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Studying earthworms (Annelida: Oligochaeta) in South Africa

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ABSTRACT

Earthworms are an important component of southern African invertebrate diversity, due both to their influential roles in soil ecosystems, and the relatively large number of species. As of 2010, there were 282 indigenous earthworm species (most endemic) known to South Africa belonging to three families: Microchaetidae, Tritogeniidae and Acanthodrilidae. In addition, 44 introduced species from six families had been recorded. However, earthworms are rarely included in environmental monitoring or conservation programmes—partly because sampling and species identification are difficult and many sampling methods are destructive and/or toxic. In this paper we review the earthworm sampling techniques most commonly used by screening data from a digitised literature collection on South African earthworms and on-line global searches. By examining a case study sampling of three vegetation types, this paper highlights taxonomic challenges and the effort required to properly curate specimens. The study provides recommendations for future sampling and highlights some key priorities for future work on the group.

From the literature review in early 2012, it is clear that collection techniques are often insufficiently recorded in published work. A total of 10 938 publications from the period 1950 to 2012 were found from the literature search and digitised collection and from these only 32 papers recorded the sampling methodology (mainly hand sorting) for South African research, pointing to the need to adopt standard sampling and reporting protocols. We also tested two of the most popular methodologies in the field. Sampling was conducted in January and February 2012 at four sites, with 24 plots at each site (12 digging and 12 using mustard extraction). A total of 2 094 earthworms collected could be assigned a species name, with introduced species predominating at both disturbed and natural sites. It took a team of three to five people digging and hand collecting all earthworm specimens encountered in a plot of 50 cm × 50 cm × 20 cm deep around 45 to 60 minutes. However, much more time was spent curating and identifying samples. While we recommend following the ISO (ISO11268-3, ISO23611-1) protocol for collecting introduced taxa, to get a complete inventory of South African earthworms a range of sampling techniques will be required; in particular, a large 1 m × 1 m × 20 cm plot is required for many large bodied native taxa, and the collection of giant earthworms will require different approaches. The identification of specimens requires skills that are scarce in the country and so there is an urgent need for training and funding for fundamental work on earthworm taxonomy. An atlas project could serve as a focal point for future research. In providing some general recommendations based on the long and fruitful history of research on earthworms in South Africa, we are optimistic that a better understanding of the group will help us to both improve our usage of natural resources and provide insights into this vitally important edaphic group.

KEYWORDS: Afrotropical Region, South Africa, megadrile, earthworms, sampling techniques, biodiversity, sampling effort, taxonomy, literature database.

INTRODUCTION

Pressures such as climate change, pollution and urbanisation mean that biodiversity research is becoming increasingly important if we are to manage ecosystem functioning

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and services such as soil fertility, clean water and waste processing (Naeem *et al.* 1994; Slotow & Hamer 2000; Crouch & Smith 2011; Hamer 2010). However, global change drivers and their synergistic effects (Millennium Ecosystem Assessment 2005) might cause extinctions of species not even known to science (Essl *et al.* 2013; Costello *et al.* 2013). One aspect that has often been overlooked is the significant contribution of soil fauna to soil health, below ground diversity, and more broadly to ecosystem functioning (Wardle 2002, Wardle *et al.* 2004, Louw *et al.* 2014).

South Africa contains one of the most diverse temperate faunas in the world (Steenkamp & Smith 2006), and has a strong history of biodiversity research (Crouch & Smith 2011). Despite this, knowledge of the soil fauna of South Africa is scattered and the fauna poorly understood (Janion-Scheepers *et al.* in prep.). Most research has focused on taxonomically well-known groups such as ants (Parr *et al.* 2005, Botes *et al.* 2006, Braschler *et al.* 2012), beetles (Davis 1997, Davis *et al.* 1999, Botes *et al.* 2007) and spiders (Dippenaar-Schoeman & Craemer 2000), with only a few recent efforts on groups such as Collembola (Janion *et al.* 2011, 2015), mites (Ermilov & Hugo-Coetzee 2012) and nematodes (Borgonie *et al.* 2011, Knoetze *et al.* 2006). Lack of current research is certainly driven by the logistics of sampling soil fauna. Sampling is often difficult and time consuming. Moreover, the lack of standardisation in sampling protocols has hampered comparisons of diversity between soil fauna studies.

The important role of earthworms as soil processors and ecosystem engineers has been widely demonstrated (Edwards & Bohlen 1996; Lavelle *et al.* 2006), and the role they play in soil fertility and health was even recognised by Darwin (1881). The activities of earthworms in the soil are vital for a healthy, fully functional system (Butt & Grigoropoulou 2010). These activities can substantially change the physical and chemical characteristics of the soil environment either directly or indirectly, with consequences for the entire soil food web, nutrient distribution and even vertebrate and understory plant communities (Edwards & Bohlen 1996). However, many ecological studies group all earthworms together, despite important functional differences between taxa reflecting a great diversity and wide range of adaptations to environmental conditions (Coleman *et al.* 2004). If the role of earthworms in ecosystems is to be quantified, a precise and accurate estimation of their diversity, abundance and biomass is needed (Valckx *et al.* 2011).

Earthworm sampling methods can be broadly divided into active, ethological (behavioural) and passive collection methods (Bartlett *et al.* 2010; Valckx *et al.* 2011). The main method of active sampling is by digging up a portion of soil (ideally of known volume) and sorting through the soil by hand to collect all earthworms and cocoons. Alternatively, earthworms can be expelled from the soil due to their behavioural response to certain stimuli by a range of chemical expellants or vermifuges (Coleman *et al.* 2004). It is also common to use both digging and hand sorting as well as chemical expellants (Baretta *et al.* 2007). Finally, indirect methods involve looking for signs of earthworms or waiting until they emerge on the surface (e.g. dispersal of some species after heavy rains).

