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Taxonomic assessment of Lumbricidae (Oligochaeta) earthworm genera using DNA barcodes

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ABSTRACT

The family Lumbricidae accounts for the most abundant earthworms in grasslands and agricultural ecosystems in the Paleartic region. Therefore, they are commonly used as model organisms in studies of soil ecology, biodiversity, biogeography, evolution, conservation, soil contamination and ecotoxicology. Despite their biological and economic importance, the taxonomic status and evolutionary relationships of several Lumbricidae genera are still under discussion. Previous studies have shown that cytochrome *c* oxidase I (COI) barcode phylogenies are informative at the intrageneric level. Here we generated 19 new COI barcodes for selected *Aporrectodea* specimens in Pérez-Losada et al. [1] including nine species and 17 populations, and combined them with all the COI sequences available in Genbank and Briones et al. [2] for Lumbricidae (435 sequences) and seven other Lumbricina families (480 sequences). Our maximum likelihood and Bayesian trees indicate that the genera *Aporrectodea*, *Allolobophora*, *Eisenia* and *Dendrobaena* (Lumbricidae) and *Diplocardia*, *Metaphire* and *Amynthas* (Megascolecidae) are polyphyletic and so invalid as currently defined. Our results also confirm that COI barcodes are a good proxy for estimating intrageneric phylogenetic diversity and relationships in earthworms.

1. Introduction

The earthworms of the family Lumbricidae are essential components of the soil biological community. They play a major role in the biogeochemical cycles of terrestrial ecosystems because of their influence on microbial activity, carbon and nitrogen cycles and alteration of soil [3–5]. Lumbricids are the most abundant invertebrate in agricultural lands from temperate regions, where they account for 90% of invertebrate biomass [3,6]. They are also prey to a wide range of invertebrate and vertebrate predators being an important link in animal food webs [3,4,7]. Several Lumbricidae species have become model organisms for ecology, toxicology, physiology and reproductive biology [3,5]. Finally, lumbricid bioresources resulting from vermiculture technology is of great economic value and provides important environmental benefits, especially in light of global concerns regarding sustainable land use, food security and climate change [8].

Surprisingly, despite their relevance and all the intense research done on lumbricids over the last 130 years, their taxonomy is still far away from being resolved [2,9]. The Lumbricidae account for ~300 described species and is considered to be a monophyletic group included in the monophyletic suborder Crassiclitellata (Oligochaeta) [10,11]. Representatives of Lumbricidae are morphologically characterized by the presence of a multilayered clitellum. However, no agreement exists about Lumbricidae classification with proposals ranging from 6 to 14 genera in classic studies [12–15] to 31 – 45 genera in modern revisions [16–19].

Lumbricidae taxonomy has been always hindered by the structural simplicity of the earthworm body plan, lacking complex appendices or highly specialized copulatory apparatuses [9]. Furthermore, variation on the few morphological features used to identify lumbricids (e.g., prostomium, arrangement of the setae, position and form of the clitellum, tubercula pubertatis, and spermathecae) usually overlaps across taxonomically close- and distant-related taxa [1,9]. This has led to morphologically similar species being lumped into a single species with various morphotypes or as a species complex that includes various taxa of uncertain taxonomic category [13,20,21], and to the creation of "catch-all" genera [2,22]. Among the Lumbricida genera, the case of

