Molecular Diagnostics and Phylogenetics of White Grubs in Sugarcane

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PREFACE

This dissertation is the result of the author's original work except where acknowledged or specifically stated to the contrary in the text. It has not been submitted for any degree or examination at any other university or academic institution.

Signed …… Date: 20/06/2008

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Dr. D. E. Conlong (Supervisor)

DEDICATION

To Karl with love.

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ABSTRACT

Scarabaeid pests in South Africa and especially KwaZulu-Natal are characterised by a very long larval life cycle and short pupal and adult periods. However, it has nearly always been the adults of the species that have been identified, with very little attention paid to the larval identification of the species. This is unfortunate as it is nearly always the larval stage that is found to be associated with crop damage. Accurate identification of the species of these larvae is important for the management of scarabaeid pest species, as it unlocks the necessary information on the biology and ecology of many species, which allows the adaptation of control methods for different species. Inadequate keys for the taxonomy of larvae of these groups, as well as the lack of morphological taxonomists working on these groups have been identified as constraints.

When a species is difficult to identify using traditional taxonomic methods, DNA diagnostic tools can be useful. Chapter 2 investigated the feasibility of identifying scarabaeid larvae using mitochondrial DNA data. Variation in the base pair sequence of the mitochondrial cytochrome c oxidase sub unit I (*cox 1*) gene was used. DNA sequences of *cox 1* from scarabaeid larvae collected from sugarcane fields were compared with sequences from scarabaeid adults of known species in order to identify the species attacking sugarcane. Neighbour-joining and maximum parsimony analyses of 658 bp *cox 1* sequences identified groups of larvae that linked to adult specimens. The major groupings delimited specimens belonging to the subfamilies Dynastinae, Melolonthinae and Rutelinae. Within-group sequence divergence ranged from 0 - 3.4 % and divergence between sister groups ranged from 2.6 - 25.1 %. The recorded divergence range within and between tribes was 0 - 21.3 % and 17.3 - 28.5% respectively. Similarly, the divergence range observed within and between genera was 0 - 19.2 % and 17.1 - 25.4% respectively. The maximum sequence divergence observed within subfamilies was 23.7 % and divergence between subfamilies ranged from 16.8 - 26.7 %. Examination of pairwise sequence divergence levels as well as node support allowed 68% of the unidentified larval specimens to be associated with identified adult specimens.

Phylogenetic analysis matched identified adult mtDNA with unidentified larval mtDNA. This allowed the identification of those larvae through morphological characteristics unique to certain species. To create a field key to the subfamilies of Dynastinae,

Melolonthinae and Rutelinae the most useful character distinguishing larvae of different species was the raster but additional morphological characteristics were included.

These relationships between larval and adult scarabaeid specimens from sugarcane were examined using various phylogenetic tools. The data set included a total of 19 morphological characters as well as 166 partial *cox 1* gene sequences. Maximum parsimony analyses were performed on morphological, molecular and combined data. The same morphological and molecular data sets were run both separately and as a combined analysis with MrBayes. In both types of analyses the morphological data performed poorly and crude groupings resulted, dividing taxa to tribe level only. Molecular data showed greater resolution than the morphological data and taxa were separated into groups equivalent to species and morphospecies designated in Chapter 2. A partition homogeneity test indicated that both data types could be combined. It is recommended that both morphological and molecular data be utilised in identification of scarabaeid sugarcane pests and that a character-based approach be implemented. Further molecular data from other genes should be included to test the accuracy of these results.

The keys produced during this study will allow workers to focus on a single species biology, and subsequently allow an analysis of between species interactions, and within species control. These advances are a start to the improvement of knowledge of the species composition of scarabaeid larvae in sugarcane fields, thus making management and biological control of these pests a greater possibility. Further recommendations for future work are discussed in Chapter 5.

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CHAPTER 1

GENERAL INTRODUCTION

Production of sugarcane in South Africa has been hampered by a number of environmental (Singels *et al*., 2005; Koonjah *et al*., 2006) and biotic factors (McFarlane *et al*., 2006). The several insect pests that have been reported from sugarcane in the industry are amongst the production constraints (McArthur & Leslie, 2004; Goebel *et al*., 2005). These sugarcane insect pests can be placed into three ecological categories (i) soil insects (ii) sap suckers and leaf feeders, and (iii) stalk borers (Carnegie & Conlong, 1994).

Of the soil pests associated with sugarcane in Africa, scarabaeids (Coleoptera: Scarabaeidae) are the dominant group both in terms of damage levels and distribution (Carnegie & Conlong, 1994). Scarabaeid larvae infested sugarcane becomes stunted, lodges easily, yields poorly and produces a poor ratoon crop (Sosa, 1984). Sugarcane severely affected by scarabaeid larval infestation is unprofitable to harvest and unwanted by the mills because of its low tonnage and low juice quality, which makes sucrose recovery difficult (Sosa, 1984).

The Scarabaeidae is a cosmopolitan family of polyphagous coleopterans, which include more than 12 000 species (Gordh & Headrick, 2000). Worldwide, scarabaeids are pests of crops such as maize (Du Toit, 1996), turf grass (Yuen-Shaung *et al*., 1983; Potter *et al*., 1996), ground nuts (Xiaoping, 1993), pineapples (Petty, 1977; Smith *et al*., 1995), grape vines (Donaldson, 1981), forestry (Hepburn, 1966), pastures (Allsopp *et al*., 1992; Rath & Worledge, 1995) and sugarcane (McArthur & Leslie, 2004).

Of the 18 subfamilies of sugarcane scarabaeids recorded from southern and eastern Africa, only Melolonthinae, Rutelinae, Dynastinae and Cetoniinae are reported as economically important pests (Scholtz & Holm, 1996). The genera include *Cochliotus* in Tanzania (Evans *et al*., 1999); *Heteronychus* Dejean 1833 (Coleoptera: Scarabaeidae), *Anomala* Samouelle 1819 (Coleoptera: Scarabaeidae) and *Adoretus* Fåhraeus 1857 (Coleoptera: Scarabaeidae) in Swaziland (Sweeney, 1967); *Schizonycha* Boheman 1857 (Coleoptera: Scarabaeidae), *Heteronychus, Asthenopholis* Brenske 1898 (Coleoptera: Scarabaeidae), *Anomala* and *Adoretus* in South Africa (Way, 1997) and *Idaecamenta* Péringuey 1904

(Coleoptera: Scarabaeoidae) in Uganda (Conlong & Mugalula, 2003; Mugalula *et al.* 2006).

Since the mid-1980's, sugarcane damage by scarabaeid larvae has become more prevalent in certain areas of the South African sugar industry. This was probably due to the fact that in the KwaZulu-Natal midlands, sugarcane was planted on land where initially black wattle, *Acacia mearnsii* de Wild, was grown (Carnegie, 1974). This has led to adults, which are insect pests of black wattle, becoming damaging to sugarcane (Carnegie, 1974). This has initiated investigations into their pest status, economic impact and possible control measures (Mansfield, 2004). Many species of indigenous Scarabaeidae are recorded from sugarcane fields in South Africa, and several have been associated with significant economic losses (Leslie, 1997; Way, 1997).

This poses a problem for the local sugar industry. The South African sugar industry (see Figure 1.1) is one of the world's leading cost-competitive producers of high quality sugar (SASA, 2005). It is a diverse industry combining sugarcane cultivation with the production of raw and refined sugar, syrups, specialized sugars and a range of by-products. It produces an average of 2,5 million tons of sugar per season (SASA, 2005). This industry makes an important contribution to the national economy, given its agricultural and industrial investments, foreign exchange earnings, its high employment numbers and its linkages with key suppliers, support industries and customers (SASA, 2005).

Most of these scarabaeids, with the exception of *Heteronychus licas* Klug 1835 (Coleoptera: Scarabaeidae) (who have adults that feed on the shoots of sugarcane), feed only as larvae on the sugarcane roots (Wilson, 1969). Investigatory work has been limited, and has focused on those species which have occurred in sufficient numbers to cause alarm, or which have been identified as important crop spoilers (Leslie, 1997; Way, 1997).

In 1995 the extent and intensity of scarabaeid larvae in the South African sugarcane industry was assessed (Way, 1995). The survey showed that scarabaeid larvae were present in a wide geographical area in sugarcane encompassing many different conditions, e.g. different soil types, altitudes and rainfall patterns (Way, 1995). These varied conditions probably accounted for the variation in numbers of scarabaeid larvae recovered in the

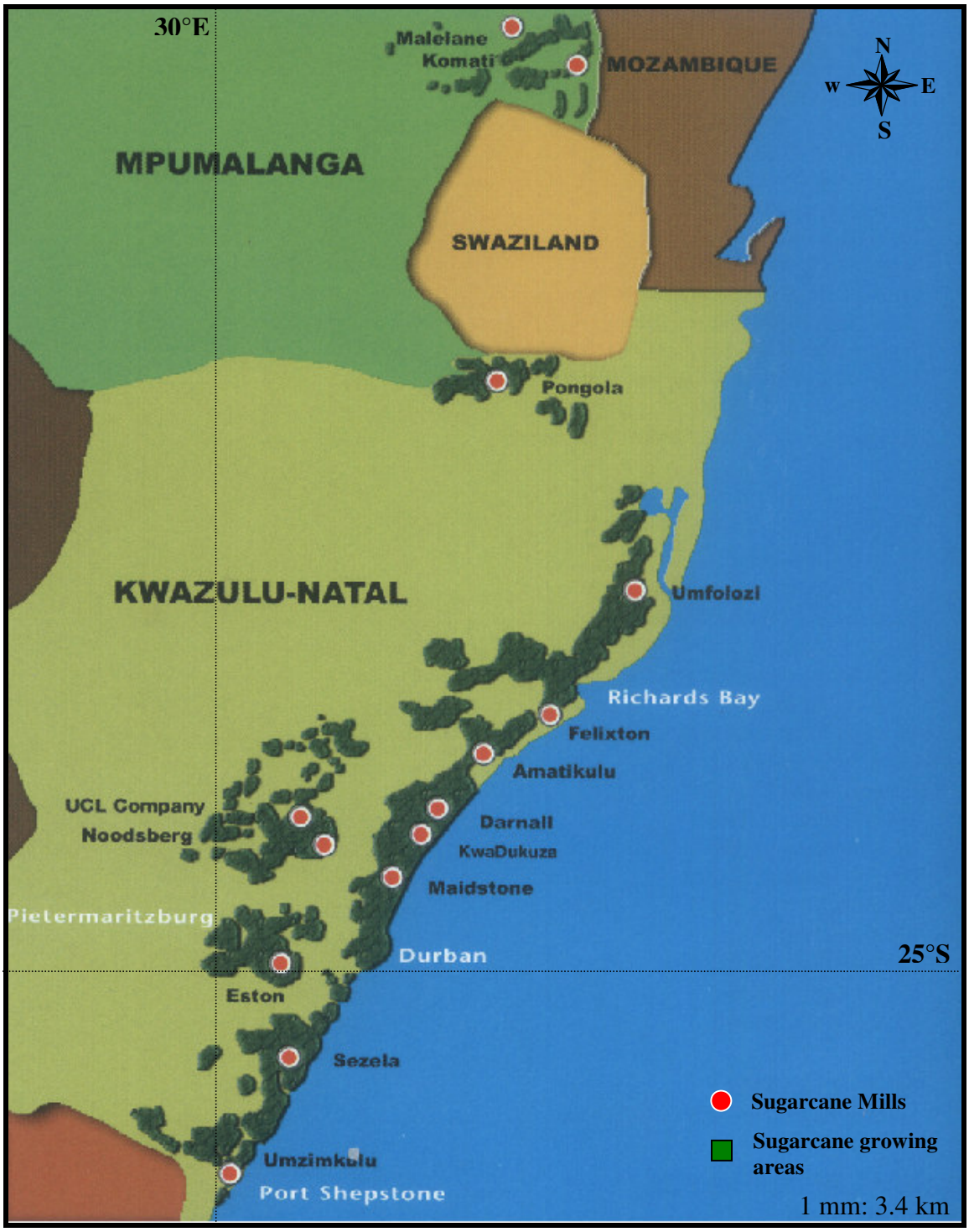

Figure1.1 Map of the South African sugar-producing regions. Mill names are indicated in lower case black fonts, city names indicated in lower case white fonts (Source: South African Sugar Industry Directory 2004/2005).

different regions (Way, 1995). *Schizonycha affinis* Boheman 1857(Coleoptera: Scarabaeidae) was the most numerous, and the most widespread scarabaeid larval species (Way, 1995). This species was particularly abundant in the KwaZulu-Natal Midlands, but present in all regions (Way, 1995). *Asthenopholis* and *Hypopholis* Erichson 1847 (Coleoptera: Scarabaeidae) were the next most abundant genera, but less widespread (Way, 1995). Fewer specimens of *H. licas*, *Heteronychus* Dejean 1833, *Adoretus fusculus* (Coleoptera: Scarabaeidae) Fåhraeus 1857 and *Anomala* were recovered (Way, 1995). *S. affinis* and *H. sommeri* are pests of sugarcane in the Midlands while *H. licas* and *Anomala* spp. are more common in the northern coastal regions of the industry (Way, 1995).

An industry-wide survey in 1996 showed how prevalent scarabaeids had become (Way, 1996). The survey showed that the eight species already recorded in sugarcane were present, in addition to 21 unknown taxa (Way, 1996). The patchy distribution of scarabaeid larvae makes them unpredictable in terms of monitoring, and capable of causing severe damage to a crop long before they are detected (Carnegie, 1988). In the case of other common species recorded so far, such as *H. sommeri*, *S. affinis*, *Anomala* spp., *Adoretus* spp., *Asthenopholis* spp. and *Temnorrhynchus* spp. (Coleoptera: Scarabaeidae), the larvae are known as crop pests (Carnegie, 1988).

In the KwaZulu-Natal midlands, much sugarcane has been grown on land that was formerly under black wattle, *Acacia mearnsii* de Wild, or wattle was grown adjacent to sugarcane (Carnegie, 1974). The larvae of the melolonthine beetles, *H. sommeri* and *S. affinis*, which are insect pests of wattle, have been associated with substantial losses to sugarcane (Hepburn, 1966; Carnegie, 1974). This association has been recorded in other insect pests of sugarcane. Pemberton and Williams (1969) related the association of insect pests with sugarcane, to adaptation of the insects to the plant consequent to its extensive cultivation. The African sugarcane borer, *Eldana saccharina* Walker 1864 (Lepidoptera: Pyralidae), and *Eoreuma loftini* Dyar 1917 (Lepidoptera: Crambidae) from Central and South America are amongst the pests that adapted to feed on sugarcane as a result of cultivation of the crop in areas previously occupied by its natural wild host plants (Conlong, 1994).

Adult scarabaeids are attracted to light and can easily be caught at a light source (Taylor, 1965). However, this method is non-selective for only sugarcane pests as the adults are

very mobile and can fly long distances. Therefore no direct links can be made between the adults caught and the larvae present. In addition, there may be more than one species affecting sugarcane in any one region (Way, 1995), further exacerbating the problem of identification, especially of the larvae.

There are thus limitations to using only morphological characteristics to identify larvae and adults, and it is difficult to link adults and larvae of the same species due to their large differences in morphology. Scarabaeids undergo total metamorphosis and thus the different life stages have no similarity (Scholtz & Holm, 1996). Adult scarabaeids can easily be identified using available morphological keys but these adult scarabaeids are only present for collection for a few months a year. Scarabaeid larvae on the other hand are available for a lengthier time period but require a large amount of labour to be collected as pits need to be dug in sugarcane fields to obtain specimens. Larval scarabaeids are problematic to identify and the lack of successful rearing methods for larva to adult (which can be identified) has prompted investigation into alternate methods of identification. It will become apparent in the following sections that alternate methods are needed to assist in identification. These will also be discussed. Such an alternative is DNA barcoding (discussed in section 1.4.1), which can be employed to identify scarabaeid larvae.

1.1 CLASSIFICATION OF SCARABAEOIDEA

The hierarchical level of families and subfamilies within the coleopteran super family Scarabaeoidea is in disarray and remains unresolved. In most American literature prior to the 1970's, such as Arnett (1968), the Scarabaeoidea included three families: Passalidae, Lucanidae, and Scarabaeidae (Ratcliffe & Jameson, 2004). This three family system of classification was the traditional North American system and has been the topic of much debate (Ratcliffe & Jameson, 2004). In some instances an author (e.g. Sanmartín & Martín-Piera, 2003) may treat the taxa Melolonthidae, Dynastidae and Rutelidae as families within the superfamily Scarabaeoidea. However, other authors (e.g. Cabrero-Sañudo & Zardoya, 2004; de Cássia de Moura *et al.*, 2003) treat the taxa as subfamilies Melolonthinae, Dynastinae and Rutelinae within the family Scarabaeidae. In this study the method of Lawrence and Newton (1995) was followed and the family Scarabaeidae was considered to include the subfamilies Aphodiinae, Scarabaeinae, Melolonthinae, Dynastinae, Rutelinae,

and Cetoniinae. Figure 1.2 indicates the hierarichal level of families and subfamilies within the Scarabaeoidea used in this study.

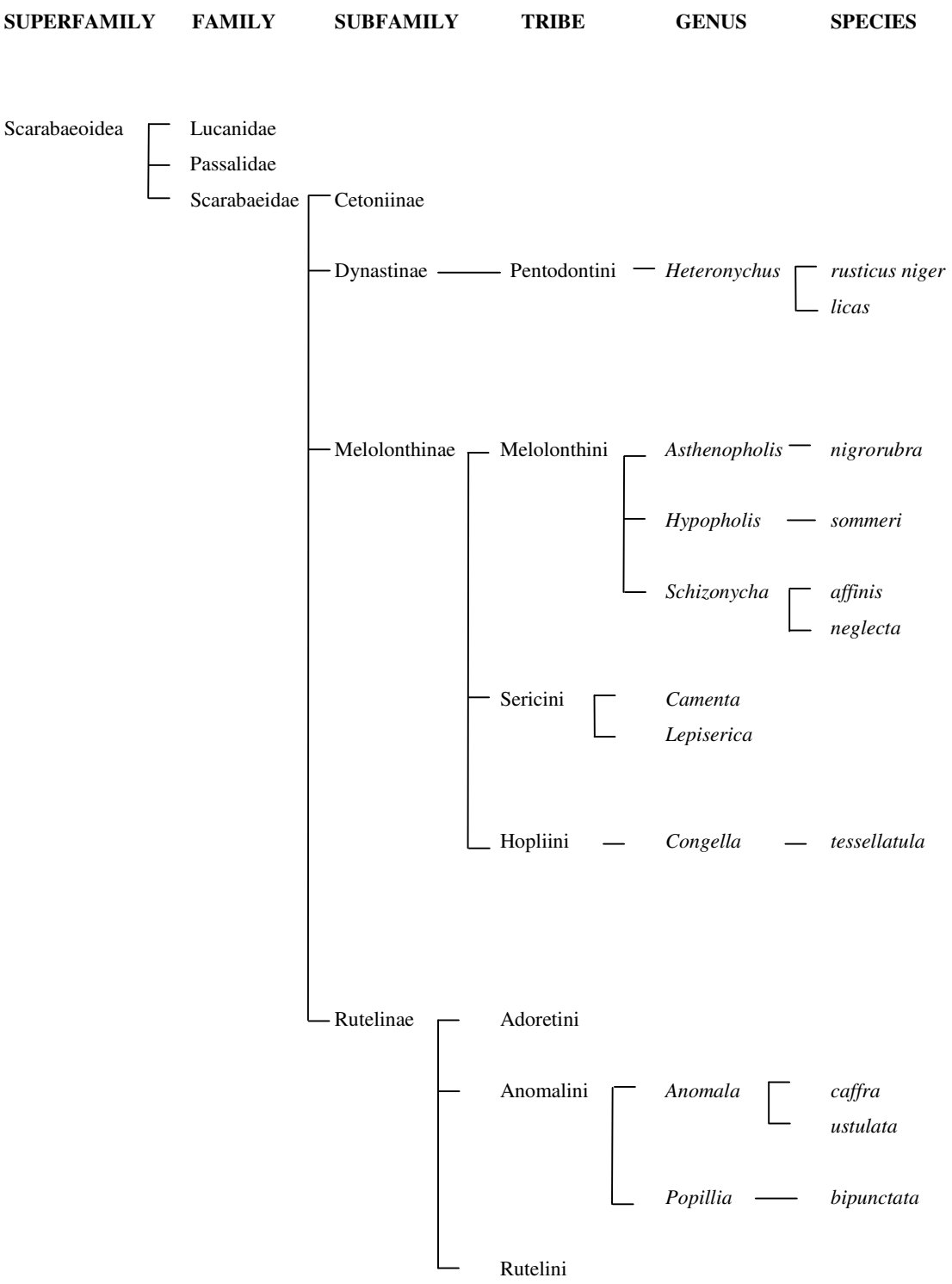

Figure 1.2 Classification and hierarchical level of families and subfamilies within the super family Scarabaeoidea used in this study (After Lawrence and Newton, 1995).

1.2 SCARABAEID BIOLOGY

Scarabaeidae are holometabolic insects with similar life cycles (Scholtz & Holm, 1996). The illustrated generalised scarabaeid life cycle is shown in Figure 1.3. Using *H. licas* occurring in southern Africa as an example throughout, mating and oviposition takes place from October to November (Taylor, 1965). The eggs are laid singly in moist soil among the roots and stools of the cane, mainly within 30 cm of the soil surface (Cackett, 1980). It is presumed that copulation usually takes place in the soil before swarming or the pre-reproductive flight of beetles, but may continue during and after the flight (Taylor, 1965). Three stages of larval growth are involved (Wilson, 1969). During each stage the thoracic and abdominal segments increase in size, but the head increases only when moulting occurs (Wilson, 1969). The first-instar first appear in late November and on hatching are pale white, with a light to dark brown head capsule, and about 6mm long (Taylor, 1965). They are active for 5-8 months during which they feed exclusively on organic detritus and humus in the soil (Cackett, 1980). The tip of the abdomen becomes a bluish-grey colour as a result of the ingested material (Taylor, 1965). The first-instar larvae moult after 14-19 days (Taylor, 1965).

Second-instar larvae feed on young root hairs to attain the final stage two to three weeks later and have all been found to damage the underground parts of the plant (Sweeney, 1967).

The third-instar is the most damaging life stage to sugarcane (Wilson, 1969). They feed on the roots and underground shoots for about six to seven months (Wilson, 1969). At the end of the third-instar larval stage, the full-grown larvae descend into the lower layers of the soil to make an earthen cell in which pupation occurs (Wilson, 1969). In the cell it evacuates its gut contents and becomes pale white again, with some wrinkling of the posterior abdominal segments. The inside of the cell is usually quite smooth, and it is presumed that the evacuated gut contents are used in plastering the inside surface (Taylor, 1965). The larva remains in the prepupal stage for about 5-6 days before pupating (Taylor, 1965).

Metamorphosis takes place in this cell and on pupation the last larval skin is pushed to one side of the pupal cell (Taylor, 1965). The pupa is at first pale yellow but turns reddish

brown towards the end of the pupal period, which ranges from 18-25 days (Taylor, 1965). Symes (1925) reported that adults after emergence remained in their pupal cases until the rains came. Adults begin to emerge from the cells early in March and reach their peak population in the soil between March and May (Taylor, 1965). During the period from April to June feeding is voracious (Taylor, 1965). This period is followed by a period of inactivity, as far as feeding is concerned, from July to early September, when the damage was negligible (Taylor, 1965). There is only one generation per year, and at the end of the 12-month cycle the adults, which have lived through the rainy season from March or April to November or December, die (Taylor, 1965). Thus their adult life-span is short and limits the time when they can be collected.

There are two flight seasons during a generation of which the first lasts from April to June and is a post-teneral flight of the new generation adults (Taylor, 1965). The second usually occurs in late October and November and is a pre-reproductive flight (Taylor, 1965). During both flights beetles are attracted to light. The practical effect of these flights is to enhance the chances of survival and reproduction of the beetles by adequate and timely dispersal (Taylor, 1965). The first flight, occurring after the mass emergence of adults, tends to disperse the beetles at a time when they might suffer from overcrowding in particular sites within the habitat, and affords chances of obtaining adequate and new sources of food (Taylor, 1965). The second flight ensures further dispersal at a time when the species is on the threshold of a substantial increase in numbers (Taylor, 1965). Numbers reach a peak in April or early May whilst the main flight season is in October (Taylor, 1965).

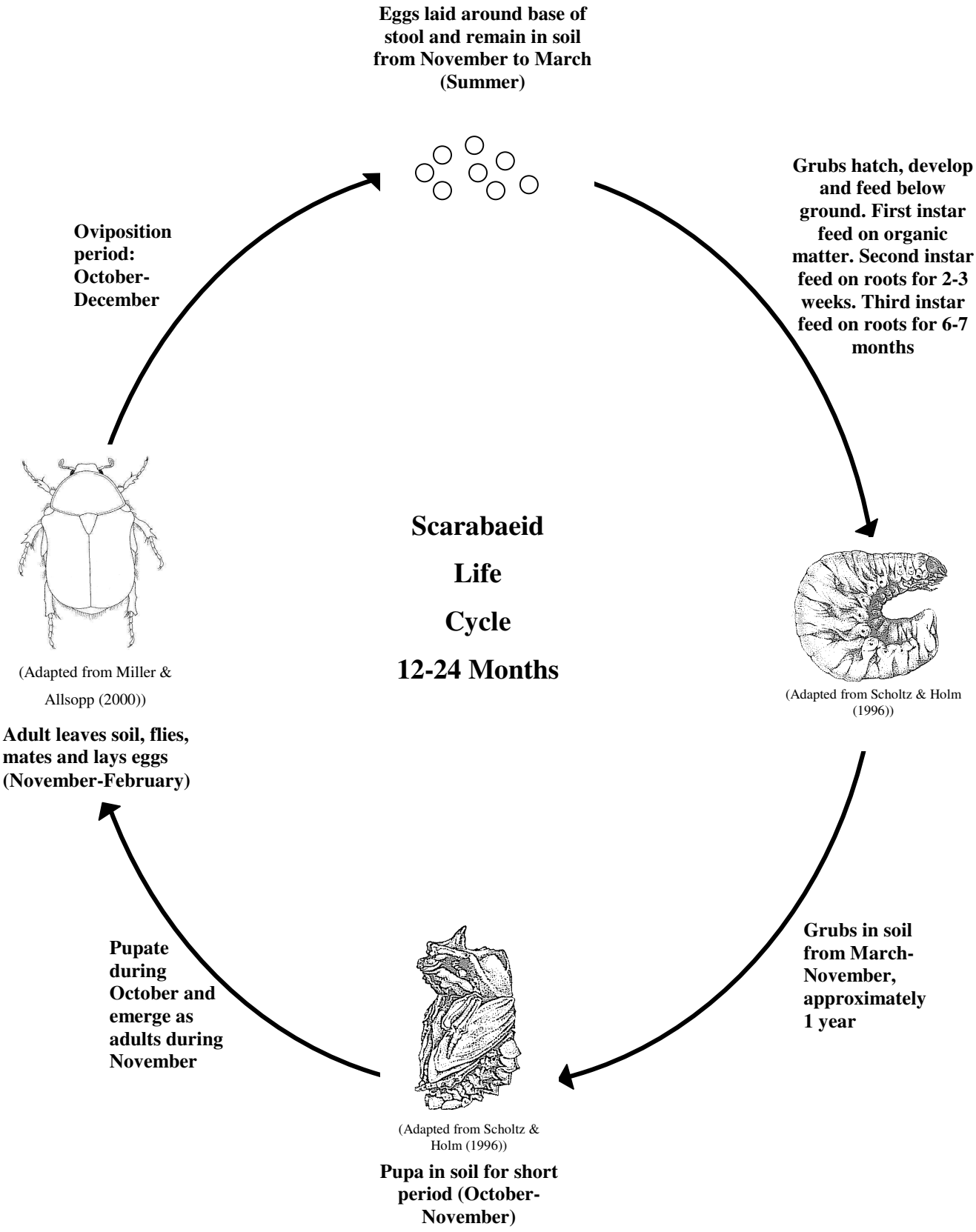

Figure 1.3 An illustrated generalised scarabaeid life cycle.

1.3 MORPHOLOGICAL IDENTIFICATION

In his work on the morphology, taxonomy and biology of larval Scarabaeidae, Hayes (1929) noted: "As with other groups of insects, our knowledge of the developmental stages lags behind our knowledge of the adults. This is especially true in the fields of morphology and taxonomy and to a lesser extent in the field of biology". The task of everyday species identification has four important limitations. Firstly, phenotypic plasticity and genetic variability present in characters used to recognise species could result in incorrect identifications. Secondly, the occurrence of morphologically cryptic taxa is unnoticed. Thirdly, many individuals cannot be identified as morphological keys are only available for certain life stages or genders. Lastly, some keys require a high level of expertise and therefore misidentifications occur frequently (Hebert *et al.,* 2003).

Scarabaeid adults have stout or compact bodies up to 60 mm long, often sombre coloured and heavily sclerotized (Figure 1.4) (Gordh & Headrick, 2000). The head is not deflexed; mouthparts visible ventrally; antenna with 7-10 segments, club lamellate with 3-7 segments; or tibia dentate; tarsal formula typically 5-5-5, rarely 0-5-5; apical sternum of abdomen exposed (Gordh & Headrick, 2000). Scarabaeid adults may be recognised by their antennae, each having 8 to 11 segments, the last 3 to 7 segments of which form a club i.e. they are greatly expanded, each dilated segment being thin and leaf-like, but when closed forming an oval-shaped ball or an elongate truncheon (Sweeney, 1967). All the tarsi have five segments (Sweeney, 1967). The front legs, especially, are often dentate keeled and flattened for digging, or in some spinose and elongated for holding on to flowers (Sweeney, 1967). Wing venation, mouthparts, genitalia (Cabrero-Sañudo & Zardoya, 2004), body length (measured from the apex of the clypeus to the apex of the elytra), body width (measured at the middle of the elytra) and setae density (dense, moderate or sparse) (Smith & Morón, 2003) are characteristics commonly used for morphological identification. Characters such as dorsal colour, clypeal apex (subcuticular, broadly rounded, rectangular), clypeal margin (reflexed, not reflexed), clypeus (glaborous, setose), frons and pronotum are also used (Smith & Morón, 2003).

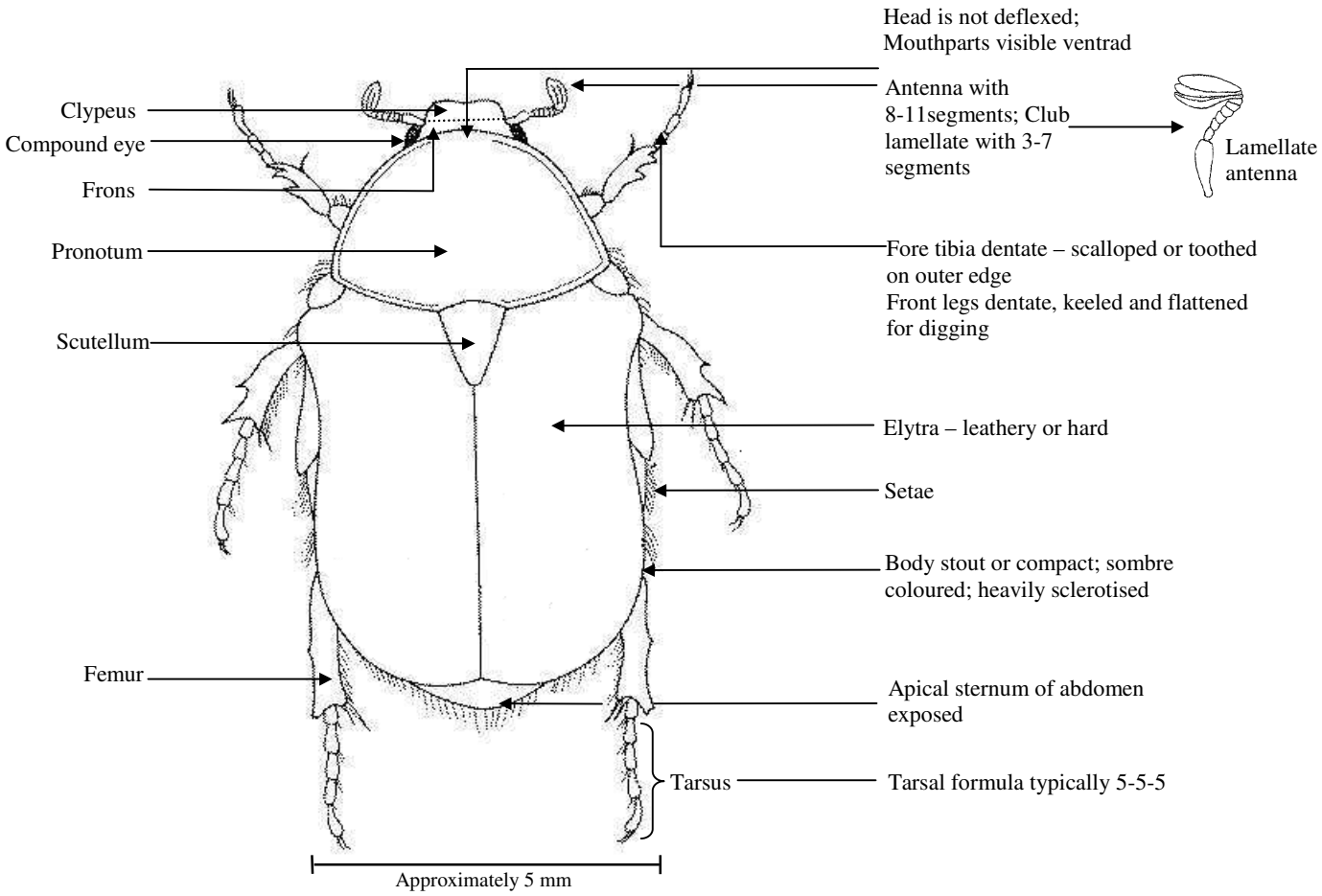

Figure 1.4 A diagram of a generalised scarabaeid adult.
(Adapted from Miller & Allsopp (2000))

Scarabaeid larvae (Figure 1.5) have been defined as larvae of Melolonthinae (Wilson, 1969) but the term has a wider usage, embracing larvae of subfamilies Rutelinae, Dynastinae, Cetoniinae and other scarabaeid subfamilies, all of which have a general similarity (Wilson, 1969). It is in this broader sense that the term scarabaeid larvae is used here. The larvae are broad and fleshy, whitish or greyish-white in colour and the body is curved in the form of the letter C; the legs are well developed but rarely used for locomotion; the head is large, downwardly inclined; strongly sclerotized, yellow, red or brown in colour, with powerful, exposed mandibles (Wilson, 1969). The head of the larva is hypognathous; stemmata are absent or one present on each side of the head; legs have five segments, two or more tarsal claws; urogomphi absent (Gordh & Headrick, 2000).