Different chemical expellants or irritants can expel earthworms from the soil. Commonly used expellants are formaldehyde (Raw 1959; ISO 11268-3, 1999; Eichinger *et al.* 2007), commercial hot mustard (Gunn 1992; East & Knight 1998; Chan & Munro 2001; Lawrence & Bowers 2002; Eisenhauer *et al.* 2008), allyl isothiocyanate (AITC) (Zarborski 2003), household detergents such as washing-up liquid (East & Knight 1998) and potassium permanganate (Evans & Guild 1947, Reinecke & Ryke 1972). Other

ethological methods used are the octet or electrical method (Čoja *et al.* 2008), heat extraction (Čoja *et al.* 2008), mechanical vibration and the use of pitfall traps (Callaham *et al.* 2003). Not all of these methods are DNA compatible and certain collection techniques can limit downstream molecular projects. When sampling earthworms formalin is often used as an expellant. However, it is not possible to recover high-quality DNA from tissue that has been exposed to formalin (Moelans *et al.* 2011), and as such the use of formalin is discouraged when tissues are to be used in molecular studies. Several authors have suggested that mustard be used as an alternative to formalin as an expellant (Gunn 1992; East & Knight 1998; Chan & Munro 2001; Lawrence & Bowers 2002; Eisenhauer *et al.* 2008; Pelosi *et al.* 2009; Valckx *et al.* 2011). Gunn (1992) found that the use of mustard was more successful than formalin, while Lawrence and Bowers (2002) found that mustard was environmentally friendly and a more efficient alternative to formalin across soil and habitat types. The use of mustard powder or prepared mustard presents some problems for standardising earthworm sampling, as the composition of the mustard, how it is applied, and the local soil conditions can vary (Zarborski 2003).

The International Organisation of Standardisation (ISO) set a standard for the sampling of soil invertebrates to address the need to standardise the sampling of terrestrial soil invertebrates (ISO 23611-1, 2006). In part 1 of the ISO document a standard is set for the hand sorting and formalin extraction of earthworms (ISO 23611-1, 2006). The isolation and hand sorting of earthworms in a soil sample of a certain area (0.25 m²) or volume (25 × 25 × 20 cm) is suggested. This method is unfortunately a very laborious procedure and is difficult when the soil has high clay content or if there is a dense root mat present, and when physical disturbance of the study site is not acceptable, meaning digging is not an option (Bouche & Garner 1984; Gunn 1992; Eisenhauer *et al.* 2007).

Although digging is the most reliable method, a true and complete account of earthworm abundance will require a range of sampling techniques (Lawrence & Bowers 2002), as the relative efficiency of different sampling methods can vary with site characteristics, season and earthworm species (Callaham & Hendrix 1997). Indeed, most studies encourage the use of more than one sampling method (Coleman *et al.* 2004). By using a combination of active, behavioural, and passive techniques (e.g. digging, formalin extraction, and cast surveys), the accuracy of estimates of earthworm populations can be significantly increased (Bouche & Gardner 1984).

Ecologists and conservationists rely on taxonomists for information regarding species identifications (Chang *et al.* 2009). Earthworm identification is, however, not straightforward. The use of morphological characters is time consuming, labour intensive and requires trained specialists (Bartlett *et al.* 2010). A high level of cryptic diversity occurs (Rougerie *et al.* 2009), and the identification of juveniles and cocoons to species level cannot be made by traditional taxonomic means alone.

Earthworm research in South Africa has been conducted since the nineteenth century (Beddard 1895). As of 2010, there were 282 described earthworm species indigenous to South Africa belonging to three families: Microchaetidae, Tritogeniidae and Acanthodrilidae (specifically to the sub-family Acanthodrilinae) (Plisko 2010). However, large areas of South Africa remain to be surveyed, and most of the groups that have been well sampled require taxonomic revision.

The reference and research collection of earthworms housed in the KwaZulu-Natal Museum (Pietermaritzburg, KwaZulu-Natal) has 138 types (Plisko 2006, 2007, 2008). All types and most specimens were fixed in formalin and so they currently cannot be

used in molecular studies. In terms of introduced fauna, 50 species had been recorded by Plisko (2010) but the number has been corrected by Plisko & Nxele (2015) to 44 due to some of the species possibly being indigenous. Many of these species are widespread and abundant, with introduced species dominating disturbed agricultural sites (Ljungström 1972; Visser & Reinecke 1977; Plisko 2000; Dlamini *et al.* 2001; Haynes *et al.* 2003; Plisko 2010). While the extent to which introduced earthworms have colonised natural sites is poorly known, results from Dlinza Forest Nature Reserve in Eshowe and from a recent survey of Queen Elizabeth Park, in Pietermaritzburg, highlighted that even at less disturbed sites introduced taxa can be more common than native taxa (Plisko 2000; Nxele 2012). It remains to be seen whether introduced earthworms are having important economic or ecological impacts in South Africa, but introduced earthworms have had major detrimental effects in several locations around the world (Hendrix *et al.* 2008), particularly in areas where earthworms were not previously present (e.g. islands, or areas post-glaciation).

The aim of this paper is to review the literature on techniques commonly used for earthworm sampling, conduct some preliminary sampling with the two most widely used methods that are compatible with genetic techniques, provide guidelines and recommendations for sampling, and highlight future research focus areas.

MATERIAL AND METHODS

Review of sampling methods

The methods used to sample earthworms were investigated by reviewing sampling techniques globally and locally. A targeted literature search was conducted using ISI Web of Science™. The search was conducted in 2012 by firstly determining the total amount of available published data on “earthworms” from 1950 to 2012 by searching for the term “earthworm*” in the title, topic or abstract. Secondly, a targeted search was done to establish the number of available research papers discussing or reviewing different earthworm sampling techniques, using the following term: “(earthworm* OR oligochaeta*) AND (sampling method* OR hand sorting* OR formalin* OR allyl isothiocyanate* OR mustard*)”. Additional data from the publications were also collected to evaluate recent and past sampling techniques, the use of different terminology when describing surveys, the collection of biogeographical data, the preservation of samples, and the use of different taxonomic identification tools. A digital literature collection in the KwaZulu-Natal Museum was also searched (donated by A. J. Reinecke and J. D. Plisko). This collection contains articles of relevance on South African earthworms, with a focus on taxonomy, ecology, vermiculture, morphology, physiology, neurology, genetics and ecotoxicology. Some papers in the collection had already been recovered in the web search and therefore were not included; only the additional papers not recovered by the web search were added.

Pilot sampling

Earthworms were sampled using digging and hand sorting at four sites in KwaZulu-Natal, South Africa, during January and February 2012 (i.e. immediately following summer rains when the collection of earthworms is most effective (Plisko 2002a; Fig. 1; Table 1). Two of the sites were in natural vegetation in protected areas (a mistbelt forest and a grassland) and two in disturbed areas (National Botanical Gardens—



Fig. 1. Earthworm sampling. (A) The most common method is for plots to be dug up, the soil sorted by hand, and earthworms removed. (B) An alternative method is to apply chemical irritants (e.g. mustard solution) to a plot and the earthworms collected once they emerge from the soil. (C) Sorting, cleaning and processing earthworms in the field is a major component of sampling. Decisions as to the size of plot used, how deep the plots are dug, and which chemicals are used in which concentrations, as well as the timing of the sampling, all need to be adjusted depending on the goals of sampling. For large individuals (and anecic species) different sampling methods are needed (see text).

