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Identifying earthworms through DNA barcodes

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Taxonomy

Summary

With almost 3000 species, earthworms provide important model systems for studying soil fauna. However, species identification of earthworms is difficult and therefore limiting. The use of DNA barcodes, which are short sequences from standardized regions of the genome, has been regarded as a promising approach to resolve this taxonomic dilemma. We evaluated sequence diversity in the mitochondrial cytochrome-*c* oxidase I (COI) gene as a tool for resolving differences among species of Chinese earthworms. Members of six genera and 28 species were examined, and species were successfully discriminated in all cases. Sequence divergence within species was generally less than 1%, whereas divergence between species was greater than 15% in all cases. Divergence among individuals of *Eisenia fetida* were much higher (up to 7.8%); however, this may represent the presence of unrecognized sibling species or subspecies. We conclude that although it cannot completely replace taxonomy, the DNA barcode is a powerful tool for identifying species of earthworms and provides a useful complement to traditional morphological taxonomy.

Introduction

Approximately 3000 described species of earthworms inhabit terrestrial ecosystems as well as freshwater and marine environments (Qiu 1999). Earthworms constitute up to 90% of the soil invertebrate biomass and are important ecosystem engineers (Tischler 1965; Edwards 2004). Species identification of adult earthworms is possible by dissection of the male genitalia (Tsai et al. 2000; Shen et al. 2003); however, this method is labor intensive, time consuming, and very difficult for non-specialists, particularly when dealing with field collections consisting of several different earthworm species. Furthermore, identification is limited to adult worms, as most life stages are unidentifiable. Furthermore, many morphological and anatomical characteristics of earthworms are

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variable, and the degree of variability can differ and features can overlap between taxa (Pop et al. 2003). Therefore, taxonomic systems often vary between researchers using different criteria or different sections of the earthworm for identification. For example, the number and location of male pores is very important in earthworm taxonomy because these characteristics are related to copulation and reproduction. Male pores in section XVIII were once used to define families of earthworms (Beddard 1895); however, this was discontinued in subsequent studies because pore location was found to vary among families and even within a single family (Michaelsen 1900; Stephenson 1930). In addition, sexual dimorphism causes problems during identification, especially given that the original descriptions for roughly half of all earthworm species were based on a single specimen (Zhong et al. 1984). Mršic (1991) and Qiu and Bouché (1998) have established many new supraspecific taxa using numeric taxonomy. However, Pop et al. (2003) indicated that this method has considerable drawbacks, particularly for ecologists, because the number of genera accepted by contemporary earthworm specialists range from 6 to 14 in classical works and from 31 to 45 in recent reports that use numerical systems.

Mitochondrial DNA (mtDNA) has been widely used in phylogenetic studies of animals because it evolves much more rapidly than nuclear DNA, thereby resulting in the accumulation of differences between closely related species (Brown et al. 1979; Moore 1995; Mindell et al. 1997). Sequence divergence is much higher among species than within species, and mtDNA genealogies generally capture the biological discontinuities recognized by taxonomists as species. The cytochrome-*c* oxidase I (COI) gene is present in all animals, and sequence comparisons are straightforward because insertions and deletions are rare. Thus, the COI gene, defined as the DNA barcode by Hebert et al. (2003a), has been used to identify species of birds (Hebert et al. 2004), spiders (Barrett and Hebert 2005), springtails (Hogg and Hebert 2004), tropical Lepidoptera (Hajibabaei et al. 2006), and insect pests (Ball and Armstrong 2006), including invasive leafminers (Scheffer et al. 2006). Although this method is not without controversy (Lipscomb et al. 2003), the taxonomic problems with earthworms are serious enough that it is critical to seek a novel solution.

Critics of DNA barcoding object to abandoning the use of traditional morphological characteristics and argue that relying on a single mitochondrial gene region for identification can be misleading, particularly in the face of widespread mitochondrial polyphyly/paraphyly (Sperling 2003; Will and Rubinoff 2004). However, DNA barcoding can be performed at any life stage; indeed, reliable identifications of juvenile or even partial specimens are possible (Palumbi and Cipriano 1998; Symondson 2002), something that morphological identification cannot accomplish. Thus far, DNA barcoding has not been applied to the identification of earthworms.

