Multigene phylogeny reveals two new isolated and relic earthworm genera (Oligochaeta: Lumbricidae)

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Phylogenetic analysis has revealed many potential new earthworm species and has led to changes in current taxonomic classifications. Here, we propose the addition of two new earthworm genera, *Galiciandrilus* gen. nov. and *Compostelandrilus* gen. nov., to the family Lumbricidae based on morphological evidence and phylogenetic analysis of nuclear 28S rRNA and mitochondrial 16S rRNA, 12S rRNA, *ND1*, *COII* and tRNA gene regions (4680 bp). *Galiciandrilus* gen. nov. comprises the species originally described as *Cernosvitovia bertae* and *Allolobophora morenoe* (later renamed *Postandrilus bertae* and *Aporrectodea morenoe*, respectively). *Compostelandrilus* gen. nov. includes two new earthworm species, *Compostelandrilus bercianus* sp. nov. and *Compostelandrilus* men*ciae* sp. nov., which are restricted to a small area of northwestern Spain. Divergence times (14.9–26.5 Mya) and branch lengths (0.7–1.45) in the species included in the Iberian clade are higher than those usually observed in most lumbricid monophyletic genera. The monophyly of these two phylogenetically distinct earthworm genera is not corroborated by any of the external or internal morphological synapomorphies commonly used in earthworm alpha taxonomy. This confirms the anatomical plasticity of the group and the need for extensive revision of the Lumbricidae taxonomy. Given the restricted geographical distribution of the earthworm species under study (< 50 km²) and their basal phylogenetic position in the Lumbricidae tree, future conservation efforts should be initiated to preserve this unique biological diversity.

ADDITIONAL KEYWORDS: Bayesian divergence time analysis – *Compostelandrilus* – earthworms – *Galiciandrilus* – Lumbricidae – molecular phylogeny – Oligochaeta.

INTRODUCTION

Earthworms constitute the largest animal biomass in most temperate terrestrial ecosystems (Lavelle & Spain, 2001). They are considered the most important soil organisms because they exert significant effects on physical, chemical and biological properties of soil and also play a key role in modifying soil structure and accelerating organic matter decomposition and nutrient turnover (Lee, 1985; Edwards & Bohlen, 1996). The Lumbricidae (Rafinesque-Schmaltz, 1815), the dominant earthworm family in the Holarctic region, is a monophyletic group included in the Crassiclitellata (James & Davidson, 2012). Lumbricidae includes ~42 genera and ~670 species (Blakemore, 2008), although current taxonomic classifications need to be revised on the basis of recent molecular phylogenetic analyses (Pop, Wink & Pop, 2003; Pop *et al.*, 2005; King *et al.*,

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2008; Briones, Morán & Posada, 2009; Pérez-Losada *et al.*, 2009, 2011, 2015; Klarica *et al.*, 2012; Domínguez *et al.*, 2015).

In 1997, the prominent earthworm taxonomist Marcel B. Bouché collected lumbricid earthworms from eight locations on the Island of Majorca (Balearic Islands, Spain). Examination of the material led to the discovery of five new earthworm species with unusual morphology regarding the posterior position of the male pore. The species were included in a new genus named Postandrilus Qiu & Bouché, 1998 (Qiu & Bouché, 1998a). Although in most lumbricids the male pore is on segment 15, in *Postandrilus* spp. the male pore is situated on any of the segments between 31 and 44. In the same publication (op. cit.), the authors also included the species Postandrilus bertae (Díaz Cosín, Mato & Mascato, 1985b) in the same genus. In the original description, this earthworm species from Galicia (NW Spain) was tentatively assigned to the eastern European genus Cernosvitovia (Díaz Cosín et al., 1985).

We have previously used a phylogenetic approach to examine the taxonomic status of Postandrilus species and their evolutionary relationships (Pérez-Losada et al., 2011; Domínguez et al., 2015). In those studies, Postandrilus was depicted as basal within the Lumbricidae, and not monophyletic, and therefore invalid as currently defined. We also found *Postandrilus* highly diverse (including multiple cryptic species in Majorca) and of vicariant origin, and we suggested that radiation of the genus began in the Late Oligocene (Pérez-Losada et al., 2011). Phylogenetic analysis of the genus Postandrilus showed that all the Majorcan taxa formed a relatively uniform and wellsupported clade clustered with another very genetically different and also well-supported clade including the species *P. bertae* and *Aporrectodea morenoe* (Díaz Cosín et al., 1985). Although A. morenoe was previously described by Díaz Cosín, Calvin & Mato (1985a), the authors were not certain about its taxonomic status and they concluded that it was difficult to assign A. morenoe to any existing earthworm genus.

