Pedobiologia **49** (2005) 317—324

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Pedobiologia

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Phylogenetic species delimitation of the earthworms *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* Bouché, 1972 (Oligochaeta, Lumbricidae) based on mitochondrial and nuclear DNA sequences

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Received 5 September 2004; accepted 5 February 2005

KEYWORDS
Earthworms;
Eisenia andrei;
Eisenia fetida;
DNA;
Phylogeny;
Species delimitations

Summary

Three samples (11 individuals) of *Eisenia fetida* (Savigny, 1826) and four samples (20 individuals) of *Eisenia andrei* Bouché, 1972 (focal species), and two samples (six individuals) of *Eisenia eiseni* (Levinsen, 1884) (nonfocal species; outgroup) were tested for species delimitation using the phylogeny-based method of Wiens and Penkrot (2002). Thirty-seven and 17 DNA sequences of the nuclear 28S (2031 bp) and mitochondrial COI (658 bp) genes, respectively, were generated. Phylogenetic hypotheses for each gene and both combined were built using maximum parsimony, maximum likelihood, and Bayesian approaches. Fully resolved and well-supported trees (>90% bootstrap proportions and 1.0 posterior probabilities) showed both *E. fetida* and *E. andrei* samples as monophyletic. Application of Wiens and Penkrot's protocol to those trees resulted in the recognition of these two taxa as different phylogenetic species. This result complements previous studies of crossability and allozyme genetics addressing their different biological status (biological species concept). Finally, our analyses also show that *E. fetida* is composed of reproductively isolated populations and suggest the presence of a hitherto unrecognized species or subspecies within this taxon.

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doi:10.1016/j.pedobi.2005.02.004

Introduction

Eisenia fetida (Savigny, 1826) and *Eisenia andrei* Bouché, 1972 (Oligochaeta, Lumbricidae) are of great importance in the vermicomposting of a wide variety of organic wastes, as a potential source of protein for animal consumption, and as fishing bait (Domínguez, 2004). Moreover, these two earthworm species are commonly used in ecotoxicology, physiology, biochemical, and genetic studies (Albani et al., 2003; Domínguez, 2004). *E. fetida* corresponds to the striped or banded morph, with the area around the intersegmental groove having no pigmentation and appearing pale or yellow, hence its common names of ''brandling'' or ''tiger'' earthworm. *E. andrei*, the common ''red'' worm, corresponds to the uniformly reddish morph. Aside from the differences in pigmentation, the two species are morphologically similar (Sims and Gerard, 1985; Reinecke and Viljoen, 1991) and their requirements, overall reproductive performances, and life cycles do not differ significantly, although growth rate and cocoon production are higher in *E. andrei* (Elvira et al., 1996).

E. andrei and *E. fetida* were first described as different morphotypes of *E. fetida* according to differences in body pigmentation (André, 1963) and Bouché (1972) gave them subspecific taxonomic status (i.e., *E. foetida foetida* and *E. foetida unicolour*). However, many authors currently accept *E. fetida* and *E. andrei* as different species (Domínguez et al., 2005). Several sources of evidence, including biochemical (Roch et al., 1980; Valembois et al., 1982), spectroscopic (Albani et al., 2003), genetic (allozyme electrophoresis; Jaenike, 1982; Henry, 1999; McElroy and Diehl, 2001), and reproductive (André, 1963; Reinecke and Viljoen, 1991; Elvira et al., 1996; Domínguez et al., 2005) studies, support this idea, although under certain limitations. Genetic studies, for example, have been focused on single populations or individuals, and do not account for intraspecific variation. Moreover, *E. fetida* and *E. andrei* are syntopic, commonly living in mixed colonies in dung and compost heaps and hence hybridization could be possible; in fact, both species already have been crossed, although the hybrid offspring is believed to be sterile (André, 1962). Hence, reproductive isolation might not be complete between both taxa (although see Domínguez et al., 2005). Moreover, abundant recent literature, for example ecotoxicological studies (OECD Draft Document, 2000), indicates continued confusion about the taxonomic status of these two earthworms, with both termed indiscriminately *E. fetida* or *E. foetida*, an inaccurate emendation of the former name (Sims, 1983; Easton, 1983; Domínguez et al., 2005).

