Biol Invasions (2015) 17:2527–2532
DOI 10.1007/s10530-015-0910-7

INVASION NOTE

DNA-barcoding of invasive European earthworms (Clitellata: Lumbricidae) in south-western Australia

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Received: 8 August 2014 / Accepted: 22 May 2015 / Published online: 31 May 2015

Abstract Using DNA-barcoding, we studied the diversity of invasive European earthworms in the south-western corner of Australia. We found six Molecular Operational Taxonomic Units belonging to five morphospecies: *Aporrectodea caliginosa*, *A. trapezoides*, *Dendrobaena* cf. *attemsi*, *Eiseniella tetraedra* and *Octolasion cyaneum*. These were variously collected from indigenous forests and/or alienated land. Two cryptic lineages were found within *A. trapezoides*, and high intraspecific genetic variation was also found within *E. tetraedra*—variation that had previously been documented in Europe. Our study demonstrates the usefulness of DNA-barcoding for the identification of earthworms, including cryptic species. Correct identification and high taxonomic resolution is crucial for the monitoring of cryptic diversity, detecting new introductions and monitoring spread of established exotic earthworms.

Keywords Oligochaeta · Annelida · Crassiclitellata · Cryptic species · COI · mt-DNA

Introduction

Introduced earthworms (Annelida: Clitellata: Crassiclitellata) are today found on all continents except Antarctica (Hendrix et al. 2008), and they can have major effects on soil processes and plant communities when introduced to non-native areas (Hale et al. 2005).

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There is some uncertainty as to whether introduced earthworms displace native species (Hendrix et al. 2006). Earthworms are sometimes problematic to identify to species due to the lack of easily observable morphological characters and a high level of phenotypic variability. Furthermore, cryptic species—taxa that are morphologically so similar to one another that they have often been classified under the same species name—are common among earthworms (e.g., Fernandez et al. 2012; James et al. 2010; King et al. 2008), as well as in other clitellates (see Erséus and Gustafsson 2009). To reliably identify specimens of cryptic species, molecular methods, such as DNA-barcoding using the COI gene, are recommended (Hebert et al. 2003). COI barcoding is useful for the identification of earthworms (Decaëns et al. 2013) and it works on juvenile specimens which usually cannot be identified morphologically (Richard et al. 2010).

Invasive species are common in the Lumbricidae, a taxon with a primarily Palearctic distribution and the only earthworm family native to northern and central Europe. Lumbricids have spread to suitable habitats around the world, including large parts of Australia (e.g., Hendrix et al. 2008), a continent with over 20 such taxa recorded (Blakemore 1999). However, to our knowledge, only one study specifically focusing on the genetic variation within and between lumbricid species in Australia has been published (Dyer et al. 1998). It concentrated on species of *Aporrectodea* Örley, 1885, using RAPD markers, and showed differences between populations of *A. trapezoides* (Dugès, 1828) up to the level found between species. Deep genetic divergence between two lineages of *A. trapezoides* was also found by Fernández et al. (2011), although their study focused mainly on the native European range of this species.

During the course of a field expedition to several sites in southwest Western Australia, with the primary purpose to collect microdrile oligochaetes (i.e., small aquatic clitellates with a single-layered clitellum), we also encountered a number of lumbricid earthworms, one of which had not previously been reported from Western Australia. This note is the first account of the genetic diversity of European earthworms in this part of Australia documented through the use of DNA-barcoding techniques. It will serve as the start of assembling a reference library of invasive lumbricids, to facilitate future identification of lumbricid earthworms in the area.

Materials and methods

A total of 32 specimens of Lumbricidae were collected from 10 localities in Western Australia, in September 2012, with habitats including gardens, agricultural land and indigenous forests (Table 1). The specimens were hand-picked and preserved in 95 % ethanol. The worms were tentatively identified based on morphology (using Sims and Gerard 1985).

DNA was extracted from a small sample of body wall tissue from the posterior part of each worm, using QuickExtract DNA Extraction Solution 1.0 (Epicentre), following the manufacturer's instructions. The remaining parts of the worms are stored in 95 % ethanol as vouchers. The DNA-barcode region of the mitochondrial Cytochrome C Oxidase subunit I (COI) was amplified as described by Martinsson and Erséus (2014). Sequencing was carried out by Eurofins mwg operon (Ebersberg, Germany). Sequences were assembled in Geneious Pro v. 6.0 (Biomatters Ltd.) and aligned using the Geneious alignment with default settings. All sequences are deposited in the database Barcoding of Life Data Systems (BOLD), and the vouchers in the Western Australian Museum (WAM), Welshpool (for accession nos., see Table 1).

