Inadequate taxonomy and highly divergent COI haplotypes in laboratory and field populations of earthworms used in ecotoxicology. A case study

Patricks Voua Otomo*, Mark S. Maboeta & Carlos Bezuidenhout

School of Environmental Sciences, North-West University, Private Bag X6001, Potchefstroom, 2520 South Africa

DNA barcoding was used to investigate the phylogenetic delimitations of *Eisenia* sp. populations used in ecotoxicological research in South Africa. A total of three focal groups (used in published works) and two non-focal groups were assessed. These focal groups, including two laboratory cultures and one field population, have been referred to as *Eisenia fetida* in the literature. A previous molecular study had already helped to establish that one of the two laboratory groups was a population of *E. fetida*'s sister species *E. andrei*. In the present contribution, analyses of the COI gene revealed that the taxonomy of the remaining laboratory and field populations had also been incorrectly assigned since all the generated sequences grouped unequivocally with published sequences of *E. andrei*. Very high sequence divergence (>25% K2P) found within *E. andrei* could signal the occurrence of hitherto undescribed cryptic species. These findings are discussed with an emphasis on the possible consequences of using poorly identified earthworms or specimens with high molecular divergence in ecotoxicological bioassays. It is not clear whether unbeknownst to the researchers, the use of cryptic species in bioassays could jeopardise the quality of ecotoxicological investigations. Early evidence suggests that cryptic oligochaete species may respond differently to metal toxicity. The need for comparative ecotoxicological studies between *E. andrei* and *E. fetida* is also evidenced, especially in the light of recent numerous reports of cryptic oligochaete species. Ecotoxicologists are consequently encouraged, whenever possible, to make use of available genomic technologies to screen their laboratory stocks and available field populations for any molecular distinctiveness.

Key words: *Eisenia fetida, Eisenia andrei*, DNA barcoding, oligochaetes, cryptic species.

INTRODUCTION

The use of DNA barcoding in confirming species identity in the field of ecotoxicology has previously been advocated (Voua Otomo *et al.* 2009). *Eisenia fetida* Savigny 1826 and *Eisenia andrei* Bouché 1972 are especially of interest since both are the recommended earthworm species for the testing of chemicals by the European Organisation of Economic Cooperation and Development (OECD 2004) and the International Standards Organisation (ISO 2005; ISO 2011). The dual recommendation of *E. andrei* and *E. fetida* by these organizations was historically based on the fact that these organisms were similar and/or considered to be conspecific.

Previous OECD guidelines (OECD 1984) for instance states that '*Eisenia foetida* exists in two races which some taxonomists have separated into

species'. Both *E. andrei* and *E. fetida* were, however, described as different species on the basis of their pigmentation (André 1963), although Savigny had originally only identified and described *Eisenia foetida* in 1826. Bouché (1972) designated them as subspecies, renaming them *E. foetida foetida* (for the current *E. fetida*) and *E. foetida unicolour* (for the current *E. andrei*). Despite earlier work by Jaenicke (1982) and Øien & Stenersen (1984) giving species status to both *E. andrei* and *E. fetida,* it is only recently that these species were conclusively proved to be different phylogenetic and biological species (Dominguez *et al.* 2005; Pérez-Losada *et al.* 2005).

There is, nonetheless, still a lack of comparative ecotoxicological studies between *E. andrei* and *E. fetida* to conclude whether they respond differently to environmental pollution since in literature both species are often referred to as *E. fetida* (Dominguez *et al.* 2005). Research has revealed

marked metabolic and reproductive differences between these species (Dominguez *et al.* 2005; Bundy *et al.* 2002; Albani *et al.* 2003). The implications of using inadequate taxonomy in the ecotoxicological context may lead to the erroneous intepretation of results and biased recommendations.

Moreover, numerous reports of cryptic oligochaete species (King *et al.* 2008; Pérez-Losada *et al.* 2009; Blakemore *et al.* 2010; James *et al.* 2010; Novo *et al.* 2010) make it a necessity to confirm species identity or authenticate phylogenetic delimitations, especially for those populations and laboratory cultures used for the testing of chemicals under the OECD and ISO frameworks. Cryptic species of the oligochaetes *Tubifex tubifex* and *Lumbricus rubellus* have already been shown to differ in terms of their tolerance to metal toxicity (Sturmbauer *et al.* 1999; Andre *et al.* 2010).