In three field surveys carried out in spring 2011 and 2014 in El Bierzo, NW Spain, we unearthed some lumbricids whose morphological appearance, habitat and ecological characteristics led us to believe that they may be *Postandrilus* relatives. Moreover, in a similar nearby location, we also collected several specimens of *Zophoscolex cyaneus* (Briones & Díaz Cosín, 1993), which also shares morphological similarities with *Postandrilus* (Pérez-Losada *et al.*, 2011). In the present study, we examine the morphology of the above-mentioned earthworm species and develop a robust phylogeny of the genus *Postandrilus* and its relatives by considering multiple gene regions. Then, we use that phylogeny to validate the taxonomic status of the new specimens and time their radiation. We also present descriptions of two new lumbricid species, *Compostelandrilus menciae* sp. nov. and *Compostelandrilus bercianus* sp. nov., and demonstrate that they comprise a distinct evolutionary lineage that deserves recognition as a new Lumbricidae genus (i.e. *Compostelandrilus*). We also propose a second new genus, *Galiciandrilus*, comprising the species originally described as *Cernosvitovia bertae* and *Allolobophora morenoe*.

MATERIAL AND METHODS

SAMPLING, EXAMINATION AND DESCRIPTIONS

Specimens of the earthworm species described in this paper were collected in sampling surveys carried out in northwestern Spain in April–May 2011, March– April 2013 and November 2014. Sampling locations were established by GPS (Garmin eTrex Legend). The earthworms were rinsed in water, anaesthetized in diluted ethanol, fixed in a mixture (1:1 v/v) of 90° ethanol and formaldehyde (4%) and placed in glass tubes containing 4% formaldehyde. Specimens and tissues (caudal portions) collected for DNA extraction were preserved in absolute ethanol and stored at -20 °C.

Species descriptions are based on external and internal examination. Internal morphological examination was conducted by dorsal dissection under a stereomicroscope (Nikon SMZ1500). Descriptions of body colour are based on living specimens, whereas body dimensions refer to fixed material.

SAMPLING POINTS

Fuente del Ciervo, Cabañas Raras (León, Castile and León, Spain). Location 1: 42°38′52″N, 6°36′35.3″W. Location 2: 42°38′34.6″N, 6°36′26.3″W.

Embalse de Bárcena, Cubillos del Sil (León, Castile and León, Spain). Location 1: 42°35.6′54″N, 6°34′36.68″W. Location 2: 42°34.59′80″N, 6°34′31.30″W.

Twenty-eight individuals of the species Z. cyaneus were collected in the surroundings of the monastery of San Miguel de Escalada, Gradefes (León, Castile and León, Spain). Holm oak (Quercus ilex) woodland interspersed with pasture. Plant species present: Cistus ladanifer, Cistus salvifolius, Cytisus striatus, Cytisus scoparius, Genista falcata, Erica australis, Anagallis monelli, Convolvulus lineatus, Ophrys lutea and Torilis nodosa. Mean precipitation, 590 mm. A sandy clay soil. Altitude 812 m.

Sampling and collection data of *Postandrilus* earthworms from the island of Majorca and on the specimens of *P. bertae* and *A. morenoe* from Galicia are described in a previous study (Pérez-Losada *et al.*, 2011).

DNA ISOLATION AND SEQUENCING

Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). Recent phylogenetic analyses (Pérez-Losada et al., 2009, 2011, 2015; Domínguez et al., 2015) have demonstrated the importance of using multiple genes for inferring evolutionary relationships in earthworms. Hence, regions of the nuclear 28S rRNA and mitochondrial 16S rRNA, 12S rRNA, NADH dehydrogenase (ND1), cytochrome oxidase subunit II (COII) and tRNA Asn, Asp, Val, Leu, Ala, Ser and Leu genes (4680 bp) were amplified using the polymerase chain reaction (PCR), as previously described (Pérez-Losada et al., 2009, 2011; Domínguez et al., 2015). PCR products were purified using a MultiScreen PCRµ96 (Millipore) kit and sequenced bidirectionally using an Applied Biosystems (ABI) 377XL automated sequencer. The ABI Big-dye Ready-Reaction kit was used following the standard cycle sequencing protocol, but with a 16th of the suggested reaction size. DNA sequences were deposited in GenBank under Accession Numbers KY609037-KY609091.

PHYLOGENETIC ANALYSIS

We first performed a phylogenetic analysis of the collected earthworm taxa and other lumbricids to determine the phylogenetic position of the new species within the Lumbricidae. The Lumbricidae data set used in this first phylogenetic analysis included 85 species and 28 genera (Supporting Information, Appendix S1). Earthworms were collected in Spain, Andorra, UK, France, Italy, Germany, Austria, Finland, Denmark, Poland, Romania, Hungary, Serbia, Israel and Turkey. Almost all currently recognized Lumbricidae genera are represented in the data set, with the exception of five monospecific genera and four small genera (less than ten species) with very restricted distributions. All earthworm specimens were identified following descriptions in Bouché (1972), Mrsic (1991), Qiu & Bouché (1998a, b, c, d), Sims & Gerard (1985), Csuzdi & Zicsi (2003) and Blakemore (2006). To root the Lumbricidae tree, we used earthworms from another two Crassiclitellata families: Criodrilidae (one species) and Hormogastridae (two species) (Supporting Information, Appendix S1).

