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An Updated Multilocus Phylogeny of the Lumbricidae (Annelida: Clitellata: Oligochaeta) Earthworms

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Abstract

Lumbricidae earthworms dominate agricultural lands and often natural terrestrial ecosystems in temperate regions in Europe. They impact soil properties and nutrient cycling, shaping plant community composition and aboveground food webs. The simplicity of the earthworm body plan has hampered morphology-based classifications and taxonomy; hence current research on Lumbricidae systematic relies mostly on molecular data from multiple or single locus [e.g., cytochrome oxidase subunit I (COI) barcodes] to infer evolutionary relationships, validate taxonomic groups and/or identify species. Here we use multiple nuclear and mitochondrial gene regions (including COI) to generate updated maximum likelihood and Bayesian phylogenies of the family Lumbricidae. We then compare these trees to new COI trees to assess the performance of COI at inferring lumbricid inter-generic relationships.

Keywords: Barcode; COI; Earthworms; Lumbricidae; Taxonomy

Introduction

Lumbricidae earthworms account for 90% of the invertebrate biomass in temperate soils [1]. They exert a large impact on soil physical, chemical and biological properties and play a major role in animal food webs and plant composition [2]. Earthworms are model organisms in ecology, toxicology, physiology and reproductive biology, and generate great economic revenue and environmental benefits in vermiculture and vermicomposting [1,3,4].

Lumbricids comprises ~300 known species, however, its taxonomy and classification has been hindered by the structural simplicity of the earthworm body plan [5,6]. As a result, no agreement exists between classifications and taxonomists about how the family should be subdivided. Only relatively recently earthworm specialists have begun to use molecular information and phylogenetic tools to validate and update Lumbricidae systematics. Phylogenetic analysis of DNA sequences has already proven useful to solve taxonomic questions within several lumbricid genera (e.g., *Eisenia* [7], *Aporrectodea* [5,8] and *Postandrilus* [9]).

Similarly, a few studies have also looked at the evolution of the family using one locus, two to three loci [6,11,12], or many more loci (13 loci) and extensive sampling [13]. These and other studies have unanimously revealed that all the proposed Lumbricidae classifications and taxonomy do not hold and do not reflect evolutionary relationships, and that a robust and exhaustive phylogeny of the family is needed. Here we propose to expand our previous Lumbricidae dataset by adding a new genetic marker, the cytochrome oxidase subunit I (COI), for a total of 75 new earthworms. The COI gene has become the standard marker for DNA barcoding (i.e., molecular taxonomic identification) of most metazoans [14-16] including earthworms (e.g., Earthworm Barcode of Life; *www.earthwormbol.org* [17-19]. COI data couple with phylogenetic approaches have aided species identification [15,17,19] and have provided valuable insights on the taxonomy and evolutionary relationships [7,15,20-23], phylogeography [20,21,24], and speciation [25,26] of earthworms. Additionally, given the popularity of this marker and large size of the COI databases (constantly expanding through barcoding initiatives), COI has often been used alone to estimate earthworm phylogenies [20,21,26]. When compared with multilocus phylogenies, COI trees have proved to be a good proxy for estimating intrageneric [7,10,12,] and sometimes intergeneric [10,12,26] evolutionary relationships in earthworms; however its potential for estimating intergeneric relationship across the Lumbricidae remains to be tested.

In the present study we update the genus-level multilocus phylogeny of the family Lumbricidae in Domínguez et al. [13] by adding the standard barcode region (COI). Then we test if this gene region is adequate for estimating intergeneric evolutionary relationships in the Lumbricidae and to assess discrepancies between gene(s) trees and Lumbricidae taxonomy.

Material and Methods

Sampling

Sampling of the family Lumbricidae for this study included 75 species representing 19 genera Figure 1. Earthworms were collected in Spain, Andorra, France, Italy, Germany, United Kingdom, Finland, Denmark, Poland, Romania, Hungary, Serbia, Israel, Austria, Turkey, South Africa, USA, Brazil, China and Vietnam. To root the Lumbricidae tree we used three representatives of the Criodrilidae and Hormogastridae. Specimens were identified as indicated in Domínguez et al. [13] and preserved in absolute ethanol and stored at -20°C. DNA extraction, amplification and sequencing.

