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Molecular assessment of commercial and laboratory stocks of *Eisenia* spp. (Oligochaeta: Lumbricidae) from South Africa

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ABSTRACT

DNA barcoding was used to investigate laboratory and commercial stocks of *Eisenia* species from four provinces of South Africa. 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 megascolecid *Perionyx excavatus*. The remaining eight haplotypes belonged to the genus *Eisenia*, although only a single *E. fetida* haplotype, represented by six specimens, was found in one of the European populations. The other seven haplotypes, all occurring in South Africa, were *E. andrei*. One of the commercial stocks from South Africa and a laboratory culture from Europe were mixtures of *E. andrei* – *P. excavatus* and *E. andrei* – *E. fetida*, respectively. Previous allozyme studies have helped to suggest that some of the populations included in this study may be suffering from inbreeding depression, which could result in adverse consequences for both the vermiculture industry and ecotoxicological research in South Africa.

KEY WORDS: Oligochaeta, Lumbricidae, South Africa, DNA barcoding, earthworms, redworm, ecotoxicology, vermiculture.

INTRODUCTION

Eisenia fetida (Savigny, 1826) and *Eisenia andrei* Bouché, 1972 have become cosmopolitan earthworm species because of their worldwide use in ecotoxicological testing and vermicomposting. Originating from Palaearctic 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 earthworm species recommended by the Organisation for Economic Co-operation and Development (OECD 1984, 2004) and the International Organization for Standardization (ISO 2008, 2012) for the testing of chemicals.

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 allozyme polymorphism, Jaenicke (1982) and Øien and Stenersen (1984) indicated that these subspecies are 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* are different biological and phylogenetic species, as judged by their reproductive isolation and DNA divergence.

In South Africa and world-wide, *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 and North-West University, respectively, 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 & van Rensburg 2003*a, b*; Maboeta *et al.* 2008; Owojori *et al.* 2009; Voua Otomo & Reinecke 2010).

Despite the interest in both species, there has been no molecular study of populations introduced locally or of cultures of *E. andrei* and *E. fetida* used in South Africa. Molecular work on selected laboratory and field populations have focused on the toxicological effects of particular toxicants on DNA integrity and allozyme polymorphism in these earthworms (Reinecke & 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 researching its taxonomic identity.

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* spp. stocks should be relevant to the breeders, potential buyers and researchers alike. The ecotoxicological literature reveals that countless researchers worldwide rely upon informally identified commercial earthworm stocks for laboratory bioassays (e.g., Beyer 1996; Fitzpatrick *et al.* 1996; Saint-Denis *et 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 investigation of earthworm stocks from selected vermiculture establishments and research laboratories in South Africa in order to confirm their taxonomic status, and assess their levels of genetic richness and differentiation.

MATERIAL AND METHODS

Earthworm populations

In the present study, the term “population” is used in an inclusive manner and thus may refer to a free-living “wild” population or to a captive breeding stock. A total of eight focal and two non-focal populations were the subject of this study. Focal populations included two vermiculture stocks from Johannesburg (Gauteng, South Africa), two vermiculture stocks and a laboratory culture from Potchefstroom (North West, South Africa), a free-living population and a laboratory culture from Stellenbosch (Western Cape, South Africa) and a vermiculture stock from Port Elizabeth (Eastern Cape, South Africa). Two non-focal laboratory cultures were acquired from Brno (Czech Republic) and Southampton (UK). 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 from the list of recent publications based upon research work carried out on the populations concerned.

Because of the economic importance of *Eisenia* spp. and considering that potential earthworm buyers are mostly unable to distinguish between different earthworm species, we decided not to sort the randomly picked local specimens according to phenotypic features, thus allowing us to identify possible mixed cultures.

TABLE 1

Localities, presumed identity, use and publications record for 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.

