian analysis supports three groups of *C. stokoei* corresponding to the three different mountain peaks from which they were collected (Fig. 3). The first group, *C. stokoei* Var. A, is separated from the other varieties by the Steenbras River valley. This is the only apparent physical barrier preventing genetic exchange within *C. stokoei* populations (Barnard, 1929). Variety A groups as sister to *C. stokoei forma typica* and *C. stokoei* Var. C, from the more northern Hottentots Holland and Drakenstein Mountains, respectively. The pairwise genetic distances between the three varieties range from 7.4% to 10.7% indicating high sequence divergence between them. This is far greater than the 3% genetic divergence suggested to delineate between invertebrate species (Lepidoptera in Herbert *et al.*, 2003, cryptorhynchine weevils in Astrin *et al.*, 2012) using COI, or the 2% suggested for vertebrate species using the cytochrome b gene (cytb) (Avice & Walker, 1999). The aim of any species delineation method is to identify reproductively isolated groups of organisms warranting classification as distinct species; thereafter it is the taxonomist's role to define and name these groups (Monaghan *et al.*, 2007). Based on the high degree of divergence, their distinct distribution on separate mountain peaks and the observed differences in morphology, it is suggested that the *C. stokoei* varieties be classified as separate species and a taxonomic revision of the species be done.

Sister to *C. stokoei* are the remaining four Plesiomorphic *Colophon* species. These are geographically separated from *C. stokoei* by a wide gap of low-lying country to the north, called the Breede River valley. *Colophon eastmani* occupies the most westerly parts of the Langeberg Range and was described by Barnard (1932) from a single locality in Keeromsberg. Endrödy-Younga (1988) included a fragmented specimen from Dassieshoek, some 30km south-east, as *C. eastmani*. However, in 1996 Mizukami described a new subspecies from the Dassieshoek locality, splitting *C. eastmani* into the subspecies *C. eastmani eastmani* (Keeromsberg) and the new *C. eastmani nagaii* (Dassieshoek). Both

subspecies were included in the Bayesian analyses and separated into two distinct, well supported groups. The genetic distance between them is high (9.6%) and, as with the *C. stokoei* varieties, it is suggested they be taxonomically revised and elevated to species level.

Barnard (1932) commented that *Colophon eastmani* appears similar to *C. cameroni*, a species occupying the mountains on the opposite side of the Hex River valley. No mention of this was made by Endrödy-Younga (1988). The Bayesian analysis groups these, albeit with only moderate support, as sister to one another. *Colophon cameroni* is reported to have a wide distribution (Barnard, 1932; Endrödy-Younga, 1988), ranging from the Waaihoek and Hex River Mountains northwards to Groot-Winterhoek peak. However, for this study only specimens from the Waaihoek Mountains were sampled and included in the analyses. Another species, *C. haughtoni*, occurs north-east of the Hex River Mountains on Matroosberg. In the approximately 35km area separating the Waaihoek Mountains from Matroosberg, there are no obvious physical barriers that could lead to isolation of the two species and Barnard (1929) suggested tracing the species' distribution to find whether there is overlap between them. However, no area of overlap has yet been recorded. A third species, which is sympatric with *C. haughtoni* on the Matroosberg Mountains was subsequently described (Mizukami, 1996). At first glance, *C. kawaii* appears very similar to *C. cameroni*, but it differs from the latter in a few relatively superficial characters (Mizukami, 1996). It was not supported as a distinct species in the Bayesian analyses based on the two specimens at our disposal. The Bayesian phylogram shows one of the *C. kawaii* specimens grouping as sister to *C. cameroni*, with a genetic distance (9.4%) suggesting it is a distinct species. The second specimen, however, collected from the same locality is nested within *C. haughtoni*. Repeated re-extraction, amplification and analyses yielded the same results. It is, thus, suggested that *C. kawaii* is a distinct species that may be outcompeted by the more common

C. haughtoni (personal observation), but is able to hybridise with it. Further sampling of *C. kawaii* is clearly needed to test this hypothesis.

Lineage II corresponds to the morphological designation of the Apomorphic group as defined by Endrödy-Younga (1988). This lineage is only moderately supported but this may be a reflection of sampling since several species from this group (*Colophon barnardi*, *C. thunbergi*, *C. oweni*, *C. cassoni* and *C. berrisfordi* (Endrödy-Younga, 1988; Bartolozzi, 1995) were not available for study. Most species of the group occupy the eastern section of the Langeberg and Swartberg Ranges; however, one species, *C. westwoodi*, is isolated on Table Mountain at the south-western extremity of the Cape Fold Mountains (Fig. 3). This grouping is surprising since the *C. westwoodi* locality is separated from that of the other species in the Apomorphic group by the Hottentots Holland-Drakenstein Mountain Ranges which are exclusively occupied by the Plesiomorphic species, *C. stokoei*.

The Langeberg Range east of Montagu provides habitat for five other species of *Colophon*, namely *C. barnardi*, *C. thunbergi*, *C. oweni* and *C. izardi*. Only *C. izardi* is included in this study. *Colophon izardi* males are characterised by large, funnel-shaped mandibles and long anterior tibia that are narrow and bent inwards at a 90° angle (Fig. 4; Barnard, 1929; Endrödy-Younga, 1988). Barnard, and subsequently, Endrödy-Younga, sampled *C. izardi* from the highest peaks in an approximate range of 45km, stretching eastwards on the Langeberg Range, suggesting that they occur in sympatry with the three other species sampled from the same mountain range. Our analyses include *C. izardi* samples from two peaks within this range. The Bayesian analysis supports the separation between the two localities (Fig. 1), showing *C. izardi** from Riversdale sister to the three *C. izardi* specimens from Grootberg although with a low genetic distance of only 2.9%. These peculiar looking beetles are even more remarkable with the existence of colour differences between the two locations. Specimens found in the Grootberg area have a pair of distinct red pronotal dots,

with specimens from the Riversdale area being entirely black (Fig. 4). Colour polymorphism is a common occurrence in many insects and may function as mechanisms of mate selection (Ueno *et al.*, 1998), camouflage through crypsis, aposematism, or the absorption of heat (Ottenheim *et al.*, 1999; Majerus & Zakharov, 2000), or might be a result of several environmental factors (Nahrung & Allen, 2005; Davis *et al.*, 2008). The reason for the differences between the two *C. izardi* colour morphs is not clear; however, the Grootberg specimens occur at a higher altitude (1 637m asl) than the Riversdale specimens (1 330m asl). The only other beetle species in the genus with colour other than black, is the spectacular *C. primosi* with orange mandibles and fore tibia (Fig. 5). They are found further north on the western section of the Swartberg Mountain Range at an altitude of 1 980m asl. Based only on the mandibles, *C. primosi* could not readily be derived from any other form. Its place within the genus was, however, secured by several morphological structures and it was placed within the Apomorphic group due to its asymmetrical aedeagus and raised clypeus (Endrödy-Younga, 1988). An interesting observation of the current study is the splitting of *C. primosi* in the Bayesian analysis as sister to all other species. Endrödy-Younga (1988) collected specimens from two localities on either side of Seweweekspoort, an extensive gorge cutting through the Swartberg Range. He observed no noteworthy morphological differences between the specimens from the two locations, raising some interesting biogeographic questions. However, only genetic material from specimens found east of the Seweweekspoort was included in the analyses and further sampling of the western mountain peaks is desirable for comparative purposes.

The Swartberg Range is drier, with less frequent cloud cover and higher summer temperatures than the Langeberg, yet it provides habitat for seven of the known *Colophon* species; namely *C. primosi*, *C. endroedyi*, *C. neli*, *C. cassoni*, *C. berrisfordi*, *C. montisatris* and *C. whitei*. Apart from *C. primosi*, the Swartberg species form a well-supported clade

within the Apomorphic lineage. This clade splits further into two distinct groups, with *C. endroedyi* and *C. neli* grouping together and the two species on either side of Meiringspoort, *C. whitei* and *C. montisatris*, forming a well-supported group.

Colophon endroedyi type material was collected from the mountain peaks east of Ossenberg. One specimen was available for genetic analysis and falls as sister to *C. neli*, a species found further east along the Swartberg Mountain Range. *Colophon neli* is the smallest known species in the genus. Type specimens were mainly collected from the peaks surrounding the Swartberg Pass; however, we found fragments of specimens as far east as Tierhoek on the Swartberg Mountain Range. *Colophon neli*, thus, has much broader distribution than originally thought and it appears to occur in sympatry with *C. cassoni*, which is also found on the Swartberg, just west of Meiringspoort. The phylogenetic analysis shows *C. neli* as a well-supported group. Two female specimens (*C. neli* *), collected approximately 15km east of Swartberg Pass branch off as sister to the other *C. neli* specimens from the Swartberg Pass peak, with a genetic distance of 8.1%. It is, thus, suggested that these females were erroneously identified and were probably *C. cassoni*. Females of both species are very similar (Endrödy-Younga, 1988). Previous *C. cassoni* locality records were listed as Meiringspoort Berg (Barnard, 1932; Endrödy-Younga, 1988). The identity of this locality could, however, not be established and no other specimens of *C. cassoni* were available for our study.

Also described from Meiringspoort Berg, is the species *C. whitei* (Barnard, 1932). We, however, found specimens of *C. whitei* on Bloupunt, a peak west of Meiringspoort. A closely related species, *C. montisatris* (Endrödy-Younga, 1988), occupies the peak east of Meiringspoort known as Blesberg. This species groups as sister to *C. whitei*, an observation made by Endrödy-Younga (1988) during his cladistic analysis. He suggested that the common ancestor of these two species must have had a distribution at the foot of the

mountain and that their separation only occurred after the present population retreated to the high altitudes of the Swartberg Pass surrounding Meiringspoort (Endrödy-Younga, 1988).

Our study is mostly congruent with what Endrödy-Younga (1988) observed in his revision of the genus, with the exceptions of one difference between the two studies. The Apomorphic, and thus derived, group as defined by Endrödy-Younga appears to be older (Fig. 2), falling as basal in the molecular phylogenetic analyses (Fig. 1). This difference might be due to Endrödy-Younga (1988) using a *Colophon* female as out-group in his cladistic analysis. Another noteworthy observation is that the genealogy of *Colophon* supports an east to west movement, something that has also been shown in *Circellium bacchus*, an ancient flightless dung beetle from the Cape Floristic Region (Sole & Scholtz 2013).