Pairwise genetic distances (uncorrected p-distances) were calculated for the COI-data set in MEGA 6.0 (Tamura et al. 2013), using pairwise deletion for missing data. The distances were analysed with the online version of Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012) with default settings, to delimit Molecular Operational Taxonomic Units (MOTUs). The MOTUs were identified by comparison with a large dataset of lumbricid barcodes from northern Europe (CE, unpublished data) as well as with publicly available databases (GenBank and BOLD). The clustering is illustrated with a NeighbourNet drawn in SplitsTree v.4.12 (Huson and Bryant 2006) (Fig. 1a).

Results and discussion

DNA extraction and sequencing were successful for all 32 specimens. The ABGD analysis divided them into six MOTUs, representing five morphospecies, with the genetic distances within clusters varying between 0 and 5.5 % and the distances between clusters between 14.7 and 23.9 % (Fig. 1b).

Table 1 List of material included in this study, with specimen identification numbers, museum voucher numbers, BOLD accession numbers for COI barcodes, collection locality data, and GPS coordinates (WGS84) for sites in Western Australia from which all specimens were collected in September 2012

Species	Specimen ID nos.	Museum voucher nos.	BOLD acc. nos.	Collection locality data: nearest town, site name	Habitat	GPS coordinates	
						S	E
A. caliginosa L3	CE16761	V 8336	LUSWA001-15	Gracetown, Cowaramup Brook	Riparian Jarrah/Marri forest	33.86690	115.01819
A. caliginosa L3	CE16762	V 8337	LUSWA002-15	Gracetown, Cowaramup Brook	Riparian Jarrah/Marri forest	33.86690	115.01819
A. caliginosa L3	CE16763	V 8338	LUSWA003-15	Gracetown, Cowaramup Brook	Riparian Jarrah/Marri forest	33.86690	115.01819
A. caliginosa L3	CE16778	V 8339	LUSWA004-15	Pemberton, Gloucester National Park	Karri forest	34.44827	116.0593
A. caliginosa L3	CE16780	V 8340	LUSWA005-15	Pemberton, Gloucester National Park	Karri forest	34.44827	116.0593
A. caliginosa L3	CE16781	V 8341	LUSWA006-15	Pemberton, Gloucester National Park	Karri forest	34.44827	116.0593
A. caliginosa L3	CE16782	V 8342	LUSWA007-15	Pemberton, Gloucester National Park	Karri forest	34.44827	116.0593
A. caliginosa L3	CE16783	V 8343	LUSWA008-15	Pemberton, Gloucester National Park	Karri forest	34.44827	116.0593
A. caliginosa L3	CE16784	V 8344	LUSWA009-15	Pemberton, Gloucester National Park	Karri forest	34.44827	116.0593
A. caliginosa L3	CE16785	V 8345	LUSWA010-15	Pemberton, Gloucester National Park	Karri forest	34.44827	116.0593
A. caliginosa L3	CE16786	V 8346	LUSWA011-15	Pemberton, Gloucester National Park	Karri forest	34.44827	116.0593
A. trapezoides L1	CE16746	V 8347	LUSWA012-15	Dunsborough, Quindalup	Agricultural land	33.63764	115.1170
A. trapezoides L1	CE16747	V 8348	LUSWA013-15	Dunsborough, Quindalup	Agricultural land	33.63764	115.1170
A. trapezoides L1	CE16748	V 8349	LUSWA014-15	Dunsborough, Quindalup	Agricultural land	33.63764	115.1170
A. trapezoides L2	CE16749	V 8350	LUSWA015-15	Dunsborough, Quindalup	Agricultural land	33.63764	115.1170
A. trapezoides L2	CE16759	V 8351	LUSWA016-15	Yallingup, Wilyabrup Brook	Agricultural land	33.79475	115.03128
A. trapezoides L2	CE16760	V 8352	LUSWA017-15	Yallingup, Wilyabrup Brook	Agricultural land	33.79475	115.03128
A. trapezoides L2	CE16769	V 8353	LUSWA018-15	Northcliffe, Watermark Kilns	Agricultural land	34.55381	116.18162
A. trapezoides L2	CE16771	V 8354	LUSWA019-15	Nannup town	Town garden	33.9771	115.7650
A. trapezoides L2	CE16775	V 8355	LUSWA020-15	Northcliffe, Boorara Brook	Riparian karri forest	34.69133	116.19049
A. trapezoides L2	CE16776	V 8356	LUSWA021-15	Northcliffe, Boorara Brook	Riparian karri forest	34.69133	116.19049
A. trapezoides L2	CE16779	V 8357	LUSWA022-15	Pemberton, Gloucester National Park	Riparian karri forest	34.44827	116.0593
D. cf. *attemsi*	CE16743	V 8358	LUSWA023-15	Fremantle, Prison Heritage Cottages	Town garden	32.05397	115.75268
D. cf. *attemsi*	CE16744	V 8359	LUSWA024-15	Fremantle, Prison Heritage Cottages	Town garden	32.05397	115.75268
D. cf. *attemsi*	CE16745	V 8360	LUSWA025-15	Fremantle, Prison Heritage Cottages	Town garden	32.05397	115.75268
E. tetraedra	CE16792	V 8361	LUSWA026-15	Pemberton, Warren River	Karri forest	34.50705	116.08729
E. tetraedra	CE17318	V 8362	LUSWA027-15	Dunsborough, Jingarmup Creek	Stream in riparian agricultural land	33.57971	115.04721
E. tetraedra	CE17374	V 8363	LUSWA028-15	Northcliffe, Boorara Brook	Riparian karri forest	34.69133	116.19049
O. cyaneum	CE16787	V 8364	LUSWA029-15	Pemberton, Gloucester National Park	Riparian karri forest	34.44827	116.0593