Here, we examined the utility of DNA barcoding for the identification of earthworm species. We targeted this group because it plays a critical role in soil food webs and because the taxonomy of earthworms has been studied extensively for at least two centuries, thereby providing a template against which to test the accuracy of DNA barcoding. Given that species identifications are often challenging and require considerable taxonomic expertise, a DNA barcode system will be helpful for immediate applications. Furthermore, the development of a universal DNA-based identification system could provide a globally important tool for the identification earthworm species.

China is home to one species of the genus *Haplotaxis* (Haplotaxidae), one species of *Desmogaster* (Moniligastridae), 19 species of *Drawida* (Moniligastridae), 168 species of *Amynthas* (Megascolecidae), 68 species of *Metaphire* (Megascolecidae), one species of *Megascolex* (Megascolecidae), one species of *Perionyx* (Megascolecidae), two species of *Pithemera* (Megascolecidae), four species of *Planapheretima* (Megascolecidae), one species of *Polypheretima* (Megascolecidae), one species of *Malabaria* (Ocnerodrilldae), one species of *Ocnerodrilus* (Ocnerodrilldae), one species of *Ilyogenia* (Ocnerodrilldae), one species of *Microscolex* (Acanthodrilidae), one species of *Plutellus* (Acanthodrilidae), three species of *Pontodrilus* (Acanthodrilidae), two species of *Dichogaster* (Octochaetidae), one species of *Ramiella* (Octochaetidae), two species of *Pontoscolex* (Glossoscolecidae), two species of *Glyphidrilus* (Mircochaetidae), 10 species of *Eisenia* (Lumbricidae), five species of *Aporrectodea* (Lumbricidae), one species of *Dendrobaena* (Lumbricidae), two species of *Dendrodrilus* (Lumbricidae), one species of *Eiseniella* (Lumbricidae), one species of *Lumbricus* (Lumbricidae), two species of *Octolasion* (Lumbricidae), and three species of *Bimastus* (Lumbricidae)(Huang et al., 2006). For this study, we collected specimens from one species of *Drawida*, 20 species of *Amynthas*, three species of *Metaphire*, one species of *Eisenia*, two species of *Aporrectodea*, and one species of *Bimastus* (Table 1).

Table 1. Mean and range of intraspecific nucleotide divergences for species collected from China, using GTR+I+R model; UD = mean percent divergence (uncorrected); CD = mean percent divergence (corrected); *N* = no. of individuals; UR = uncorrected divergence range; CR = corrected divergence range

