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DNA barcoding of earthworms (*Eisenia fetida/andrei* complex) from 28 ecotoxicological test laboratories

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ABSTRACT

Plenty of evidence indicates that the earthworms *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* Bouché, 1972 (*Lumbricidae*) can be distinguished by morphological, physiological and molecular traits. However, the morphological differences alone do not allow to correctly identify these taxa. This may be a serious problem for the reliability of the standard ecotoxicological tests for which these worms are used. Recently, DNA barcoding (i.e., sequencing of a standard mtDNA COI gene fragment for identification purposes) has been proposed as a quick and simple method to identify species, including earthworms. In order to assess the practicability and reliability of this method, an international ring test was organized by the "*Eisenia* barcoding Initiative (EBI)", a group of scientists from four public institutions and two contract laboratories. Coded samples of *E. fetida, E. andrei,* and *Eisenia* sp. were provided by 28 ecotoxicological laboratories from 15 countries on four continents. Five laboratories in Belgium, Canada, Germany, and Spain identified the specimen through DNA barcoding. All steps of the sample preparation were described by Standard Operating Procedures (SOP). The COI sequences (581 bp) obtained were used to construct a neighbor-joining tree based on the uncorrected pairwise *p*-distance. This analysis revealed three distinct haplotype clusters: one including only *E. andrei* sequences (mean within-group *p*-distance 0.026 ± 0.002) and two with only *E. fetida* sequences, referred to as *E. fetida* 1 and *E. fetida* 2. Each of the latter two in fact represented one single haplotype. The mean *p*-distance between *E. fetida* 1 and *E. fetida* 2 was 0.112, whereas the mean *p*-distances between these two taxa and *E. andrei* were 0.142 and 0.143, respectively. Such COI divergence levels are usually indicative of species level differentiation. Hence it is hypothesized that *E. fetida* 1 and *E. fetida* 2 refer to different cryptic species. Since the attribution of the individual worms to these three clusters was completely consistent among the five DNA barcoding laboratories the good applicability of DNA barcoding for the identification of these ecotoxicological test species is proven. Remarkably, specimens of the molecular *E. fetida* clusters were always identified morphologically as *E. fetida*. However, this was not true the other way round, i.e., some specimens of the molecular *E. andrei* cluster were identified morphologically as *E. fetida*. The results of this ring test are presented to standardization organizations (OECD, ISO) in order to improve the standardization and thus quality of ecotoxicological routine testing by using DNA barcoding.

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1. Introduction

Almost as long as earthworms have been used in standard ecotoxicological tests there is a discussion going on whether the earthworms recommended for these tests (e.g., OECD 1984, 2004; ISO, 1993, 1998, 2008; comparable methods have been described in national guidelines, e.g., from Brazil or Canada) belong to one species or two. Plenty of evidence suggests that two taxa, *Eisenia fetida* (Savigny, 1826) and *E. andrei* Bouché (1972) can be distinguished by morphological, physiological, and molecular traits (e.g., Jaenike, 1982; Oien and Stenersen, 1984; Reinecke and Viljoen, 1991; Jänsch et al., 2005). Pérez-Losada et al. (2005) compiled the information regarding differences between *E. fetida* and *E. andrei* and found that *E. fetid*a may be a species complex. Note that in older literature the name "fetida" is erroneously spelled "foetida" (Bouché, 1972). Domínguez et al. (2005) confirmed that *E. fetida* and *E. andrei* are reproductively isolated because no viable offspring was produced when crossed. *E. fetida* is characterized by prominent yellow transverse segmental stripes, while *E. andrei* is uniformly dark reddish. However, this difference is often not sufficient to identify these worms correctly, partly because after fixation the characteristic yellow stripes of *E. fetida* disappear (Bundy et al., 2002) and therefore the reliability of ecotoxicological test results can be questioned. This problem was ignored for quite some time, mainly because only few taxonomy experts for this group are available. Therefore, DNA barcoding (Hebert et al., 2003) of these earthworms was proposed as a quick and simple method to identify species (e.g., Voua Otomo et al., 2009, 2013a,b). DNA barcoding is especially necessary when performing standardized tests the outcome of which is used in formal environmental risk assessment procedures, e.g. as part of the registration of pesticides (EC 1107/2009). In order to assess the practicability and reliability of DNA barcoding in the context of soil ecotoxicology, an international ring test was organized by the "*Eisenia* barcoding Initiative (EBI)", a group of scientists from four public institutions and two contract laboratories. The results of this ringtest will be submitted to international organizations (mainly OECD and ISO) in order to include this information in their further standardization work, e.g., by modifying existing individual guidelines or by preparing a Guidance Document which describes the DNA barcoding procedure independently from the individual guidelines. Therefore, this contribution tries to answer three questions:

1. Is DNA barcoding reliable for species identification of the *E. fetida/andrei* complex?
2. Which species are found among the specimens routinely used for ecotoxicological testing?
3. How reliable is the morphological identification of the earthworms used in different ecotoxicological laboratories?

In sum, the EBI will improve the standardization of the earthworm tests and thus the quality of the test results by clearly identifying the test species used. This will increase the reliability and reproducibility of the ecotoxicological test results.

