A taxonomic revision of *Tritogenia* Kinberg, 1867 and *Michalakus* Plisko, 1996 (Oligochaeta, Tritogeniidae) occurring in KwaZulu-Natal Midlands, South Africa, based on morphological and DNA sequence data

By

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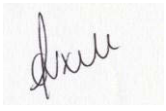
Signed:…  …… Date……18/03/2015…….

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As the candidate‟s co-supervisors we have agreed to the submission of this thesis

……. …….

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ABSTRACT

The conservation and monitoring of biodiversity depends on the knowledge of species identity and distribution. Southern Africa has a rich and characteristic megadrile fauna. Most of the fauna show high levels of endemism with closely related species often separated by subtle morphological characters. Grasslands and forests of South Africa have a diverse terrestrial earthworm fauna, but up to date systematic studies of most taxa are incomplete. Such studies are an opportunity to contribute to understanding evolutionary processes and to provide information for conservation. The genera *Tritogenia* and *Michalakus* occur in grasslands and forests in north-eastern part of South Africa in the KwaZulu-Natal province. This study investigated the taxonomic validity of the *Tritogenia* and *Michalakus* species in the KwaZulu-Natal Midlands. Ten species of *Tritogenia* and one of *Michalakus* are known from this region, with species descriptions based on morphological characters. In this study integrative taxonomy is employed, with both morphological and molecular data used to assess the reliability of traditional morphology-based techniques and the relationships among these species. Detailed comparative morphological observations from fresh *Tritogenia* material revealed a synonym (*Tritogenia soleata* Plisko, 1997 = *Tritogenia shawi* Plisko & Zicsi 1991). To gain further evidence for species level taxonomy and distribution patterns, a molecular phylogeny was constructed based on mitochondrial genes cytochrome c oxidase subunit I (COI) and 16S rRNA. A total of 146 individuals were sequenced for COI from 22 localities and 43 were sub-sampled for 16S rDNA. In most cases, the morphological and molecular data are congruent. The molecular data revealed that the genus *Tritogenia* is not monophyletic as previously thought. *Michalakus initus* Plisko 1996 nests within *Tritogenia* and this finding is observed in both morphological and molecular data. *Tritogenia shawi* is a cluster with the outgroup species not with other Midlands *Tritogenia* species. These findings demonstrate the value of using integrative taxonomy in highlighting/revealing the complexities of earthworm fauna in South Africa. The combined morphological and molecular data, though not well supported, ancestral character state reconstructions are generally in agreement with the morphological data in terms of which characters were useful in phylogeny construction.

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Abbreviations

AJA–Adrian Armstrong, BMNH–The Natural History Museum, London, UK, Cl.–clitellate, ICZN–International Code of Zoological Namenclature, JDP–J.D. Plisko, juv.–juvenile, KZN–KwaZulu-Natal, KZN wildlife–KwaZulu-Natal wildlife jurisdiction, NMSA–KwaZulu-Natal Museum, TL–Tim Liversage, ZMUH–Zoological Museum in Hamburg, Germany, PMB– Pietermaritzburg.

Chapter One

Background to study

1.1 Invertebrates and soil invertebrates

Invertebrates constitute the majority of described animals on earth (Naskrecki 2013). Only about 5% of known species are vertebrates (Barnes 1974), the remaining are invertebrates. Invertebrates vary in size, and adaptation (Banrnes 1974) and occur in both terrestrial and aquatic ecosystems. This study is focused on soil invertebrates, which may represent one quarter of all currently described biodiversity (Decaëns et al. 2006). Human societies rely on a wide variety of benefits from the environment, through ecosystem services, such as food, clean water and air. Soil is a complex ecosystem, characterised by mutual dependencies of bacteria, fungi, plants and animals. Proper description of these dependencies is essential for understanding the complexities of soil ecosystems (Cortet et al. 1999). As such, any disturbance such as unsustainable land use and management may lead to decreased soil organic matter content and decline of soil fauna (Zida et al. 2011) which could affect services such as food production. Several ecosystem services for example, nutrient cycling or soil structure maintenance (Barrios 2007; Kibblewhite et al. 2008; Lavelle et al. 2006) depend on soil. The soil biodiversity is the driving force (Lavelle et al. 2006) behind their regulation. It is therefore important for soils to remain healthy to support human activities; hence soil biodiversity should be preserved.

In general soil organisms are poorly understood but studies have suggested that factors such as plant composition (Jiménez et al. 2006), soil type variation, geology and physiochemical properties of soil (Binet et al. 1997; Chan 2001) may result in highly localised micro-climates leading to the patchy distribution of earthworm assemblages (Whalen 2004; Rossi et al. 2006; Decaëns & Rossi 2001). Patchy occurrences of species assemblages could also be explained in terms of competition (Jiménez et al. 2006), with competition for the same resources often defining faunal composition.

Most studies of soil fauna have focused on effects of soil invertebrates on soil processes, for example, soil physical processes, nutrient transformation and soil formation (Lamandé et al.

2003; Lavelle et al. 1999; Lavelle et al. 2006; Pulleman et al. 2005). Soil health is mostly assessed through soil invertebrate response (Ardestani et al. 2014; Cortet et al. 1999; Gilbert et al. 2014; Lavelle et al. 2006). For example, the presence of certain chemicals in the soil as well as the abundance of invertebrates may indicate whether or not the soil is in good condition. Less emphasis has been placed on taxonomic studies and this has resulted in a knowledge gap which this study attempts to answer by carrying out a taxonomic revision of two earthworm genera, Tritogenia and Michalakus that occur in the Midlands, using morphological and molecular data.

South Africa has diverse fauna with a long history of biodiversity research (Crouch & Smith 2011); despite this, soil fauna have not received much attention (Hamer 1999, 2000; Mwabvu et al. 2007; Mwabvu et al. 2009; Haynes et al. 2003; Vohland & Hamer 2013). In particular Hamer & Slotow (2000) indicated that the biogeography and distribution of African invertebrates are poorly studied. The authors attributed this to lack of taxonomic knowledge and expertise.

1.2 Role of macroinvertebrates in the soil

Soil invertebrates can be roughly divided into three groups: micro-, meso- and macro-invertebrates. Soil macrofauna include taxa that have a body length greater than 1cm (Wallwork 1970), have a body width greater than 2mm (Swift et al. 1979) and are visible to the naked eye (Kevan 1968). According to Ruiz et al. (2008) macroinvertebrates include groups such as earthworms, termites, beetles, ants, millipedes, spiders, scorpions, pseudo-scorpions, centipedes, earwigs, snails, crickets, true bugs, cicadas, cockroaches, isopods, mermithid nematodes, pot-worm, moth larvae and fly larvae. These organisms spend at least one part of their life cycle in or on the soil and play an essential role in healthy soil functioning. The functions of the macrofauna community in the soil are diverse and include the regulation of soil physical and chemical properties and processes such as carbon and nutrient cycles (Brussaard et al. 2007). As detritivores they feed on dead organic materials (plant and animal matter), and help to increase decomposition and mineralization rates (Ruiz et al. 2008). Furthermore, many by-products of these organisms‟ activities are used as food resources by other soil organisms (Brussaard et al. 2007).

Levels of endemism in soil macrofauna in South Africa have been discussed in other taxa such as millipedes (Hamer & Slotow 2002; Vohland & Hamer 2013), spiders (Huber & Rheims 2011) and earthworms (Plisko 2003). Given a high degree of soil macrofauna habitat specificity, and usually poor dispersal abilities (e.g. Bell et al. 2004), most soil macroinvertebrates show some degree of endemism and this makes them vulnerable to extinction (Hamer & Slotow 2000). Conservation planning bodies regard species distribution, diversity and regions of endemism as important (Forey et al. 1994) but the lack of expertise limits conservation efforts. In particular, our knowledge of diversity and distribution of many soil macroinvertebrates in South Africa is incomplete. Most conservation management strategies target plants and mammals which are more noticeable (Vohland & Hamer 2013). Unfortunately, invertebrates, including earthworms, are often neglected despite their important role in ecosystems.

1.3 Earthworms

Darwin recognised the important role of earthworms when he wrote, *"The plough is one of the most ancient and valuable of man's inventions; but long before he existed the land was in fact regularly ploughed, and still continues to be thus ploughed by earthworms. It may be doubted whether there are many other animals which have played so important a part in the history of the world, as have these lowly organized creatures"* (Darwin 1882). Earthworms contribute a significant part of biomass in the soil (Decaëns et al. 2013; Edwards 2004). Despite this, their diversity, activities and effects on soils are not completely understood. Earthworms‟ contribution to the soil ecosystems services is important to human society. They are detritivores that modify the soil and regulate resource availability and thereby act as ecosystem engineers (Jouquet *et al.* 2006). Lavelle et al. (2006) reported that earthworms convert large pieces of organic matter into rich humus in form of casts, thus improving soil fertility and quality, and have an influence on the regulation of soil formation. As such, nutrients that are released from decomposition of organic matter, including nitrates and phosphates, become available in an accessible form to plants and other organisms (Lavelle et al. 1999; Lavelle et al. 2006; Pey et al. 2014). In addition, earthworm burrows create passageways which allow aeration and drainage to take place (Salomé et al. 2011), this is important because soil microorganisms and plant roots needs air and water. The inclusion of

earthworms in ecological and soil ecosystem research is therefore vital but this requires access to accurate taxonomic information.

Anthropogenic disturbance in the soil affects the earthworm faunal composition (Callaham et al. 2003; Winsome et al. 2006). When a natural system is modified by human activities, major changes occur to the biotic and abiotic soil environment. Land conversion or habitat transformation decreases the diversity of native assemblages because indigenous species are adapted to undisturbed habitats while introduced species are often more competitive in disturbed habitats (Winsome et al. 2006). The effect introduced earthworms have on indigenous species is not well understood in South Africa; however, in Europe studies have shown that introduced earthworms tend to outcompete endemic fauna (Hendrix et al. 2008; Burtelow et al. 1998).

1.4 Earthworm taxonomy in South Africa

About 3700 earthworm species are known worldwide (Decaëns et al. 2013), of which 300 are currently known from South Africa (Plisko 2010). Of these 50 species were introduced to South Africa by humans (Plisko 2010). The 250 species indigenous to South Africa belong to the families Microchaetidae (*Microchaetus* 8 species, *Geogenia* 21 species, *Kazimierzus* 21 species, and *Proandricus* 56 species), Tritogeniidae (*Tritogenia* 35 species, *Michalakus* 1 species) and Acanthodrilidae (*Chilota* 12 species, *Eodriloides* 17 species, *Microscolex* 3 species, *Parachilota* 65 species, *Udeina* 11 species).

Traditionally, earthworm systematics has been based on morphology and anatomical characters (Bouché 1972). The taxonomy though has remained unstable with phylogenetic relationships among taxa unclear due to a low number of morphological characters available for seperating the different species (Pop et al. 2003). In addition, some characters change with developmental stages and homoplasy in many characters is high, probably reflecting high levels of phenotypic plasticity (Decaëns et al. 2013).

Morphology-based classification of earthworms have focused on characters associated with the reproductive organs, because these characters are generally considered evolutionarily more conservative and are not affected by environmental factors. The characters used have included clitella, tubercular pubertatis, spermathecae and testes (Chang et al. 2007). External

characters, like size and colouration, may vary within species because they are often affected by environmental conditions as well as specimen preservation methods used (Chang et al. 2007). An additional challenge in delimiting earthworm taxa is that it is often hard to distinguish derived characters (apomorphies) from ancestral or primitive characters (plesiomorphies) because of the lack of fossil record. Earthworms are soft bodied animals and do not fossilize well (Pérez-Losada et al. 2009).

Of the eight families of earthworms in South Africa, the indigenous Microchaetidae, Tritogeniidae and Acanthodrilidae are the well-studied. South African endemic species which belong to these families tend to have a restricted distribution and are found in natural, undisturbed biotopes, mostly in primary grasslands and forests (Plisko 1995, 2000; Nxele 2014). Species which have been introduced to South Africa, on the other hand, are usually more generalist and seem to adapt well in most biotopes even in polluted areas (Plisko 2010). Identifying earthworm species correctly is important for biodiversity and evolutionary studies (King et al. 2008; Pérez-Losada et al. 2005) because earthworms play a major role in the soil ecosystem.

1.5 *Tritogenia* and *Michalakus*

The family Tritogeniidae, with two genera *Tritogenia* and *Michalakus*, is endemic to north-eastern part of South Africa (Plisko 2003). The 35 described *Tritogenia* species (Plisko 2003) have a narrow fragmented distribution. Diversity of these earthworm taxa is negatively affected by unsustainable land use practices (Callaham et al. 2003). The current fragmented distribution pattern of *Tritogenia* may be due to human disturbance, although this has not been empirically tested. In the KZN midlands ten *Tritogenia* and one *Michalakus* species are currently known (*T. annetteae* Plisko, 1997*,* T. *debbieae* Plisko, 2003, T. *hiltonia* Plisko, 2003, T. *howickiana* (Michaelsen, 1913), T. *karkloofia* Plisko & Zicsi 1991, T. *lunata* Plisko, 1997, T. *mucosa* Plisko & Zicsi, 1991, T. *shawi* Plisko & Zicsi, 1991, T. *soleata* Plisko, 1997 and T *sulcata* Kinberg, 1867, and *Michalakus initus* Plisko 1996. These species have been identified using traditional morphological characters which is challenging because in some species morphological characters overlap. As such, some known species names may be junior synonyms of earlier described species; hence a revision of this group is necessary.

1.6 DNA in earthworm taxonomy and phylogenetics

Recently, the use of DNA-based analyses to clarify taxonomic problems has received much attention. In particular the use of DNA barcoding for identification purposes has become popular in the world (Huang et al. 2007; Meier & Wiegmann 2002; Eernisse & Kluge 1993; Fitch & Smith 1983; Hebert et al. 2004; Ward et al. 2005; Smith et al. 2007; Borisenko et al. 2008). Most molecular studies of earthworms have focused mainly on mitochondrial cytochrome c oxidase subunit I (COI) gene and its utility in integrative taxonomy (Blakemore 2013; Bantaowong et al. 2011; Huang et al. 2007; Richard et al. 2010; King et al. 2008; Rougerie et al. 2009; Chang et al. 2009; James et al. 2010; James & Davidson 2012). Given that species identifications are often challenging and require considerable taxonomic expertise, a DNA barcode system will likely speed up species identification. Additionally, the development of a universal DNA-based identification system could provide a globally important tool for the identification of earthworm species.

The usefulness of DNA data in earthworms has been highlighted in a number of recent studies. Pérez-Losada et al. (2005) used both morphological and DNA sequence data for delimitation of the earthworms, *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* Bouche, 1972, which were previously considered conspecific. Chang and Chen (2005) employed sequence data as an additional tool to re-evaluate the taxonomic status of two sibling pheretimoid earthworms. Pop et al. (2003) also confirmed that molecular data can provide additional diagnostic characters in earthworm taxonomy.

Molecular work has been done in South Africa to clarify taxonomy of several taxa including for example millipedes (Mwabvu et al. 2013), small mammals (Willows-Munro & Matthee 2009, 2011), frogs (Zimkus et al. 2010), lizards (Travers et al. 2014), fungi (Iheanacho et al. 2014), plants (Martin-Bravo et al. 2013), ants (Smith and Fisher 2009; Smith et al. 2005), moths (deWaard et al. 2011; Janzen et al. 2005) and flies (Webb et al. 2012; Zhou et al. 2009; Zhou et al. 2011) among many others.

In South Africa, despite relatively high species richness and diversity, most work on DNA has been done on earthworms in ecotoxicology (see Voua Otomo et al. 2009; Voua Otomo et al. 2013). All known earthworm taxa in South Africa have been differentiated based on morphology. However, most of the morphological and anatomical characters are not consistent and often vary between and among species which makes species diagnosis

complicated. Therefore, the number of taxa recognised by various authors depends on the degree of variability in different characters that authors accept as diagnostic, which casts doubt over the phylogenetic and diagnostic value of these morphological characters.

1.7 Conclusion

Taxonomic expertise on earthworms is scarce in South Africa with research mainly based on the study of morphological characters and proper species identification requires consultation with taxonomic experts at the KwaZulu-Natal Museum. The KwaZulu-Natal Museum collection is unique in southern Africa because it houses 138 types (Plisko 2006, 2007, 2008) and more than 50 000 specimens (KwaZulu-Natal Museum database). Because of the dearth of taxonomic expertise, other tools to accelerate species discovery are necessary. In this regard, molecular tools have great potential to provide clarification in South African earthworm taxonomy. The possibility to trace character evolution is one of the great advantages of integrating morphology into molecular phylogenetic analyses (Schols et al. 2004).

1.8 Aim and objectives of study

The aim of the study is to carry out a taxonomic revision of two earthworm genera, *Tritogenia* and *Michalakus* that occur in the KZN Midlands, using morphological and molecular data.

The objectives are:

1. To investigate phylogenetic relationships among *Tritogenia* and *Michalakus* species. In particular the phylogenetic analysis will be used to determine if *Michalakus* is a valid genus.

2. To uncover potentially cryptic lineages of *Tritogenia* and *Michalakus* in the KwaZulu-Natal midlands, and to clarify synonymy. This aspect of the project will incorporate morphological and molecular analyses.

Chapter Two

A taxonomic revision of *Tritogenia* Kinberg, 1867 and *Michalakus* Plisko, 1996 (Oligochaeta: Tritogeniidae) species occurring in the KwaZulu-Natal Midlands, South Africa

Abstract

Ten *Tritogenia* Kinberg, 1867 species, including *T. annetteae* Plisko, 1997*, T. debbieae* Plisko, 2003, *T. hiltonia* Plisko, 2003, *T. howickiana* (Michaelsen, 1913), *T. karkloofia* Plisko & Zicsi 1991, *T. lunata* Plisko, 1997, *T. mucosa* Plisko & Zicsi, 1991, *T. shawi* Plisko & Zicsi, 1991, *T. soleata* Plisko, 1997 and *T. sulcata* Kinberg, 1867, and *Michalakus initus* Plisko 1996 were revised. A new synonym is proposed: *Tritogenia soleata* Plisko, 1997 = *T. shawi* Plisko & Zicsi, 1991. *Tritogenia sulcata* Kinberg, 1867 and *T. howickiana* (Michaelsen, 1913), though similar, are left as separate species because the small parts of type material for *T. sulcata* housed at the Royal Natural History Museum, Stockholm in Sweden (NHRS) are not sufficient for physical examination (as the specimen is in pieces) but was available in a photo and the original description is limited. The rest of species treated presently are accepted as valid species. The morphological data revealed that *Tritogenia* is not monophyletic and *Michalakus initus* clustered togethet with *T. shawi*. Some morphological characters were shown to be independent and useful in separating species while others showed some correlation with each other overlapping completely in *T. lunata, T. mucosa* and *T. soleata* together.

2.1 Introduction

Earthworms are a major part of soil macrofauna. Despite this important role in soil processes their taxonomy is poorly studied and the assignment of taxa is debatable. As such, several species are probably awaiting description and described species require revision using new characters and more advanced techniques such as DNA barcoding. In South Africa 11 genera of endemic earthworms have been described including *Tritogenia* and *Michalakus*. However, genus and species boundaries remain controversial (see Plisko, 2013).

2.1.1 History of South African *Tritogenia* earthworms

Tritogenia Kinberg, 1867 and *Michalakus* Plisko, 1996 differ from other earthworm genera in their anatomy and also in geographical distribution. These two genera are endemic to the north-eastern parts of South Africa with species presently known only from Limpopo, Mpumalanga and KwaZulu-Natal (KZN) provinces (Plisko 1997; 2003; 2008). *Tritogenia* and *Michalakus* as with other endemic species occur predominantly in natural undisturbed habitatas, such as grasslands, indigenous bushes and forests. The species belonging to these genera are particularly sensitive to habitat disturbance with only *T. hiltonia* and *T. lunata* recorded from cultivated fields (Plisko 2003).

The first recorded *Tritogenia* species was *T. sulcata,* described by Kinberg in 1867. After its description 35 years passed before four new species, *T. grisea* (Michaelsen, 1902)*, T. howickiana* (Michaelsen, 1913)*, T. melmothana* (Michaelsen, 1928) and *T. zuluensis* (Beddard, 1907) were discovered and described between 1902 and 1928. In the past two decades 30 new species were added to the genus. Taxonomic classification of *Tritogenia* species has long been controversial with some authors placing them within different taxonomic groups (Plisko 2013), for example in *Brachydrilus*. Plisko (1997) described 18 new species and questioned the taxonomic position of *Tritogenia* within the Microchaetidae. Plisko (2012) discussed the systematic position of *Tritogenia* and *Michalakus* in Microchaetidae and suggested the separation of the two genera into a new family, Tritogeniidae Plisko, 2013.

2.1.2 Characters that distinguish *Tritogenia* and *Michalakus* from the other South African indigenous megardrile.

Tritogeniidae species differ from the other South African indigenous megadrile by an excretory system which is meroic (divided, nephridial tubules formed by longitudinal or transverse fragmentation of the original single pair of embryonic rudiments of each segment), with small nephridia per segment. The gizzard is located in segments 6–7 with septum 6/7 attached. The blood vessel is double in preclitellar segments and is double even when crossing septa. Although *Michalakus* share these characters with *Tritogenia*, it is distinguished from *Tritogenia* by having two gizzards.

External characters used to classify *Tritogenia* and *Michalakus* include: body dimension and shape, body colour, body segmentation, number of segments; setae arrangement; shape and location of nephridial pores; position, shape and location of female and male pores; location and number of spermathecal pores; papillae: presence or absence, number and shape, connection with genital glands; and the location and shape of clitellum and tubercula pubertatis.

Internal characters include: specific thickness of septa; location and shape of gizzard; shape and position of calciferous glands; shape, initiation and termination of typhlosole; dorsal blood vessel with its dorso-ventral vessels location; number, shape and location of nephridia; shape and location of ovaria; confirmation of holandric character, position, shape, enclosed or free state of male funnels; location, shape and characteristics of seminal vesicles; shape, number and location of spermathecae; and shape, location and characteristics of genital glands. An illustration of these characters is in appendix (Figure A2).

2.1.3 Current taxonomic status of KZN Midlands *Tritogenia* species

Currently 10 *Tritogenia* species (Table 1) are endemic to KZN Midlands. These species are *T. annetteae* Plisko, 1997*, T. debbieae* Plisko, 2003*, T. hiltonia* Plisko, 2003*, T. howickiana* (Michaelsen, 1913)*, T. karkloofia* Plisko & Zicsi, 1991*, T. lunata* Plisko, 1997*, T. mucosa* Plisko & Zicsi, 1991*, T. shawi* Plisko & Zicsi, 1991*, T. soleata* Plisko, 1997 and *T. sulcata* Kinberg, 1867. There is substantial morphological variability in these species. In some species morphological characters are so variable that they often overlap with that diagnostic of other species, for example, the position of spermathecae, in most species it is in two segments. This overlap of characters is not unusual of earthworms (Fernández et al. 2012; Pop et al. 2003).

According to Plisko (2006) the monotypic *Michalakus* occurs in the KZN Midlands often together with *Tritogenia* species. It is morphologically different from sympatric *Tritogenia karkloofia*, as well as from all other species of *Tritogenia*, by having two gizzards. It is not clear if this character is apomorphic or plesiomorphic. Given that the taxonomy of the South African earthworms has been largely based on external and internal morphological characters, the aim of this chapter is to revise the taxonomy of *Tritogenia* and *Michalakus*

species using morphological characters. These morphological data were then used to re-construct a phylogeny.

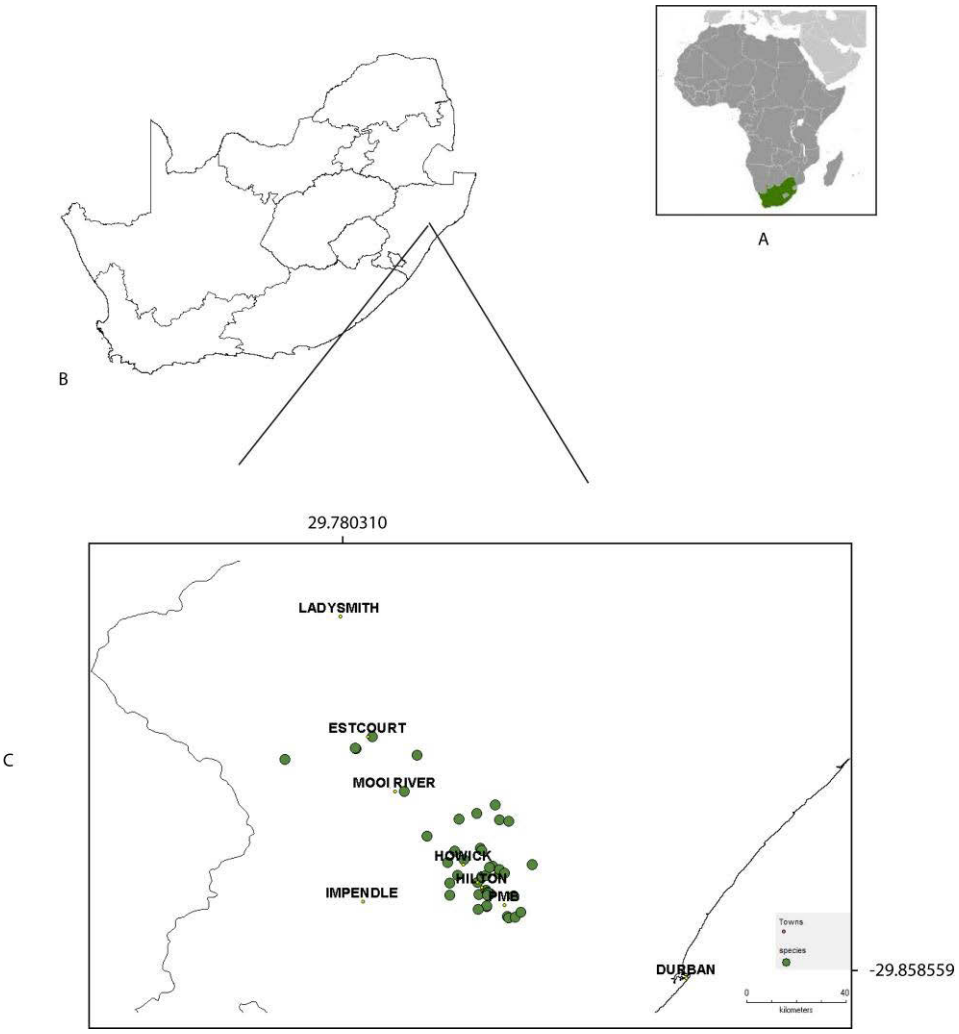

Fig. 1. Fig. 1. Distribution of *Tritogenia* and *Michalakus* species in KZN Midlands, South Africa. A–Map of Africa; B–Map of South Africa; C–Map of KZN Midlands. Dots represent sites where species were collected in different towns in the midlands.