Divergence time estimates

The CFR is famous for its unique floral diversity and topographically variable landscape. It has historically been the focus of a great deal of research and with an increased knowledge of molecular phylogenetics, new opportunities arise to explore the historical and geographical features of the region's unique diversity (Barracough, 2006; Cowling *et al.*, 2009). Combining dated phylogenies with geographical and ecological data has provided the opportunity to explore the evolutionary foundations of the region's diversity and the dominant idea emerging from various studies is that most Cape clades diversified in response to a change in climate of the late Cenozoic (Richardson *et al.*, 2001; Linder, 2005; Midgley *et al.*, 2005; Barracough, 2006; Tolley *et al.*, 2006; Verboom *et al.*, 2008; Cowling *et al.*, 2009; Swart *et al.*, 2009). The characteristic winter-rainfall, summer-arid climate of today is thought to have been initiated around the end of the Miocene due to a complex interplay between tectonic movements and a change in atmospheric circulation (Partridge & Maud, 2000). This period was characterised by renewed glaciation of the Antarctic and the

establishment of the cold Benguela upwelling system (*ca.* 14 mya) which led to the onset of drier conditions in the low lying areas of the Cape (Tankard & Rogers, 1978; Partridge & Maude, 2000; Midgley *et al.*, 2005; Cowling *et al.*, 2009; Dupont *et al.*, 2011). The period following the Miocene (5.3 mya – present) shows clade diversification and speciation corresponding to habitat shifts initiated by climatic change (Makokha *et al.*, 2007; Cowling *et al.*, 2009; Swart *et al.*, 2009; Tolley *et al.*, 2009) with fynbos emerging as the dominant vegetation type approximately 3 to 5 mya (Goldblatt & Manning, 2002; Linder, 2003; Galley & Linder, 2006).

Colophon activity patterns are mostly associated with cool, humid conditions (Barnard, 1929; Endrödy-Younga, 1988; Roets *et al.*, 2012; personal observations). It has been suggested that the genus evolved in the Great Karoo and southern coastal flats and with the gradual increase in aridity and temperatures associated with the Miocene, was forced up into the more hospitable mountains (Endrödy-Younga, 1988). The establishment of the cold Benguela upwelling system and the late afternoon south-easterly winds brought in cool moist air which condenses against the slopes creating dense orographic fog (Deacon *et al.*, 1992; Cowling *et al.*, 2009). The mountains subsequently acted as a refuge for *Colophon*, providing the ideal habitat. As time passed and the regional climates became less suitable, *Colophon* populations became more isolated and species radiated with most genetic lineages confined to specific mountains. It is thus suggested that *Colophon* experienced climatically driven allopatric speciation. A Plio-Pleistocene species radiation has been shown in a variety of other taxa in the CFR (Linder, 2003; Daniels *et al.*, 2004; Cowling *et al.*, 2009; Swart *et al.*, 2009; Tolley *et al.*, 2006; Linder *et al.*, 2010; McDonald & Daniels, 2012; Sole *et al.*, 2013) supporting the idea of climatically driven speciation. Phylogeographic studies on mammals and some reptile species in the Cape suggest that environmental fluctuations during the Plio-Pleistocene may have fragmented once continuous distribution ranges (Linder *et al.*, 2010). Our study

suggests that during this time *Colophon* distribution was widespread over adjacent mountain peaks within the four major mountain ranges and that a substantial amount of range contraction and expansion occurred corresponding to different climatic fluctuations. This would explain the occurrence of *C. izardi* on several peaks in the Langeberg Mountain range as well as the different varieties of *C. stokoei* in the Palmiet, Wellington and Hottentots Holland Mountains.

Based on our studies it is estimated that *Colophon* is an ancient flightless genus with its origin in the late Cretaceous, *ca.* 85.81 mya, a period when southern Africa was characterised by a warm, humid climate supporting extensive tropical and sub-tropical forests (Dingle *et al.*, 1983; Deacon *et al.*, 1992; Partridge & Maud, 2000). Lucanidae usually inhabit woodlands or forested areas, where larvae feed on decaying wood, so it is possible that the Cretaceous forested environment was favourable for promoting the evolution of the genus *Colophon*. The Cretaceous also saw the divergence of Angiosperms (Deacon *et al.*, 1992) with several members of the Cape floral clade appearing in the late Cretaceous and radiating in the Tertiary (Galley & Linder, 2006; Galley *et al.*, 2007). One such a clade is Restionaceae which started radiating during the late Cretaceous-Early Paleocene (70-60 mya) (Wikström *et al.*, 2001; Galley & Linder, 2006; Verboom *et al.*, 2008) with which *Colophon* larvae are associated, being found amongst their roots and tussocks.

Divergence of Lineage II was early Paleocene (*ca.* 63.94 mya) with Lineage I dating to mid Eocene (*ca.* 46.81 mya). The divergence times of the two lineages also support the east-west movement of *Colophon* as suggested by their genealogy. The orogeny of the Cape Fold Mountains (CFM) was estimated to be a Permian-Triassic event (280-235 mya) (Partridge & Maud, 2000), with the structure of the mountains being well established by the time *Colophon* evolved. The reason behind the split of the two lineages is not clear, although it might be as a consequence of the major events that triggered the end of the Cretaceous

period, better known as the K/T boundary (65 mya). The ancestral lineage of *C. westwoodi* corresponds to the Paleocene split of Lineage II. During this time, arid conditions were present in the west of the continent, persisting well into the Eocene, with an east-west climatic gradient across southern Africa temporarily established (Tyson & Partridge, 2000). It is, thus, suggested that the ancestral lineage of *C. westwoodi* was the first to move upwards with its habitat into the mountains as low altitudinal conditions became increasingly more arid. They subsequently became isolated on Table Mountain. The Cape Peninsula and Table Mountain area were also isolated by repeated marine transgressions throughout the Pleistocene (Hendey, 1983; Mucina & Rutherford, 2006; Swart *et al.*, 2009) possibly isolating *C. westwoodi* even more.

In conclusion, most species of *Colophon* showed an allopatric distribution, although contact zones between geographically adjacent species are likely as is the case between *C. kawaii* and *C. haughtoni*. Climate seems to be the main driving factor behind *Colophon* evolution and Endrödy-Younga's (1988) hypothesis appears to be supported. Addition of the five outstanding species and analysing a slower evolving gene-region could help improve the resolution of the deeper phylogenetic nodes. We suggest that the *C. stokoei* varieties as well as the *C. eastmani* subspecies be revised taxonomically due to their high degree of genetic divergence, distinct distribution on separate mountain peaks and the observed differences in morphology.

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Tables and Figures

Table 1. Summary of Primers used for PCR amplification

Locus	Primer name and sequence	Reference
CO I	C1-J-1718 (5'GGAGGATTTGGAAATTGATTAGTTCC3')	Simon <i>et al.</i> (1994)
	C1-J-2183 (5'CAACATTTATTTTGATTTTTTGG3')	Simon <i>et al.</i> (1994)
	TL2-N-3014 (5'TCCAATGCACTAATCTGCCATATTA3')	Simon <i>et al.</i> (1994)
16S rRNA	LR-J-12961 (5'TTTAATCCAACATCGAGG3')	Simon <i>et al.</i> (1994)
	LR-N-13398 (5'CGCCTGTTTAACAAAAACAT3')	Simon <i>et al.</i> (1994)
CAD	54F (5'GTNGTNTTYCARACNGGNATGGT3')	Moulton & Wiegmann (2004)
	680R (5'AANGCRTCNCGNACMACYTCRTAYTC3')	Moulton & Wiegmann (2004)
	338F (5'ATGAARTAYGGYAATCGTGGHCAYAA3')	Winterton <i>et al.</i> (2010)
	654F (5'TCYTTCCANCCYTTYARSGATTTRTC3')	Winterton <i>et al.</i> (2010)

Table 2. Estimated model parameters for COI, 16S and CAD for *Colophon* using MrModeltest Ver. 2.3

	COI	16S	CAD
Best-fit model	GTR+I+G	GTR+G	HKY+I+G
A frequency	0.3391	0.4196	0.2640
C frequency	0.1554	0.1728	0.2115
G frequency	0.1345	0.0786	0.2642
T frequency	0.3710	0.3290	0.2603
Gamma	1.0637	0.2067	0.9686
Invariable site	0.5240	0	0.5789

Table 3. Percentage pairwise genetic distances between various *Colophon* species for COI. Species indicated with * correspond to Fig. 1 and are further explained in the text.

Species	COI (%)
<i>C. stokoei</i> Var. A ; <i>C. stokoei</i> Var. C	10.7
<i>C. stokoei</i> Var. A ; <i>C. stokoei</i> forma typica	8.4
<i>C. stokoei</i> Var. C ; <i>C. stokoei</i> forma typica	7.4
<i>C. kawaii</i> * ; <i>C. cameroni</i>	9.4
<i>C. kawaii</i> ; <i>C. haughtoni</i>	3.0
<i>C. eastmani eastmani</i> ; <i>C. eastmani nagaii</i>	9.6
<i>C. neli</i> ; <i>C. neli</i> *	8.1
<i>C. izardi</i> ; <i>C. izardi</i> *	2.9