2. Material and methods

2.1. Ringtest organization

The ringtest was coordinated by ECT Oekotoxikologie GmbH (a private contract laboratory; Flörsheim, Germany), in close collaboration with the Biodiversity and Climate Research Centre (BiK-F; Frankfurt am Main, Germany) and the company All Genetics & Biology S.L. (A Coruña, Spain). The ringtest was supported by the Global Soil Advisory Group (GSAG) of the Society of Environmental Toxicology and Chemistry (SETAC). In the ringtest, 40 ecotoxicological laboratories were contacted and 31 participated (the list of participants is given at the end of this paper). However, since in three cases earthworms were destroyed during the transport, the final number of participants was 28 (16 from Europe, five from South America, three from North America, two from Africa, and two from Asia), of which 11 were affiliated to a university, 11 were governmental institutes, and six were contract laboratories. DNA barcoding was performed in parallel in five laboratories: BiK-F (Frankfurt, Germany), AllGenetics (A Coruña, Spain), the universities of Vigo (Spain) and Guelph (Canada), and the Royal Belgian Institute of Natural Sciences (Brussels, Belgium).

2.2. Ringtest performance: origin and handling of the worms

All work performed was described in Standard Operating Procedures (SOP), which covered the following steps in detail: selection and preservation of the earthworms to be analyzed, transport of the worm samples to the laboratories for DNA extraction, amplification, and sequencing. Each participating ecotoxicological laboratory had its own earthworm culture(s), about which the following information was asked (though not always completely provided):

- Origin and history of the earthworm culture (where did it come from and how long has it been kept in the lab?):
  - Four out of 28 laboratories did not know the source of their cultures. Twenty laboratories got them from different commercial breeders (only the company Minhobox, which supplied worms to various Brazilian laboratories, was named several times). In addition, the University of Coimbra and ECT had supplied worms in the past to some of the participating laboratories (details of these transfers are not available). In one laboratory, the culture exists since the late 1960s, but it was reported that several times "new" worms were added. Eight laboratories kept their worms for 20–25 years, six kept them for about 10 years, and the remaining ones started culturing worms within the last five years.

- Name of the species kept in culture: has it been identified as *E. fetida* or *E. andrei*?
- All the laboratories except three (7, 30, and 33; see Table 2) provided the species name.
- How was the species identified (e.g., by using which key) and by whom?
  - Ten laboratories did not know who had identified their worms. In the other laboratories identifications had been done by external experts, suppliers, or scientists of the respective institutions. The only key mentioned several times was Sims and Gerard (1999).
- Have individuals from the culture(s) involved already been DNA barcoded, and if so, what was the outcome?
  - Earthworms from five laboratories (all of them from outside Europe) had already been barcoded, but with one exception (Voua Otomo et al., 2009) the outcome was not published.

Each participating laboratory provided four adult individuals from one taxon (either *E. fetida* or *E. andrei*) or both (sorted by taxon) to ECT. To do so, the worms were first separated from their culture medium, rinsed with tap-water to remove soil particles from their body surface, and kept for about 24 h on moist filter-paper in a small recipient (e.g., a petri-dish) in order to empty their gut. Afterwards, individuals were killed in 70% ethanol and stored in absolute (100%) ethanol. No formalin was used during the preservation process in order to avoid DNA damage. The 100%

ethanol was exchanged after 24 h. Each recipient was labelled as follows: EBI ringtest, date, taxon name (*E. fetida, Eisenia andrei,* or *Eisenia* sp.), and name and address of the laboratory, including contact details of the person in charge. Together with the information listed above, the worms were sent to ECT. Seven laboratories provided more than four worms, so that there were 36 groups of worms (=144 worms).

At ECT each individual worm was photographed and coded. The code indicated the species name (A = *E. fetida*; B = *E. andrei*; C = unknown) as provided and the laboratory involved (a consecutive number randomizing the participants) (see Table 2). The posterior part of each worm was divided into five pieces (one for each DNA barcoding laboratory) of about 0.5 cm long. These coded pieces were sent to the five DNA barcoding laboratories (A Coruña, Brussels, Frankfurt, Guelph, and Vigo; see Table 1), while the anterior part of the body was kept at ECT as voucher specimen.

In two cases this design was modified: Laboratory 23 provided an additional group of four worms, which were tentatively referred to as *Eisenia hortensis* (Michaelsen, 1890) (23-C). This species is mostly found in composts and dung heaps, but seems to be less common than *E. fetida* or *E. andrei* (Csuzdi and Zicsi, 2003). In addition, Laboratory 39 provided also four worms of a second generation offspring produced by a cross-breeding experiment between *E. fetida* and *E. andrei* (39-C).

2.3. Ringtest performance: DNA barcoding procedure

General guidelines (SOP) for the DNA barcoding procedure were distributed among the laboratories prior to the experiment. This SOP covered primarily the documentation of the DNA barcoding work.