2.2 Material and methods

2.2.1 Sampling

In total 675 specimens accredited to the ten *Tritogenia* and one *Michalakus* KZN Midlands species (*T. annetteae* = 12, *T. debbieae* = 14, *T. hiltonia* = 44, *T. howickiana* = 163, *T. karkloofia* = 178, *T. lunata* = 135, *T. mucosa* = 7, *T. shawi* = 12, *T. soleata* = 1, *T. sulcata* = 74, *M. initus* = 35) were examined. Type material from seven *Tritogenia* and one *Michalakus* species was available in the KZN museum.

Additional field collections were conducted at eleven different localities (Table 1), where the ten *Tritogenia* and one *Michalakus* species were previously collected. Attempts to collect new material of *Tritogenia mucosa* were not successful and the type locality for *Tritogenia soleata* has been destroyed by urbanization. In addition to collection at type localities sampling was also conducted at fifteen new sites (Table A2). New material is indicated in the taxonomy section for each species under examined material. Most of the new sites fall under KZN Wildlife regulations, and the permit was obtained from their head office in Queen Elizabeth Park (permit no: OP 5247/2013). Permission was obtained from local authorities for the sites outside KZN Wildlife area of jurisdiction. New earthworm material was collected by digging out three 1m by 1m and 30cm deep soil monoliths along a 100m transect at 0m, 50m and 100m at each site. Soil was hand sorted for earthworms in large plastic trays (50cm x 50cm x 10cm). Collected specimens were narcotized using 45% ethanol solution. Some specimens were preserved in absolute ethanol (to preserve DNA integrity) for DNA analysis. The remaining specimens were fixed in 4% formalin for at least 24 hours then preserved in 75% ethanol. All new material was deposited into the KwaZulu-Natal Museum collection. A GIS referenced distribution map showing the collection localities was constructed, using available GPS co-ordinates, with DIVA 7.5 (Hijmans et al. 2012).

Table 1. Type localities resampled for *Tritogenia* and *Michalakus* species. The locality information is given as as it appears on original labels

***Tritogenia/Michalakus* spp.**	**Locality**
T. annetteae	11km SE Estcourt, 2km E Lowlands station (29°00‟S: 29°54‟E)
T. debbieae	5km N of Mooi River, grassland near N3 (29°12‟S: 30°01‟E)
T. hiltonia	Hilton College, mistbelt grassland (29°30‟47.863‟‟S:30°18‟002928‟‟E)
T. howickiana	Howick area
T. karkloofia	Karkloof Nature Reserve (29°18‟S: 30°13‟E)
T. lunata	Karkloof NR, Geekie‟s Estate (29°18‟S:30°13‟E)
T. mucosa	17km NE of Pietermaritzburg (22°29‟S: 30°23‟E)
T. shawi	PMB Cleland, 10 Lynroy Avenue
T. soleata	Pietermaritzburg, type locality doesn‟t exist anymore
T. sulcata	Port Natal
Michalakus initus	Albert falls, 2km from tourist resort Bon Accorde, grassland near small stream (29~28‟S:30~27‟E)

2.2.2 Character scoring

All specimens were examined using a Wild Heerbrugg stereo-microscope and identified according to the classifications by Plisko (1992; 1997; 2003), Plisko and Zicsi (1991) and Michaelsen (1913). The following characters were studied: body length, number of segments, prostomium, segmentation, setae, nephridial pores, female pores, spermathecal pores, clitellum, tubercula pubertatis, papillae, septa, gizzard, calciferous glands, intestine, typhlosole, blood vessels, nephridia, testicular funnels, seminal vesicles, spermathecae and genital glands (An illustration of the characters is in appendix Figure A2). Fifteen morphological characters were scored for ten *Tritogenia* and one *Michalakus* species. Clitellate specimens (386) were scored for analyses because juveniles do not have all characters developed.

2.2.3 Data analysis

Principal component analysis (PCA) of morphological characters was performed for clitellate specimens using PAST 2.17 (Hammer et al. 2001). A data matrix of 386 specimens was analysed, only clitellate specimens were used because juveniles do not have all characters. Two analyses were performed, first morphological characters were clustered to identify which morphological characters overlap and which ones are diagnostic characters. Second PCA was performed with species to observe how the species cluster based on the morphological characters. Variance and eigenvectors were calculated and data was analysed based on correlation. The scatter plot was plotted and components were chosen.

ANOVA was performed to determine whether the means of the continuous character (the number of body segments; as the other characters are not continuous) in the different species are significantly different from each other using PAST 2.17.

A phylogeny was constructed for this group with the morphological characters (Table 2). *Microchaetus papillatus* was used as an outgroup as it belongs to Microchaetidae, a closely related family (Plisko 2013). This specimen is housed at the KwaZulu-Natal Museum (NMSA/OLIG. 05012). The character matrix was analysed with PAUP* 4.0b10 (Swofford 2003) software package. The parsimony uninformative characters were excluded and 1000 bootstraps were performed. The maximum number of trees found per bootstrap replicate was limited to 500. For each bootstrap replicate tree search, 10 different starting trees were used to start branch swapping. Each of these trees were produced with a random taxon addition order. The bootstrap consensus tree (majority-rule consensus tree) was computed from the best trees found.

Table 2. Characters and character states used in the phylogenetic analysis of Midlands *Tritogenia, Michalakus* and *M. papillatus* species with absent/present states

1	Septa: 0 – thin; 1 – thickened
2	Septa: 0 – 5/6, 6/7muscular; 1 – not
3	Gizzard: 0 – one; 1 – two
4	Position of calciferous glands: 0 – in 9–10; 1 – in 10
5	Calciferous glands: 0 – stalked; 1 – not stalked
6	Calciferous glands: 0 –fused; 1 – not fused
7	Calciferous glands: 0 – ventrally widely separated; 1 – ventrally horseshoe (separated by small space)
8	Intestine: 0 – commences in segment 13; 1 – after 13
9	Typhlosole: 0 – commences in segment 17; 1 – after segment 17
10	Seminal vesicles: 0 – pair in segment 11 small; 1 – large
11	Shape of spermathecae: 0 – round and large; 1 – not round or large
12	Position of spermathecae: 0 – in two segments; 1 – in more than two segments
13	Number of spermathecae: 0 – one pair per segment; 1 – more than two per segment
14	Body length: 0 – less than 300mm; 1 – more than 300mm
15	Body segments: 0 – less than 400; 1 – more than 400

2.3 Results

Fifteen morphological characters were chosen and scored; the characters chosen were the least overlapping between species. For example some characters like tubercula pubertatis may look different on individuals depending on how a specimen was preserved giving a misleading shape.The juveniles were included in the morphological observation where specimens were dissected and characters were noted but were excluded in the scoring as they lack most adult characters.

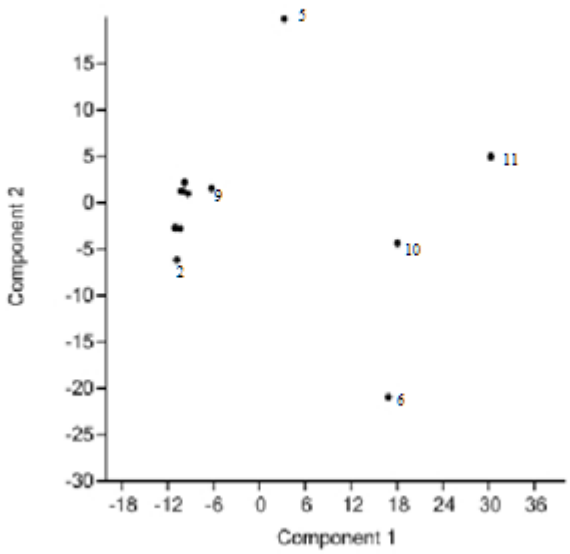

Fig. 2. Scatter plot of the first two principal components for morphological characters based on 15 morphological characters (Table 2), for 386 clitellate specimens of *Tritogenia* and *Michaalakus* from KZN Midlands. Six characters are distinct and independent showing high value for species description, the other nine characters have some degree of overlap suggesting they are not good diagnostic characters. Numbers represent characters from 1–15. The unlabelled characters overlap and labelling them causes the plot to be too congested, these are discussed below.

The result of the principal component analysis of 15 morphological characters is illustrated on the Figure 2. The first two components were plotted. The scatter plot showed four characters (char 5: stalking of calciferous glands, char 6: calciferous glands fused or not, char 10: size of seminal vesicles and char 11: shape of spermathecae) being quite distinct and independent, suggesting that each character is individually valuable in species diagnosis. Character 2 (muscularity of septa) and character 9 (commencement of typhlosole), although close to the tightly clustered characters, are nevertheless distinct, and thus are individually valuable in species diagnoses as well. Nine characters (1: thickness of septa, 3: number of gizzards, 4: position of calciferous glands, 7: separation of calciferous glands, 8: commencement of intestine, 12: position of spermathecae, 13: number of spermathecae, 14:

body length and 15: number of body segments) are tightly clustered and overlap, meaning they are correlated. These are left unlabelled in the plot, because labelling them individualy would make the plot too congested. Characters did not cluster according to any system, for example spermathecae is part of the reproductive system but char 11 (shape of spermathecae) did not group together with char 12 (position of spermathecae) or 13 (number of spermathecae) which relate to the spermathecae.

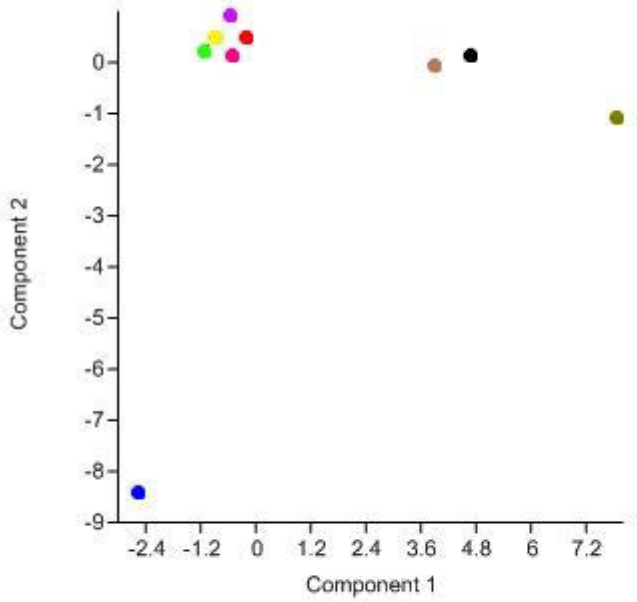

Fig. 3. Scatter plot of the first two principal components for morphological characters based on species for 386 specimens of *Tritogenia* and *Michaalakus* from KZN Midlands. Seven species show small distance between them suggesting a close relation between them, the other species are clearly distinct, scattered far from each other. Each coloured dot represents species and the codes are listed in Table 3.

Table 3. Colour coding for species used in the PCA scatter plot with some characters overlapping completely causing species to appear on top of each other, characters that overlap completely are given one colour.

Colour	**Species**
Purple	*T. annetteae*
Yellow	*T. howickiana* and *T. lunata*
Green	*T. hiltonia* and *T. mucosa*
Red	*T. karkloofia*
Pink	*T. sulcata*
Blue	*T. debbieae*
Brown	*T. shawi* and *T. soleata*
Black	*M. papillatus*
Avocado	*M. initus*

The results of a principal component analysis on species are summarized in a scatter plot, Figure 3. The scatterplot showed eight distinct groups (represented by dots) corresponding to 12 species, ten *Tritogenia,* one *Michalakus* and *M. papillatus*. Seven species (*T. annetteae, T. hiltonia, T. howickiana, T. lunata, T. mucosa, T. karkloofia* and *T. sulcata*) are close to each other indicating that these species are morphologically similar. *Michalakus initus* and *T. debbieae* scattered far from the other species suggesting that (based on coded characters) their morphological characters are not similar to the rest of the species. *Microchaetus papillatus* is placed closer to *T. shawi/ T. soleata*. Morphological characters of three species (see Table 3) overlapped completely with others (*T. lunata, T. mucosa* and *T. soleata*) explaining why only eight dots are observed.

The character means of the eleven species were found to be significantly different in the ANOVA ($F\ 9.58 = 5.808$, $p<0.000$) suggesting that this continuous character is useful in seperation of species.

2.3.1 Phylogeny

The parsimony analysis resulted in 8 most parsimonious trees with tree length of the most parsimonious tree = 7 steps (CI = 0.875, RI = 0.947). The number of parsimony informative characters = 7. Bootstrap values above 75% were considered significantly supported (Hillis & Bull 1993), while values below 50% were not shown on the tree. In general, the branches of the cladogram were weakly supported with most branches supported by less than 50% (Figure 4). There were two branches with good support (clade with *T. annetteae, T. debbieae, T. hiltonia, howickiana T. lunata, T. mucosa* and *T. sulcata* having 86% bootstrap support; and the association between the latter clade and *T. kakloofia* with 76% bootstrap value). There is no support for the monophyly of *Tritogenia* as the single *Michalakus* species is nested within the the genus. So, based on morphological characters, *Tritogenia* is not monophyletic.

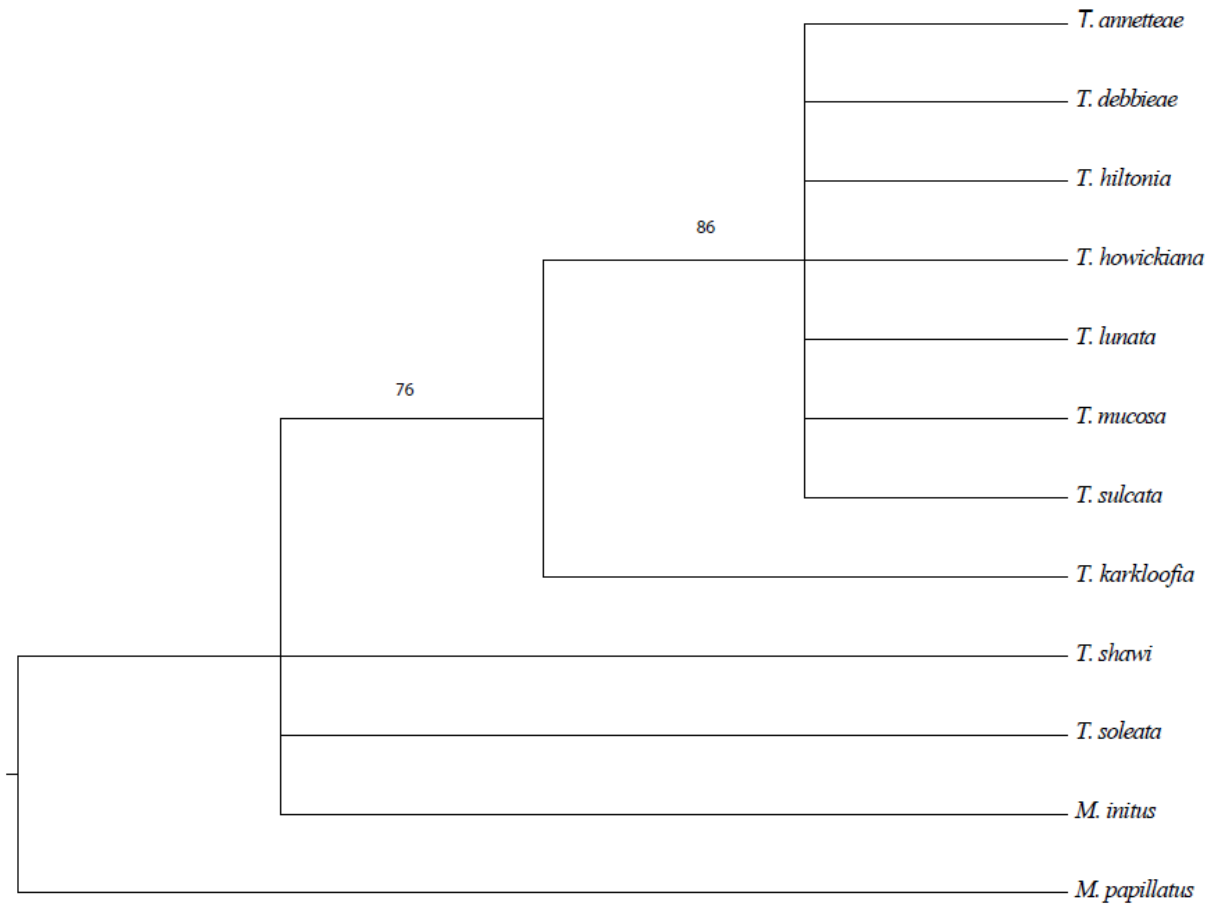

Fig. 4. The maximum Parsimony consensus tree of Midlands species belonging to the genus *Tritogenia* and *Michalakus* generated using PAUP suggested that *Tritogenia* is not monophyletic. *Tritogenia karkloofia* form a distinct lineage whilst *T. shawi* and *M. initus* seem closely related. Values annotated onto branches represent bootstrap support value, only the bootstrap support values higher than 50% are presented.

2.3.2 Taxonomy

Family Tritogeniidae Plisko 2013

Genus *Tritogenia* Kinberg, 1867

Type species: *Tritogenia sulcata* Kinberg, 1867

Tritogenia annetteae Plisko, 1997

Tritogenia annetteae: Plisko 1997: 248; 2003: 308; 2006: 59; 2013: 80.

Type locality: KZN, 11 km SE Escourt.

Description:

External: *Body length*: holotype 50 mm long, 5 mm wide at clitellum. Paratypes 11–49 mm long and 2–5 mm wide. *Number of segments*: holotype 80; paratypes: 71–81. *Prostomium*: prolobous, small. *Segmentation*: preclitellar segments with secondary annulations; 1 and 2 simple with irregular longitudinal grooves; 3 simple with slight grooves; 4–8 has two ringlets of similar size and appearance; 9 has two ringlets with second ringlet smaller than the first ringlet; from 10 and postclitellar irregularly annulated. *Setae*: difficult to trace in preclitellar segments but visible on papillae and postclitellar segments, *ab* closely paired. *Nephridial pores*: not visible. *Female pores*: not visible. *Spermathecal pores*: 11/12, 12/13 intersegmental furrows. *Clitellum*: saddle shaped, on holotype 13–1/n25; ventral border close to borders of tubercula pubertatis, segmented. *Tubercula pubertatis*: Holotype and paratypes on 19–22; glandular patches; square; close to each other but don‟t touch in the middle. *Papillae*: on 11–13, 21 paired, nipple like, in 21 very large and on tubercula pubertatis.

Internal characters: *Septa*: 4/5 slightly thickened, 5/6, 6/7 moderately thickened; 7/8, 8/9 slightly thickened; other septa very thin. *Gizzard*: 6–7, partly in 6 and more in 7, strong and well developed. *Calciferous glands*: in 9–10, highly stalked; widely separated dorsally and ventrally. *Intestine*: originates in 14. *Typhlosole*: commences in 17 and terminates in 52; V–shaped. *Blood vessels*: dorsal blood vessel double in 4–11, even so when crossing septa; single in rest of the segments; ventral vessel thin. *Nephridia*: two pairs per segment in posterior segments in dorsal and ventral parts of the body; V–shaped. Holandric; testes funnels close to seminal vesicles; funnels are closely paired. *Seminal vesicles*: in 10–11,

second pair slightly bigger. *Spermathecae*: in 11/12, 12/13; located in genital glands; variable in shape. *Genital glands*: from 11–13, large, finger-like.

Material examined: (OLD): Holotype: 11 km SE of Estcourt, 2 km E of Lowlands station, primary grassland, from sandy, moist soil and between grass-roots of various plants (29°00'S 29°54'E), 1520 m, 24.iii.1988, 1 cl., NMSA/OLIG.02335, JDP, J.G.H. Londt & A. Seymour leg.; Paratypes: same locality as holotype, 24.iii.1988, 5 with TP, 6 juv., NMSA/OLIG.00855.

(NEW): 7 km N of Mooi River, native grassland, E side of N3, sandy moist soil, (29°08.275'S 29°57.804'E), 1542 m, 31.i.2007, 1 juv., NMSA/OLIG04510, JD Plisko & S James leg.

Remarks: New material conforms to the description of this species. The species has been collected from the Estcourt area and nowhere else in the KZN Midlands.

Tritogenia debbieae Plisko, 2003

Tritogenia debbieae: Plisko 2003: 311; 2006: 59; 2013: 81.

Type locality: KZN, Mooi River.
Description:
External: *Body length*: holotype 48 mm long, 2.5 mm wide at clitellum. Paratypes 58 – 60 mm long and 4–5 mm wide. *Number of segments*: holotype not countable; paratypes: 94. *Prostomium*: prolobous. *Segmentation*: preclitellar segments with secondary annulations; 1 and 2 simple with irregular longitudinal grooves; 3 simple with slight grooves; 4–8 has two ringlets of similar size and appearance; 9 has two ringlets with second ringlet smaller than the first ringlet; from 10 and postclitellar simple. *Setae*: *ab* clearly observable in segments 10 to 17, large, closely paired. *Nephridial pores*: not visible. *Female pores*: not visible. *Spermathecal pores*: only noted in 11/12 intersegmental furrows. *Clitellum*: saddle shaped, on 1/n12–24; ventral border close to *ab* setal line, segmented. *Tubercula pubertatis*: 1/n18–1/n22; glandular patches; loose rectangle shape; occur in clitellar tissue; separated. *Papillae*: on 10–18, 23 single or paired.

Internal characters: *Septa*: all septa are thin. *Gizzard*: 6–7, partly in 6 and more in 7, large and well developed. *Calciferous glands*: in 9–10, stalked; separated slightly dorsally and widely separated ventrally. *Intestine*: originates in 13. *Typhlosole*: commences in 17 and terminates in 51; U-shaped. *Blood vessels*: dorsal blood vessel double in 4–11, even so when crossing septa; single in rest of the segments; ventral vessel thin. *Nephridia*: two pairs per segment in posterior segments in dorsal and ventral parts of the body, ventral pair near spermathecae; V-shaped. Holandric; testes funnels close to seminal vesicles; funnels are closely paired. *Seminal vesicles*: in 10–11, quite small. *Spermathecae*: large and round, tennis ball like shape; in 11/12, 12/13. *Genital glands*: not visible.

Material examined: (OLD): Holotype: Mooi-river, 5 km N of; primary grassland near M3 (29°12'S 30°01'E), 5.xii.1996, 1 cl., NMSA/OLIG.02448, JDP, TL leg.; Paratypes: same locality as holotype, 5.xii.1996, 5 cl., 6 juv., NMSA/OLIG.02449.

(NEW): Estcourt, Wagendrift Nature Reserve, grassland with few trees, ca 40m from Wagendrift dam (29°02‘35.4“‘S 29°50‘20.9“‘E), 1196 m, 16.xi.2013, 2 cl, NMSA/OLIG.06702, T Nxele, B Nxele leg.; 1 cl., NMSA/OLIG.06703a; 1 cl., NMSA/OLIG.06703b; 1 juv., NMSA/OLIG.06703c; 1 cl., NMSA/OLIG.06703d; 1 cl., NMSA/OLIG.06703e; 1 cl., NMSA/OLIG.06703f; 1 cl., NMSA/OLIG.06703g; 1 TP, NMSA/OLIG.06703h.

Remarks: The holotype is in poor condition, as such, it was very difficult to see organs. Re-description was based on paratypes and new material, which is similar to the description by Plisko (2003). The species seems to be restricted to the type locality and its close vicinity.

Tritogenia hiltonia Plisko, 2003

Tritogenia hiltonia: Plisko 2003: 312; 2006: 59; 2013: 81.

Type locality: KZN, Hilton College.

Description:

External: *Body length*: holotype 54 mm long, 5 mm wide at tubercula pubertatis. Paratypes 28–53 mm long and 4–6 mm wide. *Number of segments*: holotype 90; paratypes: 70–97.

