Bull Environ Contam Toxicol (2009) 82:261–264
DOI 10.1007/s00128-008-9585-4

Usefulness of DNA Barcoding in Ecotoxicological Investigations: Resolving Taxonomic Uncertainties Using *Eisenia* Malm 1877 as an Example

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Received: 9 May 2008 / Accepted: 8 October 2008 / Published online: 24 October 2008

Abstract Standard test species may differ in their response to toxicants. Accurate identification of test organisms is therefore of critical importance in correctly interpreting data generated from laboratory assays. This is not always possible when species are morphologically similar or where the taxonomy of the group has recently been revised. A case in hand concerns *Eisenia* sp. Based on recent genetic evidence two species, *Eisenia andrei* and *Eisenia fetida*, which were previously considered a single species, are currently recognized. In these instances, DNA barcoding, demonstrated and discussed herein, provides a method to accurately identify test organisms.

Keywords *Eisenia fetida* · *Eisenia andrei* · Mitochondrial DNA · COI

A number of soil dwelling species have been identified as standardised test species through thorough testing including *Eisenia andrei* and *Eisenia fetida* (OECD 1984; OECD 2004). Originally described as *E. foetida* by Savigny in 1862, it was later noted that two distinct forms (a uniformly pigmented and a striped form) of this species occur. Several studies, often reaching conflicting findings, were subsequently undertaken to shed more light on the recognition of two forms (e.g. André 1963; Avel 1929; Jaenike 1982; Reinecke and Viljoen 1991). Most recently, genetic evidence confirmed the presence of two distinct species (Perez-Losada et al. 2005).

Bouché (1992) stressed the importance of using genetically homogenous organisms (e.g. biologically defined species) in ecotoxicological testing since species often differ in their response to various toxic substances (Posthuma et al. 2002). Although the role of morphology in identifying and describing species is invaluable, it is of little use when dealing with cryptic species (such as *E. fetida* and *E. andrei*). DNA barcoding was proposed by Hebert et al. (2003) to deal with morphologically difficult groups or groups where taxonomic expertise is mostly lacking. Although the usefulness of DNA barcoding has been much debated (e.g. Moritz and Cicero 2004), much of this debate has centred on DNA techniques replacing traditional taxonomy rather than assisting it.

Our aim is to demonstrate the usefulness and relative ease of applying DNA techniques to identify and/or verify the taxonomy of test species. For this, we draw on a test case from our Ecotoxicology Stress Laboratory at Stellenbosch University where our original stock culture of *Eisenia* sp. was provided as *E. fetida.* Following a barcoding approach, we confirm our stock culture as *E. andrei*.

Materials and Methods

Our initial stock culture, established ~25 years ago, was obtained from Prof O. Graff (Braunschweig, Germany). The initial identification as *E. fetida* was confirmed by Prof A. Zicsi (Budapest, Hungary). To verify the species

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identification of the stock culture used at Stellenbosch University following recent advances in earthworm taxonomy, 16 worms were randomly selected for DNA barcoding. We purposefully selected such a high number of worms to ensure that we adequately capture the genetic variation present in our stock culture (Table 1).

Detailed barcoding protocols have been developed, and these are available from www.barcoding.si.edu. In short, animals are sacrificed in a humane manner typically through freezing, cyanide or immersion in ethanol. The latter has been our method of choice since it optimally preserves tissue and minimizes DNA degradation. To minimize the risk of contamination, material is always handled on a sterile work bench (benches are wiped down with ethanol) and instruments are flamed or wiped down with ethanol between samples. DNA extractions are performed using either a commercial DNA extraction kit or following phenol/chloroform (Maniatis et al. 1982) or chelex100 (Walsh et al. 1991) protocols. Although the latter two methods are more time consuming, they are far cheaper than commercial extraction kits. It is important that all barcoding studies use the same gene region to allow future comparisons. The standard region for DNA barcoding is the 5′ side of the mitochondrial DNA cytochrome oxidase I (COI) region. This region was selected through thorough screening of a wide range of taxonomic groups (Hebert et al. 2003) which typically includes a geographically representative sample that would adequately capture genetic variation within a species. The COI primers described by Folmer et al. (1994) are often used to amplify ~650 bp of the COI gene. However, optimal primer annealing is vital to obtaining good amplification and sequences, and it may be necessary to design taxon-specific primers to improve amplification quality and quantity. Sequencing is typically done using BigDye chemistry (Applied Biosystems) and run on an automated machine. Barcoding products should be sequenced bi-directionally and sequences are verified by eye using sequencing editor programs. Sequences are aligned to one another using one of several available computer software packages. DNA analysis is typically done following a distance based approach. Phylogenetic trees are constructed from Kimura-2-parameter corrected sequence distances using neighbour-joining algorithms (see Hebert et al. 2003). Sequences are deposited either as part of a barcoding project in www.barcoding.si.edu or in public data bases such as GenBank (http://www.ncbi.nlm.nih.gov) or EMBL (http://www.ebi.ac.uk/embl).

