# DNA barcoding of different earthworms' species and their response to ecotoxicological testing

**L Voua Otomo**

**23839457**

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| Supervisor: | Prof M Maboeta |
| Co-supervisor: | Prof CC Bezuidenhout |

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# PREFACE

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This work was supported by an MSc bursary from the North-West University, South Africa. I am thankful to the NWU for trusting and supporting me financially.

## DECLARATION

The experimental work conducted and discussed in this dissertation was carried out at the School of Environmental Sciences and Development, Zoology and Microbiology, North-West University, Potchefstroom Campus. This study was conducted under the supervision of Prof. Mark S. Maboeta and Prof. C. Carlos Bezuidenhout.

The study represents original work undertaken by the author and has not been previously submitted for degree purpose to any other university. Appropriate acknowledgements have been made in the text where the use of work conducted by other researchers has been included.

Laetitia Voua Otomo

November 2014

# FOREWORD

This thesis has been written in the form of two publications, with one already accepted and published (*Voua Otomo L., Voua Otomo P., Bezuidenhout C.C., Maboeta M.S. 2013. Molecular assessment of commercial and laboratory stocks of Eisenia species (Oligochaeta, Lumbricidae) from South Africa. African Invertebrates 54 (2), 499-511.*) and the other currently under review (*Voua Otomo L., Bezuidenhout C.C., Maboeta M.S., Voua Otomo P. Evidence of cadmium tolerance in metal-free stocks of the standard test species Eisenia andrei (Oligochaeta) revealed by COI haplotypes*) in Ecotoxicology and Environmental Safety. This thesis is divided into five main parts *viz.* a general introduction (section 1), a general materials and methods providing an overview of the methodology and materials used during the execution of the project (section 2), two main chapters *viz.* section 3 (Molecular assessment of commercial and laboratory stocks of *Eisenia* species (*Oligochaeta, Lumbricidae*) from South Africa) and section 4 (Evidence of cadmium tolerance in metal-free stocks of the standard test species *Eisenia andrei* (Oligochaeta) revealed by COI haplotypes) as well as a general discussion with conclusions and recommendations (section 5).

For all accepted and submitted manuscripts, the contribution of the authors was as follow: The laboratory work was carried out by the first author (Laetitia Voua Otomo) under the supervision of her co-authors Prof. Mark S. Maboeta, Prof. C. Carlos Bezuidenhout, and Dr. Patricks Voua Otomo. All the authors contributed to the writing of the two manuscripts. By signing the present declaration, the co-authors acknowledge giving me permission to submit the two manuscripts as part of my master's degree dissertation.

Prof. Mark S. Maboeta

Prof. C. Carlos Bezuidenhout

Patricks Voua Otomo

Laetitia Voua Otomo

# ABSTRACT

The ecotoxicological literature reveals that countless researchers worldwide rely upon informally identified commercial earthworm stocks for laboratory bioassays. The primary aim of this study was to investigate laboratory and commercial stocks of *Eisenia* species used in South Africa in order to confirm their taxonomy, assess their levels of genetic richness and differentiation. To do so, populations of potential *Eisenia andrei* and *Eisenia fetida* were purchased/obtained from vermiculturists and laboratories from four provinces of South Africa. DNA barcoding was used to investigate these taxonomic uncertainties. The COI gene was partially amplified and sequenced in selected earthworms from eight local populations (focal groups) and two European laboratory stocks (non-focal groups). Only nine COI haplotypes were identified from the 224 sequences generated. One of these haplotypes was found to belong to the Megascolecidae *Perionyx excavatus*. The remaining eight haplotypes belonged to the genus *Eisenia* although only a single *Eisenia fetida* haplotype, represented by six specimens, was found in one of the European populations. The other seven haplotypes, all occurring in South Africa, were *Eisenia andrei*. No *Eisenia fetida* was found in the South African based populations. One of the commercial stocks from South Africa and a laboratory culture from Europe were mixes of *E. andrei - P. excavatus* and *E. andrei – E. fetida* respectively. COI haplotype numbers were limited to two to three distinct sequences within each of the local groups. This translated into a haplotype diversity (H) lower than 0.45 in all the populations, which is very low when compared to other such earthworm studies in which COI polymorphism has been investigated. Of all the local populations investigated, only the lone field population included was genetically divergent from the other populations. This was explained by the haplotype distribution across the populations which indicated that this population was the only one not harbouring the haplotype which represented 75% or more of the COI sequences within the local populations. Because research suggests that earthworm populations with limited genetic diversity may suffer inbreeding depression which could affect traits such as reproduction and survival, the secondary aim was to test whether metal-sensitive earthworms were overly present in the populations investigated. To do so, the three most common COI haplotypes identified between the 8 local populations of *E. andrei* (called Hap1, Hap2 and Hap3) were paired up and exposed to cadmium. A total of six couples were exposed to 0, 25, 50 and 100 mg Cd/kg for 4 weeks at 20°C. The survival, biomass variation, cocoon production and cocoon hatching success were assessed for all the couples. The results indicated that couple 6 (Hap3xHap3) was the most sensitive for three of the endpoints assessed whereas couple 4 (Hap1 x Hap3) was the least sensitive. Cocoon hatching success could not help differentiate the couples. The analysis of Cd tissue contents revealed that with increasing Cd concentration, Cp6 (Hap3xHap3) could accumulate significantly more Cd than any other couple ($p \leq 0.01$). These findings indicate that earthworm populations may carry intrinsically metal-tolerant and metal-sensitive genotypes. In the context of ecotoxicological testing, the present results underline the importance of using genetically diverse populations in laboratory testing as Cp6 (Hap3xHap3) could have suffered from the deleterious effect of inbreeding. Because *E. fetida* could not be found in the local populations assessed, it is recommended that further earthworm DNA barcoding studies, covering a more representative geographical area of South Africa and including more field populations of *Eisenia spp.* be conducted. Because of the occurrence of genetic homogeneity in the populations studied, it is suggested that captive breeding initiatives be established using specimens obtained from several geographically distant field and reared populations. Further research investigating patterns of Cd accumulation/excretion kinetics between the Cd-tolerant and Cd-sensitive individuals reported in the present study, should be conducted to help determine whether inbreeding is the sole factor explaining the observed genotypic responses to Cd. Finally, the necessity of a standardised earthworm barcoding protocol that could help both to properly identify laboratory earthworm stocks and to select genetically diverse stocks suitable for laboratory testing, is discussed together with the relevance of the present work to ecotoxicological testing in general.

**Key terms**: *Eisenia spp.,* DNA barcoding, COI gene, haplotypes sensitivity, genetic homogeneity*,* Cadmium toxicity*.*

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# 1. General Introduction

## 1.1. Earthworm ecology and ecotypes

Earthworms can represent up to 90% of the invertebrate macrofauna in soil (Huang *et al.* 2007) and play an important role in soil ecology (Doube *et al.* 1994, Lazcano *et al.* 2008) and soil quality (Syers and Springett 1884). They break down organic wastes increasing mineralization of the soil, making nutrients available to plant and other soil organisms (Atiyeh *et al*. 2000).

There are three groups or ecotypes of earthworms *viz.* anecic, endogeic and the epigeic earthworms (Bouché 1977, Fraser *et al*. 1998). Each ecotype is characterised by their colour, size and their different lifestyle (Fig. 1).

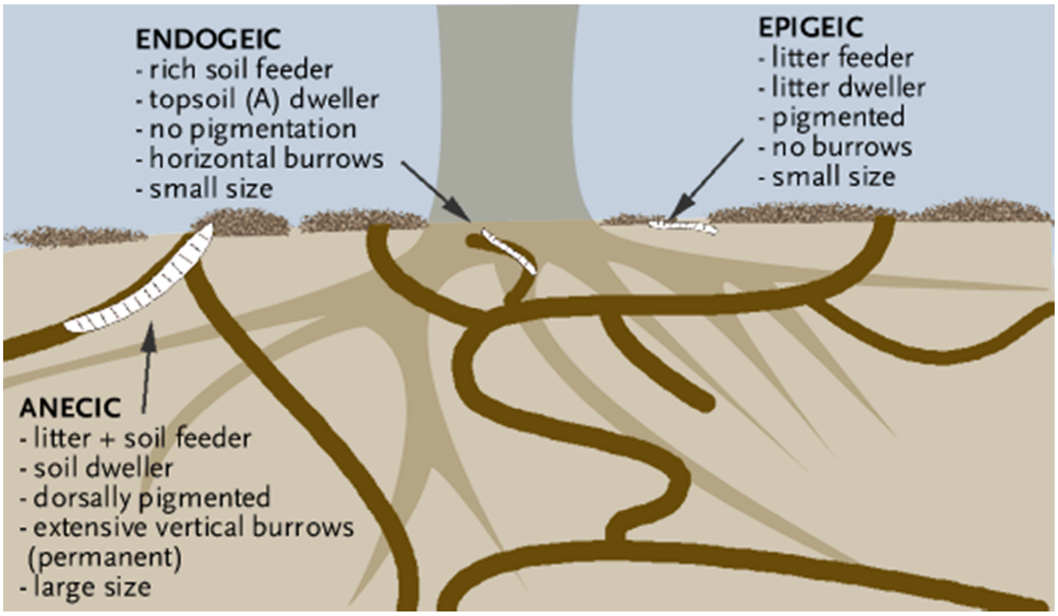

**Figure 1** Earthworms are engineers of the soil altering the forest floor (epigeic earthworms), the mineral soil horizon (endogeic earthworms) or both the forest floor and the mineral floor horizon (anecic earthworms). Source: http://www.nrri.umn.edu/worms/identification/ecology_groups.html

The anecic earthworms are deep burrowers. They make unbranched permanent deep vertical burrows in the soil as deep as two meters (Fraser *et al.* 1998, Stewart 2004). These burrows are believed to increase water infiltration and root growth (Edwards and Shipitalo 1998). Anecic earthworms mainly feed at night on fresh surface litter which they pull down in their burrow (Bouché 1977, Edwards 2004). Their activity affects both the forest floor and the mineral soil (Edwards 2004). Their casts can be found on top of their burrows. They have a long life cycle and a long life span (up to 6 years), but they do not thrive in dense population (Bouché 1977).

Anecic earthworms are big in size (10-25 cm) and like epigeic earthworms, they have a reddish-brown pigmentation (Bouché 1977).

Furthermore, earthworms that live in the top layer (rhizosphere) of the mineral soil are called endogeic (endo = in, geic = earth) (Stewart 2004). Endogeic earthworms dwell and feed on the mineral soil in the area around plant roots in which they create a web of horizontal burrows that aerate the soil thus making it easier for moisture and nutrients to flow through the soil (Stewart 2004). Endogeic earthworms have a pale colour due to the lack of skin pigmentation (they can appear dark when their gut contains soil) and they are 2 to 12 cm in size (Edwards and lofti 1977).

Another group of earthworms, is the epigeic ecotypes. Earthworms that live in leaf litter are called epigeic (epi= top, geic=earth), they do not burrow themselves in the soil but prefer to live and feed on loose decaying organic material such as leaf litter on the surface (Bouché 1977). Though bacteria and fungi are the primary decomposers of the food web (biochemical decomposition), epigeic earthworms may accelerate the decomposition when they are present by fragmenting the organic detritus, thus increasing the area exposed to microorganisms (Lazcano *et al.* 2008, Monroy *et al.* 2008). Epigeic earthworms are small bodied (1 to 7 cm) with a reddish-brown pigmentation. These earthworms are efficiently used in composting (Blakemore, 2000). The litter dwelling species red wiggler earthworms *E. fetida* and *E. andrei* are common epigeic and compost earthworms

### 1.2. Earthworms in vermicomposting

Epigeic earthworms are suitable for vermicomposting (Munroe 2007). As their name suggests the vermicomposting earthworms live in compost bin with a ready supply of organic material (Fraser *et al.* 1998). The organic material becomes vermicompost (also called worm manure) as it passes through the worm digestive system (Edwards *et al*. 2011). After ingestion, compost which contains bacteria from the worm gut, exit the worms through the end of its tail (Cleveland *et al.* 1984). This is a process known as worm composting or vermicomposting. Vermicomposting consists in turning nutrient rich organic material into water-soluble nutrient rich compost (Ndegwa *et al.* 1998). Vermicompost may be used for farming, landscaping or for sale (Kumari 2011). Several earthworm species are used for vermicomposting. They are *E. andrei* Savigny 1826, *E. fetida* Bouché 1972, *Lumbricus. rubellus* Hoffmeister 1843, *Dendrobaena veneta* Rosa 1886, *Perionyx excavatus* Perrier 1872, and *Eudrilus eugeniae* Kinberg 1867*.* However*,* because of their tolerance to temperature fluctuations (Munroe 2007) and their short life cycle (Domínguez *et al.* 2005) *E. andrei* and *E. fetida* are the most common earthworms used in vermicomposting (Sherman 2003).

Vermicomposting has gained popularity for on-site institutional management of food waste (rich in pathogenic agents for instance viruses and bacteria), such as in hospitals and shopping malls as well as for the management of industrial sludge containing toxic pollutants such as heavy metals (Pincine *et al.* 1981; Knight 1989). Developing countries such as India (Suthar *et al.* 2008), Ghana (Mainoo 2008), Malaysia (Azizi *et al.* 2013), and South Africa (Maboeta and van Rensburg 2003a), where the management of organic waste remains an important issue are turning to vermicomposting as an alternative technology for the disposal and recycling of waste.

Vermicomposting is of double interest. First, waste can be converted into good quality vermicompost (pathogens eradication) (Edwards, 1995) and second, it controls toxic pollutants (Yadav 2009). Ndegwa and Thompson (2001) reported that systems that combined conventional composting and vermicomposting were less time consuming and improved the compost quality. Vermicomposting could be more efficient than composting with a significant decrease in pH, organic carbon, C:N ratio and an increase in phosphorus and potash (Maboeta and van Rensburg 2003b; Ponmani 2014, ). Vermicomposting, could also help manage metal contamination. Garg et al (2004) reported that total potassium, total calcium and heavy metals (Fe, Zn, Pb and Cd) were lower in industrial textile mill sludge after vermicomposting.

## 1.3. Earthworms in ecotoxicology

Earthworms are considered to be suitable bioindicators of pollutants in soils. Organisations such as the OECD (Organisation of Economic Cooperation and Development) and the ISO (International Standards Organisation) have recommended selected earthworm species as standard test species for the assessment of chemical toxicity. In earthworm ecotoxicology, key protocols are the earthworms acute toxicity test (OECD 1984, ISO 2012a), the chronic toxicity test (ISO 2012b) and the avoidance behaviour test (OECD 2004a, ISO 2008). *E. andrei* and *E. fetida* are the recommended test species for these bioassays. These species are highly prolific, they reach complete maturity in only 8 weeks and are easily handled in the laboratory (OECD 1984).