DNA barcoding (through bi-directional sequencing) of the standard mtDNA COI gene fragment was performed in each of the five laboratories according to their local procedures (details available upon request). The primers used were LCO1490 (5′-ggtcaacaaatcataaagatattgg-3′) and HC02198 (5′-taaacttcagggtgaccaaaaaatca-3′) (Folmer et al., 1994), as recommended by Pérez-Losada et al. (2005) and confirmed by Pérez-Losada et al. (2012). COI sequences were compared with the sequences available at DDBJ/EMBL/GenBank and/or at Barcode of Life (IBOL). Each DNA barcoding laboratory provided a report describing their methodology and results (including results files). The alignments of all laboratories were merged at BiK-F by selecting the fragment that maximized the number of comparable positions and minimized the number of positions with missing data. A neighbor-joining (NJ) tree with 1000 bootstrap replicates was constructed from a single data set including all individuals using MEGA5 (see below).

3. Results

3.1. Agreement among DNA barcoding laboratories

A total of 668 out of the 720 samples (144 in five laboratories) were successfully DNA barcoded (overall success rate 92.8%). However, success rates differed among laboratories and varied between 75.7% and 98.6% (Table 1). For the sequence comparison, the total alignment was trimmed to a fragment of 561 bp. The possible number of bases to appear in comparisons was thus 403,920 of which 391,834 (97.0%) were non-missing data. Sequence reads were trimmed automatically by a custom Python script (quality threshold 30). Resulting alignments were trimmed by eye to minimize missing data.

Four samples were obviously confounded during the process, as in one laboratory they yielded sequences from another cluster compared to the outcome obtained by all other laboratories. Repeating the DNA barcoding process from the same tissues yielded the same results, indicating that the samples were mixed-up during distribution handling at ECT. These reads were excluded from further analysis. From the remaining 647,321 pairwise site comparisons, 91 showed discrepancies (0.00014). These discrepancies were due to 11 different alignment positions, four of which [positions 426, 429, 432, 493 of the final alignment] were multiple times read consistently different in one laboratory compared to the other laboratories. Subsequent analyses were not affected by these sequencing errors (see below).

All 144 individuals were analyzed in at least two laboratories. NJ trees of the data sets of three laboratories consistently showed four clusters with bootstrap support >95% The two other data sets revealed three of the four clusters, but did not recover the fourth cluster, because none of the individuals of this cluster had been sequenced. Anyway, the assignment of individuals to clusters was completely consistent among the laboratories.

3.2. Taxonomic identification of the studied earthworms

To associate the NJ clusters with known species, we added morphologically identified voucher specimens to the analysis. All *Eisenia* COI sequences available in GenBank were included too. We again constructed a NJ tree using the uncorrected *p*-distance with partial pairwise deletion of missing sites and 1000 bootstrap replicates on the 561 bp alignment. The results were consistent with the previous analysis in that the four clusters were recovered (Fig. 1). The largest cluster (110 sequences) included the *E. andrei* voucher sequences (mean within-group *p*-distance of 0.026 ± 0.002). The *E. fetida* voucher and GenBank sequences fell in two clusters (*E. fetida* 1 and *E. fetida* 2; 10 and 22 test sequences, respectively). Each of these two clusters represented one single haplotype. The fourth cluster (four sequences), i.e., the additional group 23-C, was classified as *Dendrobaena hortensis* (*E. hortensis* was transferred to *Dendrobaena* (Csuzdi and Zicsi, 2003)). The second generation of cross-bred *E. fetida*/*E. andrei* (No. 39-C) fell in the *E. andrei* cluster. The color of these, according to the breeder, sterile individuals was more similar to that of *E. andrei* than to that of *E. fetida* – i.e., they were dark red with very slight yellow intersegmental stripes. The mean *p*-distance between *E. fetida* 1 and *E. fetida* 2 was 0.112, whereas the mean *p*-distances between these two taxa and *E. andrei* were 0.142 and 0.143, respectively. There were no clear morphological differences (e.g., external properties such as size, segment numbers, color, segment position of clitellum and tubercula pubertatis) between worms of the clusters *E. fetida* 1 and 2.

Table 1
Overview of the success rate of COI sequencing in the earthworms studied ($n = 144 = 100\%$) and the alignment length as determined in the five DNA barcoding laboratories.

DNA barcoding laboratory	Individuals barcoded	Success rate	Alignment length [bp]	Average sequence length [bp]
A	142	98.60%	580	577.1
B	134	93.10%	623	606.4
C	140	97.20%	658	634.9
D	142	98.60%	600	599.5
E	109	75.70%	587	614.6

Table 2
Comparison of the initial (expected) identifications of the participating ecotoxicological laboratories and the results of the DNA barcoding analysis of five sequencing data sets (for details on individual worms see Appendix) (A = *E. fetida*; B = *E. andrei*; C = unknown). Two exceptions are highlighted: laboratory 23: *Eisenia/Dendrobaena hortensis*; laboratory 39: second-generation cross-breeds between *E. fetida* and *E. andrei*.