Given the phylogenetic position of the new taxa and *Postandrilus* in the Lumbricidae tree (Supporting Information, Appendix S1), we performed a second phylogenetic analysis including 20 species: *Postandrilus* 'sensu Qiu & Bouché 1998' (10 species), *A. morenoe*, *Z. cyaneus*, 2 new earthworm species (ingroup), 2 species of *Cataladrilus* (*Cataladrilus edwardsi* Qiu & Bouché, 1998 and *Cataladrilus monticola* Qiu & Bouché, 1998) and 3 species of *Prosellodrilus* [*Prosellodrilus biauriculatus* Bouché, 1972; *Prosellodrilus biserialis* Bouché, 1972; and *Prosellodrilus pyrenaicus* (Cognetti, 1904)] (outgroup). This second data set included 38 individuals of the five Majorcan *Postandrilus* species, eight individuals of *P. bertae*, five individuals of *A. morenoe*, three individuals of *Z. cyaneus*, 10 individuals of the first new species and six individuals of the second new species. All these earthworms were collected on the island of Majorca, in Galicia and in León (Spain) (see Fig. 2).

In both analyses, nucleotide sequences from each gene region (all tRNAs were combined into a single gene region) were aligned in MAFFT v6 (Katoh et al., 2005; Katoh & Toh, 2008) under the global (G-INS-i) algorithm and default settings. Phylogenetic congruence among gene regions (COII: 687 bp, 12S: 352 bp, 16S: 1354 bp, ND1: 924 bp, tRNAs: 424 bp and 28S: 939 bp) was assessed using the protocol developed by Wiens (1998). No areas of strongly supported incongruence between gene trees were observed. The gene regions were combined into four partitions: coding (COII and ND1), rRNA (12S and 16S), tRNAs and 28S. The Lumbricidae data set included an extra partition (18S). JModelTest v1.0.1 (Posada, 2009) was used to select the appropriate models of evolution for each gene partition under the Akaike information criterion (Posada & Buckley, 2004). The general time reversible model of evolution (Tavaré, 1986) was selected for each data partition, with a proportion of invariable sites and gamma distribution (GTR + Γ + I). Maximum likelihood (ML) analysis of the concatenated partitions was performed in RAxML v7.2.0 (Stamatakis, Hoover & Rougemont, 2008) with 1000 searches. Clade support was assessed using the non-parametric bootstrap procedure (Felsenstein, 1985) with 5000 bootstrap replicates. The concatenated partitions were also analysed using Bayesian methods coupled with Markov chain Monte Carlo (BMCMC) inference, as implemented in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). Two independent BMCMC analyses were run in CIPRES Science Gateway portal (Miller, Pfeiffer & Schwartz, 2010), each consisting of four chains. Each Markov chain was started from a random tree and run for 107 cycles, sampling every 1000th generation. Sequence evolution model parameters were unlinked and treated as unknown variables with uniform default priors and were estimated as part of the analysis. Convergence and mixing were monitored using Tracer v1.5 (Rambaut & Drummond, 2009). All sample points prior to the stationary point 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.

Divergence times for the clades of interest were estimated using BEAST v1.8.1 (Drummond & Rambaut, 2007), with the same partitions described above. The GTR + Γ + I model of nucleotide substitution and the relaxed lognormal model of rate of substitution (Drummond *et al.*, 2006) were chosen for all data partitions. A Yule speciation prior was used for the tree prior, as recommended by the authors. As in Pérez-Losada et al. (2011) and Domínguez et al. (2015), the cladogenic event separating the Balearic Islands from the proto-Iberian Peninsula (Late Oligocene; 30–28 Mya) (Alvarez, Cocozza & Wezel, 1974; Rosenbaum, Lister & Duboz, 2002) was used to calibrate the molecular tree. This calibration was integrated into the BEAST analysis as a normal prior (mean = 29 Myr; SD = 3; 95% interval = 24.1–33.9 Myr) to time the split of the Balearic Postandrilus from their Iberian relatives. Two runs each of 2×10^7 generations long were completed and combined using LogCombiner. All output generated by BEAST was analysed using Tracer v1.5 (Rambaut & Drummond, 2009), and a chronogram was depicted in FigTree.

RESULTS

PHYLOGENETIC INFERENCES

To determine the phylogenetic position of the earthworms collected in Fuente del Ciervo and Embalse de Bárcena and their closest relatives we conducted a phylogenetic analysis including a broad range of lumbricids and other earthworms. Individuals sampled in these localities, corresponding to two new earthworm species, fell in a well-supported clade within the Lumbricidae tree, depicted as [Z. cyaneus + (sp. nov. 1 + sp. nov. 2)] + [(A. morenoe + P. bertae) + (Postandrilus majorcanus + Postandrilus sapkarevi)] (Supporting Information, Appendix S1, green colour). This clade was sister related to another well-supported clade including the genera Cataladrilus and Prosellodrilus (Supporting Information, Appendix S1).