### *1.3.1. Earthworm acute and chronic toxicity tests*

During the tests, the earthworms are exposed to concentration range of a selected toxicant mixed in a substrate made of artificial OECD soil (OECD 1984; OECD 2004a). Alterations of the test allow the use of field collected soil (ISO 2012a,b). The endpoints assessed in these tests are mortality, reproduction and biomass variation as well as any corporal damage or behaviour change (OECD 1984, ISO 2012a,b). Recent examples of the use of these tests include Hu *et al.* (2010) who studied the acute toxicity of $TiO_2$ and ZnO NPs on *E. fetida* and found that both chemicals were harmful to the test organism with ZnO NPs being the most toxic, and Alshawish *et al*. (2004) who tested the chronic toxicity of chlorpyrifos on *Aporrectodea caliginosa*, and reported that the fecundity of *A. caliginosa* was reduced in the presence of chlorpyrifos.

### ***1.3.2. Earthworm avoidance behaviour test***

This sublethal test uses a chamber test system in which earthworms are allowed to choose between a control chamber and alternative contaminated chambers. Avoidance of the contaminated substrate by the earthworms is the endpoint assessed (ISO 2008). Avoidance test can help assess potential sublethal stress than neither acute nor chronic test can indicate (Yeardley *et al*. 1996). The first avoidance behaviour study was done on *E. fetida* by Yeardley *et al*. (1996) long before the test was standardised. Since then, many studies have assessed avoidance behaviours in other terrestrial species such as enchytraeids and collembolans (Natal da Luz *et al*. 2004, Amorim *et al*. 2005, Novais *et al*. 2010).

## 1.4. DNA barcoding

The conventional classification of earthworms based on their phenotypes (morphology, behaviour, and habitat) underrates the species diversity of these invertebrates (Pérez-Losada *et al.* 2009). Earthworms lack complex anatomic apparatus hence their morphological characteristics can be barely distinguished between and among species, to allow their classification by the use of morphological taxonomy only (Mayr 1948). The complexes *Apporectodae caliginosa* (Pérez-Losada *et al.* 2009) and *E. fetida/andrei* (Pérez-Losada *et al.* 2005) are good examples in which the traditional taxonomic method failed to delimit between species.

The need for molecular taxonomy in ecotoxicological studies in which closely related species, with often undefined phylogenetic delimitations, are used is recognized by scientists (Pérez-Losada *et al.* 2009). In her study on hormogastrid earthworms, Novo (2010) wrote: "*there is a great deal of work being conducted on the applied ecology and biology of earthworms and without the appropriate species boundaries the results could be confounding*". This, in the light of the difficulty associated to distinguish between certain species based on their morphological characteristics, and many of these species have been reported (King *et al.* 2008; Novo *et al*. 2010). The response of earthworms to chemicals might differ between cryptic species (Römbke and Moser 1999; Andre *et al*. 2010; Sturmbauer *et al*. 1999) and erroneous identification could result in the misinterpretation of the observed effects and conclusions in ecotoxological assessments (Voua Otomo *et al*. 2009).

*E. fetida* and *E. andrei* are both used in standardised ecotoxicological tests and are therefore considered to respond similarly to toxicants (Saint-Denis *et al.* 1999, Ribera *et al.* 2001).

Although cryptic species of *E. andrei* and *E. fetida* have been reported (Pérez-Losada *et al.* 2005) their comparative responses to toxicant exposure has never been investigated. Studies comparing the response of *E. fetida* and *E. andrei* to metal toxicity are scarce (Stenersen *et al.* 1992). Since *E. fetida* and *E. andrei* are both recommended test species by the OECD (OECD 1984 and 2004a) there is a need to know if they would react differently to potential environmental pollutants. Mayr (1948) was the first to report the existence of cryptic species. These are difficult to classify using morphological identification. In the case of earthworms, many of these cryptic species are being described via molecular taxonomy (Novo *et al.* 2010) using DNA barcoding.

DNA barcoding was first proposed by Hebert *et al.* (2003) for the identification of species that are morphologically difficult to characterize or species which taxonomical classification are poor. Huang *et al.* (2007) showed that the DNA barcode approach, developed by Hebert *et al.* (2003), which uses mitochondrial COI gene genotyping "*may provide a useful complement to traditional morphologic taxonomy"*. Hajibabaei *et al.* (2007) showed that barcode sequences from a given species always group together on a tree. Hence, DNA barcoding is a taxonomic method that can allow for the identification of species from a specimen, an organism's remains or environmental samples (Valentini *et al.* 2009). It differs from molecular phylogeny and population genetics in that the aim is not to establish evolution and genetic relationship, but to identify a species from a set of DNA sequences of classified species (a repertory of more than 2000 DNA sequences is available on the Barcode of Life Data Systems (BOLD); www.barcodinglife.org. Rather than determining species relationship, DNA barcode focuses on their identification and delineation (Fig. 2). It relies on a standardised DNA region. A suitable DNA region for barcoding must be short enough to be easily sequenced, must consist of a conserved region for primers to bind and must allow for genetic resolution (Zimmermann *et al.* 2011). In plants the region of choice is the chloroplast ribulose-1,5-bisphosphate carboxylase oxygenase gene (rbcL) and in animals a 658 bp fragment of the 5'end of the gene encoding the mitochondrial cytochrome C oxidase 1 (COI) (Zimmermann *et al.* 2011; Hajibabaei *et al.* 2007; Valentini *et al.* 2009).

Though DNA barcoding cannot replace molecular phylogenetics or population genetics it is a rapid and reliable assay that can provide basic knowledge that would be useful in the selection of specimen for further analyses.

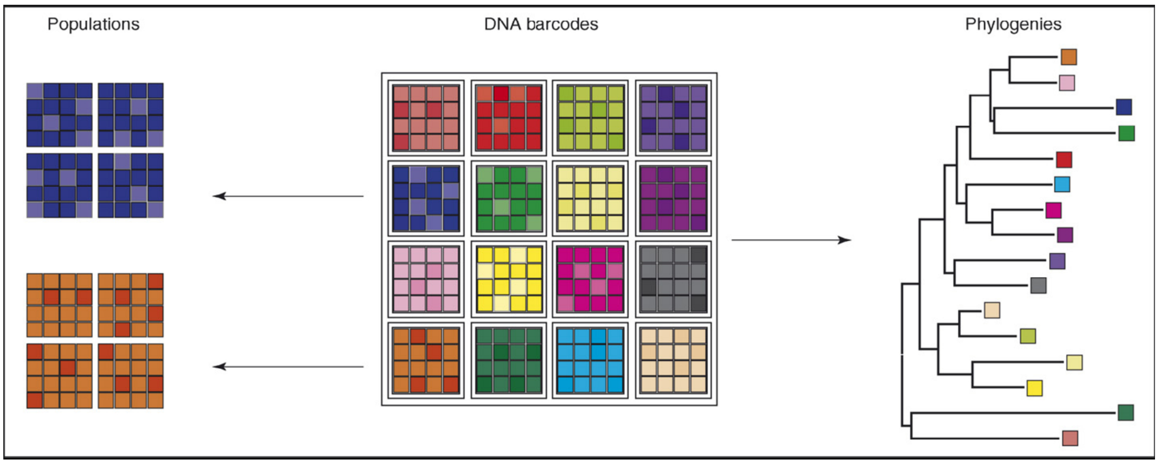

**Figure 2** Comparison of population genetics, phylogeny and DNA barcode. Rather than determining species relationship, DNA barcode focuses on their identification and delineation. From Hajibabaei *et al*. (2007).

### 1.5. Heavy metal pollution

Heavy metals are metals with molecular weight between 63.5 $g.mol^{-1}$ and 200.6 $g.mol^{-1}$ and a specific gravity superior to 5.0 (Fu and Wang 2011). Heavy metal emissions are a threat for the ecosystem because unlike many organic wastes, heavy metal cannot be degraded (Martín-González *et al*. 2006). Heavy metals can be emitted into the environment by both natural and anthropogenic activities. The main sources of metals in nature are rocks, sea-salt emissions, volcano eruptions, windblown dust, forest wildfires and vegetation (Athar and Vohora 1995).

Anthropogenic sources of heavy metals include mining, electric power stations, oil and coal-fired power plants, industrial, commercial and residential boilers (Brad 2005). Mining is the main source of heavy metals from anthropogenic activities. Heavy metal pollution that occurs as a result of mining persist for hundreds of years after the end of mining activities (Peplow 1999). Mercury (Hg), cadmium (Cd) and lead (Pb) have been listed as the most hazardous heavy metals to humans and ecosystems (Forbes and Forbes 1994). It has been estimated that during production, 5-10% of the total production of metals such as Cd, Cu, Pb, Cr and Zn are discarded as waste or part thereof and eventually loaded into soils (Nriagu & Pacyna (1988). According to these authors, this wastage rate is even higher (10-15%) for Hg and selenium (Se).

Agriculture, through the use of fertilizers, pesticides and herbicides is also a significant source of heavy metal pollution. For instance, the detrimental effects of the fungicide copper oxychloride have been established in non-target soil organisms such as earthworms (Helling *et al.* 2000;

Maboeta *et al.* 2002; Maboeta *et al.* 2004). Plachy (1997) stated that, through the use of fertilizers alone, approximately 2 600 tons of Cd are released into the ground each year.

Heavy metal pollution is a source of concern because plants and organisms including fungi, bacteria, algae and earthworms can accumulate these metals in their system (Memon *et al*. 2001) and concomitantly into the food chain resulting in environmental problems and various human illnesses (Martín-González *et al*. 2006).

### 1.6. Essential and non-essential metals

Some heavy metals are required at relatively low concentrations by living organisms. They are called micronutrients or essential metals (Blanco-Penedo *et. al.* 2006). These metals e.g. Na, K, Ca, Mn, Fe, Co, Cu, Zn, and Mo are essential for the functioning of biological systems (Alloway 1990). They occur naturally in the body and need to be continually absorbed through diet. Their deficiency triggers structural or functional abnormalities that are reversible by the re-introduction of the essential metal. However, essential heavy metals are toxic when nutritional allowance is exceeded (Alloway 1990; Srivastava 2008).

Non-essential metalshave no known nutritional benefits e.g. Pb, Li, Sr, As, Au, Sn, Ag, Be and Hg (Alloway 1990). Though until recently Cd has been thought to play no biological role and has been known only for its toxicity, it has lately been found that in the phytoplankton *Thalassiosira weissflogii*, carbonic anhydrase, exchanges Zn or Cd at its active centre. This important reaction that catalyses the hydration of carbon dioxide is essential for the growth of diatoms which grow in Zn depleted seawater (Lane 2000). Non-essential metals are harmful because they can alter biological processes. For example Cd has the possibility to replace zinc in Zn-containing metalloenzyme, this impairs the catalytic activity of the enzyme leading to serious health problems in human and other living organisms (Brzóska 2001).

### 1.7. Cadmium

In nature most of the cadmium comes from igneous rocks (containing up to 0.3 mg/kg), metamorphic rocks (containing up to 1mg/kg) and sedimentary rocks (containing up to 11 mg/kg). Being 67th in the classification of metal abundance, Cd is a rather rare element (Alloway 1990). However concentrations of cadmium in the environment have increased due to its rise in industrial use. Cadmium is used as a stabiliser in PVC materials, in the fabrication of Nickel-cadmium batteries, as an anticorrosive agent, as a colour pigment and in phosphate fertilizers (Godt *et al*. 2006). Dumping and incineration of cadmium-containing waste is the main source of the abundant Cd in the environment. Cadmium is among the most important environmental pollutants (Lee 1985; Forbes and Forbes 1994). Its half-life in soils is between 15 to 1100 years.

Cadmium has been linked to human health complications affecting the renal and respiratory systems (Nawrot *et al.* 2006). Research evidence suggested Cd is a carcinogen (Plachy 1997; Nawrot *et al.* 2006).

In nature, it could affect a wide number of organisms including plants (Carpena *et al* 2003; Nouairi *et al* 2006); insects (Cervera *et al.* 2004), amphibians (Loumbourdis *et al.* 1999) and rats (Kim *et al.*1998; Lafuente & Esquifino 2002)

Earthworms in general, have been shown to bioaccumulate Cd (Klerks & Bartholomew 1991; Morgan & Morgan 1999). Several studies have investigated the effects of Cd on earthworm species such as *D. veneta, E. fetida and E. andrei,* (Bengtsson & Rundgren 1992; Spurgeon *et al.* 1994; Reinecke & Reinecke 1996; Reinecke *et al.* 1999) and reported a range of effects including homeostatic unbalance (Reinecke *et al.* 1999); deterioration of the ovarian structure (Siekierska & Urbanska-Jasik 2002), degeneration of the nephridia (Prinsloo 1999), decrease in survival, reproduction and cocoon hatching rates (Bengtsson & Rundgren 1992; Spurgeon *et al.* 1994).

Furthermore, Reinecke *et al.* (1999) reported that *E. fetida* could develop increase resistance to Cd after long-term exposure to 0.01% $CdSO_4$. Follow-up studies found that although increased tolerance to Cd was also found at biomarker level (Voua Otomo and Reinecke 2010), this could not be confirmed at molecular level after a study of allozymes polymorphism (Voua Otomo *et al.* 2011). A biomarker can be defined as a biological response that is expressed at the physiological or molecular level following a stimulus by a toxicant (Van Gestel and Van Brummelen 1996).

### 1.8. Aims

The overall goal of this study was to utilise DNA barcoding to investigate the taxonomy of *Eisenia* species found in South Africa (vermiculture establishments and ecotoxicology laboratories) and to evaluate the response of the identified species and their genotypes to different cadmium concentrations. .

Specific objectives were as follows:

a. Utilising DNA barcoding in order to identify populations of *E. andrei*, *E. fetida* and their potential cryptic species in commercial and laboratory earthworm stocks from different provinces of South Africa.
b. Investigate the level of genetic richness and differentiation within the conspecific populations identified.

c. Assess the effects of Cd on the growth, reproduction and cocoon hatching of the identified species *viz. E. andrei* and *E. fetida*.
d. Evaluate potential differences in Cd sensitivity between conspecific earthworms carrying the genotypes identified.

# 2. General materials and methods

This chapter is an overview of the materials and methods used during this project.

## 2.1. Test earthworms

*E. fetida and E. andrei* are the two recommended earthworm species by the Organization of Economic Cooperation and Development (OECD) for the screening of chemicals (OECD 1984; OECD 2004a). These two species were first described as two morphotypes of the same species due to their close morphological resemblance. Their differences reside mainly in their pigmentation; *E. fetida* is striped, and has a yellowish coloration around the intersegmental groove which makes it appear to be pale, while *E. andrei* is uniformly red (Dominguez *et al*. 2005). André (1963) was the first to recognize them as potential different species which was confirmed later by gel electrophoresis that showed different migration patterns of their esterases (Øien and Stenersen 1984). This was conclusively corroborated by Dominguez *et al*. (2005) who showed that the offspring from *E. fetida* and *E. andrei* were not viable which meant that they were two different biological species.

### *2.1.1. The classification of Eisenia fetida and Eisenia andrei*

*Eisenia spp* are iteroparous individuals that can produce several hatchlings per cocoon. *E. fetida* and *E. andrei* are among the different species found in the *Eisenia* genus which is part of the phylum of Annelida. Annelida are further divided into three subclasses: polychaeta, Oligochaeta and the Hirudinea. *E. fetida and E. andrei* belong to the subclass Oligochaeta and like most earthworms are dioecious with a give-and-take model of insemination (Monroy *et al*. 2005).