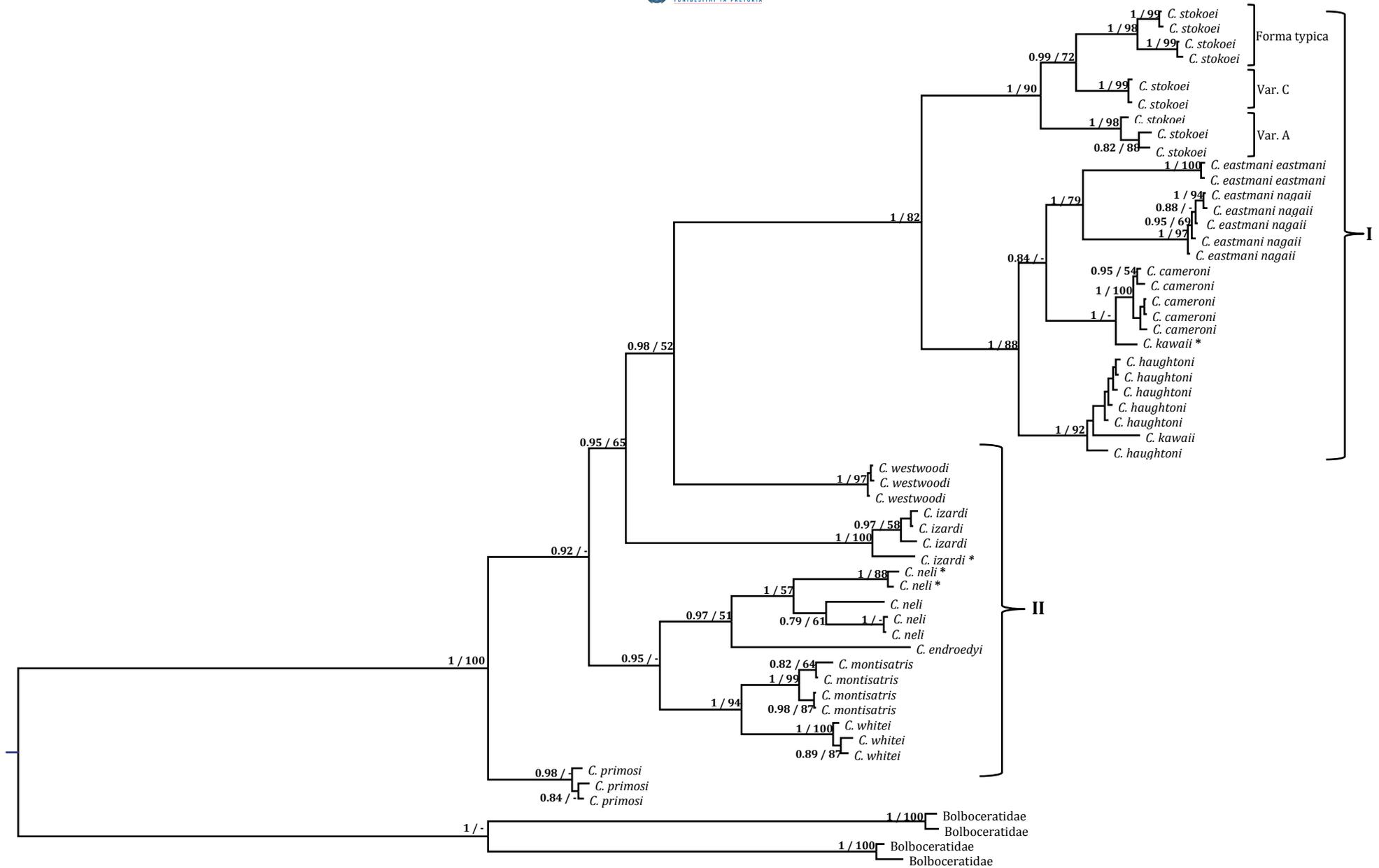


Figure 1. Bayesian phylogram of combined datasets (COI, 16S and CAD) with Posterior Probabilities and Maximum Likelihood Bootstrap given, respectively. Dashes (-) on nodes indicate weak/no support. Specimens indicated with an asterisk (*) are further discussed in the text.

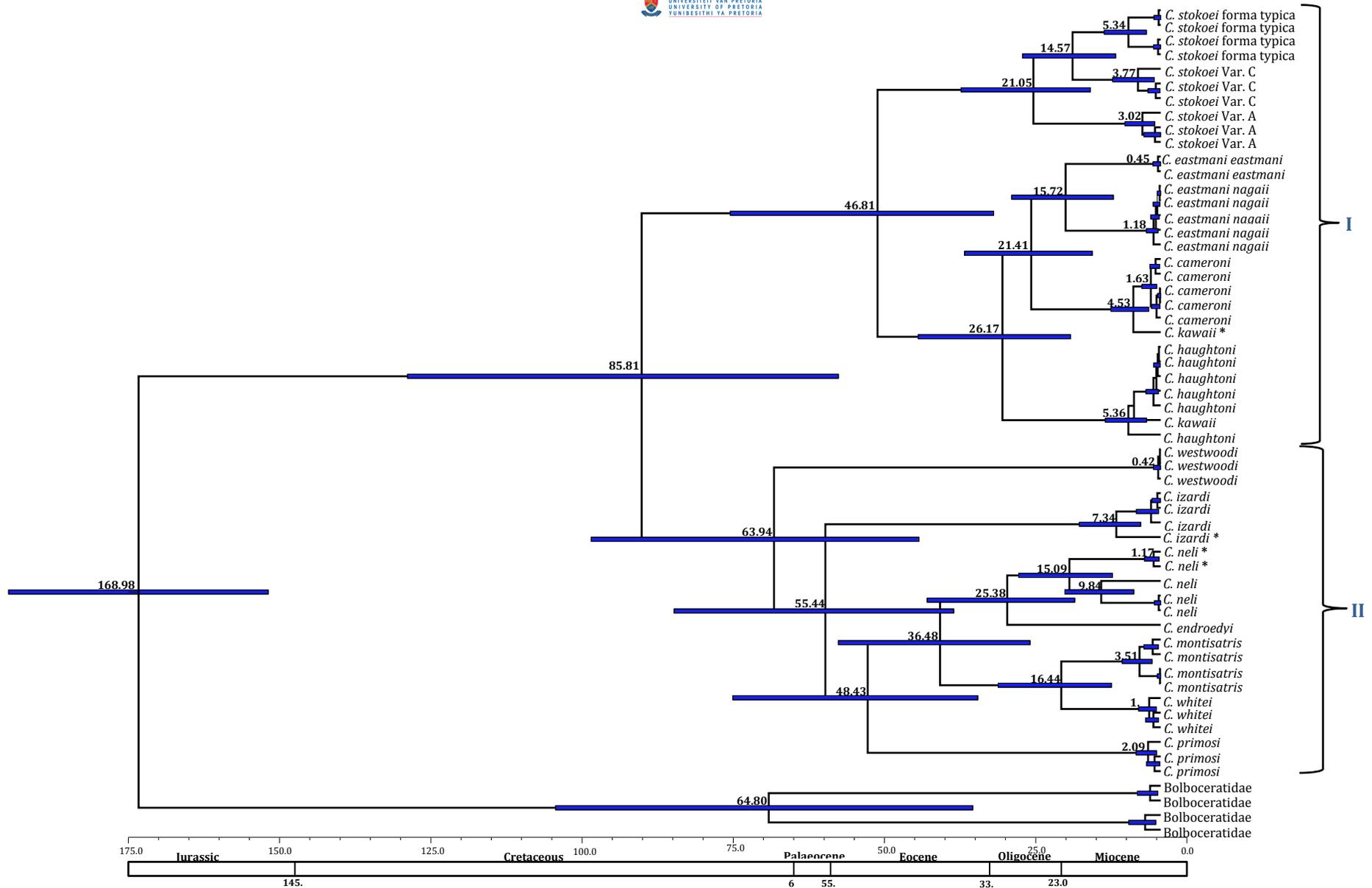


Figure 2. Estimated times of divergence for the genus *Colophon*. Mean probability distributions for node ages are indicated above branches with time intervals for the 95 % probability of actual age represented as blue bars. Specimens indicated with an asterisk (*) are further discussed in the text.

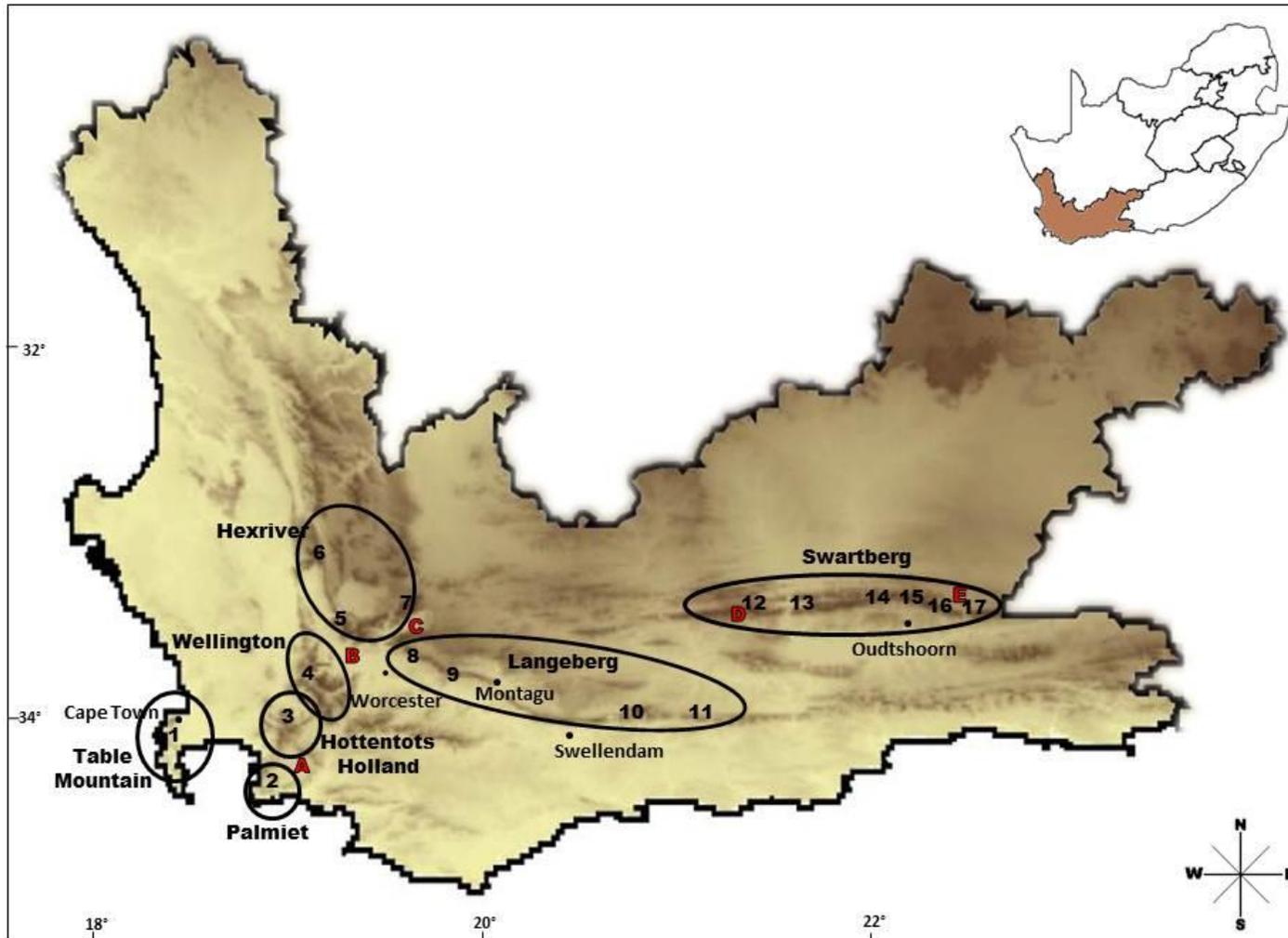


Figure 3. Map indicating the major Mountain Ranges, peaks and valleys associated with *Colophon* distribution in the Western Cape of South Africa. Numbers refer to mountain peaks; letters to valleys. (1) Table Mountain, (2) Palmietberg, (3) Sneekop, (4) Perdekop, (5) Waaiohoek, (6) Groot-Winterhoek, (7) Matroosberg, (8) Keeromsberg, (9) Dassieshoek, (10) Grootberg, (11) Riversdale, (12) Seweweekspoort Peak, (13) Ossenberg, (14) Swartberg (Swartberg Pass), (15) Tierhoek, (16) Blesberg, (17) Bloupunt. (A) Steenbras Valley, (B) Breede River Valley, (C) Hex River Valley, (D) Seweweekspoort, (E) Meiringspoort.