Population	Type/use	Origin/Locality	Code	n/ANs	Selected publications
Stellenbosch 1 Presumed ID: <i>E. fetida</i>	Laboratory	Stellenbosch University, (33°56'03.4"S 18°51'56.4"E) Western Cape, South Africa	SUN	44/JN870005–JN870024 DQ914618–DQ914633 JX912906–JX912913	Reinecke & Reinecke 2003, 2004 Maboeta <i>et al.</i> 2004 Maleri <i>et al.</i> 2007, 2008 Owojori <i>et al.</i> 2009, 2010
Stellenbosch 2 Presumed ID: <i>E. fetida</i>	Field	Middelvlei wine farm, Stellenbosch (33°55'84"S 18°49'87"E) Western Cape, South Africa	MID	19/JN870048–JN870066	Voua Otomo & Reinecke 2010 Voua Otomo <i>et al.</i> 2011
Potchefstroom 1 Presumed ID: <i>E. fetida</i>	Laboratory	North-West University, Potchefstroom (26°41'21"S 27°05'26"E) North West, South Africa	NWU	45/JN870025–JN870047 JX912898–JX912905 JX908652–JX908665	Maboeta & van Rensburg 2003a, b Maboeta <i>et al.</i> 2008
Potchefstroom 2 Presumed ID: <i>E. fetida</i>	Vermicomposting	Grimbeek Park, Potchefstroom (26°43'29"S 27°06'48"E) North West, South Africa	GRM	22/JN870067–JN870088	none
Potchefstroom 3 Presumed ID: <i>E. fetida</i> and <i>E. andrei</i>	Vermicomposting	Mieder Park, Potchefstroom (26°43'15.6"S 27°06'06"E) North West, South Africa	MPP	11/JX908641–JX908651	none
Port-Elizabeth Presumed ID: <i>E. fetida</i> and <i>E. andrei</i>	Vermicomposting	Newton Park, Port Elizabeth (33°56'57.1" S 25°33'35.0"E) Eastern Cape, South Africa	PE	21/JX908692–JX908712	none
Johannesburg 1 Presumed ID: <i>E. fetida</i>	Vermicomposting	Ferndale, Johannesburg (26°06'01.7"S 28°00'08.6"E) Gauteng, South Africa	JNB	16/JX899807–JX899822	none
Johannesburg 2 Presumed ID: <i>E. fetida</i>	Vermicomposting	Morningside, Johannesburg (26°04'52.5"S 28°03'44.4"E) Gauteng, South Africa	JOZ	10/JX912888–JX912897	none
United Kingdom Presumed ID: <i>E. fetida</i>	Laboratory	Southampton (50°54'34"N 1°24'15"W) United Kingdom	ENG	26/JX908666–JX908691	none
Czech Republic Presumed ID: <i>E. andrei</i>	Laboratory	Brno (49°11'42"N 16°36'24"E) Czech Republic	CZR	10/JN869995–JN870004	none

COI genotyping

Total genomic DNA was extracted from 224 earthworms using the NucleoSpin® Tissue kit (Macherey-Nagel). Samples of 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 (~1 µl) 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 (0.75 g 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 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 (Table 1). They were tentatively identified using the BOLD (Barcode of Life Data Systems) Identification System 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 tree was subsequently constructed. Bootstrap support was obtained from 1000 iterations. Since COI diversity is highly dependent on effective population size and because of the uneven sample sizes of the groups included in this study, we used the Contrib software of Petit *et al.* (1998) 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* spp. COI sequences occurring in South Africa, using the Median-joining method.

RESULTS

K2P-based analysis

Nine distinct sequences of the COI gene were identified amongst the 224 worms included in this study. The haplotype distributions across the populations revealed that H1 (haplotype 1) was the most widespread and H2 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).