Pietermaritzburg, and a fallow agricultural field). At the agricultural site, sampling was conducted both in ploughed cultivated areas and on contour ridges between the cultivated areas (the latter had kikuyu grass, weeds and tall reeds). At each of the four study sites,

TABLE 1
Localities sampled during the pilot survey.

Site	Habitat type	Management type	Lat / Long
Queen Elizabeth Park	Grassland	Protected nature reserve	29.57148°S 30.32235°E
Cedara	Agriculture	Tilled, fallow, previously planted with soya beans	29.54673°S 30.27778°E
Karkloof	Mistbelt forest	Protected nature reserve	29.30393°S 30.22873°E
National Botanical Gardens — Pietermaritzburg	Planted gardens and compost heaps	Landscaped gardens	29.60668°S 30.34832°E

12 sampling plots of 50 cm × 50 cm (after Keith & Schmidt 2013) randomly spaced at intervals not closer than 5 m from each other were selected, and soil to a depth of 20 cm dug out using a metal frame as a guide. The soil was placed onto a plastic sheet and hand sorted for earthworms. Where possible, leaf litter was collected and also searched for earthworms prior to the digging.

In addition, 12 more plots of the same size were sampled using a chemical irritant extraction technique. Mustard powder was prepared according to the protocol from ISO 23611-1 (ISO 2006). One hundred grams of dry mustard powder (Colman's) was dissolved in 1 litre of water. In the field, the mixture was diluted with 9 litres of water in 10 litre watering cans, to give a concentration of 10 gL⁻¹. Leaf litter was removed from each plot and then the mixture poured onto the plot. The earthworms that appeared on top of the soil were collected for about 30 minutes.

Sampled earthworms were washed twice in clean tap water and afterwards narcotised in 20% ethanol. The narcotised earthworms were then weighed (wet mass) to the nearest milligram (mg) using a field balance (Highland portable precision range field balance). Earthworms were then preserved in absolute ethanol, clearly marked with labels and transported to the KZN Museum laboratory for identification. Earthworms were identified to species level using a Wild (Heerbrugg) microscope and descriptions and keys from the published literature (Ljungström 1972; Gates 1972; Plisko 1992; Csuzdi 2010; Blakemore 2011). The collected material was deposited in the KZN Museum.

Sample-based rarefaction curves were plotted and the ACE estimator employed to examine the degree of sampling efficiency and to determine the predicted number of species in each site, using the computer programme EstimateS V9.1.0 (Colwell 2013). All unidentified earthworms (juveniles, damaged adults and other specimens that could not be identified to species level) were removed from the dataset prior to rarefaction and estimation. Sampling was considered adequate if the estimator converged at the highest observed (identified) value (Longino *et al.* 2002).

Future directions for earthworm research in South Africa

To determine future priorities, we held a workshop on earthworm research in November 2011 and again in January 2015. Using various horizon scanning exercises in agriculture, ecology and environmental sciences as a background (e.g. Sutherland *et al.* 2013), we developed various key priorities for earthworm research.

RESULTS

Review of sampling methods

A total of 10 938 publications were found using the search keyword “earthworm*”. Of these papers, only 480 (4.4%) were directly linked to earthworm sampling by using the search keywords: “(earthworm* OR oligochaeta*) AND (sampling method* OR hand sorting* OR formalin* OR allyl isothiocyanate* OR mustard*)”. Two important issues were observed. First, a vast array of different concepts and terminologies were found to describe earthworm sampling. Different authors use different terms to describe the same procedure or concept, and to avoid confusion authors need to use well defined terms (De Zorzi *et al.* 2005). One method is to develop standard protocols (e.g. Supplementary Material 1). Second, when reviewing the sampling protocol it was found that although thorough descriptions of sampling techniques were given, preservation techniques, determination of biomass and taxonomic identification were poorly documented and often not described at all. The search of the database found 32 papers that described sampling methods for South African studies, of these 13 were already recovered from the web literature search. All the publications that contained South African data were recorded in Table 2. The publications containing data from outside South Africa were recorded in Table 3. Most South African studies used random digging and hand sorting in different habitats such as forests and grasslands.

Pilot sampling

Of the earthworms collected using the two sampling methods at the four sampling sites, a total of 2 094 earthworms were assigned to 16 species belonging to five families (Table 4). Some earthworms (196) could not be identified as they were unknown juveniles or damaged specimens and were not used in the results. The species accumulation curves (Supplementary material Fig. 2 A–D) indicate that most of the earthworm species at a site were sampled where species richness was relatively low. At sites where earthworm richness was higher, undersampling of species was indicated.

In the grassland of Queen Elizabeth Park a total of 217 earthworms were collected through digging and hand sorting; no earthworms were collected when mustard was used. Only three species (*Dichogaster* sp., *Amyntas corticis* (Kinberg, 1867) and *Amyntas rodericensis* (Grube, 1879)) were found, all introduced to South Africa. Previous studies in this park collected six species in the grassland, including the indigenous *Tritogenia howickiana* (Michaelsen, 1913) and nine species when including more vegetation types in the park (Nxele 2012). In Cedara, no earthworms were found from the plots that were in the middle of the cultivated field either by digging or mustard, but 85 earthworms from five different species (*Octolasion lacteum* (Örley, 1881), *Amyntas aeruginosus* Kinberg, 1867, *Amyntas corticis*, *Amyntas gracilis* (Kinberg, 1867) and *Amyntas minimus* (Horst, 1893)) were collected from plots that were in the grass contour between the fields. All the sampled earthworms were introduced species. In addition, unknown juveniles were also collected, but could not be assigned to any species.

When using the digging and hand sorting method in the Karkloof Forest a total of 766 earthworms and nine species were collected. Eight of these species (*Pontoscolex corethrurus* (Müller, 1857), *Aporrectodea rosea* (Savigny, 1826), *Dendrobaena octaedra* (Savigny, 1826), *Dendrobaena* sp., *Dendrodrilus* sp., *Amyntas minimus*, *Amyntas* sp., unidentified lumbricid species) were introduced species, and only one indigenous specimen (*Tritogenia* sp.) was found. When using the mustard method, 485

TABLE 2

Studies that sampled earthworms in South Africa (1895–2012) combined from the literature collection database in the KwaZulu-Natal Museum and literature review. N/A = no dominant species or number of species and ecological category were mentioned.