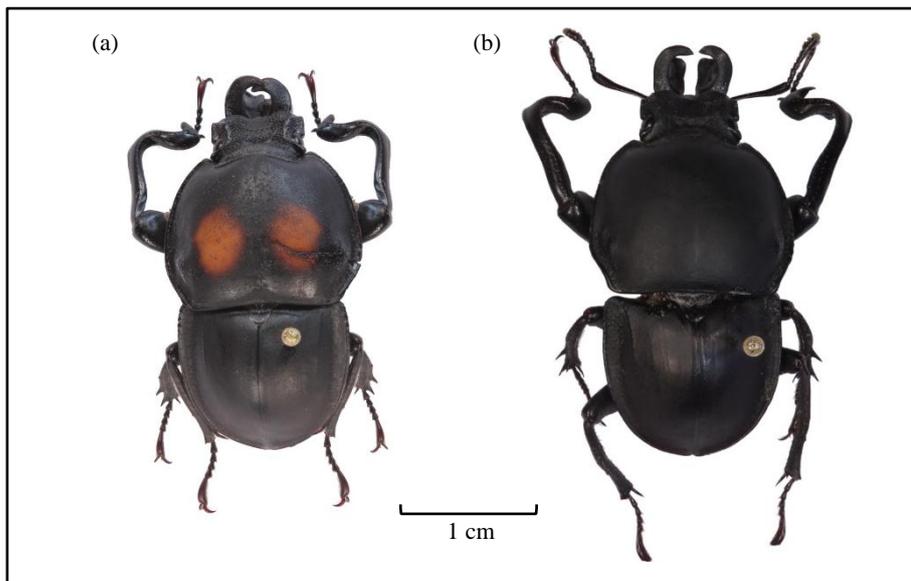


Figure 4. *Colophon izardi* showing the colour polymorphism between specimens found in the (a) Lemoenshoek-Grootberg and (b) Riversdale area.



Figure 5. *In situ* photograph of *Colophon primosi* illustrating its spectacular reddish-brown to orange mandibles and front tibia.

Chapter III:

Colophon larvae: descriptions and phylogenetic implications

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Abstract

The aim of the *Colophon* larval study was to enable researchers to identify the species found in the field; to use larvae as an alternative for adults in molecular studies; to comment on possible phylogenetic information that may contribute to the sub-familial placement of the genus; and to obtain habitat preference data. To achieve this, larvae of four *Colophon* species were examined and their main diagnostic morphological characters identified. Larvae live in a fairly homogeneous micro-habitat of moist, humus-rich soil in protected places such as under rocky overhangs and amongst the roots and tussocks of Restionaceae. This soil-living and humus-feeding habit of *Colophon* is unique in the family. *Colophon* larvae show small inter-specific differences and larval characters contributed little equivocal information from which phylogenetic support for family placement could be deduced. Clearly, as with many scarabaeoid groups, larval morphology has not diversified much from the basal ground plan and it remains for other (adult) phylogenetically significant morphological characters or DNA to provide more clarity on *Colophon*'s subfamilial placement.

Keywords: Cape Floristic Region; *Colophon*; Endangered; Larval description; Lucanidae.

Introduction

As a global biodiversity hotspot, the Cape Floristic Region (CFR) is known for its floral species richness and high levels of endemism (Cowling *et al.* 1989; Myers *et al.* 2000; Midgley *et al.* 2003). This is in large part due to both the unique reigning climate and climatic history of the area (Midgley *et al.* 2001). Associated with global warming, there has been a steady increase in temperature and decrease of rainfall within the CFR (Tyson *et al.* 2002). It is therefore becoming desirable to study the responses of species to these climatic changes. High mountain fauna and flora worldwide are especially threatened by global warming (Parmesan & Yohe 2003), more so when the mountain peaks are high enough to differ substantially in weather patterns when compared to the lower slopes. The Cape Fold Mountains (CFM) qualify as such mountains. During the summer months, strong south-easterly winds, blowing off the ocean in late afternoons, generate updrafts of cool moist air against the mountain slopes which condenses and regularly covers the peaks in a dense cold mist. During winter, cyclonic fronts and north-westerly winds bring rain and snow storms, often leaving the highest peaks covered in a blanket of snow. The CFM also provides habitat to a diversity of insect species (Cowling 1992; Linder 2003; Gilliomee 2003; Galley & Linder 2006; Cowling *et al.* 2009). One such insect species group is the enigmatic Cape High-mountain stag beetle genus *Colophon* Gray.

Endemic to the CFR and geographically isolated to the high mountains of the Western Cape, this primitive, apterous genus is represented by 17 species. *Colophon* is a relict of a past temperate climate regime that prevailed at lower elevations and which with increasing global temperatures since the Plio-Pleistocene have been forced up the mountains. This has led to speciation and the current localised distribution of individual species on more hospitable mountain tops (Endrödy-Younga 1988; Chapter 2). It has been shown that over the past century, montane species worldwide are withdrawing an average of 60m up the slopes of

their mountain habitats due to the effects of global warming (Parmesan & Yohe 2003). All *Colophon* species are restricted to the highest peaks on the mountain ranges, often in very small habitat fragments, making their survival tenuous considering the conditions predicted to prevail in future due to global warming (e.g., Lutjeharms *et al.* 2001; Meadows 2006). *Colophon* survival is not only threatened by predicted future climatic scenarios, but also by ongoing collection pressures from beetle collectors, who have traded specimens for large sums (up to US \$15 000 per specimen) of money.

Colophon species were declared “protected” by South African Cape Provincial law in 1992 and listed by CITES (Convention on International Trade in Endangered Species) in Appendix III in 2000. Fourteen of the 17 *Colophon* species are also included on the IUCN Red List (<http://www.iucnredlist.org>) and the genus as a whole was placed on the South African ToPS (Threatened or Protected Species) list in 2007. However, these assessments of *Colophon* are largely outdated and in need of revision (New 2012). Initially listed by CITES, (Anonymous 1994) to protect them from commercial exploitation, this listing has only succeeded in drawing more attention to the beetles and possibly increasing their black market value (Gess & Gess 1993; Melisch & Schütz 2000; Geertsema & Owen 2007).

Adults of all species are strongly sexually dimorphic, with males boasting large mandibles, as is typical for virtually all members of Lucanidae. The optimal weather conditions for surface activity have been defined as cool misty conditions during late afternoons and early mornings (Barnard 1929; Endrödy-Younga 1988). However, specimens of several species have been collected in the heat of day following a misty evening/morning (personal observation). A study by Roets *et al.* (2012) on the abiotic variables influencing adult *Colophon westwoodi* activity showed that their activity was significantly influenced by illuminance (suggesting they might be nocturnal) and the presence of mist (moisture) during the day. Adults appear not to feed and circumstantial evidence suggests that larvae feed on the roots of plants

(Endrödy-Younga 1988). *Colophon* larvae live in humus-rich soil beneath stones and amongst the roots of tussocks of the Restionaceae which dominate on suitable patches of soil on the mountain peaks. They are long-lived, with a 3rd-instar larva of *Colophon neli* kept in the laboratory showing no signs of growth over a six-month period (Scholtz and Endrödy-Younga 1994). Another large, possibly 3rd-instar larva, originally thought to be that of a fruit-chaffer (Scarabaeidae: Cetoniinae) and kept in humus-rich soil indoors, yielded a female specimen of *Colophon cameroni* approximately one year later (Tony Brinkman, pers. comm.).

Since adult *Colophon* beetles mainly occur on largely inaccessible mountain peaks and are mostly active during ideal conditions, finding specimens is usually difficult. Larvae, however, although sparsely distributed and usually buried in the soil, are often a more dependable source of research material. They are the topic of this paper and form part of a larger project on the phylogeny and conservation status of the various species.

Colophon larvae found in the field can be ascribed to species since in most cases only one species occurs on each mountain peak. On the basis of this, specimens of *C. neli* were collected and described by Scholtz and Endrödy-Younga (1994). This was the first, and until the current study, the only larva of any species of the genus to be described. The purpose of the present larval study is fourfold: to enable researchers to identify the species found in the field; to use larvae as an alternative for adults in molecular studies; to comment on possible phylogenetic information that may contribute to the sub-familial placement of the genus; and to obtain habitat preference data. The latter is of special importance for future studies if predicted climate change starts to impact on larval habitat availability and suitability.

Methodology

Specimens studied

Several scarabaeiform larvae were collected from various Western Cape mountains (Table 1, Fig. 1) and identified as Lucanidae based on the shape of the anal opening (Ritcher 1966). *Colophon* is the only member of the family on these mountains. Larvae were mostly found under rocky overhangs and amongst the roots and tussocks of Restionaceae, in moist, humus-rich soils. Those collected were ascribed to species based on their locality and associated adults. They were preserved in absolute alcohol for eventual description and their DNA sequence data were used for phylogenetic studies. *Colophon neli* larvae from the Scholtz & Endödy-Younga (1994) study, housed in the Ditsong Museum (formerly Transvaal Museum), were re-examined and included in the present study (Table 1).

Several characters essential to distinguish the larvae of major groups of Lucanidae (Lawrence 1981; Scholtz & Endrödy-Younga 1994) were examined under a dissecting microscope. For examination of the mouthparts, heads were dissected, main structures removed, dried, gold-coated and studied in a Scanning Electron Microscope (SEM). Ritcher's (1966) terminology was used to describe the larvae. Voucher specimens are currently deposited in the University of Pretoria Insect collection (UPSA).

General Larval Description

The four *Colophon* species' larvae differed only slightly in character from each other, thus a general description for the genus *Colophon* is provided with species differences highlighted in Table 2.

Head capsule. Maximum width: 6.3mm. Antenna 3-segmented; reduced distal segment with dorsal sensory spot (Table 2). Surface of head capsule smooth, brown, with preclypeus a

slightly lighter colour than the brown labrum and postclypeus. Labrum slightly asymmetrical. Primary frontal setae on each side consist of one or two exterior frontal setae (EFS), one anterior frontal angle seta (AA) and one anterior frontal seta (AFS). Posterior frontal setae (PFS) are absent. Isolated setae present on epicranium.

Mandibles. Left mandible with 3 distinct teeth on incisor edge, with a scissorial notch. No teeth on inner surface of mandible, with a blade-like slope between molar and scissorial area. One triangular molar tooth present (Table 2, Fig. 2a-d). Right mandible without teeth. Blade-like scissorial area present. Inner surface of mandible with a small notch and no prominent molar teeth.