In our study, total genomic DNA was extracted using the phenol/chloroform method. Five-10 mg of the tail section of worms were immersed in 250 µL lysis buffer (160 mM saccharose, 80 mM EDTA, 100 mM Tris/HCl, pH 7.8) in the presence of 10 µL proteinase K (10 mg mL^{-1}). Extractions were incubated overnight at 55°C. Following phenol/chloroform extractions, DNA was precipitated in the presence of 100 µL of a 7.5 M ammonium acetate solution and ice-cold absolute ethanol. DNA pellets were dried and re-suspended in ddH_2O.

Table 1 Specimens included in the present study. Sequences were generated for Earthworm1 through Earthworm16. Species designations, mitochondrial COI haplotypes as well as GenBank accession numbers are provided

Specimen	Species	Haplotype	Genbank
Earthworm1	*E. andrei*	SUN1	DQ914627
Earthworm2	*E. andrei*	SUN1	DQ914628
Earthworm3	*E. andrei*	SUN2	DQ914629
Earthworm4	*E. andrei*	SUN2	DQ914630
Earthworm5	*E. andrei*	SUN2	DQ914631
Earthworm6	*E. andrei*	SUN2	DQ914632
Earthworm7	*E. andrei*	SUN2	DQ914633
Earthworm8	*E. andrei*	SUN2	DQ914618
Earthworm9	*E. andrei*	SUN2	DQ914621
Earthworm10	*E. andrei*	SUN2	DQ914622
Earthworm11	*E. andrei*	SUN2	DQ914623
Earthworm12	*E. andrei*	SUN2	DQ914624
Earthworm13	*E. andrei*	SUN2	DQ914625
Earthworm14	*E. andrei*	SUN2	DQ914626
Earthworm15	*E. andrei*	SUN3	DQ914619
Earthworm16	*E. andrei*	SUN3	DQ914620
	E. fetida	*E. fetida* A	AY874520
	E. fetida	*E. fetida* A	AY874521
	E. fetida	*E. fetida* A	AY874522
	E. fetida	*E. fetida* A	AY874523
	E. fetida	*E. fetida* A	AY874515
	E. fetida	*E. fetida* A	AY874516
	E. fetida	*E. fetida* A	AY874517
	E. fetida	*E. fetida* A	AY874518
	E. fetida	*E. fetida* A	AY874519
	E. fetida	*E. fetida B*	AY874513
	E. fetida	*E. fetida B*	AY874514
	E. andrei	*E. andrei* A	AY874493
	E. andrei	*E. andrei* A	AY874502
	E. andrei	*E. andrei* A	AY874494
	E. andrei	*E. andrei* A	AY874503
	E. andrei	*E. andrei* A	AY874512
	E. andrei	*E. andrei* A	AY874495
	E. andrei	*E. andrei* A	AY874496
	E. andrei	*E. andrei* A	AY874498
	E. andrei	*E. andrei* A	AY874500
	E. andrei	*E. andrei* B	AY874511
	E. andrei	*E. andrei* B	AY874504
	E. andrei	*E. andrei* B	AY874505
	E. andrei	*E. andrei* B	AY874497

We targeted 650 bp of the COI gene using the primers LCO1490 and HCO2198 (Folmer et al. 1994). Polymerase chain reactions (PCR) were performed in a final volume of 30 μL and contained 10 ng of DNA, 1× PCR buffer, 3 μL of a 25 mM $MgCl_2$ solution, 3 μL of a 20 mM dNTP mixture, 1 unit Taq polymerase (Supertherm) and 30 pmol of each of the specified primers. PCR cycling comprised an initial denaturation step at 94°C for 5 min followed by 35 cycles of 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. To verify successful amplification, amplicons were electrophoresed in 1% agarose gels stained with ethidium bromide. PCR products were gel purified with the Wizard SV Gel and PCR clean-up system (Promega). Sequencing reactions were performed using BigDye® chemistry (Applied Biostystems). Purified sequencing products were run on an ABI 3100 automated sequencer (Applied Biosystems). All *Eisenia* sequences generated in this study were deposited in GenBank (accession numbers DQ914618-DQ914633).

To demonstrate the usefulness of a barcoding approach to resolve uncertainty regarding the taxonomy of laboratory animals, 37 *Eisenia* sequences representing three species (*E. fetida*, *E. andrei* and *E. eiseni*) available through the Barcoding of Life Database (www.barcodinglife.org) were aligned to the 16 sequences generated in the present study. A more distantly related species, *Aporrectodea handlirschi*, was also included in our analyses. We constructed a neighbour-joining (NJ) tree in PAUP* (Swofford 2000) based on Kimura-2-parameter distances (K2P). Bootstrap support was obtained from 1,000 iterations.