Therefore, in spite of all the previous evidence, we think the taxonomic status of the *E. fetida*/*andrei* complex still needs to be more rigorously addressed. Species delimitation is essential because species are used as basic units of analysis in several areas of biogeography, ecology, conservation biology, and macroevolution (Brown et al., 1996; Blackburn and Gaston, 1998; Barraclough and Nee, 2001; Sites and Crandall, 1997; Agapow et al., 2004). A better understanding of these large-scale processes requires that researchers employ methods to delimit objectively and rigorously which species are in nature (Sites and Marshall, 2003). In the last few years several methods have been proposed for empirically testing species boundaries within a statistical framework (Wiens and Servedio, 2000; Puorto et al., 2001; Templeton, 2001; Wiens and Penkrot, 2002). Recent revisions of these and other more classical methods have organized them into two general categories of tree- and nontree-based approaches (Sites and Marshall, 2003, 2004). Nontree-based methods delimit species on the basis of gene flow assessments (biological species concept), whereas tree-based methods delimit species as historical lineages (phylogenetic species concept). Previous studies of crossability and allozyme genetic differentiation have tested species boundaries in the *E. fetida*/*andrei* complex using the former approach. Systematists, however, typically favour phylogenetic methods to delimit species because of the operative functionality and statistical advantages of the phylogenetic framework (Wheeler and Meier, 2000; Wiens and Servedio, 2000). In this study we will complement previous taxonomic work on the *E. fetida*/*andrei* complex by using a tree-based approach with DNA sequence data. Molecular methods are expected to provide new and more precise means in delimiting taxonomic groups as well as in establishing phylogenetic relationships among earthworm species (Pop et al., 2004; Heethoff et al., 2004). We will apply the molecular phylogenetic procedure of Wiens and Penkrot (2002) to infer species boundaries among several populations of *E. fetida* and *E. andrei*.

Materials and methods

Twenty earthworms of *E. andrei* from Brazil, Ireland and Spain (four populations), 11 of *E. fetida* from Ireland and Spain (three populations), and six of *Eisenia eiseni* (Levinsen, 1884) (outgroup) from Spain (two populations) were collected in 2004

(Table 1). All the individuals were obtained from free-living populations and kept alive in the laboratory until required. *E. eiseni* was chosen as the outgroup based on accessibility and ecological affinities with the other two species (i.e., all of them are epigeic earthworms).

Total genomic DNA was extracted using methods described in Crandall and Fitzpatrick (1996). Polymerase-chain-reaction (PCR; Saiki et al., 1988) products for the 28S subunit of the nuclear ribosomal RNA (2031 bp; positions 105–2551 in the *Mus musculus* 28S gene; Hassouna et al., 1984) and mitochondrial cytochrome oxidase (COI; 658 bp; positions 48-705 in the *M. musculus* COI gene) genes were amplified using primers from Whiting (2001; rd1a to rd6b primers) and Folmer et al. (1994), respectively. Standard PCR conditions (5 μL 10 × *Taq* buffer; 6–8 μL 25 mM Mg_2Cl; 8 μL 10 mM dNTPs; 5 μL each of two 10 mM primers; 1.25 U *Taq*; ≈20 μL ddH_2O) were used on a Perkin-Elmer 9600 machine and comprised the following: an initial denaturation at 96 °C for 3 min followed by 40 cycles of 95 °C for 30 s, 50 °C for 45 s, 72 °C for 1 min followed by an extension at 72 °C for 5 min. PCR products were resolved by 1.5% agarose gel electrophoresis, visualized by ethidium bromide fluorescence, and purified using a GeneClean® II kit (Bio 101). 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, but using a 16th of the suggested reaction size. All PCR products gave unequivocal nucleotide chromatograms.