Species	*N*	UD	CD	UR	CR
Drawida japonica japonica (Michaelsen, 1892)	4	0	0	–	–
Amynthas plantoporophoratus (Tsai, 1964)	3	0.005	0.006	0.001-0.009	0.002-0.011
Amynthas morrisi (Beddard, 1892)	3	0.005	0.006	0.004-0.007	0.005-0.009
Amynthas loti (Chen and Hsu, 1975)	3	0.004	0.005	0.002-0.007	0.002-0.008
Amynthas tuberculatus (Gates, 1935)	3	0.001	0.001	0.000-0.002	0.000-0.003
Amynthas dactilicus (Chen, 1946)	3	0	0	–	–
Amynthas pongchii (Chen, 1936)	3	0.015	0.016	0.013-0.019	0.015-0.021
Amynthas limellus (Gates, 1935)	3	0.005	0.006	0.001-0.008	0.002-0.009
Amynthas hawayanus hawayanus (Rosa, 1891)	3	0	0	–	–
Amynthas triastriatus (Chen, 1946)	3	0	0	–	–
Amynthas incongruus (Chen, 1933)	3	0	0	–	–
Amynthas robustus (Perrier, 1872)	3	0	0	–	–
Amynthas kiangensis (Michaelsen, 1931)	3	0.007	0.008	0.004-0.010	0.005-0.011
Amynthas lautus (Ude, 1905)	3	0	0	–	–
Amynthas benignus (Chen, 1946)	3	0	0	–	–
Amynthas asacceus (Chen, 1938)	3	0.003	0.003	0.000-0.004	0.000-0.005
Amynthas hupeiensis (Michaelsen, 1895)	3	0.004	0.005	0.003-0.005	0.004-0.006
Amynthas homochaetus (Tsai, Shen and Tsai, 2002)	3	0	0	–	–
Amynthas heterochaetus (Michaelsen, 1891)	3	0	0	–	–
Amynthas diffringens (Baird, 1869)	3	0.001	0.001	0.000-0.002	0.000-0.003
Amynthas axillis (Chen, 1946)	3	0	0	–	–
Metaphire tschiliensis tschiliensis (Michaelsen, 1928)	6	0.023	0.025	0.000-0.044	0.000-0.048
Metaphire californica (Kinberg, 1867)	3	0	0	–	–
Metaphire schmardae (Horst, 1883)	3	0.006	0.007	0.002-0.013	0.002-0.015
Eisenia fetida (Savigny, 1826)	4	0.076	0.078	0.003-0.120	0.004-0.122
Aporrectodea trapezoides (Duges, 1828)	3	0	0	–	–
Aporrectodea caliginosa (Savigny, 1826)	1	–	–	–	–
Bimastus parvus (Eisen, 1874)	2	0	0	–	–

Materials and methods

Earthworms were collected from three provinces in China (Sichuan, Hebei, and Beijing), all more than 600 km apart. The adult clitellate earthworms were killed in 30% ethanol and preserved in 95% ethanol at ambient temperature for later DNA extraction. Samples were taken from caudal tissue to prevent contamination by gut contents. As a proof of taxonomic identification, the anterior part of each earthworm was kept in 100% ethanol at China Agricultural University for future studies.

We isolated total DNA from one to six individuals per species following a general extraction method as described by Palumbi et al. (1991). Briefly, single alcohol-preserved specimens were ground in homogenizing buffer and incubated at 65 °C overnight with proteinase K. DNA was then isolated by phenol–chloroform extraction followed by ethanol precipitation. A typical PCR protocol consisted of a template DNA, 1 μL; AmpliTaq polymerase, 1.25 U; primer concentration, 25 pM with dNTP concentration of 200 μM, $MgCl_2$ concentration of 2 mM, and 5 μL 10 × PCR buffer. Sterile water was added for a total volume of 50 μL. For most taxa, a 640 bp fragment of the COI gene was amplified using the flanking primers LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994). These two primer pairs did not work well and were thus modified slightly for *Metaphire schmardae*, *Amynthas incongruus*, *A. kiangensis*, *A. plantoporophoratus*, *A. benignus*, *A. limellus*, *A. homochaetus*, *A. morrisi*, *A. pongchii*, *A. asacceus*, and *A. loti*, for which primer HCO2198 was replaced with primer COI-E (5′-TATACTTCTGGGTGTCCGAAGAATCA-3′) (Bely and Wray 2004), producing a 586 bp fragment. The reaction mixtures were heated to 94 °C for 4 min and then run for 30 cycles at 94 °C for 45 s, at 49–52 °C for 40–60 s (depending on template), at 72 °C for 90 s with a final extension at 72 °C for 7 min. PCR products were purified using the QIAquick spin PCR Purification Kit (QIAGEN, Santa Clarita, CA, USA) following the protocol provided by the manufacturer. PCR products were resolved by electrophoresis through a 1.5% agarose

gel and visualized by ethidium bromide fluorescence. The target bands were cut from the gel and purified using glass beads (Geneclean II kit, Bio 101, USA). Automated sequences were generated in both directions from different runs on an Applied Biosystems (ABI) 377XL automated sequencer using the ABI Big-dye Ready-Reaction Kit, following the standard cycle sequencing protocol. All PCR products gave unequivocal nucleotide chromatograms. Some individuals were sequenced in duplicate to verify the accuracy of the sequencing. The GenBank BLAST algorithm was used to verify that all sequences were from Oligochaeta. All sequences were submitted to Genbank (accession numbers: EF077528–EF077607, DQ835672–DQ835677).