The ML and Bayesian phylogenetic analyses of Postandrilus and its relatives showed two strongly supported (bp = 100 and pP = 1.0) Iberian and Majorcan clades (Fig. 1). Formal descriptions are presented later in the paper, but we use the names of new taxa here for clarity of results interpretation. The Iberian clade comprised Galiciandrilus bertae + Galiciandrilus morenoe sister to [Z. cyaneus + (C. bercianus + C. menciae)].All the taxa in this clade were collected in northwestern Spain (Fig. 2). The Majorcan clade included all the other Postandrilus species (P. majorcanus, P. sapkarevi, Postandrilus palmensis, Postandrilus medoakus and Postandrilus lavellei) collected in Majorca. An alternative monophyletic Iberian-Majorcan Postandrilus clade was rejected by the S–H test (P < 0.001). Tree branch lengths in the Iberian clade and the Majorcan Postandrilus clade ranged from 1.16 to 1.30, whereas branch lengths in congeneric and conspecific Majorcan Postandrilus ranged from 0.08 to 0.17 and from 0 to 0.06, respectively; this highlights the genetic differences between peninsular and insular earthworm species.

Our phylogenetic trees suggested a single radiation of Postandrilus in the Balearic Islands (Fig. 1). The estimated divergence time indicated that the split between the Iberian clade and the Majorcan Postandrilus lineages took place 29.9 (27.6–32.1) Mya. The split between the two Iberian clades took place 26.5 (23.6-29.2) Mya. Within the Iberian clade, G. bertae would have diverged from G. morenoe 19.2 (15.3-23.5) Mya; the Iberian Z. cyaneus would have diverged from the two new species 21.7 (18.1–25.0) Mya and the two new species, C. bercianus and C. menciae, would have diverged from each other 14.9 (11.0–18.6) Mva. More recently. the Majorcan Postandrilus would have diversified 6.2 (4.8–7.6) Mya (Fig. 1). The former molecular time estimates overlap (i.e. they are not significantly different) with the geological ages estimated for the separation of the Balearic Islands-Kabylies microplate from the proto-Iberian Peninsula (30-28 Mya) and the duration of the Messinian salinity crisis (5.96–5.33 Mya), respectively (see Pérez-Losada et al., 2011).

Divergence times (14.9–26.5 Mya) and rates (tree length = 0.7-1.45) among the Iberian taxa are higher than the average rates estimated for most monophyletic genera within the family (see Domínguez et al., 2015; Pérez-Losada et al., 2015). Considering that all the Iberian taxa appear near the base of the lumbricid tree (ancient origin; Supporting Information, Appendix S1) and that all are geographically close (area of c. 15000 km²) (Fig. 2), we have adopted an inclusive taxonomic approach to revise the current *Lumbricidae* classification (Fig. 1). Hence, we propose that the four Iberian species should be placed in two new genera: Galiciandrilus (including G. bertae and G. morenoe) and Compostelandrilus (including C. bercianus and C. menciae). Further sampling and phylogenetic analyses of earthworms from this geographic area may suggest new splits, but at this point, we opt for a more conservative approach.

SYSTEMATICS

Phylum Annelida Lamarck, 1802 Class Oligochaeta Grube, 1850/Clitellata Michaelsen, 1919 Order Megadrili benham, 1890/Haplotaxida Michaelsen, 1900

FAMILY LUMBRICIDAE RAFINESQUE-SCHMALTZ, 1815

GENUS GALICIANDRILUS GEN. NOV.

Type species: Cernosvitovia bertae Díaz Cosín, Mato & Mascato, 1985 by original designation in this article.

Diagnosis: Galiciandrilus includes medium-sized unpigmented or flesh colour lumbricids with prolobic or proepilobic prostomium, closely paired setae,



Figure 1. ML molecular tree. Bootstrap proportions (if \geq 70%) and Bayesian posterior probabilities (if \geq 95%) are shown above and below the branches, respectively. Branch lengths are shown proportional to the amount of change along the branches. Divergence time estimates are shown on the nodes marked with clocks. Further information regarding *Postandrilus* from Mallorca can be found in Pérez-Losada *et al.* (2011).



Figure 2. Geographic distribution and phylogenetic relationships between species of the genera *Postandrilus*, *Galiciandrilus* gen. nov. and *Compostelandrilus* gen. nov.

male pores cryptic in 43 (44) or small in segment 15, nephridial pores aligned on line b; spermathecal pores lack or multiple in 14/15–19/20; gizzard in segments 19–21 (22); bifid or trifid typhlosole, calciferous glands in segments 11–13 (16); nephridial bladders bilobulated or digitoid with ampulla; two or three pairs of seminal vesicles in segments (9), 11 and 12 (Table 1). Since the morphological characters are not sufficiently distinct, the genus is also defined based on DNA sequences deposited in GenBank under Accession Numbers JN871938–JN871947, JN871988–JN872000, JN872041–JN872048, JN872084–JN872093, JN872134–JN872136, KJ911934 and KJ911947.

