IDENTIFICATION KEY TO SCARABAEID BEETLE LARVAE ATTACKING SUGARCANE IN SOUTH AFRICA USING DNA BARCODING AND INTEGRATIVE TAXONOMY

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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 larval identification. This is unfortunate, as it is mainly the larval stage that is found to be associated with crop damage. Inadequate keys for the taxonomy of larvae of these groups, as well as the lack of taxonomists working on these groups have been identified as constraints. Using mitochondrial DNA data variation in the base pair sequence of the mitochondrial cytochrome c oxidase sub unit I (*cox 1*) gene, 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. The major groupings delimited specimens belonging to the subfamilies Dynastinae, Melolonthinae and Rutelinae. Mitochondrial DNA (mtDNA) of larval specimens was linked with mtDNA of identified adult specimens through phylogenetic analysis. This allowed the identification of those larvae through morphological characteristics unique to certain species. Keys were produced during this study which will allow workers to identify larvae, in many cases to species. Obtaining species identifications for larvae will allow the application of species-specific control measures and also will facilitate analysis of interactions among species. These taxonomic 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.

Keywords: DNA barcoding, identification key, integrative taxonomy, Scarabaeidae, sugarcane

Introduction

Scarabaeids (Coleoptera) are damaging to sugarcane, both in the adult or larval stage or in both (Gordh and Headrick, 2000). Adults are either phytophagous, feeding on 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 and Headrick, 2000). In southern Africa, *Heteronychus licas* Klug 1835 (Coleoptera: Scarabaeidae) feeds as an adult

on the young sugarcane plant's meristematic region and its larva on sugarcane root hairs (Way, 1995). In general, species with root-feeding larvae are regarded as pests (Gordh and 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 and host plants) and ecology (Miller *et al*., 1999). Control measures for scarabaeid pest species in sugarcane can be developed, successfully implemented and optimised when the species have been identified (Miller *et al*., 1999). In the past, identification of species has relied on the use of morphological characteristics (Miller *et al*., 1999). Due to phenotypic variation within a single species it is not always possible to solely base identifications on morphology (Miller and Allsopp, 2000). Phenotypic variation among scarabaeid larvae collected from sugarcane fields in South Africa 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 and du Toit, 1996). The solution to this problem has been the recent use of molecular techniques to link identified adult specimens to unidentified larval specimens (Ahrens *et al*., 2007; Dittrich-Schröder, 2009; Miller *et al*., 2005). Molecular identification is needed because larvae are difficult to rear to adults in the laboratory due to their long life cycle and high mortality under laboratory conditions. Secondly, although many adults are caught in light traps, this method is non-selective as the adults are very mobile and therefore no direct links can be made between the adults caught and the larva present in the sugarcane crop.

This study aimed at creating a morphological key based on larval groupings identified through molecular techniques, which allowed unique morphological characteristics to be identified for those groupings (Dittrich-Schröder, 2009). After 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 other taxa using molecular techniques (Dittrich-Schröder, 2009) provided 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 in South Africa. The key presented in this paper is the first step in the development of a key to confirm the identification of all scarab species occurring in the soils of the South African sugarcane industry.

Materials and Methods

Linking larval and adult specimens using DNA barcodes

There are 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. Alternate methods are needed to assist in identification. Such an alternative is DNA barcoding, which can be employed to identify scarabaeid larvae. DNA barcoding is a technique suitable for all taxa, which was proposed by Hebert *et al*. (2003) 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 and Monaghan, 2006). The barcode sequences can then be compared with each other and therefore define taxa (Kurtzman, 1994; Wilson, 1969). 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 and Monaghan, 2006). This grouping can be justified by a range of studies (Hebert *et al.,* 2003; Hebert and Gregory, 2005) which showed that intraspecies variation is usually lower than interspecies variation.

Two hundred and fifteen specimens (174 larvae and 41 adults) (Appendix A) were used in this study. Variation in the DNA sequence of the *cox 1* gene was used to delineate similar groups. Neighbour-joining and maximum parsimony analyses of the 658 bp *cox 1* sequences identified groups of larvae that linked to adult specimens. 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. There was not always correspondence between mtDNA groups and morphospecies. The tree branching pattern and node support indicated possible mtDNA groupings. Similarly, morphology indicated possible groupings. In both cases discrepancies were resolved by comparing the mtDNA groupings with the morphological groupings and determining the extent of a group based on agreement of morphological and molecular data.

Construction of the key

Ritcher's (1966) keys to white grubs and their allies were used to morphologically identify the larval specimens in this study to subfamily level. These keys, as well as keys created by Sweeney (1967) and Ahrens *et al.* (2007), were used as a starting point from which the keys 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.

Dissection of the head capsule of larval specimens

The following method was adapted from the method used by James du G Harrison (personal communication[1]) 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 (Figures 1-4).

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 0.5 ml PCR tubes. These tubes were labelled and placed in the vial with the corresponding larval abdomen.