Sequences were aligned using Clustal X, version 1.8 (Thompson et al. 1997), and were improved manually using BIOEDIT 5.0.9 sequence alignment software (Hall 1999). All sequence alignments were pruned to 566 bp. Alignments of the COI sequences were possible because no insertions or deletions were detected. Alignment began at position 86 of the COI gene of *Lumbricus terrestris* (Linnaeus, 1758), which was retrieved from Genbank (accession no. U24570; Boore and Brown 1995).

NJ (Saitou and Nei 1987) and MP (Farris 1970) analyses were performed with PAUP 4.0b10 (Swofford 2002), and Bayesian inference (Huelsenbeck 2000) was carried out with MrBayes 3.0b (Huelsenbeck and Ronquist 2001). Modeltest 3.06 (Posada and Crandall 1998) was used to estimate an optimal model of evolution for the dataset using the hierarchial likelihood ratio test (hLRTs) and the Akaike information criterion (AIC). The GTR+I+G model (empirical base frequencies: $A = 0.2943$; $C = 0.2244$; $G = 0.1706$; $T = 0.3107$; substitution rates: A–$C = 0.1512$; A–$G = 2.5402$; A–$T = 0.5173$; C–$G = 0.4384$;C–$T = 4.9232$; G–$T = 1.0000$; proportion of invariable sites (I) $= 0.4260$; $\alpha = 0.4165$) was selected as the best model. Model choice was guided by the need to avoid under parameterisation (Huelsenbeck and Rannala 2004). Corrected GTR+I+G distances were calculated in PAUP* (Swofford 2002). The NJ tree was constructed with PAUP version 4.0b10 (Swofford 2002) on model of GTR+I+G with 1000 bootstrap replicates (Fig. 1). Monte Carlo Markov chains under were run for 6,000,000 generations with four Markov chains (random starting trees), one of which was cold and the remainder heated. Trees