According to Bouché (1972) and Wetzel and Reynolds (2012) the classification of *E. fetida* is as follow:

Phylum: Annelida
Subphylum: Clitellata
Class: Oligochaeta
Order: Haplotaxida
Suborder: Lumbricina
Superfamily: Lumbricodea
Family: Lumbricidae
Subfamily: Lumbricinae
Genus: *Eisenia*
Species: *Eisenia fetida*

According to Bouché (1972) the classification of *E. andrei* is as follow:

Phylum: Annelida
Subphylum: Clitellata
Class: Oligochaeta
Order: Haplotaxida
Suborder: Lumbricina
Superfamily: Lumbricodea
Family: Lumbricidae
Subfamily: Lumbricinae
Genus: *Eisenia*
Species: *Eisenia andrei*

### *2.1.2. The biology of Eisenia fetida and Eisenia andrei*

Their life cycle of both worms includes different stages namely the cocoon, juvenile, preclitellate and mature stages (Monroy *et al*. 2006). Mature earthworms possess a clitellum which produces the cocoons after mating. The number of hatchlings in a cocoon varies among species (Stephenson 1972; Monroy 2006) and seasonal changes (Stephenson 1972).

#### *2.1.2.1. Eisenia fetida*

*E. fetida* was first described by Savigny in 1826. This earthworm owes its name to the strong smell it expels when under threat. It is 35-130 mm long, weights between 200-600 mg, and has 80-120 segments (Bouché 1972). In mature specimens of *E. fetida* the clitellum (reproductive gland) a saddle-shaped (or annular in other earthworms) swelling located on the anterior part of the body is found on segment 24, 25, 26, 27-31, 32, 33 (Grove and Cowley 1927; Martin 1977); and the tubercula pubertatis, glands located on both side of the clitellum, are found on segment 27, 28-30, 31, 32 (Martin 1977). The first cocoons are released four days after copulation. Each cocoon has an incubation period of ±23 days and hatchlings reach maturity (fully developed clitellum) within 40 to 60 days (Fig. 3) (Venter and Reinecke 1988).

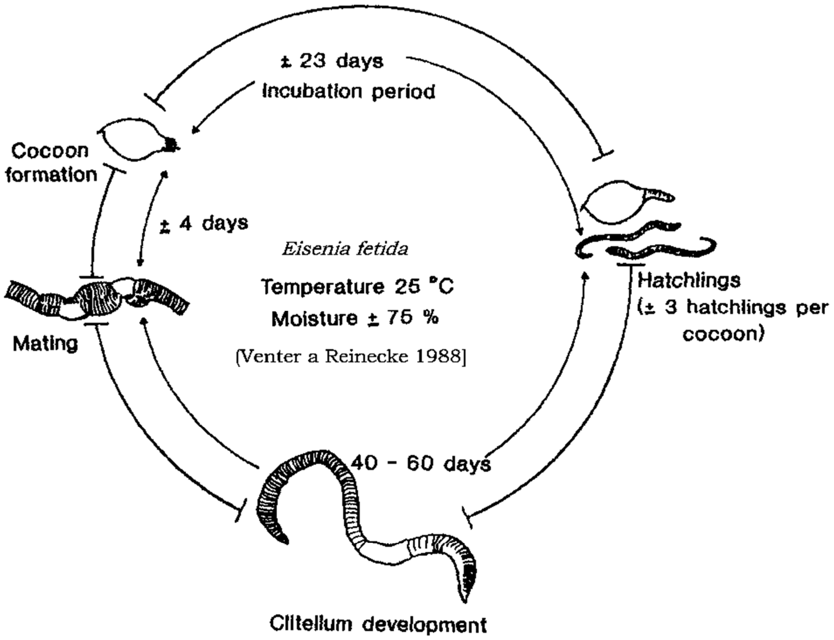

**Figure 3** The life cycle of *E. fetida.* Mature earthworms possess the clitellum which produces the cocoons after mating. The first cocoons are released four days after copulation. Each cocoon has an incubation period of ±23 days and hatchlings reach maturity (fully developed clitellum) within 40 to 60 days (Venter and Reinecke 1988).

*E. fetida* is epigeic which means that it is mainly found in compost, manure and other organic build up rather than burrowed underground (Aira *et. al.* 2008). *E. fetida* is believed to be a corticolous species originally owing to its flattened shape and its epigeic nature (Bouché 1972). It differentiates from its closely related species *E. andrei* by its light red pigmentation, its striped morphology and the apparent lack of pigmentation in its intersegmental groove and though both species are amphimictic species *E. fetida* can also self-inseminate (Dominguez *et al.* 2003).

#### *2.1.2.2. Eisenia andrei*

*E. andrei* was first described by Bouché (1972). *E. andrei* mainly differs from *E. fetida* by its wine-like red pigmentation and the absence of stripes. *E. andrei* and *E. fetida* are otherwise difficult to distinguish by the use of the morphological characteristics used in traditional taxonomy such as the number of segment, the type and position of the setae, the shape (cylindrical or flattened) and length of the body, the shape of the prostomium (a sensory device anterior to the mouth), the peristomium (the first body segment, which contains the mouth), the position of the clitellum on the body, and the type of the tubercula pubertatis. Nevertheless, their segmentation process has been recognized to be different in the early stage of their development (Devries 1968), and a few differences at the reproduction level have been reported. According to Domínguez *et al*. (2003), *E. andrei* reproduces at a higher rate than *E.fetida*.

## 2.2. DNA purification, amplification and sequencing

In order to perform DNA barcoding, DNA was extracted from selected earthworm and purified. Mitochondrial COI gene was amplified through a PCR cycle and sequenced as described further down. Specific details regarding the origin and the number of specimens studied are provided in the pertaining chapters.

### *2.2.1. DNA extraction*

DNA extraction was done according to the method described by Maniatis *et. al.* 1982. For this purpose the NucleoSpin®Tissue Kit was used. The kit comprises buffer B1, buffer B2, buffer BE, buffer T1, buffer BW, Lysis buffer B3, Wash buffer B5, Proteinase K, Proteinase buffer PB, NucleoSpin®Tissue columns and collection tubes. Approximately 0.1 to 0.2 cm of the tail of the earthworms were cut off with a surgical blade and placed separately in 2ml Eppendorf tubes. 180 µl of buffer T1 and a 25µl of proteinase K solutions were added to the Eppendorf, vortexed, and incubated overnight at 56°C using a bain-marie. The following day, the samples were vortexed and lysed by adding 200µl of buffer B3 and vigourously vortexing again. Afterwards, the samples were incubated at 70°C for 10 min on a heating block, vortexed briefly, and DNA binding conditions were adjusted by adding 210µl of ethanol (96-100%) and then vortexing vigourously. For each of the samples, one NucleoSpin®Tissue column was placed into a collection tube and the sample was applied to the column, centrifuged for 1 min at 11,000 x g, the flow-through was discarded and the column put back in the collection tube. The Silica membrane, now embedded with the DNA, was then washed: first, with 500µl of buffer BW and then, with 600µl of buffer B5. Each wash was followed by a 1 min centrifugation at 11,000 x g. After the flow-through was discarded the silica membrane was dried by centrifugating the column for 1 min at 11,000 x g. Each of the NuceloSpin®Tissue columns were placed into a new clean 1.5 ml Eppendorf tube and 100µl of pre-warmed BE buffer (70°C) were added, incubated for 1 min at room temperature and centrifuged for 1 min at 11,000 x g. The NuceloSpin®Tissue columns were discarded and the 1.5 ml Eppendorf tube containing the eluted DNA was preserved at -20°C.

### 2.2.2. DNA amplification and sequencing

DNA amplification using polymerase chain reaction (PCR) was carried out as described by Voua Otomo *et al.* 2009. For this purpose, 0.3 µl of the pure DNA from each microcentrifuge tubes were pippetted into PCR tubes and a Mix of 12.5 µl of PCR Master Mix (PCR MM) (Fermentas), 11µl of Nuclease-free $H_2O$ and, 0.5 µl of primers (Reverse:HCO2198, Sequence: TAA ACT TCA GGG TGA CCA AAA AAT CA, Forward: LCO1490, Sequence: GGT CAA CAA ATC ATA AAG) was added to each microcentrifuge tubes. The following PCR cycle was used for the amplification of the COI gene: An initial denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 30s, 50°C for 30s and 72°C for 45s. A final extension step at 72°C for 5 min completed the reactions.

Successful amplification was verified by electrophoretic means using agarose gels (0.75g SeaKem® LE Agarose, Lonza- in 50ml TAE buffer, 1.5% w/v) stained with 5µl ethidium bromide (0.1 µl EtBr /ml TAE buffer). Sequencing reactions were performed using the ABI v3.1 BigDye® kit. Purified sequences were run on an ABI 3500XL Genetic analyser.

All the sequences were aligned, edited and analysed in MEGA v5 (Tamura *et al.* 2011) using the Kimura-2-parameter (K2P) method (Kimura 1980). The Contrib software of Petit *et al.* (1998) was used to assess haplotypic richness and diversity contribution after rarefaction (used in case of different sample sizes). The software package NETWORK 4.6.1.0 (Fluxus Technology Ltd) was used to compute a haplotype network of the distinct Eisenia sp. COI sequences occurring in SA using the Median-joining (MJ) method.

## 2.3. Earthworm bioassays

### 2.3.1. Soil and selected toxicants

For ecotoxicological testing, all experiments were conducted in OECD artificial soil (OECD 1984). The OECD soil was made of 10% finely grounded sphagnum peat, 20% kaolin clay, and 70% silica sand. The soil components were mixed and ±0.6% of calcium carbonate ($CaCO_3$) was added to adjust the pH to 6 ± 0.5.

Cadmium sulfate ($CdSO_4$), the selected toxicant, was diluted in de-ionised water and mixed with the artificial soil to reach 60% of the soil water holding capacity (WHC). The WHC of the artificial soil was determined as follow: Soil samples (contained in plastic pipes sealed on one end with Whatman no. 1 filter paper) were saturated with water for three hours by immersing the sealed ends of the plastic pipes in water. Thereafter, the soil samples were put on moist sand and left to drain for two hours. Then, using an electronic moisture analyzer (Sartorius MA 35), the quantity of water needed to achieve 100% WHC was determined. Knowing the quantity of water necessary to obtain 100% WHC, the volume of water necessary to reach a final WHC of 60% was determined.

The diluted $CdSO_4$ was added to the soil, and 100 g dry mass of the artificial soil was placed in plastic containers (15 cm x 8 cm) and sealed with a perforated transparent cover.

### *2.3.2. Cd exposure*

Exposure to Cd was carried out according to the OECD acute toxicity test (1984).

Following DNA barcoding, the three most common COI haplotypes identified across populations (called Hap1, Hap2, and Hap3) were coupled. The wet weight of the individual, in each couple, was between 250 and 600 mg. The couples were made of the following COI haplotypes:

1. Couple1 (Cp1): Hap2 x Hap2
2. Couple2 (Cp2): Hap1 x Hap2
3. Couple3 (Cp3): Hap2 x Hap3
4. Couple4 (Cp4): Hap1 x Hap3
5. Couple5 (Cp5): Hap1 x Hap1
6. Couple6 (Cp6): Hap3 x Hap3

The six couples were exposed, separately, in triplicate to 0, 25, 50 and 100 mg Cd/kg for 4 weeks at 20°C. The hatching success of the collected cocoons was also monitored by keeping the cocoons in deionised water at 20°C for a further 28-day period after the Cd experiment. The concentration range of Cd was chosen to be mostly sub lethal and to permit some level of reproduction in the experimental treatments. Cadmium $LC_{50}$ in *E. fetida* has been estimated at 900 mg/kg by Song *et al.* (2002).

### *2.3.3. Endpoints*

During the experiment, the earthworm couples were fed a gram of uncontaminated horse manure weekly. The survival, biomass variation and cocoon production of each couple were assessed weekly for 4 weeks. The hatching success of the cocoons was also monitored.

Survival was assessed by scrutinising the earthworms for any damage, death or change in behaviour. Biomass variation was evaluated by comparing the earthworm actual biomass to the earthworm biomass on the first day of exposure to the metal. Cocoon production was assessed by comparing the production of cocoons at concentrations 25, 50 and 100 mg Cd/kg to the control (0 mg Cd/kg) cocoon production. The hatching success of the cocoons was also monitored by comparing the hatchings success at concentrations 25, 50 and 100 mg Cd/kg to the control (0 mg Cd/kg) hatching success.

### 2.4. Metal analysis

At the end of the exposure period, all the worms were processed for metal analysis following the protocol of Katz and Jennis (1983.). Whole worms were incubated, separately, in 10 ml (55%) nitric acid and left overnight at room temperature for digestion. The following day the samples were heated at 40 and 60°C for 2 h and then for 1 h at 120°C. The samples were then left to cool down at room temperature for 1 h, and 5 ml perchloric acid (70%) were added to each samples, and the mixture was heated to 120°C for 1 h. The mixture was left to cool down for 1 h and 5 ml of distilled water were added to each sample before being reheated to a temperature of 120°C until the emission of white fumes. The Samples were then left to cool down overnight at room temperature. Thereafter, the samples were filtrated through Whatman no. 6 filter paper into 100 ml flasks and filtered again through 0.45-lm Sartorius cellulose nitrate microfilters. Afterwards, all samples were kept in dark plastic containers until Cd tissue contents were measured in the worms exposed to the different treatments with an Agilent 7500c Inductive coupled plasma mass-spectrometer (ICP-MS). For quality control, I assessed the percent recovery of a Cd standard purchased from Sigma-Aldrich (http://www.sigmaaldrich.com). The analysis of the reference sample indicated that the percent recovery was 85.7 ± 13.4 %. The detection limit (ppb) was 0.1. The tissue concentration of the metal was calculated using the following equation:

$$[C] \times Vs / Ms$$

Where, [C] is the concentration as obtained by the ICP-MS reading, Vs is the volume of the sample and Ms the mass of the earthworm.

### 2.5. data analysis

SigmaStat® was used to analyse the data. The data were tested for normality using the Kolmogorov–Smirnov test. Normally distributed data were analyzed using a parametric multiple test (One-way ANOVA, followed with Bonferroni posttest). Non-parametric data were analyzed using the Kruskal-Wallis ANOVA followed by Dunns' test. $EC_{50}$ indices were estimated using non-linear regression analyses in IBM SPSS: IBM SPSS Statistics Version 21. The level of statistical significance was $P < 0.05$.

# 3. Molecular assessment of commercial and laboratory stocks of Eisenia species (Oligochaeta, Lumbricidae) from South Africa

*The present chapter has been published in African Invertebrates: Voua Otomo L., Voua Otomo P., Bezuidenhout C.C., Maboeta M.S. 2013. Molecular assessment of commercial and laboratory stocks of Eisenia species (Oligochaeta, Lumbricidae) from South Africa. African Invertebrates 54 (2), 499-511.*

## 3.1. Introduction

*E. fetida* Savigny 1826 and *E. andrei* Bouché 1972 have become cosmopolitan earthworm species because of their worldwide use in ecotoxicological testing and vermicomposting. Originating from Palearctic Europe, they have been successfully introduced to other ecozones mainly because of their wide temperature tolerance and robustness (Hendrix *et al*. 2008). Both *E. fetida* and *E. andrei* are the recommended earthworm species for the testing of chemicals by the European Organisation of Economic Cooperation and Development (OECD 1984, 2004a) and the International Standards Organisation (ISO 2005; 2011).