Ecotoxicological laboratory	Expected	DNA barcoding	Ecotoxicological laboratory	Expected	DNA barcoding
2-A	*E. fetida*	*E. andrei*	**23-C**	*E. hortensis*	*D. hortensis*
3-A	*E. fetida*	*E. fetida* 1	**25-B**	*E. andrei*	*E. andrei*
5-B	*E. andrei*	*E. andrei*	**27-A**	*E. fetida*	*E. andrei*
6-B	*E. andrei*	*E. andrei*	**27-B**	*E. andrei*	*E. andrei*
7-C	n.s.	*E. andrei*	**28-A**	*E. fetida*	*E. fetida* 2
8-A	*E. fetida*	*E. fetida* 2	**28-B**	*E. andrei*	*E. andrei*
9-A	*E. fetida*	*E. andrei*	**29-B**	*E. andrei*	*E. andrei*
10-B	*E. andrei*	*E. andrei*	**30-C**	n.s.	*E. andrei*
12-A	*E. fetida*	*E. fetida* 1	**31-A**	*E. fetida*	*E. andrei*
12-B	*E. andrei*	*E. andrei*	**31-B**	*E. andrei*	*E. andrei*
13-A	*E. fetida*	*E. fetida* 2	**32-B**	*E. andrei*	*E. andrei*
13-B	*E. andrei*	*E. andrei*	**33-C**	n.s.	*E. andrei*
15-A	*E. fetida*	*E. andrei*	**34-B**	*E. andrei*	*E. andrei*
16-A	*E. fetida*	*E. fetida* 2	**36-B**	*E. andrei*	*E. andrei*
18-B	*E. andrei*	*E. andrei*	**39-A**	*E. fetida*	*E. fetida* 2
20-B	*E. andrei*	*E. andrei*	**39-B**	*E. andrei*	*E. andrei*
21-A	*E. fetida*	*E. fetida* 2	**39-C**	Cross-bred	*E. andrei*
23-A	*E. fetida*	*E. andrei*[a]	**40-B**	*E. andrei*	*E. andrei*

N.s., not stated.
[a] Laboratory code 23-A: samples were mixed up.

3.3. Taxonomic assignments of world-wide laboratory cultures

It is striking that all individuals that were initially identified as *E. andrei* were correctly assigned to the *E. andrei* cluster (Table 2). Conversely, only 56% of the individuals initially identified as *E. fetida* were correctly assigned to the *E. fetida* cluster, while the remaining 44% were assigned to the *E. andrei* cluster. The additional group assumed to be *Eisenia* (now *Dendrobaena*) *hortensis* was found as an additional cluster. Only 17 of the 28 laboratories (61%) provided correct identifications of their laboratory stocks, the rate of correct identifications of the 11 remaining laboratories varied between 0 and 88% (including laboratories with unknown stocks). Most laboratories with wrong or unknown assignments actually had *E. andrei* in culture. The samples of two laboratories which claimed to keep only a single species, actually contained both *E. fetida* and *E. andrei*. Yet one of these laboratories (No. 23) did indicate that their worms (named *E. fetida*) behaved differently and came from two different breeders. The other laboratory (No. 28) provided two cultures, one identified as *E. fetida*, the other as *E. andrei*, but with the *E. fetida* culture being contaminated by the other. Still another laboratory believed to harbor two species, but apparently shipped only *E. andrei*. Finally, in one laboratory the stocks were evidently mixed or samples were erroneously assigned.

4. Discussion

4.1. Outcome of the ringtest

We use the term ringtest here as defined by the OECD (2005) in a slightly modified way: "A multi-laboratory validation study in which all laboratories test the same procedure (not substances, as in the original text), using identical test protocols, often used for eco-toxicity test method validation. The purpose .... is to determine the reproducibility of a specific method, but also whether the method (not bioassay as in the original text) is understandable and practical, and whether the inter-laboratory variability is sufficiently low as required for and known from other methods". In this subchapter we will discuss whether these criteria have been met in the EBI exercise.

The ringtest involved five DNA barcoding laboratories instead of the three to four that are required by the OECD Validation Document (OECD, 2005). These laboratories are experts in DNA barcoding (e.g., Pérez-Losada et al., 2012; Vierna et al., 2014). As required, identical protocols (called SOPs here) were used. The reproducibility and the practicability of the specific method, i.e., DNA barcoding, were proven (see discussion below). The inter-laboratory variation of the results was extremely low. Furthermore, the large number (28) and diversity of institutions which provided worms indicates that the problem (see the three questions given in Section 1) addressed by the EBI initiative is important for many potential stakeholders involved in developing or using ecotoxicological test methods with earthworms: universities, governmental institutes, and contract laboratories. Finally, because a big commercial worm breeding company (Minhobox, Brazil) joined the EBI ringtest, it is likely that the results of this ringtest may have a worldwide impact.

Base calling differences among laboratories may have been due to different interpretations by the researchers, the different sequencer models used, different sequencing chemistries, different array lengths, differences in the dye set, and different software versions at the sequencing facilities involved. However, the extremely low amount of sequence discrepancies and the consistency of the conclusions among laboratories suggest that the basal quality criteria listed above have been fulfilled, i.e., that such DNA barcoding studies can be performed by any standard sequencing laboratory. However, the four confounded samples show that sample handling and documentation must be executed and controlled rigorously.

Given that only a very limited number of specimens were typed per ecotoxicological laboratory, it is expected that still more laboratories may have unrecognized mixed stocks. Simple probability calculations show that e.g., in a situation where a stock is for 5% contaminated by another species, about 45 individuals need to be typed in order to detect this contamination with 90% probability. As an additional indication of the robustness of the outcome of the ringtest it should be noted that there was no difference in outcome between the individual DNA barcoding studies performed in the five participating DNA sequencing laboratories, i.e., DNA barcoding as performed in this ringtest delivered reproducible results.