Prostomium: prolobous. *Segmentation*: preclitellar segments with secondary annulations; 1 and 2 simple with irregular longitudinal grooves; 3 simple with slight grooves; 4–8 has two ringlets of similar size and appearance; 9 has two ringlets with second ringlet smaller than the first ringlet; from 10 and postclitellar simple. *Setae*: *ab* and *cd* visible from segments 10; closely paired, after clitellum difficult to trace. *Nephridial pores*: not visible. *Female pores*: in 14, near 14/15 intersegmental furrow, minute. *Spermathecal pores*: on 11/12 12/13 intersegmental furrows. *Clitellum*: saddle shaped, on 13–1/n24; ventral border close to tubercula pubertatis in 18–22; segmented. *Tubercula pubertatis*: 18–22; glandular patches; almost square shaped; almost touching each other ventrally; segmented. *Papillae*: on 10–16, 24, single or paired.

Internal characters: *Septa*: 4/5 slightly thickened, 5/6, 6/7 strong, muscular, 7/8, 8/9 slightly thickened, other precletella septa thin. *Gizzard*: 6–7, partly in 6 and more in 7, large and well developed. *Calciferous glands*: in 9–10, stalked; separated dorsally and ventrally. *Intestine*: originates in 13. *Typhlosole*: commences in 16 and terminates in 53; U-shaped. *Blood vessels*: dorsal blood vessel double in 4–11, even so when crossing septa; single in rest of the segments; ventral vessel thin. *Nephridia*: two pairs per segment in posterior segments in dorsal and ventral parts of the body, ventral pair near genital glands, in some specimens dorsal is larger than the ventral pair. Holandric; testes funnels are closely paired, connected to seminal vesicles. *Seminal vesicles*: in 9–10, 11, small sacs. *Spermathecae*: occurs from 9/10 to 12/13, different position in different specimens, small, variable shape, close to genital glands. *Genital glands*: 11–16, small, cluster, glands of segment 24 are large, finger -like shape.

Material examined: (OLD): Holotype: Hilton College, Mistbelt grassland, with many flowering herbs, 2-30 cm deep, and between roots (29°30'47.863"S 30°18'02.928"E), 1119 m, 13.xii.2001, 1 cl., NMSA/OLIG.03534, AJA, HM; Paratypes: same locality as holotype, 13.xii.2001, 4 cl., 2 TP, NMSA/OLIG.03644; Hilton College, med-tall grassland, with many herbs, soil very dry, ca.30 cm deep (29°30'46.820"S 30°18'02.020"E), 1109 m, 22.i.2002, 1cl., NMSA/OLIG.03546, AJA, HM leg.; Cedara, Experiment: Veld Sample 1(29°32'S 30°17'E), 25.ii.2000, 1 juv., NMSA/OLIG.02856, R.J. Haynes leg.; Cedara, Experiment: Veld; Sample 2 (29°32'S 30°17'E), 25.ii.2000, 2 juv., NMSA/OLIG.02857, R.J. Haynes leg.

(NEW): Hilton College land at, gentle slope, med-high thick grassland with many herbs; 1-20 cm below surface (29°30'48.462"S 30°18'03.719"E), 1110 m, 12.i.2005, 2cl., NMSA/OLIG.03926, AJA, M. Mlambo leg.; Hilton, Heidelheim grassland, summit, soil loam, (WGS84), (29.50687° S 30.31575° E), 29.i.2009, 1 cl., 2 juv., NMSA/OLIG.04744, AJA, R. Harrison leg.; Hilton, Deeside Farm, mistbelt grassland, newly ploughed land, at northern facing slopes above St. Joseplis (29°31'17.8"S 31°16'26.3"E), 7.i.2003, 1cl., NMSA/OLIG.03699, J. Wakelin leg.; Hilton College, med-tall grassland, with many herbs, soil very dry, ca.30 cm deep (29°30'46.820"S 30°18'02.020"E), 1109 m, 22.i.2002, 1 TP, 4 juv., NMSA/OLIG.03547, AJA, HM leg.; Hilton, grassland on side of D494 gravel road, grassland with no trees (29°30‟46.2‟‟S: 30°18‟03.5‟‟E), 1091 m, 19.xii.2012, 1 cl., 2 TP, NMSA/OLIG.06672, T. Nxele & L. Bambalele leg.; Hilton, grassland on side of D494 gravel road, grassland with no trees (29°30‟46.9‟‟S: 30°18‟03.9‟‟E), 1091 m, 19.xii.2012, 5 cl., 1 TP, 13 juv., NMSA/OLIG.06673, T. Nxele & L. Bambalele leg.; Hilton, grassland on side of D494 gravel road, grassland no trees (29°30‟46.2‟‟S 30°18‟03.5‟‟E), 1091 m, 13.xii.2012, 1 cl., NMSA/OLIG.06424, T. Nxele & L. Bambalele leg.; 1 cl., NMSA/OLIG.06425; 1 cl., NMSA/OLIG.06426; 1cl., NMSA/OLIG.06427; 1cl., NMSA/OLIG.06428; 1cl., NMSA/OLIG.06429; 1cl., NMSA/OLIG.06430; 1cl., NMSA/OLIG.06431; 1cl., NMSA/OLIG.06432; 1TP, NMSA/OLIG.06433; 1TP, NMSA/OLIG.06434; 1TP, NMSA/OLIG.06435; 1TP, NMSA/OLIG.06436; 1TP, NMSA/OLIG.06437; 1TP, NMSA/OLIG.06438; 1TP, NMSA/OLIG.06439; 1TP, NMSA/OLIG.06440; 1TP, NMSA/OLIG.06441; 1TP, NMSA/OLIG.06442; 1 juv., NMSA/OLIG.06443.

Remarks: The species is known from Hilton and Cedara area.

Tritogenia howickiana (Michalesen, 1913)

Microchaetus sulcatus var. *howickianus*: Michaelsen 1913: 432; Reynolds & Cook 1976: 115.

Tritogenia howickiana: Michaelsen 1918: 333; Plisko & Zicsi 1991: 112; Plisko 1992: 368; 1997: 280; 2003: 317, 2013: 81; Nxele 2012: 546.

Type locality: KZN, Howick.

Description:

External: Body length 57–80 mm, width 6–8 mm. Number of segments, 81–103. Prostomium

prolobous, small. Segmentation, preclitellar segments with secondary annulations: segments 1–3 simple with horizontal grooves, segments 4–8 with two ringlets of equal size, 9 with second ringlet smaller, from 10 and postclitellar simple and randomly annulated. Setae *ab* only visible from 7, 9 or 10, other setae may be seen from 10. Male pores not visible. Female pores minute, on anterior part of segment 14 near *ab* setae. Spermathecal pores minute, in 11/12 and 12/13 intersegmental furrows. Clitellum saddle-shaped and on 13–21; segmented. Tubercula pubertatis on 1/n18–21, nearly square glandular swellings. Genital papillae on 11–18, 22, 23, paired or single, variable in size, round swellings on *ab* setae.

Internal: Septa 4/5, 5/6, 6/7 strongly thickened; 7/8, 8/9 are also thickened but less so than the anterior ones; other septa thin. Gizzard well developed in 6–7, globular, muscular. Calciferous glands in 9–10, stalked. Intestinal origin in 12. Dorsal blood vessel double in the anterior segments, double even when crossing septa; simple in the posterior segments. Nephridia in posterior segments two pairs per segment, coiled; the dorsal pair larger and the ventral smaller, this varying between segments, in some the dorsal smaller and the ventral larger. Holandric, testes funnels are closely paired with the second pair somewhat differently shaped and smaller, both pairs iridescent. Seminal vesicles small, in 10 and 11; one pair per segment. Spermathecae variable in shape in 12 and 13, more than one pair per segment.

Material examined: (OLD): Lectotype: ZMUH V-7658; Pietermaritzburg, greens between Longmarket and Church Str. at corner of Boshoff Str., 20 cm deep in moist soil, after a few days of the rain (29°35'S: 30°25'E), 5.iii.1990, 8 cl., NMSA/OLIG.00363, J.D. Plisko leg.; Otto's Bluff, The Craig's Farm, on the bank of local stream (29°30'S: 30°23'E), 6.xii.1989, 1 cl., 4 juv., NMSA/OLIG.02330, J.D. Plisko leg.; Waterfall Farm, near paddock (29°18'39"S: 31°02'49"E), 2.ix.2002, 1 juv., NMSA/OLIG.03943, D. Blacklaw leg.; Waterfall Farm, near chippings (29°18'39"S: 31°02'49"E), 2.ix.2002, 2 juv., NMSA/OLIG.03944, D. Blacklaw leg.; Waterfall Farm, from garden soil (29°18'39"S: 31°02'49"E), 2.ix.2002, 3 cl., NMSA/OLIG.03942, D. Blacklaw leg.; Howick, Amberfield, from garden soil (29~27'S: 30~14'E), 15.v.1997, 1 cl., 3 TP, NMSA/OLIG.02749, J.A. Pringle leg.; Pietermaritzburg, Town Bush Valley, at left side of the road to Government Nursery, from dry black soil (29°35'S: 30°25'E), 12.x.1988, 2 juv., NMSA/OLIG.00968, J.D. Plisko leg.; Pietermaritzburg, Bisley, on bank of Umlas River (29°35'S: 30°25'E), 6.iii.1991, 7 cl., 9 juv.,

NMSA/OLIG.00860, J.D. Plisko leg.; Orient Park, near Midmar Dam, grassland (30°31'S: 30°13'E), 31.i.1991, 16 cl., 7 TP, 33 juv., NMSA/OLIG.00908, J.D. Plisko leg.

(NEW): Queen Elizabeth Park, Mixed Scrub, between plantation and grassland (29°33.683'S: 30°18.992'E), 960 m, 24.ii.2012, 2 cl., 2 TP, NMSA/OLIG.06130, T. Nxele leg.; Queen Elizabeth Park, Mixed Scrub, between plantation and grassland (29°33.683'S: 30°18.992'E), 960 m, 24.ii.2012, 5 juv., NMSA/OLIG.06194, T. Nxele leg.; Queen Elizabeth Park, Mixed Scrub, between plantation and grassland (29°33.683'S: 30°18.992'E), 960 m, 24.ii.2012, 1cl., 7 juv., NMSA/OLIG.06132, T. Nxele leg.; Queen Elizabeth Park, Mixed Scrub, between plantation and grassland (29°33.683'S: 30°18.992'E), 960 m, 24.ii.2012, 3cl., 1 juv., NMSA/OLIG.06131, T. Nxele leg.; Queen Elizabeth Park, Woodland, on the side of the road near fence (29°34.810'S: 30°19.271'E), 23.ii.2012, 2 cl., NMSA/OLIG.06106, T. Nxele leg.; Queen Elizabeth Park, open patch of grass (29°34.123'S: 30°19.153'E), 7.i.2012, 1 juv., NMSA/OLIG.06139, T. Nxele leg.; Grassland, medium grass with a few trees (29°34.252'S: 30°19.174'E), 7.i.2012, 20 juv., NMSA/Olig.06134 & 06135, T. Nxele leg.; Woodland, small grass with bush near road (29°34.345'S: 30°19.377'E), 7.i.2012, 1 cl., NMSA/Olig.06094, T. Nxele leg.; Edendale area, Smero location, Nyonithwele mountain grassland near indigenous forest (29°37.943‘‘S: 30°17.126‘‘E), 13.ix.2012, 18 cl., 4 TP, 2 juv., NMSA/OLIG.06680, T. Nxele leg.; Edendale area, Smero location, Nyonithwele mountain grassland near indigenous forest (29°37.911‘‘S: 30°17.117‘‘E), 13.ix.2012, 13 cl., 3 TP, 5 juv., NMSA/OLIG.06676, T. Nxele leg.; Howick, grassland near Howick West (29°30.417‘‘S: 30°12.631‘‘E), 21.i.2012, 1 cl., 6 TP, 20 juv., NMSA/OLIG.06675, T. Nxele & L. Bambalele leg.; Howick area, hill above Mpophomeni location, SW of Mpophomeni (29°34‘‘49.0‘‘‘S: 30°10‘‘58.0‘‘‘E), 1239 m, 20.xii.2012, 1 cl., NMSA/OLIG.06484, T. Nxele & L. Bambalele leg.; Ihlanze Private Wildlife Reserve, Saxony Section, open Acacia woodland (29°28'42.14"S: 30°19'38.29"E), 708 m, 13.ix.2012, 1 cl., NMSA/OLIG.05021, H. Grobler leg.; Pietermaritzburg, Victoria Country Club, at border Queen Elizabeth Park, grassland, dug out during land preparation for housing (29°34'27.555"S: 30°19'46.864"E), 917 m, 16.ii.2005, 3 cl., 8 TP, 9 juv., NMSA/OLIG.03950, A.J. Armstrong leg.

Remarks: This species is common in the Midlands.

Tritogenia karkloofia Plisko & Zicsi, 1991

Tritogenia karkloofia: Plisko & Zcsi 1991: 115; Reynolds & Cook 1993: 16; Plisko 1992:368; 1997: 280; 2003: 317; 2006: 59; 2013: 81.

Type locality: KZN, Karkloof Nature Reserve.

Description:

External: *Body length*: holotype 66 mm long, 4 mm wide at tubercula pubertatis. Paratypes 55–68 mm long and 4 mm wide. *Number of segments*: holotype 120; paratypes: 110–114. *Prostomium*: prolobous. *Segmentation*: preclitellar segments with secondary annulations; 1 and 2 simple with irregular longitudinal grooves; 3 simple with slight grooves; 4–8 has two ringlets of similar size and appearance; 9 has two ringlets with second ringlet smaller than the first ringlet; from 10 and postclitellar simple. *Setae*: *ab* visible from segments 8; closely paired. *Nephridial pores*: not visible. *Female pores*: not visible. *Spermathecal pores*: when observed on 10/11, 11/12 12/13 intersegmental furrows. *Clitellum*: saddle shaped, on 1/n12–1/n23; ventral border close to tubercula pubertatis in 18–22; segmented. *Tubercula pubertatis*: 18–22; glandular patches; looks like bands, separated; randomly segmented. *Papillae*: on 12, 13, 18, 23, some specimens occurs from 10–18, single or paired.

Internal characters: *Septa*: 4/5 little thickened, 5/6, 6/7 slightly thickened, 7/8, 8/9 little thickened, other precletella septa thin. *Gizzard*: 6–7, partly in 6 and more in 7, large and well developed. *Calciferous glands*: in 9–10, not stalked; fused dorsally, separated ventrally. *Intestine*: originates in 13. *Typhlosole*: commences in 18 and terminates in 61; U-shaped. *Blood vessels*: dorsal blood vessel double in 4–11, even so when crossing septa; single in rest of the segments; ventral vessel thin. *Nephridia*: two pairs per segment in posterior segments in dorsal and ventral parts of the body, ventral pair near genital glands, in some specimens the ventral pair could not be located. Holandric; testes funnels close to seminal vesicles; funnels are closely paired. *Seminal vesicles*: in 10, 11, connected to testes funnels. *Spermathecae*: not observed but Plisko & Zicsi 1991 gives it in 11/12, 12/13. *Genital glands*: 10–12, medium, paired.

Material examined: (OLD): Holotype: Karkloof Nature Reserve, Safari World, 20 cm depth of moist soil, near water reservoir (29°25'S: 30°18'E), 850 m, 4.i.1989, 1 cl, NMSA/OLIG.00369, J.D. Plisko leg.; Paratypes: Karkloof Falls Nature Reserve, Safari

World, from moist soil, near water tank; 20 cm depth (29°25'S: 30°18'E), 850 m, 4.i.1989, 3 TP, 8 juv., NMSA/OLIG.00370, J.D. Plisko leg.; Umgeni River, flooded area on bank of (29°28'S:30°29'E), 6.xii.1989, 5 cl., 2 TP, 14 juv., NMSA/OLIG.00840, J.D. Plisko leg.; Umgeni River on the bank of; crossroad to Safari World, after rain, from top layer of moist soil (29°28'S:30°29'E), 6.xii.1988, 2 TP, 5 juv., NMSA/OLIG.00467, J.D. Plisko leg.; Karkloof Nature Reserve, Safari World On the bank of Umgeni River, from moist, sandy soil (29°25'S: 30°18'E), 800 m, 4.i.1989, 7 cl., 14 juv., 3 damaged, NMSA/OLIG.00489, J.D. Plisko leg.; Wagendrift Nature Reserve in top layer of soil and among of grass-roots in sumit grassland (29°02'31.0"S: 29°50'12.7"E), 27.ii.2001, 6 cl., NMSA/OLIG.03355, A.J. Armstrong & P. Ngwenya leg.; Wagensdrift Nature Reserve in top layer of soil above rocks, medium-high grass and small shrubs (29°02'31.7"S: 29°50'14.8"E), 4.v.2001, 2 cl., 2 TP, NMSA/OLIG.03455, A.J. Armstrong & B. Kasseepursad leg.; Wagensdrift Nature Reserve in top layer of soil above rocks, medium-high grass and small shrubs (29°02'31.7"S: 29°50'14.8"E), 4.iv.2001, 11 cl., 1 TP, NMSA/OLIG.03454, A.J. Armstrong & B. Kasseepursad leg.; Otto's Bluff, the Craig's Farm, on bank of local stream (29°30'S: 30°23'E), 6.xii.1989, 1 TP, NMSA/OLIG.02334, J.D. Plisko leg.; Karkloof Nature Reserve, Safari World on bank of Umgeni river, from sandy soil (29°25'S: 30°18'E), 740 m, 1.xii.1988, 4 cl., 4 juv., NMSA/OLIG.00932, J.D. Plisko leg.; Karkfloof Falls Nature Reserve, Safari World, primary grassland (29°25'S: 30°18'E), 28.i.1991, 11 cl., 13 TP, 1 juv., NMSA/OLIG.00715, J.D. Plisko leg.;

(NEW): Ihlanze Private Wildlife Reserve, Saxony Section, open Acacia woodland (29°28'42.14"S: 30°19'38.29"E), 708 m, 13.ix.2012, 2 cl., 4 juv., NMSA/OLIG.05022a, H. Grobler leg.; Ihlanze Private Wildlife Reserve, Saxony Section, open Acacia woodland (29°28'42.14"S: 30°19'38.29"E), 708 m, 13.ix.2012, 1 cl., NMSA/OLIG.05022b, H. Grobler leg.; Ihlanze Private Wildlife Reserve, Saxony Section, open Acacia woodland (29°28'42.14"S: 30°19'38.29"E), 708 m, 13.ix.2012, 4 cl., NMSA/OLIG.05022c, H. Grobler leg.; Ihlanze Private Wildlife Reserve, Saxony Section, open Acacia woodland (29°28'42.14"S: 30°19'38.29"E), 708 m, 13.ix.2012, 2 cl., 2 TP, 2 juv., NMSA/OLIG.05022d, H. Grobler leg.; Ottoʺs Bluff area, on side of D173 road near Emanzini Private N. Reserve (29°29ʹ09.9ʺʺS: 30°21ʹ54.5ʺʺE), 799 m, 26.vi.2013, 8 TP, 12 juv., NMSA/OLIG.06685, T. Nxele, V. Ndou, S. Kave & X. Ngubane leg.; Ihlanze Private Game Reserve, Saxony Section 1 (29°28'24.6"S: 30°20'18.0"E), 717 m, 13.xii.2012, 4 cl., NMSA/OLIG.06392, T. Nxele, H.

Grobbler & Peter leg.; Ihlanze Private Game Reserve, Saxony Section 2, Acacia grassland (29°28'37.1"S: 30°19'40.0"E), 804 m, 13.xii.2012, 5 cl., 4 TP, 4 juv., NMSA/OLIG.06395, T. Nxele, H. Grobbler & Peter leg.; Ihlanze Private Game Reserve, Saxony Section 1 (29°28'24.6"S: 30°20'18.0"E), 717 m, 13.xii.2012, 10 juv., NMSA/OLIG.06391, T. Nxele, H. Grobbler & Peter leg.

Remarks: The species is common in the Midlands. Spermathecae were not visible in examined specimens but the pores outside were recorded in some specimens. It is possible that spermathecae in this species are deeply embedded in the tissue. In some cases species that lack spermathecae may reproduce parthenogenetically as it was demonstrated by Shen et al in their study in 2011. A closer look into the tissue and chromosomes of this species may show whether or not this species reproduce pathenogenetically.

Tritogenia lunata Plisko, 1997

Tritogenia lunata: Plisko 1997: 259; 2003: 317; 2006: 59; 2013: 81.

Type locality: KZN, Karkloof, Mr Geekie"s farm „Benvie".
Description:
External: *Body length*: holotype 76 mm long, 5 mm wide at clitellum. Paratypes 27–83 mm long and 4–5 mm wide. *Number of segments*: holotype 77 (Plisko 1997 counted 93 but wrote "16 last segments regenerated"); paratypes: 72–92. *Prostomium*: prolobous, small. *Segmentation*: preclitellar segments with secondary annulations; 1 and 2 simple with irregular longitudinal grooves; 3 simple with slight grooves; 4–8 has two ringlets of similar size and appearance; 9 has two ringlets with second ringlet smaller than the first ringlet; from 10 and postclitellar irregularly annulated. *Setae*: *ab* observed from 7, *cd* from the area of 10 or 12, closely paired, minute. *Nephridial pores*: not visible. *Female pores*: not visible. *Spermathecal pores*: 11/12, 12/13 intersegmental furrows. *Clitellum*: saddle shaped, on 13–21, 22, 23, 24; close to cd setal line; ventral border close to borders of tubercula pubertatis, segmented. *Tubercula pubertatis*: 1/n18–1/n22; glandular patches; randomly grooved; bean shape. *Papillae*: on 12, 13, 22, 23, single or paired small swellings in *ab* or *cd* setae.

Internal characters: *Septa*: 4/5, 5/6, 6/7 muscular; 7/8, 8/9 very strong but not to be muscular; other septa thin. *Gizzard*: 6–7, partly in 6 and more in 7, strong and well developed.

Calciferous glands: in 9–10, stalked; separated dorsally and ventrally. *Intestine*: originates in 13. *Typhlosole*: commences in 17 and terminates in area of 47; U-shaped. *Blood vessels*: dorsal blood vessel double in 4–11, even so when crossing septa; single in rest of the segments; ventral vessel thin. *Nephridia*: two pairs per segment in posterior segments in dorsal and ventral parts of the body. Holandric, testes funnels close to seminal vesicles. *Seminal vesicles*: in 10–11, second pair large. *Spermathecae*: in 11, 12 near septa; located near genital glands; variable in shape. *Genital glands*: large, finger-like.

Material examined: (OLD): Holotype: Karkloof Nature Reserve, Geeke's Estate, forest edge, mixed Podocarpus; lower part; from first 1-20 cm of wet soil (29°18'S: 30°13'E), 1260 m, 22.ii.1989, 1 cl., NMSA/OLIG.02331, J.D. Plisko & B.R. Stuckenberg leg.; Paratypes: Karkloof Mr Geekie's Farm `Benvie' lower part of Afromontane forest. From first 20 cm of moist soil, under thick litter (29°18'S: 30°13'E), 1260 m, 22.ii.1989, 18 cl., 3 TP, 27 juv., NMSA/OLIG.00242, J.D. Plisko & B.R. Stuckenberg leg.; Karkloof Nature Reserve, Melmoth section, hillside, rocky grassland, among mole-rat mounds (29°16'50.654"S: 30°16'52.420"E), 9.x.2001, 2 cl., 7 juv., NMSA/OLIG.03473, A.J. Armstrong & P. Ngwenya leg.; Karkloof Nature Reserve, Geeke's Estate, forest edge, mixed Podocarpus; lower part; from first 1-20 cm of wet soil (29°18'S: 30°13'E), 1260 m, 14.iii.1989, 1 juv., NMSA/OLIG.02333, J.D. Plisko & B.R. Stuckenberg leg.; Karkloof, Mr Geekie's Farm `Benvie', forest edge of lower part of Afromontane forest, under moss and between roots of various plants on rocks and stones of Karkloof stream (29°18'S: 30°13'E), 1260 m, 22.ii.1989, 1 juv., NMSA/OLIG.00235, J.D. Plisko & B.R. Stuckenberg leg.; Karkloof Nature Reserve, Melmoth section, valley edge of vlei, grassland, peat soil (29°17'09.232"S: 30°16'23.896"E), 10.x.2001, 1 cl., NMSA/OLIG.03477, A.J. Armstrong & P. Ngwenya leg.; Karkloof Nature Reserve, on the side of the road, on bank of muddy stream between roots, in muddy black soil (29°18'S: 30°13'E), 1250 m, 22.ii.1989, 1 juv., NMSA/OLIG.00240, J.D. Plisko & B.R. Stuckenberg leg.; Karkloof Nature Reserve, mixed Podocarpus forest edge, lower part, from first 1–20 cm of moist soil (29°18'S: 30°13'E), 1260 m, 20.xii.1988, 2 cl., 3 TP, 21 juv., NMSA/OLIG.01004, J.D. Plisko & B.R. Stuckenberg leg.; Karkloof, Mr Geekie's Farm `Benvie', higher part of Afromontane forest edge. From litter and top soil (29°18'S: 30°13'E), 1260 m, 22.ii.1989, 1 cl., 2 TP, 6 juv., NMSA/OLIG.00247, J.D. Plisko & B.R. Stuckenberg leg.; Karkloof Nature Reserve, Geeke's Estate, forest edge, mixed

Podocarpus; lower part; from first 1–20 cm of wet soil (29°18'S: 30°13'E), 1260 m, 22.ii.1989, 1 juv., NMSA/OLIG.02332, J.D. Plisko & B.R. Stuckenberg leg.; Karkloof Nature Reserve, Melmoth section valley, hillside SW, grassland, on surface (29°16'56.949"S: 30°16'33.436"E), 9.x.2001, 1 cl., NMSA/OLIG.03476, A.J. Armstrong & P. Ngwenya leg.; Karkloof Nature Reserve, Melmoth section valley, above stream, grassland (29°17'00.039"S: 30°16'31.042"E), 9.x.2001, 2 cl., 2 juv., NMSA/OLIG.03475, A.J. Armstrong & P. Ngwenya leg.; Doreen Clark Nature Reserve, medium-tall grass (29°34'41.4"S: 30°17'20.8"E), 15.i.2001, 1 juv., NMSA/OLIG.03322, A.J. Armstrong leg.; Doreen Clark Nature Reserve, grassland, tall *Digiteria eviantha* (29°34'41.8"S: 30°17'19.8"E), 25.iv.2001, 3 cl. abscised, NMSA/OLIG.03446, A.J. Armstrong & B. Kasseepursad leg.; Doreen Clark Nature Reserve, grassland, tall *Digiteria eviantha* (29°34'41.8"S: 30°17'19.8"E), 25.iv.2001, 2 TP, NMSA/OLIG.03445, A.J. Armstrong & B. Kasseepursad leg.