In an initial screening we sequenced the same one or two specimens from each sample for COI and 28S. As expected, COI showed higher genetic variability (i.e., higher mutation rate) than 28S for all the *Eisenia* taxa, hence, we sequenced three more specimens per sample for the former gene (except for EfIr and EeSa, see Table 1) to account for intraspecific variability. Therefore the total number of individuals used in our phylogenetic analyses will be 37 for COI, and the same 17 specimens for 28S and the combined 28S-COI.

Table 1. *Eisenia* samples sequenced for COI and 28S genes

Species	Locality	Code	Genes (N)	
			COI	28S
Eisenia andrei	Juiz de Fora – Brazil	EaBr	5	2
	Dublin – Ireland	EaIr	5	2
	Madrid – Spain	EaMa	5	2
	Vigo – Spain	EaVi	5	2
Eisenia fetida	Dublin – Ireland	EfIr	2	2
	Santiago – Spain	EfSa	4	2
	Vigo – Spain	EfVi	5	2
Eisenia eiseni	Santiago – Spain	EeSa	1	1
	Vigo – Spain	EeVi	5	2

Only individuals 1 and 2 from each sample were sequenced for both genes. N = specimens.

Nucleotide sequences were aligned using Clustal X (Thompson et al., 1997) under the default settings. Alignments were trivial for both genes. Only one indel was added to the 28S alignment to maintain structural homology. All DNA sequences were deposited in GenBank under the Accession Nos. AY874470–AY874523.

Incongruence between 28S and COI genes was addressed by using the methodology proposed by Wiens (1998). Separate phylogenetic analyses were conducted for both genes to detect potential areas of strongly supported incongruence (where combined analyses may fail), as indicated by conflicting nodes with bootstrap proportions (bp) ⩾ 70% or posterior probabilities (pP) ⩾ 95%.

We used the model selection procedure outlined by Huelsenbeck and Crandall (1997) as implemented in Modeltest 3.06 (Posada and Crandall, 1998) to find the best-fit model of evolution for the data. Within a maximum likelihood framework models were compared using the Akaike Information Criterion (Akaike, 1973). This approach reduces the number of unnecessary parameters that contribute little to describing the data by penalizing more complex models (Burnham and Anderson, 2002; Nylander et al., 2004). The TIM+I model ($\pi_A = 0.207$, $\pi_C = 0.275$, $\pi_G = 0.339$, and $\pi_T = 0.179$; $r_{CT} = 44.78$, $r_{CG} = 8.01$, $r_{AT} = 8.01$, $r_{AG} = 13.56$, and $r_{AC} = 1.0$; $I = 0.967$) was selected for the 28S data set, the TVM + I + Γ model ($\pi_A = 0.262$, $\pi_C = 0.239$, $\pi_G = 0.178$, and $\pi_T = 0.322$; $r_{CT} = 427.47$, $r_{CG} = 0.0$, $r_{AT} = 1548.62$, $r_{AG} = 427.47$, and $r_{AC} = 101.47$; $I = 0.395$; $\alpha = 0.535$) for the COI data set, and the GTR + Γ + I model ($\pi_A = 0.218$, $\pi_C = 0.265$, $\pi_G = 0.302$, and $\pi_T = 0.215$; $r_{CT} = 56.85$, $r_{CG} = 1.04$, $r_{AT} = 29.98$, $r_{AG} = 21.73$, and $r_{AC} = 7.33$; $I = 0.792$; $\alpha = 0.649$) for the combined 28S and COI (28S-COI) data set. We used these three models of DNA substitution, with these parameters, in all the maximum likelihood analyses, but GTR+I models with or without rate heterogeneity (Γ) were used in the Bayesian analysis.