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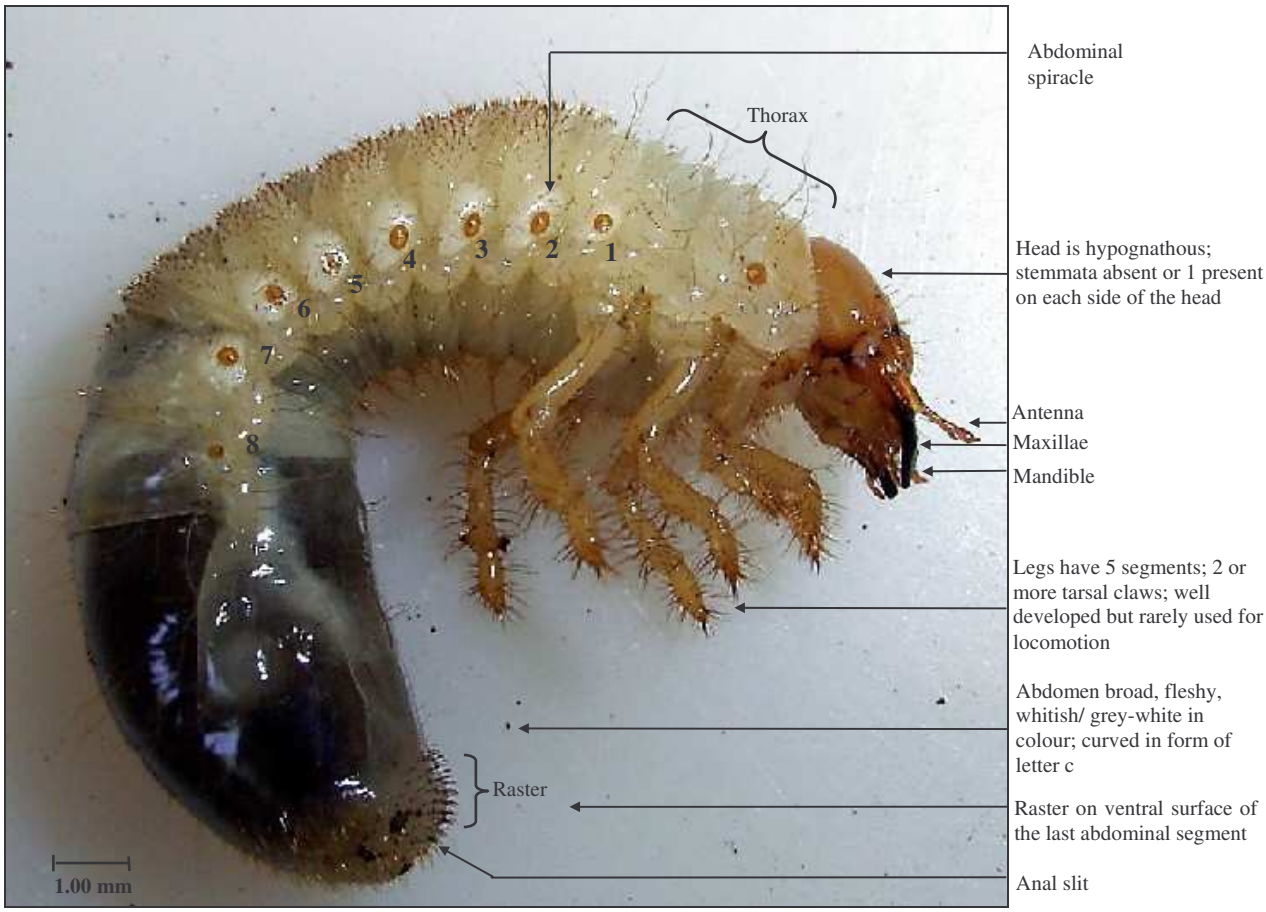


Figure 1. A photograph of a generalised scarabaeid larva.

(Photograph: Mike Way, 2004)

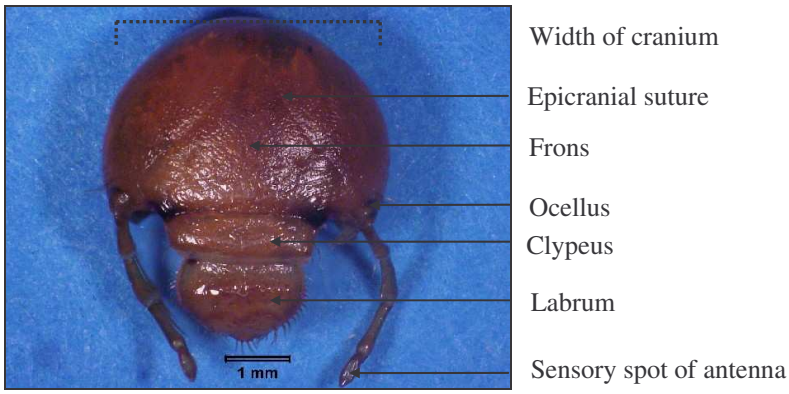


Figure 2. Generalised larval scarabaeid head indicating the dorsal characteristics that may be used for identification.

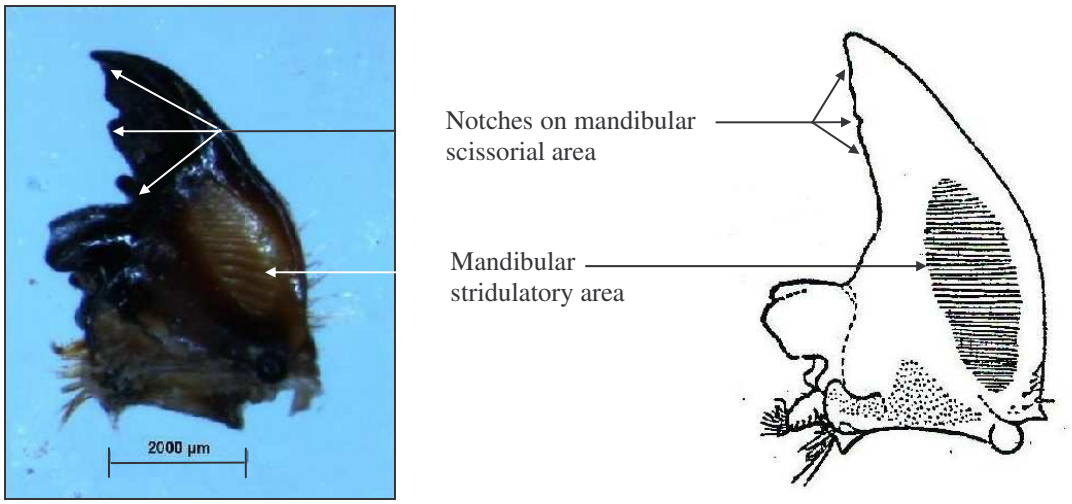


Figure 3. Generalised larval scarabaeid mandible indicating the characteristics used for identification. (Diagram adapted from Ritcher (1966))