(NEW): Karkloof, Mbona, Holbeck area, mistbelt foreston path after rain (29.30417°S: 30.36417°E), 1250 m, 19.ix.2004, 1 cl., NMSA/OLIG.03910, D.G. Herbert leg.;Umvoti Distr., Buccluech Forest (Ian Plantation) quadrat 1 (29.31030°S: 30.39908°E), 14.xii.2004, 2 cl., NMSA/OLIG.04125, M. Hamer leg.; Umvoti Distr., Buccluech Forest (Ian Plantation) (29.31030~S: 30.39908~E), 22.xii.2004, 6 cl., 1 juv., NMSA/OLIG.04123, M. Hamer leg.; York/New Hanover Road, 31.i.2014, 1cl., NMSA/OLIG.06704a, T. Nxele, L. Bambalele & S. Lamani leg.; 1 juv., NMSA/OLIG06704b; 1 juv. NMSA/OLIG.06704c.

Remarks: The species is common mostly in the Karkloof area. Calciferous glands in some of the specimens occupy segments 10–11 and 12 but in the other specimens they are only in segments 9–10.

Tritogenia mucosa Plisko & Zicsi, 1991

Tritogenia mucosa: Plisko & Zcsi 1991: 113; Plisko 1992: 369; 1997: 280; 2003: 308; 2006: 59; 2013: 81.

Type locality: KZN, 17 km NE Pietermaritzburg.

Description:

External: *Body length*: Paratypes 55–86 mm long and 5 mm wide. *Number of segments*: paratypes: 93–119. *Prostomium*: prolobous. *Segmentation*: preclitellar segments with

secondary annulations; 1 and 2 simple with irregular longitudinal grooves; 3 simple with slight grooves; 4–8 has two ringlets of similar size and appearance; 9 has two ringlets with second ringlet smaller than the first ringlet; from 10 and postclitellar simple. *Setae*: *ab* visible from segments 8; closely paired, *cd* visible from 10; closely paired, minute. *Nephridial pores*: not visible. *Female pores*: not visible. *Spermathecal pores*: not visible. *Clitellum*: saddle shaped, on 1/n12–22; ventral border close to tubercula pubertatis in 18–22; segmented. *Tubercula pubertatis*: 1/n18–1/n22; glandular patches; randomly segmented roundish, separated by small groove in the middle. *Papillae*: on 25, single or paired.

Internal characters: *Septa*: 4/5, 5/6, 6/7 muscular, 7/8, 8/9 moderately thickened, other septa thin. *Gizzard*: 6–7, partly in 6 and more in 7, large and well developed. *Calciferous glands*: in 9–10, small, separated dorsally and ventrally. *Intestine*: originates in 13. *Typhlosole*: commences in 17 and terminates in area of 61; U-shaped. *Blood vessels*: dorsal blood vessel double in 4–11, even so when crossing septa; single in rest of the segments; ventral vessel thin. *Nephridia*: two pairs per segment in posterior segments in dorsal and ventral parts of the body, in some specimens the ventral pair could not be located. Holandric; testes funnels closely paired, near to seminal vesicles. *Seminal vesicles*: small, connected to testes funnels. *Spermathecae*: in 10/11, 11/12, small, variable shape, close to septa. *Genital glands*: clustered in threes, paired.

Material examined: Paratypes: 17 km NE of Pietermaritzburg, grassland, clayey soil (22°29'S:30°23'E), 3.vi.1990, 1 cl., 3 juv., NMSA/OLIG.01056, J.D. Plisko & A. Zicsi leg.; Otto's Bluff, the Craigs Farm, from pasture (29°30'S: 30°23'E), 28.ii.1991, 2 juv., NMSA/OLIG.00711, J.D. Plisko leg.; Otto's Bluff, the Craig's Farm, primary grassland, dug out from approximately 30 cm depth of hard, but moist soil (29°30'S: 30°23'E), 31.i.1990, 1 cl., NMSA/OLIG.00347, J.D. Plisko & A. Zicsi leg.

Remarks: This species could not be examined satisfactorily because the paratypes were not in good condition, and no new specimens were found. The species is known only from type locality where it was collected between 1990 and 1991. However, the one clitellate specimen which was in good condition was examined and the characters were as described in Plisko & Zicsi (1991). Attempts to find new material were not successful.

Tritogenia shawi Plisko & Zicsi, 1991

Tritogenia shawi: Plisko & Zcsi, 1991: 117; Reynold & Cook 1993: 20; Plisko 1992: 371; 1997: 280; 2003: 318; 2006: 59; 2013: 81.
Tritogenia soleata Plisko, 1997:266; 2013: 8.

Type locality: KZN, Pietermaritzburg, Cleland.
Description:
External: *Body length*: holotype 165 mm long, 14 mm wide at clitellum. *Number of segments*: holotype 108; other specimens 95–130. *Prostomium*: prolobous. *Segmentation*: preclitellar segments with secondary annulations; 1 and 2 simple with irregular longitudinal grooves; 3 simple with slight grooves; 4–8 has two ringlets of similar size and appearance; 9 has two ringlets with second ringlet smaller than the first ringlet; from 10 and postclitellar irregularly annulated. *Setae*: *ab* observed from 3, closely paired, minute. *Nephridial pores*: not visible. *Female pores*: not visible. *Spermathecal pores*: not observed. *Clitellum*: saddle shaped, on 1/n13–27; close to cd setal line; ventral border not too close to borders of tubercula pubertatis, segmented. *Tubercula pubertatis*: 1/n17–22; glandular patches; have a pattern like grid, segmented. *Papillae*: on 14, 15, 23, single or paired small swellings in *ab* or *cd* setae.

Internal characters: *Septa*: 4/5 moderately thickened, 5/6 – 8/9 muscular; other septa thin. *Gizzard*: 6–7, partly in 6 and more in 7, large and well developed. *Calciferous glands*: in 9–10, fused together dorsally, separated ventrally by very small distance. *Intestine*: originates in 13. *Typhlosole*: commences in 22 and terminates in area of 86; U-shaped. *Blood vessels*: dorsal blood vessel double in 4–11, even so when crossing septa; single in rest of the segments; ventral vessel thin. *Nephridia*: two pairs per segment in posterior segments in dorsal and ventral parts of the body. Holandric, testes funnels are covered by seminal vesicles. *Seminal vesicles*: in 10, 11–12, second pair larger. *Spermathecae*: near septa 10/11, 11/12 in rows, three or four pairs per segment, one specimen with four pairs in 10 and six pairs in 11; variable in shape. *Genital glands*: in 15, 23, large, round.

Material examined: (OLD): Holotype: Pietermaritzburg, Cleland, 10 Lynroy Avenue, from garden soil (29°35'S: 30°25'E), 15.xii.1989, 1 TP, NMSA/OLIGO.00364, C. Shaw leg.; Pietermaritzburg, Darvill, from wet soil on the bank of the stream, at 20 cm depth (29°35'S:

30°25'E), 30.i.1991, 1 juv., NMSA/OLIG.00800, J.D. Plisko leg.; Pietermaritzburg, Darvill area, from moist soil of dry bed of local stream (29°35'S:30°25'E), 11.x.1990, 1 cl., NMSA/OLIG.00659, J.D. Plisko leg.; Pietermaritzburg, Scottsville, Golf Course, in top layer of watered grass (1-10 cm) and between the roots (29°35'S: 30°25'E), 10.xi.1989, 1 cl., 2 TP, NMSA/OLIG.00473, J.D. Plisko leg. Pietermaritzburg, 1 cl., BMNH:1893.12.16.3, Pueketi leg.

(NEW): Howick, Umgeni Valley Nature Reserve, Black Eagle trial, in litter, under log, 6.xii.2007, 1 cl., 1 juv., NMSA/OLIG.04719, D. Herbert leg.; Pietermaritzburg, Bisley Park, under log in the picnic site surrounding of bush and Acacia (29°35'S: 30°25'E), 22.xii.1993, 1 cl., NMSA/OLIG.02107, K.R. Cradok leg.; Pietermaritzburg, Town Hill (29°35'S: 30°25'E), iv.1928, 1 juv., NMSA/OLIG.02106, W.C. Rump leg.; Bisley Valley N. Reserve, savannah (29°39‟27.9‟‟S: 30°23‟31.8‟‟E), 28.vi.2013, 2 cl., NMSA/OLIG.06682, T. Nxele, V. Ndou, S. Kave & X. Ngubane leg.

Remarks: *Tritogenia shawi* is found only in PMB and surrounding areas. The spermathecae in some specimens were observed close to septa 9/10. This species is the largest of the midlands *Tritogenia* species. It was not possible to find any morphological character or combination of characters, to separate *T. soleata* from *T. shawi*. Therefore *T. soleata* is proposed to be synonimised with *T. shawi*.

Tritogenia sulcata Kinberg, 1867

Tritogenia sulcata Kinberg, 1867: 97.

Tritogenia sulcata: Perrier 1886: 876; Michaelsen 1899*b*: 415, 1900: 453, 1918: 338; Reynolds & Cook 1976: 176; Plisko 1992: 373, 1997: 280, 2003: 318, 2013: 81.

Megachaeta (Tritogenia) sulcata; Michaelsen 1891: 50.

Megachaeta? sulcata; Michaelsen 1891: 50.

Tritogenia sulcata [part.]; Michaelsen 1908: 31.

Microchaetus sulcatus f. typicus Michaelsen, 1913: 431.

Tritogenia morosa Cognetti, 1906: 13; Michaelsen 1913: 431, 1918: 338.

Microchaetus sulcatus; Reynolds & Cook 1976: 176.

Megachaeta sulcata; Reynolds & Cook 1976: 176.

Remarks: Known to have been collected from Port Natal, which is indicated as type locality. Original description is inadequate but was revised by Michaelsen in 1899b. The type specimen currently housed at the Royal Natural History Museum, Stockholm in Sweden (NHRS) is in poor condition. The photo (Figure A1) of the specimen parts was available and revision of this species was not productive. There is hope that future molecular analysis may shed some light.

Michalakus Plisko, 1996

Michalakus initus Plisko, 1996

Michalakus initus: Plisko, 1996: 289; 2006: 58; 2013: 82.

Type locality: KZN, Albert Falls, Bon Accorde Resourt.

Description:

External: *Body length*: holotype 88 mm long, 5 mm wide at clitellum; Paratypes 60–90 mm long, 6 mm wide at tubercula pubertatis. *Number of segments*: holotype 108; paratypes 94–113. *Prostomium*: prolobous. *Segmentation*: preclitellar segments with secondary annulations; 1 and 2 simple with irregular longitudinal grooves; 3 simple with slight grooves; 4–8 has two ringlets of similar size and appearance; 9 has two ringlets with second ringlet smaller than the first ringlet; from 10 and postclitellar irregularly annulated. *Setae*: *ab* observed from 7, closely paired, minute. *Nephridial pores*: not visible. *Female pores*: not visible. *Spermathecal pores*: not visible. *Clitellum*: saddle shaped, on 13–23; touches cd setal line; ventral border does not touch borders of tubercula pubertatis, segmented. *Tubercula pubertatis*: 19–1/n22; glandular rings in each segment, segmented, separated by intersegmental furrows. *Papillae*: on 10–12, 14, 22, 23, single or paired small swellings in *ab* setae.

Internal characters: *Septa*: 4/5 slightly thickened, 5/6, 6/7 moderately thickened, 7/8, 8/9 little thickened; other septa very thin. *Gizzard*: first gizzard in 6–7, partly in 6 and more in 7, strong and large; second gizzard in 9, smaller than the first one. *Calciferous glands*: in 10 fused into one dorsally, separated ventrally by small distance. *Intestine*: originates in 13. *Typhlosole*: commences in 18 and terminates in area of 57; U-shaped. *Blood vessels*: dorsal blood vessel double in 4–11, even so when crossing septa; single in rest of the segments;

ventral vessel thin. *Nephridia*: two pairs per segment in posterior segments in dorsal and ventral parts of the body. Holandric, testes funnels covered by seminal vesicles. *Seminal vesicles*: in 10–12, second pair large. *Spermathecae*: in 10/11, 11/12, 12/13 in rows, three or four pairs per segment; located near septa; variable in shape. *Genital glands*: in 9–13, 23 large, round.

Material examined: (OLD): Holotype: Albert Falls Tourist Resort Bon Accord; near small stream, grassland, moderately moist soil (29°28'S: 30°27'E), 6.iii.1991, 1 cl., NMSA/OLIG.00868, J.D. Plisko leg.; Paratypes: Albert Falls Tourist Resort Bon Accord; near small stream, grassland, moderately moist soil (29°28'S: 30°27'E), 6.iii.1991, 8 cl., 2 juv., NMSA/OLIG.00869/1-7, J.D. Plisko leg.; Albert Falls, 2 km from type locality, near Umgeni River, grassland, moist soil (29°28'S: 30°27'E), 6.iii.1991, 8 cl., 5 TP, 3 juv., NMSA/OLIG.00736, J.D. Plisko leg.; Albert Falls, 4 km from type locality, on bank of Umgeni River (29°28'S: 30°27'E), 16.xii.1991, 3 cl., 1 TP, 3 juv., 1 damaged and 3 pieces, NMSA/OLIG.01210, J.D. Plisko & A. Zicsi leg.

(NEW): On side of the road to Ihlanze Private Game Reserve, (29°30'02.3"S 30°21'50.4"E), 3087 m, 13.xii.2012, 1cl., NMSA/OLIG.06417, T. Nxele leg.

Remarks: The species is known from its type locality and close neighbourhood. The presence of two gizzards, one in segment 7 and another in segment 9 is unique to this species and its genus.

2.4 Discussion

For many years the taxonomic position of *Tritogenia* has not been properly evaluated resulting in difficulties in the classification of the South African indigenous megadrile (Plisko 2013). This is because of the simplicity and plasticity of characters used in identification of earthworm species which causes ambiguity in traditional morphology-based earthworm taxonomy (Novo et al. 2011; Csuzdi & Zicsi 2003). However, there has been progress in methods used in systematics in the last few decades (Csuzdi 2010). According to Decaëns et al. (2013), Loongyai et al. (2011) and Briones et al. (2009) the problem with taxonomic

identification of earthworms is that morphological characters, both external and internal, often show intraspecific variability.

From the fifteen morphological characters that were scored, six (thickness of septa, calciferous glands stalking, fusion of calciferous glands, commencement of typhlosole, position of seminal vesicles and shape of spermathecae) were distinct and independent meaning they are good diagnostic characters (Figure 2). These characters are not from any particular system but mixed systems for example, septa = muscular, calciferous glands = excretory, spermathecae = reproductive. The remaining characters (1, 3, 4, 7, 8, 12, 13, 14 and 15) were tightly clustered suggesting that these characters are not independent but rather are correlated to each other and would not be very informative when used on their own. These characters did not cluster according to any system; however seminal vesicles and spermathecae form part of the reproductive system, which is considered evolutionarily more conservative, but they clustered with other characters. The distinct characters (2, 5, 6, 9, 10, and 11) were consistent within species whilst the clustered characters were not, for example the number of body segments vary within species (also see detailed account in the taxonomy section). These characters grouped some *Tritogenia* species close to each other in the scatterplot whilst *T. debbieae, T. shawi, M. initus* and *M. papillatus* were scattered far apart (Figure 3). *Tritogenia debbieae* is the only *Tritogenia* in the Midlands with thin septa throughout the body and large round spermathecae, these characters were shown to be independent and useful for separating this species. The characters separating *T. shawi* from other species are characters 5 and 6; *T. shawi* has calciferous gland that is fused, with no branching from the gut which is places this species far from the others. The number of gizzards separates *M. initus*.

Morphological characters of three species overlapped completely with others (*T. lunata, T. mucosa* and *T. soleata*). The observed overlap may be due to the number of characters scored. The morphological data agrees with the authors who have noted that in some species morphological characters are so variable that they often overlap with these diagnostic of other species (Fernández et al. 2012; Pop et al. 2003), as it was shown with the characters that clustered together causing a complete overlap of some species on the PCA. The addition of more characters may help cluster the species better since only fifteen were scored for this study. In earthworm taxonomy both external and internal characters are useful in description of species therefore scoring both internal and external characters would be significant to the

analysis. It is not possible to say whether or not some of the studied characters show convergence because *Tritogenia* consistS of 35 species and only ten were studied here; a revision of all species may show if there is convergence in the morphological characters.

Most clades in the phylogeny (Figure 4) have low support values (less than 75%). This may be due to few or lack of phylogenetically informative characters and as such more characters need to be added to resolve the relationships. Despite that lack of resolution, there was strong support for a clade containing the species *T. annetteae, T. debbieae, T. hiltonia, T. howickiana, T. lunata, T. mucosa* and *T. sulcata.* There are two characters that support this clade, first is the separation of calciferous glands which is widely separated in the species in this clade, with glands mostly on the dorsal part of the gut. The second character defining this clade is the number of spermathecae; all species in this clade have one pair of spermathecae per segment. *Tritogenia karkloofia* formed a distinct well supported lineage; morphological characters support this arrangement, *T. karkloofia* has a calciferous gland that is not stalked and specimens belonging to this species have more number of body segments. In the taxonomy section, a detailed account of *T. karkloofia* is given and the spermathecae was not observed in most specimens but in some specimens the spermathecal pores were observed. A closer look into the reproduction of this species in the future is suggested as species that lack some reproductive organs usually reproduce pathenogenetically (Shen et al. 2011). More characters are needed to resolve the phylogeny of the Midlands *Tritogenia* species.

The grouping of species in the PCA is similar to that observed in the cladogram based on morphological characters with the exception of *T. debbieae* which is completely separate from the other species in the scatter plot. From the scored characters, characters 4, 8, 12, 14 and 15 seem to be of ancestral state (plesiomorphies). Thickness of septa is an autapomorphy in *T. debbieae* as *Tritogenia* species have some thickness in septa of the anterior part of the body but *T. debbieae* have thin septa throughout its body and this state might have been lost in this species. These characters provide less evidence of relationships in studied species.

Based on morphological characters *Tritogenia* is not monophyletic as the group doesn‟t include all the descendants of a common ancestor and includes *M. initus.* In the present study a synonym is proposed (*T. soleata* = *T. shawi*), their morphological characters are the same (see detailed account in the taxonomy section) and the characters of these species completely overlapped in the PCA, Figure 3.

Tritogenia sulcata is a type for *Tritogenia* which is a type for Tritogeniidae. The type material is incomplete, in poor condition and was unfortunately not available for study. *Tritogenia mucosa* was last collected in 1990/1991 and the type material is not in good condition, it is very soft and difficult to handle and no new material was available to confirm the internal characters. Recent attempts to find new material were unsuccessful.

Endemism in soil fauna has been noted in groups such as millipedes (Hamer & Slotow 2002; Vohland & Hamer 2013). This makes them vulnerable to extinction (Hamer & Slotow 2000) because they have poor dispersal ability over long distances. The transformation of habitats by humans may reduce species richness and diversity and the resulting patchiness increases possibility of the loss of endemic species (Suarez et al. 1998). The Midlands *Tritogenia* species show some level of endemism and habitat transformation poses a big threat to them since *Tritogenia* species are found primarily in natural undisturbed biotopes. According to Bell et al. (2004) mobility is limited in flightless beetles and this may increase the potential for allopatric or parapatric speciation. The same is possible for *Tritogenia* species with localised endemism in the Midlands.

To conclude, morphological data could not fully resolve the relationships in the Midlands *Tritogenia* and *Michalakus* species, therefore T. *annetteae, T. debbieae, T. hiltonia, T. karkloofia, T. lunata* and *T. shawi* as well as *M. initus* were left as valid species and will be subjected to molecular analysis which might provide better position of these species. Blakemore et al. (2010) emphasised that close resemblance of morphological characters observed in many megadrile species, missing types and lack of taxonomists are serious problems in conventional systematics and taxonomic studies of earthworms worldwide. The problem with specimens in the KwaZulu-Natal Museum is that almost all specimens have been preserved in formalin and without expensive kits DNA isolation is not possible therefore new material has been collected to verify the species using genetic data.

Chapter Three

Phylogeny of the KwaZulu-Natal Midlands *Tritogenia* Kinberg, 1867 and *Michalakus* Plisko, 1996 species (Oligochaeta: Tritogeniidae) inferred from mitochondrial DNA sequences

Abstract

The Midlands species of *Tritogenia* are difficult to distinguish morphologically and identification keys are difficult to construct. In this study molecular tools were used to reconstruct the phylogeny of the clitellates *Tritogenia* and *Michalakus* that occur in the Kwazulu-Natal Midlands. One hundred and fourty four individuals were analysed representing eleven species belonging to the *Tritogenia* and *Michalakus* genera. Two molecular markers from the mitochondrial genome, namely cytochrome c oxidase subunit I (COI) and 16S ribosomal DNA (16S rDNA) were used to reconstruct the phylogeny of the group. Analysis of the data using both Bayesian and maximum likelihood approaches revealed that *Tritogenia* is not monophyletic. The results suggest that *Michalakus* is nested within *Tritogenia*. However, a further investigation using nuclear markers was suggested to test this hypothesis. Molecular data revealed eight lineages which correlated with eight currently described species. Some lineages did not fit any known species suggesting that they may represent undescribed species. T*ritogenia shawi* does not cluster with the other *Tritogenia* species but nests within outgroup species.

3.1 Introduction

Earthworms are one of the important fauna in soil ecosystems and their contribution to soil health is well documented (Lavell et al. 2006; Barios 2007). Habitat loss, pollution and climate change are accelerating the extinction of species including those that have not been formally described (Essl et al. 2013). It is important that tools are developed which aid in species identification and that will allow more species to be delimited in a fast and accurate way. According to Chang et al. (2009), the basis of taxonomic and systematic studies is the accuracy of species names. However, it is acknowledged that identifying some taxa such as earthworms is difficult because of the lack of convenient, well-defined morphological

characters (Richard et al. 2010), particularly when the expression of these characters are often influenced by environmental factors. Traditional morphology-based identification also requires substantial taxonomic expertise in this group because it involves observation of minute morphological characters (Richard et al. 2010). Molecular analyses helps to accelerate the rate of species discovery in order to avoid the scenario of species extinction before they are discovered.

Phylogenetic relationships in megadrile fauna have been traditionally investigated using morphological characters (Csuzdi 2010; Csuzdi and Zicsi 2003; Plisko and Zicsi 1991; Chang et al. 2007). However, the intraspecific variability of morphological characters in earthworms is high (Briones et al. 2009; Huang et al. 2007). Specimens are often preserved differently and preservation may change the appearance of characters which may lead to misinterpretations, which in turn may lead to description of unfounded species (Nxele 2014). As such, the use of DNA in species diagnosis is considered an important compliment to traditional morphology-based analyses in earthworms.

In South Africa, the systematics and ecology of the indigenous megadrile is incomplete (Nxele 2012). The incorporation of new methods are essential to improve the understanding of the taxonomy of the megadrile fauna in South Africa. Plisko (2012) commented on the need for molecular information to give clarity on the position of *Tritogenia* and *Michalakus* in microchaetids. Plisko (2013) stressed that a molecular study on indigenous South African megadrile is needed to reveal the evolutionary relationships among them. In chapter 2 it was demonstrated that morphological characters on their own are not able to accurately delimit *Tritogenia* species that occur in the KZN Midlands.

The use of DNA sequences has increased in the recent past because it is less subjective than morphological characters and it is applicable at most levels (Decaëns et al. 2013; Chang & James 2011) and it allows for the analysis of many characters (Scotland et al. 2003).

Many genes have been used to identify species within invertebrates with the mitochondrial gene COI, used as the standard marker for species delimitation in DNA barcoding (Hebert et al. 2003). Most studies that applied DNA analysis to earthworms belonging to Lumbricidae and Megascolecidae (Blakemore et al. 2010) and have focused mainly on the cytochrome oxidase I (COI) mitochondrial gene (Blakemore 2013; Bantaowong et al. 2011; Huang et al. 2007; Richard et al. 2010; King et al. 2008; Rougerie et al. 2009; Chang et al. 2009; James et

al. 2010; James & Davidson 2012; Pop et al. 2003; Pérez-Losada et al. 2009; Minamiya et al. 2011a,b; Voua Otomo et al. 2009; Voua Otomo et al. 2013). The COI sequence is variable enough to differentiate between species but is less variable in individuals that belong to the same species. This means that individuals belonging to the same species will cluster closely together on a phylogeny (Stoeckle & Hebert 2008; Valentini et al. 2008) and it is possible to easily assign specimens to species clusters.