4.2. Species identity and ecotoxicological sensitivity

Our results indicate that there are three taxa within the *E. fetida/andrei* complex (*Eisenia/Dendrobaena hortensis* not included), viz. *E.*

andrei, *E. fetida* 1, and *E. fetida* 2. The latter two clusters are identical to clusters *E. fetida* 5 and 6 in Chang et al. (2009). Uncorrected *p*-distances of more than 10% usually are indicative of species level differentiation. Chang and James (2011) reviewed the literature and concluded that in the case of COI "any two specimens with Kimura 2-parameter distance lower than 9% or higher than 15% can be unambiguously assigned to the same species or two different species, respectively. This leaves an ambiguous range between 9 and 15% when morphological difference is absent between sister clades". The existence of a cryptic species pair within *E. fetida* is therefore a plausible hypothesis in need of further investigation. This suggestion is not surprising, since DNA barcoding of 87 earthworm species indicated that about 15% of the haplotypes represented cryptic taxonomic diversity (Rougerie et al., 2009). Less clear is the situation in *E. andrei*: while in our study no hint on cryptic species was found within this taxon, Voua Otomo et al. (2009, 2014); Voua Otomo et al., 2009 identified a haplotype (H 7) which was "extremely different" (i.e., more than 23%) from the remaining six haplotypes belonging to *E. andrei*. As far as we know in no other study such an observation was made.

Despite the fact that for more than 40 years (André, 1963; Bouché, 1972) more than one taxon is used in the tests, data referring to *E. fetida*,*E. andrei*, or a mixture of both are still accepted for regulatory purposes. This is strange since in many taxa (Feckler et al., 2013), including oligochaetes, congeneric species may react differently to (anthropogenic) stressors. For example, for the pesticide chlorpyrifos in artificial soil LC50-values (and 95% confidence interval) of 129 (113–148) mg/kg and 458 (403–521) mg/kg were found for the lumbricid earthworms *Lumbricus rubellus* and *Lumbricus terrestris*, respectively (Ma and Bodt, 1993). In the same study the authors did not find such a difference when comparing the sensitivity of either *Eisenia veneta* and *E. fetida* or *Aporrectodea caliginosa* and *Aporrectodea longa*. Nevertheless, "Even if two species have similar sensitivity to toxicants, the presence of two species within the same culture could result in the production of sterile hybrids that would be unacceptable for reproduction testing". (Janet McCann (Environment Canada; pers. comm.). However, sterile hybrids could be formed in cultures containing both *E. fetida* and *E. andrei* (Pérez-Losada et al., 2005; Afrânio Augusto Guimarães (Minhobox, Brazil; pers. comm.)).

Since the species – independently of how this term is defined (e.g., Bickford et al., 2007; De Queiroz, 2007) – is considered to be the biological unit for assessing the effects of chemicals on organisms, it is necessary to verify the identity of taxa used in ecotoxicological testing. Actually, neither the name, nor its taxonomic rank are important, but comparing ecotoxicological data gained in the same test for the same chemical only makes sense if the test organisms belong to the same species – otherwise these data can be questioned and the outcome of an environmental risk assessment might be wrong.

In sum, a reliable and practical identification of earthworm test species in ecotoxicological tests is recommended, preferably by combining DNA barcoding with other species-specific traits (e.g., Voua Otomo et al., 2009, 2013a,b). Our ringtest illustrated that DNA barcoding of earthworms is indeed a reliable and practical method. Further standardization of this method, for example by ISO, is advisable in order to keep a high quality standard.

4.3. Regulatory consequences

Since ecotoxicological tests are often used for environmental risk assessment, they have to comply with very stringent guidelines in order to achieve the highest quality possible needed for regulatory (legislative) decision-making. Therefore, both the test performance (usually based on OECD or ISO guidelines), and its documentation (Good Laboratory Practice rules) are