Historically, Savigny only described *E. fetida* (*E. foetida*) which was later suspected of harbouring a cryptic sister species. Bouché (1972) divided *E. fetida* into two subspecies; E. *foetida foetida* (current *E. fetida*) and E. *foetida unicolour* (current *E. andrei*). Using allozymes polymorphism, Jaenicke (1982) and Øien & Stenersen (1984) indicated that these subspecies were different species. Their findings were supported by Domínguez *et al*. (2005) and Pérez-Losada *et al*. (2005) who concluded that *E. fetida* and *E. andrei* were different biological and phylogenetic species based on their reproductive isolation and DNA divergence.

In South Africa (SA) and the rest of the world, *E. andrei* and *E. fetida* are used in the vermiculture industry and scientific research. Two South African research laboratories in the field of terrestrial ecotoxicology at Stellenbosch University (http://academic.sun.ac.za/botzoo/) and North-West University (http://www.nwu.ac.za/node/6176) have used *E. fetida* and *E. andrei* for decades and the output of their research has been published in the local and international scientific literature (Reinecke & Viljoen 1991; Reinecke & Reinecke 1997; Prinsloo *et al*. 1999; Reinecke *et al*. 2001; Reinecke *et al.* 2002; Maboeta and Van Rensburg 2003b; Maboeta *et al*. 2008; Owojori *et al*. 2009; Voua Otomo and Reinecke 2010)

Despite the interest in both these species, there has been no molecular study of locally introduced populations and cultures of *E. andrei* and *E. fetida*. Molecular studies on selected laboratory and field populations have focused on the toxicological effects of selected toxicants on the DNA integrity and allozymes polymorphism in these earthworms (Reinecke and Reinecke 2004; Voua Otomo *et al.* 2011). Voua Otomo *et al*. (2009) conducted a DNA barcoding study on an *Eisenia sp.* laboratory stock housed in the Zoology Department of Stellenbosch University as a means of confirming its taxonomy.

The need for molecular studies on these earthworms is critical for several reasons. Being economically and scientifically important, basic information such as species identity and the genetic differentiation between *Eisenia sp.* stocks should be relevant to the breeders, the potential buyers and the researchers alike. The ecotoxicological literature reveals that countless researchers worldwide rely upon informally identified commercial earthworm stocks for laboratory bioassays (see for instance Beyer 1996; Fitzpatrick *et al*. 1996; Saint-Denis e*t al.* 1998; Krauss *et al.* 2000; Gevao *et al.* 2001; Miyazaki *et al.* 2002; Gambi *et al*. 2007; Lin *et al*. 2010).

Moreover, earthworm cultures kept isolated for many generations may, with time, suffer from inbreeding depression characterized by low heterozygosity (Voua Otomo et *al.* 2011). This may undermine sustainable earthworm breeding and quality research.

The aim of this study was to conduct a DNA barcode study of earthworm stocks from selected vermiculture establishments and research laboratories from SA in order to confirm their taxonomy, assess their levels of genetic richness and differentiation.

## 3.2. Materials and Methods

### *3.2.1. Earthworm populations*

The term “population” is used, in the present study, in a more inclusive manner and may refer to a free-living “wild” population or to a captive breeding stock. A total of 8 focal and 2 non-focal populations were included in this study. Focal populations included two vermiculture stocks from Johannesburg (Gauteng, SA); two vermiculture stocks and a laboratory culture from Potchefstroom (North-West, SA), a free-living population and a laboratory culture from Stellenbosch (Western Cape, SA) and a vermiculture stock from Port Elizabeth (Eastern Cape, SA). Two non-focal laboratory cultures were acquired from Brno (Czech Republic) and Southampton (England). Table 1 provides the presumed identities (as given by the owners) of the respective earthworm groups, their geographical locality, their function/use and, when applicable, an excerpt of the list of fairly recent publications based upon research works done on the populations.

**Table 1** Localities, presumed identity, use and publications record of the earthworm groups included in the present study. n indicates the number of specimens used for COI genotyping from the respective groups. ANs indicates the Genbank accession numbers for the respective sequences.

| Population | Type/use | Origin/Locality | Code | *n/ANs* | Publication record -selected papers- |
|---|---|---|---|---|---|
| Stellenbosch 1<br>Presumed ID: *E. fetida* | Laboratory | Stellenbosch University, Stellenbosch<br>(33°56’3.4” S, 18°51’56.4” E)<br>Western Cape, South Africa | SUN | 44/JN870005-JN870024 DQ914618-DQ914633<br>JX912906- JX912913 | Reinecke and Reinecke 2003<br>Maboeta *et al.* 2004;<br>Reinecke and Reinecke 2004;<br>Maleri *et al.* 2007;<br>Maleri *et al.* 2008;<br>Owojori *et al.* 2009;<br>Owojori *et al.* 2010 |
| Stellenbosch 2<br>Presumed ID: *E. fetida* | Field | Middelvlei wine farm, Stellenbosch<br>(33°55’84” S, 18°49’87” E)<br>Western Cape, South Africa | MID | 19/JN870048-JN870066 | Voua Otomo and Reinecke 2010;<br>Voua Otomo *et al.* 2011 |
| Potchefstroom 1<br>Presumed ID: *E. fetida* | Laboratory | North-West University, Potchefstroom<br>(26°41’21” S, 27°05’26” E)<br>North-West, South Africa | NWU | 45/JN870025-JN870047<br>JX912898- JX912905<br>JX908652- JX908665 | Maboeta and Van Rensburg 2003a; Maboeta and Van Rensburg 2003b; Maboeta *et al.* 2008 |
| Potchefstroom 2<br>Presumed ID: *E. fetida* | Vermicomposting | Grimbeek Park, Potchefstroom<br>(26°43’29” S, 27°06’48” E)<br>North-West, South Africa | GRM | 22/JN870067-JN870088 | none |
| Potchefstroom *3*<br>Presumed ID: *E. fetida* and *E. andrei* | Vermicomposting | Mieder Park, Potchefstroom<br>(26°43’15.6” S, 27°06’06” E)<br>North-West, South Africa | MPP | 11/ JX908641- JX908651 | none |
| Port-Elisabeth<br>Presumed ID: *E. fetida* and *E. andrei* | Vermicomposting | Newton Park, Port-Elisabeth<br>(33°56’57.1” S 25°33’35.0”E)<br>Eastern Cape, South Africa | PE | 21/ JX908692- JX908712 | none |
| Johannesburg 1<br>Presumed ID: *E. fetida* | Vermicomposting | Ferndale, Johannesburg<br>(26°6’1.7”S 28°0’8.6”E)<br>Gauteng, South Africa | JNB | 16/ JX899807- JX899822 | none |
| Johannesburg 2<br>Presumed ID: *E. fetida* | Vermicomposting | Morningside, Johannesburg<br>(26°4’52.5”S 28°3’44.4”E)<br>Gauteng, South Africa | JOZ | 10/ JX912888- JX912897 | none |
| United Kingdom<br>Presumed ID: *E. fetida* | Laboratory | Southampton<br>(50°54’34” N, 1°24’15” W)<br>United Kingdom | ENG | 26/ JX908666- JX908691 | none |
| Czech Republic<br>Presumed ID: *E. andrei* | Laboratory | Brno<br>(49°11’42” N, 16°36’24” E)<br>Czech Republic | CZR | 10/JN869995- JN870004 | none |

Due to the economic importance of *Eisenia sp.* and because potential earthworm buyers are mostly unable to distinguish between different earthworm species, the randomly picked local specimens were not sorted according to phenotypic features, thus allowing us to identify possible mixed cultures.

### *3.2.2. COI genotyping*

Total genomic DNA was extracted from a total of 224 earthworms using the NucleoSpin® Tissue kit (Macherey-Nagel). Five to ten milligrams of the tail section of the selected specimens were treated according to the manufacturer's instructions. The universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994) were used to amplify 683 bp of the cytochrome oxidase I (COI) gene.

PCR reactions consisted of 0.3 µl (±30 ng) DNA template, 12.5 µl PCR Master Mix (Fermentas), 11 µl nuclease free water (Fermentas) and 10 pmol (~1µl) of each of the primers. PCR cycling comprised an initial denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 30s, 50°C for 30s and 72°C for 45s. A final extension step at 72°C for 5 min completed the reactions.

Successful amplification was verified by electrophoretic means using agarose gels (0.75g SeaKem® LE Agarose, Lonza- in 50ml TAE buffer, 1.5% w/v) stained with 5µl ethidium bromide. Sequencing reactions were performed using the ABI v3.1 BigDye® kit. Purified sequences were run on an ABI 3500XL Genetic analyser.

All the barcodes generated in the present study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank; accession numbers provided in Table 1). They were tentatively identified using the BOLD Identification System (Barcode of Life Data Systems, http://www.boldsystems.org) and compared to published COI sequences of *E. andrei, E. fetida* and *Allolobophoridella eiseni* deposited in GenBank by Pérez-Losada *et al.* (2005).

All the sequences were aligned, edited and analysed in MEGA v5 (Tamura *et al.* 2011) using the Kimura-2-parameter (K2P) method (Kimura 1980). A neighbour-joining (NJ) trees was subsequently constructed. Bootstrap support was obtained from 1,000 iterations. Since COI diversity would be highly dependent on effective population size and because of the uneven sample sizes of the groups included in this study, the Contrib software of Petit *et al.* (1998) was used to assess haplotypic richness and diversity contribution after rarefaction. The software package NETWORK 4.6.1.0 (Fluxus Technology Ltd) was used to compute a haplotype network of the distinct *Eisenia sp.* COI sequences occurring in SA using the Median-joining (MJ) method.

## 3.3. Results

### *3.3.1. K2P based analysis*

Nine distinct sequences of the COI gene were identified in the 224 worms investigated in this study. The haplotype distribution across the populations revealed that, H1 (haplotype 1) was the most widespread and H2 was the most frequent, representing more than 70% of all the COI sequences (Table 2). Five haplotypes were unique to their population of origin *viz.* H4 (JNB; Johannesburg), H6 (SUN; Stellenbosch University), H7 (NWU; North-West University), H8 (PE; Port Elizabeth) and H9 (ENG; Southampton).

**Table 2** Haplotype distribution and frequency across all the populations investigated. H2 was the most frequent haplotype representing more than 70% of all the COI sequences

| Haplotypes<br>Populations | H1 | H2 | H3 | H4 | H5 | H6 | H7 |
|---|---|---|---|---|---|---|---|
| GRM | 18.2% (n = 4) | 81.8% (n = 18) | 0 | 0 | 0 | 0 | 0 |
| JHB | 12.5% (n = 2) | 81.25% (n = 13) | 0 | 6.25% (n = 1) | 0 | 0 | 0 |
| JOZ | 10% (n = 1) | 80% (n = 8) | 0 | 0 | 10% (n = 1) | 0 | 0 |
| MID | 94.74% (n = 18) | 0 | 0 | 0 | 5.26% (n = 1) | 0 | 0 |
| MPP | 9.1% (n = 1) | 90.9% (n = 10) | 0 | 0 | 0 | 0 | 0 |
| NWU | 15.56% (n = 7) | 77.78% (n= 35) | 4.44% ( n = 2) | 0 | 0 | 0 | 2.22% (n = 1) |
| PE | 15% ( n = 3) | 75% (n = 15) | 10% ( n = 2) | 0 | 0 | 0 | 0 |
| SUN | 4.5% (n = 2) | 91% (n = 40) | 0 | 0 | 0 | 4.5% (n = 2) | 0 |
| ENG | 10% (n = 2) | 80% (n = 16) | 0 | 0 | 10% (n = 2) | 0 | 0 |
| CZR | 40% (n = 4) | 50% (n = 5) | 10% (n = 1) | 0 | 0 | 0 | 0 |
| Total | n = 44 | n = 160 | n = 5 | n = 1 | n = 4 | n = 2 | n = 1 |

The analysis of all the haplotypes together with previously published COI sequences of *E. andrei* and *E. fetida* revealed that the nine distinct sequences of COI identified in the ten groups could represent four different earthworm species. Haplotypes H1 to H6 grouped with previously identified sequences of *E. andrei* (K2P ≤ 8.28%) (Fig. 4). H7 grouped with BOLD sequences identified as *E. andrei.* However K2P distances revealed that sequence divergence between H7 and the other *E. andrei* haplotypes was as high as 31.10%. H7 identity is therefore considered dubious, especially considering the fact that it grouped with unpublished (i.e. potentially unverified) alleged *E. andrei* sequences from BOLD (EWSJC613-10 and EWSJC614-10) (Fig. 4). H8 grouped with Genbank sequences of the Megascolecidae *P. excavatus* (K2P ≤ 1.2%). The BOLD Identification System also identified H8 as *P. excavatus.* H9 grouped with previously identified sequences of *E. fetida* (K2P ≤ 11.7%).). The earthworm cultures from Port Elizabeth (South Africa) and Southampton (England) were mixes of *E. andrei - P. excavatus* and *E. andrei – E. fetida* respectively.

**Figure 4** Neighbour-joining (NJ) tree based on the Kimura-2-parameter (K2P) method. Bootstrap support obtained for specific nodes are reported. Genbank accession numbers or BOLD process IDs are provided in brackets for the sequences downloaded from either Genbank or BOLD. Allolobophoridella eiseni and Microscolex phosphoreus were included as outgroups. * indicate dubious *E. andrei* sequences from BOLD.

### *3.3.2. Genetic richness and differentiation of local populations*

Eight of the nine COI haplotypes (H1-H8) occurred in the selected South African earthworm stocks. H8, as established above, did not belong to the *Eisenia* genus. Consequently, only seven *Eisenia* COI haplotypes were found to occur in local populations. All these, with the exception of H7, grouped with conclusively identified specimens of *E. andrei*. Table 3 provides the Kimura 2-parameter distance matrix (%) between these haplotypes.

**Table 3** Kimura 2-parameter distance matrix (%) between the *E. andrei* COI haplotypes (H1-H7) found in the South African earthworm groups investigated. Distances between H7 and the rest of the haplotype vary between 23.28 and 31.10%; H7 identity remains uncertain

| | H 1 | H 2 | H 3 | H 4 | H 5 | H 6 | H 7 |
|---|---|---|---|---|---|---|---|
| H 1 | - | | | | | | |
| H 2 | 0.38 | - | | | | | |
| H 3 | 1.35 | 1.35 | - | | | | |
| H 4 | 7.21 | 6.79 | 8.28 | - | | | |
| H 5 | 1.16 | 1.16 | 0.57 | 8.07 | - | | |
| H 6 | 0.77 | 0.38 | 1.74 | 7.21 | 1.55 | - | |
| H 7 | 24.41 | 24.69 | 23.28 | 31.10 | 23.57 | 25.24 | - |

Prior to rarefaction analyses, PE (H = 0.426), JOZ (H = 0.378) and NWU (H = 0.377) had, in order, the three highest haplotype diversities (Table 4). After rarefaction to a common sample size of 10, this order was changed to JOZ (r(10) = 2), PE (r(10) = 1.658) and JNB (r(10) = 1.5). Table 4 also revealed that MID contributed more to the total genetic diversity amongst populations ($H_T$= 0.4498) as indicated by the only positive $C_T$ ($C_T$ = 0.322); which was mostly due to the strong divergence ($C_D$ = 0.38) of MID from the other populations. Differentiation indices $DH_T$ and $DG_{ST}$ > 0.75 for MID indeed indicated that this population was the most divergent of the local populations included in the present study. Negative $C_D$ values for the other populations indicated a lack of significant differentiation between them. This was confirmed by conventional population pairwise FSTs that showed non-significant differentiation amongst these populations.