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Aporrectodea (*Ap.*) is particularly interesting because of its species diversity and convoluted taxonomic history. First of all, aporrectodean earthworms have been nominated under three different generic names, *Aporrectodea*, *Allolobophora* and *Nicodrilus*. Eisen [23] did not indicate a type species within the *Allolobophora* but simply included seven heterogeneous species, which have been since accommodated in different genera [20]. Over the years many more species have been added to *Allolobophora* turning it into a "catch-all" genus. Omodeo [14], unwisely, designated *Allolobophoridella chlorotica*, an atypical species, as the type species. Consequently, many species previously assigned to the genus had to be excluded and moved into the genus *Aporrectodea* with *Allolobophoridella trapezoides* as the type species. Bouché [13] defined *Allolobophora sensu stricto* to include a small group of species allied to *A. chlorotica* and placed the related species *Allolobophoridella caliginosus* and *Allolobophoridella longus* in the new genus *Nicodrilus*, with *Allolobophoridella terrestris* = *Allolobophoridella giardi* as the type species [20]. This grouped together several allied species from the old "catch-all" genus, but many more still remained in a generic limbo, which he named *Allolobophora sensu lato*. Unfortunately this temporary solution to an already difficult problem did not bring stability to the nomenclature. In fact, it stimulated further subdivision within the genus. Finally, *Nicodrilus* was redefined as a junior synonym of *Aporrectodea* because its type species, *A. trapezoides*, resulted a junior synonym of *A. caliginosa*, a species originally included in the genus *Nicodrilus* [12]. Currently the genus *Aporrectodea* includes a number of species ranging from 28 [24] to 40 species [19], although the majority of them have not been cited since the original description. The type species is *Ap. trapezoides* [see 24], which is currently considered part of the *Ap. caliginosa* species complex together with *Ap. caliginosa* s.s., *Aporrectodea tuberculata*, *Aporrectodea nocturna* and *Ap. longa* [see 1].

While no morphological phylogenetic analysis has been used to assess Lumbricidae classification, molecular (mainly based on mtDNA and 18S rRNA genes) phylogenies have often revealed taxonomic incongruence in some Lumbricidae genera: *Aporrectodea* [1,2,22,25–27], *Dendrobaena* spp [2,17,28]., *Allolobophora* [22], *Eisenia* [2,17] and *Lumbricus* [2,26]. Nevertheless, most of these studies have been hampered by their reduced sequence sampling [e.g., 2, 26, 27], the inconsistence of the phylogenetic approaches used [e.g., maximum parsimony analysis in 22] or the low variation of the markers analyzed [e.g., 18S rDNA in 28]. Here we assess the taxonomic status and shallow evolutionary relationships of 17 Lumbricidae genera (paying special attention to *Aporrectodea*) and other 15 Lumbricina genera (outgroup) using parametric phylogenetic methods and a large dataset consisting of 915 cytochome oxidase (COI) barcodes from Genbank, Briones et al. [2], and 19 new barcodes generated here. Such a comprehensive dataset unites multiple molecular studies and has never been assembled before.

The COI gene is the most commonly sequenced mtDNA gene in invertebrates [29] and the selected region used here of 658 bp has become the standard marker for DNA barcoding (i.e., molecular taxonomic identification) [30–32]. Moreover, COI barcodes have been also widely used in ecology, conservation, phylogenetics and phylogeography [33–36]. Within earthworms, COI is also the most highly sequenced marker and has proved to be effective for species identification [25,27,32], taxonomy and shallow (intrageneric) molecular systematics [22,32,37–40], ecology [2], phylogeography [39–41] and for revealing cryptic speciation [26,42]. However, despite its great potential for studying soil animal biodiversity [43], phylogenetic structure estimated using COI data is rarely validated using additional mitochondrial and nuclear loci in the same or similar earthworm species. Previous studies in *Eisenia* [37] and Hormogastridae [44] have suggested that COI barcode phylogenies can recover the basic lineages depicted in trees built using multilocus datasets. Here we have generate new COI barcodes for selected specimens of the *Ap. caliginosa* species complex studied in Pérez-Losada et al. [1] to test if they are a good proxy for assessing intra and interspecies diversity and making evolutionary inferences within this group.