Total genomic DNA was extracted using the DNAeasy Tissue kit (Qiagen). The mtDNA gene region corresponding to the cytochrome oxidase subunit I (COI) was amplified using primers in [27] and conditions in [5]. PCR products were purified using a MultiScreenμ96 (Millipore) PCR kit and sequenced bidirectional using an Applied Biosystems (ABI) 377XL automated sequencer. The ABI Big-dye

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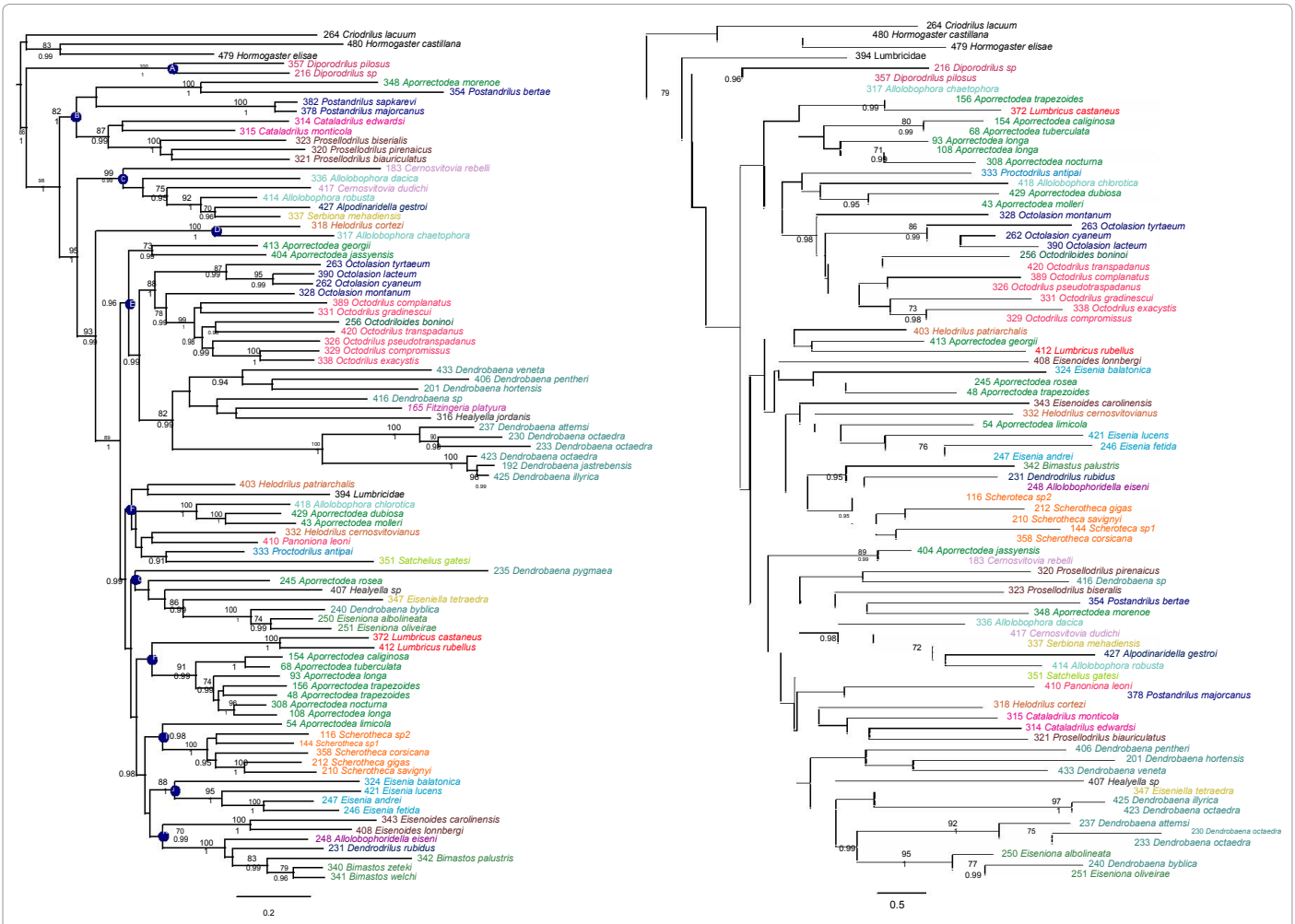

Figure 1: Multilocus (left) and COI (right) Lumbricidae maximum likelihood trees. Bootstrap proportions (if ≥ 70%) and Bayesian posterior probabilities (if ≥ 95%) are shown above and below the branches, respectively. Genera are colored with different colors.

Ready- Reaction kit was used following the standard cycle sequencing protocol, but with a 16th of the suggested reaction size.

Phylogenetic analysis

New 75 COI sequences were combined with nucleotide sequences from nuclear (28S rDNA and 18S rDNA) and mitochondrial [16S rDNA, 12S rDNA, NADH dehydrogenase (ND1), cytochrome oxidase subunit II (COII) and tRNAs Asn, Asp, Val, Leu, Ala, Ser, and Leu)] genes in [13], rendering a final dataset comprised of 85 species and 30 genera. All gene regions were aligned in MAFFT v6 [28,29] under the global (G-INS-i) algorithm and default settings. JModelTest v1.0.1 [30] was used to select the appropriate model of evolution for each DNA partition under the Akaike Information Criterion AIC [31]. The general time reversible model of evolution [32], with proportion of invariable sites and gamma distribution, was selected for each partition.