**Table 4** Measure of genetic diversity and divergence for each South African population of *E. andrei* based on COI sequence data after rarefaction to a common sample size of ten. n = number of specimens included per population; Nb. Hap = number of haplotypes; H(SE) = haplotype diversity and standard error in brackets; π (SE) = nucleotide diversity and standard error in brackets; r(10) = allelic richness after rarefaction to a common size of ten specimens per samples; DHs, DHt, DGst = divergence indices from the other populations; $C_T$, $C_S$, $C_D$ = contribution indices to total diversity; $C_{rT}$, $C_{rS}$, $C_{rD}$ = contributions indices to total allelic richness (See Petit *et al*. 1998 for more details).

| Populations | n | Nb Hap. | H(SE) | π (SE) | r(10) | $DH_S$ | $DH_T$ | $DG_{ST}$ | $C_T$ | $C_S$ | $C_D$ | $C_{rT}$ | $C_{rS}$ | $C_{rD}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GRM | 22 | 2 | 0.312 (0.106) | 0.001 (0.001) | 0.93 | 0.3 | 0.38 | 0.21 | -0.05 | 0.008 | -0.06 | -0.07 | -0.03 | -0.04 |
| JNB | 16 | 3 | 0.342 (0.14) | 0.008 (0.004) | 1.5 | 0.31 | 0.39 | 0.21 | -0.04 | 0.017 | -0.06 | -0.05 | 0.028 | -0.08 |
| JOZ | 10 | 3 | 0.378 (0.181) | 0.002 (0.002) | 2 | 0.33 | 0.41 | 0.2 | -0.03 | 0.029 | -0.06 | -0.01 | 0.078 | -0.09 |
| MID | 19 | 2 | 0.105 (0.092) | 0.001 (0.001) | 0.53 | 0.21 | 0.88 | 0.76 | 0.322 | -0.06 | 0.38 | 0.159 | -0.07 | 0.229 |
| MPP | 11 | 2 | 0.182 (0.144) | 0.0006 (0.0008) | 0.91 | 0.24 | 0.35 | 0.3 | -0.08 | -0.03 | -0.04 | -0.12 | -0.03 | -0.09 |
| NWU | 45 | 4 | 0.377 (0.082) | 0.011 (0.006) | 1.47 | 0.33 | 0.41 | 0.2 | -0.03 | 0.029 | -0.06 | 0.005 | 0.025 | -0.02 |
| PE | 20 | 3 | 0.426 (0.122) | 0.003 (0.002) | 1.66 | 0.35 | 0.43 | 0.19 | -0.02 | 0.044 | -0.06 | 0.015 | 0.043 | -0.03 |
| SUN | 44 | 3 | 0.173 (0.075) | 0.0006 (0.0007 | 0.81 | 0.24 | 0.36 | 0.33 | -0.07 | -0.04 | -0.03 | 0.002 | -0.04 | 0.044 |

Figure 5 represents a network of the *E. andrei* haplotypes found in local South African populations. The dubious haplotype H7 was excluded in this analysis. The least number of mutations was found between H1 and H2 (a single mutation) and the highest number of mutations was found between H2 and H4 (31 mutations).

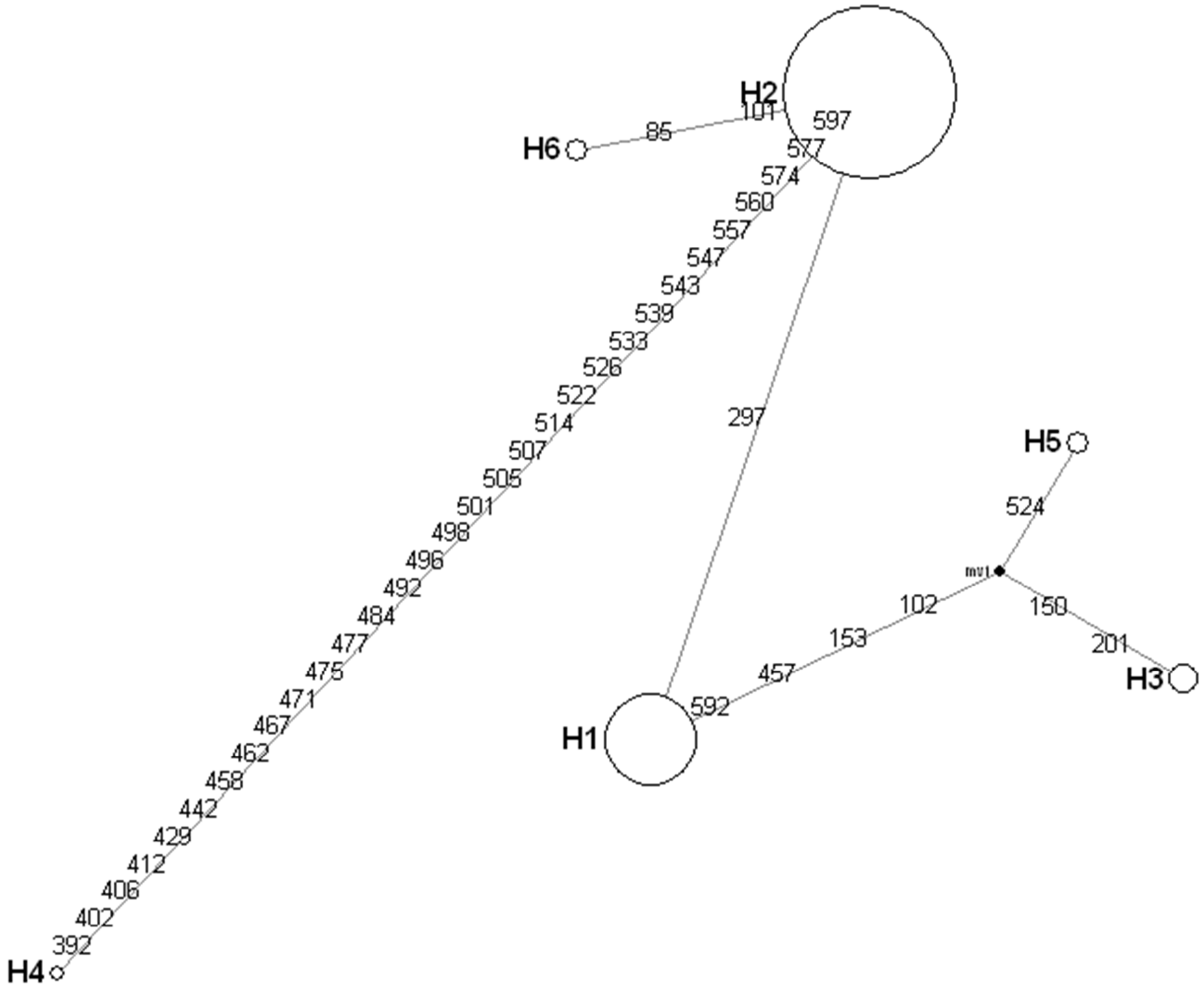

**Figure 5** Haplotype network calculated from the *E. andrei* COI haplotypes found in the South African earthworm groups investigated. The size of the circles is proportional to the number of earthworms sharing the same haplotype. The numbers on the branches indicate the positions of mutations on the COI sequences. mv1 represents a median vector (intermediate haplotypes, not found in this study)

### 3.4. Discussion

DNA analysis reveals that the sequences generated from South African based *Eisenia* populations grouped unequivocally with known sequences of *E. andrei*. Earthworm breeders and researchers have assumed that these local groups represent cultures and populations of *E. fetida*. Reinecke and Viljoen (1991) stated that local *Eisenia* populations could be a mix of *E.*

*andrei* and *E. fetida.* To date, no locally occurring *E. fetida* specimen has been formally identified using DNA markers. The occurrence of mixed local populations of *E. andrei* and *E. fetida* cannot not be excluded as it is acknowledged that both species commonly occur in mixed colonies and that *E. andrei* could outcompete *E. fetida* during periods of food abundance (Elvira *et al.* 1996). Domínguez *et al.* (2005) noted that *E. andrei* is the predominant species in commercial vermiculture establishments while *E. fetida* is mostly found in free-living populations. Considering that seven out the eight local earthworm groups investigated were bred in captivity, maybe including more field populations would have helped discover *E. fetida*.

The vermiculture stock from Port Elizabeth was a mixture of *E. andrei* and *P. excavatus*, the oriental compost worm known to be able to reproduce parthenogenetically and to thrive in similar living conditions as *E. andrei* and *E. fetida* (Hallatt *et al.* 1990). These results suggest that the untrained buyer seeking to purchase *E. fetida* in SA has a greater likelihood of acquiring *E. andrei* and occasionally together with specimens from another species such as *P. excavatus.*

The unique COI sequence (H7) identified as an *E. andrei* sequence through the BOLD identification system was extremely divergent from the other *E. andrei* sequences. Using the K2P method, the accepted threshold for species delimitation for DNA barcodes data is 15% K2P (Chang and James 2011). The divergence between H7 and the other *E. andrei* haplotypes was consistently over 23% K2P. An increasing number of cryptic Oligochaeta species have emerged from the literature with the recent advent of earthworm molecular studies (King *et al.* 2008; Pérez-Losada *et al.* 2009; Novo *et al.* 2010; Blakemore *et al.* 2010; James *et al.* 2010). H7 could represent a non-described cryptic species, however further molecular and morphological investigations would be required to shed more light on the matter.

COI haplotype numbers were limited to two to three distinct sequences within each of the local groups. This translated into a haplotype diversity (H) lower than 0.45 in all the populations. When compared to other such molecular studies in which COI polymorphism has been investigated in earthworms, the present haplotype diversity is proportionally very low. King *et al.* (2008) sequenced the COI gene in selected lineages of the European earthworm *Allolobophora chlorotica* and found H values as high as 0.95. Similarly, Novo *et al.* (2009) found H values as high as 0.92 in populations of the hormogastrid earthworm *Hormogaster elisae* from the central Iberian Peninsula. Equally high haplotypic richness have been reported in several other species of earthworms such as *Dendrobaena octaedra* (Cameron *et al.* 2008; Knott and Haimi 2010), *Amynthas wulinensis* (Chang *et al.* 2007), *Aporrectodea rosea, Octolasion lacteum,* and *L. rubellus* (Klarica *et al.* 2012).

Moreover, laboratory and vermicomposting cultures are susceptible to the founder effect (Mayr 1942) as they are usually started with a limited number of individuals. This may explain the comparatively poor haplotype diversity observed in South African *E. andrei* stocks. For *Eisenia sp.* the phenomenon could be compounded by the fact that known habitats of these species (compost heaps, manure rich soils etc.) are naturally fragmented. Despite their status as standard laboratory test species, molecular studies of free-living *E. andrei* and *E. fetida* are rare. The population genetics of these species is yet to be thoroughly investigated in Europe where they originated.

Being an introduced species to South Africa, *E. andrei* also suffered the effects of another significant factor upon entry into the country; the propagule pressure which stipulates that species introduced in large and consistent quantities would be more likely to persist in their new environment compared to those introduced in limited numbers and release events (Lockwood *et al.* 2005). This particular factor may also help explain the local predominance of *E. andrei* over *E. fetida* by assuming that larger and more consistent introduction events of *E. andrei* may have occurred.

Of all the local groups investigated MID was the only significantly divergent population. The haplotype distribution across the populations (Table 2) indicated that MID was the only population not harbouring H2, the haplotype which represented 75% or more of the COI sequences within the local populations. This perhaps indicates that H2 is rare in free-living populations of *E. andrei* or that this particular haplotype is selected against in relatively harsh environmental conditions.

Finally, Voua Otomo *et al.* (2011) established, using allozyme polymorphism, that the mean observed heterozygosity per locus ($H_o$) in two of the earthworm groups investigated in this study (SUN and MID – previously thought to be *E. fetida*) was zero. It was suspected that inbreeding could be occurring in these populations.

This may have significant implications for both the research sector and the vermiculturing industry. The SUN and MID groups have for instance been used in ecotoxicological research (Table 1). If the genetic diversity of laboratory populations is drastically reduced, the reliability of results from laboratory testing could be compromised. The lack of genetic variation has been associated with decreased fitness often affecting traits like growth reproduction and survival (Charlesworth and Charlesworth 1987; Reed and Frankham 2003). Velando *et al.* (2006) investigated the deleterious effects of inbreeding on the reproduction of *E. andrei* and reported that inbreeding causes a "*strong reduction of cocoon production*".

### 3.5. Conclusion

The use of DNA barcoding has helped to show that *E. fetida* maybe rarer than previously assumed in South Africa. *E. andrei* is the main species used in both the vermiculture industry and laboratory research. Most of these captive stocks are genetically homogenous and may in some instances suffer from inbreeding depression.

# 4. Evidence of cadmium tolerance in metal-free stocks of the standard test species *Eisenia andrei* (Oligochaeta) revealed by COI haplotypes

*The present chapter has been submitted to Ecotoxicology and Environmental Safety and is currently under review: Voua Otomo L., Bezuidenhout C.C., Maboeta M.S., Voua Otomo P. Evidence of cadmium tolerance in metal-free stocks of the standard test species Eisenia andrei (Oligochaeta) revealed by COI haplotypes.*

## 4.1. Introduction

The possibility of Cd tolerance in a standard earthworm test species was first reported by Reinecke *et al.* (1999) after 32 months exposure of *E. fetida* to 0.01% $CdSO_4$ in an artificial soil substrate. These authors found that a laboratory reared population of *E. fetida* pre-exposed to Cd performed better, with regard to growth and survival, than a control group reared on a clean substrate when exposed to higher Cd concentrations. Although *E. fetida* was then believed to have developed *resistance* to Cd after long-term exposure, the question remained whether it was a simple *physiological acclimation* (Klerks and Weis 1987) or a *genetic adaptation* (Klerks and Weis 1987; Posthuma and van1993)

Further investigations using the comet and MTT assays revealed that the abovementioned metal tolerant population of *E. fetida* had also developed cytotoxic and genotoxic tolerance to Cd (Voua Otomo and Reinecke 2010). More recently, however, a study of allozymes polymorphisms did not show a difference between the Cd tolerant laboratory population, a metal contaminated field population and a laboratory control group (Voua Otomo *et al.* 2011). These findings have strongly pointed to physiological acclimation as the most probable explanation for the Cd tolerance reported by Reinecke *et al*. (1999) after long-term exposure of *E. fetida*.