Apart from their importance as ecotoxicological test species, both *E. andrei* and *E. fetida* are also used worldwide in vermicomposting. In many places, vermiculturists have become the main suppliers of live specimens of these worms to research institutes and universities. The necessity of adequate taxonomy may also prove important for commercial earthworm stocks. Beyond the commercial value it may add to vermiculture establishments, correct taxonomic assignments, in this industry, will inevitably have a positive bearing on the reliability of research endeavours in the laboratory.

For reliable identification and genotyping of potential useful specimens of earthworms, DNA barcoding could be the method of choice as it is quick, significantly informative and both its detractors and advocates have agreed on its potential to compliment traditional taxonomy to address identification challenges (Moritz *et al.* 2004; Hajibabaei *et al.* 2007; Chang *et al.* 2009; Rougerie *et al.* 2009; Chang & James 2011). Recently, DNA barcoding was used to assess the taxonomic status of several earthworm genera and recommended an extensive revision of the presently accepted earthworm classification (Pérez-Losada *et al.* 2012).

In the present study, we have undertaken a DNA barcoding investigation of field and laboratory populations of *Eisena* sp. frequently utilized for ecotoxicological research in South Africa.

MATERIALS & METHODS

A total of five *Eisenia* sp. populations represented by 110 individuals, from North West Province and Western Cape (South Africa) and Brno (Czech Republic) were included in the present study. Three of these populations have a recorded history in recent ecotoxicological literature (Table 1). The oldest of these is a laboratory stock housed in the Stress Ecology Research Laboratory at the University of Stellenbosch. This laboratory culture was established in the early 1980s with specimens obtained from Braunschweig, Germany. Over the years, this laboratory culture has been enriched genetically through the addition of specimens from local compost heaps. Initially believed to consist of *E. fetida*, it was later found to be *E. andrei* (Voua Otomo *et al.* 2009). This laboratory stock is included mainly in order to assess the haplotype diversity amongst the different groups. In addition, to the original 16 COI sequences from an earlier study (Voua Otomo *et al.* 2009), a further 20 sequences from the same stock have been generated in the present study (Table 1). As the oldest population, it is also the one with the most extensive record in the literature (Table 1). A second laboratory culture, housed in the Unit for Environmental Research at North-West University, was started in 2001 with specimens obtained from two local commercial dealers. These commercial earthworm stocks are poorly documented as they were started two to three decades ago with specimens purchased from various sources. This culture has been referred to in the literature as *E. fetida.* The last population of interest is a field population occasionally sampled from a compost heap at Middelvlei wine farm (33°55′84″S, 49°87′18″E; Stellenbosch) and has been referred to as *E. fetida* in the literature (Table 1). Middelvlei wine farm has been in operation since 1819. We suspect that these earthworms were introduced on the farmland several decades ago through farming practices.

Two non-focal populations (with no record in the ecotoxicological literature) were also included in the present investigation. One is related to the laboratory culture housed at Stellenbosch University; it was started more than two decades ago with specimens from the Braunschweig stock. This population has been used for residential vermicomposting since its establishment. The other non-focal group recently obtained from Brno, Czech Republic, is a laboratory culture believed to be made of *E. andrei* specimens and maintained in the Department of Zoology at North-West University.

Table 1 provides a list of recent publications (last

Table 1. Origin and literature references of the field population and the laboratory cultures of earthworms included in the present study. The putative name of the species (as reported in the corresponding literature) is provided below the population name. *n*/ ANs indicates the number of specimens used for COI genotyping from the respective populations followed by their respective Genbank accession numbers. (*n*)* indicates the number of original sequences generated from the culture housed in the Stress Ecology Research Laboratory at Stellenbosch University. All the other sequences were generated in the present study.