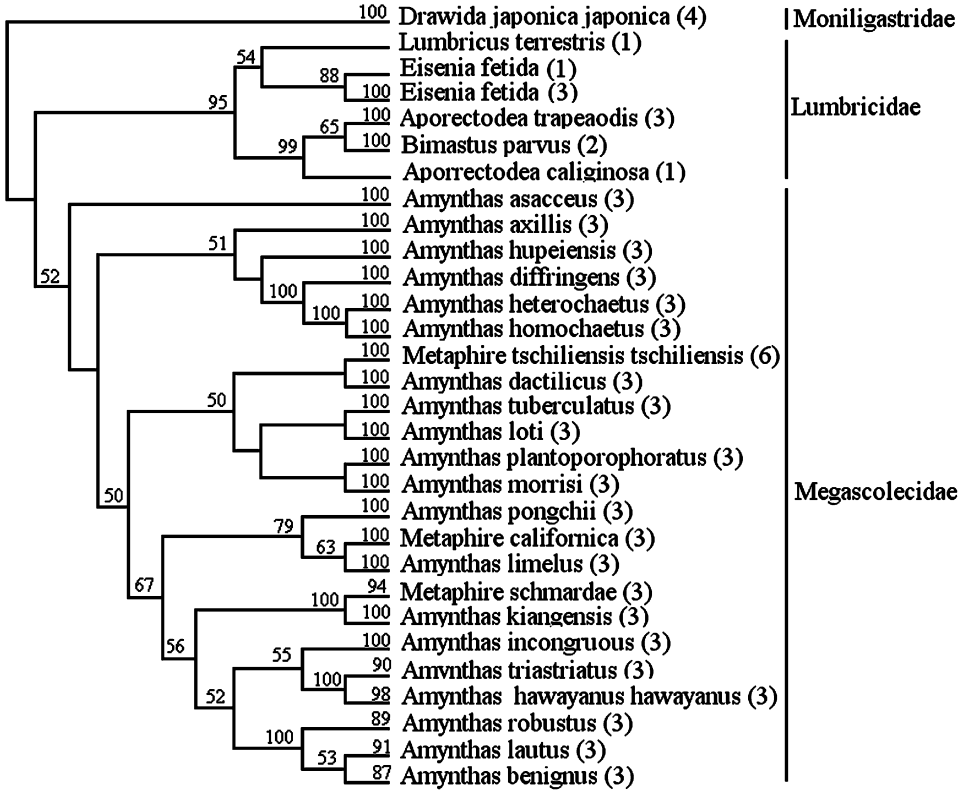

Fig. 1. Neighbour-joining analysis of a 566 bp fragment of the COI gene (for clarity, each species is listed once and the number of individuals is in brackets). NJ tree was calculated in PAUP* based on GTR+I+R chosen by hLRTs test and AIC. Numbers at nodes indicated bootstrap values from 1000 replicates. Numbers before each species meant the bootstrap values among same species. Values below 50 were not showed.

were sampled every 100 generations and the trees generated before the stabilization were discarded. Posterior probabilities were estimated using a majority-rule consensus tree based on the saved trees (Fig. 2). Parsimony trees were obtained using the heuristic search option, constructed using 40 random additions of the sequences, TBR branch swapping, 1000 bootstrap replicates, and remaining parameters set to PAUP* 4.0b10 (Swofford 2002) defaults (Fig. 3). Four-fold degenerate sites (D4) were identified in MEGA 3 (Kumar et al. 2004) and genetic distances were calculated in PAUP* 4.0b10 (Swofford 2002).

Results

We generated COI sequences from 86 adult earthworms. Three barcode sequences were obtained for most species, and only one species was represented by a single individual. Each of the 28 species included in our neighbor-joining profile, MP and BI trees, possessed a distinct COI sequence (Figs. 1–3), and none were shared between species. For clarity, each species was listed once and the number of individuals is in brackets in Figs. 1–3. COI sequences represented by two or more individuals were either identical or most similar to other sequences of the same species. Allied species clustered taxonomically, and none of the split genera were embedded in other generic groupings but instead formed distinct lineages. The three trees had similar topology; that is to say, they all supported the idea that DNA barcoding can be applied in identifying earthworms.

In most cases, the trees showed shallow intraspecific (<1% in most cases; Table 1) and deep interspecific divergence (Table 2). However, one exceptional case showed deep divergence within a species (*Eisenia fetida*). The intraspecific corrected distance in this species was 7.8%, 14-fold higher than the average distance (0.59%). Even in this exceptional case, the divergent individuals still grouped with themselves. In contrast, divergence among species exceeded 15.8% in all cases.