2. Materials and methods

2.1. Datasets

Nineteen *Aporrectodea* earthworms (Fig. 1; bold names) corresponding to nine species and 17 populations were sequenced for COI using protocols and conditions in Pérez-Losada et al. [37]. Standard COI barcoding primers were used [45]. All PCR products (658 bp) gave unequivocal nucleotide chromatograms. DNA sequences were deposited in Genbank under the Accession Numbers JN850535–JN850552. Taxon names for new barcodes follow those in Pérez-Losada et al. [1] preceded by the code PLRMD (authors' last name initials). We combined these new data with all the Lumbricidae COI sequences available in Genbank as of July 2010 (392) and in Briones et al. [2] (43), hereafter coded as "Genbank Accession Number *taxon name*" (e.g., EF653884 *Hormogaster elisae*) and "BMP (author's last name initials) *taxon name*" (e.g., BMP *Criodrilus lacuum*), respectively. To root the Lumbricidae tree, we used all the COI barcodes available in Genbank for other Lumbricina families. This rendered a final dataset of 934 sequences composed of 451 Lumbricidae barcodes (ingroup) and 2 Eudrilidae, 353 Megascolecidae, 60 Glossoscolecidae, 14 Acanthodrilidae, 41 Hormogastridae, 3 Criodrilidae (Almidae in Genbank) and 10 Ocnerodrilidae barcodes (outgroup). Short or ambiguous sequences were not included. This initial dataset was first screened using the maximum likelihood (ML) approach described below. The ML tree revealed many identical or similar (<1% divergent based on p-distances) sequences from the same species, which clustered in well-defined clades (100% bootstrap support). All these sequences but one were excluded in subsequent phylogenetic analyses, so only 190 taxa representing the intra and interspecies diversity in all the main Lumbricina clades were included. Since we are particularly interested on the taxonomic status of *Aporrectodea*, all of the 71 *Aporrectodea* barcodes in the initial phylogeny were included in the final analyses. Therefore, our final dataset consisted of 158 Lumbricidae barcodes and 32 other barcodes representing the other Lumbricina families: 2 Eudrilidae, 19 Megascolecidae, 4 Glossoscolecidae, 1 Acanthodrilidae, 2 Hormogastridae, 2 Criodrilidae and 2 Ocnerodrilidae.

2.2. Data analysis

Nucleotide sequences were aligned in MAFFT v5.7 [46] using the global (G-INS-i) algorithm and default settings. ML trees were inferred in RAxML v7.2.0 [47] using 1000 searches starting from random trees and three independent models of evolution for each codon position. Models were chosen in JModelTest v1.0 [48] under the Akaike Information Criterion. The general time reversible model of evolution (GTR), with proportion of invariable sites (I) and gamma distribution (Γ) was selected for 1st codon positions while the GTR+Γ model was selected for 2nd and 3rd positions. Clade support was assessed using the non-parametric bootstrap procedure [49] with 1000 bootstrap replicates estimated in RAxML. Bayesian–Markov chain Monte Carlo (BMCMC) trees were inferred in MrBayes v3.1.2 [50]. Two independent BMCMC analyses were run with each consisting of four chains. Each Markov chain was started from a random tree and run for 2×10^6 cycles, sampling every 1000th generation. Model parameters were treated as unknown variables with uniform default priors and were estimated