An advantage of using the molecular tools in earthworms is that it is applicable to all life stages (Rougerie et al. 2009). Juveniles in earthworm taxonomy are particularly difficult to identify using traditional morphological classifications because most characters are not well developed (Richard et al. 2010; Chang & James 2011; Decaëns et al. 2013). According to Rougerie et al. (2009) it is important to use DNA data and in particular DNA barcodes in an integrative manner, combining molecular phylogenetics with morphological taxonomy rather than to use DNA barcodes on their own. This integrative approach has been sucessfully applied to earthworms (Chang & James 2011) and is likely to produce reliable taxonomic data. Blakemore et al. (2010) went so far as to recommend that new earthworm species descriptions should be accompanied by type specimen DNA barcode information.

The aim of this investigation was to evaluate the validity of described species of *Tritogenia* and *Michalakus* in KZN Midlands, by investigating their molecular differences using two mitochondrial markers. The results will also be used to determine if *Michalakus* is a sister genus to *Tritogenia*.

3.2 Materials and methods

3.2.1 Sampling

Eleven different type localities (Table 1), where ten *Tritogenia* and one *Michalakus* species were previously collected, were resampled. In addition fifteen new sites were also sampled (see appendix Table A2). *Tritogenia* earthworms were collected in the KZN Midlands between 2011 and 2013. New earthworm material was collected by digging out three 1m by 1m and 30cm deep soil monoliths along a 100m transect at 0m, 50m and 100m at each site. Soil was hand sorted for earthworms in large plastic trays (50cm x 50cm x 10cm). Collected specimens were narcotized using 45% ethanol solution. Specimens were preserved in

absolute ethanol (to preserve DNA integrity) for DNA extraction. Tissue samples used for DNA extraction were taken from caudal tissue to avoid contamination by gut content. All specimens were examined using a Wild Heerbrugg stereo-microscope and identified according to the classifications by Plisko (1992; 1997; 2003), Plisko and Zicsi (1991) and Michaelsen (1913). Attempts to collect new material of *Tritogenia mucosa* were not successful and the type locality for *Tritogenia soleata* does not exist anymore.

Sequences of *Microchaetus papillatus, Amynthas corticis* and *Octolasion cyaneum* were included as outgroup taxa. The sequences for *Amynthas corticis* and *Octolasion cyaneum* were obtained from GenBank (Accession nos: AB542457.1, AB474283.1, HE611688.1, HE611657.1). Newly collected specimens (17) from Entumeni Nature Reserve and nine (which include *T. zuluensis*) from Hluhluwe Game Reserve (Northern KwaZulu-Natal) were included in the analysis to validate local endemism of the KZN Midlands species. Most of the new sites fall under KZN Wildlife area of jurisdiction and the permit was obtained from their head office in Queen Elizabeth Park (permit no: OP 5247/2013). Permission was obtained from local authorities for the sites outside KZN Wildlife area of jurisdiction. All new specimens were deposited at the KwaZulu-Natal Museum.

3.2.2 DNA extraction, amplification and sequencing

All DNA extractions were performed using NucleoSpin® Tissue kit by Macherey-Nagel (Genomic DNA from tissue), following the manufacturer‟s standard protocol for human or animal tissue and cultured cells. The concentration of DNA in each sample was estimated using the NanoDrop 2000 (Thermo Scientific). The isolated DNA was stored at -20^{o}C. A fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI) was amplified for 144 specimens (*T. annetteae* = 1, T. debbieae = 4, *T. hiltonia* = 24, *T. howickiana* = 12, *T. karkloofia* = 31, *T. lunata* = 1, *T. shawi* = 1, *M. initus* = 1, T. sp from Entumeni GR = 17, T. sp from Hluhluwe GR = 9). Another mitochondrial region (16S rDNA) was amplified for a subset of samples. The subset was chosen after preliminary COI data where 16S rDNA was sequenced from a representative of each monophyletic species cluster. For the COI gene, the primers LCO1490 and HCO2198 (Folmer et al. 1994) were used, whilst the primers 16Sa and 16Sb were used for 16S rDNA gene (Palumbi et al. 1991). Polymerase chain reactions (PCR) were performed in the final volume of 25µl and contained 2µl of DNA template

(approximately 35ng/ul), 1 X 2.5µl Kapa PCR buffer, 0.5µl of a 10mM dNTPs, 0.1µl of 5U/µl Kapa Taq polymerase (Kapa Biosystems) and 0.5µl of 10uM forward and reverse primers and sterile water. The thermocycler conditions were as follows: 95^{o}C for 2min.30sec for initial denaturation followed by 35 cycles at 95^{o}C for 30 sec denaturation, 50^{o}C to 52^{o}C for 45 sec annealing and 72^{o}C for 1min.25sec extension. A final extension step at 72^{o}C for 10 min completed the reactions. Successful amplifications of COI and 16S rDNA (approximately 690bp and 496bp respectively) were verified by using agarose gels (1.6g Agarose powder in 200ml TBE buffer and stained with 20µl of 4mg/ml ethidium bromide) with 3µl of 100bp molecular weight ladder (Solis BioDyne) was run with the samples. Gels were visualized under UV light. Sequencing was conducted at the University of Stellenbosch Central Analytical Facility (CAF) using Big Dye chemistry (Applied Biosystems) and the same primers used for amplifications. To verify if all sequences were Oligochaeta the BLASTn algorithm was used to BLAST sequences against GenBank (Altschul et al. 1997).

3.2.3 Sequence alignment and phylogenetic analysis

The sequences of each gene (COI and 16SrDNA) were aligned using Clustal X 2.1 (Larkin et al. 2007) using default settings. These alignments were then manually edited using BioEdit 3.3.19.0 (Hall 1998). Unreliable nucleotides (low signal strength) as well as primer sequences were trimmed off at both the 5' and 3' ends. Three data matrices were analysed. First, a data matrix including 144 COI sequences, second a data set with 41 16S rDNA sequences, last the COI data was combined with the 16S rDNA. The latter data set only included taxa that had both COI and 16S rDNA sequences.

Consistency index values, retention index values, the number of variable sites and the number of parsimony informative sites were estimated for each of the three data sets in Mega6 (Tamura et al., 2011). The program jModelTest v.0.1.1 (Darriba et al. 2012) was used to select the best-fit evolutionary model using the Akaike information criterion (AIC; Akaike 1973). Phylogenetic analyses were based on two approaches, Bayesian inference was performed using MrBayes 3.2 (Huelsenbeck & Ronquist 2001) and maximum likelihood (ML) analysis was performed using Garli (Zwickl 2011). In each case the best-fit evolutionary model selected by jModelTest was specified. Clade support was evaluated by 1000 bootstrap replicates for the maximum likelihood analysis and posterior probability

values for the Bayesian analysis. For the combined data a partitioned analysis was perfomed with two independent models (GTR+I+G and TPM3uf+I+G) For Bayesian analyses, all MrBayes analyses were run for 5000 000 generations with a sampling frequency of 1000 and a burn-in of 25%. The deviation of split frequencies was less than 0.01 at the conclusion of all analyses which confirmed that the MCMC chains had converged. The program Tracer v1.5 (Drummond & Rambaut 2007) was used to check that the Effective Sampling Size >200 and that posterior distribution for all parameters was unimodal. Consensus trees were generated using Phylip 3.69 (Felsenstein 2005) and viewed in Fig Tree v1.3.1 (Rambaut 2009).

The effect of geographical distance on the genetic divergence of populations was assessed using a Mantel test implemented in the Alleles in Space (AIS) software (Miller 2005).

3.3 Results

3.3.1 Sequencing success

A total of 144 COI and 4116S rDNA sequences were obtained for *T. annetteae, T. debbieae, T. hiltonia, T. howickiana, T. karkloofia, T. lunata, T. shawi, M. initus*. The outgroup, *Microchaetus papillatus*, was also successfully sequenced for both markers. The sequences were manually aligned and gaps edited in BioEdit. The COI alignments after primers were trimmed were 658bp and 463bp for 16SrDNA. Values of genetic variability for data sets are shown in Table 4. The COI data set had the most variable characters (n= 318, this is 48% of the total number of characters) as well as the highest number of number of parsimony informative sites (n= 282). In 16SrDNA the variable characters, n=194 which is 42% of the total number of characters and the number of parsimony informative sites, n=174. For the combined data, n=478 for variable characters (72% of the total number of characters) and n=470 for number of parsimony informative sites. The 16S rDNA dataset has the highest CI suggesting a lesser level of homoplasy compared to the other datasets. The RI is close to 1 in all datasets suggesting a good character fit on the tree, combined data dataset has slightly higher RI suggesting more synapomorphy than the others.

Table 4. Diversity values of the gene fragments used for the analyses of *Tritogenia* and *Michalakus* species

	16s rDNA	**COI**	**combined analysis**
Individual	43	146	86
No. characters	463	658	663
No. variable characters	194	318	478
No. parsimony informative sites	174	282	470
Consistency Index	0.537205	0.254395	0.376064
Retention Index	0.791837	0.858071	0.896167
Substitution Model	GTR+I+G	TPM3uf+I+G	GTR+I+G, TPM3uf+I+G

3.3.2 Molecular phylogeny

The maximum likelihood and Bayesian Inference trees were congruent therefore posterior probabilities as well as bootstrap support values were annotated onto the branches of the most likely trees generated for each of the data sets analysed (ML run with no bootstrap, Figures 5, 6, 7), which were rooted using outgroup species (*M. papillatus, A. corticis* and *O. cyaneum*).

Bootstrap values above 75% and 0.95 posterior probabilities were considered significant support, values below 50% bootstrap and 0.50 posterior probabilities were not shown on the trees. The trees recovered from the analysis of all three data sets show some lineages with good bootstrap and posterior probability support and some lineages with poor bootstrap and posterior probability suppor. Individual trees of COI and 16S rDNA (Figures 5 & 6) were supported by association recovered by the combined analysis (Figure 7). All trees share the common feature that *Tritogenia* is non-monophyletic, with both COI and 16S rDNA and the combined data placing *T. shawi* within a clade containing the outgroup taxa *M. papillatus, A. corticis, O. cyaneum* (bootstrap and posterior probabilities: 80, 0.99 for COI / 99, 0.99 for 16S rDNA / 95, 1.0 for combined data). The other Midlands *Tritogenia* species (*T. annetteae, T. debbieae, T. hiltonia, T. howickiana, T. karkloofia, T. lunata* and *T. mucosa*) together with *M. initus* are grouped together (bootstrap and posterior probabilities: 69, 0.99 for COI / 70, 0.99 for 16S rDNA / 89, 1.0 for combined data).

In the COI tree (Figure 5) specimens belonging to *T. annetteae* falls within the clade with *T. hiltonia*, which was only moderately supported in maximum likelihood analysis (ML bootstrap value; 73%) but is well supported in Bayesian tree (posterior probability 0.99). This lineage was also recovered in the 16S rDNA and combined analysis. In 16S rDNA and in combined data the bootstrap values were 96% and 97%, respectively and the posterior probabilities were 0.97 and 1.0, respectively (Figures 6 and 7). In both (COI and 16S rDNA) trees, *T. annetteae* share a recent common ancestor with *T. hiltonia*.

The specimens assigned to *T. debbieae* form a single lineage which is strongly supported in ML and Bayesian analyses (ML bootstrap support 100% and Bayesian posterior probability 1.0). This species share a recent common ancestor with *Tritogenia* from northern KZN, collected in Hluhluwe Game Reserve (Bayesian posterior probability 0.99, Figure 5).

Specimens assigned to *T. howickiana* formed two distinct clades (Figure 5), one includes specimens from Queen Elizabeth Park (QEP) and another from Howick. The clade from QEP (Figures 6 & 7) is nested within a larger clade of unidentified specimens collected from a variety of localities in the Midlands (Blackridge, Balgowan, Edendale, Mawela Game Reserve and juveniles collected on the road to Ihlanze Game Reserve) as well as with the *T. howickiana* clade from Howick which is also associated with unidentified specimens collected from Curry‟s Post and Tweedie. Specimens belonging to *T. karkloofia* seems to form the largest clade with bootstrap and posterior probabilities of 77% and 1.0 (Figure 5), 95%, 0.99 (Figure 6) and 99%, 1.0 (Figure 7), respectively. *Tritogenia karkloofia* species includes three different localities which are in close proximity to each other and share a recent common ancestor with the unidentified species from Entumeni Nature Reserve, northern KZN. The clade that combines *T. lunata* with *T. howickiana* (plus number inidentified juveniles) has poor support (no bootstrap value, only posterior probability of 0.73).

In addition there are four lineages of unidentified specimen that do not correlate with any currently described species in the genus (specimens from Curry‟s Post area, Tweedie, Balgowan and Edendale/Blackridge).

Species that are geographically close to each other are not genetically close to each other, for example *T. hiltonia* (from Hilton) genetically shares a recent ancestor with *T. annetteae* (from Mooi River) not with *T. howickiana* (from QEP). The testing of the isolation by distance in

Mantel test showed a weak but positive correlation between genetic and geographic distances ($r = 0.3778$ and $p = 0.00099$).

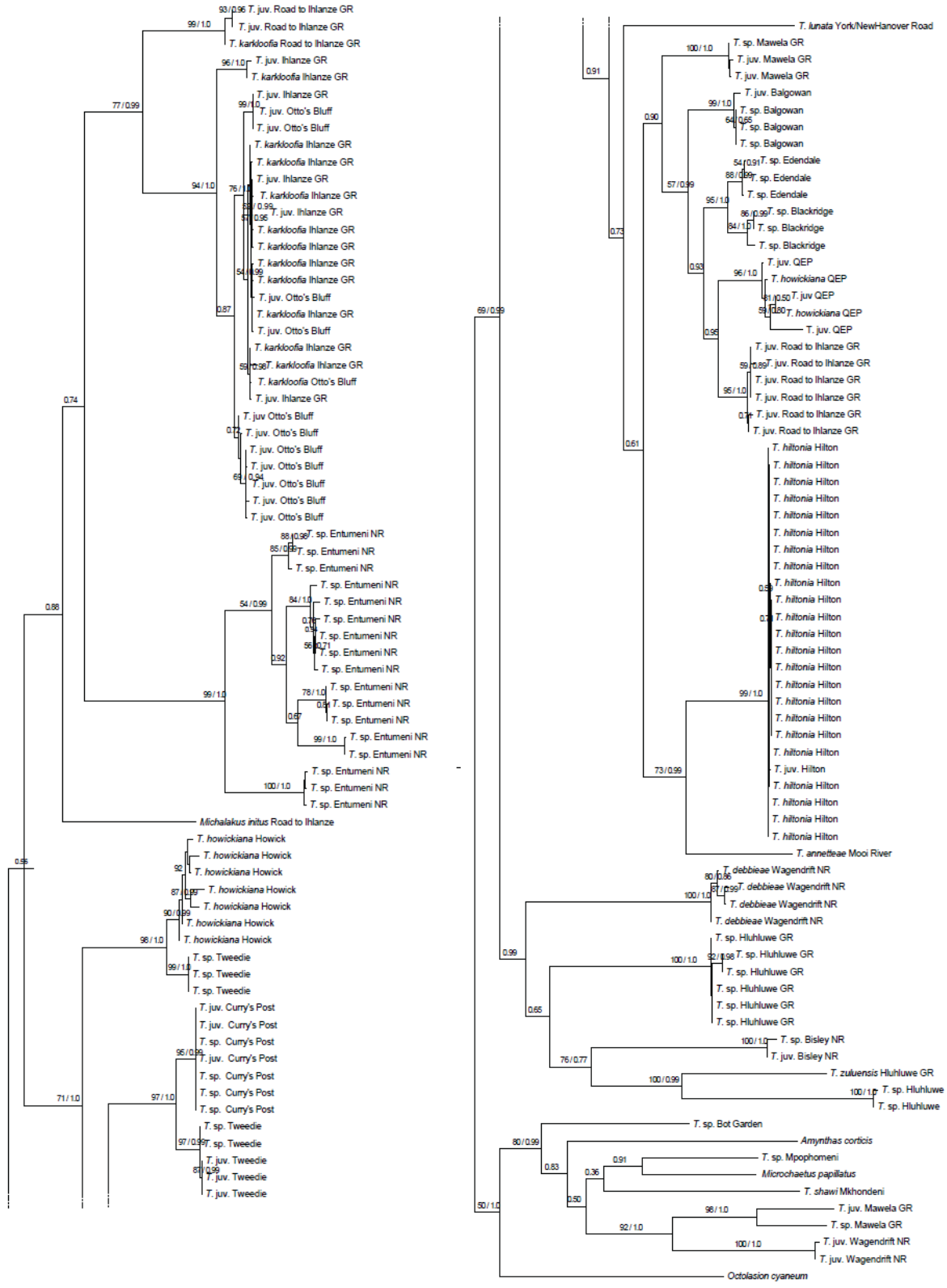

Fig. 5. The Bayesian phylogram for the COI mtDNA provided support that *Tritogenia* is not monophyletic of *Tritogenia*, *T. howickiana* forms two distinct clades and *T. shawi* is associated with species from the northern KZN. Values annotated onto the branches indicate maximum likelihood bootstrap support values followed by posterior probabilities support values. Only support values above 50% of bootstrap and 0.5 posterior probabilities are shown on the tree.

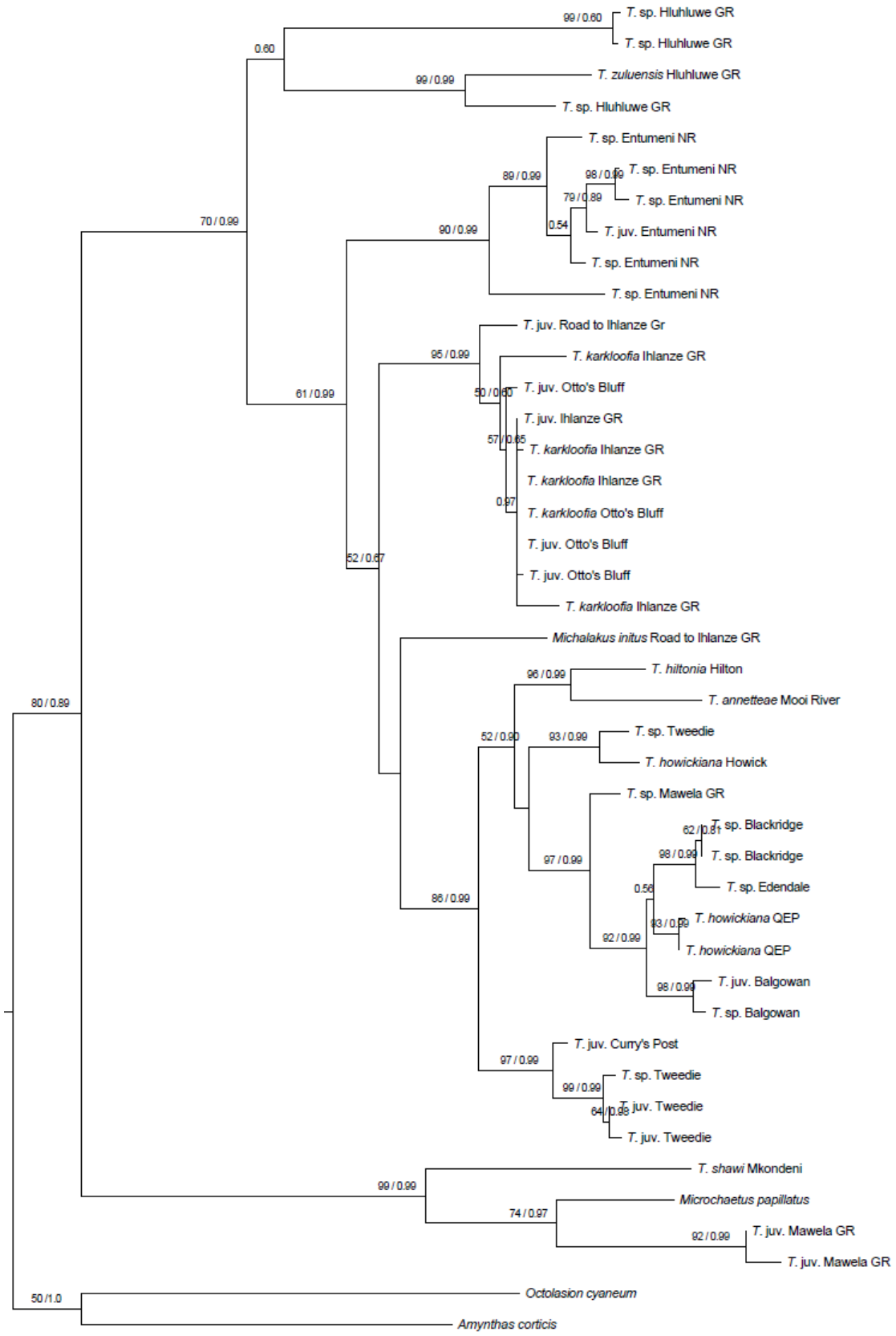

Fig. 6. The Bayesian phylogram incoporating16S rDNA provided support that *Tritogenia* is not monophyletic, *T. shawi* associates with the outgroup species and unidentified specimens form distinct clades. Values annotated at the branches indicate maximum likelihood bootstrap support values followed by posterior probabilities support values. Only support values above 50% of bootstrap and 0.5 posterior probabilities are shown on the tree.

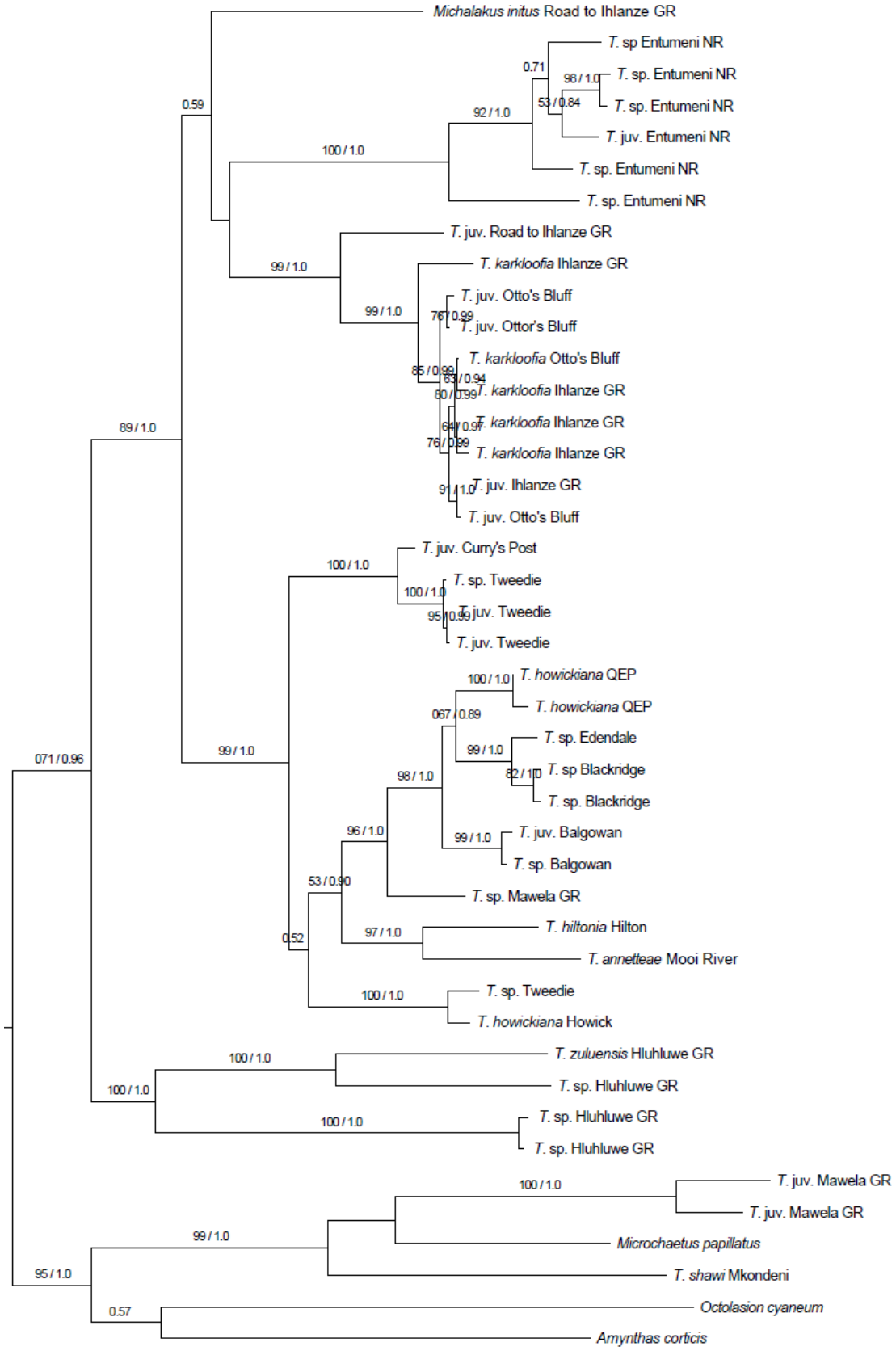

Fig. 7. The Bayesian phylogram constructed using the combined data (COI and 16S rDNA) supports the individual gene trees in that *Tritogenia* is not monophyletic and *T. howickiana* clades are possible different species. Values annotated at the branches indicate maximum likelihood followed by posterior probabilities support. Only support values above 50% of bootstrap and 0.50 of posterior probability are shown.