Table 1 continued

Species	Specimen ID nos.	Museum voucher nos.	BOLD acc. nos.	Collection locality data: nearest town, site name	Habitat	GPS coordinates	
						S	E
O. cyaneum	CE16788	V 8365	LUSWA030-15	Pemberton, Gloucester National Park	Riparian karri forest	34.44827	116.0593
O. cyaneum	CE16789	V 8366	LUSWA031-15	Pemberton, Gloucester National Park	Riparian karri forest	34.44827	116.0593
O. cyaneum	CE16790	V 8367	LUSWA032-15	Pemberton, Gloucester National Park	Riparian karri forest	34.44827	116.0593

All six MOTUs found in this study are previously known from Europe, and close matches (>99 % similarity) were found (BOLD; GenBank; CE, unpublished data)—three belong to *Aporrectodea*, two to the morphospecies *A. trapezoides* (14.9–15.3 % different), and one to *A. caliginosa* (Savigny, 1826). These morphospecies are found in all Australian states and territories except Northern Territory (Blakemore 1999). *Aporrectodea* is known to harbour cryptic diversity (Fernández et al. 2011; Fernandez et al. 2012) and the two MOTUs of *A. trapezoides* are identified as lineage 1 group C (two haplotypes, "clone 8" and a new haplotype) and lineage 2 group H (two haplotypes, "clone 1" and "clone 3") sensu Fernández et al. (2011) respectively. Clone 1 is known also from Algeria, Australia (New South Wales), Egypt, France, Italy, Portugal, Serbia, Spain, and Turkey (Fernández et al. 2011); clone 3 is known from Spain (Fernández et al. 2011); clone 8 is known from France (Fernández et al. 2011), and also found in Sweden and Norway (CE, unpublished data), whereas the new haplotype is not found in GenBank, in BOLD, nor in our non-Australian data. The MOTU of *A. caliginosa* (four haplotypes) is identified as L3 (Lineage 3) sensu Porco et al. (2013). Haplotype 1 (our individual CE16783) is found also in Germany, Norway, New Zealand and Sweden (BOLD; CE, unpublished data); Haplotype 2 (CE16761) is found also in Canada, Norway, New Zealand, Sweden and the USA (BOLD; CE, unpublished data); Haplotype 3 (CE16780, CE16784 and CE16786) is found also in Norway, Sweden and the USA (BOLD; CE, unpublished data); Haplotype 4 (CE16763, CE16778, CE16781 and CE16785) is found also in Norway, New Zealand, South Africa and Sweden (BOLD; CE, unpublished data).

The other MOTUs were identified as *Dendrobaena* cf. *attemsi* (two haplotypes), *Eiseniella tetraedra* (Savigny, 1826) (two haplotypes) and *Octolasion cyaneum* (Savigny, 1826) (one haplotype). *Dendrobaena* cf. *attemsi* is a different, but closely related species to the *D. attemsi* (Michaelsen, 1902) reported from Sweden by Rota and Erséus (1997). Haplotype 1 (CE16743 and CE16745) is not found in GenBank, in BOLD, nor in our non-Australian, unpublished data; haplotype 2 (CE16744) is found in Denmark, Norway, Spain and Sweden (BOLD; CE, unpublished data). Considering that many species of *Dendrobaena* are native to the southern part of Europe, it is likely that