Reference	Focus	Method	Location	Dominant species and ecological category
Dlamini <i>et al.</i> 2001	Quantitative & qualitative sampling of exotic earthworms on sugarcane estates	Digging and handsorting	Eshowe-Empangeni, KZN	<i>Pontoscolex corethrurus</i> ; Endogeic
Haynes <i>et al.</i> 2003	Quantitative & qualitative sampling of earthworms in agricultural land in KZN	Digging and handsorting	KZN Midlands	<i>Amyntas diffringens</i> ; Endogeic
Horn <i>et al.</i> 2007	Diversity of earthworms in forests in Limpopo	Searching and digging	Soutpansberg and Northern Drakensberg, Limpopo	<i>Amyntas diffringens</i> ; Endogeic
James & Davidson 2012	Taxonomic studies	Digging and handsorting	KZN	N/A
Jamieson 1967	Taxonomy	Digging and handsorting	Various sites	<i>Eukeria saltensis</i> ; Endogeic
Ljungström 1972	Taxonomic studies	Digging and handsorting	Various sites	<i>Pontoscolex corethrurus</i> ; Endogeic
Michaelsen 1913	Taxonomic studies	Digging and handsorting	KZN	N/A
Nxele 2012	Taxonomy & Biodiversity assessment	Digging and handsorting	Queen Elizabeth Park, Pietermaritzburg, KZN	<i>Amyntas rodericensis</i> ; Epigeic
Pickford 1937	Taxonomy	Digging	Various sites	<i>Eodriloides arundinis</i> ; Epigeic
Plisko 1991–1993, 1996a, b; 1997	Taxonomic studies	Digging and handsorting	Various sites	N/A
Plisko 2000–2004, 2006a, b 2007	Taxonomic studies	Digging and handsorting	Various sites	N/A
Reinecke & Ackerman 1977	Taxonomic studies	Digging and handsorting	Northern Kruger National Park, Limpopo	N/A
Reinecke & Ljungström 1969	Ecological studies - introduced species	Digging and handsorting	Potchefstroom	<i>Eisenia rosea</i> ; Endogeic
Reinecke & Ryke 1970	Ecophysiological study of lumbricids & microchaetids	Digging and handsorting	Potchefstroom	<i>Proandricus modestus</i> ; Endogeic
Reinecke & Ryke 1972	Ecology, sampling methods	Digging and handsorting, formalin, KMnO ₄	Potchefstroom	<i>Proandricus modestus</i> ; Endogeic

TABLE 2 (continued)

Studies that sampled earthworms in South Africa (1895–2012) combined from the literature collection database in the KwaZulu-Natal Museum and literature review. N/A = no dominant species or number of species and ecological category were mentioned.

Reference	Focus	Method	Location	Dominant species and ecological category
Reynolds 1993	Taxonomic studies	Digging and handsorting	Swaziland	N/A
Uys 2006	Biodiversity assessment	Digging and handsorting	South Africa: Drakensberg, KZN	N/A
Visser & Reinecke 1977	Quantitative & qualitative sampling in irrigation area - Population densities	Digging and handsorting	South Africa: Potchefstroom, North West Province	<i>Eisenia rosea</i> ; Endogeic
Zicsi & Plisko 1991	Taxonomy	Digging and handsorting	South Africa: KZN	N/A

earthworms were collected from seven species (*Pontoscolex corethrus*, *Aporrectodea rosea*, *Dendrobaena octaedra*, *Dendrobaena* sp., *Amyntas minimus*, *Amyntas* sp., and unidentified lumbricid species) all of which were also collected using the digging and hand sorting method. When using the digging and hand sorting method in the Botanical gardens a total of 275 earthworms from 11 species were collected. When using the mustard method 266 earthworms from 12 species were collected, including *Tritogenia* sp (Table 4). The endogeic *P. corethrus* was dominant in QEP, the epigeic *A. rodericensis* was dominant in Cedara, in the Karkloof Forest the endogeic *O. lacteum* was collected in high numbers using both methods though digging collected almost double the number of individuals than the mustard, and the endogeic *A. minimus* was dominant in the Botanical Gardens.

The earthworms collected by digging and hand sorting weighed on average 0.358 g per worm (n=294), slightly heavier than the earthworms collected using mustard extraction (average of 0.294 g each, n=273).

Estimates of the sampling effort are shown in Table 5. Four people completed the pilot study in 10 days with eight working hours in each day. This time included travelling, setting up the field workstation, sampling and travelling with equipment from plot to plot, and packing up and cleaning after each day. As field assistants became more skilled over the course of the study, the time taken to complete a task decreased. When using the digging and hand sorting method it took an average of 40 minutes for one person to sample a plot (50 × 50 × 20 cm) with an average of 44 earthworms per plot. The time taken to collect earthworms with the mustard technique averaged 13 minutes per plot (50 × 50 cm) with an average of 35 earthworms collected per plot. Standardising on 100 earthworms, preserving the earthworms (including washing and narcotising them) and the determination of the biomass took an average of 26 and 42 minutes, respectively. The time taken to identify each earthworm varied greatly depending on (a) the experience of the taxonomist and (b) the identity of the worm. It took three months to complete the classification for all the earthworms in this study, an average of two hours per specimen.

TABLE 3

Sampling methods used in earthworm studies from other countries from the literature collection database in the KwaZulu-Natal Museum.