Included species: Galiciandrilus bertae (Díaz Cosín, Mato & Mascato, 1985) comb. nov. and G. morenoe (Díaz Cosín, Calvin & Mato, 1985) comb. nov.

Etymology: Literally, earthworm from Galicia. The name of the genus is derived from Galicia, a region in northwestern Spain.

Remarks: Galiciandrilus is not clearly defined by any external or internal morphological synapomorphies commonly used in earthworm alpha taxonomy. The genus does not include any other known lumbricid species.

GENUS COMPOSTELANDRILUS GEN. NOV.

Type species: Compostelandrilus menciae sp. nov. by original designation herein.

Diagnosis: Compostelandrilus includes mediumsized pale or bluish grey lumbricids with epilobic, proepilobic prostomium, closely paired setae, male pores cryptic inside the clitellum in segment 32 or 40, nephridial pores aligned on line b or 1/3 bc; spermathecal pores multiple in 12/13-22/23 d or (17/18)18/19-23/24 cd; gizzard in segments 24-27 or (25)26-29(30); inverted T- or U-shaped typhlosole, calciferous glands large in segments (13)14-17; nephridial bladders closed U in 8, wide open V in 40(41); three pairs of seminal vesicles in 10, 11 and 12, with the last pair very large, lobulated and extended to segment 17(18) (Table 1, Figs 5, 6). Since the morphological characters are not sufficiently differential, the genus is also defined on basis on DNA sequences deposited in GenBank under Accession Numbers KY609037-KY609051, KY609055-KY609069 and KY609073-KY609088.

Included species: Compostelandrilus menciae sp. nov. and *C. bercianus* sp. nov.

Etymology: Literally, earthworm from Compostela. The name of the genus refers to the city of Santiago de Compostela and to the 'Camino de Santiago', where these earthworms were found.

Remarks: Compostelandrilus is not clearly defined by any of the external or internal morphological synapomorphies commonly used in earthworm alpha taxonomy. The genus does not include any other known

Genus species	Postandrilus palmensis	Postandrilus lavellei	Postandrilus medoakus
Length	120-200	150–175	420
Number of segments	317-363	296–398	647-737
Weight (g)	1.8-4.4	3.3–3.7	4.6-9.8
Pigmentation	Lack	Lack	Lack
Prostomium	Prolobic	Prolobic	Prolobic
Setae	Closely paired	Closely paired	Closely paired
Setal arrangement	36:3:12:2:54	36:1:6:1:30	41:1.6:8:1:41
Papillae	<i>ab</i> 11–14	<i>ab</i> 11–15(16)	<i>ab</i> 13–41
First dorsal pore	9/10	9/10	9/10
Nephridial pores	Aligned, b	Aligned, b	Aligned, b
Male pores	1/2 31, 3/5 b (cryptic)	1/2 31, 3/5 b (cryptic)	1/2 44, 1/2 b (cryptic)
Female pores	1/2 14, b	1/2 14, b	1/2 14, b
Spermathecal pores	9/10, 10/11 c	9/10, 10/11 c	9/10, 10/11 c
Clitellum	24–38 (saddle)	25(26)-(41)42 (saddle)	28–57 (saddle)
Tubercula pubertatis	30–35(36) band	31–36(37, 38) band	42–49 band
Oesophageal hearts	6–11	6–11	7–11
Morren's glands	(12)13–14, weak	12–15, weak	12–15, weak
Crop	15-16	16–17	18–20
Gizzard	17-20	18-22	21-24
Typhlosole	Bifid	Bifid, trifid	Bifid, trifid
Nephridial bladders	Digitoid with ampulla	Digitoid with ampulla	Digitoid with ampulla
Seminal vesicles	11, 12(15)	11, 12(14)	12
Spermathecae	10, 11	10, 11	10, 11
Testes	10, 11	10, 11	10, 11
Seminal funnels	10, 11	10, 11	10, 11
Ovaries	13	13	13
Female funnels	13	13	13
Ovisacs	14	14	14
Genus species	Postandrilus majorcanus	Postandrilus sapkarevi	Galiciandrilus bertae comb. nov.
Length	420	250-350	160–260
Number of segments	869	502-518	426-515
Weight (g)	10-15	3.5 - 5.7	4.5-8.4
Pigmentation	Lack	Lack	Lack
Prostomium	Prolobic	Prolobic	Prolobic
Setae	Closely paired	Closely paired	Closely paired
Setal arrangement	35:1:5:1:25	20:1:4:1:24	34:1.2:8:1:38
Papillae	ab 12–20, 31–39	<i>ab</i> 11–16	<i>ab</i> 11–20
First dorsal pore	10/11	9/10	(9/10) 10/11
Nephridial pores	Aligned, b	Aligned, b	Aligned, b
Male pores	1/3 44, 1/2 b (cryptic)	1/2 43, 3/5 b (cryptic)	43 (44) (cryptic) 1/2
Female pores	1/2 14, <i>b</i>	1/2 14, b	14, <i>b</i>
Spermathecal pores	9/10, 10/11 c	9/10, 10/11 c	14/15–19/20 c
Clitellum	30–63 (saddle)	28–54 (saddle)	(25)27 - 49(50)
Tubercula pubertatis	(43)45–53(54) band	(41)42-49(50) band	(33)36–45(48)
Oesophageal hearts	6–11	6–11	6–11
Morren's glands	13–15, weak	14–16, weak	11–16, weak
Crop	16–20	17–19	17–18
Gizzard	21-25	20-25	19–22
Typhlosole	Bifid, trifid	Bifid, trifid	Bifid, trifid
Nephridial bladders	Digitoid with ampulla	Digitoid with ampulla	Digitoid with ampulla
Seminal vesicles	12	12	11,12