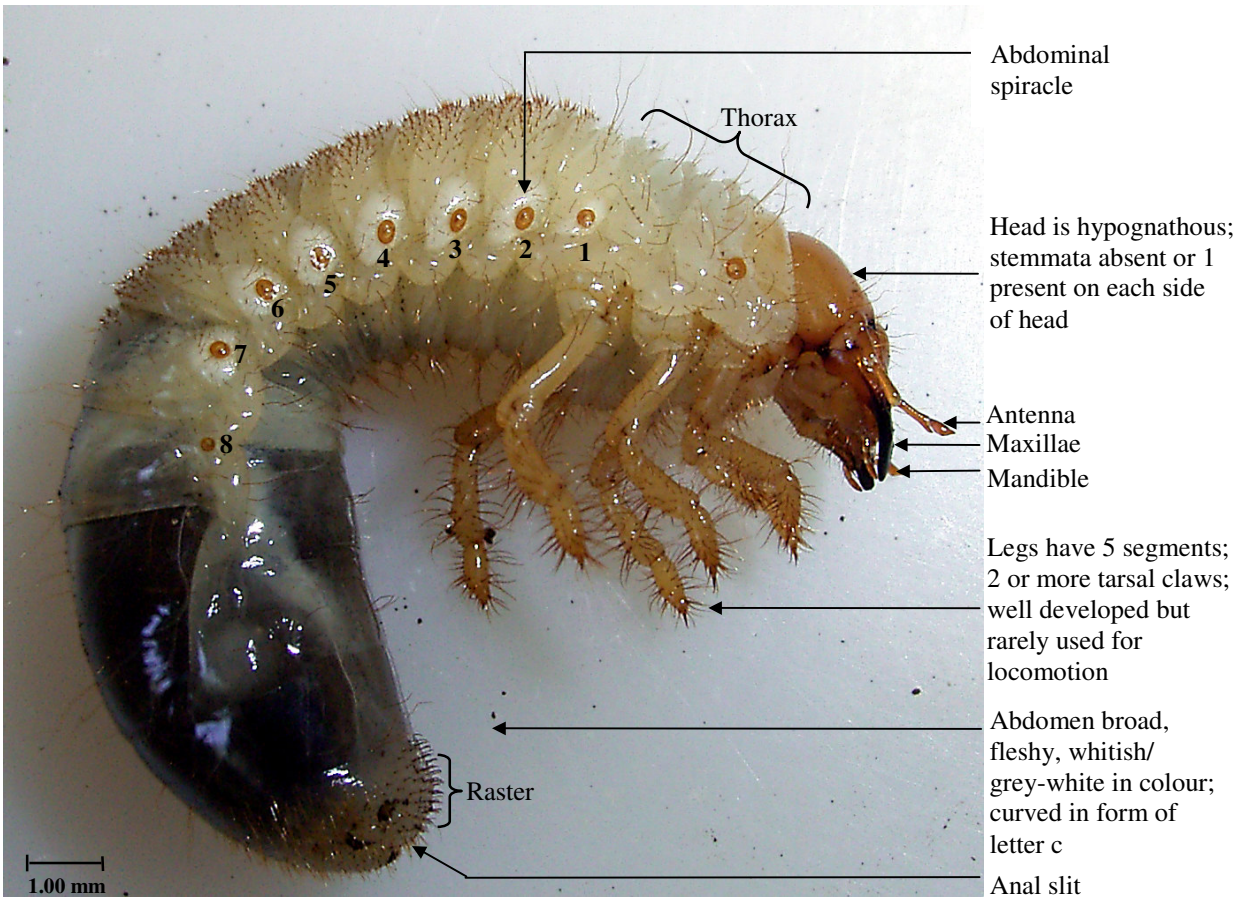

Figure 1.5 A photograph of a generalised scarabaeid larva.
(Photograph: Mike Way, 2004)

One of the most useful characters for distinguishing between larvae of different scarabaeid species is the raster, defined by Gordh & Headrick (2000) as "a complex of specifically arranged bare areas, setae and spines on the ventral surface of the last abdominal segment, anterior of the anus". The raster (Figure 1.6) is divided into the septula, palidium, teges and tegillum (Gordh & Headrick, 2000). Although many species have a unique larval raster pattern (Ritcher, 1943; Ritcher, 1945; Allsopp *et al*., 1993), the raster pattern of larvae of some species are difficult to distinguish. Additional characters of the head (Figure 1.7) such as the labrum, clypeus, ocelli, and width of cranium may also be used to confirm certain species diagnosis (Petty, 1996). Similarly, the number of notches on the left and right mandibles (Figure 1.8), the presence or absence of a stridulatory area on the ventral surface of the mandibles and the number and position of the heli (Figure 1.9) on the epipharynx are useful (Smith *et al.,* 1995). While the number and arrangement of setae on the head capsule may also be taxonomically significant, it is not always reliable for

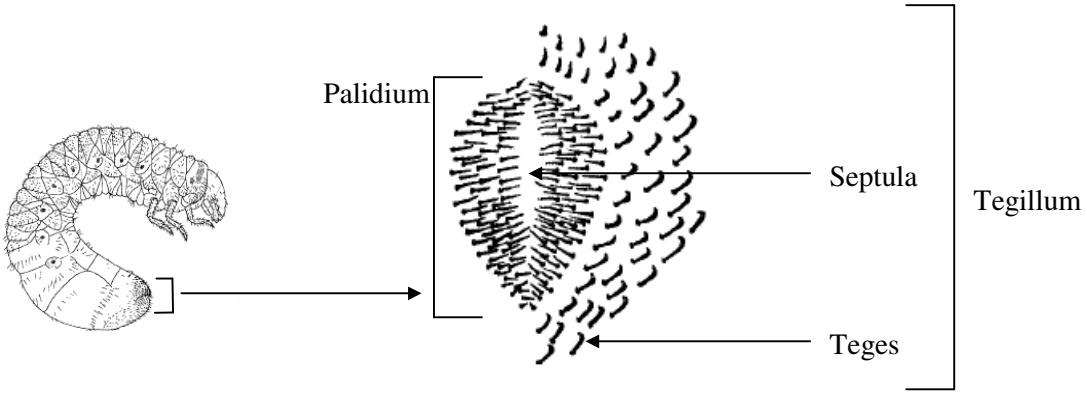

Figure 1.6 A diagram showing the components making up a generalised raster pattern of a scarabaeid larva. (Diagrams adapted from Miller & Allsopp (2000))

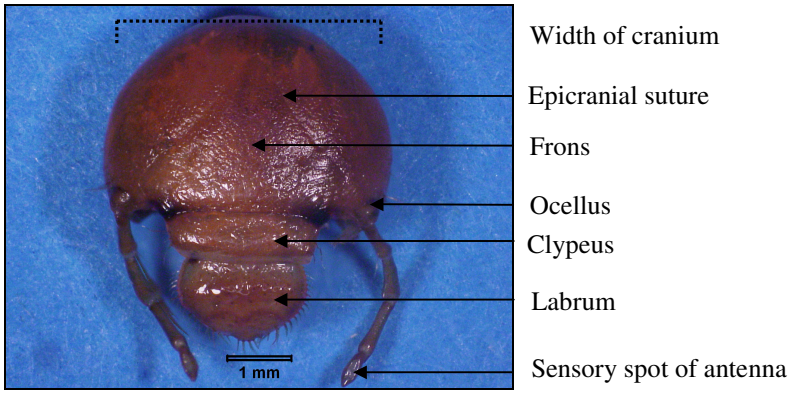

Figure 1.7 Generalised larval scarabaeid head indicating the characteristics that may be used for identification.

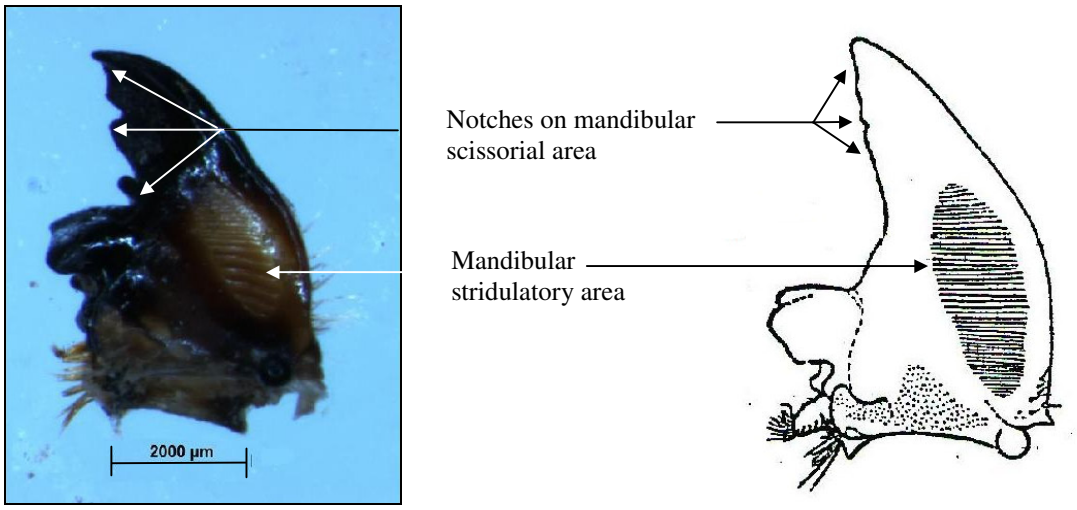

Figure 1.8 Generalised larval scarabaeid mandible indicating the characteristics used for identification. (Diagram adapted from Ritcher (1966))

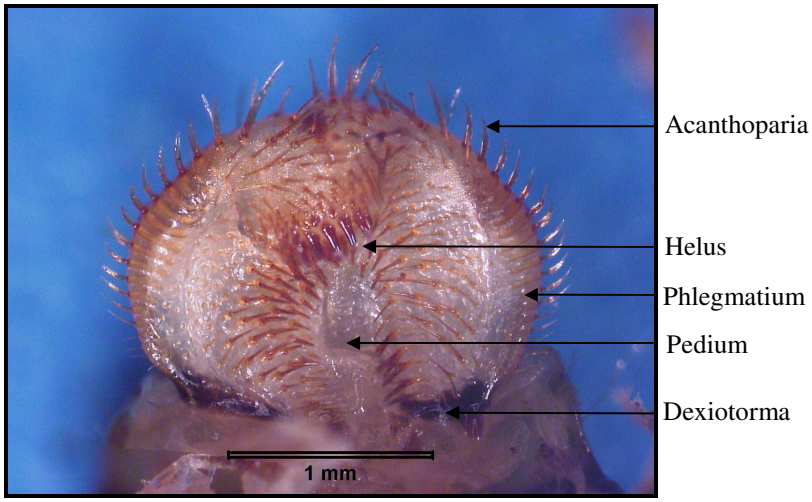

Figure 1.9 Generalised larval scarabaeid epipharynx indicating the characteristics used for identification.

identification because setae can break off as the larva moves through the soil (Scholtz & Peck, 1990).

As it is difficult to relate the more sedentary scarabaeid larvae in the soil in the field with the adults collected from the light traps (because adults are such active and mobile fliers), accurate identification of the scarabaeid larvae attacking sugarcane in the industry was not

possible. Accurate identification is important for the management of pest species, as it provides the necessary information to adapt control methods to the biologies of these species (Smith *et al*., 1995). Although the adults of white grubs are morphologically identifiable, little work has been done on the morphological identification of their larvae in South Africa (Oberholzer, 1959; Sweeney, 1967; Petty, 1976, 1977, 1978). While all scarab beetles have a general similarity, this applies much more so to their larvae. Larvae that look the same to the casual eye, and even to the expert eye, except perhaps for a size difference, may belong to quite different species that are readily distinguishable in the adult stage (Williams, 1985).

It is thus suggested that molecular techniques be used for identification of scarabaeid larvae (Miller & Allsopp, 2000), and to link identified adults with these larvae to determine their species identity.

1.4 MOLECULAR IDENTIFICATION

The use of DNA-based technologies has been recommended as the best option to "bridge" the gap between the shortage of taxonomists and the need for identification of the many unidentified species (Tautz *et al.,* 2003). Large molecular data sets are available resources that can be used to find characters useful for species diagnosis (Cognato, 2006).

Originally protein markers, especially allozymes, were the main marker type used. More recently, applications of these markers have been improved and expanded by using DNA-based techniques (Avise, 1994). Both DNA and protein markers have revolutionised biological sciences and have improved many fields of study (Loxdale & Lushai, 1998).

Entomologists now use DNA-based techniques as they offer a new level of resolution to study insect ecology and taxonomy (Loxdale & Lushai, 1998). There are many examples of where DNA-diagnostics has been used for different types of insects. As this study focuses on coleopterans it was felt that it would be necessary to include examples from another insect order. Examples from Dipterans include fruit flies (Diptera: Tephritidae) (Armstrong *et al.,* 1997), mosquitoes (Diptera: Culicidae) (Porter & Collins, 1991) and blackflies (Diptera: Simuliidae) (Krüger *et al.,* 2000; Gleeson *et al.,* 2000). In some fields of study DNA data are sometimes the only characters that can be easily used for the

taxonomy of organisms that have few morphological or ecological characters (e.g. nematodes (Nadler, 2002)). In DNA-based studies it is very important to make use of techniques that address the biological questions being asked as there are a variety of different molecular markers available for different uses (Zhang & Hewitt, 2003). These markers can be used as a marker of genetic variation within and among individuals and taxa. For this study DNA barcoding was chosen as the means to link scarabaeid larvae to identifiable scarabaeid adults.

1.4.1 DNA Barcoding

DNA barcoding is a technique suitable for all taxa (Hebert *et al*., 2003). It was proposed by Hebert *et al*. (2003a) as a method for identifying unknown specimens. DNA barcoding by definition relies on the use of the cytochrome c oxidase 1 gene *(cox1)* of the mitochondrial genome in animals (Blaxter, 2004).

DNA barcoding focuses on a short standardised segment of the genome (Hajibabaei *et al.,* 2005). In most cases short mitochondrial DNA (mtDNA) sequences (usually the 5'end of the *cox1* gene) are used to group unknown individuals with *a priori*-defined taxonomic entities based on sequence similarity, arriving at a species identification from DNA rather than from morphological characters (Vogler & Monaghan, 2006). The barcode sequences can then be compared with each other and therefore define taxa (Kurtzman, 1994; Wilson, 1995) Barcode sequences can also be compared with orthologous barcode sequences obtained from specimens earlier identified to taxon level using other methods (Blaxter, 2004). Inexact matches are either grouped with taxa in the database or identified as new to the database depending on whether they are within the threshold of sequence similarity (Vogler & Monaghan, 2006). This grouping can be justified by a range of studies (Hebert *et al.,* 2003; Hebert & Gregory, 2005) that showed that intraspecies variation is usually lower than interspecies variation.

A few advantages of DNA barcoding are as follows:

- Morphologically indistinguishable taxa can be identified without the need for live material (Blaxter, 2004).

- Complete data can be obtained from single specimens regardless of sexual morph or life stage, and later morphological identification can still be performed by using PCR sequences (Blaxter, 2004).
- The DNA barcode can be generated for single specimens, preserving parts as vouchers, or from DNA extracted from a group of organisms (Blaxter, 2004).
- A single technique can be used for all taxa: extract DNA, PCR and sequence and a standard protocol for DNA barcode determination is simple to set up and can be applied on a high-throughput scale (Blaxter, 2004).

1.4.2 Mitochondrial DNA

Mitochondrial DNA has been frequently used in the past three decades as a tool for inferring evolutionary history of populations and species (Loxdale & Lushai, 1998). This tool has been shown to be important in the fields of molecular ecology and phylogeography, and is used when investigating species limits, intraspecific relationships of populations, and haplotype distribution (Ballard & Whitlock, 2004). Comparison of mtDNA sequences can be used as a marker/measure to support the description of new species in cases where morphological differences are slight (Sperling & Hickey, 1994; Cognato *et al*., 1999).

Apart from the usefulness of its applications, potential advantages of mtDNA in this study are as follows.

- mtDNA shows maternal gene flow. MtDNA is transmitted exclusively through the female. This is an important factor in intraspecific studies as this allows DNA sequences to be traced back to a common female ancestor (Brower, 1994).
- There is no recombination due to maternal inheritance and therefore genetic variation is small within species. All variation present is due to factors other than recombination (Brower, 1994; Sperling, 1993).
- mtDNA lacks introns or non-coding regions (in animals). This means that it is a relatively simple structure (Brower, 1994).
- Robust primers make it possible to recover specific segments of the mitochondrial genome from a wide range of organisms (Brower, 1994).

- mtDNA is more abundant than nuclear DNA (the ratio of mitochondria to nuclei being greater per cell). These factors make it easier to amplify from fresh and old specimens. The double membranes surrounding mitochondria provide extra protection from degradation (Sperling, 1993; Brower, 1994).
- In animals mtDNA evolves at a rate 1-10 times faster, on average, than the typical single copy nuclear gene (Avise *et al.,* 1987; Brower, 1994). Drake *et al*. (1998) observed that the rate at which DNA mutations are accumulated is inversely proportional to the size of the genome. Mutations in nuclear DNA generally occur at a slower rate than in mtDNA (Drake *et al.,* 1998). Therefore one would require a longer nuclear DNA sequence than mtDNA sequence to be able to create a barcode that can differentiate species (Drake *et al.,* 1998). The rapid rate of mtDNA evolution allows for the characterization of population changes that have occurred in a relatively short period of time (in evolutionary terms). This makes it possible to separate recently diverged lineages from each other (Harrison, 1989; Simon *et al*., 1994) and allows variation to be studied. mtDNA is suitable for distinguishing morphologically similar species because the mutation rate is high enough to show sequence differences between closely related species (Avise, 1994).

MtDNA data have some important limitations.

- mtDNA represents only a single locus (Zhang & Hewitt, 2003). Information collected from mtDNA only represents one description of evolution (Avise, 1991, 1994). This "window" shows only the matrilineal history and could be different from the history of the population or the species (Zhang & Hewitt, 2003). Consequently conclusions we make on species/population history are probably biased (Zhang & Hewitt, 2003).
- The effective population size of mtDNA is only a quarter of that of nuclear autosomal sequences; therefore mtDNA lineages have a much faster lineage sorting rate and higher allele extinction rate (Zhang & Hewitt, 2003).
- As mitochondrial genes evolve at a fast rate they are not suitable markers for tracking deep divergences and in such cases nuclear genes would be more suitable to use (Lin & Danforth, 2004).

- mtDNA may show a high level of homoplasy due to the high proportion of the bases A/T in the third position of codons (Harrison, 1989).

The consequences of the above mentioned factors are as follows:

(i) evolutionary relationships could be oversimplified by mtDNA data. For example, scarabaeid specimens actually belonging to a sister species may be grouped together as one species indicating fewer species of scarabaeids present than truly is the case;

(ii) genetic diversity can be underestimated by mtDNA markers. Scarabaeids may exhibit less variation within species and within the subfamilies than expected;

(iii) uncertainty in genealogical analysis may increase due to the increased probability of more missing links in mitochondrial haplotypes. When analysing the sequences obtained from scarabaeids there may be uncertainty. Specimens may seem to be very distantly related due the absence of a specimen with a certain haplotype which would have linked the two groups;

(iv) remote population processes may not be detected correctly with mtDNA markers (Zhang & Hewitt, 2003). Adaptations of scarabaeids to, for example, environmental conditions may not be noticeable in the genetic composition of these scarabaeids.

The above-mentioned factors may impact this study by leading one to think that there is less genetic diversity within the Scarabaeidae in South Africa than is actually true. According to Reed (2007) populations which are smaller than a few thousand individuals tend to lose genetic variation by random genetic drift at a faster rate than genetic variation is replaced by the occurrence of mutations. This has implications for a population, as its ability to evolve in changing environmental conditions is limited.

The mtDNA *cox 1* region was chosen for use in this study and is discussed below.

1.4.2.1 Cytochrome Oxidase sub-unit I

The best gene to use mainly depends on the level of relatedness being studied and the gene's rate of evolution (Parker *et al.,* 1998; Caterino *et al.,* 2000). The *cox1* region is often used for phylogenetic study (King *et al*., 2002) and has been applied in a wide range of hierarichal levels in insects (Brower, 1996; Frati *et al.* 1997; Funk 1999; Howland & Hewitt, 1995) including closely related species (Beckenbach *et al.,* 1993; Sperling & Hickey, 1994). There is a strong reason to focus analysis on a specific gene. Inconsistency among systematists when choosing phylogenetic markers has resulted in a situation in which the whole of insect molecular systematics is only slightly greater than the sum of its parts (Caterino *et al.,* 2000). The genes and regions that have been sequenced in the past vary considerably among studies (Caterino *et al.,* 2000). This situation contrasts to that of the plant and vertebrate systematic communities (Caterino *et al.,* 2000). In these two cases a number of accepted markers have been used as standards, resulting in global phylogenies for these groups being produced from the combined data (Caterino *et al.,* 2000). The *cox1*, 16S, 18S and elongation factor-1α genes have been widely used and are useful across a wide range of divergences in insects (Caterino *et al.,* 2000). Insect molecular systematics has complemented morphological and ecological data but a more focused approach, by gathering homologous sequence data, will ensure that significant contributions are made to the evolutionary biology of insects (Caterino *et al.,* 2000).

The *cox1* gene has two important advantages.

- The universal primers for this gene are very robust, allowing sequences to be obtained from the 5' end of specimens from almost all invertebrates (Folmer *et al.,* 1994; Simmons & Weller, 2001).
- *Cox1* has a greater range of phylogenetic signal than any other mitochondrial gene (i.e. it has both fast evolving regions and highly conserved regions, suitable for the design of "universal primers" (Hebert *et al.,* 2003).

Being a protein-coding gene means that *cox1* alignments rarely show indels, making them simple to assemble (Doyle & Gaut, 2000). In common with other protein-coding genes, the third-position nucleotides show a high rate of synonymous substitutions, resulting in a rate of molecular evolution that is about three times greater than that of 12S or 16S rDNA (Knowlton & Weigt, 1998). The evolution of the *cox1* gene is fast enough to allow

identification of closely related species (Dittrich *et al*., 2006) as well as phylogeographic groups in a single species (Assefa *et al*., 2006). Other mitochondrial genes such as cytochrome *b* may seem to be just as efficient in resolving recent divergences, but changes in amino acid sequences occur more slowly in *cox1* resulting in deeper phylogenetic insight (Simmons & Weller, 2001).

Results of the DNA sequences could be presented in the form of a phylogenetic tree to illustrate the relationships between the species under study.

1.5 PHYLOGENETIC ANALYSIS

A phylogenetic analysis of nucleic acid (e.g. mtDNA) or protein sequences is a determination of how these sequences might have originated during evolution (Swofford, 1998). The aim of phylogenetic analysis is to describe the evolutionary relationships between the sequences (Holder & Lewis, 2003). Accurate models of evolution and statistical tests are necessary to extract maximum information from molecular sequence data (Whelan *et al.,* 2001). The evolutionary relationships among the sequences are represented using a graph called a tree (Baldauf, 2003). Sequences are placed at outer branches of a tree, and the branching relationships of the inner part of the tree then show the degree to which different sequences are related (Holder & Lewis, 2003). For example, two sequences that are very much alike will be located at neighbouring outside branches and will be joined to a common branch directly beneath them (Swofford, 1998). Less related sequences will be on branches that are more distant from each other on the tree (Swofford, 1998).

Phylogenetic analysis is an important area of sequence analysis (Funk, 1999). Using this type of analysis several sequences can be compared along their entire length showing which parts are changing rapidly and which parts are being influenced by natural selection (Holder & Lewis, 2003). Sequences that are the most closely related can be identified by their placement on neighbouring branches of a tree (Swofford, 1998). Therefore, when a gene family is found in an organism or group of organisms, phylogenetic relationships among the genes can help to show which genes might have a similar function which has been conserved during evolution of a related organism (Swofford, 1998). Such functional predictions can then be verified by genetic experiments (Funk, 1999).

Three major research traditions have developed which make use of this phylogenetically informative resource (Funk, 1999). In the first tradition, one or a few individuals from a number of closely related species are sampled, and their mtDNA is used to deduce a phylogeny or history of speciation events (Funk, 1999), for example work on *Sturmiopsis parasitica* Curren (Diptera: Tachinidae) by Dittrich *et al.* (2006). In the second tradition, many individuals from a variety of geographic populations of a single species are analysed (Funk, 1999), for example the study performed by Assefa *et al.* (2006) on the phylogeography of *E. saccharina*. The resulting phylogeographic tree is then used to infer historical patterns of demography and gene flow within the focal taxon, providing information on speciation, and bridging the taxon's population genetics and systematics (Avise *et al.,* 1987). In the third approach, intraspecific and interspecific studies are performed using the same data set allowing one to calculate species boundaries in a phylogenetic context (Funk & Omland, 2003). This approach is a combination of the first two traditions (Funk & Omland, 2003). An example of this type of approach can be further seen in Assefa *et al*. (2007).

In studies of the first tradition, a small number of individuals are usually sampled allowing the collection of relatively large DNA sequences (Funk, 1999). These give highly resolved, well-supported mitochondrial phylogenies (Funk, 1999). Incomplete intraspecific sampling can lead to incorrect interpretation. This could be due to undetected introgressed haplotypes. These haplotypes result from interspecific hybridisation and gene flow or from incompletely sorted ancestral polymorphisms that are still present among species (Moran & Kornfield, 1993; Parker & Kornfield, 1997).

The second tradition too has its limitations (Funk, 1999). The large number of individuals sampled often requires the use of readily collected restriction site data (e.g. Magoulas *et al.,* 1996). The information content of these data, however, is often not sufficient to provide highly resolved or strongly supported histories, limiting the confidence with which inferences can be made (Funk, 1999). Further, focusing on a single species limits explanations to within-species phenomena (Funk, 1999). Interpretations are compromised if patterns actually reflect inadequate taxonomy and, thus, an incomplete understanding of the reproductive and genetic boundaries between biological species (e.g. Su *et al.,* 1996).

The third tradition has its limitation with regards to interpreting molecular variation (Funk & Omland, 2003). It is often tempting to offer *ad lib* explanations for unusual patterns of polyphyly without fully considering alternatives (Funk & Omland, 2003). Polyphyletic groups as defined by Henning (1965) are those characterised by the possession of convergent character states. There are numerous phenomena that can produce species-level polyphyly (Funk & Omland, 2003). In some cases polyphyly is a result of misidentified specimens, species limits, and study loci, or of inadequate information (Funk & Omland, 2003). In others, it shows aspects of allelic history that supply important insights into species biology (Funk & Omland, 2003). In these studies it is important to use sampling practises that maximise the finding of important elements of intra- and interspecific variation (Funk & Omland, 2003). Increased attention to sampling and the interpretation of polyphyly across genes and taxa will offer improved insights in systematics, population genetics, and evolutionary biology in general (Funk & Omland, 2003).

In this study the third research tradition will be used. Different species, within the same scarabaeid family, from different geographical locations will be incorporated into this study. Intraspecific and interspecific divergences as well as branching patterns will be used to assign specimens to groups.

1.6 OBJECTIVES

1.6.1 General Objectives

The main aim of this project is to link larvae (which are difficult to distinguish morphologically) to adult scarabaeids (which are more easily identified by morphological techniques) from sugarcane fields by means of similarity of their mtDNA sequences. The rate of acquisition of this knowledge, especially in sugarcane, is delayed by the difficulties involved in making associations between life stages (Miller & Allsopp, 2005). Linking of known adult and unknown larval scarabaeids, by their genetic similarities, will allow the identification of the larvae to species level. Based on this foundation, the second aim is to develop a reliable morphological key that can be used by field workers to identify scarabaeid larvae.

1.6.2 Specific Objectives

- Linking of larvae to adults of Dynastinae, Melolonthinae and Rutelinae using mitochondrial DNA sequences from these life stages, and in this way identifying their species from morphologically identified adult specimens (Chapter 2).

- Determination of relationships among the families of Dynastinae, Melolonthinae and Rutelinae (Chapter 2).

- Determining the distribution of different scarabaeid larvae in the sugarcane producing areas of KwaZulu-Natal and verifying these results with adult identifications (Chapter 2).

- Development of a key for morphological identification of sugarcane scarabaeids (for adults and larvae) for use by field workers and researchers (Chapter 3).

- Development of skills in phylogenetic analysis of combined molecular and morphological data sets (Chapter 4).

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CHAPTER 2

MOLECULAR IDENTIFICATION OF SCARABAEID PEST SPECIES IN SOUTH AFRICAN SUGARCANE

2.1 INTRODUCTION

Scarabaeid beetles (Coleoptera: Scarabaeidae) are important pests of sugarcane and other cereal crops (Wilson, 1969). In sugarcane these scarabaeids, with the exception of one species, *Heteronychus licas* Klug 1855, feed only as larvae on the sugarcane roots (Wilson, 1969). In South Africa the pest species associated with sugarcane belong to the subfamilies Melolonthinae, Rutelinae, Dynastinae and Cetoniinae (Scholtz & Holm, 1996). Since the mid-1980's sugarcane damage by scarabaeid larvae has become more prevalent in certain areas of the sugar industry (Mansfield, 2004). This has initiated investigations into their pest status, economic impact and possible control measures.

For effective pest management accurate and rapid identification of these scarabaeids is crucial (Gleeson *et al.,* 2000). However one area hampering studies on scarabaeids is the ability to correctly identify the larvae in sugarcane fields. Currently it is unknown how many species of scarabaeid larvae attack sugarcane in South Africa despite the importance of this information for developing control tactics for specific pest species (Gleeson *et al.,* 2000).

Morphological identification of scarabaeid larvae requires an experienced taxonomist, and involves lengthy examination of specimens using microscopy to identify species-specific characteristics (Gleeson *et al.,* 2000). Identification of some larvae can be difficult if they have few morphologically distinguishable characteristics, which is often the case with scarabaeids. Furthermore, larvae are easily damaged during collection, leading to a large degree of uncertainty in identification. In addition, cryptic species may exist, as observed by Sperling *et al*. (1999) in their study on Lepidoptera. It should be noted that this existing reference is a single example of many. Thus, many larval forms are unidentifiable beyond genus and sometimes even beyond family level (Miller *et al*., 2005).

Linking the more easily identifiable adult and the more difficult to identify larval scarabaeids can be difficult due to a number of factors.

- The more mobile adult scarabaeids can be easily collected by means of light traps and subsequently identified. However, because of their mobility, these adult scarabaeids are not an accurate reflection of those scarabaeids attacking sugarcane. Light traps attract a range of scarabaeids, a small number of which would be from sugarcane, the others being attracted from other vegetation types (Sweeney, 1967).
- Only a few specimens have been collected as second or third instar larvae from the soil and bred through to adults and then identified (Sweeney, 1967). Thus for many groups of these scarabaeids, classifications are based largely or entirely on a single life stage (the adult) or even a single sex of a life stage (Miller *et al*., 2005).

Recent developments in deoxyribonucleic acid (DNA) technology have resulted in a range of molecular techniques being available for use in species identification (Caterino *et al.,* 2000). One of these approaches, DNA barcoding (Hebert *et al.,* 2003), promises fast and accurate species identifications by analysis of a short standardized segment of the genome. Although this approach is not without controversy (Lipscomb *et al*., 2003; Moritz & Cicero, 2004; Seberg *et al*., 2003; Tautz *et al*., 2003; Cognato, 2004; Ebach & Holdrege, 2005), the lack of adequate morphological taxonomic services makes the molecular approach an attractive alternative. Several studies have now established that sequence diversity in a ~650 bp region near the 5'-end of the mitochondrial cytochrome c oxidase subunit I (*cox* 1) gene provides strong species-level resolution for varied animal groups (Vogler & Monaghan, 2006; Waugh, 2007).

The Consortium for the Barcode of Life (CBOL; http://www.barcoding.si.edu/) aims at supporting individuals and organisations globally who are interested in utilising DNA barcoding as a means of species identification. CBOL was launched in May 2004, and is an international research alliance comprising more than 120 organizations from 45 nations. CBOL has established standards for barcode records and vouchering of specimens that must be adhered to in order to gain "reference barcode" designation. The Barcode of Life Data System (BOLD; http://www.barcodinglife.org/) is an online database and data analysis system run by the Canadian Centre for DNA Barcoding (CCDB;

http://www.dnabarcoding.ca/) and provides a means of integrating morphological, molecular and related information for every specimen. BOLD serves as a site where all specimen and corresponding sequence data can be stored, and also facilitates data management, analysis and quality evaluation. In addition, BOLD enables collaborations across the globe due to a combination of security features and the site's web-based structure. Access to BOLD is open to any researcher with interests in DNA barcoding. We therefore registered this project with BOLD.

The aim of this study was to link identified adult scarabaeids to unidentified larvae using the *cox* 1 gene. This molecular marker was chosen based on its usefulness for discrimination of closely related species, the fact that *cox 1* has become the diagnostic standard for DNA barcoding (Hebert *et al.,* 2003; Simmons & Scheffer, 2004) and the ability to link unknown scarabaeid insect larvae with identified scarabaeid adult insects of the same species (Miller *et al.,* 2005).