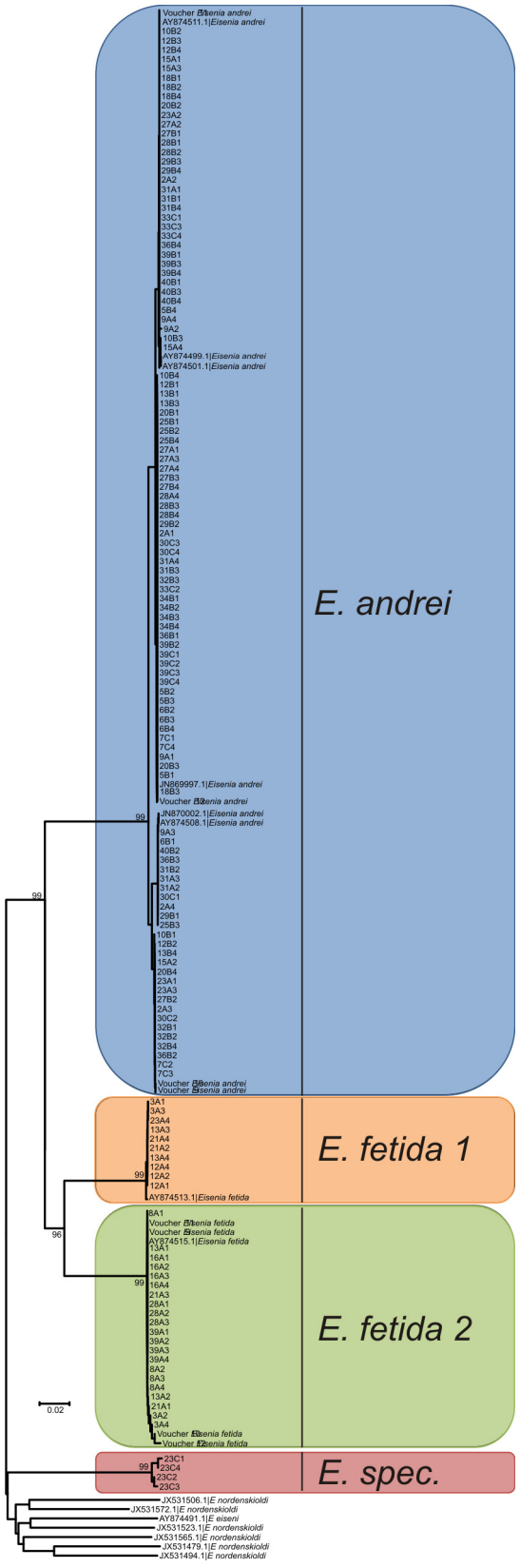

Fig. 1. Neighbor-joining tree of 154 test sequences together with morphologically identified voucher specimens and sequences from DDBJ/EMBL/GenBank. Bootstrap values (1000 replicates) >95% are shown next to the branches. The tree is drawn to scale, with branch lengths proportional with the number of base differences per site (*p*-distances). Missing positions were removed for each sequence pair.

internationally fixed. Nevertheless, these rules are unclear with respect to the identity of test species. The standardization organizations became aware of this problem when evidence accumulated (e.g., Jaenike, 1982; Oien and Stenersen, 1984; Reinecke and Viljoen, 1991; Domínguez et al., 2005; Jänsch et al., 2005; Pérez-Losada et al., 2005), but could not solve this problem, for two reasons:

- In temperate zones, there is no alternative for the earthworm species *E. fetida* and *E. andrei* in soil ecotoxicological tests. However, after it became clear that more than one species is involved, this issue was "corrected" in some guidelines by simply replacing the name *E. fetida* by the combination *E. fetida/andrei* (e.g., the ISO guideline on the Earthworm Avoidance test; ISO, 2008).
- Until recently, there were no reliable and practical tools to distinguish the different taxa within the *E. fetida/E. andrei* complex. Fortunately, this situation has changed (e.g., Briones et al., 2009).

Unfortunately, this means that almost all ecotoxicological data gained in earthworm tests in the past might be of doubtful quality. Since it is economically and politically difficult (to say the least) to repeat these hundreds, probably thousands of tests, suggestions are needed as to how to handle this problem. Firstly, most of the earthworm tests performed do not have to be repeated simply due to the fact that these data are not needed any more: the results of all acute tests performed since 1984 when the first OECD guideline was published (OECD, 1984) – they form the bulk of the existing tests – are not required any more in the environmental risk assessment of chemicals due to their low sensitivity (e.g., for veterinary chemicals (VICH, 2004). In some cases it might be possible to relate existing data to one specific species since we know from the questionnaire sent to the participants of this ringtest that many laboratories are using the same cultures for many years, partly decades. It might be possible to get general acceptance of this way of "justified guesswork". However, this will probably only cover a part of the whole available data set, meaning that research is needed. For example a selected group of chemicals, representing major use classes, mode-of-actions and/or exposure ways, has to be tested, using both *E. fetida* (preferably one of the two clades identified so far) and *E. andrei*. When doing so, the respective requirements from OECD or ISO (e.g., regarding the number of tests, their repeatability or their validity) have to be followed. Depending on the results of this study it has to be decided whether "old" test results can be used. The problem of cryptic diversity (i.e., how many more clades exist within *E. fetida*) could not be solved in this way.

We found that *E. andrei* was never erroneously identified as *E. fetida*, whereas *E. fetida* was often misidentified as *E. andrei*. Hence, *E. andrei* may be the best candidate for future test procedures. This species combines all the considered advantages of *E. fetida* as a test species (i.e., easy culturing and handling, and short generation times, even shorter than those of *E. fetida*) (Domínguez and Edwards, 2011). *E. andrei* is available all over the world (*E. fetida* was mainly used in Europe – and in one laboratory in South America and one in Canada). However, fixing a test species might be difficult because laboratories often have their "traditions" to which they like to stick. Therefore, it may be more effective to add a short paragraph in all earthworm test guidelines requiring the species identification of the test organisms used. The more so since DNA barcoding provides a relatively simple and reliable identification tool.