During a pollution event, a chosen toxicant may progressively eliminate sensitive genotypes thereby facilitating the prevalence of more tolerant ones and providing increased tolerance to the contaminated population overtime (Connell *et al*. 1999). Van Straalen and Timmermans (Van *et al*. 2002) have stated that directional selection on tolerant genotypes is one of the four ways toxicants could affect the genetic variation of contaminated populations. This process also known as *genetic erosion* designates the loss of genetic variation in a population due to genetic drift, inbreeding or exposure to a toxicant. The principle of directional selection ultimately implies that tolerant and sensitive genotypes should be an innate trait of the genetic make-up of healthy populations. In this regard, using the mitochondrial 16S rDNA gene, Sturmbauer *et al*. (1999) have been able to separate lineages of the Oligochaeta *Tubifex tubifex* based on their tolerance to Cd. Similarly, Andre *et al*. (2010) using the mitochondrial cytochrome oxidase II

(COII) showed that lineages of *L. rubellus* could be spatially grouped across a heterogonous mining area based on their tolerance to Pb/Zn polluted soils.

Sturmbauer *et al*. (1999) acknowledged that the highly divergent mitochondrial lineages, in the populations of *T. tubifex* studied indicated that the metal resistant attributes of these populations must have developed prior to any human influence. Similarly, Andre *et al*. (2010) have estimated that the youngest Pb/Zn tolerant lineage of *L. rubellus*, that they have discovered, is a least 17 000 years old, suggesting that the observed metal tolerance is an innate trait and the product of stochastic evolutionary processes rather than the cause of recent chronic exposure to Pb/Zn.

Because *E. fetida* and its sister species *E. andrei* are both standard test species (OECD 1984; OECD 2004a; ISO 2008; ISO 2012a,b), innate differences in sensitivity to toxicants within or between these species may change our current approach to earthworm ecotoxicology. From an ecotoxicological standpoint, both species have traditionally been regarded as equivalent. Furthermore, research also indicate that cryptic forms of both these species may exist (Pérez-Losada *et al*. 2005; Voua Otomo *et al*. 2013). This has prompted an ongoing international research initiative called "*Eisenia* barcoding initiative" whose aims is to improve the quality assurance of standardised ecotoxicological tests with earthworms (Roembke, Personal Communication)

Because of the history on Cd-tolerance in laboratory reared populations of earthworms, in the present contribution, I made use of DNA barcoding to identify distinct sequences of the cytochrome oxidase I (COI) gene in captive populations of *E. andrei* with no previous history of metal exposure. The worms carrying the identified COI haplotypes were subsequently paired up and exposed to Cd in order to assess the difference in sensitivity between the different couples.

## 4.2. Materials and Methods

### *4.2.1. Experimental populations*

The term "population" is used in the present study in a more inclusive manner and may refer to a free-living "wild" population or to a captive breeding stock. Eight South African based populations of *E. andrei* were included in this study. They were two vermiculture stocks from Johannesburg (Gauteng); two vermiculture stocks and a laboratory culture from Potchefstroom (North-West), a free-living population and a laboratory culture from Stellenbosch (Western Cape) and a vermiculture stock from Port Elizabeth (Eastern Cape). Table 5 provides more details on the geographical locality and the function/use of the selected earthworm populations.

**Table 5** Localities and use of the earthworm groups included in the present study. Abbreviations: n – the number of specimens used for COI genotyping from the respective groups, ANs – the Genbank accession numbers for the respective sequences.

| | Type/use | Origin/Locality | Code | *n/ANs* |
|---|---|---|---|---|
| Stellenbosch 1 | Laboratory | Stellenbosch University, Stellenbosch<br>(33°56'3.4" S, 18°51'56.4" E)<br>Western Cape, South Africa | SUN | 44/JN870005-JN870024<br>DQ914618-DQ914633<br>JX912906- JX912913 |
| Stellenbosch 2 | Field | Middelvlei wine farm, Stellenbosch<br>(33°55'84" S, 18°49'87" E)<br>Western Cape, South Africa | MID | 19/JN870048-JN870066 |
| Potchefstroom 1 | Laboratory | North-West University, Potchefstroom<br>(26°41'21" S, 27°05'26" E)<br>North-West, South Africa | NWU | 45/JN870025-JN870047<br>JX912898- JX912905<br>JX908652- JX908665 |
| Potchefstroom 2 | Vermicomposting | Grimbeek Park, Potchefstroom<br>(26°43'29" S, 27°06'48" E)<br>North-West, South Africa | GRM | 22/JN870067-JN870088 |
| Potchefstroom *3* | Vermicomposting | Mieder Park, Potchefstroom<br>(26°43'15.6" S, 27°06'06" E)<br>North-West, South Africa | MPP | 11/ JX908641- JX908651 |
| Port-Elisabeth | Vermicomposting | Newton Park, Port-Elisabeth<br>(33°56'57.1" S 25°33'35.0"E)<br>Eastern Cape, South Africa | PE | 20/ JX908692- JX908711 |
| Johannesburg 1 | Vermicomposting | Ferndale, Johannesburg<br>(26°6'1.7"S 28°0'8.6"E)<br>Gauteng, South Africa | JNB | 16/ JX899807- JX899822 |
| Johannesburg 2 | Vermicomposting | Morningside, Johannesburg<br>(26°4'52.5"S 28°3'44.4"E)<br>Gauteng, South Africa | JOZ | 10/ JX912888- JX912897 |

### 4.2.2. COI genotyping

Total genomic DNA was extracted from a total of 187 earthworms from the selected stocks of *E. andrei*. The universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994) were used to amplify and sequence 683 bp of the cytochrome oxidase I (COI) gene. PCR reactions consisted of 0.3 µl (±30 ng) DNA template, 12.5 µl PCR Master Mix (Fermentas), 11 µl nuclease free water (Fermentas) and 10 pmol (~1µl) of each of the primers. PCR cycling comprised an initial denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 30s, 50°C for 30s and 72°C for 45s. A final extension step at 72°C for 5 min completed the reactions.

Successful amplification was verified by electrophoretic means using agarose gels (0.75g SeaKem® LE Agarose, Lonza- in 50ml TAE buffer, 1.5% w/v) stained with 5µl ethidium bromide. Sequencing reactions were performed using the ABI v3.1 BigDye® kit. Purified sequences were run on an ABI 3500XL Genetic analyser. All the barcodes generated in the present study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank; accession numbers provided in Table 1).

### 4.2.3. Haplotype coupling and cadmium exposure

After tissue collection for DNA extraction, the worms were kept individually in plastic containers (5.5 cm x 3 cm) and reared on a moist horse manure substrate. Based on DNA barcode results, the worms sharing the same COI haplotype were subsequently grouped. Thereafter, the earthworms with the three most common COI haplotypes (termed Hap1, Hap2 and Hap3) were coupled and exposed to Cd in OECD artificial soil. A total of six couples (Cp1- Hap2xHap2; Cp2- Hap1xHap2; Cp3- Hap2xHap3; Cp4- Hap1xHap3; Cp5- Hap1xHap1 and Cp6- Hap3xHap3) were exposed in triplicate to 0, 25, 50 and 100 mg Cd/kg for 4 weeks at 20°C. The concentration range of Cd was chosen to be mostly sub lethal and to permit some level of reproduction in the experimental treatments. During the experiment, the earthworm couples were fed a gram of uncontaminated horse manure weekly. The survival, biomass variation and cocoon production of each couple were assessed weekly. The hatching success of the collected cocoons was also monitored by keeping the cocoons in deionised water at 20°C for a further 28-day period after the Cd experiment.

### 4.2.4. Metal analysis

At the end of the exposure period, all the worms were processed for metal analysis following the protocol of Katz and Jennis (1983.). Cd tissue contents were measured in the worms exposed to the different treatments with an Agilent 7500c Inductive coupled plasma mass-spectrometer (ICP-MS). For quality control, I assessed the percent recovery of a Cd standard purchased from

Sigma-Aldrich (http://www.sigmaaldrich.com). The analysis of the reference sample indicated that the percent recovery was 85.7 ± 13.4 %. The detection limit (ppb) was 0.1.

### *4.2.5. Statistical analysis*

SigmaStat® was used to analyse the data. The data were tested for normality using the Kolmogorov–Smirnov test. Normally distributed data were analyzed using a parametric multiple test (One-way ANOVA, followed with Bonferroni posttest). Non-parametric data were analyzed using the Kruskal-Wallis ANOVA followed by Dunns' test. $EC_{50}$ indices were estimated using non-linear regression analyses in IBM SPSS: IBM SPSS Statistics Version 21. The level of statistical significance was $P < 0.05$.

## 4.3. Results

### *4.3.1. COI haplotypes*

The 187 COI sequences generated in the present study represented only 7 distinct haplotypes. The frequency and uniqueness of these haplotypes as well as the genetic diversity of the populations studied are presented elsewhere (Voua Otomo *et al*. 2013). Regarding the three haplotypes of interest, Hap1 was present in all the populations with an overall frequency of 20.32%; Hap2 had the highest frequency (74.33%) although it was not found in the outdoor population (MID); and Hap3 (2.14%) was only present in the laboratory culture from Potchefstroom and the vermiculture stock from Port Elizabeth (Table 5). The analysis of the COI sequences generated from the earthworm specimens used in the present study, together with previously identified COI sequences by Pérez-Losada *et al*. (2005), confirmed that all sequences were unequivocally from the species *E. andrei* as discussed in details in Voua Otomo *et al*. (2013).

### *4.3.2. Survival*

After haplotype pairing and exposure to Cd for 28 days, mortality (16.66%) was only recorded in Cp6 (Hap3 x Hap3) exposed to 100 mg Cd/Kg (Fig. 6). No mortality occurred in any of the other couples. Nevertheless, survival was statistically similar in all the treatments at the end of the exposure period.

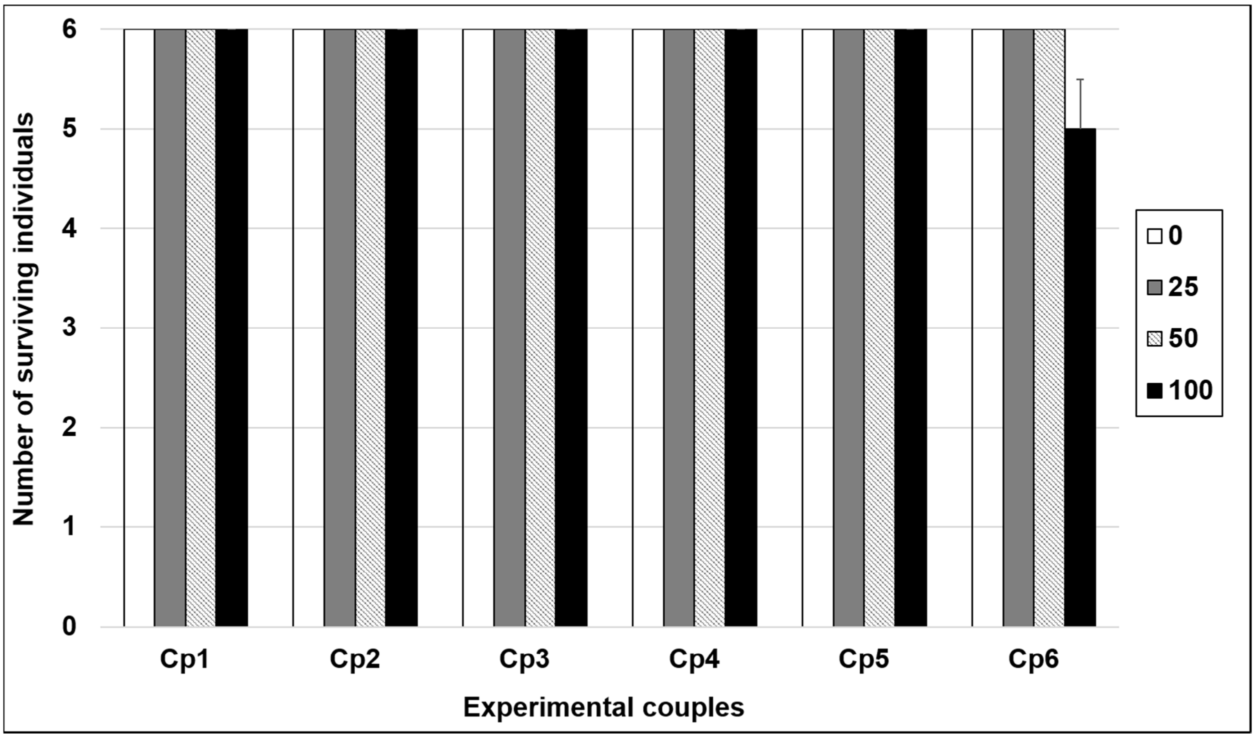

**Figure 6** Survival of couples of *E. andrei* carrying selected COI haplotypes after exposure to Cd for 28 days at 20°C.

### *4.3.3. Biomass variation*

There was no statistically significant variation in biomass in any of the couples except for Cp6 (Hap3xHap3) where biomass loss was significant ($p < 0.05$) for week 3 and week 4 in the worms exposed to 100 mg Cd/kg (Fig. 7). No Cd $EC_{50}$ could be computed for any of the couples, as over the four week the earthworm biomass remained higher than 50% of the biomass of the respective controls.

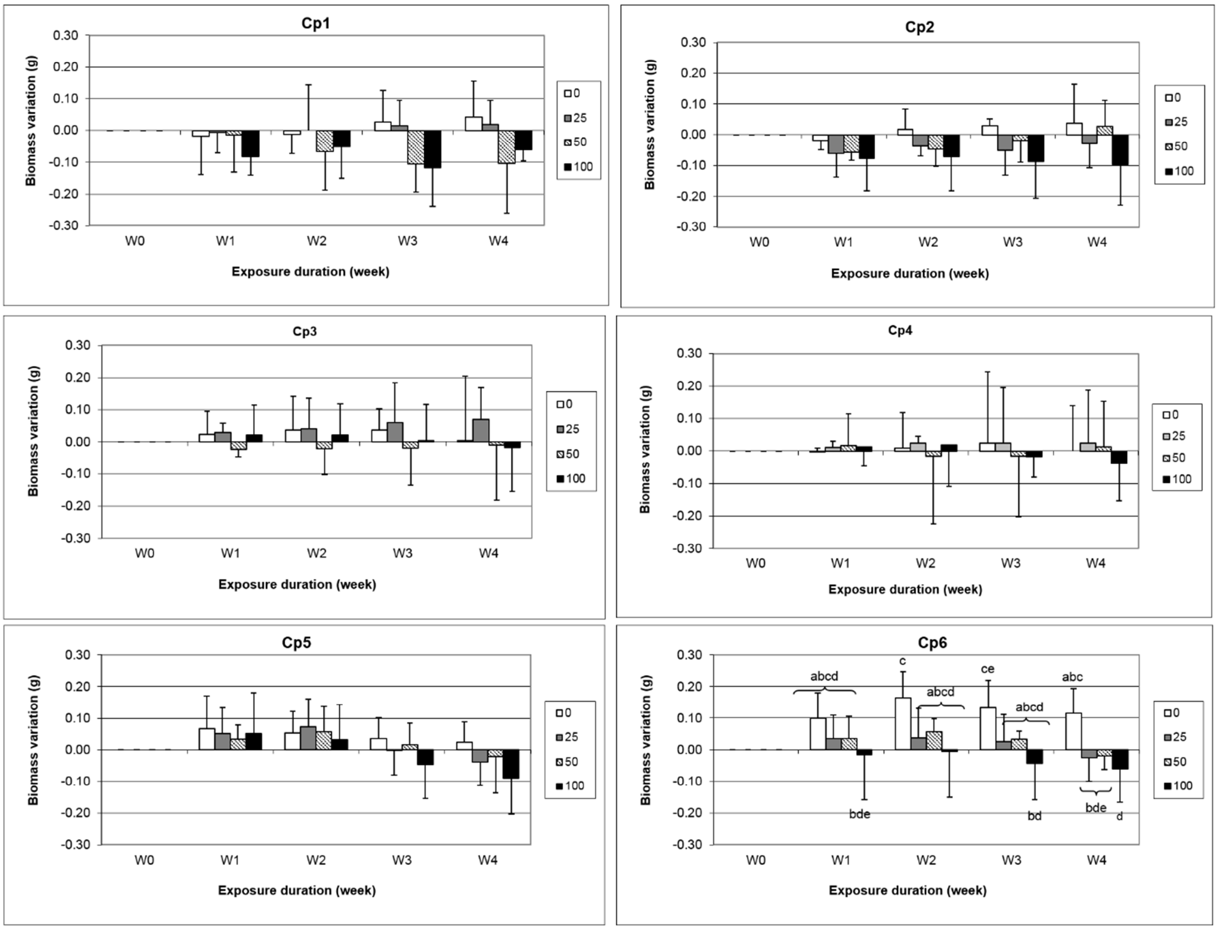

**Figure 7** Biomass variation of couples of *E. andrei* carrying selected COI haplotypes after exposure to Cd for 28 days at 20°C. The bars indicate standard deviation. The lower case letters (a, b, c, d) are used here to indicate if there is statistical significance in the biomass variations at concentrations 0, 25, 50 and 100 mg Cd/kg. There was no statistically significant variation in biomass in any of the couples except for Cp6 at week 3 and week 4 in the worms exposed to 100 mg Cd/kg.