Table 1. Morphological character matrix of the earthworm species under study

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Table 1. Continued

Genus species	Postandrilus majorcanus	Postandrilus sapkarevi	Galiciandrilus bertae comb. nov.
Spermathecae	10, 11	10, 11	(14)15–19(20)
Testes	10, 11	10, 11	10, 11
Seminal funnels	10, 11	10, 11	10, 11
Ovaries	13	13	13
Female funnels	13	13	13
Ovisacs	14	14	14
Genus species	Galiciandrilus morenoe comb. nov.	Zophoscolex cyaneus	
Length	130–185	120–190	
Number of segments	155–354	270-330	
Weight (g)	2.5-5.8	1.4–1.9	
Pigmentation	Flesh colour (clit. brown)	Bluish grey	
Prostomium	Proepilobic	Epilobic (1/3)	
Setae	Closely paired	Closely paired	
Setal arrangement	32:1:10:1:36	76:1.5:21:1:103	
Papillae	<i>ab</i> 11–16, 29–40	ab 10–15, 32–33, 44–46	
First dorsal pore	(8/9) 9/10 10/11	3/4	
Nephridial pores	Aligned, b	nd	
Male pores	15 (small)	15 (small)	
Female pores	14, <i>b</i>	14, b	
Spermathecal pores	Lack	11/12, 12/13 cd	
Clitellum	(22)23-(44)45(46)	(24)25 - (46)48(50)	
Tubercula pubertatis	(32)33–35	(35)36–42(43) line	
Oesophageal hearts	6–11	7–11	
Morren's glands	11–13, large	11 - 15	
Crop	16–18	16-17(18)	
Gizzard	19–21	19-22(23)(24)	
Typhlosole	Bifid	Inverted T	
Nephridial bladders	Bilobulated	Biscuit shaped	
Seminal vesicles	9–12	11 small, 12 large	
Spermathecae	Lack	12, 13	
Testes	10, 11	10, 11	
Seminal funnels	10, 11	10, 11	
Ovaries	13	13	
Female funnels	13	13	
Ovisacs	14	Lack	
Genus species	Compostelandrilus	Compostelandrilus	
	<i>menciae</i> sp. nov.	<i>bercianus</i> sp. nov.	
Length	140–155	150-170	
Number of segments	340–345	279–359	
Weight (g)	2.5 - 4.1	2.8 - 4.4	
Pigmentation	Pale grey (clit. brown grey)	Bluish grey	
Prostomium	Epilobic	Epilobic, proepilobic	
Setae	Closely paired	Closely paired	
Setal arrangement	85:1.8:25:1:80	45:1.5:10:1:47	
Papillae	<i>ab</i> 12 (14) 15 16	<i>ab</i> 14–24	
First dorsal pore	9/10	11/12	
Nephridial pores	Aligned, 1/3 bc	Aligned, b	
Male pores	32 (cryptic)	40 (cryptic)	

Genus species	Compostelandrilus menciae sp. nov.	Compostelandrilus bercianus sp. nov.	
Female pores	14, b	14, <i>b</i>	
Spermathecal pores	12/13-22/23 d	(17/18) 18/19–23/24 cd	
Clitellum	(27)28(29)-43(44)	30–52 (white)	
Tubercula pubertatis	$(19)21(22)-(43)45(47)^*$	(34)35-(46)47(48)	
Oesophageal hearts	6–11	6–11	
Morren's glands	(13)14–16, large	14–17, large	
Crop	18-21(23)	20-24(25)	
Gizzard	24–27	(25)26–29(30)	
Typhlosole	Inverted T, U	Inverted T	
Nephridial bladders	Closed U in 8, wide open	Closed U in 8, wide open	
	V in 40	V in 41	
Seminal vesicles	10 small, 11, 12–18 large	10 small, 11, 12–17 large	
Spermathecae	13–23	19–25	
Testes	10, 11	10, 11	
Seminal funnels	10, 11	10, 11	
Ovaries	13	13	
Female funnels	13	13	
Ovisacs	14	14	

Table 1. Continued

*Genital bands.

lumbricid species. The closest known relative among lumbricids is the species *Z. cyaneus*.