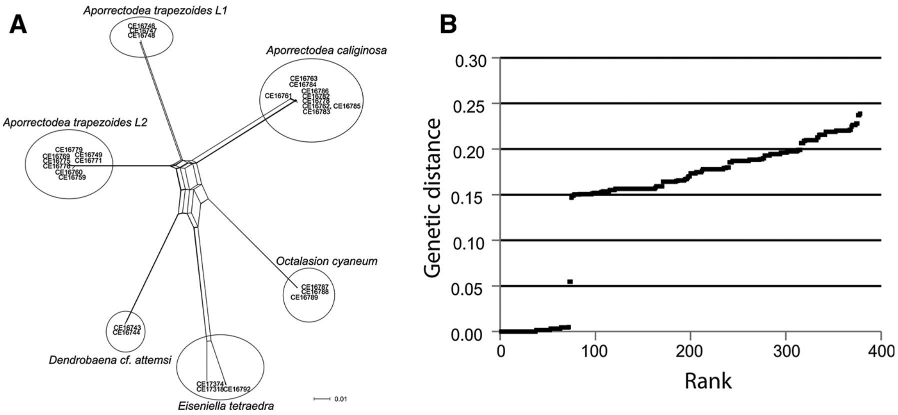

Fig. 1 **A** NeighbourNet network of COI barcodes for specimens of Australian Lumbricidae included in this study. *Scale bar* represents uncorrected p-distance. **B** Ranked pairwise genetic distances, given as uncorrected p-distances, for all specimens

this species is recently introduced also in the Scandinavian area, where it is rare. *Dendrobaena attemsi* s. str. has been reported from Australia (e.g., Baker et al. 1997), but this was questioned by Blakemore (1999), who listed it as an unconfirmed species from New South Wales, Victoria, South Australia and Tasmania. It is possible that the species previously reported as *D. attemsi* from Australia is in fact identical to our *D.* cf. *attemsi*.

A rather large genetic distance, 5.5 %, was found between the two haplotypes within *E. tetraedra*. This is within the range of variation also found in other parts of the world where several district clusters of this morphospecies, separated by 5–8 % are found (BOLD; CE, unpublished data); it is possible that *E. tetraedra* actually is a species complex, but more studies are needed to test this. Haplotype 1 (CE17318 and CE17374) is found in Canada, France, Italy, Norway, Sweden and the USA (BOLD; CE, unpublished data); haplotype 2 (CE16792) in Canada, England, Japan, New Zealand, Sweden, Taiwan, Turkey and USA (BOLD; CE, unpublished data). *Eiseniella tetraedra* has not yet been reported from the Australian Capital Territory, Northern Territory and Queensland, but it otherwise seems to be widely distributed in Australia (Blakemore 1999).

Octolasion cyaneum is already known from all Australian states and territories except the Northern Territory (Blakemore 1999). The single haplotype now found in Western Australia is not found in GenBank, in BOLD, nor our other data, but the species is widely distributed in Europe, including the Scandinavian countries (Julin 1949).

For *O. cyaneum,* only one haplotype was found, whereas all other species were polymorphic, each for which two, or more, haplotypes were found. However, with the exception of *E. tetraedra*, the intraspecific variation for all MOTUs was maximally 0.5 %. The high variation within *E. tetraedra* suggests either at least 2 separate introductions, or a single introduction on multiple individuals comprising the found genetic diversity. The presence of two lineages (with four haplotypes) of the morphospecies *A. trapezoides* also suggests more than one introduction. The wide geographical distribution of particular haplotypes can indicate frequent anthropogenic transportation.

This is the first study on invasive earthworms in Australia using DNA-barcoding. We find that the morphospecies *Aporrectodea trapezoides* is represented by two distinct lineages. We also verify the presence of one species of *Dendrobaena* close to *D. attemsi* in Western Australia. The correct assessment of specimens to species/lineages is important for the monitoring of cryptic species and also for the detection of new introductions, and DNA-barcoding has been showed to be one of the most promising methods for this purpose (Armstrong and Ball 2005). We therefore recommend that studies on invasive earthworms in Australia and elsewhere, if possible, include molecular data, e.g., DNA-barcodes, to document the genetic diversity and possible cryptic diversity.

Acknowledgments We thank Per Hjelmstedt for lab assistance. Financial support was given by Stiftelsen Paul och Marie Berghaus donationsfond (to SM), Adlerbertska Stipendiestiftelsen (to CE, SM), MJW is obliged to the Illinois Natural History Survey for partial support for this research. Earthworm specimens discussed herein were collected by the authors under the tenets of Regulation 17: Licence to Take Fauna for Scientific Purposes (Wildlife Conservation Regulations 1970) and the 'Further Conditions' associated with Licence No. SF008847, issued (September 2012) to M.J.

Wetzel by the Western Australian Department of Environment and Conservation, Kensington.

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