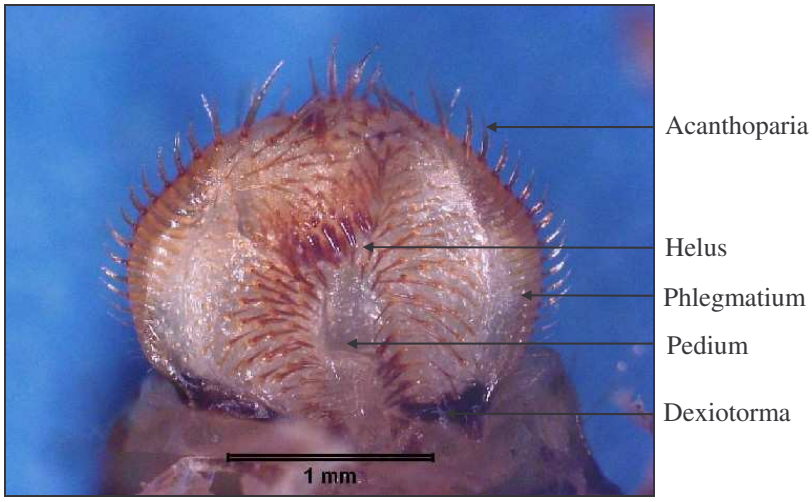


Figure 4. Generalised larval scarabaeid epipharynx indicating the characteristics used for identification.

Developing the key

The flow diagram (Figure 5) illustrates the basis of the approach used to develop the key in this study. Molecular analyses grouped specimens. Ritcher's (1966) keys were then used to identify specimens to subfamily level and thereby verify the larger groupings obtained from molecular analysis. Subsequently, specimens were examined to determine which morphological features were characteristic and/or common to the species/morphospecies within each larger grouping. Characteristic morphological features were tabulated. Molecular techniques make taxonomic groupings more reliable, and therefore this approach was performed in parallel with morphological identification in this study. Figure 6 shows the procedure followed to produce the field key. To make this key useful for individuals working in sugarcane fields, the starting point for the key (Tables 1-7) had to be at the phylum level.

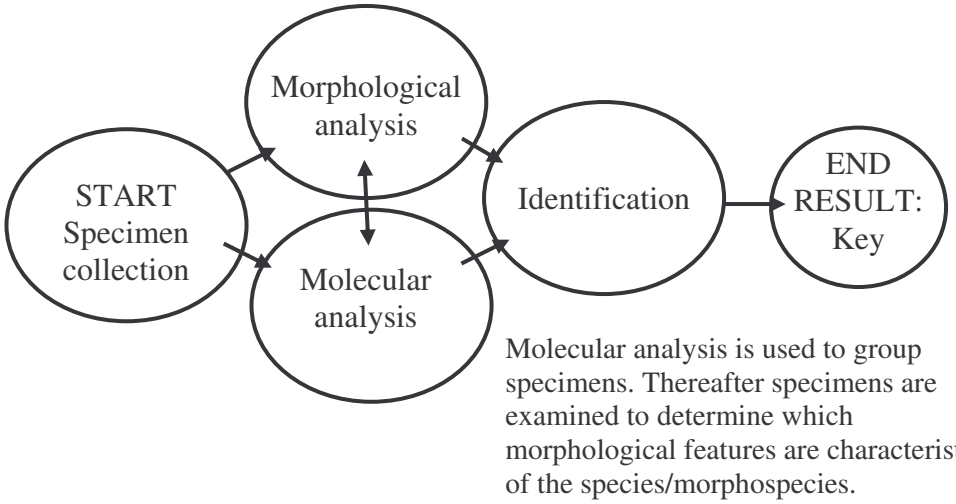


Figure 5. A schematic representation of the procedures followed to obtain the key.

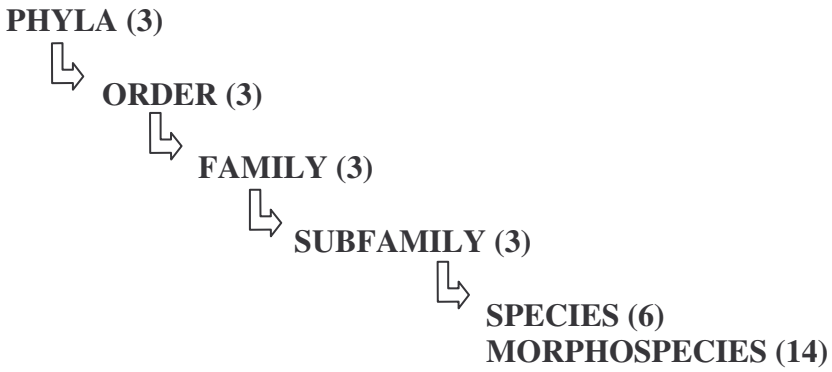


Figure 6. A diagram showing the taxonomic groupings used in this study (following Scholtz and Holm, 1996), with the number of groups at each hierarchical level used in the field key presented in this chapter given in brackets.

Tabulation of larval morphological characteristics and field key development

Scarabaeid larvae were distinguished by initially compiling a table listing the features visible mostly with a hand lens (magnification 3x) and thereafter a LEICA ZOOM 2000 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. 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. 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 as far as possible to identify larval scarabaeids found there.