COMPOSTELANDRILUS MENCIAE SP. NOV. (FIGS 3-5)

Material examined: Holotype. Adult [UVIGO (University of Vigo) 1044]. Fuente del Ciervo, Cabañas Raras (León, Castile and León, Spain). Cork oak (Quercus suber) forest with a dense, rich understory of shrubs and lianas. Plant species present: Arbutus unedo, Phyllirea angustifolia, Pistacia terebinthus, Lonicera etrusca, L. periclymenum, Tamus communis, Hedera helix and Ruscus aculeatus. Mean precipitation, 675 mm. A sandy clay loam soil developed over slate. Altitude 630-650 m. Location 1: 42°38′52″N, 6°36′35.3″W [18 specimens (6 subadults and 12 juveniles)]. Location 2: 42°38′34.6″N, 6°36′26.3″W [20 specimens (11 adults, 6 subadults and 3 juveniles)], leg. J. Domínguez, M. Aira, Pablo G. Porto, Lorena San Martín. February 2011. Paratypes. 37 specimens (UVIGO 1045), same collection data as for holotype.

Etymology: The species name is derived from Mencía, a Spanish variety of grape primarily found in the northwestern part of the country.

Morphological description: External morphology (Figs 3, 4). Body pigmentation is pale grey, turning to brownish grey in the clitellum and also in the first segments. Length 140–155 mm (n = 11 adults); diameter 6–7 mm

(n = 11 adults); body cylindrical in cross-section; 340–345 segments (n = 11 adults). Weight (fixed specimens): 2.5– 4.1 g (*n* = 11 adults). Prostomium epilobic. Peristomium has longitudinal furrows that extend to first segments. Segments 7–27 have two weak transversal furrows that may appear as one or two transversal furrows posterior to the clitellum. Clitellar segments have only one furrow per segment ventrally. First dorsal pore at the intersegmental furrow 9/10. Nephridial pores aligned 1/3 of the distance bc above C, the first pore opens at the intersegmental furrow 10/11, although the first visible nephridia occur in segment 5. Spermathecal pores minute, at the intersegmental furrows 12/13 and 22/23, at level of chaetae d. Male pores (32) are cryptic and only recognized after internal dissection. Female pores on segment 14 slightly above chaetae b. Clitellum saddleshaped extending from (27) 28 (29) to 43 (44). Genital band from (19, 20) 21 (22) to (43, 44) 45 (46, 47) with not clearly distinguished tubercula pubertatis extended as whitish expanded patches (Table 1).

Chaetae very small and closely paired, with the interchaetal ratio aa: 80, ab: 1.6, bc: 23, cd: 1, dd: 83 at segment 62. Chaetae cd (ab?) on whitish expanded patches on segments 12, (14), 15 and 16 (Table 1).

Internal anatomy. Septa 5/6–10/11 thickened and muscular. Hearts in segments 6 (reduced) to 11, oesophageal. Large calciferous glands in segments (13) 14–16. Crop in 18–21 (23), gizzard in segments 24–27. First section of the intestine (29–39) very pleated. Erect bifid typhlosole begins in segment 29 and gradually changes to an inverted U-shaped (Table 1, Fig. 5).



Figure 3. Photographs and drawings (lateral views) of *Compostelandrilus menciae* sp. nov. and *Compostelandrilus bercianus* sp. nov.

Male sexual system holandric, testes and funnels (not enclosed in testes sacs, but with sperm present) located ventrally in segments 10 and 11. Three pairs of seminal vesicles in segments 10, 11 and 12. The last pair is very large, lobulated and extended to segment 18. Ovaries and female funnels in segment 13, ovarian receptacles (ovisacs) in 14. Eleven pairs of sessile spermathecae in segments 13–23. Nephridial vesicles are rounded to sausage shaped, with the first pair appearing in segment 5. In segments 5–11 nephridia are larger and joined to the posterior septa; from segment 12 forward they are joined to the body wall; the nephridial bladders are U-shaped in segment 8 and V-shaped in segment 40 (Table 1, Fig. 5).