Of course, adding species identification via DNA barcoding in ecotoxicological guidelines would increase the efforts and costs needed for testing. However, in order to limit this additional burden, we propose to identify the test worms regularly, e.g., once a year and each time a new culture is started or an old one is mixed with "new" worms. Of course, in such case it must be secured that no "contamination" by other worms is occurring. The present ringtest showed that the practical part of DNA barcoding (i.e., selecting some worms from the culture, preparing them for DNA barcoding, sending small tissue samples to a DNA barcoding laboratory) does not require more than one hour of work in the laboratory plus a waiting time of about one day for gut purging. Results from a commercial or public DNA barcoding laboratory are available within a few weeks. Data interpretation is also quickly done since fortunately for these earthworms many sequences are available in places like GenBank. No detailed survey of the costs required for DNA barcoding individual worms was made, but it is estimated that extraction, PCR and sequencing (in both directions) of one specimen would cost about 10 (without working time), between 2 and 5 working days (depending on the degree of automation available). In relation to the other costs of such tests they are surely not prohibitive.

Actually, in all test guidelines currently used, the same approach is used in order to guarantee the sensitivity of the tested worms: Usually once a year the worms are exposed to a chemical of which the toxicity towards *E. fetida/andrei* is known, the so-called reference substance (e.g., the fungicide carbendazim in the earthworm reproduction test (OECD, 2004; ISO, 1998)). The result of this test has to be in a certain range, specified in the respective guideline. By doing so, it could be secured that the sensitivity of these worms kept often for decades in the laboratory does not change over time.

In parallel to the ringtest, both the OECD and the ISO have been informed about the EBI initiative: The Technical Committee (TC) 190 "Soil" of the International Organization of Standardization has already agreed that the DNA identification of test specimens is required for all test guidelines with soil invertebrates. This addition will be made within the next five years. In the case of the OECD the situation is more complex, because there is no "automatic" procedure to modify OECD Guidelines or Guidance Documents, but decisions are expected in spring 2015. Probably the biggest problem for both organizations is clarifying the question whether other test species should also be DNA barcoded (or by using another advanced technique). Indeed, identification problems with test organisms are not limited to earthworms, but have also been reported in tubificid and lumbriculid oligochaetes or amphipod crustaceans (Feckler et al., 2013).

4.4. Taxonomic side note

As was mentioned above, it is necessary to verify the identity of taxa used in ecotoxicological studies. Identity of taxa also means that the same species always receives the same name and that the name is the correct (=valid) one in nomenclatural terms (ICZN, 1999). For that purpose, COI fragments of the name-bearing types of *E. fetida* and of *E. andrei* should be sequenced in the near future and compared with the other published sequences. Types are extant at the Muséum National d'Histoire Naturelle Paris (*E. fetida*) and the Station Biologique de Paimpont (*E. andrei*) but they were not accessible to us during the EBI campaign. Furthermore, the validity of *E. andrei* has been questioned (e.g., Blakemore, 2013), because older names – synonyms of *E. fetida* (see lists in Csuzdi and Zicsi, 2003; Blakemore, 2013) – may have priority over *E. andrei* and replace it. We reviewed these names (13 altogether) and found only one possible candidate to replace *E. andrei*, *E. nordenskioldi caucasica* Michalsen 1903. Types of this subspecies, however, are lost (I. Tsiplenkina, Natural History Museum, St. Petersburg, pers. com), so DNA barcodes cannot be obtained. The other 12 names go with a coloration pattern typical of *E. fetida*, or their use is

prohibited for nomenclatural reasons (ICZN, 1999; Art. 23.9.1). A replacement of the name *E. andrei* by a different one is therefore considered unlikely here. Should it occur in the future, an application can be forwarded to the International Commission on Zoological Nomenclature to conserve the common and widely used name *E. andrei* (ICZN, 1999; Art. 23.9.3).

5. Summary, conclusions and outlook

- DNA barcoding is a reliable and practical method for identifying *Eisenia* species.
- Current morphological separation of the *E. fetida/andrei* complex into two species contrasts with the three clusters recovered by DNA barcoding: *E. andrei*, *E. fetida* 1, and *E. fetida* 2.
- The prior taxonomic assignments of 17 out of 28 ecotoxicological laboratories were completely correct. Most laboratories with wrong or unknown assignments actually have *E. andrei* in stock.
- Earthworms used for ecotoxicological tests should regularly be checked by DNA barcoding. Further research is needed to assess to what extent the (cryptic) species in the *E. fetida/andrei* complex differ in terms of their sensitivity toward various ecotoxicological agents or stressors.
- The existence of a cryptic species pair within *E. fetida* is therefore a plausible hypothesis which requires further investigation.

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Name	Country
Flemish Institute for Technological Research (VITO)	Belgium
Minhobox	Brazil
Centro de Tecnologia Mineral (CETEM)	Brazil
EMBRAPA Floresta	Brazil
North-West University	Brazil
Universidade do Estado de Santa Catarina	Brazil
EMBRAPA Agrobiologia	Brazil
Environment Canada	Canada
STANTEC Consulting	Canada
Research Ctr. Environ. Chemistry + Ecotoxicology (RECETOX)	Czech Republic
University of Aarhus	Denmark
Institut National de l'Environnement Industriel et des Risques (INERIS)	France
BioChemAgrar	Germany
University of Bremen	Germany
Fraunhofer Institute for Molecular Biology and Applied Ecology	Germany
ECT Oekotoxikologie GmbH	Germany
Julius-Kühn-Institut	Germany
Eurofins-GAB GmbH	Germany
Yokohama National University	Japan
University of Coimbra	Portugal
University of Aveiro	Portugal
University of Stellenbosch	South Africa
Instituto Nacional de Investigación y Tecnología Agraria (INIA)	Spain
Autonomous University of Barcelona	Spain
University of Ruhuna	Sri Lanka
	The Netherlands
Huntingdon Life Scienecs	United Kingdom
Edgewood Chemistry and Biology Center	USA

Appendix.