### *4.3.4. Reproduction*

Cocoon production indicated that Cp1 (Hap2xHap2), Cp2 (Hap1xHap2) and Cp5 (Hap1xHap1) produced significantly lower cocoon numbers in all the Cd treatments ($p \leq 0.05$) when compared to their respective controls (Fig. 8). The number of cocoons produced by Cp3 (Hap2xHap3) was statistically lower than the number produced by its respective control only in Cd treatments ≥ 50 mg/kg. For Cp6 (Hap3xHap3), in the lowest 25 mg Cd/kg treatment, cocoon production was significantly lower than in its respective control ($p < 0.05$) and was totally suppressed in all the other treatments. Contrariwise, Cp4 (Hap1xHap3) showed a significant decrease in reproduction, when compared to its respective control, only in the highest 100 mg Cd/kg treatment.

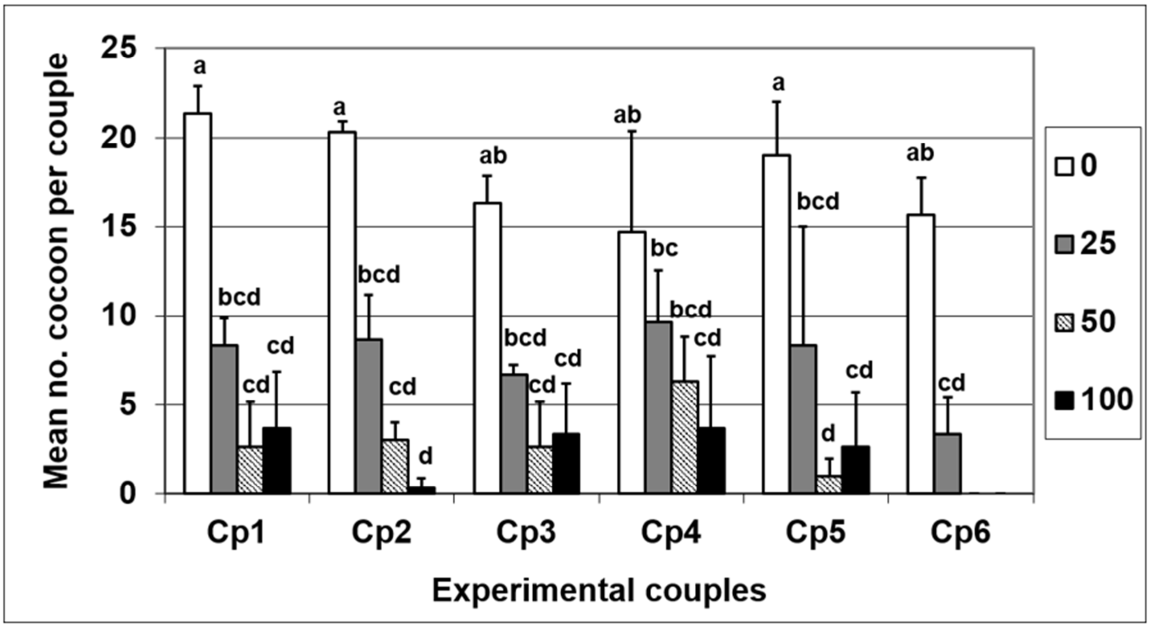

**Figure 8** Mean number of cocoons per couple of *E. andrei* carrying selected COI haplotypes after exposure to Cd for 28 days at 20°C. The bars indicate standard deviation. The lower case letters (a, b, c, d) are used here to indicate if there is statistical significance in the variations of the number of cocoon produced at concentrations 0, 25, 50 and 100 mg Cd/kg. In most of the couples cocoon production was significantly affected in the presence of the metal (compared to the control).

The computing of $EC_{50s}$ for reproduction revealed that Cp6 (Hap3xHap3) and Cp4 (Hap1xHap3) had the lowest and highest $EC_{50s}$, respectively (Table 6).

**Table 6** Effective concentrations ($EC_{50s}$) for the effects of Cd on the reproduction of couples of *E. andrei* carrying selected COI haplotypes after exposure to Cd for 28 days at 20°C. The values are provided in mg/kg soil. The numbers in brackets indicate confidence intervals. For Cp4, the bootstrap estimates mean was 43.0957 mg/kg, with a standard deviation of 14.7683, no confidence intervals could be determined.

| $EC_{50s}$ | | | | | |
|---|---|---|---|---|---|
| Cp1 | Cp2 | Cp3 | Cp4 | Cp5 | Cp6 |
| 20.63 (18.75-22.50) | 21.92 (17.85-26.04) | 21.05 (19.53-22.59) | 43.09 (not determined) | 22.26 (14.61-33.68) | 15.91 (13.43-18.33) |

### 4.3.5. Hatching success

The assessment of the hatching success revealed that there was no significant difference in the number of hatchlings per cocoon between the haplotype couples assessed (Fig. 9). Comparisons within the respective couples however, revealed some of the deleterious effects of Cd to Cp2 (Hap1xHap2) and Cp3 (Hap2xHap3). For Cp2 (Hap1xHap2), cocoons produced in the 50 and 100 mg Cd/Kg treatments did not hatch at all. For Cp3 (Hap2xHap3), the hatching rates of cocoons produced in all Cd treatments were significantly lower than the rate in their respective control.

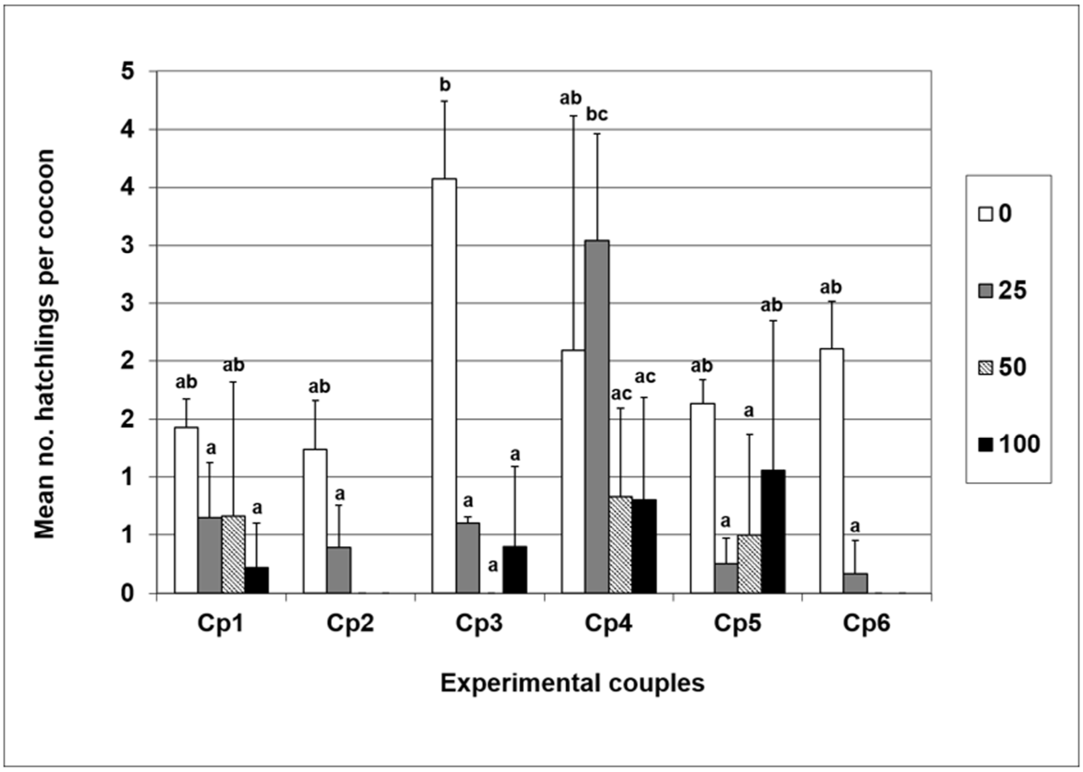

**Figure 9** Mean number of hatchlings per cocoon produced by couples of *E. andrei* carrying selected COI haplotypes after exposure to Cd for 28 days at 20°C. The bars indicate standard deviation. The lower case letters (a, b, c, d) are used here to indicate if there is statistical significance in the variations of the mean number of hatchlings per cocoon at concentrations 0, 25, 50 and 100 mg Cd/kg.

### 4.3.6. *Metal analysis*

The assessment of Cd tissue content in the experimental organisms revealed that except for Cp5 (Hap1xHap1) and Cp6 (Hap3xHap3), patterns of Cd accumulation were similar for all couples (Fig. 10). In the 100 mg Cd/kg treatment, Cp5 (Hap1xHap1) accumulated significantly more Cd than Cp2 (Hap1xHap2), Cp3 (Hap2xHap3) and Cp4 (Hap1xHap3) ($p \leq 0.5$). In the same treatment, Cp6 (Hap3xHap3) accumulated significantly more Cd than any other couple ($p \leq 0.01$).

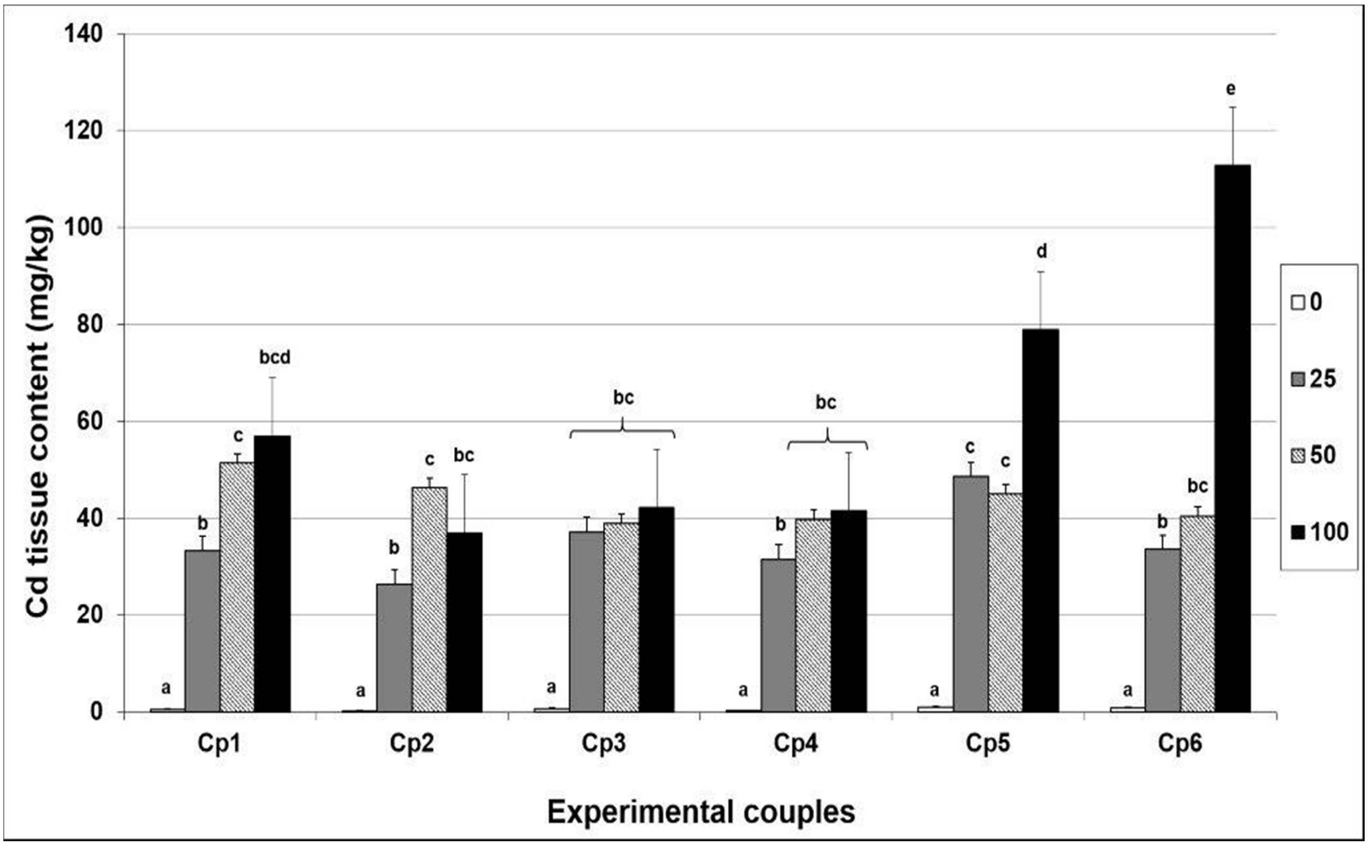

**Figure 10** Cd tissue content per couple of *E. andrei* carrying selected COI haplotypes after exposure to Cd for 28 days at 20°C. The bars indicate standard deviation. The lower case letters (a, b, c, d) are used here to indicate if there is statistical significance in the Cd tissue content variations at concentrations 0, 25, 50 and 100 mg Cd/kg.