Population Putative name	Type/use	Locality	Code	*n*/ ANs	Publication record -selected papers-
Stellenbosch 1 *E. fetida*	Laboratory	Stress Ecology Research Laboratory, Stellenbosch University	SUN	20/ JN870005–JN870024 (16)*/ DQ914618–DQ914633	Vermeulen *et al.* 2001; Reinecke *et al.* 2001; Reinecke *et al.* 2002; Reinecke & Reinecke 2003; Reinecke & Reinecke 2004; Maboeta *et al.* 2004; Maleri *et al.* 2007; Maleri *et al.* 2008; Owojori *et al.* 2009; Owojori *et al.* 2010; Voua Otomo *et al.* 2009.
Stellenbosch 2 *E. fetida*	Field	Middelvlei wine farm (33°55′84″S, 18°49′87″E)	MIDD	19/ JN870048–JN870066	Voua Otomo & Reinecke 2010; Voua Otomo *et al.* 2011.
Potchefstroom 1 *E. fetida*	Laboratory	Unit for Environmental Sciences Laboratory, North-West University	POTCH	23/ JN870025–JN870047	Maboeta & Van Rensburg 2003a; Maboeta & Van Rensburg 2003b; Maboeta *et al.* 2008.
Potchefstroom 2 *E. andrei*	Laboratory	Department of Zoology, North-West University	ZOO	10/ JN869995–JN870004	None
Potchefstroom 3 *E. fetida*	Vermicomposting	Grimbeek Park, Potchefstroom (26°43′29″S, 27°06′48″E)	VERM	22/ JN870067–JN870088	None

decade) utilizing the focal populations discussed as well as the number of specimens per population/laboratory culture included in the present study.

COI genotyping

Total genomic DNA was extracted using the NucleoSpin® Tissue kit (Macherey-Nagel) from a varying number of earthworms per group (Table 1). We used the universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994) to amplify 650 bp of the cytochrome oxidase I (COI) gene.

PCR reactions consisted of 0.3 μl ($\pm$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 30 s, 50°C for 30 s and 72°C for 45 s. 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 50 ml TAE buffer, 1.5% w/v) stained with 5 μl ethidium bromide. Sequencing reactions were performed using the ABIv3.1 BigDye® kit. Purified sequences were run on an ABI 3500XL Genetic analyser.

All the new barcodes generated in the present study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank; ANs: JN869995–JN870088). These *Eisenia* sequences were compared to conclusively identified COI sequences of *E. fetida* and *E. andrei* deposited in GenBank and additional sequences (EWSJC613-10 and EWSJC614-10) from the Barcode of Life Data Systems (BOLD, http://www.boldsystems.org). *Allolobophoridella eiseni* (ANs: AY874492 and AY874487) and *Microscolex phosphoreus* (AN: AB608785) were used as outgroups.

All the sequences were aligned, edited and
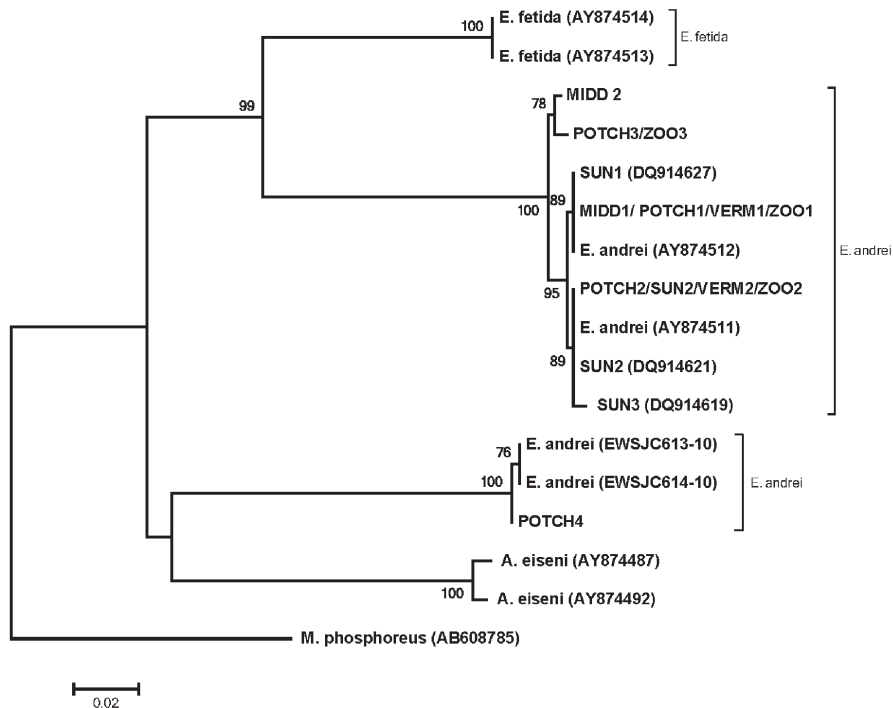
Fig. 1. Phylogenetic tree based on Kimura-2-parameter distances separating all haplotypes identified in the field population and laboratory cultures of *Eisenia* sp. Bootstrap support obtained for specific nodes are reported. Genbank accession numbers or BOLD process IDs are provided in brackets for the sequences downloaded from either Genbank or BOLD. *Allolobophoridella eiseni* and *Microscolex phosphoreus* were included as close and distant relatives to *Eisenia* sp., respectively. *The grouping of POTCH4 and the BOLD samples from Brazil represents a potential unidentified earthworm species. The '*E. andrei*' ID is therefore considered dubious.