Both maximum likelihood (ML) and Bayesian methods of phylogenetic inference were applied to both the COI dataset alone and all the genes combined (ALLgenes). ML analysis was performed in GARLI under default settings for the genetic algorithm, except that searchreps = 10. Clade support was assessed using the non-parametric bootstrap procedure [33] with 1,000 bootstrap replicates. Bayesian analysis coupled with Markov chain Monte Carlo (BMCMC) inference was performed in MrBayes v3.1.2 [34]. Four independent BMCMC analyses were run in the CIPRES Science Gateway portal [35], each consisting of four chains. Each Markov chain was started from a random tree and run for 2x107 cycles, with sampling every 1,000th generation. Sequence evolution model parameters were estimated independently for each data partition starting as unknown variables with uniform default priors. Convergence and mixing were monitored using Tracer v1.5 [36]. All sample points prior to reaching stationary levels were discarded as burn-in. The posterior probabilities for individual clades obtained from 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 was tested under both likelihood and Bayesian frameworks. Likelihood topological tests were conducted using the Shimodaira and Hasegawa (S-H) [37] test as implemented in RAxML v7.2.0 [38]. Bayesian topological tests were performed as described in Huelsenbeck et al. [39].

Results and Discussion

ML and Bayesian trees Figure 1 for COI and ALLgenes showed minor topological differences between phylogenies of the same dataset, but no inconsistency (i.e., highly supported clades composed

of different taxa) was detected between them. However, COI and ALLgenes ML and Bayesian trees were significantly different between datasets (P<0.001 for the S-H test; P<0.001), showing major differences in their topologies with some clades being inconsistent between the two datasets (e.g., 404 *Aporrectodea jasseyensis* and 183 *Cernosvitovia rebelli;* 156 *Aporrectodea trapezoids* and 372 *Lumbricus castaneus;* 248 *Allolobophoridella eiseni,* 342 *Bimastus palustris and* 231 *Dendrodrilus rubidus*). These differences highlight the low power (i.e., phylogenetic signal) of COI to solve intergeneric relationships in the Lumbricidae (as suggested in [10]), unless combined with other genes [12]. In the ALLgenes trees, the 28 analyzed Lumbricidae genera were distributed in 11 clades (A to K in Figure 1) as in Domínguez et al. [13] and support for some clades was increased in the new tree.

No major differences were observed between both studies for the backbone of the Lumbricidae tree, although some minor differences including the position of 336 *Allolobophora dacica*, 245 *Aporrectodea rosea* and 407 *Healyella sp*., and relationships among a few taxa in clades B, E, F, H and I were observed. No inconsistent topological differences were observed among trees. The monophyly of Lumbricidae genera represented species was confirmed for *Bimastos, Diporodrilus, Eiseniona,Eisenia, Eisenoides, Lumbricus, Prosellodrilus, Scherotheca and Cataladrilus* (this analysis); *while Allolobophora, Aporrectodea, Cernosvitovia, Dendrobaena, Healyella, Helodrilus, Octodrilus, Octolasion, and Postandrilus* were still para or polyphyletic as currently defined, in agreement with assemblages in Domínguez et al. [13] and Pérez- Losada et al. [10]. Within the non-monophyletic taxa, some genera were scattered throughout the phylogeny falling in different singular or polyspecies clades (*Allolobophora, Aporrectodea, Healyella and Helodrilus*), while others were more closely clustered, although intermixed with species from other genera (*Cernosvitovia, Dendrobaena, Octodrilus, Octolasion and Postandrilus*). Systematically, these topological rearrangements suggest that the more dispersed genera may have to be split into new genera, while the less dispersed genera may only require to be redefined to accommodate new species.

The ALLgenes trees also confirmed that *Dendrobaena octaedra* may constitute a species complex. Overall, our new analyses reinforce the statement made by others [10-13] of that the Lumbricidae taxonomy needs extensive revision, and that phylogenetic relatedness must be used to delineate the next Lumbricidae classification.

Conclusions

This study presents a well-supported phylogeny of the family Lumbricidae and updates previous phylogenetic analyses in Domínguez et al. [13]. Our comparison of multilocus and COI trees highlights the low phylogenetic signal of COI to infer lumbricid intergeneric relationships, unless combined with other genes. If used alone, this gene should only be used for species identification (barcoding) and assessing evolutionary relationships below the genus level. Our results also confirm that the Lumbricidae taxonomy needs extensive revision.

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