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 1 deals with higher level taxonomy to separate phyla that could be found in the soil, and Table 5 the taxonomy required to key out to the scarabaeid species and morphospecies.

The key is colour coded to further aid ease of identification. In Tables 1-3 sections referring to the Scarabaeidae are shaded in light grey. Tables 4-7 use colour for the scarabaeid taxa corresponding to the three subfamilies of interest (i.e. red = Dynastinae; blue = Melolonthinae; green = Rutelinae).

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 for individuals who may 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.

Results

Minimal information and detail regarding molecular methods as well as phylogenetics trees are given in this paper, as this work is being prepared for publication elsewhere. DNA barcoding grouped specimens into 29 provisional groups (Group A to Group AC) based on phylogenetic tree branching patterns resulting from maximum parsimony and neighbour joining, per cent sequence divergence as well as comparison of morphological data. The major groupings delimited specimens belonging to the subfamilies Dynastinae, Melolonthinae and Rutelinae. For six species the authors 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. The strong support for the nodes also confirms the clustering of specimens into groups.

Figure 7 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.

A variety of arthropods are found at or near the soil surface. They could belong to four phyla: Insecta, Arachnida, Acari and Diplopoda (Table 1). It is important to note that scale bars on images are merely to provide an example, and specimens in these categories may vary in size. If the arthropod collected belongs to the phylum Insecta, identification can proceed by using Table 2. Should this not be the case the identification process ends. Table 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 (sclerotization, no visible head capsule, well developed head), segmentation and colour of the abdomen and legs (weakly developed, absence of legs, well-developed thoracic legs). Table 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 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 5-7) separated specimens within the subfamily Dynastinae, Rutelinae and Melolonthinae respectively. Table 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 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 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 (different patterns in terms of arrangement and number of pali). Additionally Morphospecies 10 and 11 can be separated from the other melolonthines by the presence of enlarged stipes which are bulbous.

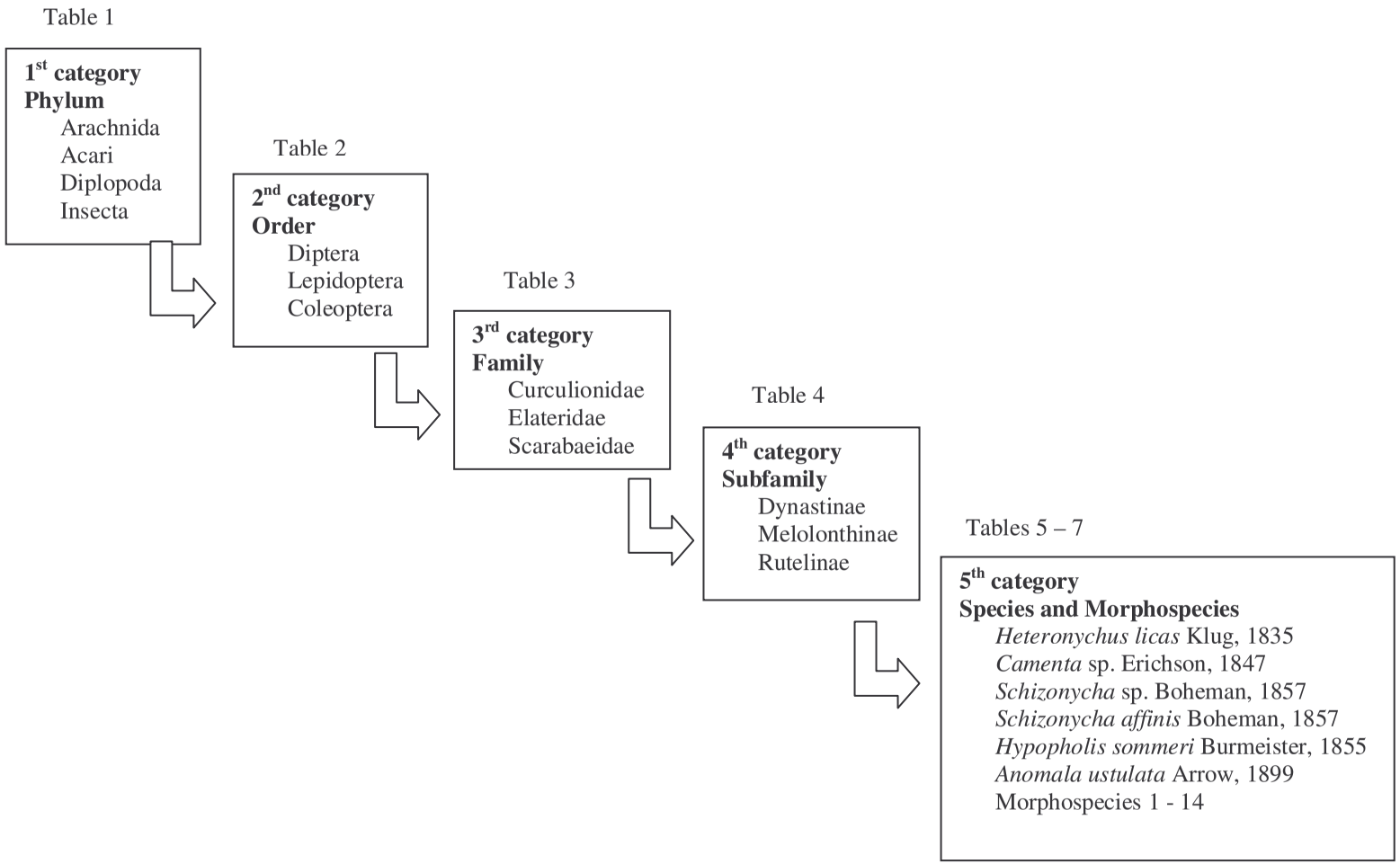


Figure 7. The breakdown of the species and morphospecies determined from the specimens found in soils of the sugarcane areas sampled in this study, according to the system of nomenclature described in Figure 2.