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. 1). 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%. The identity of H7 is therefore uncertain, especially considering the fact that it grouped with unpublished (i.e. potentially unverified), alleged *E. andrei* sequences from

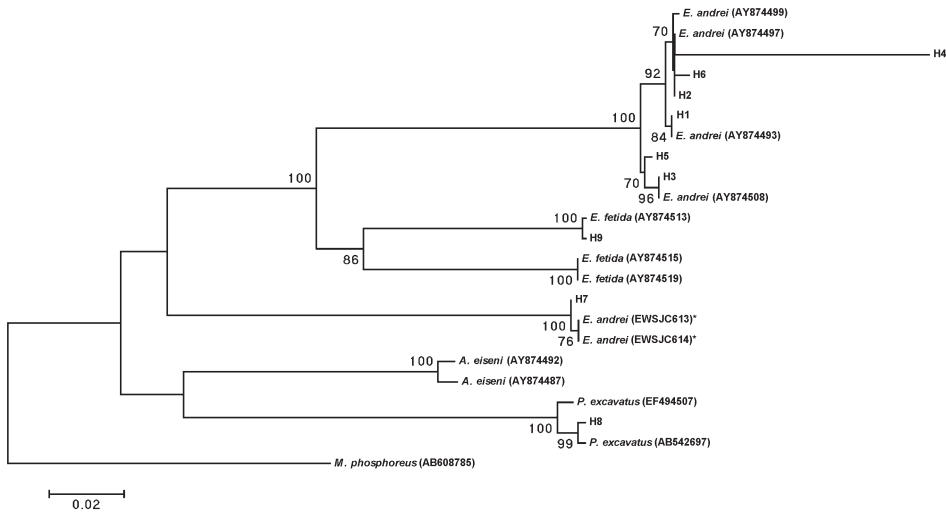


Fig. 1. Neighbour-joining tree based on the K2P method. Bootstrap support obtained for specific nodes are reported. Genbank accession numbers or BOLD process IDs are provided in brackets for the sequences downloaded from either Genbank or BOLD. *Allolobophoridella eiseni* and *Microscolex phosphoreus* were included as outgroups. Asterisk indicates dubious *E. andrei* sequences from BOLD.

BOLD (EWSJC613-10, EWSJC614-10) (Fig. 1). H8 grouped with GenBank sequences of the megascolecid *Perionyx excavatus* ($K2P \leq 1.2\%$). The BOLD system also identified H8 as *P. excavatus*. H9 grouped with previously identified sequences of *E. fetida* ($K2P \leq 11.7\%$). The earthworm cultures from Port Elizabeth and Southampton were mixtures of *E. andrei* – *P. excavatus* and *E. andrei* – *E. fetida*, respectively.

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, does not belong to the genus *Eisenia*. Consequently, only seven *Eisenia* COI haplotypes were found to occur in local populations. All of 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. 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 changed to JOZ ($r(10) = 2$), PE ($r(10) = 1.658$) and JNB ($r(10) = 1.5$). MID also 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 (Table 4). Differentiation indices DH_T and $DG_{ST} > 0.75$ for MID revealed that this population was indeed the most divergent of the local populations included in the present study. Negative C_D values for the other populations reflected a lack of significant differentiation between them. This was confirmed by conventional population pairwise FSTs that showed non-significant differentiation amongst these populations.

Figure 2 represents a network of the *E. andrei* haplotypes found in local South African populations. The dubious haplotype H7 was excluded from this analysis. The least

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/ Populations	H1	H2	H3	H4	H5	H6	H7
GRM	18.20% (n = 4)	81.80% (n = 18)	0	0	0	0	0
JHB	12.50% (n = 2)	81.25% (n = 13)	0	6.25% (n = 1)	0	0	0
JOZ	10.00% (n = 1)	80.00% (n = 8)	0	0	10.00% (n = 1)	0	0
MID	94.74% (n = 18)	0	0	0	5.26% (n = 1)	0	0
MPP	9.10% (n = 1)	90.90% (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	15.00% (n = 3)	75.00% (n = 15)	10.00% (n = 2)	0	0	0	0
SUN	4.50% (n = 2)	91.00% (n = 40)	0	0	0	4.50% (n = 2)	0
ENG	10.00% (n = 2)	80.00% (n = 16)	0	0	10.00% (n = 2)	0	0
CZR	40.00% (n = 4)	50.00% (n = 5)	10.00% (n = 1)	0	0	0	0
Total	n = 44	n = 160	n = 5	n = 1	n = 4	n = 2	n = 1