List of the DDBJ/EMBL/GenBank accession numbers of the *Eisenia* COI sequences used in the study with their respective taxonomic designation. To avoid inflation by redundant information only unique haplotypes of these reference sequences were used in the neighbor-joining analysis.

Accession number	Identical haplotypes	Accession number	Identical haplotypes
AY874511.1| *Eisenia andrei*	AY874509.1| *Eisenia andrei*	JN869997.1|*Eisenia andrei*	JN870070.1| *Eisenia andrei*
	AY874507.1| *Eisenia andrei*		JN870074.1| *Eisenia andrei*
	AY874506.1| *Eisenia andrei*		JN870078.1| *Eisenia andrei*
	AY874505.1| *Eisenia andrei*		JN870081.1| *Eisenia andrei*
	AY874504.1| *Eisenia andrei*		AY874493.1| *Eisenia andrei*
	AY874497.1| *Eisenia andrei*		AY874494.1| *Eisenia andrei*
	JN870088.1| *Eisenia andrei*		AY874495.1| *Eisenia andrei*
	JN870087.1| Eisenia andrei		AY874496.1| *Eisenia andrei*
	JN870086.1| *Eisenia andrei*		AY874498.1| *Eisenia andrei*
	JN870085.1| *Eisenia andrei*		AY874500.1| *Eisenia andrei*
	JN870084.1| *Eisenia andrei*		AY874502.1| *Eisenia andrei*
	JN870083.1| *Eisenia andrei*		AY874503.1| *Eisenia andrei*
	JN870082.1| *Eisenia andrei*		AY874512.1| *Eisenia andrei*
	JN870080.1| *Eisenia andrei*		
	JN870079.1| *Eisenia andrei*	AY874513.1|*Eisenia fetida*	AY874514.1| *Eisenia fetida*
	JN870077.1| *Eisenia andrei*		
	JN870076.1| *Eisenia andrei*	AY874515.1|*Eisenia fetida*	AY874516.1| *Eisenia fetida*
	JN870075.1| *Eisenia andrei*		AY874517.1| *Eisenia fetida*
	JN870073.1| *Eisenia andrei*		AY874518.1| *Eisenia fetida*
	JN870072.1| *Eisenia andrei*		AY874519.1| *Eisenia fetida*
	JN870071.1| *Eisenia andrei*		AY874520.1| *Eisenia fetida*
	JN870069.1| *Eisenia andrei*		AY874521.1| *Eisenia fetida*
	JN870068.1| *Eisenia andrei*		AY874522.1| *Eisenia fetida*
	JN870067.1| *Eisenia andrei*		AY874523.1| *Eisenia fetida*
	JN870032.1| *Eisenia andrei*		
	JN870030.1| *Eisenia andrei*	JX531506.1|E nordenskioldi	

(*Continued*)

Accession number	Identical haplotypes	Accession number	Identical haplotypes
	JN870028.1| *Eisenia andrei*	JX531572.1|E nordenskioldi	
	JN870026.1| *Eisenia andrei*	AY874491.1|E eiseni	
	JN870024.1| *Eisenia andrei*	JX531523.1|E nordenskioldi	
	JN870022.1| *Eisenia andrei*	JX531565.1|E nordenskioldi	
	JN870020.1| *Eisenia andrei*	JX531479.1|E nordenskioldi	
	JN870018.1| *Eisenia andrei*	JX531494.1|E nordenskioldi	
	JN870016.1| *Eisenia andrei*		
	JN870014.1| *Eisenia andrei*		
	JN870010.1| *Eisenia andrei*		
	JN870008.1| *Eisenia andrei*		
	JN870007.1| *Eisenia andrei*		
	JN870006.1| *Eisenia andrei*		
	JN870005.1| *Eisenia andrei*		
	JN870001.1| *Eisenia andrei*		
	JN870000.1| *Eisenia andrei*		
	JN869999.1| *Eisenia andrei*		
	JN869998.1| *Eisenia andrei*		
	JN869996.1| *Eisenia andrei*		
	AY874510.1| *Eisenia andrei*		
JN869997.1| *Eisenia andrei*	JN870003.1| *Eisenia andrei*		
	JN870004.1| *Eisenia andrei*		
	JN870034.1| *Eisenia andrei*		
	JN870049.1| *Eisenia andrei*		
	JN870051.1| *Eisenia andrei*		
	JN870053.1| *Eisenia andrei*		
	JN870055.1| *Eisenia andrei*		
	JN870057.1| *Eisenia andrei*		
	JN870059.1| *Eisenia andrei*		
	JN870061.1| *Eisenia andrei*		
	JN870063.1| *Eisenia andrei*		
	JN870064.1| *Eisenia andrei*		
	JN870065.1| *Eisenia andrei*		
	JN870066.1| *Eisenia andrei*		

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