Table 1. Key features of common arthropod Phyla encountered on or in soil in South African sugarcane fields.

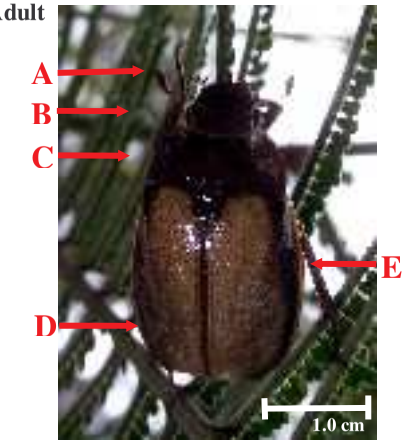
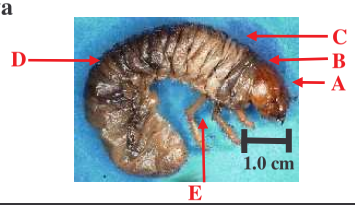
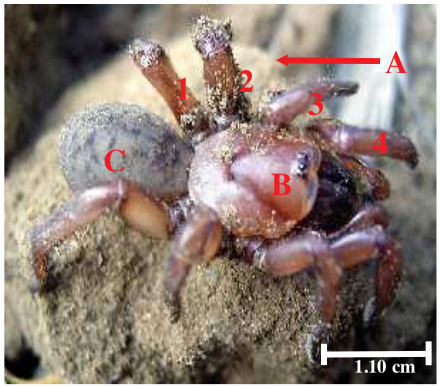
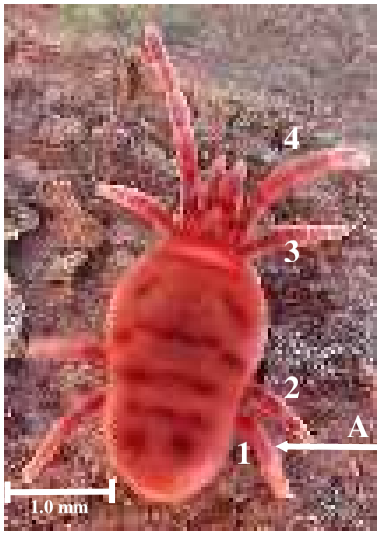
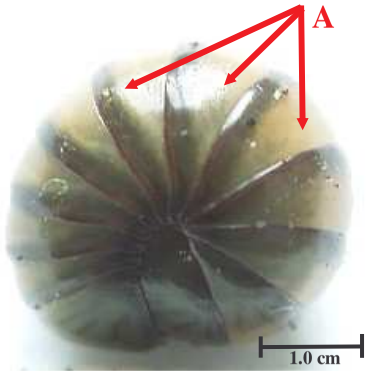
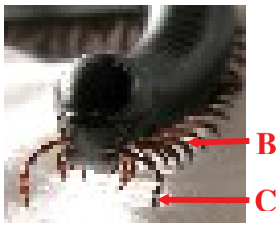
Insecta (Insects) Go to Table 2	**Arachnida (Spiders)**	**Acari (Mites)**	**Diplopoda (Millipedes)**
Adult  Photograph by Way (2005) Larva  	 Photograph by Way (2007)	 http://magickcanoe.com/blog/2006/05/09/she-wore-red-velvet/	 Photograph by Way (2007)  http://www.seattlebugsafari.com/millipedes_centipedes.htm
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; rounded; many segmented; one or two pairs of legs on each segment; Antennae 7-segmented A = many segments; B = legs; C = antenna

Table 2. Key features of larvae of common insect Orders found on or in soil in South African sugarcane fields.

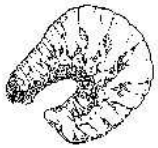
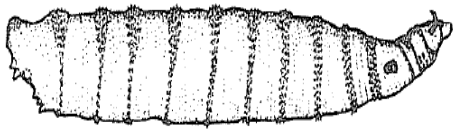
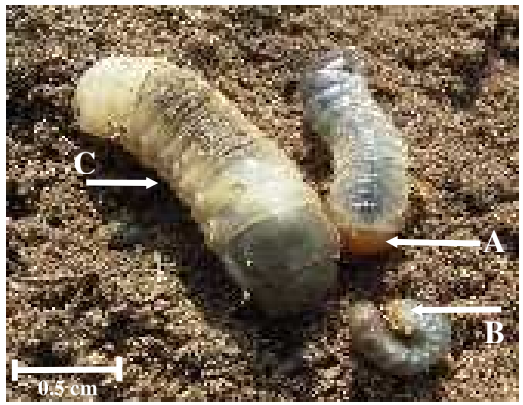
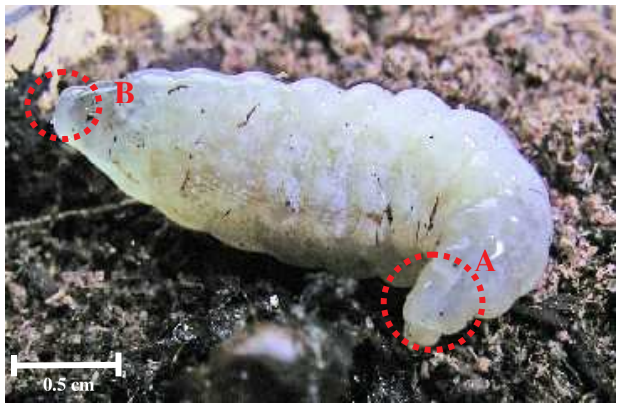
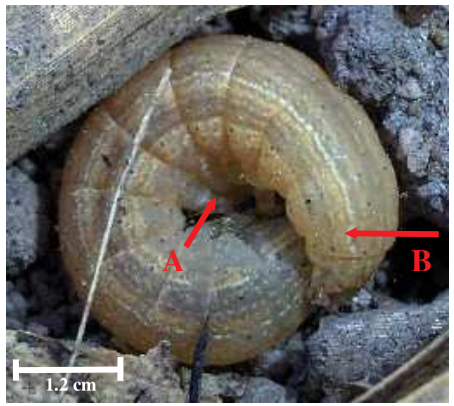
Beetles (Order Coleoptera) Go to Table 3	**Flies (Order Diptera)**	**Caterpillars (Order Lepidoptera)**
 Adapted from Scholtz & Holm (1996)	 Adapted from Scholtz & Holm (1996)	 Adapted from Scholtz & Holm (1996)
 Photograph by Way (2007)	 Photograph by Way (2007)	 **Photograph by Way (2007)**
C-shaped, Stout cylindrical, soft-bodied, Head strongly sclerotised (yellow, red or brown); Thorax and abdomen 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. Key features of the larvae of the most common coleopteran families encountered on or in soil in South African sugarcane fields.

