Genetic methods to characterize test species in ecotoxicology: The *Eisenia* Barcoding Initiative (EBI)

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Background:

Almost as long as earthworms have been used in standard ecotoxicological tests there is a discussion going on whether the compost worms recommended for these tests belong to one species or two. There is plenty of evidence that two taxa, *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* Bouché, 1972 (Lumbricidae), can be distinguished by morphological, physiological, behavioral and molecular traits. However, it is unclear whether these differences are sufficient to interpret both taxa as separate species. Therefore, two questions arise in this context:

1. Which earthworm species are used in standard ecotoxicological tests?
2. Is DNA barcoding practical and robust enough for the characterization of the species *Eisenia fetida* and *Eisenia andrei*,?

In order to answer these questions, an international ring test was organized.

Material and Methods:

All steps performed are described in Standard Operation Procedures (SOP).

(1). **Ring test participation:** 28 laboratories: 16 from Europe, 5 from South America, 3 from North America, 2 from Africa and 2 from Asia.

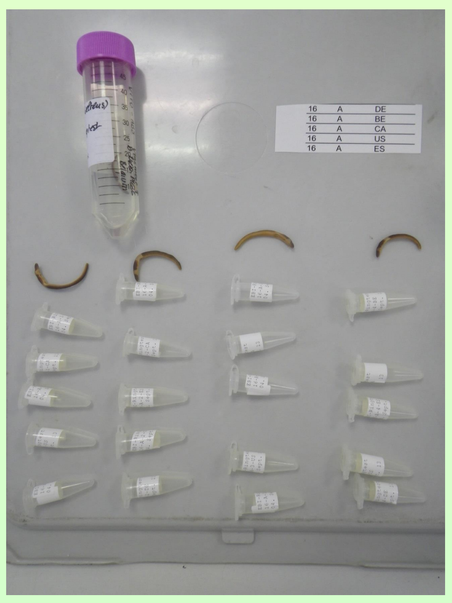
(2) Background of these laboratories: 11 from universities, 11 from governmental institutes and 6 contract laboratories.

(3) Earthworm material: Since 7 laboratories provided more than one group, the total number of groups was 36 (= 144 worms). Before sending the worms to ECT, they were kept 24 h on moist filter-paper in order to empty their gut. Earthworms were killed in 70% ethanol and then transferred to 100% ethanol.

(4) Work at ECT: Photographic characterization of live worms. Anterior parts of all worms were kept as voucher specimen at ECT. A fragment of approximately 0.5 cm of the tail of each of the 144 specimens were shipped to five DNA barcoding laboratories (A Coruña, Brussels, Frankfurt, Guelph and Vigo; see coded **Table** below).

(5). **Genetic characterization:**

The DNA barcoding laboratories carried out DNA isolations of the coded earthworm fragments, amplification of a fragment of the mitochondrial COI gene, bi-directional sequencing, electropherogram analysis, and phylogenetic analyses. The primers used were LCO1490 and HC02198 (Folmer et al. 1994; Mol Mar Biol Biotechnol 3:294-299), as recommended by Pérez-Losada et al. 2005 (Pedobiologia 49:317-324)**.**



Results

Agreement among DNA barcoding laboratories

DNA barcoding success rate varied between 75.7% and 98.6% **(Table).** A separate neighbor-joining analysis of all data sets (long and short data set from each lab, p-distance, partial deletion, 100 bootstraps) yielded consistent results. We found four divergent clusters. The taxonomic assignment of individuals to the respective clades was consistent among the laboratories.

Barcoding Laboratory	Individuals barcoded	Success rate	Alignment length [bp]	Average sequence length [bp]
A	142	98.6 %	580	577.1
B	109	75.7 %	587	614.6
C	134	93.1 %	623	606.4
D	140	97.2 %	658	634.9
E	142	98.6 %	600	599.5

Results and Discussion:

Taxonomic assignments and identity of world-wide lab cultures

To assign the inferred clades to described species, we added morphologically identified voucher specimens to the analysis. All *Eisenia* COI sequences available in GenBank were included too. Again, four clades were recovered. The largest clade (108 test sequences) included the *E. andrei* voucher sequences (mean within group distance of 0.026). The *E. fetida* voucher and GenBank sequences fell in two clades (*E. fetida*_A and *E. fetida*_B (10 and 22 test sequences, respectively), both with a mean within-group distance of zero). The two clades *E. fetida* A and B are identical to the two clades E. fetida 5 and 6 described by Chang et al. 2009; Pedobiol 52: 171-180).The fourth cluster (four sequences) was classified as *Dendrobaena hortensis* (= *Eisenia hortensis*). When comparing the prior opinions of the 28 ecotoxicological laboratories on the taxonomic affiliation of their worms with the DNA barcoding analysis, it is striking that all individuals initially identified as *E. andrei* belonged to the *E. andrei* clade **(Table)**. However, only 56% of the individuals believed to be *E. fetida* were assigned to the respective *E. fetida* clades; 44% were genetically assigned to the *E. andrei* clade.

Lab Code	Expected	Barcoding
2	*E. fetida*	*E. andrei*
3	*E. fetida*	*E. fetida A* [1]
5	*E. andrei*	*E. andrei*
6	*E. andrei*	*E. andrei*
7	n.s.	*E. andrei*
8	*E. fetida*	*E. fetida B*
9	*E. fetida*	*E. andrei*
10	*E. andrei*	*E. andrei*
12	*E. fetida*	*E. fetida A* [1]
12	*E. andrei*	*E. andrei*
13	*E. fetida*	*E. fetida B* [1]
13	*E. andrei*	*E. andrei*
15	*E. fetida*	*E. andrei*
16	*E. fetida*	*E. fetida B* [2]
18	*E. andrei*	*E. andrei*
20	*E. andrei*	*E. andrei*
21	*E. fetida*	*E. fetida B* [1]
23	*E. fetida*	*E. andrei* [2]

Lab Code	Expected	Barcoding
23	*D. hortensis*	*D. hortensis*
25	*E. andrei*	*E. andrei*
27	*E. fetida*	*E. andrei* [2]
27	*E. andrei*	*E. andrei*
28	*E. fetida*	*E. fetida B* [2]
28	*E. andrei*	*E. andrei*
29	*E. andrei*	*E. andrei*
30	n.s.	*E. andrei*
31	*E. fetida*	*E. andrei*
31	*E. andrei*	*E. andrei*
32	*E. andrei*	*E. andrei*
33	n.s.	*E. andrei*
34	*E. andrei*	*E. andrei*
36	*E. andrei*	*E. andrei*
39	*E. fetida*	*E. fetida B*
39	*E. andrei*	*E. andrei*
39	n.s.	*E. andrei*
40	*E. andrei*	*E. andrei*

Notes: n.s. = not specified; [1] = *E. fetida* A and B; [2] = *E. fetida* and *E. andrei* mixed

Summary and Outlook

- COI-Sequencing is a robust method for molecular characterization of *Eisenia* (the 5 laboratories revealed almost identical outcomes).
- Morphological separation of earthworms into 2 species contrasts with the three genetic clades recovered: *E. andrei* , *E. fetida* A , *E. fetida B*.
- P-distances of more than 10% are usually found only among species. The existence of a cryptic species pair within *E. fetida* is therefore a plausible hypothesis which requires further investigation.
- 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 in ecotoxicological laboratories should regularly be characterized. Research is needed to clarify how much the three genetic species differ in terms of their sensitivity towards chemicals.

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