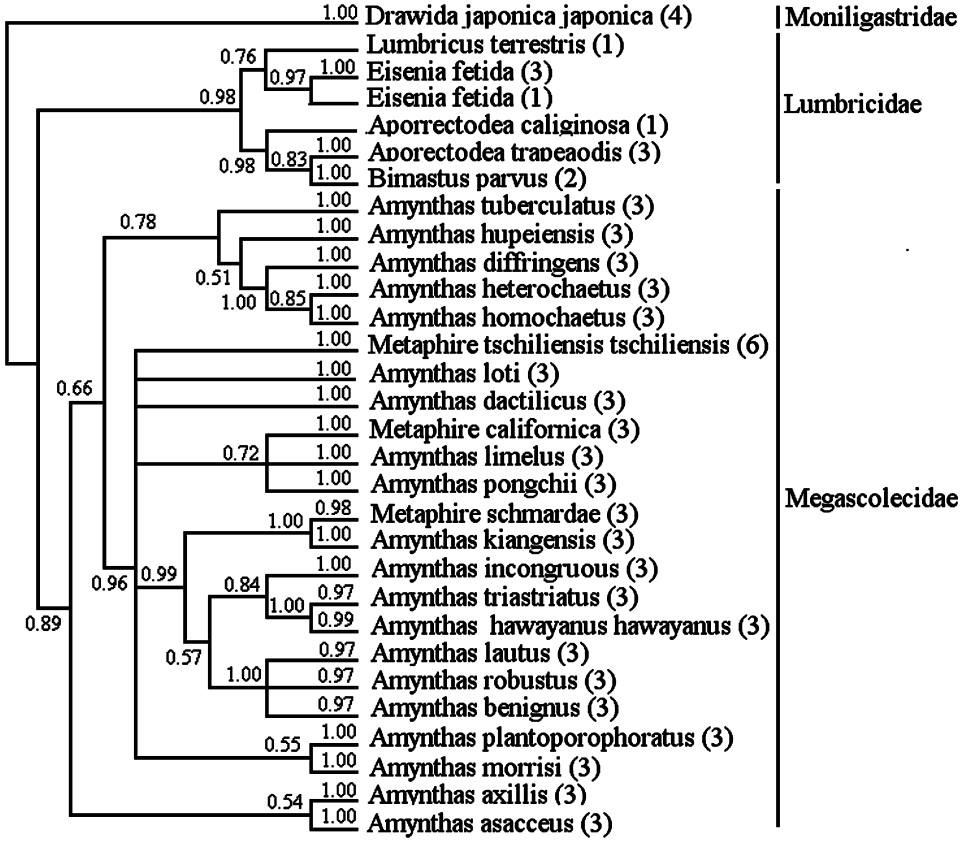

Fig. 2. Bayesian inference analysis of a 566 bp fragment of the COI gene (For clarity, each species is listed once and the number of individuals is in brackets) Parameters were estimated from GTR+I+G chosen by hLRTs test and AIC. Numbers at nodes indicated posterior probabilities from 6,000,000 generations. Numbers before each species meant the posterior probabilities among same species. Values below 0.5 were not showed.

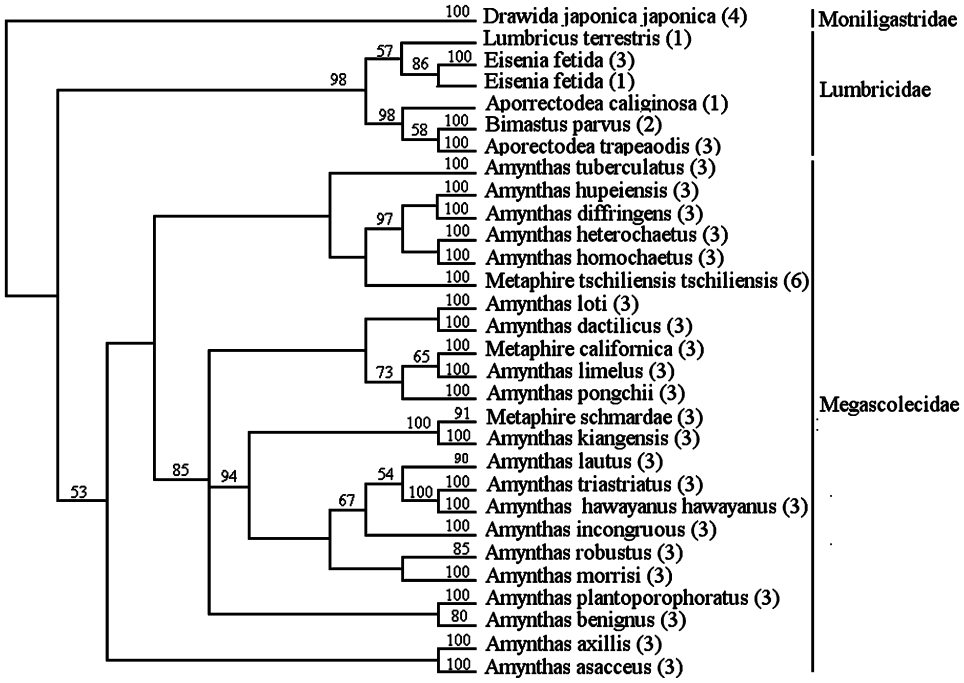

Fig. 3. Maximum parsimony analysis of a 566 bp fragment of the COI gene (for clarity, each species is listed once and the number of individuals is in brackests) Parameters estimated see text. Numbers at nodes indicated bootstrap values from 1000 replicates. Numbers before each species meant the bootstrap values among same species. Values below 50 were not showed.