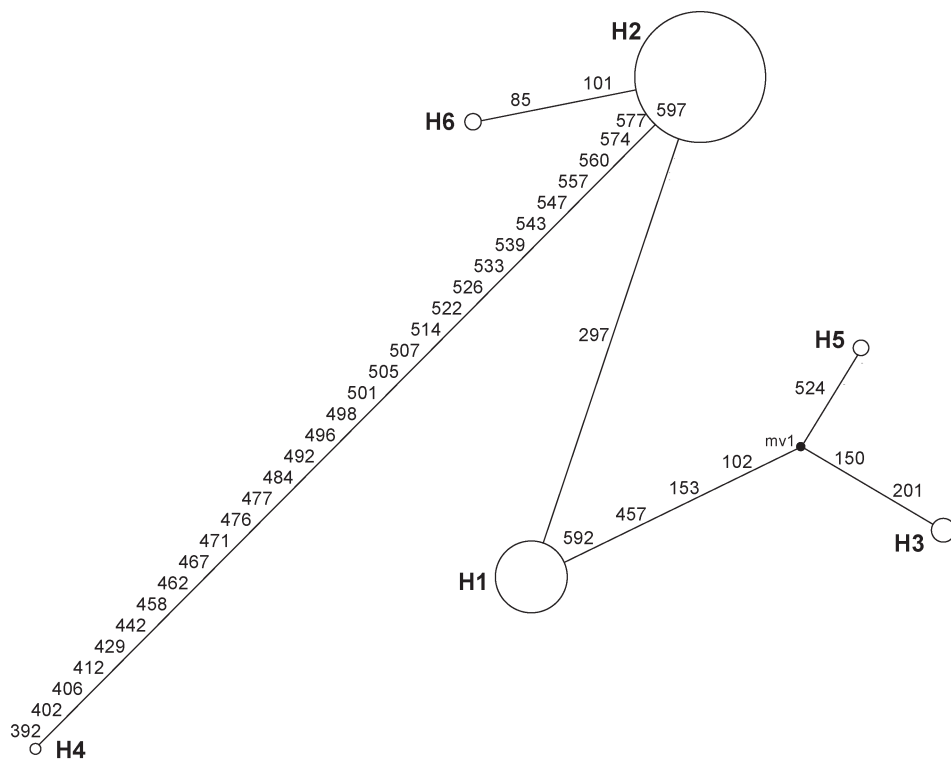


Fig. 2. Haplotype network calculated from the *E. andrei* COI haplotypes found in the South African earthworm groups investigated. The size of the circles is proportional to the number of earthworms sharing the same haplotype. The numbers on the branches indicate the positions of mutations on the COI sequences, mv1 represents a median vector (intermediate haplotypes, not found in this study).

number of mutations found was between H1 and H2 (a single mutation) and the highest number of mutations was between H2 and H4 (31 mutations).

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 mixture 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 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 of the eight local earthworm groups investigated were bred in captivity, perhaps the inclusion of more field populations would have helped to detect the presence of *E. fetida*.

TABLE 3

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

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 South Africa has a greater likelihood of acquiring *E. andrei*; and occasionally together with individuals of another species such as *P. excavatus*.