### 4.4. Discussion

Most of the endpoints assessed in the present study pointed to Cp6 (Hap3xHap3) as the most sensitive couple, with significant toxic effects of Cd recorded for biomass (Fig. 7) and reproduction (Fig. 8). This was confirmed by Cp6 lowest $EC_{50}$ value [15.91 (13.43-18.33) mg Cd/kg] for reproduction (Table 6). Cp6 was also the only couple to suffer mortality (although limitedly so) within the Cd concentration range used in this this study (Fig. 6). These results indicate that Hap3 may be particularly susceptible to the deleterious effects of inbreeding as opposed to Hap1 and Hap2 who did not show similar results in Cp5 (Hap1xHap1) and Cp1 (Hap2xHap2) respectively. Inbreeding is known, in several organisms, to decrease the fitness of individuals, affecting such traits as growth, reproduction and survival (Charlesworth and Charlesworth 1987; Reed and Frankham 2003). This is, to a certain extent, exemplified in the present study, which also supports the findings of Velando *et al.* (2006) who reported that inbreeding could cause a significant reduction in cocoon production in *E. andrei.*

Hap3 was part of both the most sensitive (Cp6 - Hap3xHap3) and the least sensitive (Cp4 - Hap1xHap3) couples. Cp4 was identified as the least Cd-sensitive couple because it had the highest $EC_{50}$ (43.09 mg Cd/kg) for reproduction (Table 6). This indicated that of all the haplotypes tested for sensitivity, Hap3 was particularly sensitive when inbred whereas it performed better when outcrossed, especially with Hap1. Cocoons produced from the crossing of Hap2 and Hap3 (Cp3), for instance, had significantly lower hatching rates in all Cd treatments (Fig. 9). This was not the case for the cocoons produced from (Cp4 - Hap1xHap3).

Metal analysis indicated that perhaps other than inbreeding, Cp6 (Hap3xHap3) was as sensitive to Cd because it had the ability to accumulate significantly more Cd at high concentrations than the other couples (Fig. 10). Such an ability to accumulate comparatively greater metal content in earthworm specimens of the same species has previously been reported (Corp and Morgan 1991; Mariño and Morgan 1999) In these studies, however, the worms accumulating more metals had a history of long-term exposure to metals or were inhabiting metal contaminated areas. In the present contribution, novel evidence is presented. This evidence suggests that, given the conditions, such differential accumulations can occur in laboratory stocks with no prior incident of metal contamination.

The singular tolerance of Cp4 (Hap1xHap3) to Cd toxicity could have been interpreted as “genetic adaptation” in a study of long-term metal exposure conducted unknowingly on a sub-samples of individuals carrying Hap1 and Hap3. Although a number of studies that have investigated the question of metal tolerance in earthworms have documented evidence for metal induced physiological acclimation (Voua Otomo and Reinecke 2010; Voua Otomo *et al.*

2011; Bengtsson and Rundgren 1992; Aziz *et al.* 1999), as many studies have reported finding evidence for genetic adaption or genetically inherited tolerance to metals. For instance, Martinez and Levington (1996) reported that *Lumbricus hoffmeisteri* from a metal contaminated area had evolved genetic based resistance to heavy metal after estimating the minimum number of genes contributing to the difference in resistance between individuals from the contaminated population and a control group. They followed the method of Lande (1981), which uses parameters such as phenotypic differences and genetic variance among populations to deduce the number of genes contributing to a specific character. Spurgeon and Hopkin (2000) found increasing resistance to Zn in F1 and F2 generations of *E. fetida* reared on a Zn-contaminated medium but originally sampled from a metal free population. Toxicokinetic experiments undertaken to verify whether changes in Zn kinetics could be the cause of the increased resistance to Zn failed to indicate any consistent change between the generations. It was thus suggested that *E. fetida* had developed genetically inherited resistance to Zn under laboratory conditions. Likewise, Langdon *et al*. (2003) studied cocoon production over two generations *L. rubellus* collected from abandoned As/Cu and tungsten (W) mines. The F1 individuals from each population were allowed to reach sexual maturity on a clean substrate before being exposed to either sodium arsenate or copper chloride. Results indicated greater tolerance to As but not to Cu. The F1 (adults) and F2 (cocoons) from the investigated groups did not have significantly high body burdens of As when compared to the control groups. The authors concluded that resistance to As was probably genetically inherited. More recently, Fisker *et al*. (2011) conducted a similar multigenerational study on *Dendrobaena octaedra* using Cu and reached a similar conclusion as the studies mentioned above, stating that "natural selection has resulted in genetic adaptation to Cu pollution in the exposed populations". The crucial drawback to these studies is that they never performed any molecular investigations of their experimental populations to support the reported genetic basis for the alleged genetically inherited tolerance or genetic adaptation. Evidence for such genetic adaptation or genetically inherited resistance to metals in earthworms has remained objectively weak.

The presents study may suggests that such cases of "genetically inherited tolerance" or "genetic adaptation" to metals in earthworms could be expression of innate traits which do not necessarily require prior exposure to metals to develop. Exposure to heavy metals seems to initiate biochemical reactions in earthworms that aim to reduce the toxicological effects of these metals on target organs. The induction of metallothionein (MT) has been observed in many organisms exposed to heavy metals as well as in earthworms (Stürzenbaum et al., 2001). Earthworms' species such as *Lumbricus mauritii* or *Terrestris Lumbricus* induce MT production when exposed to Pb and Zn or to Cd, Cu and Hg in contaminated soil (Maity et al., 2011 and Calisi et al., 2011a), likewise *L.* rubellus, *E.* fetida, and *E.* andrei have been reported to produce

MT in response to cd exposure (Calisi et al., 2009; Ndayibagira et al., 2007; Brulle et al., 2007; Demuynck et al., 2006; Gruber et al., 2000).

Evidence for innate metal tolerance has now been found in the freshwater Oligochaeta *T. tubifex* using 16S rDNA (Sturmbauer *et al.* 1999), in the earthworm *L. rubellus* using COII (Andre *et al.* 2010) and herein in *E. andrei* using COI.

The present contribution also highlights the potential shortcomings of the use of genetically homogenous earthworm stocks in laboratory testing. Voua Otomo *et al.* (2013) have documented cases of stocks of *E. andrei*, used for laboratory testing, having only two or three distinct COI sequences. This translated into: 0.10 ≤ (H) ≤ 0.42 [where (H) is the COI haplotype diversity]. This was lower than COI haplotype diversity of free living European earthworms such as *Allolobophora chlorotica* (H ≤ 0.95, (King *et al.* 2008)) and *Hormogaster elisae* (H ≤ 0.92, (Novo *et al.* 2009)). Voua Otomo *et al.* (2013) stipulated that the comparatively poor haplotype diversity observed in these *E. andrei* stocks was perhaps caused by the fact that laboratory cultures are susceptible to the founder effect (Mayr 1942) as they are usually started with a limited number of individuals thus limiting *de facto* their genetic diversity.

Amid cases of mistaken identity (Voua Otomo *et al.* 2009; Voua Otomo *et al.* 2013) and relatively limited genetic diversity in earthworm stocks used in laboratory testing, the "*Eisenia* barcoding initiative" is justified. A standardised earthworm barcoding protocol could help (i) to confirm the taxonomy of laboratory stocks and (ii) to identify genetically diverse stocks suitable for laboratory testing.

### 4.5. Conclusions

The present study shows that earthworm populations may intrinsically carry metal-tolerant and metal-sensitive genotypes. In the context of ecotoxicological testing, the present results underline the importance of using genetically diverse earthworm laboratory stocks because conducting testing on stocks with a limited gene pool may not reflect the range of responses that could be exhibited by a healthy population and cause misleading conclusions and recommendations.

## 5. General discussion, conclusions and recommendations.

The principal aims of this study were to make use of DNA barcoding to investigate the taxonomy of *Eisenia* species found in vermiculture establishments and ecotoxicology research laboratories in South Africa, and to evaluate their responses to Cd contamination. Specific objectives were centred around (i) identifying populations of *E. andrei*, *E. fetida* and their potential cryptic species, (ii) inferring the level of genetic richness and differentiation within the conspecific populations identified, (iii) assessing the effects of Cd on the growth, reproduction and cocoon hatching of the identified species and (iv) assessing potential differences in Cd sensitivity between conspecific earthworms genotypes. Other than the fact that *E. fetida* could not be found in the local populations investigated, all the specific objectives listed above were successfully reached as elaborated further below.

Regarding specific objective (i) the present research endeavour has helped to established that the vermiculture industry and ecotoxicology research laboratories in South Africa make use of *E. andrei* rather than *E. fetida*. DNA barcoding has revealed that *E. fetida* may be less conspicuous than researchers and vermiculturists have presumed. From earlier local sources such as Reinecke and Viljoen 1991 it is possible that some earthworm stocks were mix colonies of *E. andrei* and *E. fetida*. Research evidence suggests that *E. andrei* matures and reproduces at a greater rate when in captive mix colonies with *E. fetida* (Elvira *et al.* 1996). This means that the current predominance of *E. andrei* could be the consequence of long-term population shifts in some of the prime captive colonies established in South Africa.

The present study only sampled populations from four of the eleven provinces of South Africa. Sampling within the selected provinces was also far from representative. According to the website of the Earthworm Interest Group of South Africa (EIGSA- http://www.eigsa.co.za), vermiculturists are present in six provinces of South Africa (Western Cape; Eastern Cape, Northwest Province, Kwa-zulu Natal, Mpumalanga and Gauteng). A wider and more representative sampling would have perhaps generated additional results about the extent of the genetic diversity of *E. andrei* and the potential presence of *E. fetida*. Finding *E. fetida* would also help conducting comparative ecotoxicological studies between the two species, as such studies are crucially lacking.

In this regard, it is recommended that further earthworm DNA barcoding studies, covering a more representative geographical area of South Africa and including more field populations of *Eisenia spp.* be conducted. Other than only looking for local representatives of *E. fetida,* further earthworm DNA barcoding studies will also improve our understanding of the molecular dynamics within *Eisenia spp.* A significant finding reported in the present work was the identification of a possible unknown species. The single highly divergent haplotype (H7) from the ecotoxicology laboratory of North-West University was indubitably from a different species, perhaps even remotely related to *Eisenia spp*.

In terms of objective (ii), it was found that all the populations included in the present study possessed only two to three COI haplotypes representing an haplotype diversity (H) < 0.45; which is less than half the diversity reported in other such earthworm studies (Novo *et al.* 2009; King *et al.* 2008). This particular finding may undermine the quality assurance of ecotoxicological testing in South African laboratories because less genetic diversity in test populations implies a greater likelihood of inbreeding depression which affects endpoints of interest in earthworm bioassays (Velando *et al*. 2006). In South Africa, as anywhere outside of Eurasia, the challenge of doing research with *E. andrei* and *E fetida* is that both are introduced species with *de facto* limited local genetic diversity within their respective populations.

Until more haplotypes of *E. andrei* are discovered and isolated in South Africa, it is recommended - as a way to circumvent the lack of genetic variation in *E. andrei* laboratory and vermiculture stocks- that captive breeding initiatives be established using specimens obtained from several geographically distant field and reared populations. In the case of South Africa, the EIGSA could assist in this regard. Its regional representatives are listed on the website of the group (http://www.eigsa.co.za/CONVENORS.htm). They can be reached via email and would usually offer worms at no cost for research.

Regarding practical objectives (iii) and (iv), the assessment of the ecotoxicity of Cd on some genotypic couples of *E. andrei* led to a novel and significant finding: the occurrence of innate Cd-tolerance and Cd-sensitivity in some of the couples. Differences in tolerance to toxicant between species (and conspecific individuals) is a natural attribute, which has been the topic of research. The species sensitivity distribution (SSD) concept, exploit differences in sensitivity between organisms to infer safe environmental concentrations of toxicants and to help setting environmental quality criteria (Posthuma *et al*. 2002). Here, it is shown that significant differences in responses to metal contamination should be expected during the grouping of earthworms for laboratory testing and that to avoid biased results, laboratory stocks should have some level of genetic diversity. This finding further support the need for more genetically diverse laboratory (and vermiculture) stocks, as indicated above.

In the present study, I used mtDNA *viz*. COI haplotypes as a means to identify and categorize individual earthworm specimens based on their response to Cd toxicity and do not regard the present mitochondrial haplotypes as proxy for possibly nuclear encoded traits. An important number of studies have documented the possible selective pressure of Cd toxicity on the mitochondrion. Dorta *et al.* (2003) showed that Cd could alter the transmembrane electrical potential of mitochondria, thus inducing premature depression of mitochondrial ATP levels. Cd exposure could inhibit mitochondrial electron transfer chain and trigger the formation of reactive oxygen species, causing the opening of membrane permeability pores which could lead to apoptosis (Li *et al.* ., 2003). Cd has also been shown to impair the function of important mitochondrial enzymes such as citrate synthase and NADP-dependent isocitrate dehydrogenases which are involved in Krebs cycle (Ivanina *et al.* 2008). Notwithstanding, there is as yet no clear evidence that Cd tolerance could be encoded by the mitochondrion.

Furthermore, metal analysis, indicated that Cp6 (Hap3xHap3) could have been more sensitive to Cd because it had the ability to accumulate significantly more Cd at high concentrations than the other breeding pairs (Fig. 10). This could point to differences in metal accumulation kinetics rather than inbreeding as the probable cause of the observed Cd sensitivity. In this regard, it is recommended that further research be conducted in order to investigate Cd accumulation/excretion kinetics between the Cd-tolerant and Cd-sensitive individuals reported in the present study. Such a study would be innovative because previous studies such as Klerks and Bartholomew (1991) and Morgan and Morgan (1999) which assessed patterns of Cd (bio)accumulation in earthworms did not attempt to separate the test organisms based on their genotypic features. A larger experiment with a greater number of haplotypes is required and bioconcentration factors (BCF) could be determined as in Maboeta *et. al.* (2008).
BCF = [e/w]/ [substrate]
Where e/w is the metal concentration in the earthworm, and [susbtrate] is the metal concentration of the soil.

The key implication of the overall findings presented in this study is that there is a pressing need to render earthworm ecotoxicology more reliable and ecologically relevant by making sure that laboratory tests are performed on conclusively identified and genetically representative organisms rather than subsets of recurrent genotypes. In that regard, a standardised earthworm barcoding protocol could come as a prerequisite to the conventional earthworm bioassays currently recommended by the OECD and the ISO. Such a barcoding protocol would help (i) to confirm the taxonomy of laboratory stocks and (ii) to identify genetically diverse stocks suitable for laboratory testing. The results presented here support the necessity of using advances in DNA technologies to come alongside and reinforce current ecotoxicological approaches. DNA barcoding in earthworm ecotoxicology needs to evolve from a simple tool used to address taxonomic uncertainties to one that would help make sure that laboratory populations have enough genetic diversity to stand in proxy for non-captive earthworm populations and other terrestrial organisms.

Furthermore, the issues at hand, *viz.* taxonomic uncertainties and/or genetic homogeneity could potentially affect other laboratory bioassays that make use of hard-to-differentiate test species. For instance, the OECD enchytraeid reproduction test (OECD 2004b) state that the "*test guideline is designed to be used for assessing the effects of chemicals on the reproductive output of the enchytraeid worm Enchytraeus albidus Henle 1873…*" and that "*other species of the Enchytraeus genus are also suitable, for example E. buchholzi Vejdovsky 1879 or E. crypticus Westheide & Graefe 1992…"* It carries on saying "*if other species of Enchytraeus are used, they must be clearly identified…"* This protocol makes provision for the use of even more species than the mere two recommended by the earthworm reproduction test (OECD 2004a). It is therefore reasonable to expect similar issues as the ones highlighted in this study to be present in potworm ecotoxicology. Thus, beyond earthworm ecotoxicology, lessons drawn from the present research endeavour have the potential to benefit ecotoxicological testing at large.

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