We conducted equally weighted maximum parsimony (MP) heuristic searches with 100 random addition (RA) replicates and tree bisection and

reconnection (TBR) branch-swapping using PAUP* v4.0b10 (Swofford, 2002). Confidence in the resulting relationships was assessed using the nonparametric bootstrap procedure (Felsenstein, 1985) with 1000 bootstrap replicates, TBR branch-swapping, and ten RA replicates. Maximum likelihood (ML) heuristic searches were performed using PAUP*, with 10 RA replicates and TBR branch-swapping. One hundred replications, TBR branch-swapping, and one RA replicate were used for the bootstrap analysis. Bayesian phylogeny estimation was performed using MrBayes v3.0 (Ronquist and Huelsenbeck, 2003) under single models for 28S and COI and mixed models (all model parameters unlinked) for 28S-COI. Each Markov chain was started from a random tree and run for 4.0×10^6 cycles with every 500th cycle sampled from the chain. Model parameters were treated as unknown variables with uniform priors and were estimated as part of the analysis. We ran four chains simultaneously, three heated (temperature = 0.2) and one cold, using Metropolis coupled Markov chain Monte Carlo (BMCMC) to enhance the mixing capabilities of the Markov chains. Each analysis was repeated three times. Stationarity was checked as suggested in Huelsenbeck et al. (2002) and Nylander et al. (2004). All sample points prior to reaching the plateau phase were discarded as "burn in" and the remaining trees combined to find the maximum a posteriori probability (MAP) estimate of phylogeny. If not found, a 50% majority-rule consensus tree was estimated. Branch lengths were represented as means of the posterior probability density.

We used the Wiens and Penkrot's (2002) protocol to test species boundaries between DNA sequences (haplotypes) of *E. andrei* and *E. fetida*. This method is based on a sampling design that includes: (a) focal species (the species of interest in the study; here the *E. andrei*/*fetida* complex) and nonfocal species (closely related reference species; *E. eiseni*) to apply the exclusivity test (i.e., all members of a group share a more recent common ancestor with every other member of that group than any of them does with any non-member) for the focal species, and (b) at least two individuals per locality to make the between-population gene flow inferences. It requires a phylogeny of haplotypes (or individuals) of known locality and taxonomic designation. A topology that fails to recover haplotypes from a given locality as a clade is taken as evidence for potential gene flow between populations (i.e., focal species = single species). The method is implemented using dichotomous flow charts that lead to several alternatives for making species-level decisions.

Results

No areas of strongly supported conflict were identified between single gene phylogenies (Fig. 1); hence 28S and COI sequences were then combined for phylogenetic analyses. The MP analysis found three, six and seven most parsimonious trees for 28S ($L = 37$), COI ($L = 253$), and 28S-COI ($L = 293$), respectively. The ML analyses of the same data sets resulted in single most likely trees for each gene and both combined, with likelihood scores −3012.6 (28S), −1948.7 (COI), and −5252.6 (28S-COI). The BMCMC analyses of the 28S and 28S-COI data set generated MAP trees (pP = 0.001 and 0.029, respectively), but 50% majority-rule consensus trees were estimated for COI. A total of 21, 146, and 25 trees were discarded (burn-in) in the analyses of the 28S, COI, and 28S-COI genes, respectively.

In all the MP, ML, and BMCMC phylogenetic analyses of 28S and COI each *Eisenia* species was shown as monophyletic (Fig. 1), although clade supported varied among gene trees. The phylogenetic analyses of the combined data set showed the same topological pattern, but with the monophyly of the three *Eisenia* species supported by high bp (>90%) and pP (1.0). Intraspecific relationships were mostly unresolved in all the analyses except for the EfIr sample, which was clearly separated from the other two *E. fetida* samples in all the gene trees. Interspecific corrected genetic distances (Table 2) ranged between 0.005 and 0.011 for the slow-evolving nuclear 28S (lower triangle) and between 0.312 and 0.569 for the fast-evolving mitochondrial COI (upper triangle). Intraspecific genetic distances (Table 2) were ⩽0.002 and ⩽0.005 for the same data sets, except for the two pairs EfIr and EfSa or EfVi in COI, which presented exceptionally high values of ∼0.180.