Epipharynx. Elevated pedium. Chaetoparia well developed with about 20 pointed setae. Haptolachus with three setae. Distinct, united, symmetrical pternotorma (Table 2, Fig. 2e).

Hypopharynx. Distinct asymmetrical sclerome, right side with prominent truncate process (Fig. 2f).

Maxilla. Palpus 4-segmented with slight dark colouration around anterior end of second segment and without setae on last segment. Distinctly separated galea and lacinia. Lacinia with single, terminal unicus and a fringe of about 10 stiff setae; galea with two subequal unci and a fringe of stiff setae. No maxillary stridulation area on stipes (Fig. 2f).

Thorax. Transverse row of sparse long and short setae on dorsal side of thorax, with no anterior process on prothorax.

Abdomen. Row of sparse long setae on tergites of segments 1-3, third segment with a broader band of short, stiff setae. Large field of short stiff, and long isolated, setae on segments 4-7. Segments 8-9 with isolated long setae. Abdominal segment 10 greatly constricted. Vertical anal slit with bulbous lateral lobes and a well-developed raster.

Spiracles. Thoracic and abdominal spiracles with reniform plate, concavity facing cephalad. Spiracles on abdominal segment 1-3 larger than spiracles on abdominal segments 4-8.

Tarsangulus. Well-developed claw with 2 setae.

Stridulatory organs. Mesocoxal stridulatory organ (pars stridens) consisting of a main row of large, dark tubercles, with less defined area of small, pale granules outside this (Fig. 2g). Metatrochanteral stridulatory organ (plectrum) consisting of a row of about 50 granular carinae, close together on the anterior surface (Fig. 2h).

Discussion and conclusion

The larvae of each of the species collected were found in similar micro-habitats on the mountains. These consisted of moist, humus-rich soil in protected places such as under rocky overhangs and amongst the dense roots and bases of plants, mainly Restionaceae. From this study, and the little information provided by Scholtz and Endrödy-Younga (1994) and Tony Brinkman's unpublished record, there is fairly compelling evidence to suggest that the larvae are humus-feeders that are dependent on an accumulation of protected, moist decomposing vegetation. Moisture is obviously critical for larval survival; hence their occurrence in sheltered places where desiccation is less severe than in exposed places. Increased temperatures and drier conditions predicted by global warming scenarios are likely to have far-reaching effects for larvae and it is perhaps in this area that future studies of habitat suitability and change should concentrate.

Colophon larvae show only small inter-specific differences, something in common with the larvae of several scarabaeoid groups. This has been ascribed to the fact that the larvae of most of the groups live in fairly homogeneous micro-habitats such as humus, rotting wood and dung, somewhat removed from the selective pressures that necessitate major change from the

basal larval type in the group. That *Colophon* larvae of the different species are geographically isolated also precludes inter-species competition that might necessitate habitat shift and corresponding morphological adaptive change.

Scholtz and Endrödy-Younga (1994) discussed in some detail *Colophon* (and the east coast forest species, *Prosopocoilus natalensis*) larval morphology from a Lucanidae phylogenetic perspective so only major points pertinent to this study are discussed here. *Colophon* larvae are characterised by 3-segmented antennae (this is the basal condition in Lucanidae (and Scarabaeoidea)) and precludes them from inclusion in the Lucaninae based on it – Lucaninae have 4-segmented antennae, with the last segment greatly reduced in size. The epipharynx is generally similar in all studied species but distinct from other known lucanid species. The left mandible has teeth on the incisor edge between the apical teeth and mola, a character that places *Colophon* amongst other members of the subfamily Lucaninae. All Lucaninae have teeth in this position, a character unique to the group. Scholtz and Endrödy-Younga (1994) appear to have misinterpreted the extent of the scissorial area, leading to the conclusion that there are no teeth in this area, which, they concluded also placed *Colophon* outside of the Lucaninae. The meso- and meta-trochanteral stridulatory organs are similar to those of various other basal and lucanine groups so are of little phylogenetic use. The soil-living and humus-feeding habit of *Colophon* larvae is unique in the family. An ongoing study by David Hawks (University of California, Riverside) and Matt Paulsen (University of Nebraska, Nebraska), on the phylogeny of the world Lucanidae will give more insight into the family placement of *Colophon*.

In conclusion, *Colophon* larval characters contribute little equivocal information from which phylogenetic support can be deduced. Clearly, as with many scarabaeoid groups, larval morphology has not diversified much from the basal ground-plan and it remains for other

(adult) phylogenetically significant morphological characters or DNA to provide more clarity on *Colophon*'s subfamilial placement.

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Table and Figures

Table 1. *Colophon* species and larval specimens examined and used in the analyses.

Species	Location	Second instar	Third instar
<i>Colophon haughtoni</i>	Matroosberg Mountain	-	2
<i>Colophon cameroni</i>	Waaihoek Mountain	4	7
<i>Colophon eastmani</i>	Ben Heatlie Mountain	-	1
<i>Colophon neli</i>	Swartberg Mountain	-	2

Table 2. Structural differences between larvae of four *Colophon* species.

	<i>Colophon haughtoni</i>	<i>Colophon cameroni</i>	<i>Colophon eastmani</i>	<i>Colophon neli</i>
Left mandible	3 distinct teeth on incisor edge	3 distinct teeth on incisor edge	S2 and S3 semi fused.	3 distinct teeth on incisor edge
	Prominent scissorial notch	Prominent scissorial notch	Small scissorial notch	Prominent scissorial notch
	Sloping (>90°) angle between molar and scissorial area	Sloping (>90°) angle between molar and scissorial area	Strong bladelike slope between molar and scissorial area	Region between molar and scissorial area slightly more angulated
	One prominent triangular molar tooth	One prominent triangular molar tooth	Small rounded molar tooth	One prominent triangular molar tooth
Antenna	2 setae on distal segment	No setae on distal segment	2 setae on distal segment	2 setae on distal segment
Epipharynx	Proto-, dexio- and laephoba present	Proto-, dexio- and laephoba present	Proto-, dexio- and laephoba present	No phoba
	No epitorma	No epitorma	No epitorma	Faint epitorma
	Haptomerum with 2 sensilla	Haptomerum with 2 sensilla	Haptomerum with 2 sensilla	No sensilla on haptomerum

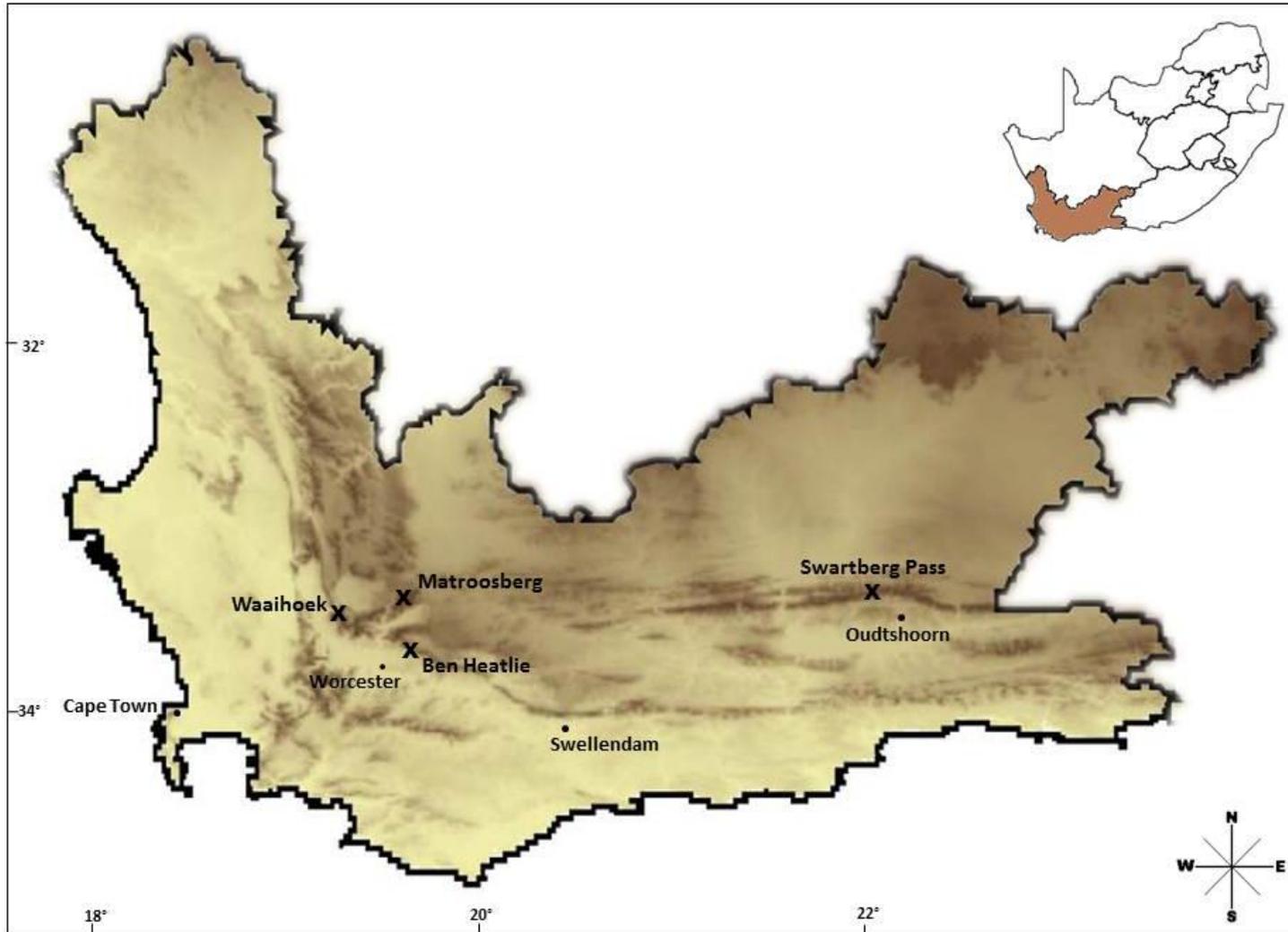


Figure 1. Map indicating the Mountain Peaks where *Colophon* larvae have been collected in the Western Cape of South Africa.