analysed in MEGA v5 (Tamura *et al.* 2011). Neighbour-joining (NJ) trees were constructed using the Kimura-2-parameter (K2P) method (Kimura 1980). Bootstrap support was obtained from 1000 iterations.

RESULTS

Ninety-four new *Eisenia* sequences were generated in the present study. Of these, 59 (62.77%) were identical to the SUN2 haplotype found in a previous study (Voua Otomo *et al.* 2009). This shared haplotype was found in all the *Eisenia* populations/cultures (POTCH2/SUN2/VERM2/ZOO2) except for the field population collected at Middelvlei farm. SUN2 was also the only haplotype found in the 20 additional individuals from the stock culture of the Stress Ecology Research Laboratory at Stellenbosch University. The second most frequent sequence (MIDD1/POTCH1/VERM1/ZOO1; 31.91%) was found in all *Eisenia* populations/cultures except for the SUN laboratory culture [it had however been previously found to occur in that culture – SUN1 haplotype–

Voua Otomo *et al.* (2009)]. A rare shared haplotype (POTCH3/ZOO3; 3.19%) was found in both laboratory populations from North-West University and two unique haplotypes (MIDD2 and POTCH4; 1.65%) were found in the MIDD field population and the POTCH laboratory population, respectively. The least diverse of all the groups were both the MIDD and the VERM groups with two haplotypes each, while the POTCH culture harbouring four haplotypes was the most diverse.

Phylogenetic analyses showed that all the local *Eisenia* sequences grouped unequivocally with 100% bootstrap support, to identified sequences of *E. andrei* (Fig. 1). The unique haplotype POTCH4 was considerably divergent from the other local sequences (> 23% K2P). It grouped, nonetheless, with identified BOLD sequences of *E. andrei*. Excluding POTCH4, sequence divergences smaller than 1.75% and greater than 16.5% K2P separated the new *Eisenia* sequences with previously identified sequences of *E. andrei* and *E. fetida*, respectively.

DISCUSSION

None of the populations investigated were found to be *E. fetida.* This is in contrast to literature evidence from the last decade stating that South African ecotoxicologists have utilized this species. Prior to the widespread use of genomic technologies, a local study compared the growth and reproduction of local earthworms, identified as *E. fetida*, to *E. andrei* and *E. fetida* specimens imported from France (Reinecke & Viljoen 1991). This was done in an attempt to confirm the identify of the populations occurring in South Africa. Maturation time (development of well-defined clitellia), indicated that these worms 'resembled neither *E. fetida* nor *E. andrei*' from France while cumulative cocoon production indicated they 'more closely resembled *E. fetida*' (Reinecke & Viljoen 1991). However, with regard to appearance, growth and cocoon hatching success, the local earthworms 'resembled *E. andrei* more closely'. Consequently, the authors could not conclusively rule on the question and stated that these observations could be either due to the occurrence of mixed local populations or to geographical isolation of these local populations from either of the conspecific populations from France (Reinecke & Viljoen 1991). There is a strong possibility that these worms may have been *E. andrei* all along.