Reference	Focus	Method	Location
Beyer <i>et al.</i> 1980	Ecotoxicology	Digging and hand sorting	USA
Beyer & Cromartie 1987	Ecotoxicology	Digging and hand sorting	USA
Boyd 1957	Ecology	Active searching, KMnO ₄ , Digging and hand sorting	Scotland
Butt 1991	Vermiculture	Formalin	UK
Butt <i>et al.</i> 2006	Ecology	Digging and hand sorting	England
Chaudhuri & Bhattacharjee 1999	Ecology	Digging and hand sorting	India
Cook <i>et al.</i> 1980	Ecotoxicology	Formalin	Nigeria
Cuendet 1983	Ecology	Formalin, Digging and hand sorting	Switzerland
Damoff & Reynolds, 2004	Taxonomy	Digging and hand sorting	USA
Daniel <i>et al.</i> 1992	Sampling techniques	Formalin, Chloroacetophenone, Digging and hand sorting	Switzerland
Edwards & Lofty 1982	Vermiculture	Formalin	UK
Enckell & Rundgren 1988	Ecology	Formalin	Faroe Island
Enckell <i>et al.</i> 1986	Ecology	Formalin	Faroe Island
Evans & Guild 1947	Ecology	Vermifuge	UK
Fragoso & Reynolds 1997	Taxonomy	Digging and hand sorting, Active searching	Mexico
Guild 1951	Ecology	KMnO ₄	Scotland
Guild 1952a	Ecology	KMnO ₄	UK
Guild 1952b	Ecology	KMnO ₄	Scotland
Guild 1957	Ecology	Digging and hand sorting	Scotland
Haimi & Boucelham 1991	Ecology	Active searching	Finland
Hendriksen 1990	Ecology	Formalin	Denmark
Hopp 1947	Ecology	Soil corer	USA
Joannes & Kretzschmar 1983	Physiology	Formalin	France
Laverack 1960	Physiology	Digging and hand sorting	England
Laverack 1961	Physiology	Formalin	UK
Martin 1980	Ecology	Digging and hand sorting	New Zealand
Mischis <i>et al.</i> 1997	Taxonomy	Digging and hand sorting, Active searching	Argentina
Moreno <i>et al.</i> 1982	Taxonomy	Formalin, Digging	Spain
Murchie 1958	Ecology	KMnO ₄ , Digging and hand sorting	USA
Nelson & Satchell 1962	Sampling techniques	Digging and hand sorting	England

TABLE 3 (*continued*)

Sampling methods used in earthworm studies from other countries from the literature collection database in KwaZulu-Natal Museum.

Reference	Focus	Method	Location
Nordström & Rundgren 1972	Sampling techniques	Formalin, Formalin + detergent, KMnO ₄ , Digging and hand sorting	Sweden
Nordström & Rundgren 1973	Ecological	Formalin	Sweden
Nuutinen & Haukka 1990	Ecological	Formalin	Finland
Omedeo & Martinucci 1987	Taxonomy	Digging and hand sorting, Active searching	Italy & Algeria
Pavliček <i>et al.</i> 1996	Biodiversity	Formalin	Israel
Pop 1987	Ecology	Formalin	Carpathians
Pop & Postolache 1987	Ecology	Formalin	Romania
Raw 1962	Ecology	Formalin	UK
Reeves & Reynolds 2004	Taxonomy	Digging and hand sorting	Cayman Islands
Reynolds 1972	Ecology	Digging and hand sorting	USA
Reynolds <i>et al.</i> 1995	Taxonomy	Digging and hand sorting	Belize
Reynolds & Hanel 2005	Taxonomy	Digging and hand sorting	South Atlantic ocean
Reynoldson 1955	Ecology	KMnO ₄ , Digging and hand sorting	Wales
Reynoldson <i>et al.</i> 1955	Ecology	Active searching	UK
Satchell 1970	Ecology	KMnO ₄ , Formalin, Digging and hand sorting, Heat extraction, Floating	UK
Scheu 1987	Ecology	Active searching	Canada
Scheu 1993	Ecology	Active searching	Canada
Schwert 1977	Taxonomy	Digging and hand sorting	Canada
Sims 1967	Taxonomy	Formalin	Gambia
Tomlin & Gore 1974	Ecotoxicology	Formalin	Canada

Future directions for earthworm research in South Africa

The key priorities for research identified during the workshop on earthworms are summarised in Box 1. These included foundational biodiversity goals such as preparing an atlas, taxonomic keys, field guides and a DNA barcode library. Other goals included increasing outreach efforts and public interest (e.g. through a roadshow). The latter are considered important in terms of engaging with stakeholders that already have an interest in earthworms as well as providing exposure to taxonomic experts, thereby assisting with the potential enrolment of future taxonomists. There are many urgent needs, but it is clear that these goals must be underpinned by foundational biodiversity survey and description work, which in turn requires capacity building in taxonomy in South Africa.

TABLE 4
Species abundance for each sampled site.

Family	Species	Status	Queen Elizabeth Park (natural grassland)		Cedara (Agricultural field)		Karkloof (natural forest)		Botanic Gardens (transformed)		Total
			Digging	Mustard	Digging	Mustard	Digging	Mustard	Digging	Mustard	
Acanthodrilidae	<i>Acanthodrilidae</i> sp.	?	0		0		0	0	0	5	5
Acanthodrilidae	<i>Dichogaster</i> sp.	Introduced	4		0		0	0	2	0	6
Pontoscolicidae	<i>Pontoscolex corethrurus</i>	Introduced; Endogeic	212		0		0	0	2	0	214
Lumbricidae	<i>Aporrectodea rosea</i>	Introduced; Endogeic	0		0		48	7	4	1	60
Lumbricidae	<i>Dendrobaena octaedra</i>	Introduced; Epigeic	0		0		7	0	0	0	7
Lumbricidae	<i>Dendrobaena</i> sp.	Introduced	0		0		64	68	0	1	133
Lumbricidae	<i>Dendrotrilus</i> sp.	Introduced	0		0		1	0	0	0	1
Lumbricidae	Lumbricidae sp.	Introduced	0		0		159	143	7	19	328
Lumbricidae	<i>Octolasion lacteum</i>	Introduced; Endogeic	0		0		474	258	11	14	757
Megascolicidae	<i>Amyntas aeruginosus</i>	Introduced; Epigeic	0		1		0	0	30	15	46
Megascolicidae	<i>A. corticis</i>	Introduced; Epigeic	0		22		9	7	5	4	47
Megascolicidae	<i>A. gracilis</i>	Introduced; Epigeic	0		2		0	0	9	1	12
Megascolicidae	<i>A. minimus</i>	Introduced; Endogeic	0		14		0	0	100	74	188
Megascolicidae	<i>A. rodericensis</i>	Introduced; Epigeic	1		46		0	0	29	32	108
Megascolicidae	<i>Amyntas</i> sp.	Introduced	0		0		3	2	76	99	180
Tritogeniidae	<i>Tritogenia</i> sp.	Native; Endogeic	0		0		1	0	0	1	2
	TOTAL		217	N/A	85	N/A	766	485	275	266	2094