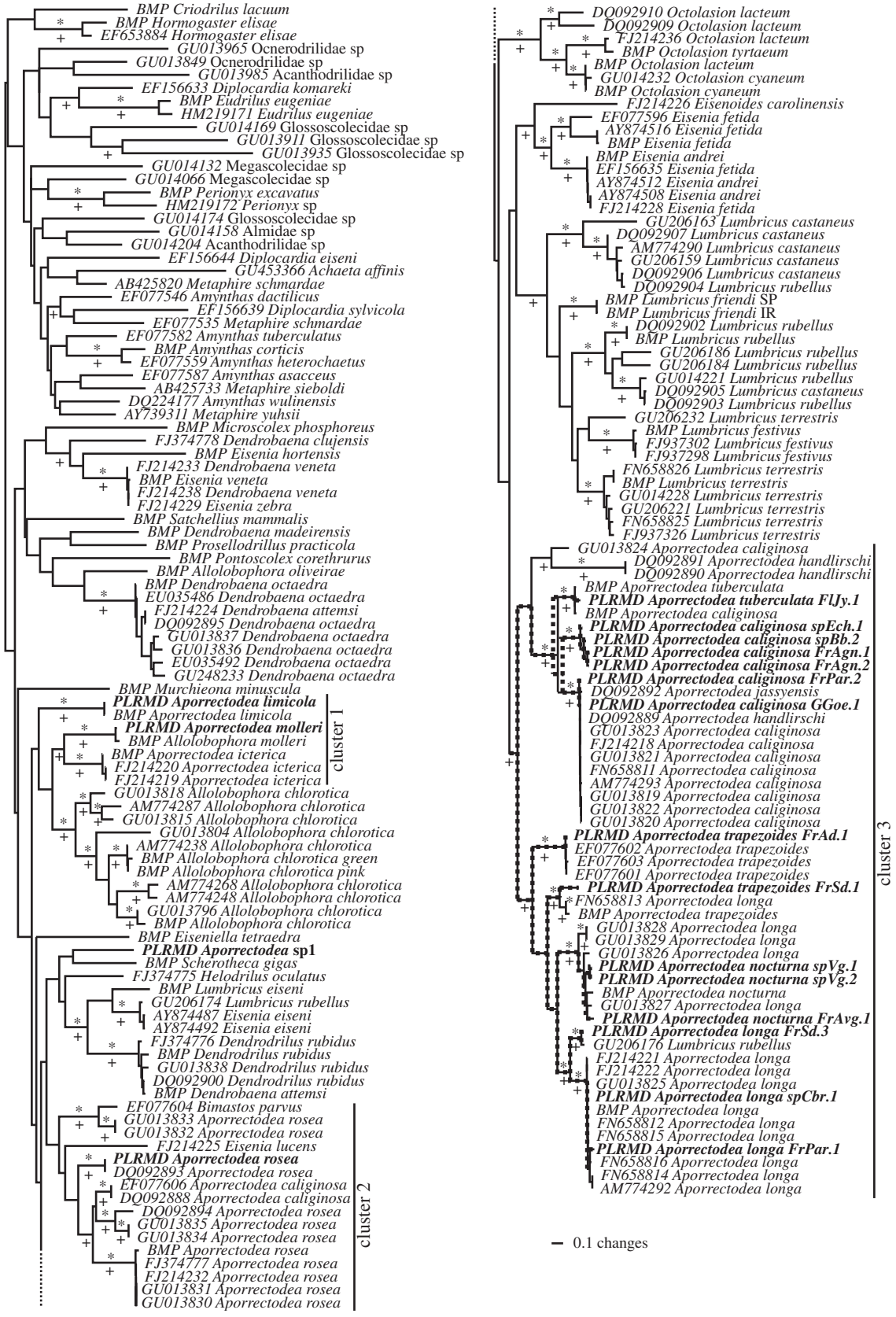

Fig. 1. COI ML tree of Lumbricina earthworms. Bootstrap proportions ≥70% and Bayesian posterior probabilities ≥0.95 are indicated with * and + above and below branches, respectively. Sequences generated in this study are highlighted in bold. *Aporrectodea* clusters are also indicated. The section of the multilocus tree in Pérez-Losada et al. [1] involving the specimens of the *Ap. caliginosa* species complex sequenced here is shown in dotted lines.

as part of the analysis for each codon position. Convergence and mixing were monitored using Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/). All sample points prior to reaching stationarity were discarded as burn-in. The posterior probabilities (p*P*) for individual clades obtained from two separate analyses were compared for congruence and then combined and summarized on a 50% majority-rule consensus tree.