Table 2. Mean percentage divergence values (range given in parentheses, where applicable) between earthworm taxa collected from China, using GTR+I+R model; UD = uncorrected *p*-distance; CD = corrected *p*-distance

Family	Megascolecidae	Lumbricidae	Moniligastridae
Individuals within species			
n	23	3	1
UD	0.5 (0–4.7)	3.4 (0–12.3)	0
CD	0.5 (0–4.9)	3.5 (0–13.4)	0
Species within genus			
n	2	2	
UD	17.7 (15.4–22.5)	18.1 (17.7–20.8)	
CD	18.4 (15.8–23.1)	19.5 (18.2–21.9)	
Species between genera			
n	2	3	
UD	17.7 (15.4–22.5)	18.2 (17.7–22.1)	
CD	18.3 (15.8–23.1)	19.6 (18.3–22.8)	
Species between families			
n	23	4	1
UD	18 (15.4–22.5)	18.6 (17.7–22.3)	27.6 (25.2–33.5)
CD	19.3 (15.8–23.1)	19.9 (18.3–23)	29.1 (26.6–34.6)

Note: For individuals within species, *n* = number of species that had two or more individuals; for species within genus, *n* = number of genera with two or more species; for species between genera, *n* = number of genera analyzed within a family; and for species between families, *n* = total number of species analyzed.

The most divergent taxon was *Drawida japonica japonica* (Michaelsen, 1892), which had a mean sequence divergence of >29% (range 26.6–34.6%) from the other taxa (Table 2.). Excluding the polytypic species, the average intraspecific distance was very low at 0.35%.

The mean percent sequence divergence between congeneric species was 20.4% (SE = 0.02). Mean

intraspecific distances ranged from a low of 0% in several species to a high of 7.8% in *E. fetida* (Savigny, 1826) (Table 1). Conversely, mean nucleotide divergence among the four genera with multiple species ranged from a low of 9.8% in *Aporrectodea* to a high of 18.1% in *Amynthas*. Mean nucleotide divergence among the three families was 22.8% (Table 2).

Discussion

This study provides evidence that a COI-based identification system would be effective for identifying earthworm species. The simplest test of species identification by DNA barcodes is whether any sequences are found in two species. This did not occur in our study, indicating that there is much lower sequence variation among members of a species than between closely allied species. Indeed, the mean corrected divergence value of 20.4% for congeneric species indicates that most pairs are separated by more than 90 diagnostic substitutions in the 566 bp of the COI gene that we examined. This high level of divergence contrasted with the low intraspecific values that we observed. The dichotomy in divergences allowed us to successfully conduct identification tests using the neighbor-joining tree, and should enable the reliable delineation of the 86 tested specimens. That is, for 86 specimens, 100% of the COI identifications agreed with the morphological identifications provided by expert taxonomists.

Hebert et al. (2003b) found that over 98% of animal species show greater than 2% divergence and suggested that this was the threshold for spider identification. They proposed a standard sequence threshold of 10 $\times$ the mean intraspecific variation for the group (Hebert et al. 2004). In our study, the average intraspecific corrected distance was 0.35%, giving a sequence threshold of 3.5%. All congeneric species pairs showed at least 15.8% corrected divergence, while levels of corrected divergence within a species were $<1\%$ (Table 1). This suggests that a sequence-corrected divergence greater than 15% can reliably distinguish recognized species of earthworms. This result also meets the standard of 10 $\times$ the mean intraspecific variation for the group as proposed by Hebert et al. (2004). Thus, all of the 28 known species were successfully identified. Corrected divergences of *E. fetida* were much higher (up to 7.8%); however, this may represent the presence of an unrecognized sibling species or subspecies. Three *E. fetida* individuals were highly similar with close bootstrap values; another individual had lower similarity and bootstrap values compared to those three individuals. These results demonstrate that DNA barcoding can be an efficient tool for earthworm species identification.