DISCUSSION

A wide variety of techniques are used for the sampling of earthworms in the field (Bouché & Gardner 1984, Druce *et al.* 2004; Čoja *et al.* 2008), reflecting in part the different research questions asked, but also because earthworm species have a variety of ecological strategies, patterns of behaviour and habitat preferences (Lee 1985; Blakemore 2002). Epigeic and endogeic species can often be collected by hand; whereas anecic species might require chemical irritants to expel the earthworms from the soil. In our sampling, epigeic earthworms dominated in Cedara and endogeic earthworms dominated in the Botanical Gardens. In addition, earthworm populations are usually spatially aggregated (Valckx *et al.* 2011). This complicates sampling efforts and attempts to estimate population sizes, particularly as the factors that determine this patchy spatial distribution are not yet sufficiently understood (Valckx *et al.* 2009). Thus, sampling techniques need to account for the spatial distribution of earthworms in order to get an accurate estimate of species richness and abundance. At the indigenous forest in Karkloof, a greater abundance of earthworms was obtained by digging in comparison to the mustard solution method, whereas almost equal abundances were obtained using the two methods in the transformed habitat of the Botanical Gardens. Despite the existence of international standards (ISO 23611-1 and ISO 11268-3), many studies are not comparable due to the lack of standardisation of sampling protocols. While there has been a substantial and long history of earthworm research in South Africa, for various purposes, most sampling in South Africa has been via digging and hand sorting (Table 2).

In this study we used hand digging with manual sorting and mustard extraction. Only one indigenous earthworm was collected during the whole study (*Tritogenia* sp.: Table 4).

TABLE 5
Table indicating the average sampling effort and costs per task or activity.*

Task/ activity	Effort/ costs
Logistics (excluding accommodation for 1 person):	
Travel to all sites	R1 261 (R3.30/km for 382 km)
Car rental	R1 886
Stipends	R2 250 (3 field assistants, R150/per person/day)
Air ticket	R1 573
Food & tolls	R2 364
Digging	Average of 55–60 seconds per worm
Mustard	Average of 21–30 seconds per worm
Preservation	Average of 15.5 seconds per worm
Determination of biomass	Average of 21–29 seconds per worm
Worm sampling and preservation	R100 per plot — sampling 30 earthworms per plot
Identification	2hr/worm, R80/hr

* To measure the amount of time, costs and effort needed per sample, detailed notes were kept on each of the different activities or tasks. These included: digging and collection of earthworms by hand, using the mustard solution collection technique and collecting earthworms, processing of samples (killing, labelling, adding to preservative, etc.), taking of soil samples and other environmental notes and measurements, preparation of plate for barcoding (including photographing specimens), weighing of specimens and identification of specimens. A detailed budget was kept for the entire project in order to estimate the logistical costs, the cost per sampling plot and the cost per soil sample. The values are as per South African Rand, January 2015

While this might be expected for disturbed areas such as gardens and agricultural areas, it was disconcerting to see a similar pattern in protected areas. This is likely the result of the human activities adjacent to and even in the protected areas. Similarly, Nxele (2012) collected five species of earthworm in grassland in Queen Elizabeth Park by digging 1 m × 1 m quadrats to a depth of 0.5 m: the indigenous *Tritogenia howickiana* and the non-indigenous *Pontoscolex corethrurus*, *Amyntas gracilis*, *Amyntas minimus*, and *Amyntas rodericensis*. These results are different from results obtained by Baretta *et al.* (2007) who also used digging and handsorting plus a chemical expellent, dilute Formol (0.5%). These authors found four native species and one exotic species when all methods were combined; however the most abundant was the exotic *Amyntas corticis*. The natural forests have high earthworm diversity because of high food availability and variability (Fragoso *et al.* 2006). In Karkloof forest, nine species were found and this is the second highest richness of all sampled sites. A native species was collected in this forest, similar to other studies in terms of diversity but completely different in terms of the number of natives found. For example, Huerta *et al.* (2007) studied the diversity of earthworms in heavily deforested Tabasco in Mexico. They sampled tropical rain forest and plantations of mango (*Mangifera indica*) and cacao (*Cacao theobroma*). These authors found 14 native species and five exotics, the tropical rain forest having the greater earthworm diversity.

The method of digging and hand-sorting soil from a 25 × 25 cm quadrat to 20 cm depth seems to be more suitable for sampling epigeic and endogeic earthworm species, probably as a result of their much higher densities in the upper soil layers. As our survey showed, no anecic earthworms were collected with our quadrat size. The size of each plot is a trade-off between collecting individuals and work. Based on available data and experience, we suggest following the ISO standard (i.e. a sample plot size of 50 cm × 50 cm and 20 cm deep) for smaller sized earthworms in South Africa, and digging larger and deeper quadrats for quantitatively sampling larger sized earthworms (i.e. plots of at least 1 m × 1 m × 0.5 m) (Nxele 2012).

Twelve soil samples appeared sufficient to sample most species of non-indigenous earthworms at species-poor sites, but not at species-rich sites as observed in the species accumulative curves (Supplementary material 2). The number of samples per site must be large enough to represent communities at the site adequately, but not so large that they are too time-consuming to process (Stark *et al.* 2001). From the literature review, the number of samples varied from five or six samples (Chaudhuri & Bhattacharjee 1999; Uys 2006) to 46 samples (Cuendet 1983) depending on the research purpose. Our literature survey indicates that given the high spatial and temporal variability in earthworm communities and variability introduced by different sampling techniques, giving a specific guidance on the sample size is difficult, and will likely be quite context specific.

Additional modifications are required for very large bodied taxa, as they were not collected with our quadrat size in our survey. First, one can dig even larger areas (e.g. approximately 2 m × 2 m × 0.5 m deep for earthworms up to around 100 cm long). Second, sampling of larger earthworms, especially giant earthworms, can be accomplished by searching for fresh casts (indicated by active deposition of fresh mud above ground), in a defined larger area, quickly topping the casts and grabbing the earthworm by the tail end, after which the tail end will anastomose and can immediately be preserved in 95 % ethanol. The species might then be identified using DNA barcoding, and the density

per unit area estimated. However, it should be noted that specimens can't be kept for morphological examination with this technique, and as such it is of limited use. Finally, some sampling will need to be opportunistic (e.g. sampling of giant earthworms that cross roads after rains). Owing to the sporadic nature of overland movement, a network of volunteers in various parts of the survey region might be able to track the weather conditions and collect specimens when conditions are right. The specimens would be useful for taxonomic study and a future atlas project.