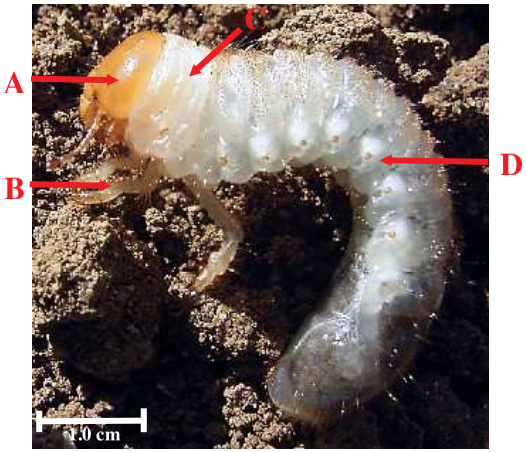
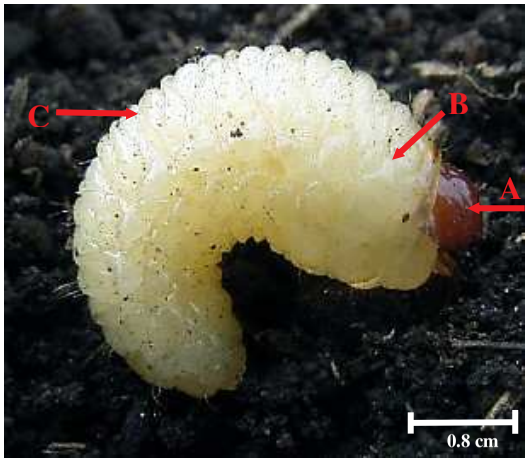
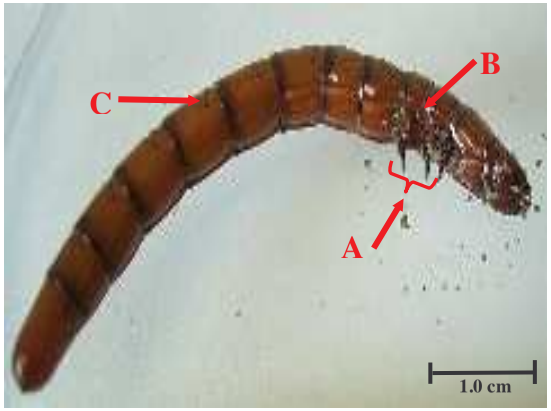
Scarabaeidae (leaf chafers, dung beetles) Go to Table 4	**Curculionidae (weevils)**	**Elateridae (click beetles)**
 Photograph by Way (2007)	 Photograph by Way (2007)	 Photograph by Way (2007)
C-shaped, Stout, cylindrical, soft-bodied; Strongly sclerotised head capsule; Weakly developed legs; Whitish/greyish thoracic and abdominal 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 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)).

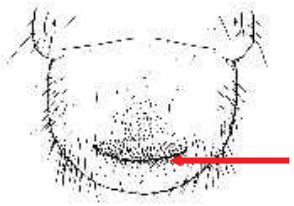
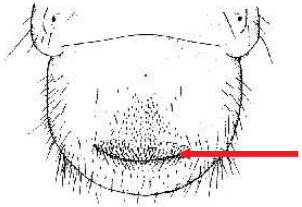
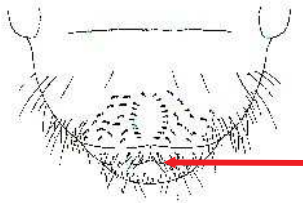
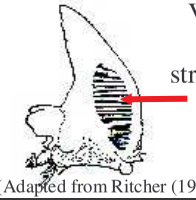
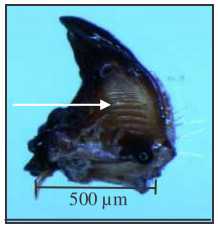
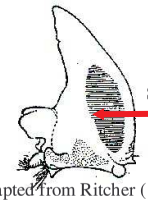
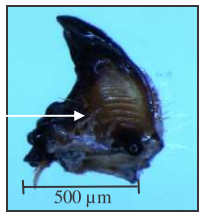
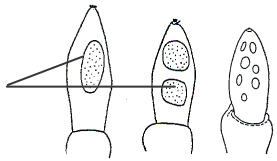
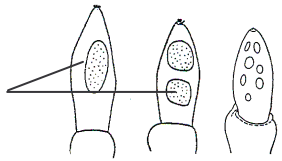
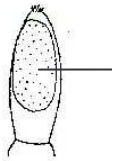
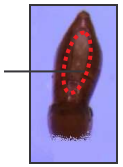
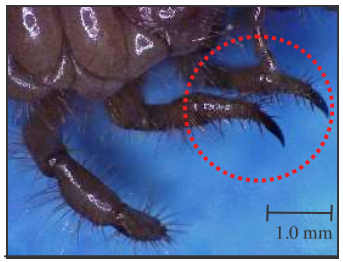
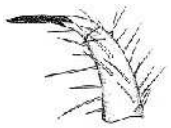
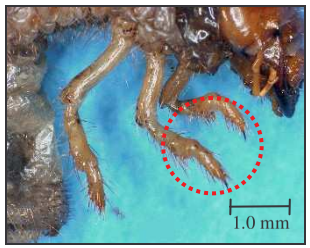
	Dynastinae Go to Table 5	**Rutelinae** Go to Table 6	**Melolonthinae** Go to Table 7
Anal opening (on last abdominal segment)	 Anal opening a transverse arc (Adapted from Oberholzer (1959))	 Anal opening a transverse arc (Adapted from Oberholzer (1959))	 Anal opening inverted Y-shaped (Adapted from Ritcher (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 5. Key features of larvae identified to genus and species/morphospecies levels within the Dynastinae encountered in soils of South African sugarcane fields.