3.4 Discussion

In all trees, some lineages were not well supported. In particular, nodes associated with older divergence events (deeper nodes) were recovered with weak branch support. Combining data from two mtDNA genes did improve branch support values confirming previous studies which have advocated the use of multi-gene phylogenies (Perez-Losada 2009; James & Davidson 2012; Pop et al. 2003). Eight lineages within the molecular phylogeny represented the described species, *T. annetteae, T. debbieae, T. hiltonia, T. howickiana, T. karkloofia, Tritogenia lunata, Tritogenia shawi and Michalakus initus* (Figure 5).

From the newly collected material, no specimens could morphologically be assigned to *T. sulcata* as there was no type material to compare the specimens to. The molecular data however recovered two completely separate clades of specimens belonging to *T. howickiana* (Figure 5); one is for specimens from QEP and another from Howick. It is known from literature (Michaelsen 1913; Plisko & Zicsi 1991) that *Tritogenia sulcata* and *Tritogenia howickiana* are similar morphologically; the presence of two clades for specimens of *T. howickiana* could be that one of these clades actually belongs to *T. sulcata*. As the type is not available for *T. sulcata*, it is difficult to assign the specimens of the second clade to a new species. It is also possible that one clade is another population of *T. howickiana* and the other clade belongs to specimens of an undescribed cryptic species of *T. howickiana*. A molecular phylogeny of other *Tritogenia* species outside of the Midlands may be helpful in assigning these specimens to *T. sulcata* because if they do not fit any other species outside the Midlands then they may be assigned to *T. sulcata*.

Tritogenia debbieae is monophyletic and well supported and this is in agreement with the PCA results in chapter 2 where morphological characters placed this species far from the others. Of all the Midlands *Tritogenia* species *T. debbieae* is the only one with very large spermathecae that has a shape like a „tennis ball" and this character was showed to be independent in the chapter 2. *Tritogenia karkloofia* was recovered in accordance with the phylogeny based on morphological data, forming a well supported lineage. Morphological characters support this arrangement, *T. karkloofia* has a calciferous gland that is not stalked and specimens belonging to this species have more segments.

Morphological characters overlapped *T. howickiana* and *T. lunata* in the PCA but the molecular data separated each in its own lineage. The clitellum in *T. lunata* may reach

segment 24 whilst in *T. howickiana* extends to segment 23 (as explained in the taxonomy section of chapter 2) even though the anterior segments overlap, the posterior segments are the main difference between these species. *Tritogenia hiltonia* is also recovered as monophyletic group and is well supported

The hypothesis that *Tritogenia* is monophyletic is not supported. *Tritogenia shawi* is nested within the outgroup and *Michalakus* nests within one of the *Tritogenia* clades. *Michalakus* is monotypic and the main character, amongst others that separate this genus from *Tritogenia* is the presence of the second gizzard which appears to be an autapomorphy. According to the present taxonomy, since *M. initus* is nested within *Tritogenia* in all trees, its generic rank should be re-evaluated. More markers are necessary to properly distinguish whether *Michalakus* is indeed a distinct genus from *Tritogenia*. For example in a study by Novo et al. (2011) the molecular data revealed that the *Hormogaster* is paraphyletic. However, these authors suggested that a dense sampling effort is required to further test the relationships within the Hormogastridae. *Michalakus* initus was recently collected on the road between Pietermaritzburg and Ihlanze Game Reserve, and this is new record for the species.

Some populations (with specimens from Tweedie, Curry‟s Post, Mawela GR, Balgowan and Blackridge/Edendale) did not fit any known species and could thus represent undescribed species. However, there are not enough morphological differences, to describe them as new distinct species and they could represent distinct populations rather than distinct species. The observed branching and poor support for some lineages is common in earthworm phylogenetics, Chang and James (2011) discussed the challenge of achieving reasonable phylogenetic resolution in earthworm molecular phylogenetics and that morphological change may proceed slowly unlike genetic change. Only mitochondrial genes were used in this study and these genes are said to be inherited only through the maternal lineage, therefore only a limited part of the evolutionary history is revealed (Dasmahapatra & Mallet, 2006). Therefore, inclusion of nuclear markers may be an essential necessity in further studies of phylogenetic relationships within Tritogeniidae.

Tritogenia soleata was proposed to be a synonym of *T. shawi* in chapter 2 but unfortunately the type locality for *T. soleata* does not exist anymore and this could not be tested through molecular data.

The isolation by distance test revealed a weak correlation between genetic and geographic distances in the *Tritogenia* species from Midlands. The known distribution of *Tritogenia* and *Michalakus* species in the Midlands shows localised endemism; this may be because earthworms are known to have low potential to disperse (Fernández et al. 2013). *Tritogenia* species in general are restricted to the north–eastern part of South Africa. This may be due to soil properties or geology but more study on this is needed. A study by Novo et al. (2011) in the Mediterranean region revealed a highly isolated earthworm species and they could not establish whether this distribution is ancient or is a result of recent extinction; this is also true for the Midlands *Tritogenia* because competition with other species may cause a non-uniform distribution in a population. A closer look into vegetation and soil type and properties may bring some light into the distribution of this group. According to Lavelle (1988) and Salome *et al.* (2011), food supply, soil texture, vegetation type and pH values play a major role in governing the earthworm densities. The quality and amount of above and below ground litter produced by plants influence earthworm populations (Campana *et al.* 2002; Whalen 2004; Nxele 2012) and this may be observed in some forests and native grasslands.

From the ten species of *Tritogenia* in the Midlands, seven were confirmed by the molecular data (T. annetteae, T. debbieae, T. hiltonia, T. howickiana, T. karkloofia, T. lunata and T. shawi). The status of *Tritogenia sulcata* and *T. mucosa* remains to be confirmed as no specimens were available for molecular analysis. The data suggests that the taxonomic rank of *Michalakus initus* should be revised as the species currently nests within *Tritogenia*.

Chapter Four

Morphological characters useful for phylogeny reconstruction

Abstract

Phylogenies of earthworms based on characters derived from morphological data have been controversial. Here we look at which morphological characters are useful in phylogeny reconstruction. Molecular data was added to the morphological data to create a phylogeny of seven *Tritogenia* and one *Michalakus* species that are found in the KwaZulu-Natal Midlands. Ancestral character state reconstruction was used to infer the evolution of morphological characters. The combined morphological and molecular data phylogeny was not well supported and this may be due to the limited resolving power of the morphological data. Mapping of the morphological characters onto the molecular phylogeny showed that seven morphological characters are good for phylogeny reconstruction.

4.1 Introduction

As the field of genomics is increasing, so more molecular characters are being used to answer phylogenetic questions (Wheeler et al. 2006; Wiens 2004). Some authors (for example Baker & Gatesy 2002; Scotland et al. 2003) believe that the role of morphological characters in phylogeny reconstruction is diminishing as more molecular data becomes available. Wiens (2004), however, argued that although there are many advantages of molecular data the necessity to continue to collect morphological data for phylogenetic analysis is vital but methods for morphology-based phylogenies need to be improved. A case in point, the phylogenetic relationships of fossil taxa as well as their connection to the living taxa needs to be resolved. For this reason morphological data need to be collected (Jenner 2004; Novacek 1992), as the Tree of Life cannot be reconstructed without the fossil taxa (Novick et al. 2010; Wiley 2010; Wiens 2004). Some earthworm species are known from only one specimen fixed in formalin, as such, obtaining molecular data may be difficult, thus morphological data still has an important role in phylogenetics. According to Ortiz et al. (2007) the comparison of

both morphology and molecular phylogenies provide the most robust estimation of phylogeny.

Scotland et al. (2003) highlighted that the differences between morphological and molecular data are the number of potentially unambiguous characters available, speed of character discovery and the suitability of characters for analysis. Hillis (1987) agreed that what makes molecular data more reliable and accurate in phylogeny reconstruction is the increased number of characters. However, the number of characters needed for accurate phylogeny reconstruction is difficult to estimate (Hillis 1996). Increasing the number of characters generally increases accuracy (Huelsenbeck & Hillis 1993; Charleston et al. 1994; Rosenberg & Kumar 2001), but the addition of some character sets does not lead to improvement. Support measures such as bootstrap values have been used in most phylogenetic studies (Richard et al. 2010; King et al. 2008; Rougerie et al. 2009; Nürk et al. 2013) and this increases the reliability and confidence in the trees. Although molecular data have many advantages, morphological data are particularly useful not only in describing species but also in ecology, behaviour and physiology studies (Maddison 1996). Morphology data also allows the reconstruction of ancestral states to understand patterns of morphological evolution. However, in earthworms some characters change with developmental stages and homoplasy in many characters is high, reflecting high levels of phenotypic plasticity (Decaëns et al. 2013; Chapter 2). Deciding which morphological characters are phylogenetically useful is challenging.

The aim of this chapter was to evaluate which morphological characters are useful in phylogeny reconstruction by tracing morphological traits onto the molecular phylogeny. The objectives were (1) to construct a phylogeny by using morphology and molecular data in one supermatrix analysis and (2) to superimposing morphological onto molecular phylogeny and measure the amount of homoplasy for each character to determine which traits are tracing the evolutionary history of the group.

4.2 Materials and methods

In this chapter morphological character data matrix as well as molecular sequences data matrix were combined for eight species (*T. annetteae, T. debbieae, T. hiltonia, T. howickiana,*

T. karkloofia, T. lunata, T. shawi, M. initus). *Tritogenia mucosa* and *T. sulcata* were not included because their molecular data are not available. Maximum likelihood and Bayesian analyses were performed as outlined in Chapter 3 using a partitioned analysis with GTR model for nucleotide data and Mkv model for morphological data.

Parsimony Ancestral States Reconstruction as implemented in Mesquite version 2.75 (Maddison & Maddison 2008) was used to trace the selected fifteen informative morphological characters scored for *Tritogenia* and *Michalakus* (Chapter 2) onto the molecular phylogeny. Traits for taxa represented in the phylogeny were scored using information based on observations and literature (mainly Michaelsen 1900; Plisko 1992, 1997; see Chapter 2). These morphological characters were superimposed onto the most likely molecular tree (ML). Characters were treated as unordered and reconstructed onto the tree inferred from the combined (COI & 16S rDNA) molecular data. The consistency index (CI) was then calculated for each character in Mesquite. The CI measures the amount of homoplasy with CI=1 if there is no homoplasy.

4.3 Results

The tree of combined molecular and morphological data was overall poorly supported with only two well-supported branches (Figure 8). The first well supported branch is the one that seperates *M. initus* (90% bootstrap and 1.0 posterior probability values) from *T. karkloofia, T. debbieae, T. howickiana, T. lunata, T. hiltonia* and *T. annetteae.* This first branch has characters 3 (number of gizzards) and 4 (position of calciferous glands) supporting the association. The second branch is the one that seperates *T. debbieae* (80% bootstrap and 0.99 posterior probability values) from *T. howickiana, T. lunata, T. hiltonia* and *T. annetteae*. This second branch has character 6 (fusion of calciferous glands) supporting the association. One of the reasons for the lack of resolution in the Supermatrix tree could be that the morphological characters are contributing large amounts of homoplasy. Only seven morphological characters (of the 15 characters examined) had a CI = 1. Morphological characters that were scored in *Tritogenia* and *Michalakus* can be divided into two classes of characters depending on phylogenetic informativeness according to Figure 9. The first group includes characters that are useful for phylogenetic analysis, these characters have a CI = 1 which suggests that they are useful for phylogenetic analysis. These include the number of

gizzards, position of calciferous glands, calciferous glands stalking, calciferous glands fused/not, commencement of intestine, commencement of typhlosole and size of seminal vesicles.

The second class of characters includes characters that all had CI values close to zero. These characters does not provide information for phylogenetic analysis and include septa thickness (CI = 0), muscularity of septa (CI = 0.3), distance between calciferous glands (CI = 0.5), shape of spermathecae (CI = 0), position of spermathecae (CI = 0.3), number of spermathecae (CI = 0.5), body length (CI = 0.3) and number of body segments (CI = 0.3). Characters 1: thickness of septa and 11: shape of spermathecae; seem to be autapormorphic. Although these characters are diagnostic for species they provide no information on the evolutionary relationships among species. This analysis highlights characters that should be used with caution when constructing phylogeny.

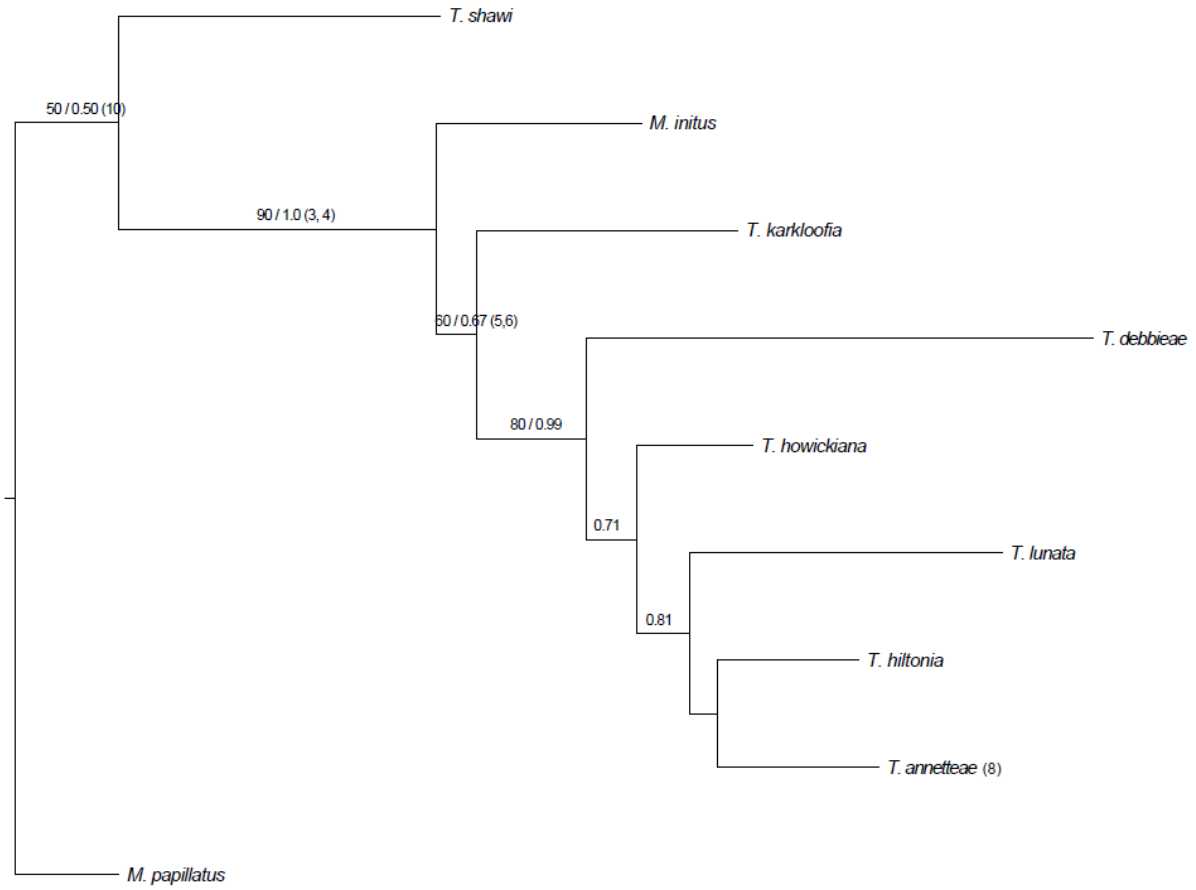

Fig. 8. Combined morphology and molecular data phylogram with *M. initus* nested within *Tritogenia* species. The monophyly of *Tritogenia* is not supported. Values annotated onto the branches indicate maximum likelihood bootstrap support values followed by posterior probabilities support values. Only support values above 50% of bootstrap and 0.50 posterior probabilities are shown on the tree. Morphological characters which had CI = 1 are plotted in brackets.

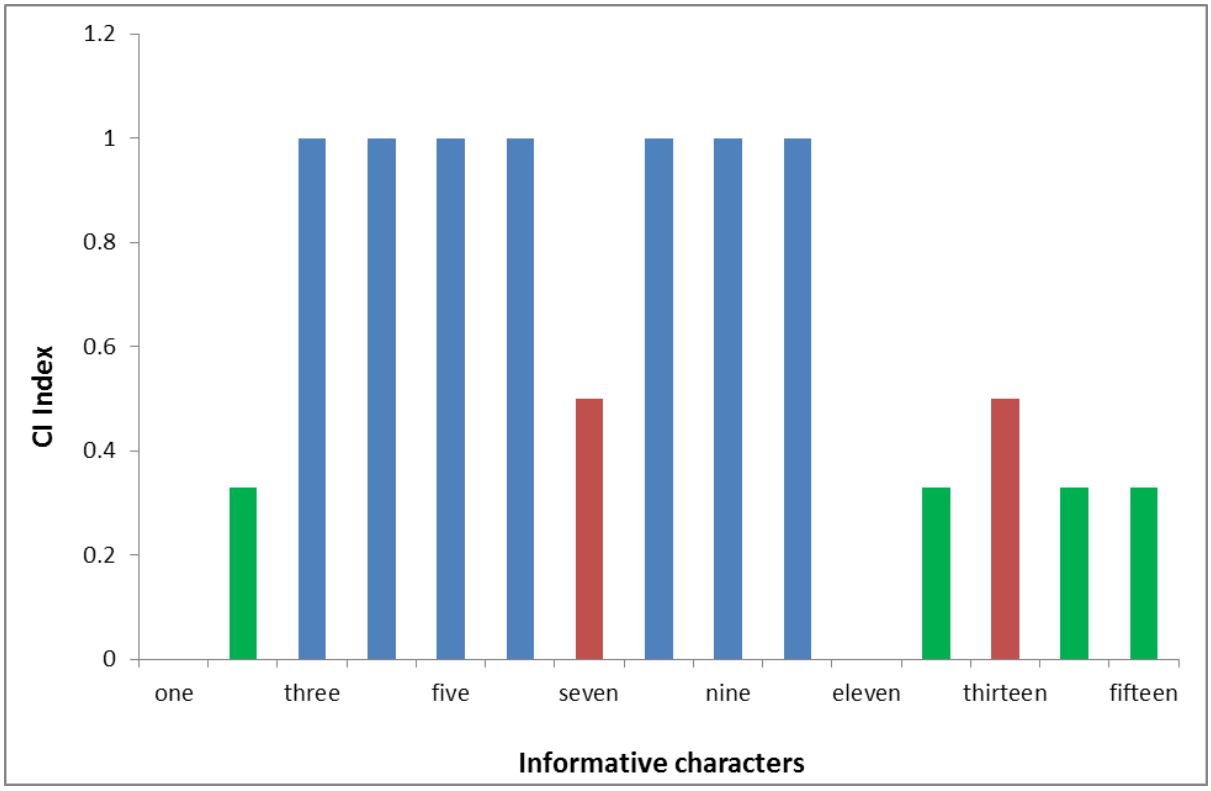

Fig. 9. Informative morphological characters with seven characters that are useful in phylogenetic analysis of *Tritogenia* and *Michalakus* species, the remaining eight characters are not informative for phylogenetic analyses. The characters used are the same as those in Table 2. Characters are coloured according to the CI values: in blue have CI = 1, brown have CI = 0.5, green have CI = 0.3.

4.4 Discussion

Phylogenetic trees that depict evolutionary relationships among a set of taxa are a powerful predictive tool and can be used to describe and understand character evolution (Wiley 2010). The phylogeny based on combined morphological and molecular data was not well supported, with many branches with support of less than 50% bootstrap values (Figure 8). This is in contrast to what other authors (for example Kupriyanova et al. 2006; Meier & Wiegmann 2002) observed where the combined morphological and molecular data trees have relatively high support. The lack of support in our study may be due to the morphological characters having limited power to resolve relationships (53% of characters had a CI < 0.5).

Distance between calciferous glands, position of spermathecae, number and shape of spermathecae, septa thickness, body length and number of body segments are plesiomorphic in *Tritogenia* and *Michalakus* species as shown by Figure 9. These characters provide less evidence of relationships in the phylogeny as they have low CI values. From the eight characters with CI < 0.5, six of them (1: thickness of septa, 7: distance between calciferous glands, 12: position of spermathecae, 13: number of spermathecae, 14: body length and 15: number of body segments) were also found to be less useful in the separation of species in chapter 2. Ortiz et al. (2007) conducted a similar study in plants where they looked at implications for morphological diversification and found that within a moonseed family few characters had unambiguous changes while others were clearly ambiguous.

Characters 1: thickness of septa and 11: shape of spermathecae; seem to be autapomorphies as only *T. debbieae* has thin septa and very large ball like spermathecae. Although these characters are diagnostic for *T. debbieae* they provide no information on the evolutionary relationships among species; these characters in this species might have occurred by reversal. The thickness of septa may be an adaptive character in *Tritogenia* and *Michalakus*, the thickened septa in the anterior part of these organisms may be an adaptation for burrowing through hard soils and this may explain why this character had CI = 0, it gives no information on relatedness. Studies have shown that ecological adaptations to similar habitats and diet may produce the same character (Koepfli et al. 2007; Hoffmann 1988; Kays 2000).

The body length and number of body segments overlap in the Midlands species (see taxonomy section) and this may explain why they are not good characters (CI = 0.3) for construction of the phylogeny, although may be good in individual species.

The position of the gizzard has been one of the historically important characters separating the *Tritogenia* and *Michalakus* species from other genera of the Microchaetidae (Kinbeg 1867; Michaelsen 1913; Plisko 1992, 1996, 1997, 2003). When Tritogeniidae was established by Plisko (2013), the gizzard remained key character in separating the two genera. Unlike *Tritogenia*, *Michalakus* has two gizzards in two different segments and this character should be used in the construction of phylogeny as it shows no homoplasy (CI = 1). The calciferous glands in the Tritogeniidae are in different positions compared to other South African oligochaetes, in *M. initus, T. karkloofia* and *T. shawi* the glands are fused dorsally forming a horseshoe shape (Plisko & Zicsi 1991; Plisko 1996). This trait is informative and has been

used in oligochaetes species diagnosis; in this study this character appeared as a good character for phylogeny construction. The start of intestine as well as its typhlosole is distinct in different families and in the ancestral state reconstruction these characters showed to be important in phylogeny analysis.

To conclude, some of the characters that had CI =1 in this chapter were also independent and good diagnostic characters in chapter 2. A look into all species of *Tritogenia* might give a better conclusion as to whether some of these characters are convergences or not. In this study (chapter 2 and 4) there was no evidence that the reproductive characters are the most valuable as some of the characters were not independent some with low CI values. An addition of more characters, both external and internal, might also give a better understanding of the species. However, from the currently analysed characters we have an idea of which characters should be used with caution when reconstructing phylogeny of *Tritogenia* and *Michalakus* species.

Chapter Five

Conclusions

Dobzhansky (1973) once said there is no logic in biology except in the light of evolution. Therefore all similarities and differences among organisms are the result of cladogenesis (lineage splitting) and anagenesis (character change), and phylogenetic trees should be useful to many users (Wiley 2010). In this study the phylogeny inferred from morphology is not well resolved but that inferred from molecular data (Figures 4; 5) has well lineages. The molecular analysis had good support especially the combined analyses of COI and 16S rDNA had stable nodes (Figure 7).

Thorough comparative morphological observations of fresh *Tritogenia* material revealed a synonym, *T. soleata =T. shawi*, and revealed high localised endemism in this group. *Tritogenia shawi* does not cluster with other *Tritogenia* species but nests with the outgroup species. Erecting a new genus for this species at this point is unwarranted until the taxonomy of all species of Tritogeniidae can be critically revised using DNA sequence data.

Although future studies would benefit from the addition of other indigenous earthworm representatives, and from the analysis of other genes, the phylogenetic analysis of COI and 16S rDNA recovered several well supported phylogenetic relationships, some of which were congruent with existing classification. *Tritogenia* is non-monophyletic and this was recovered by the morphological and molecular data. Although *Michalakus* nests within *Tritogenia*, more markers are required to resolve the relationship between the two genera before any taxonomic changes are proposed. Even though the mitochondrial and morphological data is a strong data set, the combination of a nuclear and mitochondrial genes as well as the morphology data-sets may provide a better conclusion.

Molecular data demonstrated that *T. howickiana* might consist of two independently evolving lineages, one lineage may belong to *T. sulcata* but more geographic sampling is needed to make a final conclusion. Some undescribed specimens did not fit any described species suggesting that they may be new species. However there are no morphological differences among these species, describing these species at this stage may be premature. A nuclear marker needs to be used to further assess these species. The phylogeny based on combined

morphological and molecular data was not well supported and this may be due to the limited resolving power of morphological characters. Ancestral character state reconstruction revealed seven morphological characters that are good for constructing phylogeny.

Nuclear and mitochondrial markers combined with morphological characters may provide a better understanding when all species of Tritogenia are used. Most work around the world has put more emphasis on plants and vertebrates and less focus on invertebrates (Hamer 2010). There is now a growing awareness of the importance of including invertebrate surveys and taxonomic descriptions when considering conservation priorities (Essl et al 2013).
Information is needed to assist in understanding the abiotic conditions that determine the spatial distribution of earthworms at the local scale and an assessment of soil quality that includes biological, chemical and physical properties can provide valuable information for evaluation of the sustainability of land management practices (Doran & Parkin 1994).