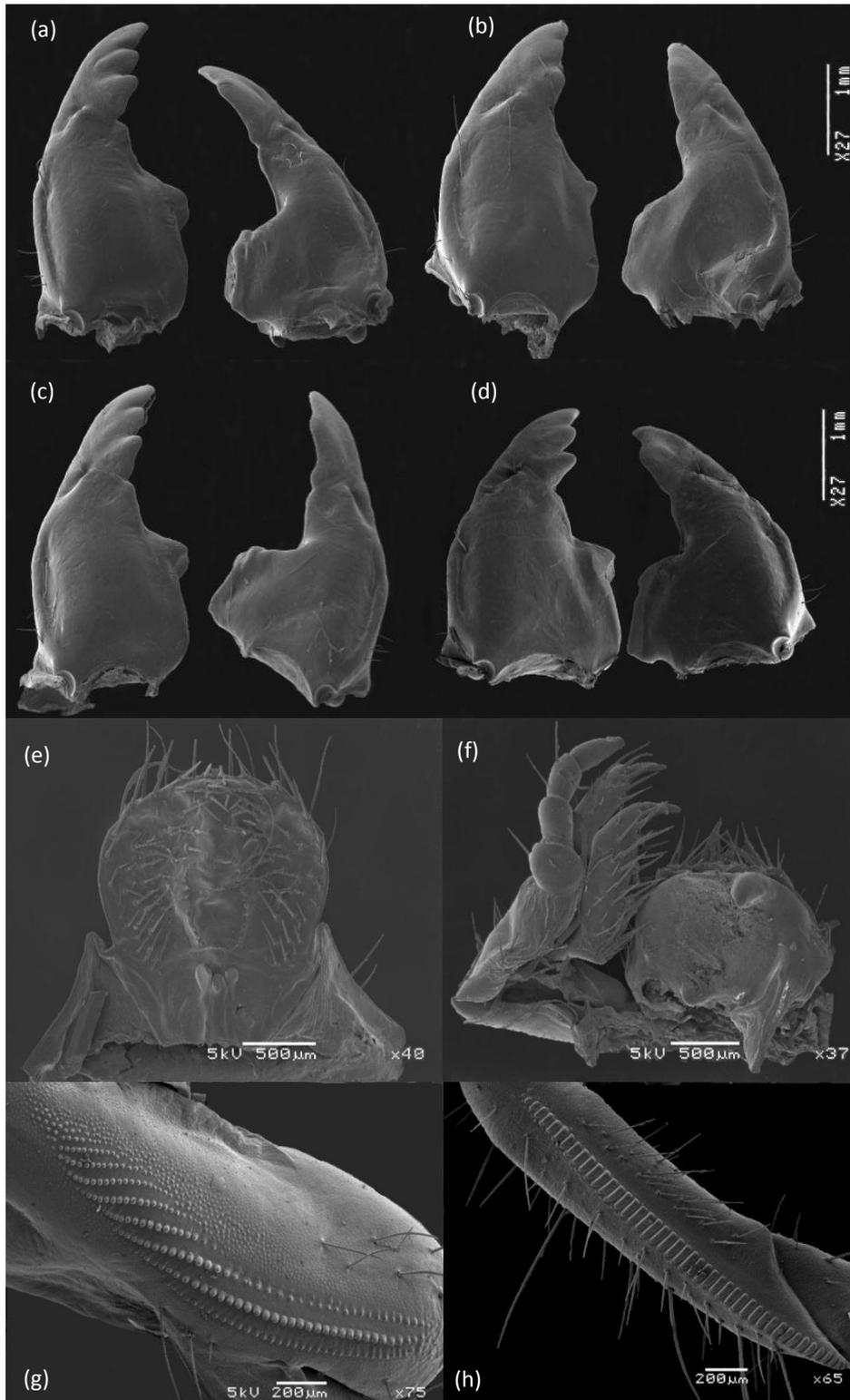


Figure 2. Structures of *Colophon* larvae. (a-d) Left and right larval mandibles of (a) *C. cameroni*; (b) *C. eastmani*; (c) *C. haughtoni* and (d) *C. neli*; (e) epipharynx of *C. cameroni*; (f) hypopharynx and maxilla of *C. cameroni*; (g) posterior view of the Mesocoxal stridulatory organ (pars stidens) of *C. eastmani*; (h) anterior view of the Metatrochanteral stridulatory organ (plectrum) of *C. eastmani*.

Chapter IV:

Notes on conserving South Africa's Mountain Peak dweller, *Colophon* Gray (Coleoptera: Lucanidae)

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South Africa has been rated as the third most biologically diverse country in the world, with three of the 34 biodiversity hotspots falling within its borders (Mittermeier *et al.*, 2004). The contribution of the insect fauna to this biodiversity is, however, not well known with conservation focus mainly falling on South Africa's rich floral diversity, followed by the variety of large mammal and bird fauna, all of which play a significant role in the ecotourism industry (Samways, 2002; Samways *et al.*, 2012). The first formal protection of an insect species in South Africa dates back to 1976 (McGeogh, 2002), yet, insect conservation in South Africa remains a somewhat neglected and relatively new discipline facing many challenges and opportunities (Samways *et al.*, 2012). The inclusion of insects on Threatened Species Lists, such as the IUCN (International Union for Conservation and Nature) Red List and the more local Threatened or Protected Species (ToPS) list, is promising for the development of insect conservation. These lists can then be passed on to conservation managers and policy makers to be used in measuring biodiversity loss and quantifying the rates of species extinction, with the intention of ultimately aiding in biodiversity management (Samways, 2002; Friedmann, 2009). One of the South African insect species already featuring on such a ToPS list is the Cape High-mountain stag beetle genus *Colophon*.

Endemic to South Africa, the genus *Colophon* is a relict of an ancient Gondwanaland temperate lineage of flightless beetles adapted to high montane conditions of the Western Cape Fold Mountains (Endrödy-Younga, 1988; Chapter 1; 2). Already threatened by warming conditions induced by a change in climate during the Miocene (Chapter 2), the

species have gradually been forced higher up mountains until each of the 17 extant species now only exist in tiny fragments of temperate habitat on the highest mountain peaks. *Colophon* were given provincial legal protection (Cape Nature and Environmental Conservation Ordinance #24) in 1992 and listed in CITES (Convention on International Trade in Endangered Species) Appendix III in 2000, mainly due to their perceived rarity and desirability by beetle collectors. Although they were listed by CITES (Anonymous, 1994) to protect them from commercial exploitation, this listing has only succeeded in drawing more attention to the beetles and possibly increased their black market value (Gess & Gess, 1993; Melisch & Schütz, 2000; Geertsema & Owen, 2007). They are currently the only South African insect species to be CITES listed (www.cites.org >2013). Fourteen of the 17 described species have also been listed on the IUCN Red List due to their restricted distribution (Table 1; B1) and their perceived decline in numbers (Table 1; 2e) (<http://www.iucnredlist.org> >2013). The genus as a whole was also placed on the South African ToPS list in 2007. These assessments are, however, mostly based on speculation regarding *Colophon* species' rarity and their commercial value in the beetle collectors' trade, since no study, until recently, has attempted to determine accurate species' distribution, habitat specificity, phylogenetic relationships, phylogeography and population size. In order to make informed decisions regarding conservation and for the effective implementation of management programmes, sound knowledge of the species' habitat requirements and biology is needed (New, 2009; McGeoch *et al.*, 2011; New, 2012). This is often lacking for invertebrate species and is particularly true for *Colophon*. The current study thus undertook to reassess the conservation status of *Colophon* in terms of its ToPS and IUCN listing, as current assessments are largely outdated and in need of revision (Samways *et al.*, 2012).

Threatened Species Lists in South Africa

The National Environmental Management Biodiversity Act (No. 10 of 2004, Chapter 4 Part 2 Section 56) (NEMBA) provides for the listing of threatened or protected species. The first ToPS list was published in the government gazette on 23 February 2007 (<http://www.speciesstatus.sanbi.org/threatened.aspx> >2013). The primary role of the list is to catalogue species in danger of extinction or unsustainable decline induced or aggravated by “restricted activities” (Republic of South Africa, 2007: Section 57) and therefore provide authorities with the necessary tools to enforce legislation pertaining to anthropogenic activities related to the listed species (Friedmann, 2009). All restricted activities pertain to direct use and include activities such as hunting, collecting, exporting and trading (comprehensive list available at <http://www.speciesstatus.sanbi.org/threatened.aspx> >2013). The list does not take habitat loss or decline in habitat quality into consideration as these are dealt with in a different section of NEMBA, the listing of threatened and protected ecosystems in Section 52 Act 10 of 2004.

Species are placed in one of four categories on the ToPS list using a scientifically objective and robust system that can reliably and consistently be applied across various taxonomic groups. Specialists and taxonomic experts in the fields of Zoology, Entomology and Conservation participate by submitting data, reviewing assessments and/or assisting in the assessment process. The South African Biodiversity Institute (SANBI), a local government authority, provides the platform for the collection of such species information to ultimately be used in the threat assessment processes. SANBI aids in the exploration, conservation, sustainable use, appreciation and enjoyment of South Africa’s rich biodiversity (<http://www.sanbi.org> >2013). As part of their mandate to monitor and report on the conservation status of South Africa’s indigenous plant and animal species, SANBI implemented a Threatened Species Programme to manage the collection of species

information for use in the assessment of species status according to IUCN Red List Categories and Criteria. The programme also promotes the use of species' information in all aspects of biodiversity conservation, which includes the formulation of threatened species lists.

Proposed Conservation Status of *Colophon*

We recognise that our knowledge of *Colophon* is incomplete, mainly because monitoring of the beetles proves to be a particular challenge due to their restricted distribution and the irregular weather conditions during which they are active. A recent study by Roets *et al.* (2012) studied the abiotic variables influencing adult activity of one of the species, *C. westwoodi*, but it still remains to be determined whether these variables actually do dictate the activity of all *Colophon* species. Little is known about population size, responses to disturbances and life cycle. However, information we gathered on *Colophon* and presented in the earlier chapters, as well as personal observations made during fieldwork and data gleaned from museum specimens, private collections and published literature serve as baseline information for assessing the conservation status of *Colophon*. This was done according to ToPS (Table 2) and IUCN Ver. 3.1 criteria (criteria pertaining to *Colophon* in Table 3; for the comprehensive list of criteria see <http://www.iucnredlist.org>) and suggestions are presented here for the revised conservation listing of *Colophon* species. Differences between the threat assessments and resultant conservation status for each species are listed below (Red List IUCN criteria (Table 3) presented in brackets); to protect the *Colophon* populations precise locality data is withheld and mountain ranges refer to those presented in Chapter 2: Fig 3.

Colophon barnardi Endrödy-Younga, 1988

- Red List Status: Critically Endangered (B1ab(v) & B2ab(v))
- ToPS Status: Critically Endangered

Known from only one location on the Langeberg Mountain with an Extent of Occurrence (EoO) and Area of Occupancy (AoO) <5km².

Colophon berrisfordi Barnard, 1932

- Red List Status: Critically Endangered (B1ab(v) & B2ab(v))
- ToPS Status: Critically Endangered

Known from only one location on the Swartberg Mountains with an EoO and AoO <10km².