2.2 MATERIALS AND METHODS

2.2.1 Taxa

Two hundred and fifteen specimens (174 larvae and 41 adults) (Appendix A) were used in this study. Larvae of all instars were used as morphological characters are standard throughout instars. Most of the adult and larval scarabaeid specimens had already been collected during surveys in the sugar industry. Scarabaeid larvae were collected by sampling an area in the soil of 300 x 300 x 300 mm below the sugarcane stool. Larvae were placed in plastic trays containing soil and transported to the laboratory. Both adult scarabaeids, which had been pinned, and larvae, preserved in 70% alcohol, were present in the South African Sugarcane Research Institute (SASRI) collection. As a portion of the larval specimens had been collected between five to ten years ago and the DNA had degraded, field trips were undertaken to collect additional specimens. Adult specimens were collected from sugarcane fields using light traps and after being identified by taxonomist James du G. Harrison from the Transvaal Museum, Pretoria, were used for DNA sequencing. Larvae were collected from sugarcane fields in the KwaZulu-Natal province of South Africa. Adult specimens of *Camenta* Erichson 1847 (Coleoptera: Scarabaeidae) and *Lepiserica* Brenske 1900 (Coleoptera: Scarabaeidae) were identified by

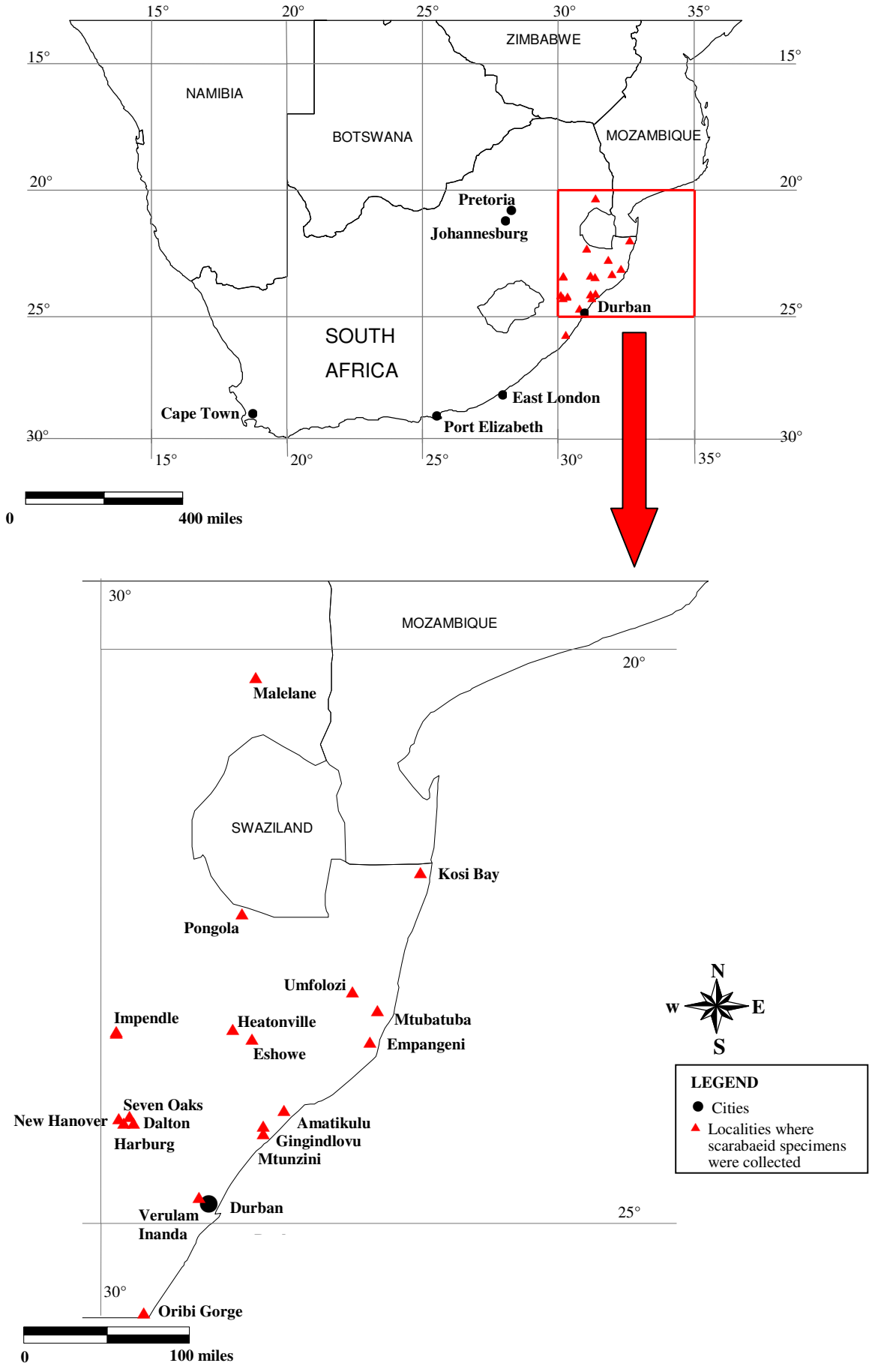


Figure 2.1 Map of South Africa and KwaZulu-Natal showing the collection localities of scarabaeids in this study.

taxonomist Dr. Dirk Ahrens from the Zoologische Staatssammlung in Munich, Germany. Larvae were identified to subfamily and tribe level, using Ritcher's (1966) key, based on characters of the head capsule, mouthparts, spiracles and raster (Chapter 3). Freshly collected larvae were preserved by placing them in boiling water for 2 minutes, then removing and storing them in absolute ethanol.

Hajibabaei et *al.* (2005) suggested preservation of specimens in absolute ethanol. This allows specimens to be easily analysed at a later stage provided that the ethanol is replaced regularly. This procedure also prevented colour change of the specimens, allowing subsequent examination of morphological characteristics to be more effective. Thirty three of the specimens in this study were sequenced at SASRI and the remaining 182 specimens by the Canadian Centre for DNA Barcoding, University of Guelph, Guelph, Ontario, Canada. All voucher specimens are housed at SASRI.

2.2.2 DNA extraction, PCR amplification and DNA sequencing

For specimens sequenced at SASRI, three legs (prothoracic, mesothoracic and metathoracic) were removed from adult and larval specimens for DNA extraction. DNA was extracted from individual specimens using the Qiagen DNeasy^{TM} Tissue Kit (Qiagen Inc., Santa Clara, CA) and the protocol for animal tissue (pp. 18-20). Partial fragments (658bp) of the mitochondrial *cox* 1 gene were amplified via the polymerase chain reaction (PCR) using primers C1-J-1514 (GGTCAACAAATCATAAAGATATTGG) and C1-N-2173 (TAAACTTCAGGGTGACCAAAAAATCA) (Simon *et al.,* 1994) or alternatively amplifying two smaller fragments with primers BC1Fm (GTAAAACGACGGCCAGTTCWACWAAYCAYAARGAYATYGG) and Scar-2RDm (CAGGAAACAGCTATGACGADARWGGDGGRTANACDGTTC), Scar-4Fm (GTAAAACGACGGCCAGTGAAAGAGGTGCWGGWACNGG) Scar-3RDm (CAGGAAACAGCTATGACAAAATRTAWACTTCDGGRTGNCC). BC1Fm, Scar-2RDm, Scar4F and Scar-3RDm are degenerate primers designed by Dr. Andrew Mitchell (Wagga Wagga Agricultural Institute, Wagga Wagga, Australia). The primers with names ending in "m" include non-degenerate M13 sequences of 17 nucleotides at their 5'-ends to facilitate DNA sequencing. BC1Fm is a modified version of LCO1490 (Folmer *et al.,* 1994) while Scar-2RDm, Scar-3RDm and Scar-4Fm were designed specifically for Scarabaeidae. PCR amplifications were performed on a Perkin Elmer GeneAmp PCR

System 2400 under the following conditions: 95°C for 10 minutes, 35 cycles of (94°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes), 72°C for 7 minutes, 4°C hold. Each 50 µl PCR reaction mix was prepared using 1 x PCR Buffer (JMR Holdings, United Kingdom), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 15 pmol of each PCR primer, 1 unit of Supertherm Gold DNA Polymerase (JMR Holdings, United Kingdom), and approximately 4µl of genomic DNA/RNA mix. Contamination was checked for using negative controls. PCR products were examined using agarose gel electrophoresis and purified using a QIAquick™ PCR Purification Kit (Qiagen), following the manufacturer's protocol. PCR products were sequenced in both directions. DNA sequencing was performed using an ABI PRISM® BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) under the following conditions: 32 cycles of (96°C for 10 seconds, 50°C for 30 seconds, 60°C for 4 minutes), 4°C hold. Sequencing reaction products were purified using the manufacturer's ethanol/sodium acetate/EDTA precipitation protocol. Sequences were visualized on an ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, CA). DNA sequence chromatograms were checked for base-calling errors and edited using the Staden package (Staden, 1996). The remaining specimens were sequenced at the Canadian Centre for DNA Barcoding, University of Guelph, Guelph, Ontario, Canada (http://www.barcodinglife.org/views/login.php).

2.2.3 Alignment and phylogenetic analysis

Sequences were aligned using ClustalX version 1.81 (Thompson *et al.,* 1997) and then manually corrected using BioEdit version 7.0.1 (Hall, 1999). As one check for the presence of nuclear mitochondrial pseudogenes ("numts") nucleotide sequences were translated to amino acids using MEGA version 3.1 (Kumar *et al.,* 2004) and checked for frameshifts and stop codons. Maximum parsimony (MP) analysis was performed using PAUP*4.0b10 (Swofford, 1998) and comprised a heuristic search with 100 random addition sequences of taxa, with 1 tree being held at each step and all characters weighted equally. A 200 replicate MP bootstrap analysis was performed using a single random taxon addition sequence for each bootstrap replicate, and TBR branch-swapping on completion. Due to time constraints only 200 bootstrap replicates were performed although Hedges (1992) recommended performing 400-2000 bootstrap replicates if one would like statistically meaningful results. Uncorrected pairwise DNA distances were calculated.

This project was registered on the BOLD system and subsequently all sequences sequenced at SASRI were uploaded to BOLD. In BOLD, all submitted sequences are first translated into amino acids and then compared against a Hidden Markov Model (HMM) of the *cox* 1 protein to validate their derivation from the *cox* 1 gene (Ratnasingham & Hebert, 2007). If this is correct, sequences are examined for stop codons to detect pseudogenes and then compared against a database of possible contaminants such as human and *E. coli* sequences (http://www.barcodinglife.org/views/login.php). Should any errors be detected the submitter is informed and the sequence removed. Once a trace file is submitted BOLD supplies a PHRED score for each nucleotide position and an average for the whole sequence (Ratnasingham & Hebert, 2007). Based on these results the sequence is placed into one of four categories ranging from, failed (no sequence) to high quality (mean PHRED >40) (Ratnasingham & Hebert, 2007). The Management and Analysis System (MAS) of BOLD offers a variety of tools for data analysis. The "Taxon ID Tree" option was used to generate a neighbour-joining (NJ) tree from the 208 nucleotide sequences as well as from the *Schizonycha affinis* Boheman 1857 (Coleoptera: Scarabaeidae) species group using the Kimura 2-Parameter method. Users do not have the option to implement a model which best fits their data on the BOLD website. Three distance methods were available to chose from namely, the pairwise distance method, The Jukes Cantor method and the Kimura 2-parameter method. The Kimura 2-parameter methods was chosen due to this method being the most complex of the three available methods. A filter was applied to disregard sequences shorter than 500bp as well as ambiguities. The resulting NJ tree was downloaded from BOLD in Newick format edited in TreeView (Page, 2001). Additionally MEGA 3.1 was used to generate a NJ tree under the Kimura 2-parameter model with 1000 bootstraps.

2.2.4 Base Composition

The frequency of the bases A, C, G and T was calculated using MEGA version 3.1 (Kumar *et al*., 2004).

2.2.5 Geographical distribution of specimens

Identified adult scarabaeids, belonging to the same species as those in this study, at the South African National Collection of Insects (SANC), Pretoria were examined and

collection localities were recorded. This procedure was subsequently repeated at the Transvaal Museum in Pretoria. The purpose of this task was to identify the probable distribution of the scarabaeids in this study.

2.3 RESULTS

A 658bp DNA sequence alignment was obtained from 208 scarabaeid adult beetle specimens. There were 303 variable characters and 277 parsimony informative characters. Across all sequences there was an excess of Thymine (T) (33%) and Adenine (A) (29%) over Cytosine (C) (21%) and Guanine (G) (17%).

2.3.1 Phylogenetic Analysis

Trees obtained from NJ and MP were similar in overall topology and sequences clustered into overlapping groups with similar groups being recovered by NJ and MP.

2.3.1.1 Neighbour Joining Analysis

The neighbour joining (NJ) tree was constructed implementing the Kimura 2-parameter distance using BOLD software. Of the initial 211 sequences, three were excluded due to the sequences being less than 500bp in length. The resulting NJ tree (Figure 2.2) clearly grouped the specimens into one of three subfamilies Dynastinae, Melolonthinae or Rutelinae. Subsequently an unrooted tree (Figure 2.3) was generated using MEGA to compare grouping of individual specimens to those groups assigned based on morphology, sequence divergence and branching pattern. The unrooted tree confirmed the groupings observed in the NJ analysis.

2.3.1.2 Maximum Parsimony Analysis

Parsimony searches resulted in one tree of length = 2221 (CI = 0.2409; RI= 0.870; HI = 0.7591; RC = 0.2096). This tree was divided into 28 preliminary groups based on branching pattern and sequence divergence. Two main differences were visible between the MP tree and the NJ tree, namely arrangement of scarabaeid groups within the tree and bootstrap support. The arrangement of three groups differed from the NJ analysis. Group

H, *Popillia bipunctata* Fabricius 1787 (Coleoptera: Scarabaeoidae), was placed at the lower most end of the tree next to Group P. Group O was placed between individuals of Group P. Bootstrap support values for groups were in most cases lower than those of the NJ analysis.

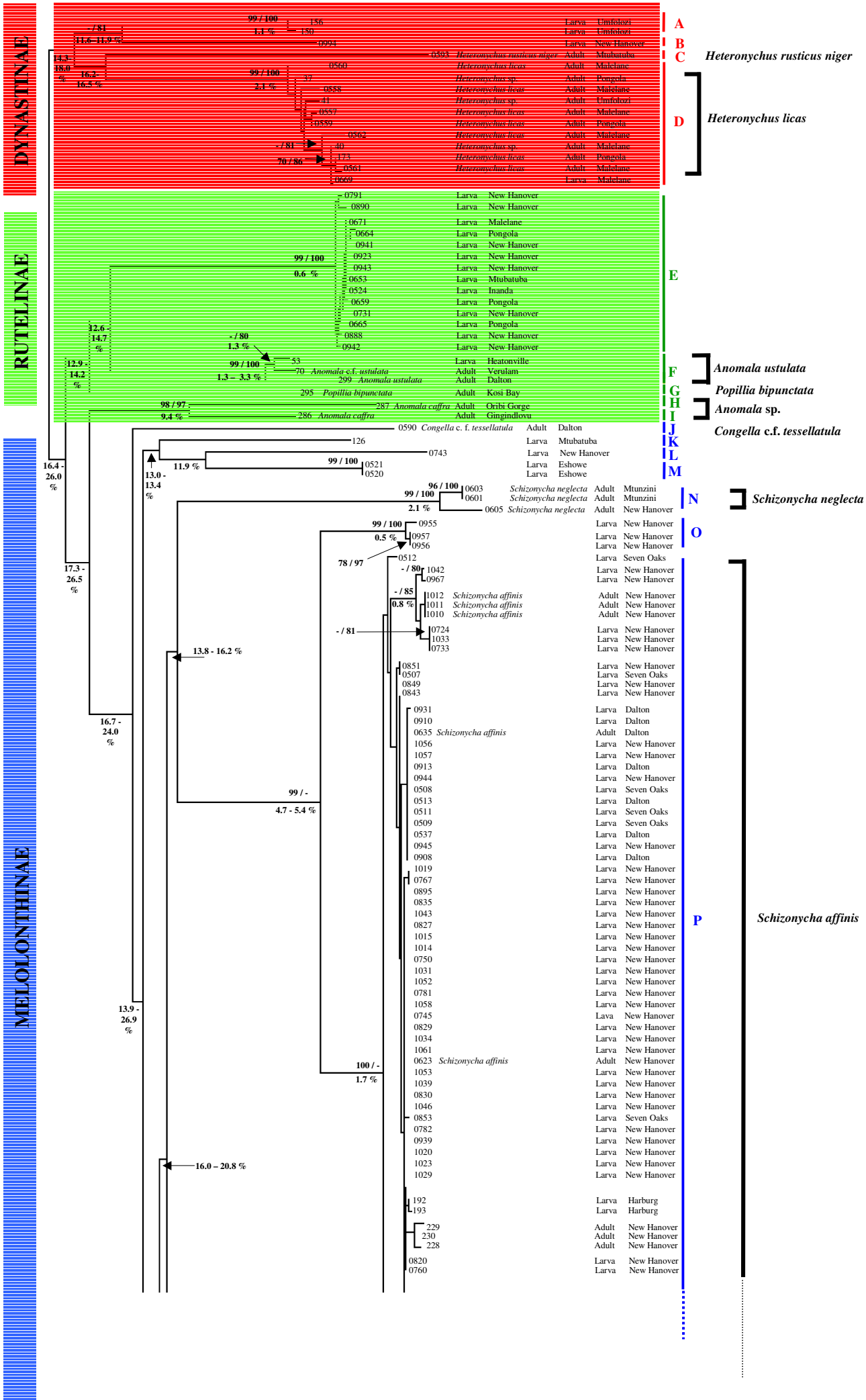
2.3.2 DNA sequence variation

Specimens were grouped into 29 provisional groups (Group A – Group AC) based on phylogenetic tree branching patterns resulting from MP and NJ, percent sequence divergence as well as comparison of morphological data (see chapter 1). Within-group sequence divergence ranged from 0 – 3.4 % and divergence between sister groups ranged from 2.6 - 25.1 %. The recorded divergence range within and between tribes was 0 – 21.3 % and 17.3 - 28.5% respectively. Similarly, the divergence range observed within and between genera was 0 – 19.2 % and 17.1 – 25.4% respectively. The maximum sequence divergence observed within subfamilies was 23.7 % and divergence between subfamilies ranged from 16.8 – 26.7 %.

For six species we were able to link unidentified larvae to positively identified adults. In five instances no larvae in the study linked to identified adult specimens and for nine groups no identified adult specimens linked to clusters containing only larvae.

Groups A and B contained two and one larvae respectively with a between-group divergence of 11.6% - 11.9%. These larvae were identified as Dynastinae using morphology. Examination of morphological characteristics showed no differences between the larvae of the two groups. Average sequence divergence values for this data were of a magnitude greater than those observed within groups and would therefore indicate that these relationships present those exhibited within tribes or genera.

Group C contained a single *Heteronychus rusticus niger* Harrison 2007 (Coleoptera: Scarabaeoidae) adult and Group D contained several *Heteronychus* sp. adults, *H. licas* adults and a single larva. The among-group divergence between Groups C and D was 16.2% - 16.5%. Group D showed a maximum within-group divergence of 2.1%. The


DYNASTINAE
RUTELINAE
MELOLONTHINAE
A
B
C
Heteronychus rusticus niger
D
Heteronychus licas
E
F
Anomala ustulata
G
Popillia bipunctata
H
I
Anomala sp.
J
Congella c.f. tessellatula
K
L
M
N
Schizonycha neglecta
O
P
Schizonycha affinis
99 / 100
1.1 %
- / 81
11.6 - 11.9 %
14.3 - 18.0 %
16.2 - 16.5 %
99 / 100
2.1 %
- / 81
70 / 86
99 / 100
0.6 %
12.6 - 14.7 %
12.9 - 14.2 %
- / 80
1.3 %
99 / 100
1.3 – 3.3 %
98 / 97
9.4 %
16.4 - 26.0 %
13.0 - 13.4 %
11.9 %
99 / 100
96 / 100
99 / 100
2.1 %
99 / 100
0.5 %
78 / 97
- / 80
- / 85
0.8 %
- / 81
17.3 - 26.5 %
13.8 - 16.2 %
16.7 - 24.0 %
99 / -
4.7 - 5.4 %
13.9 - 26.9 %
100 / -
1.7 %
16.0 – 20.8 %
0593 Heteronychus rusticus niger Adult Mtubatuba
0560 Heteronychus licas Adult Matelane
37 Heteronychus sp. Adult Pongola
0558 Heteronychus licas Adult Matelane
41 Heteronychus sp. Adult Umfolozi
0557 Heteronychus licas Adult Matelane
0559 Heteronychus licas Adult Pongola
0562 Heteronychus licas Adult Matelane
40 Heteronychus sp. Adult Matelane
173 Heteronychus licas Adult Pongola
0561 Heteronychus licas Adult Matelane
0669 Larva Matelane
0791 Larva New Hanover
0890 Larva New Hanover
0671 Larva Matelane
0664 Larva Pongola
0941 Larva New Hanover
0923 Larva New Hanover
0943 Larva New Hanover
0653 Larva Mtubatuba
0524 Larva Inanda
0659 Larva Pongola
0731 Larva New Hanover
0665 Larva Pongola
0888 Larva New Hanover
0942 Larva New Hanover
53 Larva Heatonville
70 Anomala c.f. ustulata Adult Verulam
299 Anomala ustulata Adult Dalton
295 Popillia bipunctata Adult Kosi Bay
287 Anomala caffra Adult Oribi Gorge
286 Anomala caffra Adult Gingindlovu
0590 Congella c. f. tessellatula Adult Dalton
126 Larva Mtubatuba
0743 Larva New Hanover
0521 Larva Eshowe
0520 Larva Eshowe
0603 Schizonycha neglecta Adult Mtunzini
0601 Schizonycha neglecta Adult Mtunzini
0605 Schizonycha neglecta Adult New Hanover
0955 Larva New Hanover
0957 Larva New Hanover
0956 Larva New Hanover
0512 Larva Seven Oaks
1042 Larva New Hanover
0967 Larva New Hanover
1012 Schizonycha affinis Adult New Hanover
1011 Schizonycha affinis Adult New Hanover
1010 Schizonycha affinis Adult New Hanover
0724 Larva New Hanover
1033 Larva New Hanover
0733 Larva New Hanover
0851 Larva New Hanover
0507 Larva Seven Oaks
0849 Larva New Hanover
0843 Larva New Hanover
0931 Larva Dalton
0910 Larva Dalton
0635 Schizonycha affinis Adult Dalton
1056 Larva New Hanover
1057 Larva New Hanover
0913 Larva Dalton
0944 Larva New Hanover
0508 Larva Seven Oaks
0513 Larva Dalton
0511 Larva Seven Oaks
0509 Larva Seven Oaks
0537 Larva Dalton
0945 Larva New Hanover
0908 Larva Dalton
1019 Larva New Hanover
0767 Larva New Hanover
0895 Larva New Hanover
0835 Larva New Hanover
1043 Larva New Hanover
0827 Larva New Hanover
1015 Larva New Hanover
1014 Larva New Hanover
0750 Larva New Hanover
1031 Larva New Hanover
1052 Larva New Hanover
0781 Larva New Hanover
1058 Larva New Hanover
0745 Lava New Hanover
0829 Larva New Hanover
1034 Larva New Hanover
1061 Larva New Hanover
0623 Schizonycha affinis Adult New Hanover
1053 Larva New Hanover
1039 Larva New Hanover
0830 Larva New Hanover
1046 Larva New Hanover
0853 Larva Seven Oaks
0782 Larva New Hanover
0939 Larva New Hanover
1020 Larva New Hanover
1023 Larva New Hanover
1029 Larva New Hanover
192 Larva Harburg
193 Larva Harburg
229 Adult New Hanover
230 Adult New Hanover
228 Adult New Hanover
0820 Larva New Hanover
0760 Larva New Hanover

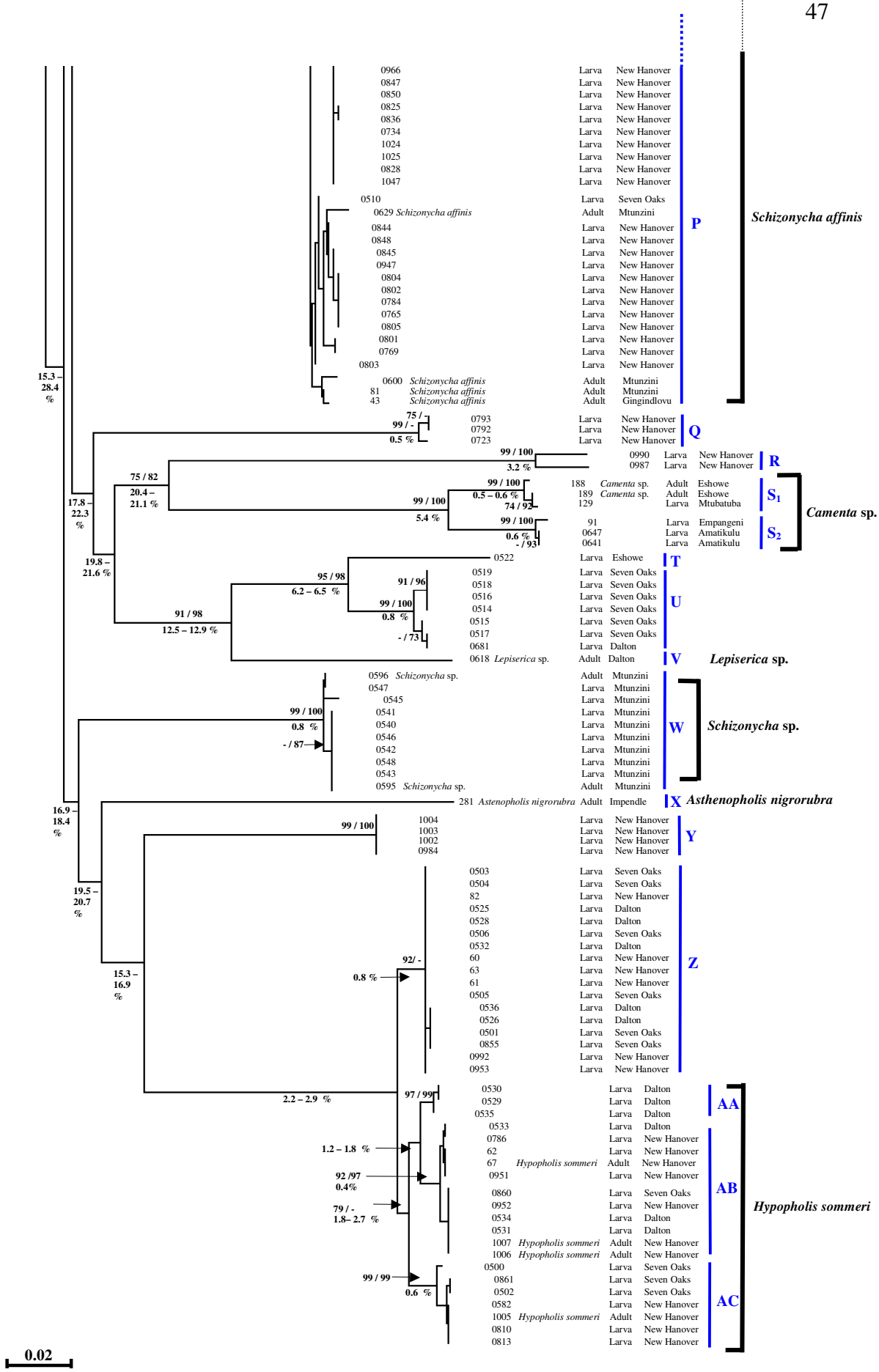


Figure 2.2 Phylogram using NJ. Group names are colour coded according to subfamily designation (Blue = Melolonthinae; Green = Rutelinae; Red = Dynastinae). Group identities written in bold black colour indicate inferences made using molecular and morphological data. Values above/below branches indicate divergence percentages as well as bootstrap support values for maximum parsimony and neighbour joining respectively.

sequence divergence between the larval specimen from Malelane and the adult *H. licas* specimen from Malelane was sufficiently low (0.2%) to suggest that this larva is *H. licas*.

Group E consisted of 15 unidentified larvae and showed low within-group variation (maximum 0.6%). Larvae were identified based on morphological characteristics of the mouthparts as belonging to the ruteline tribe Adoretini.

Group F showed divergence values of 1.3% - 3.3%. Group F was represented by two adult *Anomala ustulata* Arrow 1899 (Coleoptera: Scarabaeoidae) individuals and a larval specimen. The sequence divergence between the adult specimens from Verulam and larval specimens was 1.3%. This was further supported by the high bootstrap value (100%) in the NJ analysis (Figure 2.2). Group G consisted of a single *Popillia bipunctata* (tribe Anomalini) adult specimen from Kosi Bay although it was not collected from sugarcane. This species occurs almost ubiquitously throughout South Africa extending through Swaziland, Mozambique and Zimbabwe and up to East Africa.

Groups H and I each contained an adult specimen of *Anomala caffra* Burmeister 1844 (Coleoptera: Scarabaeoidea) exhibiting a sequence divergence of 9.4%.

Group J was a single *Congella* c. f. *tessellatula* (tribe Hopliini) specimen from Dalton.

Group K consists of a single larval specimen from Mtubatuba. Comparing percent sequence divergence of Group K and the remaining groups indicated no closely related specimens were sampled. Divergence shown between Group K and L and Group K and M was recorded as 13.4% and 13.1% respectively. Group L and M differed by 11.8%. Group N, containing three adult specimens identified as *S. neglecta,* exhibited a maximum within-group divergence of 2.1%. Two specimens were collected from Mtunzini and one from New Hanover.

The maximum sequence divergence within Group O was 0.5%. This group was represented by three larval specimens from New Hanover.

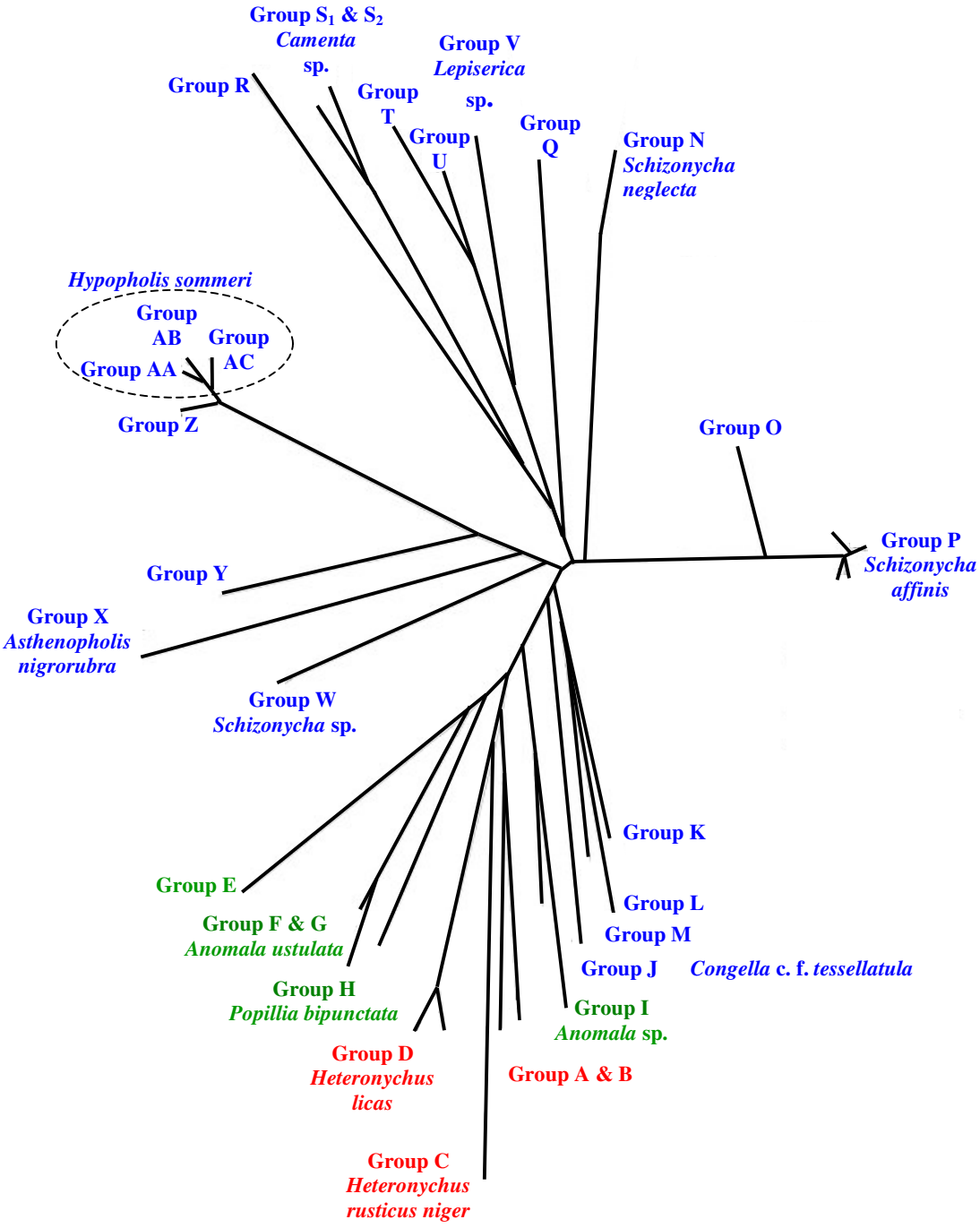


Figure 2.3 Unrooted phylogenetic tree derived from neighbour joining analysis using BOLD software. Group names are colour coded according to subfamily designation (Blue = Melolonthinae; Green = Rutelinae; Red = Dynastinae).

Group P clustered *S. affinis* adult specimens with larvae and was the largest group with 89 specimens, nine of which were identified *S. affinis* adults. The maximum within-group sequence divergence observed was 1.7%.

Group Q comprised only larvae collected from New Hanover with an among-group sequence divergence of 0.5%.

Both larval specimens of Group R were collected from the same area and showed a within-group sequence divergence of 3.2%. Group T consisted of a single larval specimen from Dalton and differed from Group U by 6.2% - 6.5%.

Group V contained a single *Lepiserica* sp. (tribe Sericini) specimen from Dalton.

Group W consisted of two *Schizonycha* sp. adult specimens as well as larvae exhibiting a maximum within-group divergence of 0.8%. Group X contained a single *A. nigrorubra* specimen from Impendle.

Group Y contained four larval specimens from New Hanover whose morphological characteristics were not dissimilar to those of the larvae in groups Z, AA and AB. The among-group divergence range of group Y and AA, and, Y and Z was 14.1 – 15.2 % and 13.8 – 14.2 % respectively. These values fall within the boundaries of the mean divergence observed in this study within tribes and genera. Morphological identification indicated that specimens in group Y are in the tribe Melolonthini.

Group Z consisted of larvae only from the KwaZulu-Natal Midlands. Within-group divergence ranged from 0 – 0.8 % and confirmation of morphological characteristics suggests that these specimens are members of one species. The mean divergence between groups Z and AA was 2.19%.