Confidence in our best hypotheses of phylogenetic relationships were tested by first creating alternative constrained hypotheses and then performing new searches in RAxML under those constrains (e.g., *Aporrectodea* is monophyletic). Likelihoods were then compared using the Shimodaira and Hasegawa (S–H) [51] topological test implemented in RAxML. Bayesian topological tests were also performed as described in Huelsenbeck et al. [52].

Genetic divergence among congeneric and conspecific earthworm taxa was estimated using uncorrected p-distances. This distance was chosen to make our estimates comparable to those reported in previous studies of earthworm COI barcodes.

3. Results

ML and Bayesian approaches generated almost identical topologies. All the outgroup taxa formed a clade in our phylogenetic trees (Fig. 1) except for two sequences from Briones et al. [2]: *Microscolex phosphoreus* (Megascolecidae) and *Pontoscolex corethrurus* (Glossoscolecidae). Within the outgroup, three Megascolecidae genera did not form monophyletic assemblages in our phylogenetic analyses: *Diplocardia*, *Metaphire* and *Amynthas*. The alternative monophyletic hypotheses for these three genera were rejected by the S–H test in all cases ($P < 0.0001$) and had p$P < 0.0001$ (Table 1). Similarly, five ingroup Lumbricidae genera did not form monophyletic assemblages either: *Dendrobaena*, *Eisenia*, *Allolobophora*, *Aporrectodea*, *Lumbricus* and *Dendrodrilus* (Fig. 1). All the alternative monophyletic hypotheses for these groups except for *Lumbricus* and *Dendrodrilus*, which were not tested (see below), were rejected by the S–H test ($P < 0.0001$) and had p$P < 0.002$ (Table 1). All potential taxon misidentifications (see below) were removed before the tests were carried out. Within the *Aporrectodea*, the *Ap. caliginosa* species complex and the species within (*Ap. caliginosa*, *Ap. tuberculata*, *Ap. longa*, *Ap. nocturna* and *Ap. trapezoides*) did not form monophyletic assemblages. *Octolasion* formed a clade, but *Octolasion lacteum* did not. As in the outgroup, some of these taxa were mutually polyphyletic. These conflicting results are discussed in detail below.

Within the *Ap. caliginosa* species complex, our COI tree (Fig. 1) recovered all the main lineages and relationships described in Pérez-Losada et al. [1] for this group using multiple mtDNA genes (3567 bp) different from COI and the 28S rDNA gene (809 bp). Clade support, however, was lower in our COI tree because of the fewer synapomorphies in the COI dataset.

Table 1
Shimodaira and Hasegawa [51] test results and Bayesian posterior probability estimates (p*P*) for monophyletic hypotheses of earthworm genera and species. Δ = difference −ln *L*. *n* = No of monophyletic trees.

	S–H test		BMCMC	
Taxon	Δ	*P*	*n*	p*P*
Lumbricidae				
Allolobophora	10.2	<0.0001	41	0.0014
Aporrectodea	12.7	<0.0001	0	<0.0001
Dendrobaena	17.0	<0.0001	0	<0.0001
Eisenia	30.0	<0.0001	0	<0.0001
Megascolecidae				
Diplocardia	77.7	<0.0001	0	<0.0001
Metaphire	138.7	<0.0001	0	<0.0001
Amynthas	340.1	<0.0001	0	<0.0001

Uncorrected p-genetic distances among congeneric species ranged from 9.8% (*Aporrectodea*) to 25.4% (*Dendrobaena*), while conspecific distances in putative species ranged from 0.1% (e.g., *Ap. nocturna*) to 16.5% (*Latirus castaneus*) (Table 2). Earthworm congeneric divergences reported here were similar to those reported in other barcode studies [25–27], but conspecific divergences in several taxa forming non-monophyletic (*Ap. caliginosa*, *Ap. trapezoides* and *Lumbricus terrestris*) and monophyletic (*A. chlorotica*, *Allolobophoridella eiseni*, *Aporrectodea rosea*, *Eisenia fetida*, *L. castaneus* and *Lumbricus rubellus*) assemblages were higher than the value reported for conspecific taxa (10%) [25]. This supports the existence of cryptic species in those lineages as suggested before for some of these and other earthworm taxa [1,25–27,37,42].