Colophon cameroni Barnard, 1929

- Red List Status: Endangered (B1ab(v))
- ToPS Status: Endangered

Known from five locations on the Hex River Mountains with an EoO of 40km².

Colophon cassoni Barnard, 1932

- Red List Status: Data Deficient
- ToPS Status: Protected

The locality on the Swartberg Mountain for the type specimens, collected in 1932, is vague (Meiringspoort Berg) and impossible to locate. Other specimens collected since are only present in private collections, and these also state the same vague locality as the type. This lack of information does not allow for assessment of the species' threat status. The species should, however, be placed on ToPS as protected due to possible collection pressures.

Colophon eastmani Barnard, 1932

- Red List Status: Endangered (B1ab(v))
- ToPS Status: Endangered

Known from two locations on the Langeberg Mountains with an EoO <60km². *Colophon eastmani* was split into the disjunct subspecies *C. eastmani eastmani* and *C. eastmani nagaii* (Mizukami 1996). Our phylogenetic study suggests these should be taxonomically revised and both subspecies be elevated to species level (Chapter 2). Their respective threat statuses would thus change to Critically Endangered, because each is only known from a single location with an EoO and AoO <10km².

Colophon endroedyi Bartolozzi, 2005

- Red List Status: Critically Endangered (B1ab(v))
- ToPS Status: Critically Endangered

The species is only known from a single location on the Swartberg Mountains with an EoO <50km².

Colophon haughtoni Barnard, 1929

- Red List Status: Endangered (B1ab(v) & B2ab(v))
- ToPS Status: Endangered

Known from two locations on the Hex River Mountains with an EoO of 13 km² and AoO <10km².

Colophon izardi Barnard, 1929

- Red List Status: Endangered (B1ab(v))
- ToPS Status: Endangered

Known from three locations on the Langeberg Mountains with an EoO of 13km². The species is highly desirable to collectors due to their unique front legs (Chapter 2: Fig. 4).

Colophon kawaii Mizukami, 1997

- Red List Status: Critically Endangered (B1ab(v))
- ToPS Status: Critically Endangered

Known from only a single location on the Hex River Mountains with an EoO and AoO <1km².

Colophon montisatris Endrödy-Younga, 1988

- Red List Status: Critically Endangered (B1ab(v) & B2ab(v))
- ToPS Status: Critically Endangered

The species is known from only a single location on the Swartberg Mountains with an EoO and AoO <1km².

Colophon neli Barnard, 1932

- Red List Status: Endangered (B1ab(v) & B2ab(v))
- ToPS Status: Endangered

Known from only two locations on the Swartberg Mountains with an EoO and AoO <4km².

Colophon oweni Bartolozzi, 1995

- Red List Status: Critically Endangered (B1ab(v) & B2ab(v))
- ToPS Status: Critically Endangered

This species is known from only a single location on the Langeberg Mountains with an EoO and AoO <10km².

Colophon primosi Barnard, 1929

- Red List Status: Endangered (B1ab(v))
- ToPS Status: Endangered

This species is known from two locations with an EoO <80km² but is suspected to occur on three more unexplored mountain peaks within the Swartberg range. The males of this species are highly desirable because of the unique, unusually enlarged, orange mandibles (Chapter 2: Fig. 5).

Colophon stokoei Barnard, 1929

- Red List Status: Not Threatened (B1ab(v))
- ToPS Status: Protected

This species has an EoO of 349km² and has been recorded from seven locations from the Palmietriver in the south to the Wellington Mountains in the north. However, they are likely to occur on at least five other mountain peaks of suitable altitude within the range of this species. Five varieties of *C stokoei* were described (Barnard, 1929; Endrödy-Younga, 1988). Three of the varieties are suggested to be described as separate species based on their phylogenetic divergence, distinct distribution on separate mountain peaks and the observed differences in morphology (Chapter 2). This taxonomic revision would possibly result in

each of the newly described species (old varieties) having a threat status of Endangered or Critically Endangered.

Colophon thunbergi Westwood, 1855

- Red List Status: Critically Endangered (B1ab(v) & B2ab(v))
- ToPS Status: Critically Endangered

Known from only a single location on the Langeberg Mountains with an EoO and AoO <10km².

Colophon westwoodi Gray, 1832

- Red List Status: Critically Endangered (B1ab(v) & B2ab(v))
- ToPS Status: Critically Endangered

Known from only a single location on Table Mountain with an EoO and AoO <6km². They occur on a commonly visited mountain peak and are often trampled by tourists (Geertsema & Owen, 2007; Roets *et al.*, 2012).

Colophon whitei Barnard, 1932

- Red List Status: Endangered (B1ab(v))
- ToPS Status: Endangered

These species are known from two locations on the Swartberg Mountains but is likely to occur at another three, as there are at least three other mountain peaks of suitable altitude within the species range. They have an EoO of 50-100km².

Apart from the threat posed to *Colophon* through overexploitation by commercial collectors (Gess & Gess, 1993; Melisch & Schütz, 2000), habitat loss and decline in habitat quality also pose a risk to their survival. Restricted to the high mountain peaks of the Western Cape Floristic Region (CFR), most of the known *Colophon* localities fall within a protected area of sorts (personal observation). Roughly 20% of the CFR is protected by some form of conservation area or reserve (Rouget *et al.*, 2003) although in a recent review regarding the state of biodiversity in the Western Cape, it was found that only 8% of the land area is being secured for conservation, with the remaining areas falling on privately owned land (le Roux *et al.*, 2007). Conservation requirements of invertebrate fauna is not always met within these reserves (Clark & Samways, 1996; Van Rensburg *et al.*, 1999) which is evident in the construction of telecommunication towers on several of the mountain peaks within the protected areas. The construction of these towers result in the damage of part of the specialised habitat occupied by *Colophon* as well as several other montane species.

Colophon habitat is also at risk due to global warming. With various projected global warming scenarios (Lutjeharms *et al.*, 2001; Meadows, 2006), and the imminent change in climate, despite uncertainties involving the details and rate, it is fairly certain that changing temperature and rainfall regimes will have an influence on the welfare and distribution of many insect species (Erasmus *et al.*, 2002; Thomas *et al.*, 2004; New, 2012). High altitudinal insects, such as *Colophon* are possibly most at risk due to climate change, as movement of species' distributions towards higher elevations progressively isolates populations and makes the eventual escape to other mountain peaks more unlikely (New 2012). *Colophon* species' current distribution on tiny fragments of habitat on the highest mountain peaks which accommodate low ambient temperatures, pose a threat to their extinction should ambient temperatures increase any further.

In conclusion, as research funding is limited and with the management of threatened and protected species increasingly difficult, it is becoming more and more critical to prioritise conservation efforts (Field *et al.*, 2005). With little modification or additional effort to the already existing conservation programme, *Colophon* is able to confer protection to a large number of naturally co-occurring species (Roberge & Angelstam, 2004) for which threat assessments are too costly or unachievable (Pearson, 1994, New, 2012)

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Tables

Table 1. Current IUCN Red List status for Colophon (<http://www.iucnredlist.org> >2013)

Species	IUCN Red List Status
<i>Colophon berrisfordi</i>	Critically Endangered B1+2e
<i>Colophon cassoni</i>	Critically Endangered B1+2e
<i>Colophon montisatrix</i>	Critically Endangered B1+2e
<i>Colophon primosi</i>	Critically Endangered B1+2e
<i>Colophon barnardi</i>	Endangered B1+2e
<i>Colophon eastmani</i>	Endangered B1+2e
<i>Colophon haughtoni</i>	Endangered B1+2e
<i>Colophon thunbergi</i>	Endangered B1+2e
<i>Colophon whitei</i>	Endangered B1+2e
<i>Colophon cameroni</i>	Vulnerable B1+2e
<i>Colophon neli</i>	Vulnerable B1+2e
<i>Colophon stokoei</i>	Vulnerable B1+2e
<i>Colophon westwoodi</i>	Vulnerable B1+2e
<i>Colophon izardi</i>	Lower Risk/near threatened

Table 2. Criteria for listing species as threatened or protected in terms of NEMBA (No. 10 Section 56 of 2004). IUCN 2001 criteria available from www.redlist.org >2013.

CATEGORY	CRITERIA
Critically	All South African species
Endangered (CR)	<p>Evaluated as Extinct, Extinct in the Wild or Critically Endangered according to the IUCN 2001 Red List system (national assessment) and threatened by direct use or</p> <p>Evaluated as Critically Endangered due to an extremely limited geographic range / area of occupancy (IUCN criterion B), an extremely small and declining population (IUCN criterion C) or a very small or restricted population (IUCN criterion D)</p>
Endangered (EN)	<p>All South African species</p> <p>Evaluated as Endangered according to the IUCN 2001 Red List system (national assessment) and threatened by direct use</p>
Vulnerable (VU)	<p>All South African species</p> <p>Evaluated as Vulnerable according to the IUCN 2001 Red List system (national assessment) and threatened by direct use</p>
Protected (PR)	<p>All South African species</p> <p>Evaluated as Near Threatened according to the IUCN 2001 Red List system and threatened by direct use or</p> <p>In need of regulation/management as current utilisation may result in a significant decline in wild populations of the species</p>

Table 3. Summary of criteria B (IUCN Ver. 3.1) used in the *Colophon* assessment (comprehensive list of criteria available from <http://www.iucnredlist.org/technical-documents/categories-and-criteria/2001-categories-criteria> >2013).

Criteria	Critically Endangered (CR)	Endangered (EN)	Vulnerable (VU)
B. Geographic range			
B1. Area of occurrence	<100km ²	<5 000km ²	<20 000km ²
B2. Area of occupancy	<10km ²	<500km ²	<2 000km ²
And any 2 of the following 3:			
(a) Severely fragmented or number of locations	=1	≤5	≤10
(b) Continuing decline in any of: (i) extent of occurrence; (ii) area of occupancy; (iii) area, extent and/or quality of habitat; (iv) number of mature individuals			
(c) Extreme fluctuations in any of: (i) extent of occurrence; (ii) area of occupancy; (iii) number of locations or subpopulations; (iv) number of mature individuals			

Chapter V:

General Conclusion

This section serves as a summary of the key findings of each of the preceding chapters, which address the three main aims set out at the start of the project on the systematics and conservation of the Cape High-mountain stag beetle genus *Colophon*.

Phylogeny, historical biogeography and divergence time estimates

This was the first molecular, and most comprehensive, phylogenetic study conducted on the relationships within the genus *Colophon*. One of the aims was to determine the specific and phylogenetic status of the 17 described species of the genus. This was achieved by analysing DNA sequences from three gene regions. The molecular phylogenetic study is complementary to the morphological phylogeny hypothesised by Endrödy-Younga in 1988. He examined 14 of the 17 currently recognised species of *Colophon* and, based on general morphological structures, suggested that the genus is monophyletic and that the species separated into two distinct evolutionary lineages. The current study supported his findings.