Equipment requirements for earthworm sampling techniques and protocols are relatively modest (Supplementary Material 1). However, it takes a lot of time to sample and process samples. Time, resources and expensive equipment are limiting factors to zoological research globally (Bartlett *et al.* 2010; Butt & Grigoropoulou 2010, Uys *et al.* 2010). For these reasons the design inventories, sampling methods and planning and monitoring needs to be as effective and efficient as possible (Uys *et al.* 2010). Two people take 20 minutes to sample a plot of 50 × 50 × 20 cm by digging, and 13 minutes using mustard solution because of the waiting time involved. Mustard may be a useful and quicker method to sample alien earthworm species in leaf litter and topsoil. When using mustard solution, there is less labour involved compared to digging and hand sorting and using mustard solution does not affect the DNA of the specimens, which makes it suitable for molecular analyses. However, the results appear to be variable, and it is hard to control the amount that penetrates into the soil.

Setting a standard for the determination of earthworm biomass would make it possible to compare different datasets, but the biomass of an individual species or of a population as a whole can be used to estimate production and productivity of an ecosystem only when comparing ecosystems of the same or similar structure and species composition. The biomass of earthworm populations varies greatly and in most soils it exceeds the biomass of all other soil-inhabiting invertebrates (Edwards 1994). One practical issue is that the gut contents are a substantial proportion of an earthworm's biomass, and earthworms lose about 10–20% of their mass during fixation (Lee 1985), thus the point at which individuals are weighed must be made clear.

The major limitation to sampling is the ability to identify all the specimens collected to species level. With few taxonomists trained to identify South African earthworms (South Africa has one retired taxonomist and a full-time junior taxonomist at the KwaZulu-Natal Museum), additional capacity and new methods to facilitate species discovery and description are necessary. Earthworms are well suited to combining DNA-based analysis with morphological taxonomy (Hogg & Hebert 2003; Rougerie *et al.* 2009; Vernooy *et al.* 2010), an approach that has been used successfully in other parts of the world (Boyer *et al.* 2011, Porco *et al.* 2013). This approach involves training taxonomists in morphological identification, ensuring collections are developed and linked to DNA samples, cataloguing all relevant information and making it accessible.

The need to enlarge South Africa's capacity to answer applied and basic research questions on the roles and impacts of earthworms in the functioning of natural ecosystems was unanimously agreed upon during a workshop on soil organisms at the XVII Congress of the Entomological Society of Southern Africa (July 2011), and separately by the CAPE Invasive Alien Animal Working Group (May 2011) (Louw *et al.* 2014).

DNA bar-coding and development of user-friendly identification keys are needed to facilitate identification to species level. Given that DNA barcoding has been shown to be

extremely useful in the delimitation of difficult to identify species (Pop *et al.* 2003), the collection of samples in a DNA-compatible way should be included in future sampling protocols. In addition, juvenile specimens can be barcoded to verify their identifications (Richard *et al.* 2010). The earthworm barcoding library is growing on the Barcode of Life Data System (BOLD), also revealing interesting cryptic diversity (James & Davidson 2012, Porco *et al.* 2013, Decaëns *et al.* 2013).

The way ahead

Documenting the diversity of invertebrate groups should be a priority (Box 1) as invertebrate surveys, collections, and taxonomic descriptions should form an important part of setting conservation priorities (Essl *et al.* 2013). South Africa has achieved major success with atlas projects which have led to red-listing, volunteer networks and conservation actions (Harrison *et al.* 1997; Minter *et al.* 2004; Mecenero *et al.* 2013; Bates *et al.* 2014). A South African earthworm Atlas and Conservation Assessment would allow indigenous species of earthworms to be considered for conservation action through conservation plans, Environmental Impact Assessments (EIAs), and the like. Although South Africa is seen as a leader in the area of conservation planning (Balmford 2003), the number of EIAs or Biodiversity Assessments that have considered earthworms are few (McGeoch *et al.* 2011). Owing to the importance of earthworms in soil ecosystems, and their clear diversity importance in South Africa, this deficiency should be rectified. As a prelude to the Atlas project, we plan to run sampling and identification workshops in various provinces. The Atlas project would include both indigenous and non-indigenous species.

Data on diversity and distribution of earthworms have mostly been collected by taxonomists, building up large reference collections of study material (e.g. KZN Museum). These collections adequately serve the purpose of taxonomists but have limited use for environmental management, planning and conservation. Quantified, consolidated surveys and data for earthworms are patchy and scarce. Large amounts of material are collected but resources in terms of capacity and funds required to process the samples are limited. Although the plethora of different methodologies of earthworm sampling in use around the world could be a cause for inconsistencies in comparing different sets of data, the focus should be on efficiency, consistency, ease of use and practicability. When acquiring data during field trips, sampling trials needs to be maximised and optimised: i.e. gathering of soil data and biogeographic information, and measures of the effectiveness of the sampling techniques, as well as sampling all taxonomic groups present. These would be key sampling needs during surveys if questions on biodiversity, abundance and distributions are to be addressed. Surveys should be well planned and executed, and quantitative data collected, which in return will assist in obtaining diversity and distribution patterns for earthworms.

In addition to the integration of sampling techniques to extract earthworms from the soil, taxonomic identification and compiling a DNA barcode reference library, information is needed to assist in understanding the abiotic conditions that determine the spatial distribution of earthworms at the local scale. 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), the introduction of earthworms in areas for soil rehabilitation (Fründ *et al.* 2004), and

Box 1: Key priorities for work on megadrile earthworms in South Africa

- Compile an Atlas & Red List assessment.
- Develop a field guide to roughly separate specimens which will include pictures of live earthworms.
- Map and model species distributions based on climatic conditions, soils and land use.
- Engender public interest through outreach and participation to create awareness, expose and increase public interest on the subject.
- Develop a DNA barcode reference library.
- Construct a dated molecular phylogeny of indigenous South African species to enable a determination of key biogeographic breaks and explore evolutionary history.
- Develop a standardised protocol for sampling, collecting and curating earthworms (See Supplementary Material 1).
- Train future expertise for the identification of earthworm species in South Africa. This will be in the form of the training of students and farmers, annual “maintenance” training courses and the provision of identification services.
- Assess the risks of future introductions to South Africa and around South Africa via the various dispersal pathways.
- Determine the status and invasive potential of introduced species.
- Research the value of ecosystem services provided by earthworms under South African conditions, and the potential of species as bio-indicators of soil health.

the management of areas susceptible to earthworm invasions (Gundale 2002, Gundale *et al.* 2005).

To build our ecological and taxonomic knowledge of soil fauna in South Africa, studies of soil organisms need more coordination (Louw *et al.* 2014). In order to do this, one needs to move away from traditional *ad hoc* sampling to more coordinated, standardised sampling for comparison with other studies. The development of a survey plan should be interdisciplinary (involvement of soil scientists and biologists), thereby maximising sampling data and minimising effort, and thus will save time and money and assist with knowledge and information generation across disciplines and taxonomic groups. Also, increasing sampling intensity (effort) will increase detection probability.