Results and Discussion

The accurate identification of laboratory test animals is central to ecotoxicological studies. In this respect, the confusion surrounding the use of *E. fetida*/*E. andrei* in laboratory experiments may be hugely problematic for accurate interpretation of test results. This is perhaps best illustrated by the indiscriminate use of *E. fetida* (or *E. foetida*) without the necessary consideration for accurate taxonomy (Perez-Losada et al. 2005). The emergence of DNA barcoding as a means of species identification (Hebert et al. 2003) holds much promise for identification of ecotoxicological laboratory test species. The methodology developed for DNA barcoding is universal, based on a single gene region (COI although ribosomal genes are sometimes used for specific groups) and standardized across a wide range of taxa.

For DNA barcoding to be successful, certain criteria must be met. For example, the genetic divergence within species (intra-specific divergences) must be smaller than the separation between species (inter-specific divergences) (the 10× rule). Three unique DNA sequences (or haplotypes), separated by <1% K2P sequence divergence, characterized the 16 Stellenbosch specimens included in the present study. On average, <1% K2P sequence divergence separated specimens within species with >16% K2P sequence divergence between species (the highest K2P sequence distance in this study was 25.2% between *E. fetida* and *A. handlirschi*). However, two very divergent haplotypes were detected within *E. fetida* separated by 11.6% K2P sequence divergence which might represent an additional and yet undescribed *Eisenia* species (Perez-Losada et al. 2005).

The results of our phylogenetic analyses are shown in Fig. 1. The monophyly of the three species (*E. fetida, E. andrei* and *E. eiseni*) were confirmed by 100% bootstrap support. The three haplotypes detected for the Stellenbosch specimens (SUN1, SUN2 and SUN3) grouped with 100% support within *E. andrei*. Indeed, two of the haplotypes detected in this study were identical to published *E. andrei* sequence data.

One of the critical assumptions of a DNA barcoding approach concerns the availability of genetic data that will reliably discriminate between taxa at species level (Hebert et al. 2003). Perez-Losada et al. (2005) generated such a data set for the three *Eisenia* sp. (their study included *E. eiseni* from Spain, *E. fetida* from Spain and Ireland and

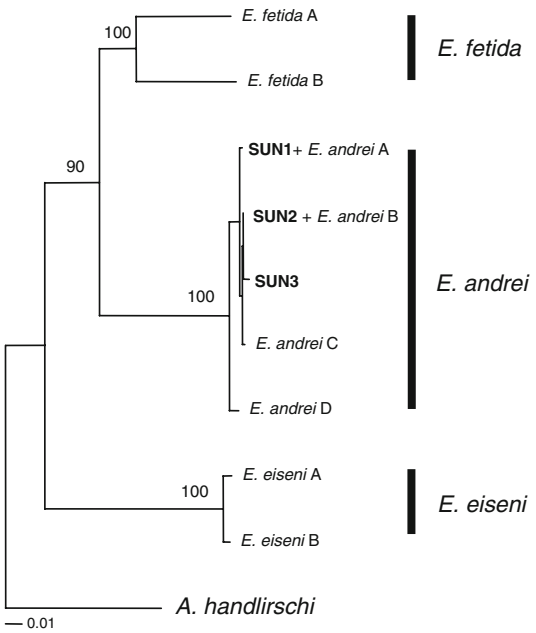

Fig. 1 Distance (neighbour-joining) tree based on Kimura-2-parameter distances separating all haplotypes identified in this study. Values above branches denote bootstrap support obtained for specific nodes. *Aporrectodea handlirschi* was included as a distant relative to *Eisenia* sp.

E. andrei from Spain, Ireland and Brazil). They found no shared haplotypes between *E. fetida* and *E. andrei* (both taxa were mutually exclusive) which, when taken with reproductive isolation (Dominguez et al. 2005), qualifies these taxa as distinct phylogenetic and biological species.

In conclusion, reliable species identification is essential in ecotoxicological studies in that it prevents discrepancies between comparative studies where different test species are used as well as misleading recommendations and/or conclusions. Given the ease and reliability of the approach outlined herein, we urge all ecotoxicologists working with cultures of uncertain provenance, to investigate and establish the taxonomic affinities of their test species.

Acknowledgments This work was funded through the DST•NRF Centre for Invasion Biology (BvV) and National Research Foundation (SAR). Any opinions, findings and conclusions or recommendations expressed in this material are those of the authors and the NRF does not accept any liability in regard thereto. PVO was funded by the government of Gabon. Prof A.J. Reinecke provided the material for this study as well as valuable comments and discussions on the project. Prof H.N. Nigg and an anonymous reviewer are acknowledged for comments on the manuscript.

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