Most species showed an allopatric distribution pattern, with genetic lineages confined to specific mountain ranges. However, contact zones between geographically adjacent species are likely, as is the case between two of the species, *C. kawaii* and *C. haughtoni*. Eleven of the 12 studied *Colophon* species are supported in the molecular phylogeny. Addition of the five outstanding species and analysing a slower evolving gene-region will likely improve the resolution of the deeper phylogenetic nodes. We also suggest that a taxonomic revision be done on the *C. stokoei* varieties as well as the *C. eastmani* subspecies, due to their high degree of genetic divergence, distinct distribution on separate mountain peaks and the observed differences in their morphology.

Another major aim of the project was to estimate the timing of key biogeographical events in the diversification of the genus *Colophon* and thereby determining the main factors driving their evolution, and to test the theory of a lowland origin for the genus as proposed by Endrödy-Younga (1988). It was hypothesised that *Colophon* is an ancient flightless genus, with its origin in the late Cretaceous, a period when southern Africa was characterised by a warm, humid climate supporting extensive tropical and sub-tropical forests (Dingle *et al.*, 1983; Deacon *et al.*, 1992; Partridge & Maud, 2000) and the more typical lucanid habitat. The split between the two lineages occurred during the early Paleocene and mid-Eocene respectively. The reason behind the split is unclear, but could be as a consequence of the major events that triggered the end of the Cretaceous period, better known as the K/T boundary.

Climate seems to be the main driving factor behind *Colophon* evolution and the lowland origin of the genus appears to be supported. Results of the current study suggest that the genus was forced up into the more hospitable mountains with the gradual increase in aridity associated with the renewed glaciation of the Antarctic and the establishment of the cold Benguela upwelling system around the end of the Miocene (Tankard & Rogers, 1978; Endrödy-Younga, 1988; Partridge & Maude, 2000; Midgley *et al.*, 2005; Cowling *et al.*, 2009; Dupont *et al.*, 2011). Environmental fluctuations during the Plio-Pleistocene may also have fragmented a once widespread *Colophon* distribution, with a substantial amount of range contraction and expansion occurring during that time, leading to their current localised distribution on the mountain peaks.

Colophon larvae: descriptions and phylogenetic implications

The purpose of the larval study was fourfold: to enable researchers to identify the species found in the field; to use larvae as an alternative for adults in molecular studies; to comment

on possible phylogenetic information that may contribute to the sub-familial placement of the genus; and to obtain habitat preference data. To achieve this, several *Colophon* larvae were examined. They live in a fairly homogeneous micro-habitat of moist, humus-rich soil in protected places such as under rocky overhangs and amongst the roots and tussocks of Restionaceae. This soil-living and humus-feeding habit of *Colophon* is unique in the family. *Colophon* larvae show small inter-specific differences; however, larval characters contributed little equivocal information from which phylogenetic support could be deduced. Clearly, as with many scarabaeoid groups, larval morphology has not diversified much from the basal ground-plan and it remains for other (adult) phylogenetically significant morphological characters or DNA to provide more clarity on *Colophon*'s subfamilial placement. A study on the topic by collaborators in the USA is currently underway.

Conserving South Africa's Mountain Peak dweller

A combination of all the information obtained during this study with observations made in the field and the published literature, contributed to developing recommendations for the revised conservation status of *Colophon* under ToPS (Threatened or Protected Species) and IUCN (International Union for Conservation and Nature) criteria. The main threats to *Colophon* survival include overexploitation by commercial collectors (Gess & Gess 1993; Melisch & Schütz 2000), decline in habitat quality and habitat loss due to the projected change in climate. Thus, it is suggested that out of the 17 currently described species, eight are listed as Critically Endangered, seven as Endangered and one as Data Deficient. *Colophon stokoei* was listed as Not Threatened due to its wide distribution range. However, *C. stokoei* is in need of taxonomic revision which will probably result in all varieties being described as full species with each having a threat status of Critically Endangered or Endangered.

Future considerations

In light of this project's findings, it is suggested that future considerations in terms of *Colophon* research should focus on obtaining more information on their biology, behaviour and population size and in so doing contribute knowledge for the effective conservation management of each species. A taxonomic revision of the species, focusing on the *C. stokoei* varieties and *C. eastmani* subspecies, should be done and a complete taxonomic key of all described species compiled. Future fieldwork should focus on sampling the five species that remained elusive during the project, to eventually be included in phylogenetic analyses.

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Appendix I:

Table S1: Localities for *Colophon* species with associated GenBank Accession numbers for the three gene regions analysed in the molecular phylogeny.

Species collected	Location	Adult	Larvae	GenBank Accession Number		
				COI	16S	CAD
<i>Colophon cameroni</i>	Waaiohoek		x	KC763199	KC763249	KC763292
<i>Colophon cameroni</i>	Waaiohoek		x	KC763200	KC763250	KC763293
<i>Colophon cameroni</i>	Waaiohoek		x	KC763202	KC763251	KC763294
<i>Colophon cameroni</i>	Waaiohoek		x	KC763201	-	KC763327
<i>Colophon cameroni</i>	Waaiohoek		x	KC763203	KC763252	KC763328
<i>Colophon eastmani eastmani</i>	Ben Heatlie	x		KC763212	KC763279	KC763297
<i>Colophon eastmani eastmani</i>	Ben Heatlie		x	KC763213	KC763280	KC763321
<i>Colophon eastmani nagaii</i>	Dassieshoek	x		KC763214	KC763278	-
<i>Colophon eastmani nagaii</i>	Dassieshoek	x		KC763216	KC763281	KC763323
<i>Colophon eastmani nagaii</i>	Dassieshoek	x		KC763217	KC763282	KC763324
<i>Colophon eastmani nagaii</i>	Dassieshoek	x		KC763215	KC763283	KC763325
<i>Colophon eastmani nagaii</i>	Dassieshoek	x		KC763218	KC763284	KC763326
<i>Colophon endrodyi</i>	Ossenbergr	x		KC763232	KC763288	KC763318
<i>Colophon haughtoni</i>	Matroosbergr	x		KC763207	KC763256	KC763296
<i>Colophon haughtoni</i>	Matroosbergr	x		KC763210	-	KC763320
<i>Colophon haughtoni</i>	Matroosbergr	x		KC763206	KC763257	-
<i>Colophon haughtoni</i>	Matroosbergr		x	KC763208	KC763258	KC763301
<i>Colophon haughtoni</i>	Matroosbergr		x	KC763211	-	KC763295
<i>Colophon haughtoni</i>	Matroosbergr	x		KC763209	KC763259	-
<i>Colophon izardi</i>	Grootbergr	x		-	KC763260	KC763330
<i>Colophon izardi</i>	Grootbergr	x		KC763224	KC763261	KC763300
<i>Colophon izardi</i>	Grootbergr	x		KC763225	KC763262	-
<i>Colophon izardi</i>	Riversdale	x		KC763226	KC763263	KC763322
<i>Colophon kawaii</i>	Matroosbergr	x		KC763205	KC763269	KC763305
<i>Colophon kawaii</i>	Matroosbergr	x		KC763204	KC763270	KC763306
<i>Colophon montisatris</i>	Blesbergr	x		KC763233	KC763271	KC763307
<i>Colophon montisatris</i>	Blesbergr	x		KC763234	KC763272	KC763312
<i>Colophon montisatris</i>	Blesbergr	x		KC763235	KC763273	KC763313
<i>Colophon montisatris</i>	Blesbergr	x		KC763236	KC763274	KC763314
<i>Colophon neli</i>	Swartbergr	x		KC763229	KC763266	-
<i>Colophon neli</i>	Swartbergr	x		KC763230	KC763267	-
<i>Colophon neli</i>	Swartbergr	x		KC763231	KC763268	KC763311
<i>Colophon neli</i> (female)	Swartbergr	x		KC763227	KC763264	KC763302
<i>Colophon neli</i> (female)	Swartbergr	x		KC763228	KC763265	KC763303
<i>Colophon primosi</i>	Seweweekspoort	x		KC763222	KC763285	KC763316
<i>Colophon primosi</i>	Seweweekspoort	x		-	KC763286	KC763331

<i>Colophon primosi</i>	Seweweekspoort	x		KC763223	KC763287	KC763317
<i>Colophon stokoei forma typica</i>	Sneeukop	x		KC763191	KC763240	-
<i>Colophon stokoei forma typica</i>	Sneeukop	x		KC763192	KC763241	KC763289
<i>Colophon stokoei forma typica</i>	Sneeukop	x		KC763193	KC763242	KC763290
<i>Colophon stokoei forma typica</i>	Sneeukop	x		KC763194	KC763243	KC763291
<i>Colophon stokoei</i> Var. A	Platberg	x		KC763197	KC763246	-
<i>Colophon stokoei</i> Var. A	Platberg	x		-	KC763247	KC763329
<i>Colophon stokoei</i> Var. A	Platberg	x		KC763198	KC763248	KC763315
<i>Colophon stokoei</i> Var. C	Perdekop	x		KC763195	KC763244	KC763298
<i>Colophon stokoei</i> Var. C	Perdekop	x		KC763196	KC763245	KC763299
<i>Colophon westwoodi</i>	Table Mountain	x		KC763220	KC763253	KC763304
<i>Colophon westwoodi</i>	Table Mountain	x		KC763219	KC763254	-
<i>Colophon westwoodi</i>	Table Mountain	x		KC763221	KC763255	KC763319
<i>Colophon whitei</i>	Bloupunt	x		KC763237	KC763275	KC763308
<i>Colophon whitei</i>	Bloupunt	x		KC763238	KC763276	KC763309
<i>Colophon whitei</i>	Bloupunt	x		KC763239	KC763277	KC763310

Table S2: Percentage pairwise genetic distances between various *Colophon* species for 16S and CAD. Species indicated with * correspond to Fig. 1 and are further explained in the text.

Species	16S (%)	CAD (%)
<i>C. stokoei</i> Var. A ; <i>C. stokoei</i> Var. C	2.6	0.6
<i>C. stokoei</i> Var. A ; <i>C. stokoei</i> forma typica	4.6	1.4
<i>C. stokoei</i> Var. C ; <i>C. stokoei</i> forma typica	2.6	1.1
<i>C. kawaii</i> * ; <i>C. cameroni</i>	4.1	0.9
<i>C. kawaii</i> ; <i>C. haughtoni</i>	0.3	0.8
<i>C. eastmani eastmani</i> ; <i>C. eastmani nagaii</i>	3.8	0.9
<i>C. neli</i> ; <i>C. neli</i> *	8.1	0.5
<i>C. izardi</i> ; <i>C. izardi</i> *	2.5	0.5