CONCLUSION

We consider that the ISO sampling methodology augmented with more qualitative sampling is suitable for sampling most non-indigenous species of earthworm in South Africa. Sampling of indigenous species apparently requires a larger quadrat size or the use of other methods. Sampling and preservation techniques need to allow DNA-based studies to assist, complement and inform traditional morphology-based taxonomy. However, if we are to understand, conserve and manage earthworms we require more foundational knowledge. This will not happen without capacity-building and substantial resources.

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SUPPLEMENTARY MATERIAL 1: A PROPOSED PROTOCOL FOR SAMPLING EARTHWORMS

Equipment list

- 2 × measuring tape (5 m).
- String or rope.
- markers.
- distilled water (amount depends on how many plots needed; for 60 g of mustard, 1L of water is needed).
- mustard powder (60 g per 1 L of water).
- watering can.
- cleansing tray.
- GPS.
- sorting trays.
- collecting vials (*ca.* 10 per plot).
- spade.
- fork.
- pick axe.
- ethanol (*ca.* 0.5 L per plot).

Site information

Take and database photos of each sampling site.

Make notes of the:

- Site type (grassland, forest etc.).
- Topography.
- Date.
- Time.
- Weather conditions.
- Rainfall.
- Humidity (Lutron humidity meter).
- Ambient air temperature (Lutron air temperature meter).
- Vegetation type.
- % of bare soil.
- Condition of the site e.g. burnt or not.

Plot

- Place plot frame on selected plot.
- Photograph area.

- Search plot for casts (physical count).
- Collect casts (if needed)– label and store.

Soil sampling

- Make note of litter component of soil (depth) in the plot.
- Soil samples should be taken from inside the same plot once the earthworm sample has been removed.
- Ideally one sample per plot should be taken but due to the analysis cost, a minimum of 5 samples should be taken from a site. If the sampling site is against a slope, 5 soil samples should be taken at the top of the slope, 5 in the middle and 5 at the bottom.
- Remove plot vegetation by cutting the grass and any other vegetation to ground
- Measure soil temperature with a digital soil thermometer.
- Soil temperature to be taken at:
 - Just under the soil surface.
 - At 10 cm.
 - Between 20–30 cm.
 - At 50 cm.
- Take soil density measurements with Kingtest DCP (as prescribed by product manual) to a depth of 50 cm.
- Take soil sample with 75 mm soil auger to a depth of 50 cm.
- Take soil moisture measurements with the Lutron Soil Moisture meter from the soil sample in the soil auger.
- Moisture measurements to be taken at:
 - Just under the soil surface.
 - 0–10 cm.
 - 10–20 cm.
 - 50 cm.
- Label and store the soil sample in a paper bag for further analysis.

Plot size

- According to ISO 23611-1 plot size has been established at 50 × 50 cm. Larger plots (at least 1 × 1 m) are required for indigenous species.
- A pre-made steel frame is used to demarcate the plot area. It can be pressed into the soil if possible but should not be hammered into the soil which will cause disturbance.

Number of plots

- From our study, 12 randomly selected plots more than 5 m apart were mostly sufficient, but this will be highly context specific.

Digging and hand sorting

- Search through the litter component of the soil (2–5 cm).
- Collect earthworms, mark and record them correctly (epigeic or litter species).
- Dig out the soil in the area of the plot frame to the standard depth.
- Place the soil on a plastic sheet and search for earthworms.
- Move the sorted soil to one area for replacement into the hole.

Mustard extraction

- Remove the epigeic (topsoil) species – living and feeding on soil surface.
- Dissolve 60 g of dry mustard powder in 1 liter of distilled water. (ISO 11268-3).
- On site: Mix the mustard emulsion into 9 litres of water = 6.67 g/L.
- Pour the mixture onto the test site in 2 or 3 portions according to seepage capacity.
- The total duration of the extraction may be 30 mins.
- Allow the mustard solution to deeply seep into the ground without damming up in the plot area.
- Collect all emerging earthworms for approximately 30 mins.

Preservation of earthworms after collection

Although formalin is widely used as a fixative for biological specimens, nucleic acids extracted from formalin-preserved specimens are not available for downstream DNA use (Lehmann and Kreipe 2001, Gugic *et al.* 2007; James *et al.* 2010); therefore fixation in formalin is not recommended for molecular studies. Absolute ethanol is an excellent agent for field preservation of DNA within earthworm samples (Thakuria *et al.* 2008) and is recommended for future use in earthworm sampling in South Africa.

- Earthworms to be kept in 2 groups: (a) epigeic/litter species and (b) soil/endogeic & anecic species.
- Earthworms are washed in tap water.
- 20% ethanol is then used to kill them.
- 4% formalin is used to fix the specimens and after 24 hours transferred to 70% ethanol. Earthworms to be used for molecular studies are not fixed with formalin but are transferred straight to 100% ethanol.
- Specimens are identified in the lab.

Labelling

Important information is written on the label but more information is recorded in a field book and may be later transferred into a computerized database. Paper and pencil should be used for the labelling of samples in the field but a printed label on a good quality paper or hand written by permanent ink pen may be used for long-term storage. Laser printing is not permanent and should not be used but permanent pigment ink pens may be used. An example of a suitable label is provided below:

Museum accession no. (NMSA/OLIG.06364)
Country & province (RSA, KZN)
Locality (Queen Elizabeth Park)
Brief site info (grassland with few shrubs)
GPS coordinates (29.57148°S 30.32235°E)
Elevation (118 m a.s.l.)
Date (2 February 2012)
Collectors (JD Plisko, A. Mamlala leg.)
Person who classified (JDP det.)

SUPPLEMENTARY MATERIAL 2

Species richness estimates for the sites from the data collected by the method producing the highest number of species: Cedara, Queen Elizabeth Park and Karkloof via digging [A to C] and Botanic Gardens via the mustard solution method [D]).

The analyses were carried out in EstimateS (Colwell 2013) using the default settings. Only specimens that could be identified to species level were included in the analyses. S(est) = estimated number of earthworm species (95% confidence intervals (CI) for S(est) indicated) using rarefaction; ACE = Abundance Coverage-based Estimator (Chazdon *et al.* 1998). The CIs are wider where species richness is higher (Karkloof and Botanic Garden) and do not converge on identified species richness. This, together with the relatively higher ACE mean estimates, indicates undersampling of species.