Group AA consisted of three larvae with a mean within-group divergence of 0.2%. Mean observed among-group divergence between group AA and AB was 1.5%. The mean within-group sequence divergences shown for groups AB and AC was 0.4% and 0.6% respectively. Groups AB and AC both consisted of *H. sommeri* adult specimens and larval specimens. The mean between-group divergence recorded was 2.5% between groups AB

and AC. Examination of larval morphological characteristics indicated no differences between specimens in these two groups.

2.4 DISCUSSION

In this study, phylogenetic relationships were inferred from the nucleotide sequences using both parsimony and distance based methods, grouping the specimens into 28 groups.

2.4.1 Base Composition

In this study the base frequency of A and T exceeded that of GC with the percentage of the nucleotides A and T present comprising 62% of the total nucleotides. In four other studies based on Coleopterans (Table 2.1) the percentage of AT ranged from 68% - 72%. In this study the average percentage AT observed was lower than that observed in studies on other Coleopterans. Three groups (Group R, S_1 and S_2) showed a base frequency of AT which was below 60%, ranging from 52 – 57 %. Sixteen groups showed an AT base frequency ranging between 60-65% (Groups AA, AB, AC, B, C, L, N, O, P, Q, T, U, W, X, Y, and Z). The remaining 11 groups (Groups A, D, E, F, G, H, I, J, K, M and V) showed an AT base frequency of above 65%.

Table 2.1 Base frequency of A and T of the mtDNA *cox*1 gene in six different insect orders

Insect Order	Family	Genera	% AT	Source
Coleoptera	Scarabaeidae	*Ammoecius* sp.	72	Cabrero-Sañudo & Zardoya, 2004
Coleoptera	Scarabaeidae	*Scarabaeus* sp.	71	Forgie *et al*., 2006
Coleoptera	Scarabaeidae	*Prodontria* sp.	69	Emerson & Wallis, 1995
Coleoptera	Silphidae	*Nicrophorus* sp.	68	Dobler & Müller, 2000
Diptera	Calliphoridae	*Chrysomya* sp.	77	Junqueira *et al*., 2004
Hymenoptera	Braconidae	*Apanteles* sp.	74	Mardulyn & Whitfield, 1999
Isoptera	Rhinotermitidae	*Reticulermes* sp.	63	Ye *et al.*, 2004
Lepidoptera	Nymphalidae	*Aglais* sp.	71	Vandewoestijne *et al*., 2004
Orthoptera	Rhaphidophoridae	*Dolichopoda* sp.	70	Allegrucci *et al.,* 2005

Different organisms show varied proportions of base composition with the frequency of AT and GC in their genome seldom being equal (Mooers & Holmes, 2000). Two main processes, natural selection and mutation, are believed to influence the variation of base composition in species (Mooers & Holmes, 2000). Comparing the values obtained in this study on scarabaeids with those of other insect orders, such as Diptera, Hymenoptera, Isoptera and Orthoptera, we see that for all other orders considered here, with the exception of Isoptera, the base frequency of AT exceeds 70%. In the Coleoptera the base frequency seems to be distributed more evenly compared to Diptera which shows a compositional AT bias of 77%.

Compositional bias in DNA sequences can negatively affect phylogenetic analysis based on those sequences. Hasegawa and Hashimoto (1993) showed that phylogenetic analyses based on rRNA genes could be unreliable due to the AT or GC bias in the rRNA genes of some taxa. They recommended that inferred amino acid sequences of encoded proteins be used as these provide more reliable phylogenies. However, Foster and Hickey (1999) showed that compositional biases in nucleotides are noticeable in amino acids derived from those nucleotides. According to Rosenberger & Kumar (2003) methods have been developed to overcome problems associated with compositional heterogeneity among sequences. These include distance approaches, parsimony methods as well as maximum-likelihood. It is thought that incorrect phylogenetic inferences arise from the grouping of unrelated organisms who have similar nucleotide frequencies (Rosenberger & Kumar, 2003). This grouping can occur due to convergence rather than common ancestry and in some instances these incorrect groupings may even have strong statistical support (Rosenberger & Kumar, 2003). Furthermore, Rosenberger & Kumar (2003) stress the importance of accuracy in phylogenetic analyses as other studies often utilise the results generated from these studies. Of the factors leading to phylogenetic inaccuracies the use of an appropriate evolutionary model is thought to be the most important (Rosenberger & Kumar, 2003). These models generally assume (i) unequal rates of transition and transversion (ii) rate heterogeneity among sites, and (iii) homogeneity of nucleotide frequencies (Rosenberger & Kumar, 2003).

In this study on scarabaeids two different approaches were used to analyse the resulting data – neighbour joining (a distance approach) and maximum parsimony. Evaluation and comparison of results obtained from both approaches should ensure that grouping of

organisms into putative species groups is accurate and not skewed by base composition bias. Due to the relatively even base composition exhibited in the scarabaeids in this study it is not expected that base composition should have a large influence on results generated from phylogenetic analyses.

2.4.2 Percent Sequence Divergence

A useful measure for specimen identification is percent within-group sequence variation. This means that a specimen could be identified as the same species as a reference specimen if the two were linked by parsimony or NJ analysis and if they differed by less than the maximum sequence divergence value observed within that group.

In 1998, Johns and Avise showed that on average more than 2% divergence was observed between closely related vertebrate species. These results were obtained using the mitochondrial gene, cytochrome b. Results from a later study (Avise, 2000) showed that intraspecific divergences in most instances were lower than 1% and hardly ever greater than 2%. Hebert *et al*. (2003) investigated this further and considered various animal phyla.

From their study using the mtDNA *cox 1* gene, Hebert *et al.* (2003) concluded that congeneric species exhibited a considerable sequence divergence such that species could be easily separated. Some researchers state that there is a "threshold" or genetic divergence among and within species that can be used to define species boundaries (Zhou *et al.,* 2007). Note should be taken that these typical thresholds cannot necessarily be used for all species and taxa as this "threshold" might only be applicable to a certain species (Zhou *et al.,* 2007). This is due to differing rates of evolution within lineages of certain species (Zhou *et al.,* 2007). Researchers such as Miller *et al.* (2005) used genetic distances/threshold calculated from their own data. This method has some disadvantages. Firstly, the distance criterion is used to identify the species boundaries. Both distance criteria and species boundaries thus are obtained from the same data (Zhou *et al.,* 2007). Secondly, when taxa are widely sampled the average interspecific divergence decreases (Zhou *et al.,* 2007). Thirdly, recently diverged species might not be differentiable if a single gene is used (Zhou *et al.,* 2007). Fourthly, instances where sequence divergence is very low could be due to incorrect specimen identification or mitochondrial introgression (Hebert *et al.,* 2003). When low sequence divergence is due to introgression Hebert *et al*. (2003) suggest the use

of additional data to aid in species discrimination. For example, if mtDNA is used initially these results can be verified using the internal transcribed spacer (ITS) region, which evolves at a faster rate than mtDNA (Hebert *et al.,* 2003). When DNA data is used, species boundaries are best predicted through phylogenetic analysis, consideration of additional biological evidence, and evaluation with taxonomic expertise (Wheeler, 2004). Thus, dependence only on standard percent sequence divergence alone for species diagnoses and assessment of species limits is not recommended (Cognato, 2004; Vogler & Monaghan, 2006; Waugh, 2007).

Table 2.2 Within-group and among-group divergences in the c*ox 1* gene for Coleoptera (source Cognato, 2004)

Species	**Family**	**Within-group divergence (%)**	**Divergence between sister species (%)**
Aramigus tessellates	Curculionidae	0.1 – 9.0	9.8 – 14.3
Blepharida flavocostas	Chrysomelidae	0.1 – 2.4	4.4 – 5.6
Curculio caryae	Curculionidae	0.2 – 1.7	10.8 – 11.3
Ips confusus	Curculionidae	0.3 – 1.6	3.2 – 4.3
Ips pini	Curculionidae	0.3 – 3.4	10.0 – 12.6
Liparthrum pilosum	Curculionidae	2.0 – 4.7	10.1 – 11.5
Ophraella communa	Chrysomelidae	0.3 – 3.8	4.9 – 7.4
Canthon sp.	Scarabaeidae	0.0 – 2.0	10.0 – 19.0 *
Tomicus piniperda	Curculionidae	0.3 – 1.5	11.0 – 12.7
Trirhabda lewisii	Chrysomelidae	0.3 – 1.8	3.6 – 5.6
Trirhabda sericotrachyla	Chrysomelidae	0.3 – 0.9	1.7 – 3.9

* Source Monaghan *et al.,* 2005

In this study on scarabaeids, numerous approaches were utilized before species boundaries were defined. Table 2.2 was used as a guideline when deciding on the grouping of specimens in this study. Groups were defined on the basis of tree branching as well as sequence divergence. Adult and larval morphology was used additionally.

2.4.3 DNA Sequence Variation and Phylogenetic Analysis

2.4.3.1 *Heteronychus licas*

In the past *H. licas* was believed to be the only problematic species of *Heteronychus* in sugarcane (Carnegie, 1974; Cackett, 1992; Rajabalee, 1994). The molecular analysis, however, thus indicating either that *H. licas* actually comprises a species complex or that previous authors misidentified *Heteronychus* species in sugarcane. In South Africa approximately 12 species of *Heteronychus* have been recorded (James du G. Harrison 2007, pers. comm.) and it is therefore recommended to further study the genetic differentiation within the genus *Heteronychus* to identify the economically important group/s.

2.4.3.2 *Anomala* species group

The sequence divergence between the adult specimens from Verulam and larval specimens was 1.3% which is less than that observed between the two *A. ustulata* adults indicating that the larval specimen belongs to the same species.

The species *A. ustulata* is widespread in southern Africa, being recorded from South Africa (KwaZulu-Natal; Gauteng; Limpopo), Swaziland, Mozambique, Zimbabwe (James du G. Harrison 2007, pers. comm.) and Botswana (data from SANC collection). Congeneric ruteline species in Groups H and I each consisted of a single adult *Anomala* sp. specimen. This group is a sister-group to the Melolonthinae (Figure 2.2) as its basal point does not form part of the Rutelinae. DNA barcoding is a diagnostic tool and utilizes terminal groupings to assign identities to specimens and not higher-phylogeny (Greenstone *et al*., 2005). Group I was collected at Gingindlovu and group H was collected at Oribi Gorge. These locations are widely separated geographically and climatically. Divergence between these specimens was recorded as 9.4%. From visual inspection the two specimens appear to be different species. According to Machatschke's (1972) *Coleopterum Catalogus* there are 29 species of *Anomala* occurring in South Africa with seven species (*Anomala basalis* Guérin 1847; *Anomala dita* Péringuey 1902; *Anomala fusciceps* Fåhraeus 1857; *Anomala lutea* Klug 1855; *Anomala probative* Péringuey 1902; *Anomala bohemani* Fåhraeus 1857 and *Anomala calcarata durbana* Machatschke 1954, all occurring in the sugarcane

growing areas of South Africa. This possibly indicates that these two *Anomala* specimens are different species. Correspondence with Dr. Pol Limbourg from the Royal Belgian Institute of Natural Sciences confirmed this assumption. He stated that *Anomala caffra* belongs to the basalis group of *Anomala* species, whose genitalia are very similar between different taxa.

2.4.3.3 Groups K, L and M

Examination of the raster pattern of all larval specimens in this study suggested that Group L and M would be very closely related to Group K as the raster patterns of all specimens were alike. According to the mean divergence values calculated for within and between groups, tribes, genera and subfamilies the divergence between groups K, L and M indicate that the divergence is of a magnitude observed within tribes or genera. This would indicate that groups K, L and M are possibly members of the same tribe or even of the same genus.

2.4.3.4 Group O

Comparison of morphological characters showed that the only difference between specimens in Groups O and P was the slight variation in raster pattern of the two groups. The mean divergence observed between Group O and Group P (5.5%) as well as the morphological difference justified treating these groups as two different species, probably within the same genus.

2.4.3.5 *Schizonycha* species group

Specimens 228, 229 and 230 were unidentified adult specimens from New Hanover. To confirm the association made by the molecular data, these three specimens were sent for identification by taxonomist James du G. Harrison. The identities were verified to be *S. affinis* based on examination of the genitalia, general morphology and collection locality.

Comparison of the raster of the larval specimens in group P as well as all other larval specimens included in the study showed that group P (*S. affinis*) as well as Group W (*Schizonycha* sp.) have similar raster patterns. The low within-group divergence, strong bootstrap support (85% for MP and 100% for NJ) as well as the consistency between

morphological characters of the larvae in this group would indicate that all specimens are members of the same *Schizonycha* species. This result led to the question whether or not the *Schizonycha* sp. group is actually *S. affinis*? To answer this question among-group sequence divergence values were compared between Group W and group P. These ranged from 13.5% - 16.7%. From this we can conclude that the *Schizonycha* sp. and *S. affinis* are separate species. Identified adult specimens in this study included *S. affinis* and *S. neglecta* but the specimens in group W could not be identified to species level. According to Pope (1960) *Schizonycha* is among the largest genera of Melolonthinae. Of the more than 300 species described, all but eight are of African origin and about 120 occur in southern Africa (Pope, 1960). Pope (1960) noted the distribution of *S. affinis* being the former Natal (now KwaZulu-Natal) and Cape Province, and *S. neglecta* being the former Cape Province, Natal, Transvaal and Rhodesia. The *S. affinis* material examined by Pope (1960), before revising the species of *Schizonycha,* was collected from Indaleni, Durban, Weenen, Pietermaritzburg, Pinetown, Eshowe and Zululand in KwaZulu-Natal. Similarly the *S. neglecta* material was collected from Zululand, Richmond, Estcourt, Weenen, Malvern, New Hanover, Southbroom, Melmoth and Durban, also all in KwaZulu-Natal. The *cox* 1 data clearly suggests that there are multiple species represented in this sample and group W is possibly a third species of *Schizonycha*, as Pope (1960) recorded a further 25 species of *Schizonycha* occurring in KwaZulu-Natal. Similarly, work done by Harrison (unpublished) indicates that this species is widespread in KwaZulu-Natal and Swaziland but has a limited distribution in Mpumalanga, Limpopo, Eastern Cape and Western Cape.

2.4.3.6 Groups Q, R, S, T and U

Group Q comprised only larvae collected from New Hanover. The among-group sequence divergence of 0.5% as well as the branching pattern of the NJ tree would suggest that these specimens could be one species. (Table 2.3 gives examples of intra- and among-group divergence values obtained in other Coleoptera, for comparison). For clarification morphological characters were examined. Firstly, using Ritcher's key (1966) to identify the larvae, all three specimens belong to the subfamily Melolonthinae. Secondly, the arrangement of pali constituting the raster as well as the number of heli present in the haptomerum of the epipharynx were examined and slight variations observed. Specimen 0723 differed from specimen 0792 and 0793 by the presence of a single helus in the epipharynx. This difference does not necessarily warrant separation of group Q into two

species especially when considering that all other characters showed no variation. Thirdly, other characters examined such as the spiracles and the mandibles showed no variation between the two subgroups (1 = 0793 & 0792; 2 = 0723) making up group Q. Thus it is probable that the specimens in group Q are one species and the differences in morphology are due to within species variation.

Although both larval specimens of Group R were collected from the same area, the within-group sequence divergence was 3.2%. All other groups differed from Group R by more than 19.0%. This value could indicate separate species. Both specimens were morphologically identified as belonging to the tribe Sericini. The maximum divergence between Group R and Group S was 21.1%. Group S consisted of two subgroups S_1 and S_2. Group S_1 consisted of an adult *Camenta* sp. (tribe Sericini) and one larval specimen. The within-group divergence ranged form 0.5% - 0.6%. The divergence between the two *Camenta sp*. specimens was 0.5% and that between the adult specimens and the larva was recorded as 0.6%. These values are within the limit expected between individuals of the same species and would infer that the larvae would be a *Camenta* sp. belonging to the tribe Sericini. Morphological identification suggested the larva belong to the tribe Sericini. A similar raster pattern was exhibited by individuals in Group S_2 but in this case the divergence between these two sub-groups ranged from 5.0% - 5.8%. These values are suggestive of among-species divergences. Strong bootstrap support (100%) for these relationships in the MP analysis for both subgroups as well as for the branch from which these groups emerged substantiate this.

Morphological identification of specimens in groups T and U put these specimens into the tribe Sericini, but further comparisons of morphology between these two groups could yield no other differences. Despite this it is concluded that groups T and U are most likely separate species based on their sequence divergence.

2.4.3.7 *Hypopholis* species group

The maximum among-group divergence of group AA and AB (1.8%) is less than that observed between groups AB and AC (2.5%) which are conspecific. Looking at the NJ tree we see that group AA and AB are more closely related than group AB and AC which leads us to think that groups AA and AB are either one species or are very closely related species

within the same genus. It is uncertain whether group AA is a separate species as all the specimens in this group are larvae and therefore one would most likely not be able to separate these specimens from specimens in group AB and AC based on morphology alone. The presence of identified adult specimens (*H. sommeri*) in both groups AB and AC as well as the divergence of 2.7% between these groups causes some uncertainty. If specimens belonged to the same species one would expect the sequence divergence to be less than that which is presently observed (2.7%). Reference to the distribution of the genus *Hypopholis* throughout South Africa showed that it is widespread throughout KwaZulu-Natal and well represented along the eastern parts of the Eastern Cape province (James du G. Harrison 2007, pers. comm.). Two colour forms exist within the adult specimens of *Hypopholis sommeri* - a light brown form and a black colour form. The segregation of groups AA, AB and AC could be attributed to the presence of different colours in the adult specimens of *H. sommeri.* As all specimens in groups AA, AB and AC were from the same region, one does not expect these morphological differences to be due to geography. All adult *H. sommeri* specimens in group AB and AC were light brown in colour but no adults were in group AA and therefore this inference cannot be substantiated at this stage. To investigate this hypothesis a dark form of *H. sommeri*, possibly from a museum collection, should be sequenced and the resulting sequence be compared to those of groups AA, AB and AC. This could then determine whether all specimens in groups AA, AB and AC are actually one species.

Alternatively, this grouping could be indicative of cryptic species. To resolve this uncertainty more adult specimens would need to be collected, identified morphologically and sequenced.

2.4.4 Neighbour Joining Tree versus Maximum Parsimony Tree

In this study, a slight difference was observed in the trees resulting from NJ and MP analysis. All groups recovered in the NJ analysis were recovered in the MP analysis with exactly the same specimens present in each group. The only difference being the placement of the different major clades on the different trees.

Parsimony methods compare all the possible trees and give each tree a score that is used to choose between different trees (Golding & Morton, 2003). In MP the number of

evolutionary changes necessary to explain the observed data are compared. The most parsimonious tree is the one with the least number of evolutionary changes (Golding & Morton, 2003). Distance methods, such as neighbour joining (NJ), calculate evolutionary distances between all taxa (Nei & Kumar, 2000). These distances are then compared and a phylogenetic tree is built based on the relationship between these values (Nei & Kumar, 2000).

Differences observed in tree topology could be due to the fact that during maximum parsimony only parsimony informative characters are used. This means that not all the sequence information is utilised when analysing the data. In this study 277 characters were parsimony informative. In effect, 42% of the information present in the sequences of 658 bp is utilised to construct the MP tree. However, NJ analysis uses all sites to calculate evolutionary distances between specimens.

2.4.5 The c*ox 1* region in DNA barcoding unidentified specimens

The purpose of any method of species separation is to identify reproductively isolated groups that are worth classifying as distinct species (Monaghan *et al*., 2005). Knowledge of species identities and biology is imperative when developing control methods for an agricultural pest. This process cannot be initiated if problematic species cannot be identified (Waugh, 2007). Assigning individuals to a particular species based on morphology is possible for well-known taxa but for uncommon taxa it can be difficult (Dalebout *et al*., 2004). This becomes problematic as taxonomic expertise are limited. The main problem is the ability to link the different morphological character systems of larvae and adults (Dirk Ahrens 2008, pers. comm.). Larval stages have few diagnostic characters which can be used to diagnose the huge diversity present in the tropics (Dirk Ahrens 2008, pers. comm.). Furthermore, data are not available for the majority of taxa for both larval and adult life stages as most species are described as adult (Dirk Ahrens 2008, pers. comm.). The use of molecular approaches overcomes this gap allowing rapid output of data for use of species identification and subsequently the design and implementation of control methods. In this study as well as other studies (Miller *et al.,* 2005; Webb *et al*., 2006; Ahrens *et al*., 2007) the use of mtDNA *cox*1 region for barcoding of insects has proven to be effective in linking unknown larvae with already identified adults. In general these studies reveal that DNA barcoding determines most species identities although some taxa are more difficult to identify than others. Hebert *et al.* (2003) proposed that the

analysis of sequence diversity in the *cox* 1 gene of mitochondrial DNA can serve as a global bioidentification system for all animals. Tautz *et al,.*(2003) suggested that many of the problems currently faced by traditional morphology-based taxonomy could be further resolved by using DNA sequences as a universal reference standard. To quote John Waugh (2007) DNA barcoding "may prove a useful tool for taxonomists and the many other agencies and individuals interested in species identification."

In this study DNA barcoding was used to initiate the process of identifying unknown species of Scarabaeidae larvae from areas of the South African sugar industry. In the past separation of larval species has relied on morphological differences. In some instances these morphological differences overlap between different groups. Therefore it is not always certain which specimens belong to which species group. To resolve this uncertainty molecular techniques were employed to group specimens based on molecular data. Similarly, Paquin & Hedin (2004) initially experienced difficulty differentiating between specimens of cave-dwelling spiders using solely morphology. They subsequently used DNA barcoding to differentiate between instars of cave-dwelling spiders. In this study on scarabaeids, prior to molecular work, larval specimens could be identified only to family level based on morphology. Afterwards we concluded that DNA barcoding substantiated these identities and linked unidentified larvae to identified adult specimens. As observed in Waugh's study (2007) and in this study, in most instances species differentiation was easily possible because intraspecific variation was an order of magnitude less than that which was observed interspecifically. DNA barcoding is likely to achieve the four scientific goals of taxonomy as defined by Seberg *et al* (2003) "to name, circumscribe, describe and classify species". Once the identity of a species is known, knowledge of its ecology and behaviour becomes more easily determined, and, should it be a pest species, this increased knowledge will prove invaluable in determining and implementing management strategies against it (Miller *et al.,* 1998). Furthermore Miller *et al.* (1998) stated that accurate identification of scarabaeid sugarcane pests at the larval stage is of importance before the development and implementation of pest management strategies can even commence.

Knowledge of larval life stages and their identification will allow timely detection of changes in population structure as well as insight into dominant species (Ahrens *et al.,* 2007). This information could be used to reduce the presence of dominant species by

applying control strategies before potential agricultural damage may occur (Ahrens *et al.,* 2007). The rate of acquisition of this knowledge, especially in sugarcane, is however delayed by the difficulties involved in making associations between life stages (Miller & Allsopp, 2005). The idea of molecular barcoding for taxonomic purposes is already a reality and the current study as well as others (Blaxter, 2004; Miller & Allsopp, 2005; Ahrens *et al.,* 2007) show its success. Miller *et al* (1997, 1998, 1999) developed strategies for the identification of larvae of Australian canegrubs (Coleoptera: Scarabaeidae) using traditional techniques for those species that were amenable to these data, and DNA sequence data for those that were indistinguishable. Pfenninger *et al.* (2007) agreed that DNA barcoding can be a valuable tool to increase accuracy, objectivity and comparability of the taxonomic assessment in ecological studies.

2.4.6 Proposed method when using larval specimens for both morphological and molecular analyses

There are a number of methods reported in the literature for the killing and preservation of insect larvae. Tantawi and Greenberg (1993) tested a range of different preservatives from the disciplines of entomology and pathology. They showed that all preservatives caused some degree of shrinkage when live larvae were placed into them and that heating larvae in boiling water prior to placing them in preservative prevented this shrinkage from occurring.

There are various killing solutions that also aid in fixing larval specimens. Fixation is the first stage of preservation and involves stabilizing the freshly killed specimen by either protein coagulation or the action of the fixative chemically combining with the specimen (Adams & Hall, 2003). The most widely used fixative and preservative solutions include Hood's solution, Kahle's solution and acetic alcohol (Adams & Hall, 2003). Fixation prevents cellular lysis, which would be induced by bacterial fauna and enzymes present in the gut of the larval specimen (Adams & Hall, 2003). An alternate way of killing is to immerse the larvae in very hot water for a short period (hot water killer (HWK)) which also fixes the specimen (Adams & Hall, 2003). Several methodologies on how to HWK larvae with respect to temperature of the water used and duration of immersion exist. Adams & Hall (1993) assessed a range of methods available in the literature. They concluded that the best method, with regards to maintaining specimens integrity, included

immersion in water at more than 80°C for at least 30 seconds and preservation in 80% ethanol.

In this study three legs (prothoracic, mesothoracic and metathoracic) of the larval specimens were used for DNA extraction but it was also imperative that specimens remained intact for later morphological analysis to create the larval field key. Various methods of preservation were tested. Firstly, living field collected larvae were rinsed twice in 100 ml cold water for 5 minutes and placed in 30 ml labelled vials before freezing them at –20^{o}C. This method was thought to be the best for molecular work but examination of the specimens at a later stage revealed that freezing caused these specimens to shrink and turn black in colour prohibiting accurate morphological examination at a later stage. In the second method, live field collected larvae were placed in 100 ml boiling water for 2 minutes and then preserved in 30 ml vials containing absolute ethanol. This method was favourable as specimens remained intact and it did not prevent isolation of DNA for possible molecular analysis. Based on the results at hand I recommend taxonomists to use this method for morphological work in the future and to preserve material for possible DNA work.

2.5 CONCLUSIONS

In this chapter we show the first association of larvae and adults, collected from sugarcane in South Africa, within the subfamilies Dynastinae, Melolonthinae and Rutelinae based on mtDNA sequences. These findings show the power of the *cox 1* region in barcoding unidentified scarabaeid pest species. The strong support for the nodes also confirms the clustering of specimens into groups. Whether these clusters accurately represent species boundaries needs to be investigated further by complementing this study with information from field studies of the sampled populations and broader genetic surveys that include identified adults of closely related species. This information would be required to confirm whether the groups defined by these nodes are defining species.

In five instances no larvae in the study linked to identified adult specimens. For nine groups no identified adult specimens linked to clusters containing only larvae. These identities could be resolved by:

(i) setting up light traps in sugarcane fields during the flight season of adult beetles to collect a range of specimens to which the unidentified larvae could possibly be linked; and

(ii) barcoding selected museum specimens whose collection locality corresponds to areas within the sugar industry.

These results will enable linking of larvae to adults by DNA barcoding. Larval morphological characters unique to the sequenced group can then be sought. If such characters can be established, morphological keys can be developed to identify these larvae to species level. A reliable morphological key could be used by field workers to identify scarabaeid larvae in the field. This would allow for the first time a clear understanding of the scarabaeid species diversity in sugarcane in South Africa. The ability to use mtDNA sequence data to associate the morphologically indistinguishable larvae to their respective adults will play a key role in planning and implementing management strategies of these pests within the sugar industry of South Africa. This will be due to the fact that once species can be correctly identified a more dependable study can be made of the species biology. Once this knowledge is ascertained it is easier to develop integrated control measures (using biological, cultural or chemical control parameters) that target certain ‘weak’ stages in the biology of the specific species.

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CHAPTER 3

MORPHOLOGICAL IDENTIFICATION OF SCARABAEID (COLEOPTERA) LARVAE IN SOUTH AFRICAN SUGARCANE

3.1 INTRODUCTION

Scarabaeids (Coleoptera) are damaging to sugarcane either in the adult or larval stage or in both (Gordh & Headrick, 2000). Adults are phytophagous, feeding upon the foliage and flowers of many species of plants, or feed as scavengers. Larvae are soil dwelling and feed on roots of plants sometimes used as food by the adult stage (Gordh & Headrick, 2000). In southern Africa *Heteronychus licas* Klug 1835 (Coleoptera: Scarabaeidae) feeds as an adult on the young sugarcane plants meristematic region and its larva on sugarcane root hairs (Way, 1995). In general, species with root-feeding larvae are regarded as pests (Gordh & Headrick, 2000).

Accurate identification of scarabaeid larvae is essential for understanding larval species biology (e.g. soil type and depth they occur in and at, oviposition preferences, host plants) and ecology (Miller *et al*., 19990). Control measures for scarabaeid pest species in sugarcane can be developed, successfully implemented and optimised once species have been identified (Miller *et al*., 1999). In the past identification of species has relied on the use of morphological characteristics for species discrimination (Miller *et al*., 1999). Due to phenotypic variation within a single species it is not always possible to solely base identifications on morphology (Hebert *et al.,* 2003). Phenotypic variation within scarabaeid larvae collected from sugarcane fields has caused difficulty in separation of presumed species. Absence of taxonomic keys for South African larval scarabaeid pests of sugarcane has exacerbated this problem (Way & du Toit, 1996). The solution to this problem has been the use of molecular techniques to link identified adult specimens to unidentified larval specimens (Chapter 2). The need for this work exists, as larvae are difficult to rear to adults in the laboratory due to their long life cycle and high mortality in captivity. Secondly, many adults are caught in light traps but this method is non-selective for only sugarcane pests as the adults are very mobile and therefore no direct links can be made between the adults caught and the larva present.

This study aimed at creating a morphological key based on larval groupings identified through molecular techniques (Chapter 2), which allowed unique morphological characteristic to be identified for those groupings. Once these characteristics had been defined they could be used in a key to separate specimens from each other. This grouping of larvae into species or subfamilies using molecular techniques provides the basis for the construction of a reliable morphological key, especially for larvae, that can be used by field workers to identify scarabaeid larvae found in the soil of sugarcane fields.

3.2 MATERIALS AND METHODS

3.2.1 Construction of the key

At the start of this study the possibility of creating a key using Lucid 3.4 was unknown. This only came to my attention while developing the field key at Transvaal Museum with James du G Harrison. It soon became apparent that because of its ease of use, it would be more appropriate to develop a key for larval scarabaeid species using Lucid 3.4 rather than a bulky field key. The idea behind the Lucid 3.4 key was to create a key which could be useful to scarabaeid specialists as well as the layman.

Ritcher's (1966) keys to white grubs and their allies were used to morphologically identify the specimens in this study to subfamily level. These keys as well as keys created by Sweeney (1967) and Ahrens *et al.*, (2006) were used as a starting point from which the keys (in this chapter) for South African scarabaeid larvae were created. However, to be able to use Ritcher's (1966) key, the head capsule of all larval specimens had to be removed from the abdomen. Mandibles and maxilla needed to be dissected out for inspection. These procedures are explained in section 3.2.1.1.

The flow diagram (Figure 3.1) illustrates the basis of the approach used to develop the key in this study. Molecular analyses grouped specimens (Chapter 2). Ritcher's (1966) keys were then used to identify specimens to subfamily level and thereby verify groupings obtained from molecular analysis. Subsequently, specimens were examined to determine which morphological features were characteristic and/or common to the species/morphospecies. Morphological features of each individual were tabulated. Using

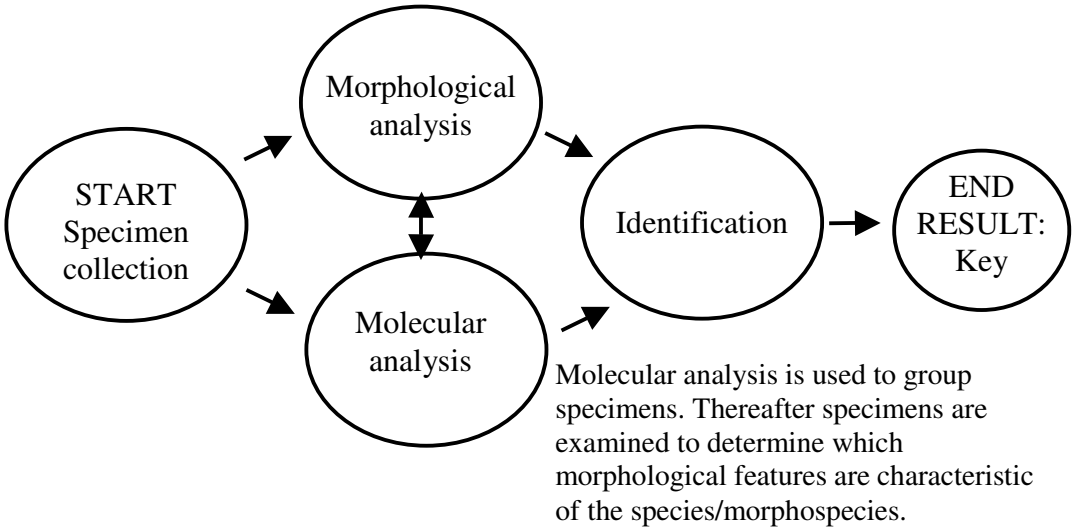


Figure 3.1 A schematic representation of the procedures followed to obtain the key.

molecular techniques it is more reliable to distinguish taxonomic groups and therefore this approach was performed in parallel with morphological techniques in this study.

Figure 3.2 shows the basic structure of the field key. To make this key useful for individuals working in sugarcane fields, the starting point for the key had to be at the phylum level. The key consists of a number of tables (Tables 3.1 – 3.7). Quite different arthropods belonging to different phyla are found on or in sugarcane soil in South Africa and these need to be identified to verify they are not scarabaeids. The arthropods were differentiated based on characters of the head, thorax, legs and antennae. Once these arthropods could be identified as belonging to the order Insecta, other characteristics could then be used to separate the Coleoptera from the remaining insects, and hence separate the family Scarabaeidae from the Curculionidae and Elateridae. From here the Scarabaeidae could be separated further into subfamilies based on characteristics of their anal openings, mandibles, antennae and tarsal claws.

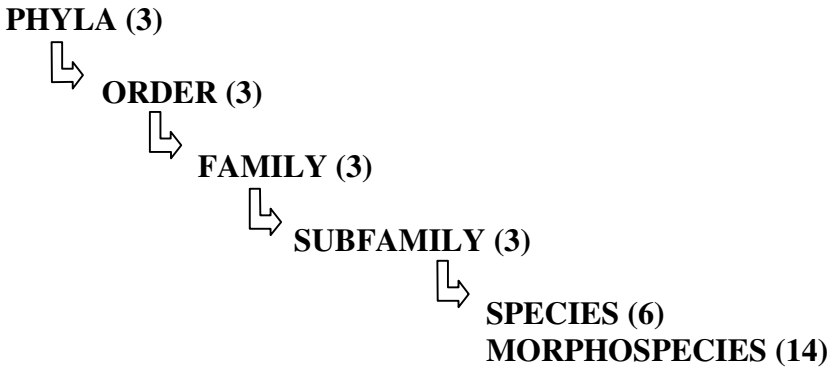


Figure 3.2. A diagram showing the taxonomic groupings used in this study (following Scholtz & Holm, 1996), with the number of groups at each hierarchical level used in the field key presented in this chapter given in brackets.