4. Discussion

Five Lumbricidae and three Megascolecidae genera formed non-monophyletic assemblages in our phylogenetic analyses. Some of those genera are likely the result of the inadequacy of the extant earthworm taxonomic classification, but others seem more likely to be taxonomic misidentifications or barcode mislabels (both will be considered misidentifications here). We will rely on phylogenetic cross-comparisons of barcodes from the same species or genus to distinguish between those two cases. Considering the nature of the analyzed data (COI barcodes exclusively), other factors that may also cause discrepancies between gene and species trees (sampling and systematic error) will not be considered here. Below we will evaluate each taxon in detail.

Table 2
Congeneric and conspecific uncorrected p-genetic distances. Clusters of p-distance=0 are considered as one single terminal.

Taxon	Interspecies[a]	Intraspecies[b]
Outgroup		
Amynthas	19	
Eudrilus eugeniae		7.0
Hormogaster elisae		8.4
Metaphire	17.2–19.6	
Perionyx	15.6	
Ingroup		
Allolobophora	17.6–23.0	
A. chlorotica		1.6–16.4
Allolobophoridella eiseni		0.9–11.0
Aporrectodea	9.8–19.7	
Ap. caliginosa	12.4–17.1	0.6–3.1
Ap. icterica		1.2
Ap. limicola		0.2
Ap. longa		0.3–9.7
Ap. molleri		2.0
Ap. nocturna		0.1–6.3
Ap. rosea	19.4	1.4–15.6
Ap. trapezoides	13.8–15.8	0.4–7.0
Ap. tuberculata		2.0–2.5
Eisenia	22.1–23.3	
E. andrei		1.2–1.6
E. eiseni		0.7–11.8
E. fetida		0.7–11.8
Dendrobaena	18.2–25.4	
D. octaedra		0.1–7.3
D. veneta		0.2–0.9
Dendrodrilus rubidus		1.0–3.9
Lumbricus	15.0–20.6	
L. castaneus		1.9–16.5
L. festivus		1.1–2.6
L. friendi		0.3
L. rubellus		0.9–13.9
L. terrestris	16.6	0.2–6.1
Octolasion	18.4	

[a] Species forming non-monophyletic clades are also considered.
[b] Only species forming monophyletic clades are considered.

Aporrectodea did not form a monophylum in any of our trees but three separate clusters with variable nodal support (Fig. 1). All alternative phylogenetic hypotheses grouping these *Aporrectodea* clusters in clades were rejected by our ML topological test ($P < 0.0001$) and showed p$P < 0.001$. Our comprehensive molecular analysis, therefore, extends previous results [2,22,25–27] and confirms that *Aporrectodea* is not a valid genus as currently stated. New phylogenetic analyses including more *Aporrectodea* species may reveal even more subdivisions within the group [e.g., 22]. It is clear that the genus *Aporrectodea* needs extensive taxonomic revision [17,24]. Future systematic work aiming to classify *Aporrectodea* or list the species within should rely on estimated phylogenetic relationships such as those presented here.

Aporrectodea cluster 1 (Fig. 1) was not monophyletic and included *Aporrectodea limicola* and a well supported subclade of *Aporrectodea molleri* and *Aporrectodea icterica*, which was sister to *A. chlorotica* specimens. *Aporrectodea* sp1 in Pérez-Losada et al. [1] did not cluster with the other *Aporrectodea* species but with *Scherotheca gigas*. This material is currently under revision and since its taxonomic affinities have not been established yet we will not discuss it here. *Ap. molleri* in Briones et al. [2] and *Ap. molleri* here are synonyms. The former was placed first in the genus *Eophila* by Easton [53], considered valid by Blakemore [24] and then moved to the genus *Aporrectodea* in the web project Fauna Europea [54].