We were able to successfully discriminate known species of Chinese earthworms using COI sequences in all cases. Nucleotide composition averaged over all taxa showed an A+T bias (T = 31.1%, C = 22.4%, A = 29.4%, G = 17.1%), which is commonly observed in earthworms. Interestingly, Heethoff et al. (2004) found that the A+T content was different in large (60.4%) versus small (62.9%) *Octolasion tyrtaeum*, indicating the A+T content of earthworm was biased. However, the 60% A+T content observed in our study is considerably lower than that reported for some insect taxa (70–75%; Nardi et al. 2003).

Measures of genetic divergence are often used to infer species boundaries because of the strong correlation between genetic divergence and reproductive isolation (Coyne and Orr 1989; Gleason and Ritchie 1998; Sasa et al. 1998; Ferguson 2002). Levels of divergence between congeneric species of Chinese earthworms appear higher than those reported for animals in general in that congeneric taxa showed an average of 11.2% sequence divergence (Hebert et al. 2003b). Our data support previous work (Pérez-Losada et al. 2005) showing that the sequencing of mitochondrial genes is useful for discriminating closely related species. For example, Chang and Chen (2005) used the cytochrome-*c* oxidase subunit I gene to identify two sibling species, *Amynthas formosae* and *A. yuhsii*, and transferred them from the genus *Amynthas* to the genus *Metaphire*. Collectively, these results demonstrate the effectiveness of mtDNA and, in particular, COI sequences for detecting species-level differences even in cases of recent divergence. Furthermore, they indicate that COI divergence may help to clarify misleading morphological classifications. In addition, mitochondrial genes (COII) were previously used to differentiate between different morphotypes of the earthworm *O. tyrtaeum* (Heethoff et al., 2004). The researchers stated that there was a strong correlation between the size of the earthworms and the COII sequences, and also suggested that *O. tyrtaeum* consisted of two morphologically and genetically different lineages. That is, COII can be used to measure genetic distances between *O. tyrtaeum* specimens of different size classes. Besides the genetic distinction of the two lineages, the authors observed almost identical sequences within the second lineage. These findings will be helpful in analyzing genetic variation in earthworms.

A non-parametric distance estimation for nucleotide sequences is the measurement of percent distances in four-fold degenerate sites (D4) (Heethoff et al., 2007). D4 sites do not affect the protein sequence and are less sensitive to transition–transversion bias (Li 1993). In this study, pair-wise distances of the D4 sites at 43% did not reach saturation.

The rate of evolutionary change of the COI gene might affect species identification by DNA barcoding. Avise (2000) stated that a common rate of change of animal mitochondrial genomes is 0.02 million years $(Myr)^{-1}\,bp^{-1}$. However, Chang and Chen (2005) found that the evolutionary rate of change in the COI gene of *Metaphire yuhsii* varied with geographic site in Taiwan (0.11 and $0.035\,Myr^{-1}\,bp^{-1}$). They hypothesized that this was a result of geographical barriers and could therefore be explained by geological history and ancient topography. This should be considered when identifying earthworms using DNA barcoding, particularly if the samples are collected at different geographical sites.

Given our results, we suggest that COI barcoding provides a powerful tool for species discrimination in earthworms. We were able to successfully identify 86 individuals representing 28 species, or approximately one-tenth of all Chinese earthworm species (28/306) (Huang et al. 2006). This work could represent the first step toward a DNA barcode system for earthworms. Future studies should examine larger sample sizes, increased taxonomic diversity, and more extensive geographic coverage. With DNA barcoding, new taxa can be easily added to the profile data set, thus continually broadening the taxonomic coverage in the database. Furthermore, the probability of identifying an unknown species increases as more taxa are added to the dataset. Such work will enable the creation of a DNA-based identification system for earthworms, thus allowing for identification to be performed by anyone with access to a basic DNA sequencing laboratory. Although DNA barcoding is not meant to replace traditional morphological species identification, the combined use of the two approaches can provide a powerful tool for identifying earthworm species across a diversity of taxa. Therefore, this practical method of species identification has broad scientific implications.

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