Future research could include:

1. An inclusion of nuclear markers may be essential for further testing the relationships of species in Tritogeniidae.

2. Create a phylogeny of all Tritogenidae species and more sampling of closely related families to see the relationships in the indigenous megadrile of South Africa.

3. Soil analysis could explain the pattern of distribution of these species.

REFERENCES

AKAIKE, H. 1973. Information theory and an extention of the maximum likelihood principle. In: Petrov, B.N., Csaki, F., (Eds), Socond International Symposium on Information Theory. Akademiai Kiado, Budapest (Hungary): 267–281.

ALTSCHUL, S.F., MADDEN, T.L., SCHAFFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W. & LIPMAN, D.J. 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programmes. *Nucleic Acids Research* **25**: 3389–3402.

ARDESTANI, M.M., VAN STRAALEN, N.M. & VAN GESTEL C.A.M. 2014. Uptake and elimination kinetics of metals in soil invertebrates: a review. *Environmental Pollution* **193**: 277–295.

BAKER, R. H., & GATESY, J. 2002. Is morphology still relevant? In: DeSalle, R. Giribet, G. & Wheeler, W., (Eds). *Molecular systematics and evolution: theory and practice*. Birkhäuser Verlag, Basel, Switzerland, 163–174.

BANTAOWONG, U., CHANABUN, R., TONGKERD, P., SUTCHARIT, C., JAMES, S.W. & PANHA, S. 2011. New earthworm species of the genus Amynthas Kinberg, 1867 from Thailand (Clitellata, Oligochaeta, Megascolecidae). *Zookeys* **90**: 35–62.

BARNES, R.D. 1974. Invertebrates Zoology, third edition. W.B. Saunders Company

BARRIOS, E. 2007. Soil biota ecosystem services and land produc-tivity. *Ecological Economics* **64**: 269–285.

BEDDARD, F.E. 1907. On two new species of the African genus (*Microchaetus*) belonging to the collection of Oligochaeta in the Museum of Christiania. *Proceedings of the Zoological Society of London* **77**: 277–281.

BELL, K.L., YEATES, D.K., MORITZ, C. & MONTEITH, G.B. 2004. Molecular phylogeny and biogeography of the dung beetle genus *Temnoplectron* Westwood (Scarabaeidae: Scarabaeinae) from Australia‟s wet tropics. *Molecular Phylogenetics and Evolution* **31**: 741–753.

BINET, F., HALLAIRE, V. & CURMI, P. 1997. Agricultural practices and the spatial distribution of earthworms in maize fields. Relationships between earthworm abundance, maize plants and soil compaction Soil Bid. *Biochemistry* **29** (3/4): 511–583.

BLAKEMORE, R.J., KUPRIYANOVA, E.K. & GRYGIER, M.J. 2010. Neotypification of *Drawida hattamimizu* Hatai, 1930 (Annelida, Oligochaeta, Megadrili, Moniligastridae) as a model linking mtDNA (COI) sequences to an earthworm type, with a response to the „Can of Worms" theory of cryptic species. *ZooKeys* **41**: 1–29.

BLAKEMORE, R. 2013. Earthworms newly from Mongolia (Oligochaeta, Lumbricidae, Eisenia). *Zookeys* **285**: 1–21.

BORISENKO, A.V., LIM, B.K., IVANOVA, N.V., HANNER, R.H. & HEBERT, P.D.N. 2008. DNA barcoding in surveys of small mammal communities: a field study in Suriname. *Molecular Ecology Resources* **8**: 471–479.

BOUCHÉ, M.B. 1972. *Lombriciens de France: écologie et systématique*. Paris: Institut National des Recherche Agronomique.

BRIONES, I.M.J., MORÁN, P. & POSADA, D. 2009. Are the sexual, somatic and genetic characters enough to solve nomenclatural problems in lumbricid taxonomy? *Soil Biology and Biochemistry* **41**: 2257–2271.

BRUSSAARD, L., DE RUITER, P.C. & BROWN, G.G. 2007. Soil biodiversity for agricultural sustainability. *Agriculture, Ecosystem & Environment* **121** (3): 233–244.

BURTELOW, A.E., BOHLEN, P.J. & GROFFMAN, P.M. 1998. Influence of exotic earthworm invasion on soil organic matter, microbial biomass and dinitrification potential in forest soils of the northeastern United States. *Applied Soil Ecology* **9**: 197–202.

CALLAHAM, M.A., HENDRIX, P.F. & PHILLIPS, R.J. 2003. Occurrence of an exotic earthworm (*Amynthas agrestis*) in undisturbed soils of the southern Appalachian Mountains, USA. *Pedobiologia* **47**: 466–470.

CAMPANA, C., GAUVIN, S. & PONGE, J.F. 2002. Influence of ground cover on earthworm communities in an unmanaged beech forest: linear gradient studies. *European Journal of Soil Biology* **38**: 213–224.

CHAN, K.Y., 2001. An overview of some tillage impacts on earthworm population abundance and diversity-implications for functioning in soils. *Soil and Tillage Research* **57**: 179–191.

CHANG, C.H. & CHEN, J.H. 2005. Taxonomic status and intraspecific phylogeography of two sibling species of *Metaphire* (Oligochaeta: Megascolecidae) in Taiwan. *Pedobiologia* **49**: 591–600.

CHANG, C., LIN, Y., CHEN, I., CHUANG, S. & CHEN, J. 2007. Taxonomic re-evaluation of the Taiwanese montane earthworm *Amynthas wulinensis* Tsai, Shen &Tsai, 2001 (Oligochaeta: Megascolecidae): polytypic species or species complex? *Organisms Diversity & Evolution* **7**: 231–240.

CHANG, C.H, ROUGERIE, R. & CHEN, J.H. 2009. Identifying earthworms through DNA barcodes: pitfalls and promise. *Pedobiologia* **52**: 171–180.

CHANG, C. & JAMES, S. 2011. A critique of earthworm molecular phylogenetics. *Pedobiologia* **54**S: (S3-S9).

CHARLESTON, M. A., HENDY, M. D. & PENNY, D. 1994. The effects of sequence length, tree topology, and number of taxa on the perfor- mance of phylogenetic methods. *Journal of Comparative Biology* **1**: 133–151.

CORTET, J., GOMOT-DE VAUFLERY, A., POINSOT-BALAGUER, N., GOMOT, L., TEXIER, C. & CLUZEAU, D. 1999. The use of invertebrate soil fauna in monitoring pollutant effects. *European Journal of Soil Biology* **35** (3): 115–134.

CROUCH, N.R. & SMITH, G.F. 2011. Informing and influencing the interface between biodiversity science and biodiversity policy in South Africa. *Botanical Journal of the Linnaean Society* **166**: 301–309.

CSUZDI, C. 2010. A monograph of the Paleotropical Benhamiinae earthworms. (Annelida: Oligochaeta, Acanthodrilidae). *In*: Csudi Cs.& Mahunka S. (Eds.), *Pedozoologica Hungarica,* Taxonomic, zoogeographic and faunistic studies on soil animals, No 6. Budapest: Hungarian Natural History Museum.

CSUZDI, C. & ZICSI, A. 2003. Earthworms of Hungary (Annelida: Oligochaeta, Lumbricidae). *In*: Csudi Cs. & Mahunka S. (Eds.), *Pedozoologica Hungarica*, Taxonomic, zoogeographic and faunistic studies on soil animals, No 1. - Budapest: Hungarian Natural History Museum.

DARRIBA, D., TABOADA, G.L., DOALLO, R. & POSADA, D. 2012. jModeltest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**: 772.

DARWIN, C. R. 1882. The formation of vegetable mould, through the action of worms with observations of their habits. London: John Murray.

DASMAHAPATRA, K.K. & MALLET, J. 2006 DNA barcodes: recent successes and future prospects. *Heredity* **97**: 254–255.

DECAËNS, T. & ROSSI, J.P. 2001. Spatiotemporal structure of an earthworm community and soil heterogeneity in a tropical pasture. *Ecography* **24**: 671–682.

DECAËNS, T., JIMÉNEZ, J. J., GIOIA, C., MEASEY, G. J. & LAVELLE, P. 2006. The values of soil animals for conservation biology. *European Journal of Soil Biology* S23–S38.

DECAËNS, T., PORCO, D., ROUGERIE R., BROWN, G.G. & JAMES, S.W. 2013. Potential of DNA barcoding for earthworm research in taxonomy and ecology. *Applied Soil Ecology* **65**: 35–42.

DEWAARD, J.R., HEBERT, P.D.N. & HUMBLE, L.M. 2011. A Comprehensive DNA Barcode Library for the Looper Moths (Lepidoptera: Geometridae) of British Columbia, Canada. *PLoS ONE* **6**: e18290.

DOBZHANSKY, T. 1973. "Nothing in Biology Makes Any Sense Except in the Light of Evolution." *American Biology Teacher* **35**: 125–29.

DORAN, J.W. & Parkin, T.B. 1994. Defining and assessing soil quality. In: J.W. Doran et al., (ed.) *Defining Soil Quality for a Sustainable Environment*, pp 3-21, SSSA Spec. Publ. No. 35, Soil Sci. Soc. Am., Inc. and Am. Soc. Agron., Inc., Madison, WI.

DRUMMOND, A.J. & RAMBAUT, A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC. *Evolutionary Biology* **7**: 214. Also available from http://beast.bio.ed.ac.uk/Tracer

EDWARDS, C.A. 2004. The importance of earthworms as key representatives of soil fauna. In: Edwards, C.A. (Ed.), Earthworm Ecology, 2nd ed. CRC Press, Boca Raton, pp. 3–11.

EERNISSE, D.J. & KLUGE, A.G. 1993. Taxonomic congruence versus total evidence and amniote phylogeny inferred from fossils, molecules and morphology. *Molecular Biology and Evolution* **10**: 1170–1195.

ESSL, F., MOSER, D., DIRNBÖCK, T., DULLINGER, S., MILASOWSZKY, N., WINTER, M. & RABITSCH, W. 2013. Native, alien, endemic, threatened and extinct species diversity in European countries. *Biological Conservation* **164**: 90–97.

FELSENSTEIN, J. 2005. PHYLIP (Phylogeny Inference Package) version 3.6. *Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.*

FERNÁNDEZ, R., ALMODÓVAR, A., NOVO, M., SIMANCAS, B. & COSÍN, D.J.D. 2012. Adding complexity to the complex: New insights into the phylogeny, diversification and origin of parthenogenesis in the Aporrectodea caliginosa species complex (Oligochaeta, Lumbricidae). *Molecular Phylogenetics and Evolution* **64**: 368–79.

FERNÁNDEZ, R., ALMODÓVAR, A., NOVO, M., GUTIÉRREZ, M. & COSÍN, D.J.D. 2013. Earthworms, good indicators for palaeogeographical studies? Testing the genetic structure and demographic history in the peregrine earthworm *Aporrectodea trapezoids* (Duges, 1828) in southern Europe. *Soil Biology & Biochemistry* **58**: 127–135.

FITCH, W.M. & SMITH, T.F. 1983. Optimal sequence alignments. *Procceedings of Natural Academy of Science USA* **80**: 1382–1386.

FOLMER, O., BLACK, M., HOEH, W., LUTZ, R. & VRIJENHOEK, R. 1994. DNA Primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294–299.

FOREY, P.L., HUMPHRIES, C.J. & VANE-WRIGHT, R.I. 1994. Systematics and conservation evaluation, Clarendon Press Oxford.

GILBERT, K.J., FAHEY, T.J., MAERZ, J.C., SHERMAN, R.E., BOHLEN, P., DOMBROSKIE, J.J., GROFFMAN, P.M. & YAVITT, J.B. 2014. Exploring carbon flow through the root channel in a temperate forest soil food web. *Soil Biology & Biochemistry* **76**: 45–52.

HALL, T.A. 1998. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acid Symposium* Ser., **41**: 95–98.

HAMER, M. 1999. An illustrated key to the spirostreptidian (Diplopoda: Spirostreptida) genera of Southern Africa. *Annals of the Natal Museum* **40**: 1–22.

HAMER, M. 2000. Review of the millipede genus *Doratogonus*, with description of fifteen new species from Southern Africa (Diplopoda, Spirostreptida, Spirostreptidae). *Annals of the Natal Museum* **41**: 1–76.

HAMER, M. 2010. African Invertebrates in the International Year of Biodiversity. *African Invertebrates* **51**: 223-230.

HAMER, M. & SLOTOW, R. 2000. Patterns of distribution and speciation in the genus *Doratogonus* (Diplopoda: Spirostreptidae). In: Wytwer, J. & Golovatch, S. (Eds). *Progress in studies on Myriapoda and Onchophora. Fragmenta Faunistica* **43** (Supplement): 295–311.

HAMER, M. & SLOTOW, R. 2002. Conservation application of existing data for South African millipedes (Diplopoda). *African Entomology* **10** (1): 29–42.

HAMMER, Ø., HARPER, D.A.T. & RYAN, P.D. 2001. PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4** (1): 9pp. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

HAYNES, R.J., DOMINY, C.S. & GRAHAM, M.H. 2003. Effect of agricultural land use on soil organic matter status and the composition of earthworm communities in KwaZulu_Natal, South Africa. *Agriculture, Ecosystems and Environment* **95**: 453–464.

HEBERT, P.D.N., CYWINSKA, A., BALL, S.L. & deWAARD, J.R. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B-Biological Sciences* **270**: 313–321.

HEBERT, P.D.N., PENTON, E.H., BURNS, J.M., JANZEN, D.H. & HALLWACHS, W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *PNAS* **101**: 14812-14817.

HENDRIX, P. F., CALLAHAM, M.A., DRAKE, J.M., HUANG, C., JAMES, S.W., SNYDER, B.A. & ZHANG, W. 2008. Pandora‟s Box contained bait: The global problem of introduced earthworms. *The Annual Review of Ecology, Evolution and Systematics*. **39**: 593–613.

HIJMANS, R.J., GUARINO, L. & MATHUR, P. 2012. DIVA-GIS. Version 7.5.

HILLIS, D. M. 1996. Inferring complex phylogenies. *Nature* **383**:140–141.

HILLIS, D. M. 1987. Molecular versus morphological approaches to systematics. Annual *Review of Ecology & Systematics* **18**: 23–42.

HILLIS, D.M. & BULL, J.J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42** (2): 182–192.

HUANG, J., XU, Q., SUN, Z.J., TANG, G.L. & SU, Z.U. 2007. Identifying earthworms through DNA barcodes. *Pedobiologia* **51**: 301–309.

HUBER, B.A. & RHEIMS, C.A. 2011. Diversity and endemism of pholcid spiders in Brazil's Atlantic Forest, with descriptions of four new species of the Atlantic Forest endemic genus *Tupigea* (Araneae: Pholcidae). *Journal of Natural History* **45** (5-6): 275–301.

HUELSENBECK, J. P. & HILLIS, D. M. 1993. Success of phylogenetic methods in the four-taxon case. *Systematic Biology* **42**: 247–264.

HUELSENBECK, J.P. & RONQUIST, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.

IHEANACHO, H.E., NJOBEH, P.B., DUTTON, F.M., STEENKAMP, P.A., STEENKAMP, L., MTHOMBENI, J.Q., DARU, B.H. & MAKUN, A.H. 2014. Morphological and molecular identification of filamentous *Aspergillus flavus* and *Aspergillus parasiticus* isolated from compound feeds in South Africa. *Food Microbiology* **44**: 180–184.

JAMES, S.W., PORCO, D., DECAËNS, T., RICHARD, B., ROUGERIE, R. & ERSEUS, C. 2010. DNA barcoding reveals cryptic diversity in Lumbricus terrestris L., 1758 (Clitellata): resurrection of L. herculeus (Savigny, 1826). *PLoS ONE* **5:** (12) e15629.

JAMES, S.W. & DAVIDSON, S.K. 2012. Molecular phylogeny of earthworms (Annelida: Crassiclitellata) based on 28S, 18S and 16S gene sequences. *Invertebrate Systematics* **26**: 213–229.

JANZEN, D.H., HAJIBABAEI, M., BURNS, J.M., HALLWACHS, W., REMIGIO, E. & HEBERT, P.D.N. 2005. Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. *Philosophical Transactions of the Royal Society Biological Sciences* **360**: 1835–1845.

JENNER, R. 2004. Accepting partnership by submission? Morphological phylogenetics in a molecular millennium. *Systematic Biology* **53**: 333–342.

JIMÉNEZ, J., DECAËNS, T. & ROSSI, J. 2006. Stability of the spatio-temporal distribution and niche overlap in neotropical earthworm assemblages. *Acta Oecologica* **30**: 299–311.

JOUQUET, P., DAUBER, J., LAGERLÖF, J., LAVELLE, P. & LEPAGE, M. 2006. Soil invertebrates as ecosystem engineers: intended and accidental effects on soil and feedback loops. *Applied Soil Ecology* **32**: 153–164.

KAYS, R.W. 2000. The behavior and ecology of olingos (*Bassaricyon gabbii*) and their competition with kinkajous (*Potos Xavus*) in central Panama. *Mammalia* **64**: 1–10.

KEVAN, D.K.McE. 1968. Soil animals. Northumberland Press, Gateshead on Tyne, UK.

KIBBLEWHITE, M. G., RITZ, K. & SWIFT, M. J. 2008. Soil health in agricultural systems. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363:** 685–701.

KINBERG, J.G.H. 1867. Annulata nova. *Öfversigt af Kongliga Vetenskaps-Akademiens Förhandlingar* **23**: 97–103.

KING, R.A, TIBBLE, A.L. & SYMONSON, W.O.C. 2008. Opening a can of worms: unprecedented sympatric cryptic diversity within British lumbricid earthworms. *Molecular Ecology* **17**: 4684–4698.

KOEPFLI, K., GOMPPER, M.E., EIZIRIK, E., HO, C., LINDEN, L., MALDONADO, J.E. & WAYNE, R.K. 2007. Phylogeny of the Procyonidae (Mammalia: Carnivora): Molecules, morphology and the Great American Interchange. *Molecular Phylogenetics and Evolution* **43**: 1076–1095.

KUPRIYANOVA, E.K., MACDONALD, T.A. & ROUSE, G.W. 2006. Phylogenetic relationships within Serpulidae (Sebellida, Annelida) inferred from molecular and morphological data. *Zoological Scripta* **35** (5): 421–439.

LAMANDÉ, M., HALLAIRE, V., CURMI, P., PERES, G. & CLUZEAU, D. 2003. Changes of pore mor-phology, infiltration and earthworm community in a loamy soil under different agricultural managements. *Catena* **54**: 637–649.

LARKIN, M.A., BLACKSHIELDS, G., BROWN, N.P., CHENNA, R., MCGETTIGAN, P.A., MCWILLIAM, H., VALENTIN, F., WALLACE, I.M., WILM, A., LOPEZ, R., THOMPSON, J.D., GIBSON, T.J., HIGGINS, D.G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948.

LAVELLE, P. 1988. Earthworms and the soil system. *Biology and Fertility of Soils* **6**: 237–251.

LAVELLE, P., BRUSSAARD, L. & HENDRIX, P. 1999. Earthworm management in tropical agroecosystems. CABI, Wallingford, 300 pp.

LAVELLE, P., DECAËNS, T., AUBERT, M., BAROT, S., BLOUIN, M., BUREAU, F., MARGERIE, P., MORA, P. & ROSSI, J.P. 2006. Soil invertebrates and ecosystem services. *European Journal of Soil Biology* **42**: S3–S15.

LOONGYAI, W., BANGRAK, P. & CHANTSAVANG, S. 2011. External morphological comparison, taxonomic revision and molecular differentiation of the four economically important species of earthworm in Thailand. *International Journal of Agriculture & Biology* **13**: 553–558.

MADDISON, W.P. 1996. Molecular approaches and the growth of phylogenetic biology. In Ferraris, J.D. & Palumbi, S.R. (Eds), *Molecular zoology: Advances, stratergies and protocols*. National Academy of Sciences Press, Washington DC. 47–63.

MADDISON, W.P. & MADDISON, D.R. 2008. Mesquite: a modular system for evolutionary analysis. Version 2.5 http://mesquiteproject.org.

MARTIN-BRAVO, S., ESCUDERO, M., MINGUEZ, M., JIMÉNEZ-MEJIAS, P. & LUCEÑO, M. 2013. Molecular and morphological evidence for a new species from South Africa: *Carex rainbowii* (Cyperaceae). *South African Journal of Botany* **87**: 85–91.

MEIER, R. & WIEGMANN, B. 2002. A phylogenetic analysis of Coelopidae (Diptera) based on morphological and DNA sequence data. *Molecular Phylogenetics and Evolution* **25**: 393–407.

MICHAELSEN, W. 1891. Terricolen der Berliner Zoologischen Sammlung. I. Afrika. *Archiv für Naturgeschichte* **57**: (12): 205–228.

MICHAELSEN, W. 1899a. Terricolen von verschiedenen Gebieten der Erde. Mitteilungen aus dem *Naturhistorischen Museum in Hamburg* **16**: 1–122.

MICHAELSEN, W. 1899b. Revision der Kinberg'schen Oligochaeten-Typen. *Öfversigt af Kongliga Vetenskaps-Akademiens Förhandlingar* **56**: 413–448.

MICHAELSEN, W. 1900. *Oligochaeta*. *In: Das Tierreich.* Berlin: R. Friedlander. Lief. 10: I–XXIX 1–575.

MICHAELSEN, W. 1902. Neue Oligochaeten und neue Fundorte alt-bekannter. *Mitteilungen aus dem Naturhistorischen Museum in Hamburg* **19**: 1–54.

MICHAELSEN, W. 1908. III. Annelida. A. Oligochaeten aus dem Westlichen Kapland. *In*: Schultze, L. *Zoologische und antropologische Ergebnisse e. Forschungsreise im Südafrika.* Bd 1. Lief. 2. *Denkschriften der medizinisch-naturwissenchaftlichen Gesselschaft zu Jena* **13**: 30–42.

MICHAELSEN, W. 1913. The Oligochaeta of Natal and Zululand. *Annals of the Natal Museum* **2** (4): 397–457.

MICHAELSEN, W. 1918. Die Lumbriciden, mit besonderer Berücksichtigung der bisher als Familie Glossoscolecidae zusammengefassten Unterfamilien. *Zoologische Jahrbücher, Abteilung für Systematik* **41**: 1–398.

MICHAELSEN, W. 1928. Miscellanea Oligochaetologica. *Arkiv för Zoologi* **20** A (2): 1–15.

MILLER, M.P. 2005. Alleles In Space: computer software for the joint-analysis of interindividual spatial and genetic information. *Journal of Heredity* **96**: 722–724.

MINAMIYA, Y., OHGA, K., HAYAKAWA, H., ITO, K. & FUKUDA, T. 2011a. Coelomic fluid: a noninvasive source of DNA in earthworms. *Molecular Ecology Resources* **11**: 645–49.

MINAMIYA, Y., HAYAKAWA, H., OHGA, K., SHIMANO, S., ITO, M.T. & FUKUDA T. 2011b. Variability of sexual organ possession rates and phylogenetic analyses of a parthenogenetic Japanese earthworm, *Amynthas vittatus* (Oligochaeta: Megascolecidae). *Genes, Genetics & Systematics* **86**: 27–35.

MWABVU, T., HAMER, M. & SLOTOW, R. 2007. A taxonomic revision of the southern African millipede genus, *Bicoxidens* Attems, 1928 (Diplopoda: Spirostreptida: Spirostreptidae), with the description of three new species and a tentative phylogeny. *Zootaxa* **1452**: 1–23.

MWABVU, T., HAMER, M., SLOTOW, R. & BARRACLOUGH, D. 2009. A revision of the taxonomy and distribution of *Spirostreptus* Brandt 1833 (Diplopoda, Spirostreptida, Spirostreptidae) with description of a new species and a new genus of spirostreptid millipede. *Zootaxa* **2211**: 36–56.

MWABVU, T., LAMB, J., SLOTOW, R., HAMER, M. & BARRACLOUGH, D. 2013. Is millipede taxonomy based on gonopod morphology too inclusive? Observations on genetic variation and cryptic speciation in *Bicoxidens flavicollis* (Diplopoda: Spirostreptida: Spirostreptidae). *African Invertebrates* **54** (2): 349–356.

NASKRECKI, P. 2013. Endangered Terrestrial Invertebrates. *Encyclopedia of Biodiversity (Second Edition).* Pg 219–227.

NOVACEK, M.J. 1992. Fossils as critical data for phylogeny. In: Novacek, M.J. & Wheeler, Q.D. (Eds). Extinction and Phylogeny. Columbia University Press.

NOVICK, L.R., CATLEY, K.M. & FUNK, D.J. 2010. Characters are key: the effect of synapomorphies on cladogram comprehenshion. *Evolution: Education and Outreach* **3**: 539–547.

NOVO, M., ALMODOVAR, A., FERNÁNDEZ, R., GIRIBET, G. & COSÍN, D.J.D. 2011. Understanding the biogeography of a grouo of earthworms in the Mediterranean basin-The phylogenetic puzzle of Hormogastridae (Clitellata: Oligochaeta). *Molecular Phylogenetics and Evolution* **61:** 125–135.