3.2.1.1 Dissection of the head capsule of larval specimens

The following method was adapted from the method used by James du G Harrison (pers. comm.) Before dissection, larval specimens were rinsed in water to remove any soil from the specimen. Using a scalpel the head capsule was removed from the thorax by carefully cutting around the head capsule where it joins the thorax. Subsequently the head capsule was placed in a glass vial containing lactic acid for 24 hours. Lactic acid degrades the muscles which are responsible for maintaining the position of the mandibles, maxilla and epipharynx within the head capsule. This allowed for removal of the intact mandibles, maxilla and epipharynx from the head capsule (Refer to Figures 1.5 and 1.7-1.9 in Chapter 1 for clarification of these terms). Closed forceps (number 5) were inserted from the anterior side of the head capsule between the two mandibles. The forceps were gradually opened forcing the mandibles apart. Mandibles were then removed from the head capsule and excess muscle strands were removed using a scalpel. Following this an incision was made below the labium to remove the maxillae and labium from the epipharynx. Antennae remained attached to the head capsule to avoid confusion between dorsal and ventral orientation of sensory spots. Dissected components of the head capsule were stored in absolute ethanol in 0.5ml PCR tubes. These tubes were placed in the vial with the corresponding larval abdomen.

3.2.1.2 Tabulation of larval morphological characteristics

Scarabaeid larvae were distinguished by initially compiling a table (Appendix B) listing the features visible mostly with a hand lens (magnification 3x) and thereafter a stereo microscope (magnification ranging from 10x to 40x). As many larval specimens as available, belonging to the same species, were examined and characters that were easily seen with the stereo microscope or hand lens were used to create the key. The purpose of the key was to aid larval identification during field work and therefore features readily seen with the naked eye or a hand lens were preferably sought. Microscopic larval characteristics were only used when necessary. Therefore some of the characters used in the Lucid 3.4 key cannot be used by field workers. A LEICA ZOOM 2000 stereo microscope was used to examine the microscopic features. The anatomical terminology used in both keys was based on Böving (1936) and Ritcher (1966). Photographs of morphologically important features, such as mandibles, raster and head capsule, were taken using a LEICA MZ16 Light Microscope. These photographs were used in developing the key.

3.2.2 Field Key

3.2.2.1 Background

A key is a tool that can be used for the identification of an unknown organism (Anonymous, 2004). Usually a specialist in a certain field creates a key by putting together information about a certain organisms (Anonymous, 2004). Two main types of keys exist (i) dichotomous keys and (ii) matrix keys.

The field key is presented in the results section of this chapter. Line drawings as well as photographs are included as they supplement the written descriptions, thereby circumventing the need to have knowledge of Coleopteran taxonomy. The key was designed in A4 format, so that pages can be laminated, bound and then used in the field to identify larval scarabaeids found there.

3.2.2.2 How to use the field key

The main objective of this study was to produce a key for use as a tool for non-entomological field workers. For this reason the key in this document starts at a simplistic level, that more experienced entomologists could ignore. Table 3.1 deals with higher level taxonomy to separate phyla that could be found in the soil, and Table 3.4 - 3.7 the lower level taxonomy required to key out to the scarabaeid species and morphospecies, which are the subject of this dissertation.

In Tables 3.1 – 3.3 the Scarabaeidae is shaded in light grey. Tables 3.4 – 3.7 use colour for the scarabaeid taxa corresponding to the respective colours used in Chapter 2 for the three subfamilies of interest (i.e. Red = Dynastinae; Blue = Melolonthinae; Green = Rutelinae). The shaded groups are those of particular interest in this study, but other groups were included for completeness as these groups are encountered in sugarcane fields. In Table 3.1 insects, spiders, mites and millipedes can be separate from one another based on characters of their respective head, thorax, abdomen, legs and antennae. Table 3.2 separates larvae of the three insect groups commonly occurring in sugarcane field soils namely beetles (Coleoptera), flies (Diptera) and caterpillars (Lepidoptera). These are separated on characteristics of the head, abdomen segmentation and legs. Table 3.3 focuses on separating larvae belonging to Coleopteran families commonly found in sugarcane soils. Here the Scarabaeidae (leaf chafers and dung beetles), Curculionidae (weevils) and Elateridae (click beetles) are separated based on the body form and consistency and presence or absence of legs. Table 3.4 groups specimens of the Scarabaeidae into the subfamilies Dynastinae, Melolonthinae or Rutelinae. This table makes use of morphological characteristics used in Ritcher's (1966) key to identify larvae to subfamily level. Characters such as the shape of the anal opening, presence or absence of a stridulatory area on the mandible, number of dorsal sensory spots present on the antenna and shape and length of tarsal claws were used. Tables 3.5- 3.7 separated specimens within the subfamilies Dynastinae, Rutelinae and Melolonthinae respectively.

There is value in using line drawings together with photographs when showing morphological features in keys as they complement each other. The tables include line drawings as well as photographs because workers in sugarcane fields generally have limited knowledge of the groups. The shaded columns of the field key indicate the

arthropod groups of interest in this study but other groups were included as these organisms may be encountered in sugarcane fields.

By developing the field key, it aided in deciding which characters would be most useful to use for the Lucid 3.4 key, and how they could be found.

3.2.3 The Lucid 3.4 Key

The type of key created using Lucid 3.4 has numerous advantages:

(i) it can contain electronic images and line drawings to make the key easy to use for any individual, especially those not familiar with the relevant terminology;

(ii) the user can select any character state to initiate the identification process and need not start the identification process with a certain character as in traditional dichotomous keys;

(iii) the key can be made accessible to all individuals by placing it on a website,

(iv) updating of information and elaboration of the key is simple due to its electronic nature.

An added advantage is that this Lucid 3.4 key can be placed on the South African Sugar Association website for all farmers and Pest and Disease Officers to access and use. Most sugarcane growers and Pest and Disease Officers have access to computers. This means that they would be able key out scarabaeid larvae found in the sugarcane field in their offices at the computer with the specimen in their hand, where they could also have access to a microscope. Finally the Lucid 3.4 key allows continual development and additions by specialists at the South African Sugarcane Research Institute, universities and taxonomists at museums working on scarabaeid taxonomy. An alternate aim would be to have keys to all sugarcane insect pests in the Lucid 3.4 database.

3.2.3.1 Background

Lucid 3.4 belongs to a group of products designed for the development, deployment and use of interactive computer-based identification keys (Anonymous, 2004). Lucid 3.4 was developed in 1994 at the Cooperative Research Centre for Tropical Pest Management (University of Queensland, Australia) (Anonymous, 2004). This was in an effort to train

entomology students to identify insect Orders using a computer based key (Anonymous, 2004). It was clear that creating a key only to identify insect Orders would require much time and effort, and therefore a more generic key structure was developed, which could be used as the basis when creating any more detailed taxonomic key (Anonymous, 2004). The Lucid 3.4 suite of products consists of a Lucid 3.4 Builder and a Lucid 3.4 Player. The Lucid 3.4 Builder is used to create the specific taxonomic key whereas the Lucid 3.4 Player is distributed to users who then use the player to identify their specimen or organism.

3.2.3.1.1 Dichotomous keys

Dichotomous keys are the most widely used keys which rely on the user answering questions about the specimen (Anonymous, 2004). These questions are arranged in a series of couplets. When a question is answered the user proceeds to a number indicating the following question to be answered. The key continues in this way until the name of a species or taxon is given (Anonymous, 2004). A common problem with these type of keys is the unanswerable couplet – the user has no choice about the order in which characters have to be dealt with and in instances where the question is difficult or impossible to answer, the user cannot continue and the identification process ends (Anonymous, 2004). This problem can be solved by using matrix keys such as used in Lucid Phoenix.

3.2.3.1.2 Matrix keys

The main advantage of this type of key is that the user can decide to start at any point and advance in any order (Anonymous, 2004). The user therefore avoids features which are difficult to resolve and those characters which are not applicable to the specimen (Anonymous, 2004). Computers are mainly used for these types of keys (Anonymous, 2004). By using a matrix key the user might be able to eliminate all the taxa except those that match the unidentified specimen (Anonymous, 2004).

An example of a matrix key is Lucid 3.4. Lucid 3.4 is a software package which allows the creation of a computer-based, multi-media, matrix key (Anonymous, 2004). The builder of the key chooses features that best describe and differentiate between the taxa included (Anonymous, 2004). The database contains all the information about the taxa that are to be

identified. When the user chooses a state or feature, the key discards those taxa which do not have that state or feature and only those taxa having that state or feature are retained (Anonymous, 2004). The Lucid package consists of a "builder" and a "player". The "builder" allows the developer of the key to build the key and the" player" enables the users to view and interact with the key (Anonymous, 2004). Users wanting to identify an entity using the key for identification choose features that match their specimen (Anonymous, 2004).

3.2.3.2 How to use the Lucid 3.4 key

When commencing identification and the Lucid 3.4 player is opened the user sees a window with four lists (Figure 3.3):

(i) **Features available** – features (characteristics of the entities in the key that can be used to describe each entity) that describe the specimen

(ii) **Features chosen** – features that have been chosen by the user

(iii) **Entities remaining** – all entities (the items the key aims to identify e.g. taxon; species of flies) in the key that can still be used in identification

(iv) **Entities discarded** – entities that have been excluded from the key

Initially, when the user starts the key the 'features chosen' and 'entities discarded' windows are empty (Figure 3.3) (Anonymous, 2004). To commence identification the user chooses a feature by placing a tick in the box alongside the appropriate state (Figure 3.4). The Lucid 3.4 player then removes entities that do not match the chosen feature from the 'entities remaining' window. As identification progresses and more features are selected the 'entities remaining' list will be reduced until eventually a single entity remains and the specimen is identified (Figure 3.4) (Anonymous, 2004). Additionally, the key also assists users to identify specimens with the aid of pictures and images, which clarify features and their states (Anonymous, 2004).

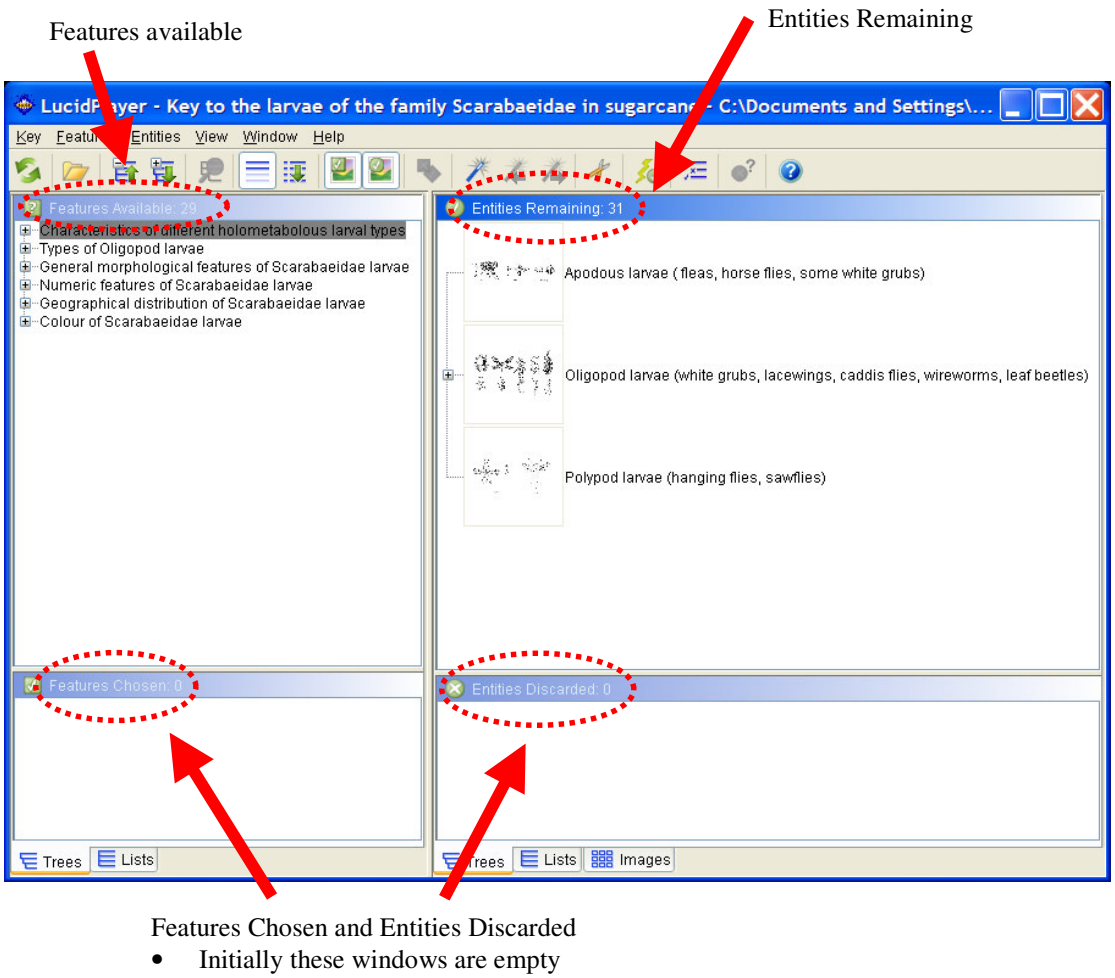


Figure 3.3 The initial screen when opening the *Key to the larvae of the family Scarabaeidae in sugarcane* showing the four lists (Features available, Entities Remaining, Features Chosen and Entities Discarded).

The *Open all* button and the *Collapse all* button (Figure 3.4) will either open all nodes or collapse all nodes. The open all is useful when starting identification as the user is able to see all the features available to choose before commencing identification. This allows the user to start the identification process by selecting features that are easily identifiable to him/her. To use these two buttons the user must select one of the four windows (Features available, Features chosen, Entities remaining or Entities discarded) which he/she would like to 'open' or 'close'.

Users are able to adjust the size of font which the key is displayed in to allow for easy readability (Figure 3.5). By selecting the *View* pull-down menu and the *Font Size* option, the user can select a range of font sizes from smallest to largest.

More detailed options available in Lucid 3.4 player are accessible from the "Help" section within the Lucid 3.4 player or from the following web site – www.lucidcentral.org.

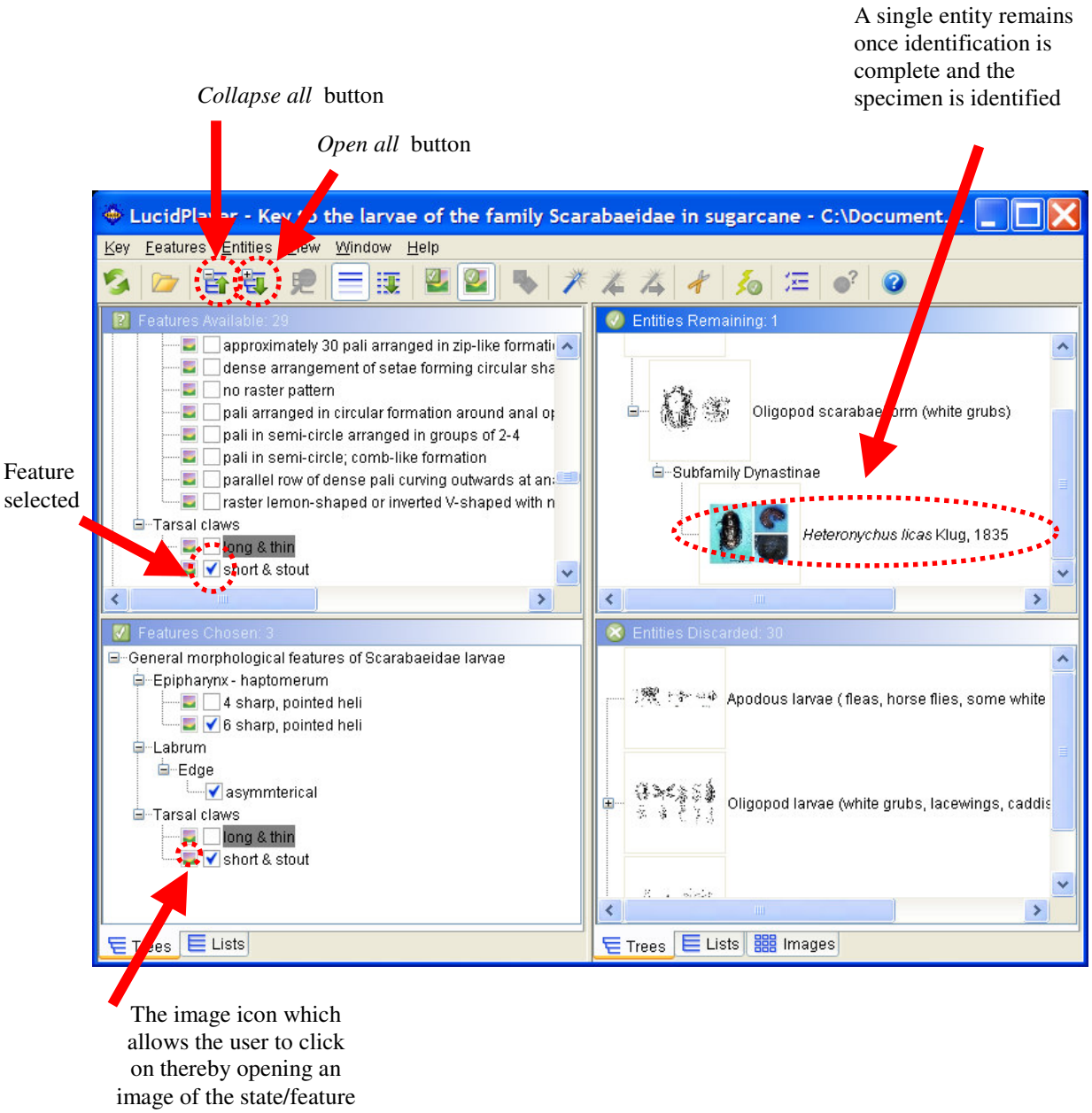
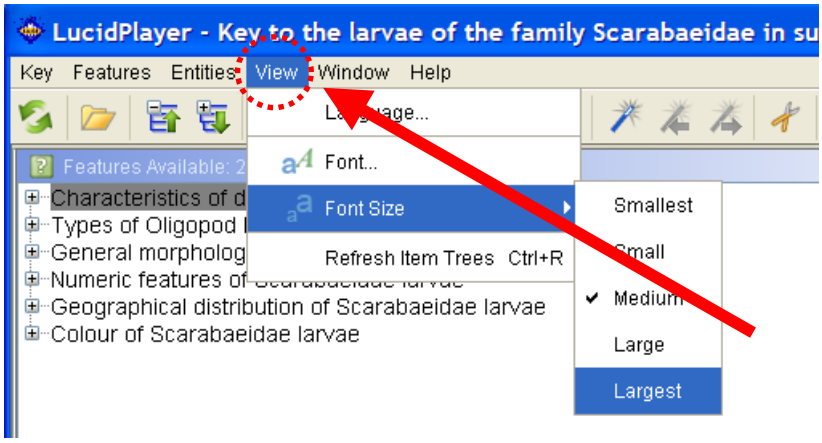


Figure 3.4 The screen visible once a larva has been identified using the *Key to the larvae of the family Scarabaeidae in sugarcane*.



The *Font size* option from the *View* pull down menu allows the user to adjust the font for easy readability

Figure 3.5 The *View* pull down menu where the font size can be adjusted.

3.2.3.3 Guidelines for making an Identification *(adapted from the Lucid 3.4 manual)*

Lucid 3.4 allows the user to choose any feature and its state to start identification from, but working through the key in an ordered manner makes identification easier. The following are advised (Anonymous, 2004).

(i) **Familiarity with the specimen** – familiarity with the characteristics of the specimen as well as the key will make identification easier.

(ii) **Note and use distinctive feature** – Some taxa possess distinctive features which may allow the taxon to be identified quickly. It is suggested that these be used first.

(iii) **Answer easy features first** – examine the list of features available and answer those that are easily seen on the specimen to be identified first. In the opening window of Lucid 3.4 the list of features appear in an initial order. The user can select any feature from any position in the list. It is recommended that the user looks at the list of 'Features available' and starts the identification by using features that are obvious.

(iv) **Choosing multiple states** – if the user is uncertain which state is the correct one it is recommended that multiple states are chosen so that the target taxon remains in the 'Entities remaining' list.

3.3 RESULTS

3.3.1 Field Keys

Figure 3.6 gives an overview of the taxa which the key identifies in phylum, order, family, subfamily, species and morphospecies categories and refers the reader to the relevant table. The morphospecies designation correspond to the group names from Chapter 2 (Morphospecies 1 = Group A; Morphospecies 2 = Group B; Morphospecies 3 = Group E; Morphospecies 4 = Group K; Morphospecies 5 = Group L; Morphospecies 6 = Group M; Morphospecies 7 = Group O; Morphospecies 8 = Group Q; Morphospecies 9 = Group R; Morphospecies 10 = Group T; Morphospecies 11 = Group U; Morphospecies 12 = Group Y; Morphospecies 13 = Group Z; Morphospecies 14 = Group AA).

A variety of arthropods are found at or near the soil surface. They could belong to four phyla: Insecta; Arachnida; Acari and Diplopoda (Table 3.1) identified by the following differences: number of body parts, number of legs and antennae. If the arthropod collected belongs to the phylum Insecta identification can proceed by using Table 3.2. Should this not be the case the identification process ends. Table 3.2 differentiates the three insect larval groups commonly occurring in the soil of sugarcane fields, namely Coleoptera (beetles), Diptera (flies) and Lepidoptera (caterpillars). These are differentiated based on characteristics of the head, segmentation of the abdomen and legs. Table 3.3 focuses on separating larvae belonging to the Coleopteran families. Here the Scarabaeidae (leaf chafers and dung beetles), Curculionidae (weevils) and Elateridae (click beetles) are separated based on their body form and consistency and presence or absence of legs. Table 3.4 groups specimens of the Scarabaeidae into the subfamilies Dynastinae, Melolonthinae or Rutelinae. This table uses morphological characteristics described in Ritcher's (1966) key to identify larvae to subfamily level. Characters such as the shape of the anal opening, presence or absence of a stridulatory area on the mandible, number of dorsal, sensory spots present on the antenna and shape and length of tarsal claws. The following three tables (Table 3.5- 3.7) separated specimens within the subfamily Dynastinae, Rutelinae and Melolonthinae respectively. Table 3.5 identifies specimens belonging to the subfamily Dynastinae to species level, (*H. licas*, and to Morphospecies 1 and 2). In this table *H. licas* specimens can be separated from specimens belonging to Morphospecies 1 and 2 by the presence or absence of pigmented spots on the head laterad to each epicranial suture, the

length of the mandibular setae and the width of the base of the mandible. Table 3.6 identifies ruteline specimens to either *A. ustulata* (tribe Anomalini) or Morphospecies 3 (tribe Adoretini). Only specimens belonging to the subfamily Rutelinae could be identified to tribe level. Ruteline specimens are separated based on characters of mandible and the raster. Specimens belonging to the species *A. ustulata* exhibit a raster in zip-like formation and a square molar lobe whereas specimens belonging to Morphospecies 3 have a raster which is an inverted v-shape and a trilobed molar lobe. Table 3.7 differentiates melolonthine specimens into seven categories : *H. sommeri* and Morphospecies 12, 13 and 14; *Camenta* sp.; *S. affinis*; *Schizonycha* sp.; Morphospecies 4, 5 and 6; Morphospecies 9, and, Morphospecies 10 and 11. These specimens are differentiated using the raster. Additionally Morphospecies 10 and 11 can be separated from the other melolonthines by the presence of enlarged stipes which are bulbous.

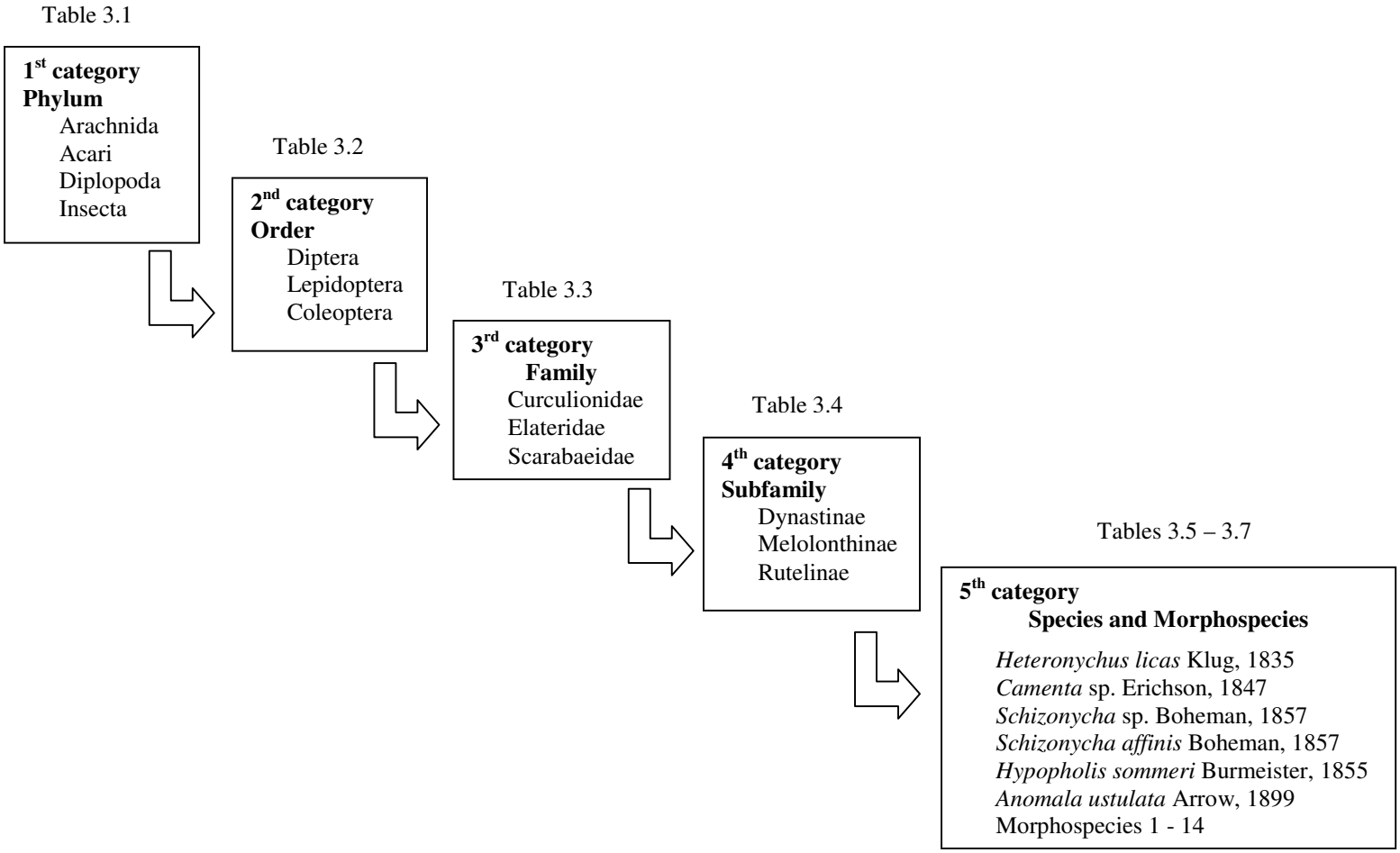


Figure 3.6 The breakdown of the species and morphospecies determined from the specimens found in soils of the sugarcane areas sampled in this study, according to the system of nomenclature described in Figure 3.2.

Table 3.1. Key features of common arthropod Phyla encountered on or in soil in South African sugarcane fields

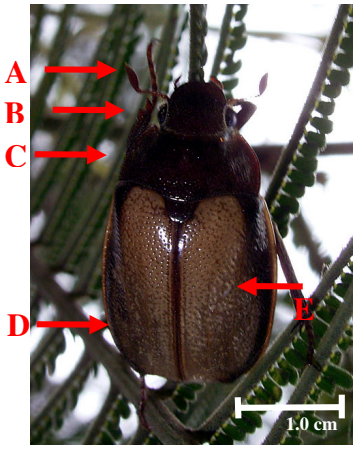
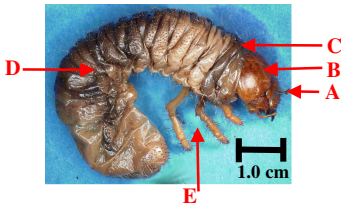
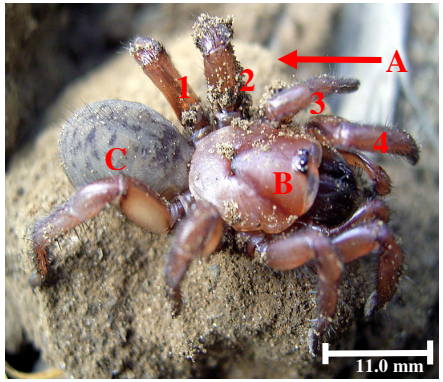
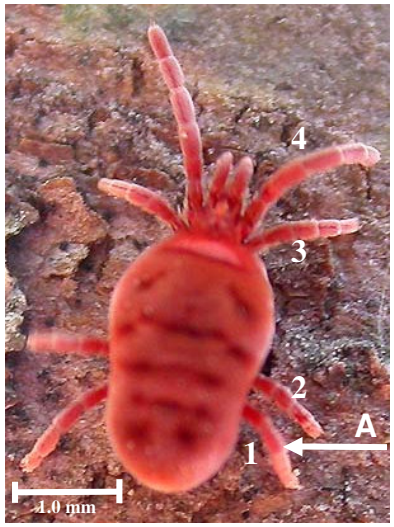
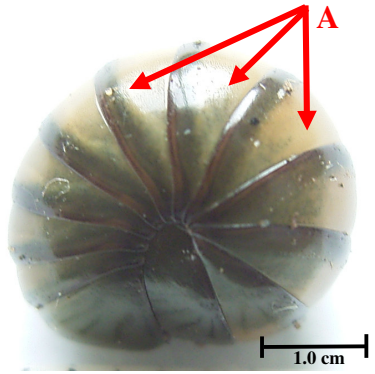
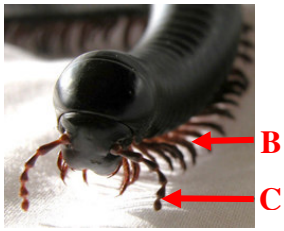
Insecta (Insects) *Go to Table 3.2*	**Arachnida (Spiders)**	**Acari (Mites)**	**Diplopoda (Millipedes)**
 Photograph by Way (2005) 	 Photograph by Way (2007)	 http://magickcanoe.com/blog/2006/05/09/she-wore-red-velvet/	 Photograph by Way (2007)  http://www.seattlebugsafari.com/millipedes_centipedes.htm
3 body parts (head, thorax, abdomen); 3 pairs of legs; Antennae A= antennae; B= head; C= thorax; D= abdomen; E=leg	2 body parts (cephalothorax & abdomen); 4 pairs of legs; No antennae A= legs (1-4); B= cephalothorax; C= abdomen	1 body part; 4 pairs of legs; No antennae A = legs (1-4)	Short wide bodies; flattened or rounded; many segmented; one or two pairs of legs on each segment; Antennae 7-segmented A = many segments; B = legs; C = antenna

Table 3.2. Key features of larvae of common insect Orders found on or in soil in South African sugarcane fields

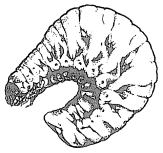
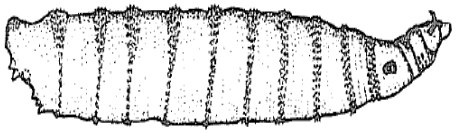
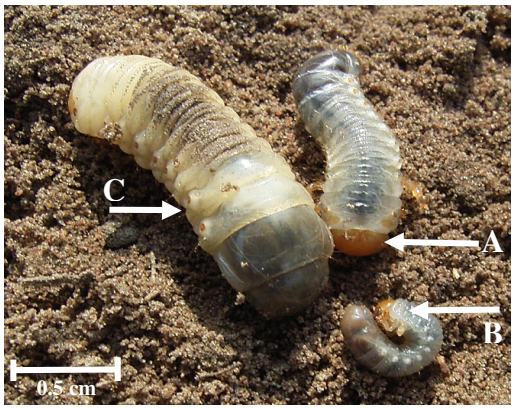
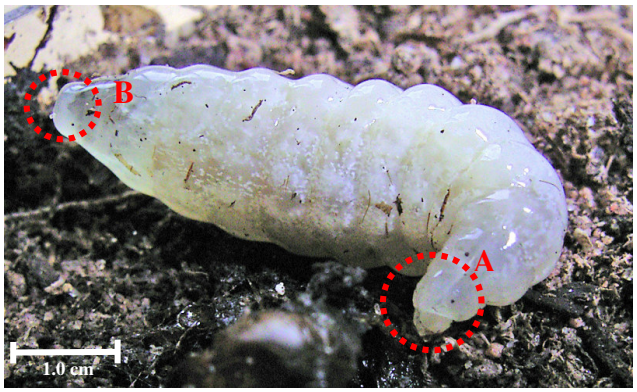
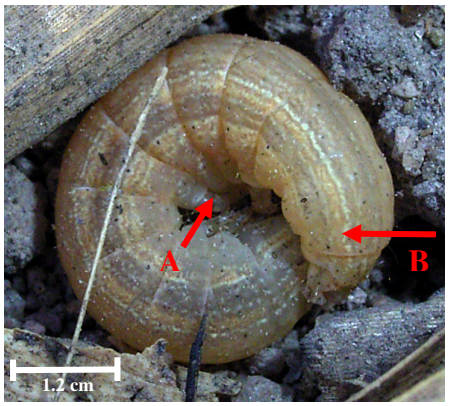
Beetles (Order Coleoptera) *Go to Table 3.3*	**Flies** (Order Diptera)	**Caterpillars** (Order Lepidoptera)
 Adapted from Scholtz & Holm (1996)	 Adapted from Scholtz & Holm (1996)	 Adapted from Scholtz & Holm (1996)
 Photograph by Way (2007)	 Photograph by Way (2007)	 Photograph by Way (2007)
C-shaped, Stout cylindrical, soft-bodied, Head strongly sclerotised (yellow, red or brown); Body whitish/greyish; Legs weakly developed A = head; B = thorax; C = abdomen	Soft bodied, no visible head capsule, lacks true legs. Sclerotised mouth hooks at anterior end. Sometimes pair of sclerotised spiracles visible at posterior end A = mouth hooks; B = spiracles	Soft bodied, Well-developed head, 3 pairs of well-developed thoracic legs; abdominal prolegs present A = abdominal prolegs; B = abdomen