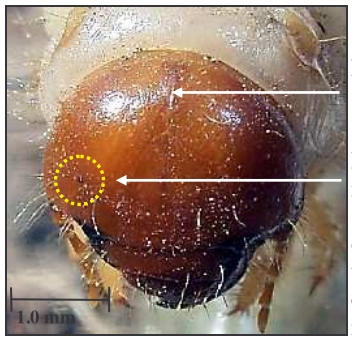
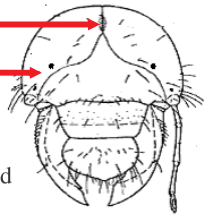
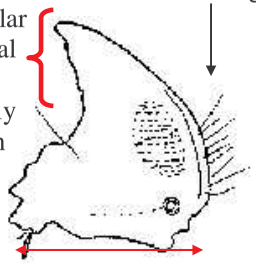
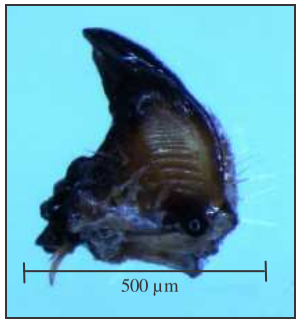
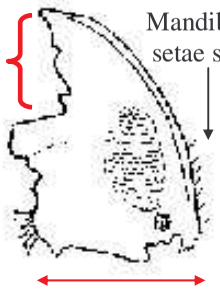
	Heteronychus licas	Morphospecies 1 & 2
Head capsule	 Photograph by Way (2007) Epicranial suture Pigmented, indented spots visible on head laterad to each; head capsule smooth in texture  (Adapted from Ritcher (1966))	 Epicranial suture distinct Head capsule rough/ pitted
Mandible	Few notches on mandibular 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 6. Key features of larvae identified to tribe, genus and species/morphospecies levels within the Rutelinae encountered in soils of South African sugarcane fields

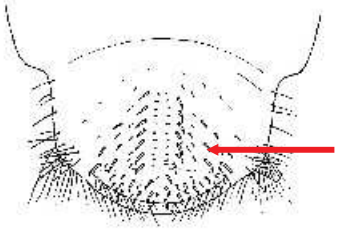
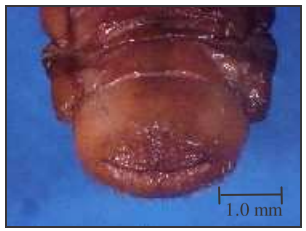
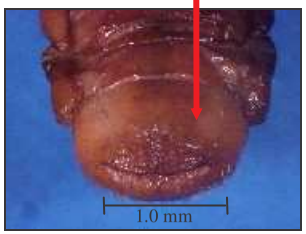
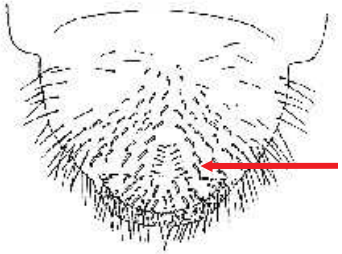
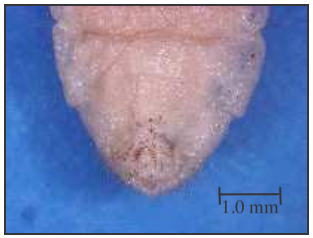
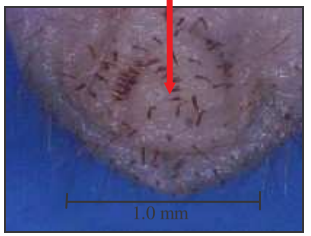
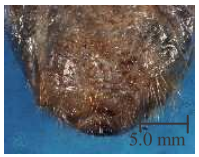
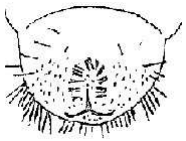
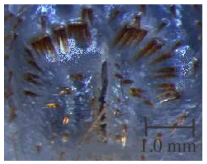
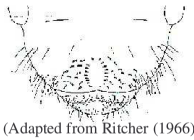
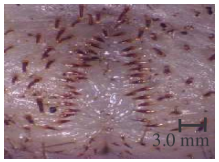
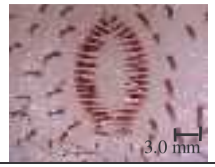
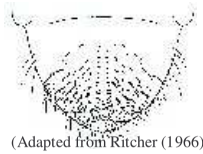
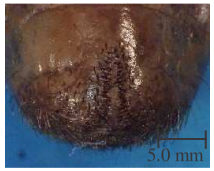
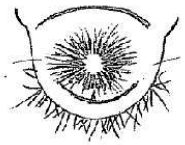
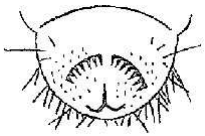
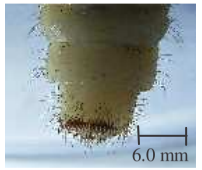
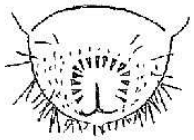
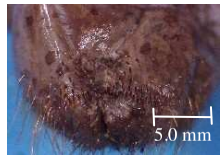
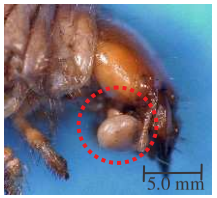
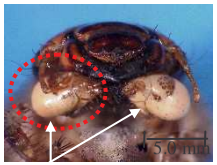
	Anomala ustulata (Tribe Anomalini)	Morphospecies 3 (Tribe Adoretini)
Mandible	  Molar lobe square	  Molar lobe trilobed
Raster pattern (on last abdominal segment)	 (Adapted from Ritcher (1966)) Approximately 30 pali arranged in a zip-like formation; approximately 60 hamate (hooked) setae  Hamate setae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