Nevertheless, these local populations could have been a mixture of the two species since it is acknowledged that both commonly occur in mixed colonies and that *E. andrei* could outcompete *E. fetida* during periods of food abundance (Elvira *et al.* 1996). *E. andrei* is believed to be the dominant species in commercial vermiculture establishments (Dominguez *et al.* 2005) as a consequence of favourable ambient conditions (typified by regular nutrient availability). It is therefore possible that initial mixed populations of *E. fetida* and *E. andrei* might have occurred locally (as suggested by Reinecke & Viljoen 1991) and that over time, *E. andrei* became the most dominant species. This observation is particularly important for the earthworm laboratory stock housed in the Stress Ecology Research Laboratory at the University of Stellenbosch. This laboratory culture has been enriched with specimens from local sources. This practice could have ultimately resulted in a shift from a predominantly *E. fetida* culture to the present *E. andrei* stock. Such a practice if not rigorously controlled could, in the long run, jeopardise the integrity of long-term laboratory earthworm cultures.

Accurate taxonomy in the field of ecotoxicology is important since without reliable taxonomy, eco(toxico)logical studies would be irrelevant (Dominguez *et al.* 2005). The present results would hardly be those of an isolated case study. Ecotoxicological literature reveals that worldwide, countless researchers rely upon informally identified commercial earthworm stocks for laboratory bioassays. In many instances, earthworms are simply collected from the environment and brought to the laboratory prior to ecotoxicological testing. Lowe & Butt (2007) rightfully argued that the validity of results derived from earthworms of unknown origin must be questioned. Dupont (2009) has acknowledged that 'for the molecular ecologist the problematic taxonomy of earthworms is a critical drawback'.

Our results suggest the occurrence of two distant sequence groups identified as *E. andrei*: one composed of most of the sequences generated in the present study, the other composed of POTCH4 haplotype and other BOLD sequences. High sequence divergence (as high as 25.24% K2P) within *E. andrei* sequences may signal the occurrence of undescribed cryptic species. Such high sequence divergence is beyond the 15% K2P accepted boundary for species separation when using DNA barcodes (Chang & James 2011). This particular finding adds up to the list of similar abundant reports, in oligochaete species, from the literature (King *et al.* 2008; Pérez-Losada *et al.* 2009; Blakemore *et al.* 2010; James *et al.* 2010; Novo *et al.* 2010). It is therefore possible that ecotoxicological testing at the Unit for Environmental Sciences Laboratory at North-West University is being performed on at least two different earthworm species masquerading as *E. fetida.*

The occurrence of high sequence divergence amongst conspecific earthworms is not surprising considering the fact that these organisms have poor dispersal ability which would make them prone to settle in fragmented populations with little or no gene flow between them (Field *et al.* 2007; Novo *et al.* 2009; Mathieu *et al.* 2010). In the case of the *Eisenia* sp. the phenomenon could be compounded by the fact that the known habitats of the species (compost heaps, manure-rich soils, etc.) are naturally fragmented. However, it is worthwhile to note that except for the single rare POTCH4 haplotype (found in the Unit for Environmental Sciences Laboratory, North-West University), all the *E. andrei* COI sequences were in general similar (<1.8% K2P) across populations.

The origin of the divergent POTCH4 haplotype may not be pinpointed until a more geographically inclusive study is conducted. Local commercial earthworm populations have been poorly documented and often started or were enriched with specimens from several local or international sources. We suspect that the putative *E. andrei* sequences (of Brazilian origin) retrieved from BOLD and closely related to POTCH4 (0.19% K2P) may have been misfiled or simply misidentified. This grouping represents a potentially unidentified earthworm species.

In conclusion, this study highlights a pressing need for reliable traditional and molecular taxonomy in the field of earthworm ecotoxicology. It also uncovers the need for comparative ecotoxicological studies between *E. andrei* and *E. fetida*. It is not clear as to how the use of conspecific individuals with increased molecular divergence will impact on the reliability of ecotoxicological testing. Similarly, it is not clear whether unbeknownst to the researchers, the use of cryptic species in bioassays could jeopardise comparative studies using earthworm populations or cultures of diverse origins. Early evidence already suggests that cryptic oligochaete species may respond differently to metal toxicity (Sturmbauer *et al.*1999; Andre *et al.* 2010). Consequently, ecotoxicologists are encouraged, whenever possible, to make use of available genomic technologies to screen their laboratory stocks and available field populations for any molecular distinctiveness.

ACKNOWLEDGEMENTS

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