Table 3.3 Key features of the larvae of the most common Coleopteran families encountered on or in soil in South African sugarcane fields

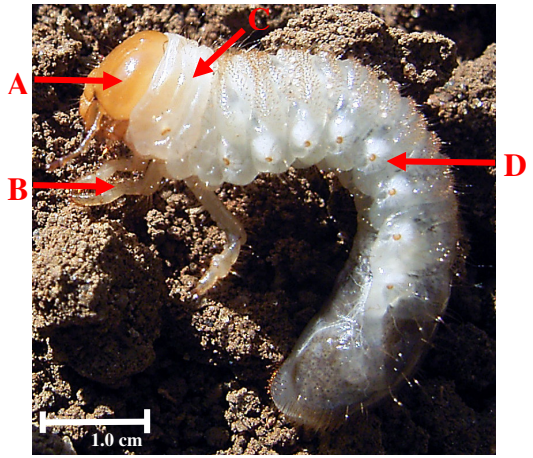
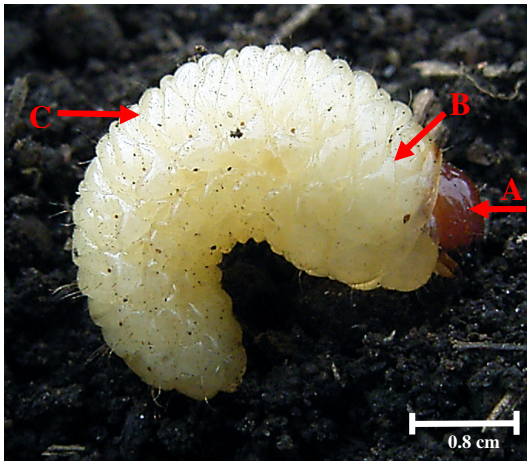
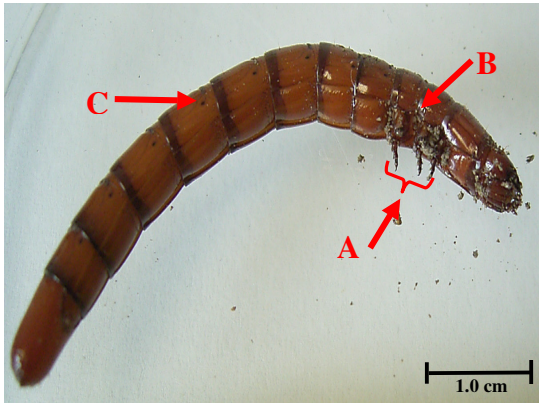
Scarabaeidae (leaf chafers, dung beetles) *Go to Table 3.4*	**Curculionidae** (weevils)	**Elateridae** (click beetles)
 Photograph by Way (2007)	 Photograph by Way (2007)	 Photograph by Way (2007)
C-shaped, Stout, cylindrical, soft-bodied; Strongly sclerotised head capsule; Weakly developed legs; Whitish/greyish body colour A = head capsule; B = legs; C = thorax; D = abdomen	C-shaped soft body; Sclerotised head capsule; Legless; Whitish body colour A = sclerotised head; B = thorax; C = whitish abdomen	Smooth, hard and shiny body; Short legs A= legs; B = thorax; C = abdomen;

Table 3.4. Key features of the larvae of the most common subfamilies of Scarabeidae encountered on or in soil in South African sugarcane fields (Developed from the keys of Richter (1966))

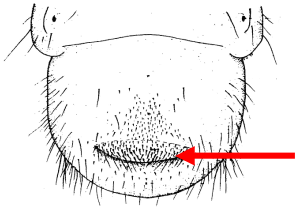
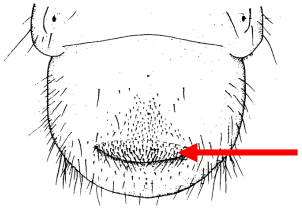
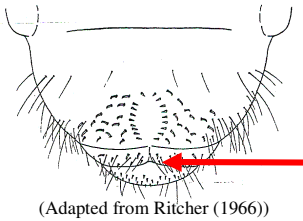
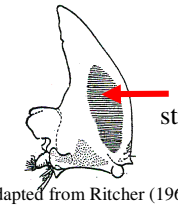
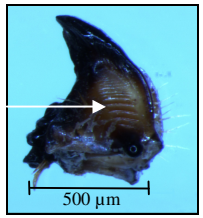
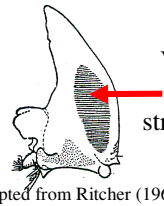
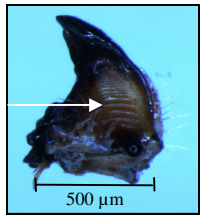
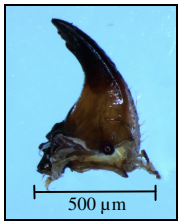
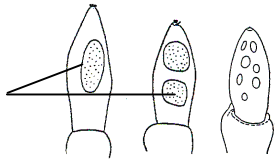
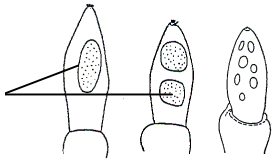
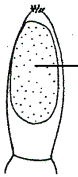
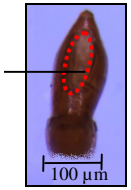
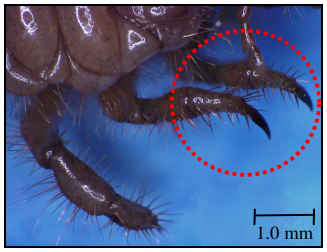
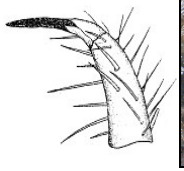
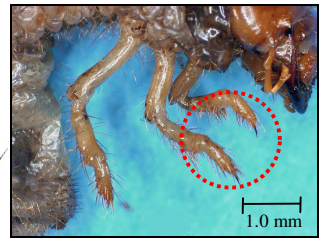
	Dynastinae *Go to Table 3.5*	**Rutelinae** *Go to Table 3.6*	**Melolonthinae** *Go to Table 3.7*
Anal opening (on last abdominal segment)	 Anal opening a transverse arc (Adapted from Oberholzer (1963))	 Anal opening a transverse arc (Adapted from Oberholzer (1963))	 Anal opening inverted Y-shaped (Adapted from Ritcher (1966))
Mandible	 Ventral, oval, stridulatory area  (Adapted from Ritcher (1966))	 Ventral, oval, stridulatory area  (Adapted from Ritcher (1966))	 No stridulatory area  (Adapted from Ritcher (1966))
Antennae	Last antennal segment 1 or more dorsal sensory spots  (Adapted from Ritcher (1966))	Last antennal segment 1 or more dorsal sensory spots  (Adapted from Ritcher (1966))	 One dorsal sensory spot  (Adapted from Ritcher (1966))
Tarsal claws	Claws short and stout  (Adapted from Sweeney (1967)) 	Claws long & thin  (Adapted from Sweeney (1967)) 	

Table 3.5. Key features of larvae identified to genus and species/morphospecies levels within the Dynastinae encountered in soils of South African sugarcane fields

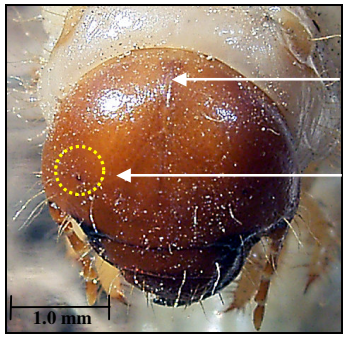
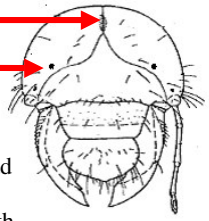
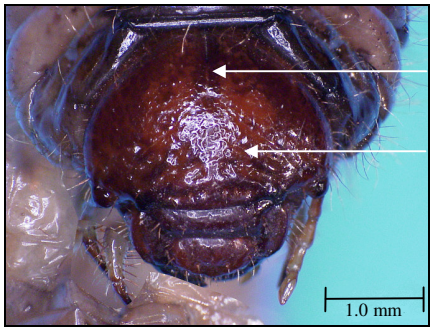
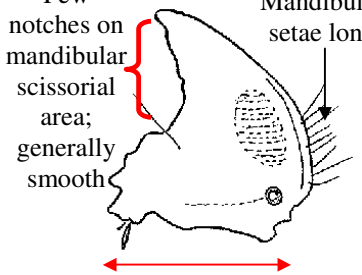
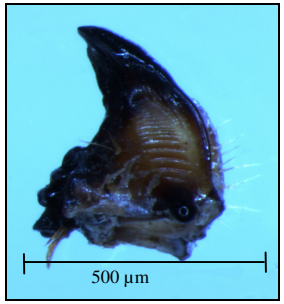
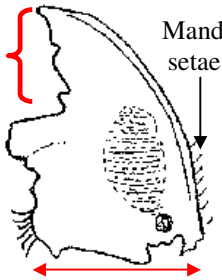
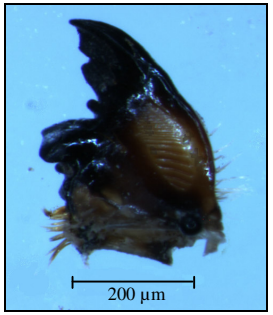
	Heteronychus licas	**Morphospecies 1 & 2**
Head capsule	 Photograph by Way (2007) Epicranial suture Pigmented, indented spots visible on head laterad to each; head capsule smooth in texture  (Adapted from Ritcher (1966))	 Epicranial suture distinct Head capsule rough/ pitted
Mandible	Few notches on mandibular scissorial area; generally smooth Mandibular setae long  Base of mandible broad 	Many notches on mandibular scissorial area Mandibular setae short  Base of mandible narrow 

Table 3.6. Key features of larvae identified to tribe, genus and species/morphospecies levels within the Rutelinae encountered in soils of South African sugarcane fields

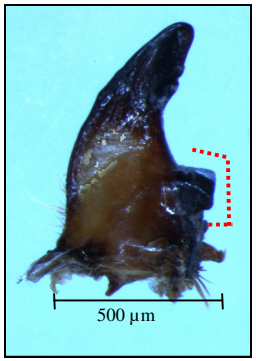
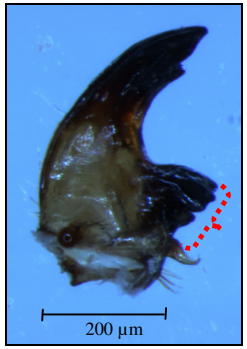
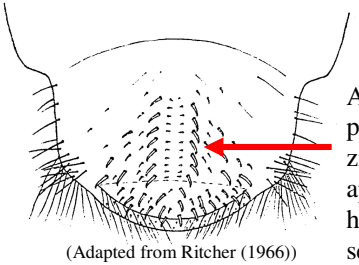
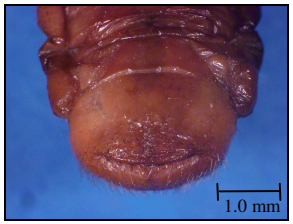
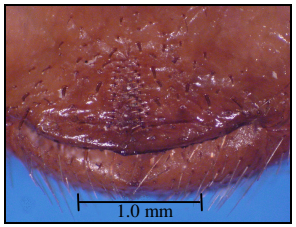
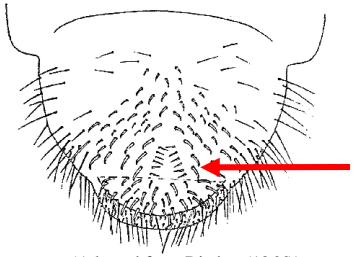
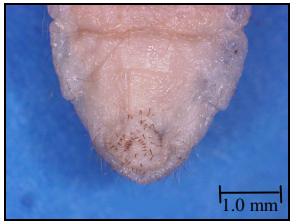
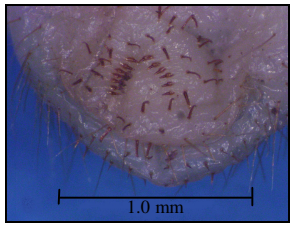
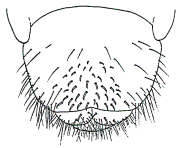
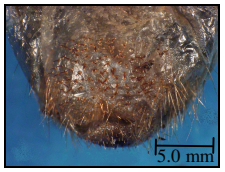
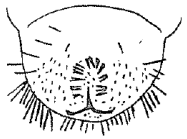
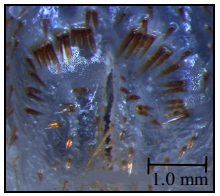
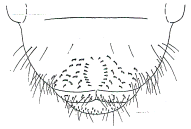
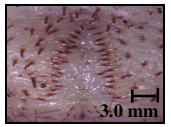
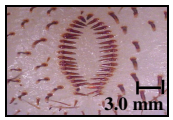
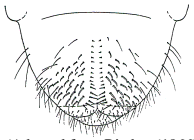
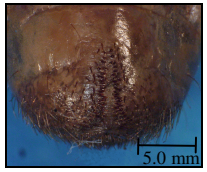
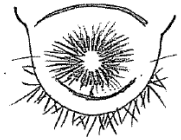
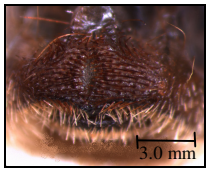
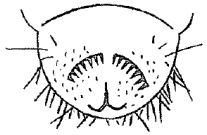
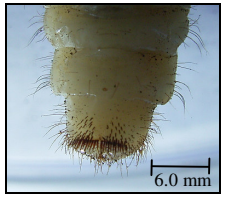
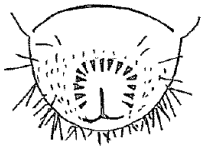
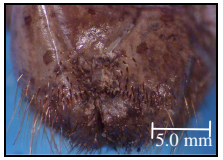
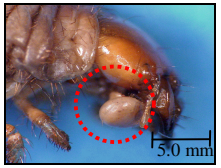
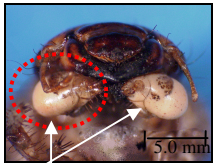
	***Anomala ustulata* (Tribe Anomlini)**	**Morphospecies 3 (Tribe Adoretini)**
Mandible	  Molar lobe square	  Molar lobe trilobed
Raster pattern (on last abdominal segment)	 (Adapted from Ritcher (1966)) Approximately 30 pali arranged in a zip-like formation; approximately 60 hamate (hooked) setae  Hamate setae  	 (Adapted from Ritcher (1966)) 12-14 pali arranged in an inverted V-shape; 50-60 hamate (hooked) setae  Hamate setae  

Table 3.7. Key features of larvae identified to tribe, genus and species/morphospecies levels within the Melolonthinae encountered in soils of South African sugarcane fields

	***H. sommeri* Morphospecies 12, 13 & 14**	***Camenta* sp.**	***S. affinis***	***Schizonycha* sp.**	***Morphospecies* 4, 5 & 6**	***Morphospecies* 9**	***Morphospecies* 10 & 11**
Raster Pattern (on last abdominal segment)	No raster patter, setae scattered randomly around anal opening  (Adapted from Ritcher (1966)) 	Pali arranged in a semi circle; Pali in groups of 2-4 and some single  	Raster lemon shaped or inverted v-shaped; number of pali ranging from 12-22; pali dense  (Adapted from Ritcher (1966))  	Parallel row of dense pali curving outwards at anal opening and becoming double; 17-24 pali;  (Adapted from Ritcher (1966)) 	Raster consisting of a dense arrangement of setae forming a circular shape  	Pali arranged in a semi circle; comb-like formation; above the anal opening  	Raster consisting of pali arranged in a circular formation around the anal opening  
Maxilla							  Enlarged stipes which are bulbous

Glossary of terms used in the key

Cranium: the sclerotized portion of the head capsule except the neck (Gordh & Headrick, 2000).

Hamate: barbed; pertaining to structures furnished with hooks or barbs (Gordh & Headrick, 2000).

Heli: (helus) a coarse, fixed spine without a cup (Gordh & Headrick, 2000).

Ocelli: lateral simple eyes in larval holometabolous insects (Gordh & Headrick, 2000).

Palidia: found on scarabaeid larvae, a group of pali arranged in one or more rows. Medially placed across the venter in front of the lower anal lip; paired and extending forward and inward from one of the ends of the anal split; paired and extending straight, acutely or obliquely forward from inside of one of the ends of the anal slit. Pali usually recumbent with apices directed toward the septula (Gordh & Headrick, 2000).

Setae: hollow, often slender, hair-like cuticular projections produced by epidermal cells of the integument (Gordh & Headrick, 2000).

Tarsi: the insect's foot (Gordh & Headrick, 2000).

3.3.2 Lucid 3.4 Key

The *Key to the larvae of the family Scarabaeidae in sugarcane* can be accessed from the Lucid central web page at the following link http://idlifedev.cbit.uq.edu.au/server-player/player.jsp?datasetId=scarab. This key combines general features of larval specimens, which can be observed with the naked eye or a hand lens, as well as taxonomically specific features, which require dissection of the head capsule and use of a microscope for examination. The advantage of this matrix type key is that the user is able to select features according to his/her knowledge or level of expertise and is able to use these for identification of a specimen.

This key starts with six features: Characteristics of different holometabolous larval types; Types of Oligopod larvae; General morphological features of Scarabaeidae larvae; Numeric features of Scarabaeidae larvae; Geographical distribution of Scarabaeidae larvae; Colour of Scarabaeidae larvae. These features have a group of further features which can be arrived at by using the *Open all* button. Should a user be unfamiliar with entomological taxa he/she would be recommended to begin identification by determining whether the specimen belongs to the family Scarabaeidae. This would be achieved by comparing the stated criteria of the *Characteristics of different holometabolous larval types* to those of the unidentified specimen. Should the specimen belong to the group of Oligopod larvae, containing the Scarabaeidae, further examination could then lead to a further, more specific identification. If the specimen still belongs to the family Scarabaeidae the further *Features Available* could be used in order of personal preference to arrive at an identification. Users with more entomological experience are able to omit these initial steps and have the option of commencing identification by comparing any of the listed *Features Available* to those of the unidentified specimen.

This Lucid 3.4 Key is more reliable than the field key due to the incorporation of a variety of characteristics of scarabaeid larvae occurring in South African sugarcane. Here morphological features are included - general and specific, geographical locality, size features, colour, and images of larvae and adult species.

3.4 DISCUSSION

Although the scarabaeid adults are identifiable, little work has been done on the identification of their larvae to below family level in South Africa (Oberholzer, 1959; Petty, 1978; Petty, 1976; Petty 1977), especially in sugarcane. There was thus a need for taxonomic descriptions, to as close to species level as possible, of the most economically important groups within the sugar industry. The shortcoming in the taxonomy of these groups was identified at a White Grub Working Group meeting in 1996 (Way & du Toit, 1996).

There are published keys available such as Richter (1966), Sweeney (1967) and more recently Ahrens *et al.* (2006). However, they deal in general with scarabaeids found in North America, Swaziland and Nepal. This is not a problem if determining the identity of a specimen to family and subfamily level, because the Scarabaeidae are widely distributed (Ritcher, 1966) and characters used to identify specimens to this level are robust and thus reliable to be used world-wide. Keys published in France (Paulian, 1941), India (Gardner, 1935), Russia (Golovianko, 1936) and the Philippine Islands (Viado, 1939) are examples of comprehensive studies separating scarabaeid larvae to family, subfamily and genus level (Ritcher, 1966). It is however problematic when scarabaeid larval specimens from South Africa need to be identified to species level, as no such keys presently exist.

To address this problem, a field key as well as the Lucid 3.4 key were developed. These concentrated on scarabaeid species occurring mainly in the sugarcane growing areas of the KwaZulu-Natal midlands.

Chapter 2 linked larvae to adults by means of their mtDNA sequences. Once these relationships were established based on genetics, larval morphological characteristics unique to these certain groupings and/or species were used as a means to identify field collected individuals. A morphological key was developed based on the results of these findings. This key was developed with the intention of being a user-friendly tool to enable identification of scarabaeid larvae as close to species level as possible using easily seen characteristics. This is important as accurate identification is essential for pest management because it provides the necessary information to adapt control methods to the biologies of these species (Smith *et al.,* 1995).

3.4.1 Field key

The presented key (Tables 3.1-3.7) identifies six species and 14 morphospecies of scarabaeid larvae collected from the soil of sugarcane fields. The aim was that this key could be used by the lay person but also be scientifically accurate. The key utilises mainly external morphological characteristics, but in some instances relies on minute characters of the mandible and maxilla to differentiate between different species or morphospecies. The use of minute characters for identification is not always ideal especially when aiming to develop a field key. This was however unavoidable, as these minute characters are necessary to differentiate between species or morphospecies. This key was developed from keys created by Ritcher (1966), Sweeney (1967) and Ahrens *et al.,* (2006).

There are numerous reasons for developing a key for South African scarabaeids associated with sugarcane. Sweeney's (1967) key 'The Scarabaeidae associated with sugarcane in Swaziland' would be the most suitable key to use as it was created for scarabaeid larvae from sugarcane. Several reasons exist though as to why this key is not the most accurate key to use. Firstly, this key was created based only on morphological characteristics of larvae and is not based on additional data such as molecular data. Identification of specimens based purely on morphology can lead to incorrect identification as some specimens may be morphologically indistinguishable yet at the molecular level different species. Miller *et al.* (1999) encountered this problem and utilised molecular techniques which then reliably identified morphologically indistinguishable larvae. Secondly, the key is intended for identification of scarabaeid larvae of sugarcane in Swaziland. One cannot assume that morphological characteristics of species present in Swaziland and South African sugarcane fields will be identical. Thirdly, the key dates back to 1966 and is possibly outdated. This means that with the work that had been performed on taxonomy of scarabaeids since 1966 these morphological characteristics used in the key may not be the most reliable characters for species identification and other more effective methods for species identification may exist. Fourthly, the key is difficult to use as characteristics are not indicated on images with labels. The key makes use of entomological terminology specific to scarabaeids. This results in only individuals with a good understanding of the larval morphology and terminology using this key. The lay person wanting to identify scarabaeid larvae would find this key too complex for use and thus not use it. Lastly,

unreliable characters such as number of setae is used and these can be damaged thereby possibly leading to inaccurate identification.

The key created by Ahrens *et al.,* (2006) identified scarabaeid adults and larvae of Nepal. Although this key cannot be used to identify South African scarabaeid species (as his species are not present in South Africa) this key was very useful as it provided a guideline and example of a simple, user-friendly key and characters which may be useful to differentiate species.

The key to scarabaeid larvae of sugarcane in South Africa resulting from this study does have some drawbacks. Individuals using the key require knowledge of how to dissect the head capsule of larvae in order to use the characteristics of the mandibles and maxilla. Access to a microscope is necessary to inspect characters such as the mandibular stridulatory organs, unci on maxilla, antennal dorsal sensory spots and the number of heli present on the epipharynx. For *A. ustulata*, *H. licas* and SASRI Morphospecies 9 there were very few specimens available for examination when creating the key. Similar problems were experienced by Miller & Allsopp (2000) who in some instances had five specimens per taxa and therefore examined less than five specimens when constructing their key. This means that for these specimens the key is not very reliable and more development is required for confirmation. Future recommendations would include sequencing more specimens collected from the field. It is recommended that field collected specimens identified as *A. ustulata*, *H. licas* and SASRI Morphospecies 9 using this key be sequenced. Once this has been performed, phylogenetic analysis will indicate with which species these specimens group. Should these specimens group with *A. ustulata*, *H. licas* and SASRI Morphospecies 9, morphological characteristics used in the key can be compared with those of the additional specimens for congruency and reliability. More adult specimens should be collected, sequenced and analysed together with the existing sequences of the larvae. This procedure should be continued until unidentified larvae can be identified due to the association of these larvae with identified adult specimens.

3.4.2 Lucid 3.4 Key

The development of Lucid 3.4 in 1994 allowed for the first time users of these keys to start identification using any character. This has allowed a larger group of people to identify taxa.

The Lucid 3.4 computer generated key has many advantages: (i) it contains electronic images and line drawings to make the key easy to use for any individual, especially those not familiar with entomological terminology (ii) the user can select any character state to initiate the identification process of the larva collected, and need not start the identification process with a certain character as in traditional dichotomous keys (iii) the key is accessible to all individuals, who have access to the internet, in the case of this key, from the website of the South African Sugar Industry (iv) updating of information and elaboration of the key is simple due to its electronic nature, and (v) geographical distribution information is incorporated into the key. This key is more reliable than a dichotomous key and specimens can be accurately identified as it is based on molecular and morphological data. This key has the possibility of being expanded in various ways. Identification of scarabaeid species from other crops could be included in this key. Insect pests in sugarcane and other agricultural crops could possibly also be included.

Similarly, Miller & Allsopp (2000) have used the Lucid 3.4 program to re-describe the scarabaeid pest species in Australia and to create an electronic key in an effort to enable field extension officers and researchers to easily identify problematic scarabaeid species in Australian sugarcane growing areas.

3.5 CONCLUSIONS

In this chapter we created a morphological key which can be used in two different ways. The field key, which is in the form of a booklet, can be taken into the field to identify scarabaeid larvae there. The Lucid 3.4 key can be used by farmers and/or field officers with computers to identify scarabaeid larvae. The Lucid 3.4 key is user-friendly and accessible to all via the Lucid central web page at the following link http://idlifedev.cbit.uq.edu.au/server-player/player.jsp?datasetId=scarab. These keys will allow for the first time accurate identification of field collected larvae in South African

sugarcane fields and provide an estimate of the diversity of species within South Africa. To increase the reliability of the key it is suggested that more specimens be examined. Following this, species biology can be studied in more depth and subsequently control measures can be developed.

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CHAPTER 4

PHYLOGENETIC TECHNIQUES FOR ASSOCIATION OF SCARABAEID (COLEOPTERA) LARVAE AND ADULTS IN SOUTH AFRICAN SUGARCANE

4.1 INTRODUCTION

Taxonomy or systematics, "the science of identification and classification", originates from a system of classification developed in the 18th century by Carl Linnaeus (Dorit *et al.,* 1991). Different data (or character) fields are used to verify the phylogenetic history of a group. These include data from five areas of study, namely comparative anatomy, ultrastructure, development, biochemistry and palaeontology (Dorit *et al.,* 1991). These data types can be analysed using three approaches: evolutionary systematics, phenetics and phylogenetic systematics (Dorit *et al.,* 1991). Controversy around the definition of taxonomic characters is the main factor separating these three approaches (Dorit *et al.,* 1991). Phylogenetic systematics (the third approach), arose in the 1960's because of inadequacies of the first two approaches, namely evolutionary systematic and phenetics (Dorit *et al.,* 1991). The subjectivity of the evolutionary systematists when selecting taxonomic characters, and the combining of analogy and homology by the pheneticists caused dissatisfaction (Dorit *et al.,* 1991). Phylogenetic systematics aims to reconstruct evolutionary events by grouping organisms that share a common ancestor (Dorit *et al.,* 1991).

Most of our knowledge about phylogeny is from classifications based on morphological data (Scotland *et al*., 2003). Presently, molecular data are more frequently used than morphological data because of the increased number of characters, the simplicity and speed of discovery and their suitability for analysis using models (Hillis, 1987). With the growth of molecular phylogenetics a debate on the use of morphological and molecular data has taken place (Jenner, 2004). This has partly been due to contradictory results obtained from molecular and morphological analyses (Jenner, 2004). In most instances the reason has been that it is unknown what proportion of the molecular and morphological trees are incongruent and whether this incongruence is true or fictitious (Jenner, 2004).

This has led to a debate concerning the use of different sources of information to estimate phylogenetic history. These sources include molecular, morphological, biogeographical, behavioural and ecological data (Allsopp & Lambkin, 2006). Three main methods exist which are used: the total evidence approach (Kluge, 1989), separate analysis approach (Miyamoto & Fitch, 1995) and the conditional combination approach (Bull *et al*., 1993; Rodrigo *et al*., 1993; de Queiroz, 1993). Many researchers find it advantageous to analyse molecular and morphological data separately and in a combined format (Jeffrey *et al.*, 2003; Schulmeister, 2003; Wahlberg & Nylin, 2003) and subsequently compare the results (Jenner, 2004).

It was felt that the morphological and phylogenetic data generated in Chapters 2 and 3 of this dissertation would provide good material to test the robustness of phylogenetic systematic analyses. This chapter discusses a few phylogenetic methods that can be used for phylogenetic species identification of scarabaeid larvae in sugarcane. Distance-based and character-based methods for use in species discrimination in DNA barcoding studies are also discussed.

4.2 MATERIALS AND METHODS

4.2.1 Molecular data

4.2.1.1 Specimens examined

The sequence data generated in Chapter 2 were used in this chapter. However, all sequences from adult specimens were excluded, as only larval morphological characters were scored, to leave only the sequences of the 166 larval specimens for the current analyses. Scarabaeid species and specimens used in the analysis, their SASRI or BOLD voucher number, life stage, identity if known and collection locality in South Africa are presented in Appendix A.

4.2.1.2 Phylogenetic analysis

Maximum Parsimony (MP) analysis was performed using the molecular data set with a heuristic search option (100 random taxon-addition replicates) with tree-bisection

recognition (TBR) branch swapping, all characters equally with one tree held at each step. Bootstrap values were calculated from 200 replicates in parsimony analyses. Due to time constraints only 200 bootstrap replicates were performed although Hedges (1992) recommended performing 400 - 2000 bootstrap replicates if one would like statistically meaningful results.

A Bayesian inference of Scarabaeidae was performed with MrBayes 3.1.2 under the GTR+I+G model of evolution (Huelsenbeck & Ronquist, 2001). Bayesian searches were run with four chains running simultaneously for 3 000 000 generations. A tree was sampled every 500 generations. To determine when chain stationarity was achieved the likelihood values were plotted as a function of generation. Chain stationarity was achieved after 150 000 generations thus discarding 300 trees. The resulting tree in Newick format was edited in MEGA 3.1 (Kumar *et al.,* 2004).

4.2.2 Morphological data

4.2.2.1 Specimens examined

Refer to section 4.2.1.1.

4.2.2.2 Characters examined

A total of 19 morphological characters were included in the analysis, and are listed in Appendix C. The morphological characters considered included those that were used to separate specimens into species and morphospecies groups in Chapter 3. Morphological characteristics used in the key (Chapter 3) were scored by assigning a number to different states (e.g. presence or absence) of a character (e.g. stridulatory area on mandible) observed within the group of larvae in this study (Appendix D).

4.2.2.3 Phylogenetic analysis

MP analyses were performed on these scored morphological data with a heuristic search option (100 random taxon-addition replicates) with tree-bisection recognition (TBR) branch swapping all characters equally. Bootstrap values were calculated from 200

replicates in parsimony analyses. Due to time constraints only 200 bootstrap replicates were performed although Hedges (1992) recommended performing 400 - 2000 bootstrap replicates if one would like statistically meaningful results.

In Bayesian analysis, morphological data were analysed using the model for "standard" discrete data, which considers equal probability for all character state changes. All Bayesian analyses were initiated from random starting trees. Bayesian searches were run with four chains running simultaneously for 3 000 000 generations. A tree was sampled every 500 generations. To determine when chain stationarity was achieved the likelihood values were plotted as function of generation. Chain stationarity was achieved after 200 000 generations thus discarding 400 trees. The resulting tree in Newick format was edited in MEGA 3.1 (Kumar *et al.,* 2004).

4.2.3 Combined analysis

4.2.3.1 Partition homogeneity test

In the partition homogeneity test (Farris *et al*., 1995) the morphological and molecular data are subjected to statistical tests. If the result is non-significant then the data can be combined. This test was applied to both sets of data in PAUP*version 4.0b8 (Swofford, 1998) with 1000 replications.

4.2.3.2 Phylogenetic analysis

MP analysis was performed in PAUP*version 4.0b8 (Swofford, 1998) with representative sequences (94 sequences) from each of the groups (A-AC) in Chapter 2. The heuristic search option (100 random taxon-addition replicates) with tree-bisection recognition (TBR) branch swapping, all characters equally, was used. Bootstrap values were calculated from 200 replicates in parsimony analyses. Due to time constraints only 200 bootstrap replicates were performed although Hedges (1992) recommended performing 400-2000 bootstrap replicates if one would like statistically meaningful results.

Bayesian analysis using the combined morphological and molecular data was performed using the program MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Different evolutionary

models were used to analyse the morphological and molecular data. All Bayesian analyses were initiated from random starting trees. The molecular data were analysed using the GTR+I+G model of evolution. This model allows different rates of substitution types among the nucleotides and unequal nucleotide frequencies. Morphological data were analysed using the model for "standard" discrete data. Bayesian searches were run with four chains running simultaneously for 3 000 000 generations. A tree was sampled every 500 generations. To determine when chain stationarity was achieved the likelihood values were plotted as a function of generation. Chain stationarity was achieved after 200 000 generations thus discarding 400 trees. The posterior probability was calculated by including a model generated from learning about the data. Once the model is specified the program will search for the best trees that are consistent with the data and the model. A 50% majority rule consensus tree was constructed from the remaining trees. The percentage of times a clade occurred among this sampling of trees was interpreted as its posterior probability. Four independently repeated analyses resulted in similar tree topologies and similar clade probabilities. The resulting tree in Newick format was edited in MEGA 3.1 (Kumar *et al.,* 2004).

4.3 RESULTS

4.3.1 Molecular Data

The Akaike Information Criterion (AIC) test implemented in Modeltest selected the GTR+I+G evolutionary model (proportion of invariable sites = 0.47; $\alpha = 0.57$; empirical base frequencies: A = 0.37; C = 0.119; G = 0.06; T = 0.38; and substitution rates: A-C = 0.28; A-G = 7.00; A-T = 0.57; C-G = 0.98; C-T = 3.77; G-T = 1.00).