Table 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 pattern, 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 (from Gordh and Headrick, 2000)

Cranium:	the sclerotized portion of the head capsule except the neck.
Hamate:	barbed; pertaining to structures furnished with hooks or barbs.
Heli:	(helus) a coarse, fixed spine without a cup.
Hypognathous:	descriptive of insects with the head vertically orientated and the mouth directed ventrad.
Ocelli:	lateral simple eyes in larval holometabolous insects.
Palidia:	rows of setae arranged in a unique pattern on the last abdominal segment.
Raster:	setae and spines on the ventral surface of the last abdominal segment.
Setae:	hair-like structures projecting from the body.
Stemmata:	a simple eye or single-lens optical device found on the head of most holometabolous larvae in the region of the head where the compound eye will develop.
Tarsi:	the segments of the insect's foot.

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, 1976, 1977, 1978), especially in sugarcane. There is thus a need for taxonomic descriptions of the most economically important groups within the sugar industry. The shortcomings in the taxonomy of these groups was identified at a White Grub Working Group meeting in 1996 (Way and du Toit, 1996).

There are published keys available such as Richter (1966), Sweeney (1967) and more recently Ahrens *et al.* (2007). 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 throughout the world (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 because all the species found are endemic to southern Africa. To address this problem, a field key concentrating on scarabaeid species occurring mainly in the sugarcane growing areas of the KwaZulu-Natal midlands, was developed.

Molecular work linked larvae to adults by means of their mtDNA sequences (Dittrich-Schröder, 2009). 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.

Field key

The presented key (Tables 1-7) identifies six species and 14 morphospecies of scarabaeid larvae collected from the soil of sugarcane fields in the KwaZulu-Natal midlands. A morphospecies is a typological species recognised on the basis of morphological differences (Gordh and Headrick, 2000). This means that variation in morphological features groups certain specimens together and this grouping is further supported by genetic data such as tree branching patterns. This category is a preliminary step indicating a potential species but which requires further work, such as ecological or behavioural data, to confirm with certainty the presence of a species.

Although Sweeney's (1967) key 'The Scarabaeidae associated with sugarcane in Swaziland' was available, several reasons exist as to why it is not the most accurate key to use. Because it was created over 40 years ago, it was based only on morphological characteristics of larvae, without the addition of more modern 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 shown to be 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 is difficult to use as characteristics are not indicated on images with labels. The key makes use of entomological terminology specific to

scarabaeids. As a result, only workers with a good understanding of larval morphological terminology will be adept at using this key. The lay person wanting to identify scarabaeid larvae would find this key too complex for use and would thus not use it. Lastly, unreliable characters such as number of setae on body parts and segments are used. Setae can be damaged or broken off preserved specimens, thereby possibly leading to inaccurate identification.

More modern keys, such as that created by Ahrens *et al.* (2007) to identify scarabaeid adults and larvae of Nepal was very useful as a model on which to develop the presented key. It provided the guidelines on which this key was based.

The presented key resulting from this study does have some drawbacks. Individuals using the key need to dissect the head capsule of larvae in order to use the characteristics of the mandibles and maxilla (following the methods given). Access to a microscope is thus 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. In addition, very few *A. ustulata*, *H. licas* and SASRI Morphospecies 9 specimens were available for examination when creating the key. Similar problems were experienced by Miller and 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 those specimens the key is not very reliable and more specimens of these species need to be examined to confirm species identification from the key.

An electronic key called Lucid 3.4 was produced from the field key. The purpose behind Lucid 3.4 was to create a key which could be useful to scarabaeid specialists as well as the layman. 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 Lucid 3.4 can be placed on a 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. 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.

Future recommendations would thus include collecting and sequencing more specimens collected from the field. Once this has been performed, phylogenetic analysis, as completed by Dittrich-Schröder (2009), will indicate the species with which 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.

Conclusions

This paper describes a user-friendly morphological key which can be used in the form of a booklet, and can be taken into the field to identify scarabaeid larvae. The key will allow for the first time the accurate identification of field collected larvae in South African sugarcane fields, initially in the KwaZulu-Natal midlands area. Its use to identify species of scarabaeidae through their larvae, will allow for the first time an accurate estimate of the diversity of species in the KwaZulu-Natal midlands within South Africa. When further developed with specimens from other sugarcane growing regions, it is suggested that more specimens be examined to increase the reliability of the key. Particular species biology can be studied in more depth and subsequently crop loss can be attributed to particular species, after which control measures for that particular species can be developed, a study which cannot currently be done.

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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.