NÜRK, N.M, MADRINAN, S., CARINE, M.A., CHASE, M.W. & BLATTNER, F.R. 2013. Molecular phylogenetics and morphological evolution of St. John‟s wort (*Hypericum*; Hypericaceae). *Molecular Phylogenetics and Evolution* **66**: 1–16.

NXELE, T.C. 2012. The megadrile fauna (Annelida: Oligochaeta) of Queen Elizabeth Park, South Africa: species composition and distribution within different vegetation types. *African Invertebrates* **53** (2): 543–558.

NXELE, T.C. 2014. Comments on problems appearing during identification of *Tritogenia* species (Oligochaeta: Tritogeniidae). In: Pavlíček, T, Cardet, P., Almeida, M. T., Pascoal, C., Cássio F. (Eds.): Advances in Earthworm Taxonomy VI (Annelida: Oligochaeta). – Proceedings of the 6th International Oligochaete Taxonomy Meeting (6th IOTM), Palmeira de Faro, Portugal, 22- 25 April, 2013. *Zoology in the Middle East* **60** (2): 32–37.

ORTIZ, R.D.C., KELLOGG, E.A. & VAN DER WERFF, H. 2007. Molecular phylogeny of the moonseed family (Menispermaceae) : implications for morphological diversification. *American Journal of Botany* **94** (8) : 1425–1438.

PÉREZ-LOSADA, M., EIROA, J., MATO, S. & DOMÍNGUEZ. J. 2005. Phylogenetic species delimitation of the earthworm Eisenia fetida (Savigny, 1826) and Eisenia Andrei (Bouché, 1972) (Oligochaeta, Lumbricidae) based on mitochondrial and nuclear DNA genes. Pedobiologia **49**: 317–324.

PÉREZ-LOSADA, M., RICOY, M., MARSHALL, J.C. & DOMÍNGUEZ, J. 2009. Phylogenetic assessment of the earthworm Aporrectodea caliginosa species complex (Oligochaeta: Lumbricidae) based on mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution* **52**: 293–302.

PERRIER, E. 1886. Sur les generes de lombriciens terrestris de Kinberg. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences* **102**: 857–877.

PEY, B., NAHMANI, J., AUCLERC, A., CAPOWIEZ, Y., CLUZEAU, D., CORTET, J., DECAËNS, T., DEHARVENG, L., DUBS, F., JOIMEL, S., BRIARD, C., GRUMIAUX, F., LAPORTE, M., PASQUET, A., PELOSI, C., PEMIN, C., PONGE, J., SALMON, S., SANTORUFO, L. & HEDDE, M. 2014. Current use of and future needs for soil invertebrate functional traits in community ecology. *Basic and Applied Ecology* **15** (3): 194–206.

PLISKO, J.D. 1992. The Microchaetidae of Natal, with description of new species of *Microchaetus* Rapp and *Tritogenia* Kinberg, and the new genus *Proandricus* (Oligochaeta). *Annals of the Natal Museum* **33**: 337–378.

PLISKO, J.D. 1995. New data on the biosystematics and distribution of *Microchaetus natalensis* (Kinberg, 1867) in north-eastern South Africa (Oligochaeta: Microchaetidae). *Annals of the Natal Museum* **36**: 281–291.

PLISKO, J.D. 1996. *Michalakus*, a remarkable new genus of microchaetids earthworm from South Africa (Oligochaeta: Microchaetidae). *African Invertebrate* **37**: 287–293.

PLISKO, J.D. 1997. New species of the genus *Tritogenia* Kinberg, 1867 from southern Africa (Oligochaeta: Microchaetidae). *African Invertebrates* **38**: 241–281.

PLISKO, J.D. 2000. The role of Nature Reserves in the protection of the terrestrial earthworm fauna (Oligochaeta), based on the material from Dlinza Forest Nature Reserve (KwaZulu-Natal, South Africa). *Lammergeyer* **46**: 75–80.

PLISKO, J.D. 2003. Eleven new South African earthworms (Oligochaeta: Microchaetidae) with new information on some known species, and an inventory of the microchaetids of KwaZulu-Natal. *African Invertebrates* **44** (2): 279–325.

PLISKO, J.D. 2006. The Oligochaeta type material housed at the Natal Museum, South Africa. *African Invertebrates* **47**: 57–61.

PLISKO, J.D. 2007. New species of South African acanthodriline earthworms of the genera *Eodriloides* and *Chilota*, with a redescription of *Chilota quindecimus* (Oligochaeta: Acanthodrilidae). *African Invertebrates* **48**: 33-40.

PLISKO, J.D. 2008. A re-assessment of the South African *Tritogenia zuluensis* species-group, with remarks on included species (Ologochaeta: Microchaetidae). In: Pavlicek, T. & Cardet, P., eds, *Advances in earthworm taxonomy III (Annelida: Oligochaeta). Proceedings of the 3rd International Oligochaeta Taxonomy Meeting (3rd IOTM)* Platres, Cyprus, April 2nd to 6th 2007. Imprinta LTD, Nicosia, 97–107.

PLISKO, J.D. 2010. Megadrile earthworm taxa introduced to South African soils (Oligochaeta: Acanthodrilidae, Eudrilidae, Glossoscolecidae, Lumbricidae, Megascolecidae, Ocnerodrilidae). *African Invertebrates* **51** (2): 289–312.

PLISKO, J.D. 2012. Notes on the status of the family Microchaetidae (Oligochaeta). *Zoology in the Middle East* **58** (Suppl. 4): 47–58.

PLISKO, J.D. 2013. A new family Tritogeniidae for the genera *Tritogenia* and *Michalakus*, earlier accredited to the composite Microchaetidae (Annelida: Oligochaeta). *African Invertebrates* **54** (1): 69–92.

PLISKO, J.D. & ZICSI, A. 1991. Über neue *Tritogenia*-Arten aus Süd-Afrika (Oligochaeta: Microchaetidae). *Mitteilungen aus dem Naturhistorischen Museum in Hamburg* **88**: 111–123.

POP, A.A., WINK, M. & POP, V.V. 2003. Use of 18S, 16S rDNA and cytochrome c oxidase sequences in earthworm taxonomy (Oligochaeta, Lumbricidae). *Pedobiologia* **47**: 428–433.

PULLEMAN, M.M., SIX, J., UYL, A., MANNISSEN J.C.Y. & JONGMANS, A.G. 2005. Earthworms and management affect organic matter incorporation and microagrigate formation in agricultural soils. *Applied Soil Ecology* **29** (1): 1–15.

RAMBAUT, A. 2009. http://tree.bio.ed.ac.uk/software/figtree/

REYNOLDS, J. W. & COOK, D. G. 1976. *Nomenclatura Oligochaetologica. A catalogue of names, descriptions and type specimens of the Oligochaeta.* Ottawa: Runge Press.

REYNOLDS, J. W. & COOK, D. G. 1993. *Nomenclatura Oligochaetologica. A catalogue of names, descriptions and type specimens of the Oligochaeta.Suplementum tertium*. Lindsey: Blewett Printing.

RICHARD, B., DECAËNS, T., ROUGERIE, R., JAMES, S.W., PORCO, D. & HEBERT, P.D. 2010. Re-integrating earthworm juveniles into soil biodiversity studies: species identification through DNA barcoding. *Molecular Ecology Resources* **10**: 606–614.

ROSENBERG, M. S. & KUMAR, S. 2001. Incomplete taxon sampling is not a problem for phylogenetic inference. *Proceedings of the National Academy of Sciences in USA* **98**: 10751–10756.

ROSSI, J. P., HUERTA, E, FRAGOSO, C. & LAVELLE, P. 2006. Soil properties inside earthworm patches and gaps in a tropical grassland (La Mancha, Veracruz, Mexico). *European Journal of Soil Biology* **42**: S284–S288.

ROUGERIE, R., DECAËNS, T., DEHARVENG, L., PORCO, D., JAMES, S.W., CHANG, C., RICHARD, D., POTAPOV, M., SUHARDJONO, Y. & HEBERT, P.D.N. 2009. DNA barcodes for soil animal taxonomy. *Pesquisa Agropecuaria Brasileira* **44** (8): 789–802.

RUIZ, N., LAVELLE, P. & JIMÉNEZ, J. 2008. Soil macrofauna field manual, technical level. Food and Agriculture Organization of the United Nations, Rome, Italy

SALOMÉ, C., GUENAT, C., BULLINGER-WEBER, G., GOBAT, J.M. & LE BAYON, R.C. 2011. Earthworm communities in alluvial forests: influence of altitude, vegetation stages and soil parameters. *Pedobiologia* **54S**: S89–S98.

SAVIGNY, J.C. 1826. [La multiplicite des espècies de ver de tere]. *In*: Cuvier, G: Analyse des Travaux de l‟Academie royale des Sciences, pendant l‟année 1821, partie physique. *Mémoires de l'Académie des Sciences de l'Institut de France* (*Physique*)*, Paris* **5**: 176–84.

SCHOLS, P., D‟HONDT, C., GEUTEN, K., MERCKX, V., JANSSENS, S. & SMETS, E. 2004. MorphoCode: coding quantitative data for phylogenetic analysis. *Phyloinformatics* **4**: 1–4.

SCOTLAND, R.W., OLMSTEAD, R.G. & BENNET, J.R. 2003. Phylogeny reconstruction: the role of morphology. *Systematic Biology* **52**: 539–548.

Shen, H., Tsai, C., Fang, Y. & Chen, J. 2011. Parthenogenesis, polyploidy and reproductive seasonality in the Taiwanese mountain earthworm *Amynthas catenus* Tsai et al., 2001 (Oligochaeta, Megascolecidae). *Pedobiologia* **54**: 133–139.

SMITH, M.A. & FISHER, B.L. 2009. Invasions, DNA barcodes, and rapid biodiversity assessment using ants of Mauritius. *Frontiers in Zoology* **6**: doi: 10.1186/1742–9994–1186–1131.

SMITH, M.A., FISHER, B.L. & HEBERT, P.D.N. 2005. DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Philosophical Transactions of the Royal Society Biological Sciences* **360**: 1825–1834.

SMITH, M.A., WOOD, D.M., JANZEN, D.H., HALLWACHS, W. & HEBERT, P.D.N. 2007. DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 4967–4972.

STOECKLE, M.Y. & HEBERT, P.D.N. 2008. Barcode of Life: DNA tags help classify animals. *Scientific American* **298**: 39–43.

SUAREZ, A.V., BOLGER, D.T. & CASE, T.J. 1998. Effects of Fragmentation and Invasion on Native Ant Communities in Coastal Southern California. *Ecology **79***: 2041–2056.

SWIFT, M.J., HEAL, W. & ANDERSON, J.M. 1979. Decomposition in terrestrial ecosystems. University of California Press, Berkeley, USA.

SWOFFORD, D.L. 2003. PAUP* Phylogenetic Analysis Using Parsimony. Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts, USA.

TAMURA, K., PETERSON, D.N.P., STECHER, G., NEI, M. & KUMAR, S. 2011. MEGA 5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular Biology and Evolution* **28***:* 2731–2739.

TRAVERS, S., JACKMAN, T.R. & BAUER, A.M. 2014. A molecular phylogeny of Afromontane dwarf geckos (*Lygodactylus*) reveals a single radiation and increased species diversity on a South African montane center of endemism. *Molecular Phylogenetics and Evolution* **80**: 31–42.

VALENTINI, A., POMPANON, F. & TABERLET, P. 2008. DNA barcoding for ecologists. *Trends in Ecology and Evolution* **24**: 110–117.

VOHLAND, K. & HAMER, M. 2013. A review of the millipedes (Diplopoda) of Namibia, with identification keys and descriptions of two new genera and five new species. *African Invertebrates* **54** (1): 251–304.

VOUA OTOMO, P.V., VAN VUUREN, B.J. & REINECKE, S.A. 2009. Usefulness of DNA barcoding in ecotoxicological investigations: resolving taxonomic uncertainties using *Eisenia* Malm 1877 as an example. *Bulletin of Environmental Contamination and Toxicology* **82**: 261–264.

VOUA OTOMO, L., VOUA OTOMO, P., BEZUIDENHOUT, C. & MABOETA, M.S. 2013. Molecular assessment of commercial and laboratory stocks of *Eisenia* spp. (Oligochaeta: Lumbricidae) from South Africa. *African Invertebrates* **54** (2): 499–511.

WALLWORK, J.A. 1970. Ecology of soil animals. McGraw-Hill, London, UK.

WARD, R.D., ZEMLAK, T.S., INNES, B.H., LAST, P.R. & HEBERT, P.D.N. 2005. DNA barcoding Australia‟s fish species. *Philosophical Transactions of the Royal Society B-Biological Sciences* **360**: 1847–1857.

WEBB, J.M., JACOBUS, L.M., FUNK, D.H., ZHOU, X., KONDRATIEFF, B., GERACI, C.J., DeWALT, E., BAIRD, D.J., RICHARD, B. & PHILLIPS, I. 2012. A DNA Barcode Library for North American Ephemeroptera: Progress and Prospects. *PLoS ONE* **7**: e38063.

WHALEN, J.K. 2004. Spatial and temporal distribution of earthworm patches in corn field, hayfield and forest systems of southwestern Quebec, Canada. *Applied Soil Ecology* **27**: 143–151.

WHEELER, W., AAGESEN, L., ARANGO, C.P., FAIVOVICH, J., GRANT, T., D‟HAESE, C., JANIES, D., SMITH, W.L., VARON, A. & GIRIBET, G. 2006. Dynamic homology and phylogenetic systematics: A unified approach using POY. American Museum of Natural History, USA.

WILEY, E.O. 2010. Why trees are important. *Evolution: Education and Outreach* **3**: 499–505.

WILLOWS-MUNRO, S. & MATTHEE, C.A. 2009. The evolution of the southern African members of the shrew genus *Myosorex*: Understanding the origin and diversification of a morphologically cryptic group. *Molecular Phylogenetics and Evolution* **51**: 394–398.

WILLOWS-MUNRO, S. & MATTHEE, C.A. 2011. Linking lineage diversification to climate and habitat heterogeneity: phylogeography of the southern African shrew *Myosorex varius*. *Journal of Biogeography* **38**: 1976–1991.

WINSOME, T., EPSTEIN, L., HENDRIX, P.F. & HORWATH, W.R. 2006. Competitive interactions between native and exotic earthworm species as influenced by habitat quality in a California grassland. *Applied Soil Ecology* **32**: 38–53.

WIENS, J.J. 2004. The Role of Morphological Data in Phylogeny Reconstruction. *Systematic Biology* **53** (4): 653–661.

ZHOU, X., ADAMOWICZ, S.J., JACOBUS, L.M., DEWALT, E. & HEBERT, P.D.N. 2009. Towards a comprehensive barcode library for arctic life - Ephemeroptera, Plecoptera, and Trichoptera of Churchill, Manitoba, Canada. *Frontiers in Zoology* **6**.

ZHOU, X., ROBINSON, J.L., GERACI, C.J., PARKER, C.R., FLINT, O.S., ETNIER, D.A., RUITER, D., DEWALT, E., JACOBUS, L.M. & HEBERT, P.D.N. 2011. Accelerated construction of a regional DNA-barcode reference library: caddisflies (Trichoptera) in the Great Smoky Mountains National Park. *Journal of the North American Benthological Society* **30**: 131–162.

ZIDA, Z., OUÉDRAOGO, E., MANDO, A. & STROOSNIJDER, L. 2011. Termite and earthworm abundance and taxonomic richness under long-term conservation soil management in Saria, Burkina Faso, West Africa. *Applied Soil Ecology* **51**: 122–129.

ZIMKUS, B.M., RÖDEL, M. & HILLERS, A. 2010. Complex patterns of continental speciation: Molecular phylogenetics and biogeography of sub-Saharan puddle frogs (*Phrynobatrachus*). *Molecular Phylogenetics and Evolution* **55** (3): 883–900.

ZWICKL, D.J. 2011. Garli available from (http://www.nescent.org/wg/garli).

APPENDIX

Table A1. Data matrix of morphology characters used in the morphology analyses of the Midlands *Tritogenia* and *Michalakus* species

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Tritogenia annetteae	1	0	0	0	0	1	0	1	0	1	1	0	0	0	0
Tritogenia debbieae	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Tritogenia hiltonia	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0
Tritogenia howickiana	1	0	0	0	0	1	0	0	0	1	1	0	0	0	0
Tritogenia karkloofia	1	0	0	0	1	0	0	0	1	0	1	0	0	0	0
Tritogenia lunata	1	0	0	0	0	1	0	0	0	1	1	0	0	0	0
Tritogenia mucosa	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0
Tritogenia shawi	1	0	0	0	1	0	1	0	1	1	1	0	1	0	0
Tritogenia soleata	1	0	0	0	1	0	1	0	1	1	1	0	1	0	0
Tritogenia sulcata	1	0	0	0	?	1	?	0	?	?	1	0	0	0	0
Michalakus initus	1	0	1	1	1	0	1	0	1	1	1	1	1	0	0
Microchaetus papillatus	1	1	0	0	1	0	0	0	1	1	1	1	1	1	1

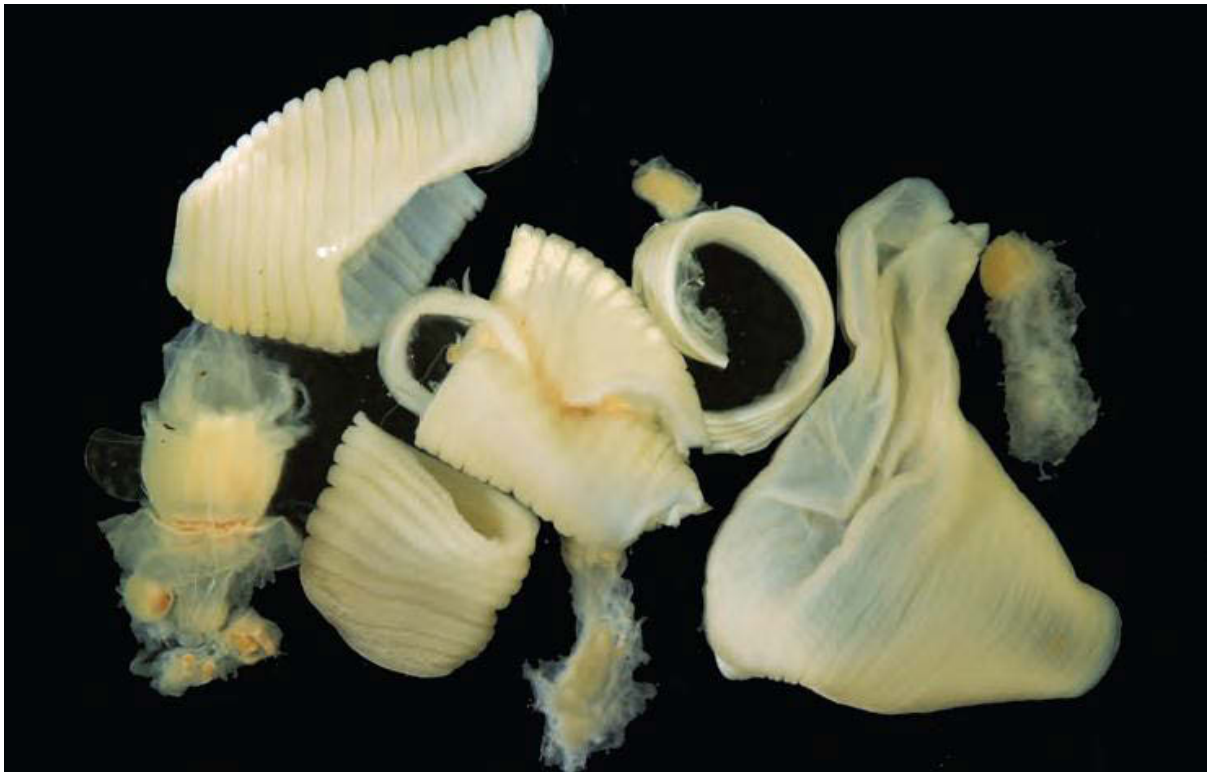

Fig. A1. *Tritogenia sulcata* Kinberg, 1867, type material fragments (NHRS 157; photo by E. Sigvaldadóttir).

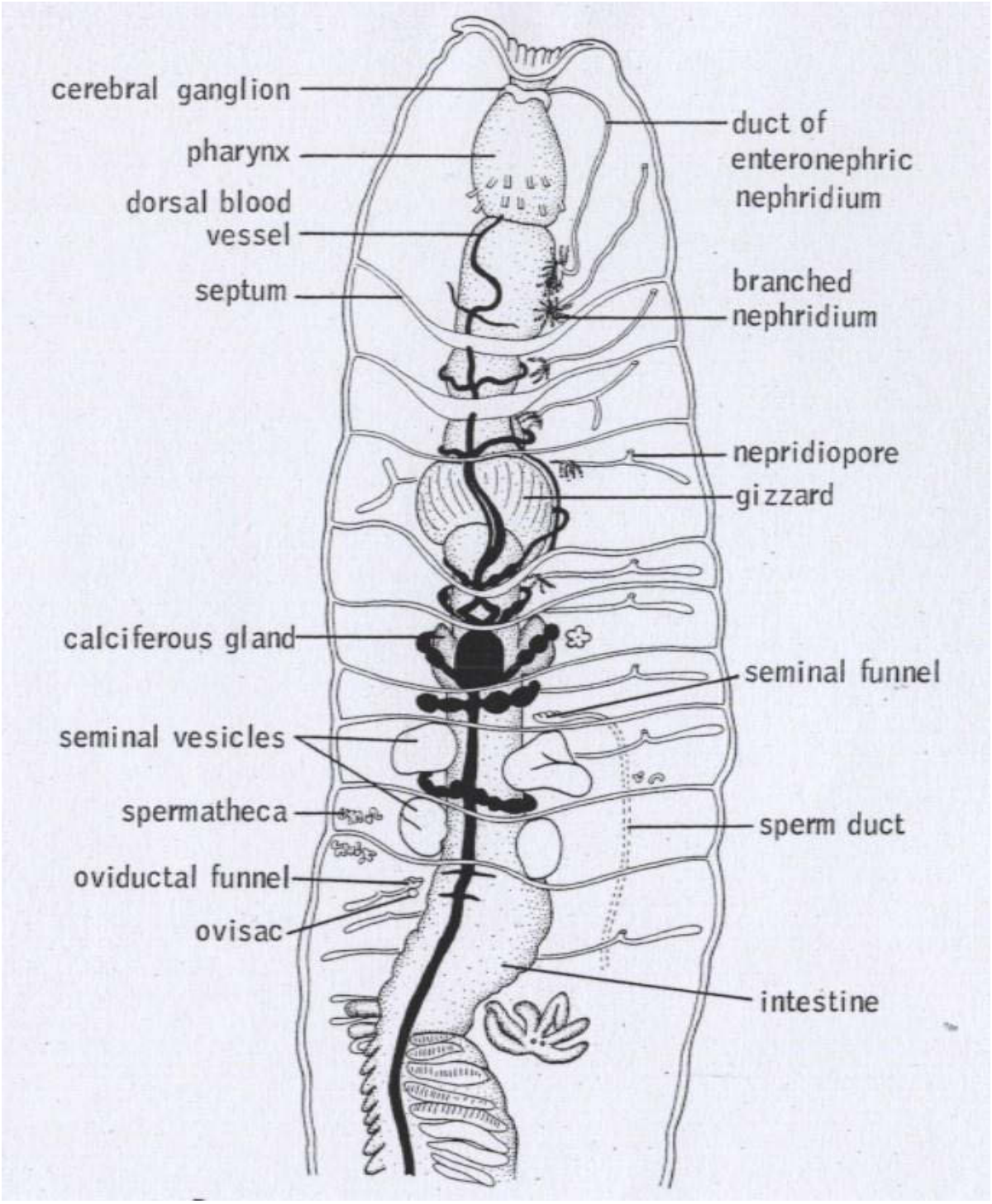

Fig. A2. Dorsal view of earthworm, *Microchaetus* showing internal characters (After Barnes 1974)

Table A2. New localities sampled in the KZN Midlands

Locality	GPS coordinates
Blackridge	29°37.146'S: 30°19.067'E
Edendale, Smero	29°37.943‘‘S: 30°17.126‘‘E
Bisley Nature Reserve	29°39‘‘27.9‘‘‘S: 30°23‘‘31.8‘‘‘E
Queen Elizabeth Park	29°34.252'S: 30°19.174'E
Hilton	29°30‘‘46.2‘‘‘S: 30°18‘‘03.5‘‘‘E
Howick	29°30.417‘‘S: 30°12.631‘‘E
Otto‘‘s Bluff	29°29‘‘09.9‘‘‘S: 30°21‘‘54.5‘‘‘E
Karkloof	29°15‘‘15.7‘‘‘S: 30°21‘‘34.7‘‘‘E
Ihlanze private Game Reserve	29°28'24.6"S: 30°20'18.0"E
Road to Ihlanze Game Reserve	
Curry‘‘s Post Area	29°25‘‘02.2‘‘‘S: 30°12‘‘07.3‘‘‘E
Tweedie	29°23‘‘54.3‘‘‘S: 30°04‘‘06.9‘‘‘E; 29°27‘‘37.1‘‘‘S: 30°10‘‘30.9‘‘‘E
Balgowan	29°21‘‘48.2‘‘‘S: 30°05‘‘57.5‘‘‘E
Wagendrift	29°02‘‘35.4‘‘‘S: 29°50‘‘20.9‘‘‘E
Mawela Game Reserve	29°04'00.08"S: 30°03'44.35"E