An unweighted parsimony heuristic search resulted in a single most parsimonious tree (Figure 4.1) of 1795 steps (CI = 0.325; RI = 0.882). Bayesian analysis under the GTR+I+G model of evolution resulted in a consensus tree with high posterior probability values for most nodes that were also highly supported in the MP bootstrap tree (Figure 4.1). Posterior probabilities were only indicated on branches if they were higher than 0.95. All groups recovered in Bayesian analysis and MP and NJ (Chapter 2) consisted of the same specimens. Arrangement of groups differed slightly but individual members of groups did not vary.

In general, the Melolonthinae were well supported by MP and Bayesian analysis whereas the Dynastinae were only supported by Bayesian analysis. The Rutelinae as a whole were supported by Bayesian analysis but only Morphospecies 3 was supported by MP. Melolonthine groups K, L, M, O, P, Q, R, S, W, AA, AB and AC were highly supported by the Bayesian analysis. All melolonthine groups with the exception of group T and U were supported by MP.

4.3.2 Morphological Data

Nineteen morphological characters were scored in 166 scarabaeid taxa of which 18 were parsimony informative. Parsimony analyses in PAUP resulted in a single most parsimonious tree (Figure 4.2) of length 59 steps (CI = 0.5254; RI = 0.9369). Although support for most nodes was relatively weak, and several were unresolved, seven main groups are evident in the consensus tree. Bayesian analysis also resulted in a poorly resolved consensus tree. Posterior probabilities were only indicated on branches if they were higher than 0.95 and bootstrap support is only indicated if higher than 70%. Morphospecies 3, 12, 13 & 14 , *H. sommeri* group and *Camenta* sp. group were the only groups supported by both MP and Bayesian analyses. The Dynastinae were only supported by MP analysis. The resulting tree recovered the same specimens in each group as in the NJ analysis in Chapter 2.

4.3.3 Combined data analysis

4.3.3.1 Partition homogeneity test

The partition homogeneity test showed that the morphological and molecular data sets could be combined as they were not significantly different (p = 0.64 at $P>0.05$). The total length of the combined data set (molecules (658 bp) + 19 morphological characters) was 677 characters.

4.3.3.2 Phylogenetic analysis

MP search resulted in a single most parsimonious tree of 1828 steps (CI= 0.3315; RI= 0.8277; Figure 4.3). The subfamily Melolonthinae was well supported with all groups,

except Morphospecies 10 (Group T) and 11 (Group U), having high bootstrap support. The Dynastinae and Rutelinae were poorly supported. In the groups recovered by MP the same specimens with one exception represented all groups. Morphospecies 13 (Group Z) contained one specimen from the *H. sommeri* group (Group AB) namely DNA number BOLD 0786.

The 50% majority-rule consensus tree recovered from Bayesian analysis of a combined molecular and morphological data set is depicted in Figure 4.4. The combined data set and the molecular data resulted in similar results. In general, support for most nodes of the combined analysis was similar with a few nodes exhibiting slightly higher support than in the molecular analysis. Bayesian analysis resulted in a tree where the three subfamilies formed monophyletic groups.

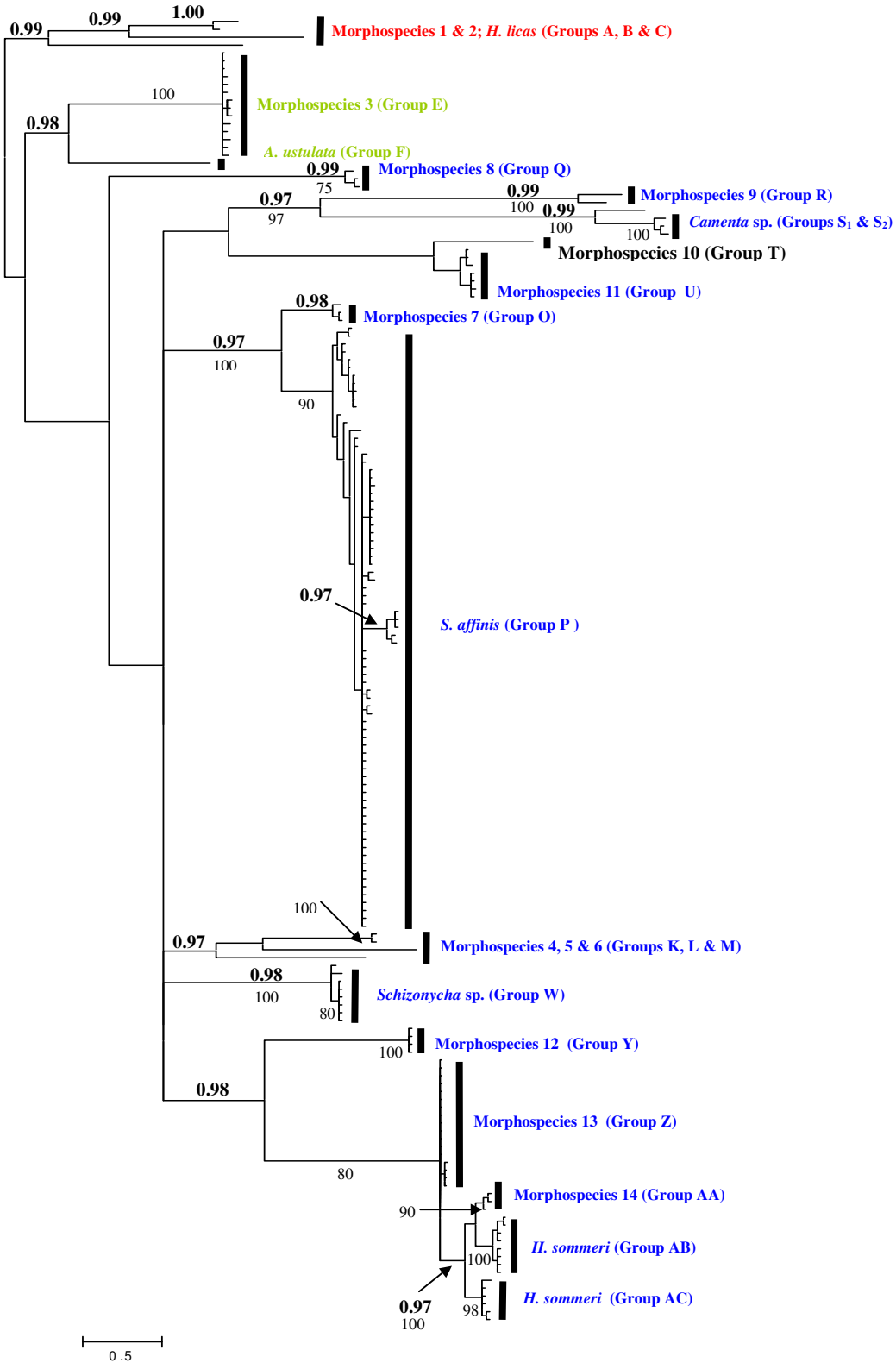

Figure 4.1 The 50% majority-rule consensus tree resulting from the Bayesian inference based on the molecular data set under the GTR+I+G model. The numbers above branches are posterior probabilities corresponding to the Bayesian inference. Bootstrap values resulting from the maximum parsimony analysis are only indicated if greater than 70%. Group labels are coloured according to subfamily status (Red = Dynastinae; Blue = Melolonthinae; Green = Rutelinae).

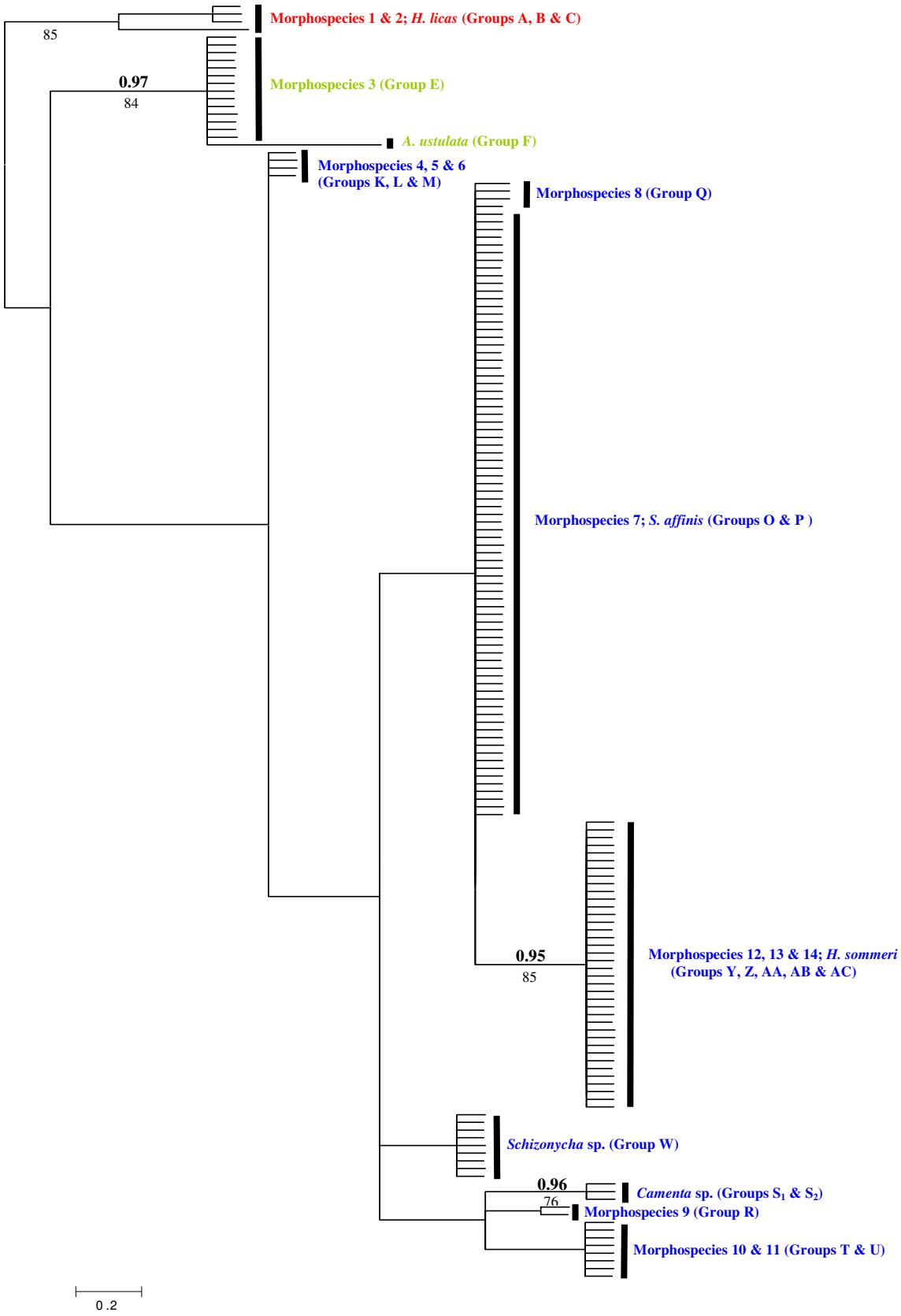

Figure 4.2 The 50% majority-rule consensus tree resulting from the Bayesian inference based on the morphological data set under the Markov model. The numbers above branches are posterior probabilities corresponding to the Bayesian inference. Group labels are coloured according to subfamily status (Red = Dynastinae; Blue = Melolonthinae; Green = Rutelinae).

Figure 4.3 Parsimony analysis of combined morphological and molecular data sets. The single most parsimonious tree is shown. The numbers indicate bootstrap values corresponding to the maximum parsimony analysis, only shown if greater than 70%. Group labels are coloured according to subfamily status (Red = Dynastinae; Blue = Melolonthinae; Green = Rutelinae).

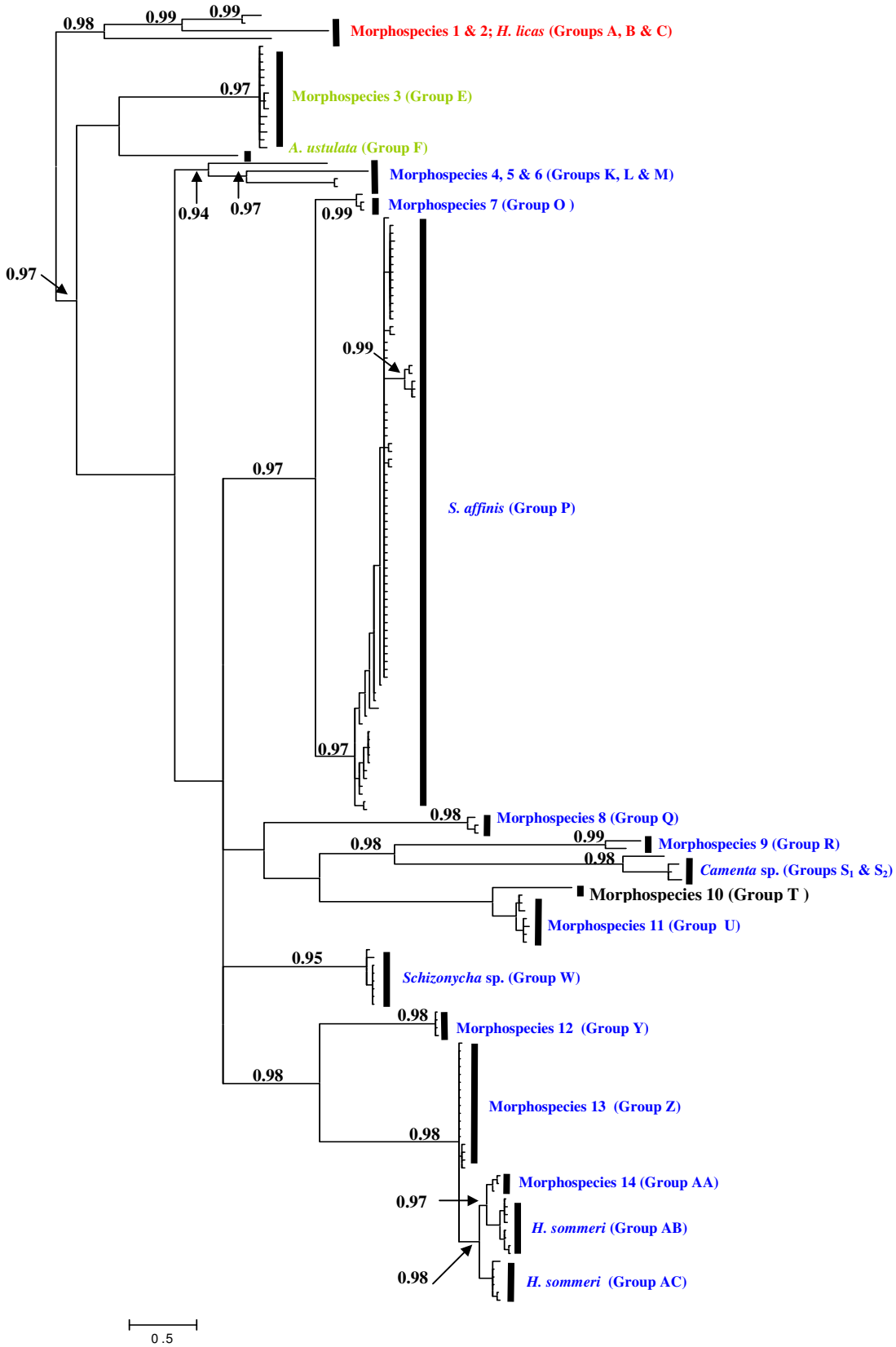

Figure 4.4. The 50% majority-rule consensus tree resulting from the Bayesian inference based on the combined morphological and molecular data sets. The numbers above branches are posterior probabilities corresponding to the Bayesian inference. Bootstrap values resulting from the maximum parsimony analysis are only indicated if greater than 70%. Group labels are coloured according to subfamily status (Red = Dynastinae; Blue = Melolonthinae; Green = Rutelinae).

4.4 DISCUSSION

In this study, phylogenetic tools were utilised to determine whether morphological and molecular data provided the same results in terms of the groupings of the scarabaeid larvae.

4.4.1 Molecular and Combined Data

The resulting trees generated from Bayesian and MP analyses for the molecular and combined data sets were very similar to those generated from NJ analysis in Chapter 2. In Figures 4.1 and 4.4 the terminal groupings were represented by exactly the same specimens in each group as the NJ analysis. Figure 4.3 showed a single change where a specimen (DNA number BOLD 0786) from Group AB (*H. sommeri* group) was clustered with specimens from Group Z (Morphospecies 13). For clarification sequence 0786 was compared to the neighbouring sequence from Group Z (DNA number BOLD 0503) as well as sequences belonging to Group Z. This comparison indicated that sequence 0786 differed from sequence 0503 and other sequences from Group Z by 11 nucleotides. This would strongly suggest that sequence 0786 has been incorrectly grouped with sequences in Group Z. A possible explanation for this incorrect grouping could be due to the presence of unknown nucleotides (N's) at the beginning of both sequences 0786 and 0503.

If this incorrect grouping is truly associated with the unknown nucleotides at the beginning of the sequences then there are no differences in terms of the terminal groups between the trees resulting from NJ, MP and Bayesian analysis. This could suggest that either of the methods be used.

Bayesian inference is based on the posterior probability of trees in tree space (Holder & Lewis, 2003). The posterior probability is calculated by including a model that has been generated from learning something about the data (Holder & Lewis, 2003). Once the model has been included the program searches for the best trees which fit the model and the data (Holder & Lewis, 2003). Bayesian methods have primarily been used for the analysis of mixed data sets, such as morphology and genes, which were initially analysed using parsimony (Nylander *et al.,* 2004). The development of the Markov chain Monte Carlo (MCMC) algorithm allowed incorporation of complex models into Bayesian

analyses (Nylander *et al.,* 2004); this methods was seen as a faster method to incorporate these complex models (Holder & Lewis, 2003). This analysis process is nevertheless lengthy although it is faster than maximum likelihood (Holder & Lewis, 2003).

Neighbour Joining (NJ) is a popular algorithm that it is relatively fast and easily applicable in instances where sequence divergences are low (Holder & Lewis, 2003). In this method sequences are converted into a distance matrix (Holder & Lewis, 2003). This distance matrix is representative of the evolutionary distance (number of changes), which has occurred over time between any two sequences (Holder & Lewis, 2003).

According to the results obtained from this study it would not be necessary to choose Bayesian analysis over NJ. As the resulting terminal groups and their members are the same using either method, results could be generated faster using NJ and provide the same accuracy.

4.4.2 Morphological Data

Trees resulting from morphological data alone showed few groupings and these were only resolved up to the tribe level in most instances. A possible explanation for this crude grouping of taxa could be due to the relatively few morphological characters (19) used. Similar studies combining morphological and molecular data used considerably more morphological characters. These studies focused on a range of organisms such as spiders (Bruvo-Mađarić *et al*., 2005)(45 characters), beetles (Cabrero-Sañudo & Zardoya, 2004)(84 characters), butterflies and skippers (Wahlberg *et al*., 2005)(99 characters) and lizards (Giugliano *et al*., 2007)(163 characters). The main reason for the low resolution of the morphological tree is the low variability of the morphological characters. In this study on three subfamilies of the Scarabaeidae, the aim was to use DNA barcoding methods to link adult and larval specimens. Based on these groupings as well as morphological identifications to subfamily level, identities of specimens were assigned. In Chapter 3 morphological characters were examined to create a key that could be used to separate a range of collected specimens into species/morphospecies groups. This process involving the examining of morphological characters which differed among species/morphospecies clearly showed the small amount of morphological variation especially between closely related species/groups. It is thus not surprising that the tree resulting from Bayesian and

MP analysis using morphological data only showed unresolved terminal groups. An alternative approach to using morphological data in this manner would be to utilise a character-based approach. This is discussed in the subsequent section.

4.4.3 Character-based methods for DNA barcoding

To be able to understand the merits of character-based DNA barcoding one needs to discuss distance-based methods that have been previously used and are still used in analysis of DNA barcoding. DNA barcoding is a process in which an amplified segment of the mtDNA COI gene is sequenced (Waugh, 2007). This sequence is then compared with existing sequences from voucher specimens (Waugh, 2007). When sequences are compared using the software on the BOLD website, the Kimura 2-parameter (K2P) distance measure is implemented. This method calculates the sequence divergence among individuals (Waugh, 2007). The K2P model is the most effective to use when using the COI region to generate DNA barcodes, as distances within species are usually low (Waugh, 2007).

During the early stages of DNA barcoding, distance measures were used to determine species identities (Hebert *et al*., 2003). Distance measures can be used in two different ways. The first is the BLAST (Altschul *et al*., 1990) hit where a similarity score is generated for two sequences - the query sequence and the closest sequence in the database, thereby determining the nearest neighbour. In the second method distances are utilised in tree-building (Hebert *et al*., 2003). Both approaches have shortcomings:

- Classical studies/taxonomy make use of diagnostic characters and not distances to delimit species (DeSalle *et al*., 2005).
- Similarity scores, such as the results obtained from a BLAST hit, can provide a fast analysis of many genes at once but these results often lead one to think that two specimens are closely related (Koski & Golding, 2001). This is not always true as this “score” is dependant on the number of sequences in the database to which the query sequence can be compared and on the number of homologs in that gene (Koski & Golding, 2001).
- Distance methods are unable to provide a cut-off threshold because of the overlap of intra- and interspecific distances (Goldstein *et al*., 2000). Threshold limits will vary for species and can thus not be applied broadly (Goldstein *et al*., 2000).

Character-based information is a practical alternative. These methods are more reliable as they can only group specimens when certain diagnostic characters are present in both specimens (DeSalle *et al*., 2005). This approach allows classical, morphological, behavioural and ecological information to be combined (DeSalle *et al*., 2005) before a species identity is generated. Using the above mentioned characters, a hierarchy can be constructed using shared, derived characters and these characters can be used to deduce common ancestry (DeSalle *et al*., 2005). Neither neighbour-joining (NJ) nor BLAST allow analysis of character information but rather use pairwise distances only (DeSalle *et al*., 2005). Instead Maximum Likelihood (ML) or maximum parsimony (MP) are preferred alternatives. These methods avoid the loss of information, which occurs when characters are converted to distances (DeSalle *et al*., 2005).

One such method of character-based assessment is the character attribute organization system (CAOS) developed by Sakar *et al*. (2002). This method is rapid, avoids false positive results, uses the evolutionary information present in character-state data, and is accurate even though the DNA barcoding gap of different studies may vary (Kelly *et al.,* 2007). CAOS is based on the concept that individuals of a certain taxonomic group have shared characters, which are not present in other groups (Rach *et al.,* 2008). How the CAOS methods are applied is explained below in a simplified way. Initially a guide tree is produced, either using maximum parsimony (MP) or maximum likelihood (ML), from the existing DNA sequences in the study (Kelly *et al.,* 2007). The purpose of the guide tree is to identify diagnostic characters (Rach *et al.,* 2008). "Rule sets" are generated by examining the diagnostic character states that are present at each node of the MP or ML tree (Kelly *et al.,* 2007). The CAOS algorithm identifies characteristic attributes (CA's), which separate each node from the other (Rach *et al.,* 2008). Examples of CA's are genes, amino acids, base pairs, morphological or behavioural attributes (Rach *et al.,* 2008). It is important to realise that these CA's are only present in one group and not in other groups that descended from the same ancestral node (Rach *et al.,* 2008). These "rule sets" can then be applied to the sequences once an unknown sequence has been aligned to sequences in the data set (Kelly *et al.,* 2007). If this unknown sequence contains sufficient diagnostic information it will be linked to sequences in the data set that share the same diagnostic characters (Kelly *et al.,* 2007). Should the unknown sequence contain insufficient diagnostic information and can therefore not be placed in a group, in which the sequences share the same diagnostic characters, the CAOS analysis will be terminated (Kelly *et al.,*

2007). This has the advantages that false-positives are eliminated and a sequence will not be assigned an identity if the character data does not support this identity (Kelly *et al.,* 2007).

This character-based method is consistent with the phylogenetic species concept (Baum & Donoghue, 1995). In this species concept, individuals of the same species are thought to be more closely related than individuals from other closely related species. Individuals of the same species should have fewer nucleotide differences in their sequences compared to the sequences of individuals of other species. Libscombe *et al.* (2003) state that a character-based method of species identification is more accurate than a distance-based method. This is because a distance-based approach identifies clusters of similar entities but does not have a linking component where species are identified and named using taxonomy (Libscombe *et al.*, 2003).

In their study on DNA barcoding using chitons Kelly *et al.* (2007) suggest that character-based DNA barcodes should be used to list diagnostic markers of different species, which can then be used to separate species from one another. Furthermore, it is suggested that the species specific diagnostic markers are used together with traditional morphological taxonomy. Kelly *et al.* (2007) found that CAOS was a useful tool to identify intraspecific divergences but species descriptions should nevertheless be confirmed from numerous sources.

Rach *et al.* (2008) used character-based DNA barcoding to discriminate between genera and species of Odonata. They discussed the problem of the currently used genetic distance approach when using DNA barcodes to define species boundaries. Using genetic distance to separate species is not practical. The rates of evolution of mtDNA vary between, within and among species. This means that there is overlapping of intraspecific and interspecific distances. This overlapping is problematic as an unknown sequence cannot be linked confidently to a known sequence using distance-based methods and this is especially difficult when sample size is small (Rach *et al.,* 2008). The character-based approach uses diagnostic characters or short DNA sequences to group specimens into taxonomic entities (Rach *et al.,* 2008). The character-based approach can therefore be used in conjunction with traditional morphological taxonomy (Rach *et al.,* 2008). The main difference between

the character-based approach and the molecular approach is that the number of diagnostic characters are almost unlimited when using the molecular approach (Rach *et al.,* 2008).

Using the data from this study on scarabaeids it would be recommended to apply the character attribute organization system (CAOS) to determine whether the same results in terms of groupings are achieved as those which have been obtained from NJ, MP and combined analyses. The CAOS approach may be faster and more reliable and work on this data set using CAOS could indicate the feasibility of using this approach for future work with similar objectives.

4.5 CONCLUSION

In this study on scarabaeids in sugarcane, larval groupings were examined using various phylogenetic tools. Morphological and molecular data were analysed separately and in a combined analysis using Bayesian and MP methods. The resulting trees were compared to those obtained from NJ analysis in Chapter 2. No significant differences in results were observed between these methods for this study. This has indicated that fast NJ methods may be just as efficient as lengthy Bayesian analyses. It is suggested that character-based methods be used in future rather than distance-based methods due to the ability of this methods to reduce the occurrence of false positives, incorporate information from a range of sources, and the possibility of complementing morphological taxonomy.

For identification of scarabaeid sugarcane pests it is recommended that fresh specimens be used when available and identified using the key created in Chapter 3. Should there be uncertainty, molecular methods can be utilised to confirm the identification. This can be performed by removing a leg from the specimen and following the procedures outlined in Chapter 2. The resulting sequence can then be compared to those of identified specimens and assigned an identification based on which taxa this sequence groups with.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 INTRODUCTION

This chapter sums up the results presented in chapters 2, 3 and 4 and discusses future research opportunities. The main aim of this project was to link larvae to identified adult scarabaeids from sugarcane fields by means of their mtDNA sequences. Based on this foundation, the second aim was to develop a reliable morphological key, which could be used by field workers to identify scarabaeid larvae.

Aspects are also discussed which could supplement the results from this study, thereby improving the understanding of the taxonomy of scarabaeid sugarcane larvae and improving identification methods of these larvae. These advances are a start to the improvement of knowledge of the species composition of scarabaeid larvae in sugarcane fields, thus making management and biological control of these pests a greater possibility.

5.2 MOLECULAR IDENTIFICATION OF SCARABAEID (COLEOPTERA) PEST SPECIES IN SOUTH AFRICAN SUGARCANE

We showed the first linking of larvae and adults of the same species, collected from sugarcane in South Africa, within the subfamilies Dynastinae, Melolonthinae and Rutelinae, based on mtDNA sequences. DNA-based methods in this case were found to be an efficient method to identify the various scarabaeid pest species, especially the larvae, which do not have as many diagnostic morphological characters as their adults. These results showed the strength of using the *cox* 1 region in DNA barcoding of unidentified scarabaeid pest species. The magnitude of the differences observed between within-group and among-group sequence divergence levels, as well as the strong support for the nodes, confirmed the clustering of specimens into groups. These results enabled the linking of larvae to adults by DNA barcoding. Whether this represents the species boundaries needs to be investigated further by complementing this study with more information from field studies of the sampled populations and broader genetic surveys which include identified adults of closely related species. This information would be required to confirm whether

the groups defined by these nodes are defining species. Further work, such as DNA barcoding of additional museum specimens and collection of beetles from light traps, is recommended to link unidentified larval specimens (Morphospecies 1-14) to identified adult specimens and to resolve the identities of unknown groups. Additional molecular data from nuclear genes could be generated in future. The *cox* 1, 16S, 18S and elongation factor-1α genes have been widely used and are informative across a broad range of divergences in insects (Caterino *et al.,* 2000).

The ability to use mtDNA sequence data to link the morphologically indistinguishable larvae to their respective adults will be important in planning and implementing management strategies of these pests within the sugar industry of South Africa. Once all scarabaeid species in sugarcane can be correctly identified a reliable study can be made of each species biology. Currently, obtaining this knowledge is hampered by difficulties involved in making associations between life stages (Miller & Allsopp, 2005). Understanding the larval life stages biology, through knowing their identification will allow timely detection of changes in population structure and thereby understanding of dominant species (Ahrens *et al.,* 2007). Once this knowledge is ascertained it is easier to develop control measures, either biological, cultural or chemical, or an integration of these, that target certain life stages in the biology of the specific species. This information could be used to reduce the presence of dominant species by applying control strategies before potential agricultural damage may occur (Ahrens *et al.,* 2007).

Chemical control of scarabaeids in South Africa in the past has been unsuccessful (Cackett, 1992) and was later banned (Baker, 1986). A promising control method remains the action of entomopathogens, especially fungi (Rajabalee, 1994). The choice of two particular strains of *Beauveria brongniartii* (Saccardo) Petch 1924 collected in Madagascar has helped in a large way to formulate an effective biopesticide specific to *Hoplochelus marginalis* Fairmaire 1889 (Coleoptera: Melolonthinae) and is now proving to be a breakthrough in the control of this major sugarcane pest in Réunion Island (Rajabalee, 1994). In Tanzania, the fungus *Cordyceps barnesii* Thw. (Hocking, 1966) attacks third instar *Cochliotis melolonthoides* Gerstaecker 1867 (Coleoptera: Melolonthinae) larval pests of sugarcane eventually causing death (Evans *et al.,* 1999). Having the correct species identification of the scarabaeid larvae has helped obtain the most effective fungal strain against the larvae of that particular pest species. Similarly, in South Africa research

on entomopathogens can readily be conducted as a start has been made to the identification of scarabaeid larvae in sugarcane.

5.3 MORPHOLOGICAL IDENTIFICATION OF SCARABAEID (COLEOPTERA) PEST SPECIES IN SOUTH AFRICAN SUGARCANE

Many of the scarabaeid larvae are closely related and are difficult to distinguish from each other morphologically. Misidentifications of pests, such as stalk borer larvae, have occurred often, resulting in publication of incorrect data (Polaszek, 2001). This is especially the case when immature life stages such as larvae are considered. Morphologically characteristic features such as setae are easily damaged or even broken, which make identification difficult and results unreliable (Meijermann & Ulenberg, 1998).

In this study on scarabaeid larvae, larval morphological characters unique to the sequenced groups were tabulated and subsequently used to establish morphological keys to identify these larvae to species level. Two types of keys were generated. A simplistic morphological key was generated for use by field workers with a limited knowledge of scarabaeid taxonomy to identify scarabaeid larvae in the field. This key was constructed in a tabular format and only used characteristics visible with the naked eye or a hand lens. This was necessary in order for quick preliminary identifications in the field. This key contained mainly line drawings and images thereby circumventing the need for knowledge of taxonomic terminology.

The second key was in the form of an electronic key using the software Lucid 3.4. This key uses a combination of easily visible morphological characteristics as well as microscopic characteristics for accuracy. The electronic key is available to public users from the Lucid central web page at the following link http://idlifedev.cbit.uq.edu.au/server-player/player.jsp?datasetId=scarab.

The electronic key provides a new means of identifying specimens. This key does not restrict identification of certain taxa to taxonomists but rather allows most individuals with some biological knowledge to identify a specimen. This is achieved by the use of a variety of information of the species described in the key, such as photos, line drawings, easily visible external morphological characteristics, minute microscopic characteristics, head

capsule measurements and incorporation of geographic information. Most importantly, the user has the ability to choose which characters he/she would like to utilize to commence identification. This factor makes the key available for use to a wide audience. This is so because the key can contain taxonomically specific information as well as basic information and therefore the key is useful to a scarabaeid taxonomy expert as well as an individual with limited knowledge of scarabaeids who wishes to identify a specimen. The advantage of using the Lucid key for identification is that the information can be updated and modified at any time without having to re-develop the key.

To increase the reliability and accuracy of the key it is suggested that more specimens from each species and morphospecies be examined from all areas of the sugar industry. This study mainly considered scarabaeids from the KwaZulu-Natal midlands area. Only those larvae and adult scarabaeid specimens that were linked by mtDNA studies were included in the key. There are other species which still need to be included in the key (both in the study area, and the wider South African sugarcane growing regions) once they have been identified using molecular techniques. Future work should aim at collecting specimens from all areas of the South African sugar industry, identifying these using the developed molecular techniques and adding the characteristic information to the Lucid key so that these specimens can be identified. More specimens per morphospecies /species should be collected such that head capsule size measurements, indicative of instar, can be generated.

This will allow faster identification of field collected larvae and thereby provide an estimate of the diversity of species within South Africa. Following this, species biology can be studied in more depth and subsequently control measures can be developed. The concept of creating a key using Lucid 3.4 can later be used for other insect pests of sugarcane.

5.4 PHYLOGENETIC TECHNIQUES FOR ASSOCIATION OF SCARABAEID (COLEOPTERA) LARVAE AND ADULTS IN SOUTH AFRICAN SUGARCANE

It was felt that the morphological and phylogenetic data generated in Chapters 2 and 3 of this dissertation would provide good material to test the robustness of phylogenetic systematic analyses. This chapter discussed a few phylogenetic methods that can be used

for phylogenetic species identification of scarabaeid larvae in sugarcane. Distance-based and character-based methods for use in species discrimination in DNA barcoding studies were also discussed.