Discussion

According to Wiens and Penkrot's (2002) protocol, failure of haplotypes from the same locality to cluster together is potential evidence for gene flow between localities (i.e., focal species = single species). However, all the trees showed that haplotypes of the focal species *E. andrei* and *E. fetida* are exclusive and there is no gene flow between their basal lineages. Hence, it can be concluded that, based on Wiens and Penkrot's method, *E. andrei* and *E. fetida* are two different phylogenetic species. This result supports previous allozyme genetic studies that revealed fixed allelic differences at some loci and Nei's (1978) unbiased

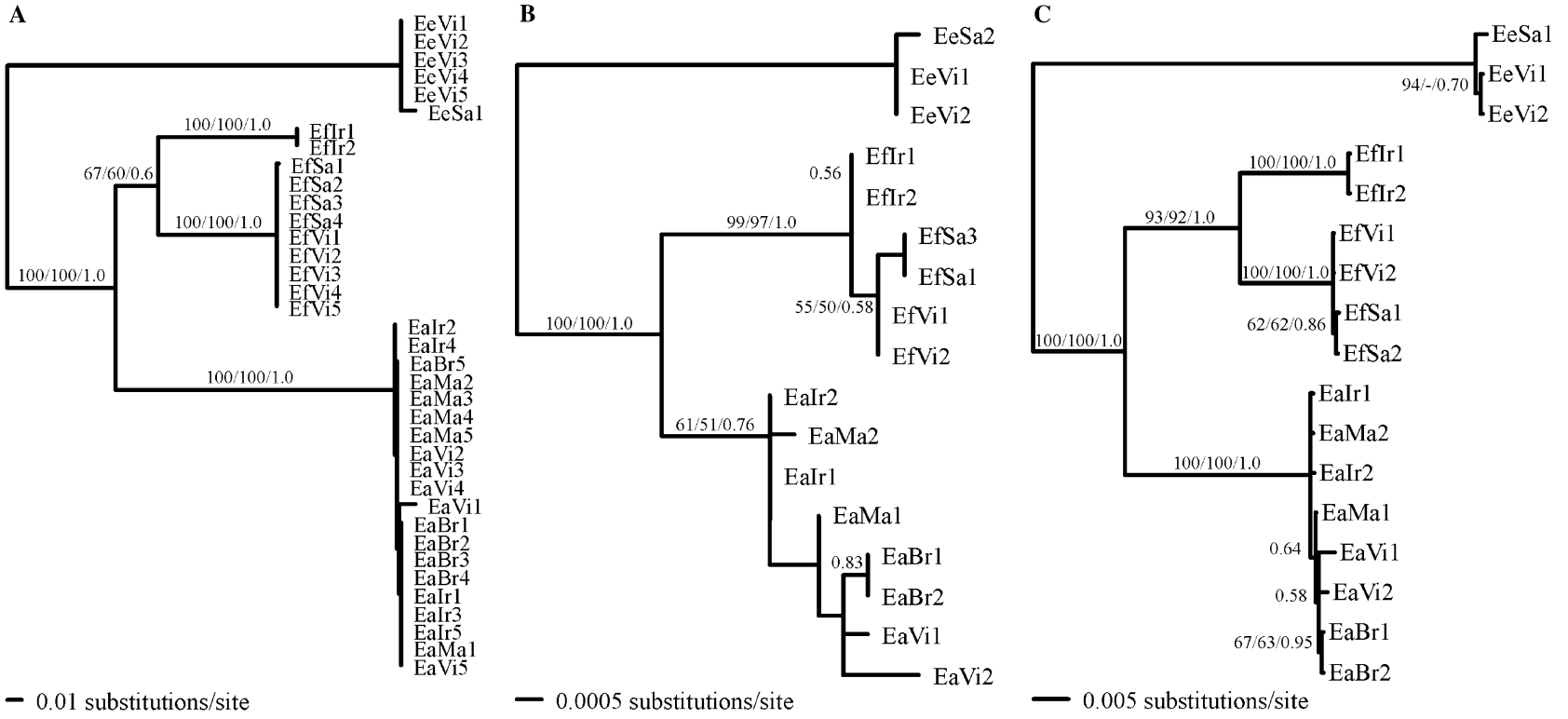

Figure 1. Phylogenetic trees: (A) Bayesian 50% majority-rule consensus tree for COI; (B) maximum a posteriori probability estimate of phylogeny for 28S; (C) maximum a posteriori probability estimate of phylogeny for 28S-COI. Branch lengths are shown proportional to the amount of change along the branches. No major discrepancies were observed between the trees presented here and those obtained under the MP and ML approaches. Bootstrap support (MP and ML, respectively) and posterior probabilities are presented over the branches.

Table 2. Corrected genetic distances between samples (averaged among specimens) for the 28S (lower triangle) and COI genes (upper triangle)

	EaBr	EaIr	EaMa	EaVi	EfIr	EfSa	EfVi	EeSa	EeVi
EaBr	–	0.004	0.002	0.004	0.333	0.315	0.314	0.569	0.569
EaIr	0.002	–	0.002	0.005	0.331	0.313	0.312	0.565	0.564
EaMa	0.001	0.001	–	0.003	0.333	0.315	0.314	0.562	0.561
EaVi	0.001	0.002	0.001	–	0.335	0.315	0.314	0.553	0.553
EfIr	0.006	0.005	0.005	0.007	–	0.182	0.181	0.493	0.473
EfSa	0.006	0.005	0.005	0.007	0.001	–	0.000	0.487	0.462
EfVi	0.007	0.005	0.006	0.007	0.001	0.001	–	0.485	0.461
EeSa	0.009	0.010	0.010	0.010	0.011	0.011	0.011	–	0.009
EeVi	0.009	0.010	0.010	0.010	0.010	0.011	0.011	0.001	–