Aporrectodea cluster 2 was also not monophyletic and included *Ap. rosea* and two *Ap. caliginosa* specimens from Genbank, which clustered with two specimens of *Bimastos parvus* and *Eisenia lucens*. DQ09288 *Ap. caliginosa* [55] and EF077606 *Ap. caliginosa* [27] were likely misidentified since all the other *Ap. caliginosa* in our analysis clustered together and fell in a very different position in the tree. Ten species identifications in Huang et al. [27] needed reconsideration based on a more extensive analysis of COI data by Chang et al. [25]. We now add one more species to that list. We cannot determine if GU013832 and GU013833 *Ap. rosea* represent a new species or if EF077604 *B. parvus* [27] and FJ214225 *E. lucens* are also misidentifications.

Aporrectodea cluster 3 was monophyletic and well supported and included the *Ap. caliginosa* species complex (*Ap. caliginosa*, *Ap. trapezoides*, *Ap. longa*, *Ap. tuberculata* and *Ap. nocturna*), plus *Aporrectodea handlirschi* and *Ap. jassyensis* and GU206176 *L. rubellus*, which fell in a subclade of *Ap. longa* specimens. *L. rubellus* is most likely a misidentification since all the other barcodes from the same species formed a well supported clade (see below). DQ092892 *Aporrectodea jassyensis* and DQ092890 and DQ092891 *Ap. handlirschi* are also likely misidentified considering the short branches connecting these three specimens to *Ap. caliginosa* and the fact that the other two *Ap. handlirschi* specimens in the tree are very different from any other *Aporrectodea* taxa. As for the species within the *Ap. caliginosa* complex, GU013824 *Ap. caliginosa* may represent a new species or a misidentification, BMP *Ap. caliginosa* was likely mistaken for *Ap. tuberculata*, FN658813 *Ap. longa* is likely *Ap. trapezoides*, and GU013826–GU013829 *Ap. longa* are likely *Ap. nocturna*.

Our *Ap. caliginosa* species complex COI tree recovered the same lineages and basic relationships previously described in Pérez-Losada et al. [1] using multiple mtDNA and nDNA gene regions (4376 bp) (Fig. 1). As in *Eisenia* [37] and Hormogastridae [44], these results indicate that COI barcodes are a good proxy for assessing intra and interspecies diversity and making evolutionary inferences in earthworms.

Dendrobaena spp. did not form a monophyletic clade either (Fig. 1). Several inconsistencies seem to occur here. The two *Dendrodrilus attemsi* specimens were depicted in two different places in the COI tree: FJ214224 *D. attemsi* clustered with *Dendrodrilus octaedra* specimens in a well supported clade, while BMP *D. attemsi* clustered with *Dendrodrilus rubidus* specimens in another well supported clade. This suggests BMP *D. attemsi* is a misidentification. Similarly, the well supported clade and short branches connecting *Dendrodrilus veneta*, *Eudrilus veneta* and *Eudrilus zebra* suggests that they are the same species. This partially supports the French classification of these two genera [13], which considers *E. veneta* as part of the *Dendrobaena* group [24]. *E. veneta* was described as *D. veneta veneta* and *D. veneta zebra* is synonymous. Other three Lumbricidae (*Satchellius*, *Prosellodrilus* and *Allolobophora*), one Megascolecidae (*Microscolex*) and one Glossoscoleidae (*Pontoscolex*) genera clustered with *Dendrobaena* species. Except for *Allolobophora* no other species from these five genera has been included in the analysis, so cross-comparisons could not be performed to assess their taxonomic status; nonetheless, all the other Megascolecidae and Glossoscoleidae genera as well as all the other outgoup taxa included in our analysis formed a separated clade, which may indicate that *Microscolex* and *Pontoscolex* have been misidentified. As for *Allolobophora*, this genus has been already described as polyphyletic and its taxonomic validity questioned [22]. Assuming *Satchelius*, *Prosellodrilus*, *Allolobophora* and *Eisenia hortensis* have been correctly identified, our trees (Fig. 1) and topological tests (Table 1) suggest that, in agreement with previous phylogenetic analyses [2,28], *Dendrobaena* is not a valid genus and needs extensive revision [17].