In this study on scarabaeids in sugarcane, larval groupings were examined using various phylogenetic tools. Morphological and molecular data were analysed separately and in a combined analysis using Bayesian and MP methods. The resulting trees were compared to those obtained from NJ analysis in Chapter 2. No significant differences in results were observed between these methods for this study. Based on the results obtained from this study it has indicated that fast NJ methods may be just as efficient as lengthy Bayesian analyses. It is suggested that character-based methods be used in future rather than distance-based methods due to the ability of this method to reduce the occurrence of false positives, incorporate information from a range of sources, and the possibility of complementing morphological taxonomy.

With the shortage of conventional taxonomists becoming more apparent, DNA barcoding and DNA taxonomy are methods presently available for species identification (Pfenniger *et al.,* 2007). Monaghan *et al*., (2005) recommend that DNA data be used as the first step in testing taxonomic hypotheses and that additional data types be employed to verify these hypotheses. Monaghan *et al*., (2005) list a few benefits of this method which can also be applied to this study. Firstly, specimens can be quickly assigned to probable groups. Unidentified scarabaeid larvae can be quickly assigned to one of the three subfamilies. Secondly, once the first step has been completed distinctive morphological characters can easily be determined by comparing unidentified specimens with already identified specimens falling into the same temporarily defined species group. Thirdly, the study of temporarily defined species can proceed although the taxonomic identification has not yet been verified. Fourthly, specimens collected from different localities throughout the sugarcane growing regions of South Africa and belonging to the same species can be linked. Similarly, Pfenniger *et al.* (2007) stated that DNA barcoding is valuable in species identification when specimens are damaged, only larval instars are present and/or when only part of a specimen is available.

In this case the approach of combining morphological and molecular data is effective. In such instances data from different sources is utilised. This allows the phylogenetic history

of organisms to be estimated based on independent data sources. Further work is recommended. This would include adding information from nuclear genes to confirm the consistency of the obtained results. The internal transcribed spacer (ITS) of the nuclear ribosomal DNA repeat (nrDNA) would be suggested for use (Simmons & Scheffer, 2004).

5.5 SPECIES COMPOSITION WITHIN THE SOUTH AFRICAN SUGARCANE INDUSTRY

One of the earliest records of scarabaeids in South African sugarcane by Carnegie (1974 & 1988) already noted the presence of *H. sommeri* and *S. affinis* as abundant. Later it became evident that *H. sommeri* and *S. affinis* were present mainly in the KwaZulu-Natal midlands and that *H. licas* was confined to the Zululand area, namely Nkwalini, Pongola and Umfolozi (SASA information sheet, 1993).

In 1995 the extent and intensity of scarabaeids in the South African sugarcane industry was monitored (Way, 1995). The survey showed that scarabaeids were present in a wide geographical area in sugarcane encompassing many different conditions, e.g. different soil types, altitudes and rainfall patterns etc. (Way, 1995). *S. affinis* was the most numerous, and the most widespread scarabaeid species, particularly abundant in the KwaZulu-Natal Midlands, but present in all regions (Way, 1995). *Astenopholis* sp. and *Hypopholis* were the second most abundant genera, but less widespread (Way, 1995). Fewer specimens of *Heteronychus licas*, *Heteronychus* sp., *Adoretus fusculus* and *Anomala* sp. were recovered (Way, 1995). *S. affinis* and *H. sommeri* are pests of sugarcane in the Midlands while *H. licas* and *Anomala* spp. are more common in the northern lowland regions of the industry (Way, 1995).

Surveys in the following year, 1996, showed that scarabaeid numbers had increased (Way, 1996). Eight species already recorded in sugarcane were present, in addition to the approximate 21 unknown taxa (Way, 1996). The larvae of common species recorded in the past, such as *H. sommeri*, *S. affinis*, *Anomala* spp., *Adoretus* spp., *Astenopholis* spp. and *Temnorrhynchus* spp., are considered to be crop pests (Carnegie, 1988).

Mansfield (2004) focused studies mainly on scarabaeids within the KwaZulu-Natal midlands. This study showed that *H. sommeri, S. affinis* and *Adoretus ictericus* Burmeister

1844 (Coleoptera: Scarabaeidae) were the predominant species. Similarly to studies conducted in the past by other researchers, *H. sommeri* and *S. affinis* were identified as species most abundant in the study by Mansfield (2004).

This study concentrated mainly on scarabaeids from the KwaZulu-Natal midlands. The results obtained here agreed with those obtained in the surveys conducted by Carnegie (1974 & 1988), Way (1995 & 1996) and Mansfield (2004). The Melolonthinae are well represented in the KwaZulu-Natal midlands with the Dynastinae and Rutelinae being more abundant in the KwaZulu-Natal North Coast and even Malelane areas. The most abundant scarabaeids were *S. affinis* followed by *H. sommeri*. Since these two species, *S. affinis* and *H. sommeri,* have been and still are the most dominant scarabaeid species in sugarcane it is recommended that the biology of these two species be studied first. In doing so the life cycle of these scarabaeids can be understood and later control measures implemented. Once this process has been performed it will be easier to understand the biologies of the remaining problematic scarabaeid species and subsequently design control strategies for those species.

5.6 CONCLUSIONS

In order to manage and control scarabaeid sugarcane pests, knowledge of their identity is imperative. This will allow workers to focus on a single species biology, which will allow an analysis of between species interactions, and within species control. To improve further studies it is suggested that more specimens be used for molecular and morphological work. These studies could focus on species composition of sugarcane scarabaeids, their natural enemies, and the genetic differentiation of natural enemies will be important for the improved production of sugarcane.

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APPENDIX A. Scarabaeid species and specimens used in the analysis, their SASRI or BOLD voucher number, life stage, identity if known and collection locality in South Africa

Voucher Number	Life Stage	Subfamily [1]	Collection Locality	Latitude, Longitude	Species Name
37	Adult	Dynastinae	Pongola	27°11'S 31°21'E	*Heteronychus* sp. Klug, 1835
40	Adult	Dynastinae	Malelane	25°25'S 31°34'E	*Heteronychus* sp. Klug, 1835
41	Adult	Dynastinae	Umfolozi	28°42'S 32°19'E	*Heteronychus* sp. Klug, 1835
43	Adult	Melolonthinae	Gingindlovu	29°01'S 31°34'E	*Schizonycha affinis* Boheman, 1857
53	Larva	Rutelinae	Heatonville	28°53'S 31°28'E	Unidentified
60, 61, 62, 63	Larva	Melolonthinae	New Hanover	29°06'S 30°24'E	Unidentified
67	Adult	Melolonthinae	New Hanover	29°06'S 30°24'E	*Hypopholis sommeri* Burmeister, 1855
70	Adult	Rutelinae	Verulam	29°21'S 31°03'E	*Anomala* c.f. *ustulata* Arrow, 1899
81	Adult	Melolonthinae	Mtunzini	28°34'S 31°24'E	*Schizonycha affinis* Boheman, 1857
82	Larva	Melolonthinae	New Hanover	29°06'S 30°24'E	Unidentified
91	Larva	Melolonthinae	Empangeni	28°30'S 31°31'E	Unidentified
126, 129	Larva	Melolonthinae	Mtubatuba	28°33'S 31°16'E	Unidentified
150, 156	Larva	Dynastinae	Umfolozi	28°42'S 32°19'E	Unknown
173	Adult	Dynastinae	Pongola	27°11'S 31°21 'E	*Heteronychus licas* Klug, 1835
188, 189	Adult	Melolonthinae	Eshowe	28°30'S 31°14'E	*Camenta* sp. Erichson, 1847
192, 193	Larva	Melolonthinae	Harburg	29°29'S 30°41'E	Unidentified
228, 229, 230	Adult	Melolonthinae	New Hanover	29°15'S 30°23'E	Unidentified
281	Adult	Melolonthinae	Impendle	29°50'S 30°10'E	*Asthenopholis nigrorubra* Harrison, unpublished
286	Adult	Rutelinae	Gingindlovu	29°01'S 31°34'E	*Anomala caffra* Burmeister, 1844
287	Adult	Rutelinae	Oribi Gorge	30°41'S 30°20'E	*Anomala caffra* Burmeister, 1844
295	Adult	Rutelinae	Kosi Bay	26°54'S 32°50'E	*Popillia bipunctata* Fabricius, 1787
299	Adult	Rutelinae	Dalton	29°45'S 30°43'E	*Anomala usulata* Arrow, 1899
BOLD 0500 – BOLD 0519	Larva	Melolonthinae	Seven Oaks	29°07'S 30°05'E	Unidentified
BOLD 0520 – BOLD 0522	Larva	Melolonthinae	Eshowe	28°33'S 31°16'E	Unidentified
BOLD 0524	Larva	Rutelinae	Inanda	29'21'S 31°03'E	Unidentified
BOLD 0525, BOLD 0526, BOLD 0528	Larva	Melolonthinae	Dalton	29°11'S 30°22'E	Unidentified
BOLD 0529 – BOLD 0530	Larva	Dynastinae	Dalton	29°11'S 30°22'E	Unidentified
BOLD 0531 – BOLD 0537	Larva	Melolonthinae	Dalton	29°11'S 30°22'E	Unidentified
BOLD 0540 – BOLD 0548	Larva	Melolonthinae	Mtunzini	28°34'S 31°24'E	Unidentified
BOLD 0557 – BOLD 0558	Adult	Dynastinae	Malelane	25°25'S 31°34'E	*Heteronychus licas* Klug, 1835
BOLD 0559	Adult	Dynastinae	Pongola	27°25'S 31°31'E	*Heteronychus licas* Klug, 1835
BOLD 0560 – BOLD 0562	Adult	Dynastinae	Malelane	25°25'S 31°34'E	*Heteronychus licas* Klug, 1835

BOLD 0582	Larva	Melolonthinae	New Hanover	29°15'S 30°23'E	Unidentified
BOLD 0590	Adult	Melolonthinae	Dalton	29°45'S 30°43'E	*Congella* c. f. *tessellatula* Péringuey, 1902
BOLD 0593	Adult	Dynastinae	Mtubatuba	28°02'S 32°01'E	*Heteronychus rusticus niger* Klug, 1855
BOLD 0595 – BOLD 0596	Adult	Melolonthinae	Mtunzini	28°34'S 31°24'E	*Schizonycha* sp. Boheman, 1857
BOLD 0600	Adult	Melolonthinae	Mtunzini	28°34'S 31°24'E	*Schizonycha affinis*, Boheman, 1857
BOLD 0601, BOLD 0603, BOLD 0605	Adult	Melolonthinae	Mtunzini	28°34'S 31°24'E	*Schizonycha neglecta*, Boheman, 1857
BOLD 0618	Adult	Melolonthinae	Dalton	29°45'S 30°43'E	*Lepiserica* sp., Brenske, 1900
BOLD 0623	Adult	Melolonthinae	New Hanover	29°'12'S 30°28'E	*Schizonycha affinis*, Boheman, 1857
BOLD 0629	Adult	Melolonthinae	Mtunzini	28°34'S 31°24'E	*Schizonycha affinis*, Boheman, 1857
BOLD 0635	Adult	Melolonthinae	Dalton	29°11'S 30°22'E	*Schizonycha affinis*, Boheman, 1857
BOLD 0641, BOLD 0647	Larva	Melolonthinae	Amatikulu	28°39'S 31°31'E	Unidentified
BOLD 0653	Larva	Rutelinae	Mtubatuba	28°02'S 32°01'E	Unidentified
BOLD 0659, BOLD 0664, BOLD 0665	Larva	Rutelinae	Pongola	27°25'S 31°31'E	Unidentified
BOLD 0669	Larva	Dynastinae	Malelane	25°25'S 31°34'E	Unidentified
BOLD 0671	Larva	Rutelinae	Malelane	25°25'S 31°34'E	Unidentified
BOLD 0681	Larva	Melolonthinae	Dalton	29°10'S 30°23'E	Unidentified
BOLD 0723 – BOLD 0724	Larva	Melolonthinae	New Hanover	28°33'S 30°13'E	Unidentified
BOLD 0731	Larva	Rutelinae	New Hanover	29°13'S 30°26'E	Unidentified
BOLD 0733 – BOLD 0734, BOLD 0743, BOLD 0745	Larva	Melolonthinae	New Hanover	29°13'S 30°26'E	Unidentified
BOLD 0750	Larva	Melolonthinae	New Hanover	29°15'S 30°25'E	Unidentified
BOLD 0760	Larva	Melolonthinae	New Hanover	29°12'S 30°27'E	Unidentified
BOLD 0761, BOLD 0765	Larva	Melolonthinae	New Hanover	29°12'S 30°26'E	Unidentified
BOLD 0767, BOLD 0769	Larva	Melolonthinae	New Hanover	29°12'S 30°20'E	Unidentified
BOLD 0781 – BOLD 0782	Larva	Melolonthinae	New Hanover	29°15'S 30°25'E	Unidentified
BOLD 0784, BOLD 0786	Larva	Melolonthinae	New Hanover	29°17'S 30°21'E	Unidentified
BOLD 0791	Larva	Rutelinae	New Hanover	29°18'S 30°14'E	Unidentified
BOLD 0792	Larva	Melolonthinae	New Hanover	29°14'S 30°26'E	Unidentified
BOLD 0793	Larva	Melolonthinae	New Hanover	29°14'S 30°26'E	Unidentified
BOLD 0801	Larva	Melolonthinae	New Hanover	29°14'S 30°28'E	Unidentified
BOLD 0802	Larva	Melolonthinae	New Hanover	29°14'S 30°26'E	Unidentified
BOLD 0803	Larva	Melolonthinae	New Hanover	29°14'S 30°28'E	Unidentified
BOLD 0804	Larva	Melolonthinae	New Hanover	29°14'S 30°26'E	Unidentified
BOLD 0805	Larva	Melolonthinae	New Hanover	29°14'S 30°28'E	Unidentified
BOLD 0810	Larva	Melolonthinae	New Hanover	29°11'S 30°11'E	Unidentified
BOLD 0813	Larva	Melolonthinae	New Hanover	28°35'S 30°32'E	Unidentified
BOLD 0820	Larva	Melolonthinae	New Hanover	29°15'S 30°25'E	Unidentified
BOLD 0825	Larva	Melolonthinae	New Hanover	29°12'S 30°26'E	Unidentified
BOLD 0827 - BOLD 0830	Larva	Melolonthinae	New Hanover	29°17'S 30°21'E	Unidentified
BOLD 0835 – BOLD 0836 ; BOLD 0843 – BOLD 0844	Larva	Melolonthinae	New Hanover	29°15'S 30°25'E	Unidentified

BOLD 0845	Larva	Melolonthinae	New Hanover	29°12'S 30°26'E	Unidentified
BOLD 0847 – BOLD 0848	Larva	Melolonthinae	New Hanover	29°15'S 30°25'E	Unidentified
BOLD 0849 – BOLD 0851	Larva	Melolonthinae	New Hanover	29°12'S 30°26'E	Unidentified
BOLD 0853	Larva	Melolonthinae	Seven Oaks	29°17'S 30°21'E	Unidentified
BOLD 0855 ; BOLD 0860 – BOLD 0861	Larva	Melolonthinae	Seven Oaks	29°06'S 30°24'E	Unidentified
BOLD 0888 ; BOLD 0890	Larva	Rutelinae	New Hanover	29°15'S 30°25'E	Unidentified
BOLD 0895	Larva	Melolonthinae	New Hanover	28°35'S 30°32'E	Unidentified
BOLD 0908 ; BOLD 0910 ; BOLD 0913	Larva	Melolonthinae	Dalton	29°11'S 30°22'E	Unidentified
BOLD 0923	Larva	Rutelinae	New Hanover	28°35'S 30°32'E	Unidentified
BOLD 0931	Larva	Melolonthinae	New Hanover	29°13'S 30°20'E	Unidentified
BOLD 0939	Larva	Melolonthinae	New Hanover	28°35'S 30°32'E	Unidentified
BOLD 0941 – BOLD 0943	Larva	Rutelinae	New Hanover	28°34'S 30°13'E	Unidentified
BOLD 0944 - BOLD 0945 ; BOLD 0947 ; BOLD 0951 – BOLD 0953	Larva	Melolonthinae	New Hanover	29°06'S 30°24'E	Unidentified
BOLD 0955 – BOLS 0957	Larva	Melolonthinae	New Hanover	29°46'S 30°52'E	Unidentified
BOLD 0966 – BOLD 0967	Larva	Melolonthinae	New Hanover	29°13'S 30°19'E	Unidentified
BOLD 0984 ; BOLD 0987	Larva	Melolonthinae	New Hanover	29°11'S 30°27'E	Unidentified
BOLD 0990	Larva	Melolonthinae	New Hanover	29°10'S 30°28'E	Unidentified
BOLD 0992	Larva	Melolonthinae	New Hanover	29°13'S 30°19'E	Unidentified
BOLD 0994	Larva	Dynastinae	New Hanover	29°13'S 30°19'E	Unidentified
BOLD 1002 – BOLD 1003	Larva	Melolonthinae	New Hanover	29°14'S 30°22'E	Unidentified
BOLD 1004	Larva	Melolonthinae	New Hanover	29°16'S 30°24'E	Unidentified
BOLD 1005 – BOLD 1007	Adult	Melolonthinae	New Hanover	29°06'S 30°24'E	*Hypopholis sommeri* Burmeister, 1855
BOLD 1010	Adult	Melolonthinae	Dalton	28°35'S 30°32'E	*Schizonycha affinis*, Boheman, 1857
BOLD 1011 – BOLD 1012	Adult	Melolonthinae	New Hanover	29°18'S 30°14'E	*Schizonycha affinis*, Boheman, 1857
BOLD 1014 – BOLD 1015	Larva	Melolonthinae	New Hanover	29°16'S 30°24'E	Unidentified
BOLD 1019	Larva	Melolonthinae	New Hanover	29°11'S 30°27'E	Unidentified
BOLD 1020; BOLD 1023	Larva	Melolonthinae	New Hanover	29°16'S 30°18'E	Unidentified
BOLD 1024 – BOLD 1025	Larva	Melolonthinae	New Hanover	28°35'S 30°32'E	Unidentified
BOLD 1029 ; BOLD 1031 ; BOLD 1033 – BOLD 1034	Larva	Melolonthinae	New Hanover	29°19'S 30°17'E	Unidentified
BOLD 1039	Larva	Melolonthinae	New Hanover	29°11'S 30°27'E	Unidentified
BOLD 1042 – BOLD 1043	Larva	Melolonthinae	New Hanover	29°14'S 30°22'E	Unidentified
BOLD 1046 – BOLD 1047	Larva	Melolonthinae	New Hanover	29°13'S 30°19'E	Unidentified
BOLD 1052 – BOLD 1053	Larva	Melolonthinae	New Hanover	29°19'S 30°18'E	Unidentified
BOLD 1056	Larva	Melolonthinae	New Hanover	29°11'S 30°27'E	Unidentified
BOLD 1057	Larva	Melolonthinae	New Hanover	29°16'S 30°24'E	Unidentified
BOLD 1058 ; BOLD 1061	Larva	Melolonthinae	New Hanover	29°16'S 30°23'E	Unidentified

1 Subfamily names were assigned to larval specimens after morphological examination.

APPENDIX B. Morphological characteristics grouping larval specimens into species and morphospecies groups used to develop the Field key and Lucid 3.4 key

General morphological features of Scarabaeidae larvae	A M-species 1 Dynastinae	B M-species 2 Dynastinae	D *Heteronychus licas* Dynastinae	E M-species 3 Rutelinae	F *Anomala ustulata* Rutelinae	K M-species 4 Melolonthinae	L M-species 5 Melolonthinae	M M-species 6 Melolonthinae	O M-species 7 Melolonthinae	P *Schizonycha affinis* Melolonthinae	Q M-species 8 Melolonthinae	R M-species 9 Melolonthinae	S_1 *Camenta* sp. Melolonthinae	S_2 *Camenta* sp. Melolonthinae	T M-species 10 Melolonthinae	U M-species 11 Melolonthinae	W *Schizonycha* sp. Melolonthinae	Y M-species 12 Melolonthinae	Z M-species 13 Melolonthinae	AA M-species 14 Melolonthinae	AB *Hypopholis sommeri* Melolonthinae	AC *Hypopholis sommeri* Melolonthinae
Anal opening • An even transverse arc • An inverted Y-shape or angulate	Even, transverse arc	Even, transverse arc	Even, transverse arc	Even, transverse arc	Even, transverse arc	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped	Angulate/inverted y-shaped
Antennae - Last antennal segment – number of dorsal sensory spots • One or more • one	One or more	One or more	One or more	One or more	One or more	one	one	one	one	one	one	one	one	one	one	one	one	one	one	one	one	one	one
Epipharynx – Haptomerum • 3 broad, comb-like heli • 3 sharp, pointed heli • 4 sharp, pointed heli • 6 sharp, pointed heli • with a row of 6-9 pointed heli • with a row of short, stout heli	3 broad, comb-like heli	3 broad, comb-like heli	6 sharp, pointed heli	A row of 6-9 pointed heli	A row of short, stout, heli	6 sharp, pointed heli	6 sharp, pointed heli	6 sharp, pointed heli	8 heli	8 heli	8 heli	3 sharp, pointed heli	3 sharp, pointed heli	3 sharp, pointed heli	4 sharp, pointed heli	4 sharp, pointed heli	8 heli	8 heli	8 heli	8 heli	8 heli	8 heli
Head capsule - Epicranial suture • Distinct • Indistinct	Distinct	Distinct	Indistinct	Distinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct
Head capsule - Surface • Rough / pitted • Smooth	Rough/ pitted	Rough/ pitted	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Rough/ pitted	Rough/ pitted	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Labrum - Edge • Asymmetrical • Symmetrical	Symmetrical	Symmetrical	Asymmetrical	Symmetrical	Asymmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical	Symmetrical
Labrum - Shape • Oval • Pointed • Trilobed	Oval	Oval	Pointed	Oval	Oval	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed
Mandible - Molar lobe • Irregular • Square • Trilobed	Square	Square	Irregular	Trilobed	Square	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Square	Square	Square	Square	Square
Mandible - Scissorial area • Broad • Narrow	Narrow	Narrow	Narrow	Broad	Broad	Broad	Broad	Broad	Narrow	Narrow	Narrow	Broad	Broad	Broad	Broad	Broad	Broad	Narrow	Narrow	Narrow	Narrow	Narrow
Mandible - Setae • Long • Short	Short	Short	Long	Short	Short	Short	Short	Short	Short	Short	Short	Short	Short	Short	Short	Short	Short	Long	Long	Long	Long	Long
Mandible - Ventral stridulatory area • Absent • Oval in shape	Present	Present	Present	Present	Present	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Mandible - Width of mandible at base • Broad • Narrow	Narrow	Narrow	Broad	Narrow	Narrow	Broad	Narrow	Broad	Narrow	Narrow	Broad	Narrow	Narrow	Narrow	Broad	Broad	Broad	Broad	Broad	Broad	Broad	Broad
Maxilla - Cardo-maxillary articulating membrane • Bearing numerous black dots • No black dots	No black dots	No black dots	No black dots	No black dots	No black dots	No black dots	No black dots	No black dots	No black dots	No black dots	No black dots	Bearing numerous black dots	Bearing numerous black dots	Bearing numerous black dots	Bearing numerous black dots	Bearing numerous black dots	No black dots	No black dots	No black dots	No black dots	No black dots	No black dots
Maxilla - Lacinia • 1 or 2 well developed terminal unci • 3 well developed terminal unci	3	3	1 or 2	3	1 or 2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Maxilla - Stipes • Enlarged and bulbous • Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Enlarged	Enlarged	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal	Small / normal
Maxilla - Stridulatory teeth • Pointed • Truncated	Truncate	Truncate	Truncate	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed
Raster pattern • 12-14 pali arranged in an inverted V-shape; 50-60 hamate setae • Approximately 30 pali arranged in zip-like formation; approximately 60 hamate setae • Dense arrangement of setae forming circular shape • No raster pattern • Pali arranged in circular formation around anal opening • Pali in semi-circle arranged in groups of 2-4 • Pali in semi-circle; comb-like formation • Parallel row of dense pali curving outwards at anal opening and becoming a double row; number of pali ranging from 17-24 • Raster lemon-shaped or inverted V-shaped with number of pali ranging from 12-22	No raster pattern	No raster pattern	No raster pattern	12-14 pali arranged in an inverted v-shape; 50-60 hamate setae	Approximately 30 pali arranged in zip-like formation; 60 hamate setae	Dense arrangement of setae forming a circular shape	Dense arrangement of setae forming a circular shape	Dense arrangement of setae forming a circular shape	Raster lemon-shaped or inverted v-shaped with number of pali ranging from 12-22	Raster lemon-shaped or inverted v-shaped with number of pali ranging from 12-22	Raster lemon-shaped with the number of pali ranging from 12-22	Pali in semi circle, comb-like formation	Pali in semi-circle; groups of 2-4	Pali in semi-circle; groups of 2-4	Pali arranged in circular formation around anal opening	Pali arranged in circular formation around anal opening	Parallel row of dense pali curving outwards at anal opening and becoming a double row; 16-24 pali	No raster pattern	No raster pattern	No raster pattern	No raster pattern	No raster pattern
Tarsal claws • Long and thin • Short and stout	Short and stout	Short and stout	Short and stout	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin	Long and thin
Average specimen length (head to last abdominal segment) • 20.25 mm • 6.90 – 11.90 mm • 19.60 mm • 10.50 – 14.50 mm • 12.40 – 28.55 mm • 8.25 – 20.65 mm • 14.90 – 23.60 mm • 8.65 – 12.40 mm • 13.45 – 18.90 mm • 16.60 – 21.45 mm	18.95 – 27.30 mm	18.95 – 27.30 mm	19.60 mm	6.90 – 11.90 mm	20.25 mm	16.60 – 21.45 mm	16.60 – 21.45 mm	16.60 – 21.45 mm	8.25 – 20.65 mm	8.25 – 20.65 mm	8.25 – 20.65 mm	8.65 – 12.40 mm	13.45 – 18.90 mm	13.45 – 18.90 mm	1`0.5 – 14.50 mm	1`0.5 – 14.50 mm	14.90 – 23.60 mm	12.40 – 28.55 mm	12.40 – 28.55 mm	12.40 – 28.55 mm	12.40 – 28.55 mm	12.40 – 28.55 mm
Width of head (between most distant points) • 1.80 – 2.55 mm • 2.00 – 3.90 mm • 2.80 – 3.25 mm • 2.95 – 3.90 mm • 3.00 – 5.95 mm • 3.30 mm • 3.45 – 3.89 mm • 3.55 – 4.40 mm • 3.6 mm • 3.95 – 5.00 mm • 4.15 – 4.35 mm	3.95 – 5.00 mm	3.95 – 5.00 mm	3.30 mm	1.80 – 2.55 mm	3.6 mm	3.55 – 4.40 mm	3.55 – 4.40 mm	3.55 – 4.40 mm	2.00 – 3.90 mm	2.00 – 3.90 mm	2.00 – 3.90 mm	2.95 – 3.90 mm	3.45 – 3.89 mm	3.45 – 3.89 mm	2.80 –3.25 mm	2.80 –3.25 mm	4.15 – 4.35 mm	3.00 – 5.95 mm	3.00 – 5.95 mm	3.00 – 5.95 mm	3.00 – 5.95 mm	3.00 – 5.95 mm
Geographical distribution of Scarabaeidae larvae • Amatikulu • Dalton • Empangeni • Eshowe • Gingindlovu • Harburg • Heatonville • Inanda • Kosi Bay • Malelane • Mtubatuba • Mtunzini • New Hanover • Oribi Gorge • Pongola • Seven Oaks • Umfolozi • Verulam	Umfolozi	New Hanover	Mtubatuba Malelane Pongola Umfolozi	New Hanover Malelane Pongola Mtubatuba Inanda	Heatonville Verulam Dalton	Mtubatuba	New Hanover	Eshowe	New Hanover	Seven Oaks New Hanover Dalton Harburg Mtunzini Gingindlovu	New Hanover	New Hanover	Eshowe Mtubatuba	Empangeni Amatikulu	Eshowe	Seven Oaks Dalton	Mtunzini	New Hanover	Seven Oaks New Hanover Dalton	Dalton	New Hanover Seven Oaks Dalton	New Hanover Seven Oaks
Colour of Scarabaeidae larvae - Head • Light brown • Dark brown • Yellow to light brown • Dark brown to black • White to yellow	Light brown	Dark brown	Light brown	Yellow-light brown	Light brown	Dark Brown	Yellow-light brown	Dark brown-black	Light brown	Yellow-light brown	Light brown	Light brown	Dark brown	Dark Brown	Dark Brown	Light Brown	Dark Brown	Whitish – yellow	Dark brown	Yellow-light brown	Yellow-light brown	Yellow-light brown
Colour of Scarabaeidae larvae Body • Dark brown • Light brown • Dark brown to black • Translucent with dark interior • Whitish • Whitish-yellow • Brown-grey • Translucent whitish with dark brown • Light brown to dark brown • Whitish yellow	Dark brown	Dark brown	Light brown	Yellow-light white	Light brown	Dark brown	Dark Brown	Dark Brown-black	Translucent with dark interior	Translucent, whitish with dark brown	White	Whitish – Yellow	Brown-grey	Dark brown- black	Dark brown	Light brown –dark brown	Dark brown	Translucent-white	Dark brown-black	Brown-grey	Dark Brown-Grey	Light brown-dark brown

APPENDIX C. Morphological Characters, their character number as used in Appendix D and scoring of characters used in the matrix of Appendix D

1. ***Anal opening****: an even transverse arc = 0; an inverted Y-shape = 1*
2. ***Mandible ventral, oval, stridulatory area****: absent = 0; present = 1*
3. ***Last antennal segment dorsal sensory spot*** *: 1 dorsal sensory spot = 0; 1 or more dorsal sensory spots = 1*
4. ***Maxillary stridulatory teeth****: truncate = 0; pointed = 1*
5. ***Labrum:*** *symmetrical = 0; asymmetrical = 1*
6. ***Labrum shape****: oval = 0; trilobed = 1; pointed = 2;*
7. ***Tarsal claws****: long and thin = 0; short and stout = 1*
8. ***Cardo-maxillary articulating membrane****: no black dots = 0; bearing black dots = 1*
9. ***Stipes of maxilla****: small/normal = 0; enlarged and bulbous = 1*
10. ***Raster pattern****:*

    no raster = 0;

    ± 30 pali arranged in zip-like formation, approximately 60 hamate setae = 1;

    12-14 pali arranged in an inverted V-shape, 50 - 60 hamate setae = 2;

    Pali arranged in circular formation around anal opening = 3;

    Pali in semi-circle, comb-like formation = 4;

    Pali in semi-circle arranged in groups of 2 - 4 = 5;

    Dense arrangement of setae forming circular shape = 6;

    Raster lemon-shaped or inverted v-shaped with number of pali ranging from 12- 22 = 7;

    Parallel row of dense pali curving outwards at anal opening and becoming a double row, pali ranging from 17-24 = 8
11. ***Maxillary palpus****: 4-segmented = 0; 3-segmented = 1;*
12. ***Galea and lacinia of maxilla****: distinctly separated = 0; entirely fused or fused proximally but distally free = 1*
13. ***Lacinia of maxilla****: 1or 2 well-developed terminal unci = 0; 3 well-developed terminal unci = 1; no unci = 2*
14. ***Molar lobe of mandible****: square = 0; trilobed = 1; irregular = 2*

15. ***Haptomerum of epipharynx****:*

    with a row of short, stout heli = 0;

    with a row of 6-9 pointed heli = 1;

    3 broad comb-like heli = 2;

    6 sharp pointed heli = 3;

    3 sharp and pointed heli = 4;

    4 sharp pointed heli = 5;

    8 heli = 6;

16. ***Scissorial area of mandible****: narrow = 0; broad = 1*

17. ***Mandibular setae****: long = 0; short = 1*

18. ***Surface of head capsule****: smooth = 0; rough/pitted = 1*

19. ***Epicranial suture****: indistinct = 0; distinct = 1*

APPENDIX D. Morphological data matrix with character scores within the matrix as assigned to each species or morphospecies group based on the presence of certain characters

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Anomala ustulata	0	1	1	1	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0
Camenta sp.	1	0	0	1	0	2	0	1	0	5	0	0	1	2	0	1	1	1	0
Heteronychus licas	0	1	1	0	1	2	0	0	0	0	0	1	0	2	3	0	1	1	0
Hypopholis sommeri	1	0	0	1	0	2	0	0	0	0	0	0	2	0	6	0	0	0	0
Schizonycha affinis	1	0	0	1	0	2	0	0	0	7	0	0	2	2	6	0	1	0	0
Schizonycha sp.	1	0	0	1	0	2	0	0	0	8	0	0	1	2	6	1	1	0	1
Morphospecies 1	0	1	1	0	0	0	0	0	0	0	0	1	1	0	2	0	1	1	1
Morphospecies 2	0	1	1	0	0	0	0	0	0	0	0	1	1	0	2	0	1	1	1
Morphospecies 3	0	1	1	1	0	0	1	0	0	2	0	1	1	1	1	1	1	0	1
Morphospecies 4	0	0	0	1	0	2	0	0	0	6	0	0	1	2	3	1	1	0	0
Morphospecies 5	0	0	0	1	0	2	0	0	0	6	0	0	1	2	3	1	1	0	0
Morphospecies 6	0	0	0	1	0	2	0	0	0	6	0	0	1	2	3	1	1	0	0
Morphospecies 7	1	0	0	1	0	2	0	0	0	7	0	0	2	2	6	0	1	0	0
Morphospecies 8	1	0	0	1	0	2	0	0	0	7	0	0	1	2	6	0	1	0	0
Morphospecies 9	1	0	0	1	0	2	0	1	0	4	0	0	1	2	2	1	1	0	0
Morphospecies 10	1	0	0	1	0	2	0	1	1	3	0	0	1	2	5	1	1	0	0
Morphospecies 11	1	0	0	1	0	2	0	1	1	3	0	0	1	2	5	1	1	0	0
Morphospecies 12	1	0	0	1	0	2	0	0	0	0	0	0	2	0	6	0	0	0	0
Morphospecies 13	1	0	0	1	0	2	0	0	0	0	0	0	2	0	6	0	0	0	0
Morphospecies 14	1	0	0	1	0	2	0	0	0	0	0	0	2	0	6	0	0	0	0