genetic distances greater than 0.45 between both species (Jaenike, 1982; Henry, 1999; McElroy and Diehl, 2001). Additionally, it agrees with previous evidence indicating that both earthworms exhibit different biochemical particularities of the antibacterial factor (Roch et al., 1980), bacteriostatic activity of a chloragogen cell secretion (Valembois et al., 1982), and fluorescent fingerprints (Albani et al., 2003), and confirms recent reproductive isolation results by Elvira et al. (1996) and Domínguez et al. (2005). In conclusion, all of this evidence proves that *E. andrei* and *E. fetida* are two different phylogenetic and biological species (sensu Mayr, 1942). All future research on micro or macroevolution, biogeography, ecology, conservation, and studies of more applied aspects (e.g., vermiculture, vermicomposting, and ecotoxicology) on these two taxa should be aware of their specific status and the corresponding implications.

Our phylogenetic trees also showed that the population of *E. fetida* from Ireland (EfIr) does not exchange alleles with any other populations from the same or different species; hence according to Wiens and Penkrot's procedure, EfIr also qualifies as a different *Eisenia* species. This result is also supported by the high COI genetic distances (~0.180) between EfIr and other *E. fetida* populations compared to the averaged values obtained among intraspecific samples (0.003). Previous allozyme population genetic studies by Jaenike (1982) and McElroy and Diehl (2001) found very low levels of genetic differentiation among populations of each *Eisenia* species, similar to those reported here. This implies that the mitochondrial genetic characteristics of the Irish population of *E. fetida* could be the result of reproductive isolation and so suggests that this sample may constitute an unrecognized species or subspecies of *E. fetida*. Future study by our group will address this question in more detail.

Acknowledgements

This research was supported by CICYT (AGL2003-01570) and Xunta de Galicia (PGIDIT03P-XIB30102PR) Grants and funds from Keith A. Crandall's laboratory.

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