Eisenia species were scattered throughout the tree (Fig. 1). We discussed already *E. veneta* and *E. zebra*. Considering that the other five *Eisenia* species are clustered in four different clades (three well supported) separated from other lumbricids and that the topological tests are highly significant (Table 1), we must assume that *Eisenia* is not a valid taxon (see also *Lumbricus* discussion below). EF156635 and FJ214228 *E. fetida* are likely *Eisenia andrei*. This confirms previous results by Briones et al. [2] and indicates that this genus needs to be urgently revised as suggested before [17].

Lumbricus formed a well supported monophylum except for BMP *L. eiseni* and GU206174 *L. rubellus* (GU206176 *L. rubellus* was discussed already), which were clustered with *Eudrilus eiseni*. Both *L. eiseni* and *E. eiseni* are now considered *A. eiseni*. Considering that all the other *L. rubellus* [except DQ092904 *L. rubellus* [55], which has been also likely misidentified] also formed a well supported clade, we must conclude that GU206174 *L. rubellus* is also *A. eiseni*. DQ092905 *L. castaneus* [55] seems to have been also misidentified. Morphologically, *Lumbricus* is considered the most homogeneous Lumbricidae genus [17,56], and this and other previous molecular phylogenetic analyses [2] seem to confirm that view.

Octolasion formed a well supported clade, however non-monophyletic assemblages, also well supported, were observed among two of the species within. BMP *O. lacteum* clustered very closely with BMP and GU014232 *Octolasion cyaneum* and FJ214236 *O. lacteum* clustered with BMP *Octolasion tyrtaeum* instead of doing it with the other two *O. lacteum* in the clade. Hence we think that both BMP [2] and FJ214236 [57] *O. lacteum* are misidentifications.

Within the Megascolecidae, *Metaphire* (three species), *Amynthas* (six species) and *Diplocardia* (three species), did not form monophyletic assemblages (Fig. 1). Inconsistencies in the Megascolecidae COI barcodes [27] have been reported before [25] but they were all intraspecific. Our trees hence suggest that these three genera are not valid and need further revision. Chang and Chen [40] and Chang et al. [39] have recently revised the former two genera and Leontieva et al. [58] have studied *Diplocardia*. All of them have suggested already taxonomic rearrangements based on molecular phylogenetic analyses. Considering the reciprocal polyphyly of these three groups as presented here, more extensive work is needed within the Megascolecidae to assess the taxonomic status of these three genera altogether.

The reliability of the taxonomic information attached to the records in Genbank has been questioned already [59–62]. Recently, several inconsistencies and doubtful identifications in

Megascolecidae COI barcodes have been detected [25]. Our results revealed new misidentifications and confirmed several potential taxonomic inconsistencies within the Lumbricidae and Megascolecidae. Ideally these results should be confirmed with more markers and specimens so potential sampling and systematic error could be evaluated. Nevertheless, considering the effectiveness of COI barcoding for identifying earthworm species [25,32,40,58] and assessing intrageneric relationships [22, 32, 37 – 39; this study], our trees strongly suggest a careful re-examination of the involved specimens or the quality of the generated sequences (e.g., nuclear copies of COI barcodes [63]). Despite those potential misidentifications, our results put into question the current taxonomic status of several Lumbricidae and Megascolecidae genera and species and suggest an extensive taxonomic revision of the current earthworm classification.

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