The unique COI sequence (H7) identified as an *E. andrei* sequence through the BOLD system was extremely divergent from the other *E. andrei* sequences. Using the K2P method, the accepted threshold for species delimitation on the basis of DNA barcode data is 15% K2P (Chang & James 2011). The divergence between H7 and the other *E. andrei* haplotypes was consistently more than 23% K2P. An increasing number of cryptic oligochaete species have been reported in the literature since the recent advent of earthworm molecular studies (King *et al.* 2008; Pérez-Losada *et al.* 2009; Blakemore *et al.* 2010; James *et al.* 2010; Novo *et al.* 2010). H7 could represent an as yet undescribed species. However, additional molecular and morphological investigations would be required to shed further light on the matter.

COI haplotype numbers were limited to two or 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 in earthworms has been investigated, 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) obtained H values as high as 0.92 in populations of the hormogastrid earthworm *Hormogaster elisae* from the central Iberian Peninsula. Equally high haplotypic richness has been reported in several other species of earthworms such as *Dendrobaena octaedra* (Cameron *et al.* 2008; Knott & Haimi 2010), *Amyntas wulinensis* (Chang *et al.* 2007), *Aporrectodea rosea*, *Octolasion lacteum*, and *Lumbricus 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* spp., the phenomenon could be compounded by the fact that

TABLE 4

Measure of genetic diversity and divergence for each South African population of *Eisenia andrei* based on COI sequence data after rarefaction to a common sample size of ten. Abbreviations: n – number of specimens included per population; Nb Hap. – number of haplotypes; H (SE) – haplotype diversity with standard error in brackets; π (SE) – nucleotide diversity with standard error in brackets; r (10) – allelic richness after rarefaction to a common size of ten specimens per sample; DH_s , DH_p , DG_{ST} – divergence indices from the other populations; C_p , C_s , C_d – contribution indices to total diversity; C_{rT} , C_{rS} , C_{rD} – contribution 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_p	DG_{ST}	C_T	C_S	C_D	C_{rT}	C_{rS}	C_{rD}
GRM	22	2	0.312 (0.106)	0.0010 (0.0010)	0.93	0.30	0.38	0.21	-0.050	0.008	-0.06	-0.070	-0.030	-0.040
JNB	16	3	0.342 (0.140)	0.0080 (0.0040)	1.50	0.31	0.39	0.21	-0.040	0.017	-0.06	-0.050	0.028	-0.080
JOZ	10	3	0.378 (0.181)	0.0020 (0.0020)	2.00	0.33	0.41	0.20	-0.030	0.029	-0.06	-0.010	0.078	-0.090
MID	19	2	0.105 (0.092)	0.0010 (0.0010)	0.53	0.21	0.88	0.76	0.322	-0.060	0.38	0.159	-0.070	0.229
MPP	11	2	0.182 (0.144)	0.0006 (0.0008)	0.91	0.24	0.35	0.30	-0.080	-0.030	-0.04	-0.120	-0.030	-0.090
NWU	45	4	0.377 (0.082)	0.0110 (0.0060)	1.47	0.33	0.41	0.20	-0.030	0.029	-0.06	0.005	0.025	-0.020
PE	20	3	0.426 (0.122)	0.0030 (0.0020)	1.66	0.35	0.43	0.19	-0.020	0.044	-0.06	0.015	0.043	-0.030
SUN	44	3	0.173 (0.075)	0.0006 (0.0007)	0.81	0.24	0.36	0.33	-0.070	-0.040	-0.03	0.002	-0.040	0.044

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 has yet to be thoroughly investigated in Europe, where they originated.

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

Of all the local groups investigated, MID was the only significantly divergent population. The haplotype distributions across the populations (Table 2) show 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 under 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 is suspected that inbreeding could be occurring in these populations.

This may have significant implications for both the research sector and the vermiculture 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 such as growth, reproduction and survival (Charlesworth & Charlesworth 1987; Reed & Frankham 2003). Velando *et al.* (2006) researched the deleterious effects of inbreeding on the reproduction of *E. andrei* and reported that inbreeding causes a “*strong reduction of cocoon production*”.

CONCLUSION

The use of DNA barcoding has helped to show that *E. fetida* may be rarer in South Africa than previously assumed. *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.

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