COMPOSTELANDRILUS BERCIANUS SP. NOV. (FIGS 3, 4, 6)

Material examined: Holotype. Adult (UVIGO 1114). Embalse de Bárcena, Cubillos del Sil (León, Castile and León, Spain). Mixed woodland of cork oak (Q. suber), holm oak (Q. ilex) and Pyrenean oak (Quercus pyrenaica) interspersed with strawberry trees (A. unedo). Plant species present: C. ladanifer, C. salvifolius, C. striatus, C. scoparius, G. falcata, E. australis, Halimiun alyissoides, P. angustifolia and P. terebinthus. Mean precipitation, 675 mm. A sandy clay loam soil developed over slate. Altitude 740–780 m. Location 1: 42°35.6′54″N, 6°34′36.68″W [38 specimens (4 adults, 13 subadults and 21 juveniles)]. Location 2:



Clitellum

- clitellum variation
- tubercula pubertatis
- tubercula pubertatis variation
- male pore position
- spermathecae positition

Figure 4. Diagram of the external morphology of the genera Postandrilus, Galiciandrilus and Compostelandrilus.

42°34.59′80″N, 6°34′31.30″W [15 specimens (8 adults, 2 subadults and 5 juveniles)], leg. Jorge Domínguez, M. Aira, Pablo G. Porto, March and April 2013 and November 2014. Paratypes. 52 specimens (UVIGO 1175), same collection data as for holotype.

Etymology: The species name is derived from El Bierzo, a region in the west of the province of León, in northwestern Spain.

Morphological description: External morphology (Figs 3, 4). Body pigmentation is pale whitish grey, turning to brown in the clitellum. Length 150–170 mm (n = 6); diameter 6–8 mm (n = 6); body cylindrical in cross-section with the posterior region more flattened; 279–359 segments (n = 6). Weight (fixed specimens): 2.8–4.4 g (n = 6). Prostomium proepilobic.

Peristomium has longitudinal furrows that extend to segment 2. Segments from the ninth forward have one transverse furrow in the middle that can occur one or two transversal furrows after the clitellum, dividing the segments in two or three sections. First dorsal pore at the intersegmental furrow 10/11. Nephridial pores aligned above B. Spermathecal pores minute, at the intersegmental furrows between (17/18) 19/20 and 23/24. Male pores cryptic (40). Female pores in 14 slightly above chaetae *b*. Clitellum saddle-shaped extending from (30) 31 (32) to 51 (52) 53. Tubercula pubertatis extended as a band from (34) 35 to (46) 47 (48) (Table 1).

Chaetae very small and closely paired, with interchaetal ratio at segment 62, aa: 45, ab: 1.5, bc: 10, cd: 1, dd: 47. Marked papillae on chaetae ab from segments 13, 14 to 30, 31 (Table 1).



Figure 5. Compostelandrilus menciae sp. nov. (UVIGO 1044). (A) Lateral view of prostomium, peristomium and segments 1–6. (B) Ovary, ovisacs, female funnels, male funnels and spermiductes. (C) Crop, gizzard and stomach. (D) Stomach and typhlosole. (E) Spermathecae, 11 pairs from segment 13–23. (F) Posterior nephridium, showing the nephridial curved bladder.

Internal anatomy. Septa 5/6–10/11 thickened and muscular. Hearts in 6 (reduced) to 11, oesophageal. Large calciferous glands in 14–17. Crop in 20–24 (25), gizzard in 25, 26–29, 30. Simple typhlosole begins in 31, 32 and gradually changes to erect bifid inverted T-shaped and again to simple finishing at segment 160, 170, leaving more than 100 atyphlosolate segments (Table 1, Fig. 6).

Fraying testes and iridescent and very large funnels in segments 10 and 11. Three pairs of seminal vesicles in 10, 11 and 12. The last pair is very large, lobulated and extended to segment 17 (21, 22). Ovaries and large female funnels in 13, ovarian receptacles (ovisacs) racemose in 14. Variable number (5–7 pairs) of oval spermathecae in segments 17, 19–23. Nephridial vesicles are flattened, sausage shaped, with the first pair appearing in segment 5. In segments 5–11, nephridia are larger and jointed to the posterior septa, and from segment 12 forward are jointed to the body wall; the nephridial bladders are



Figure 6. Compostelandrilus bercianus sp. nov. (UVIGO 1114). (A) Lateral view of anterior segments. (B) Seminal vesicles, male funnels, pharynx, crop, gizzard and Morren's glands. (C) Crop, gizzard and Morren's glands. (D) Internal anatomy of crop and gizzard. (E) Spermathecae, seven pairs from segment 19–25. (F) Anterior nephridium, showing the nephridial curved bladder.

U-shaped in segment 8 and V-shaped in segment 40 (Table 1, Fig. 6).

DISCUSSION

SYSTEMATICS OF THE PROPOSED NEW GENERA GALICIANDRILUS AND COMPOSTELANDRILUS

The results of the phylogenetic analysis carried out in the present study strongly support the hypothesis that the genus *Postandrilus*, as described by Qiu & Bouché (1998a), is not monophyletic and must be redefined (Supporting Information, Appendix S1). Although in the great majority of earthworm species from the family Lumbricidae the male pores occur in an anterior position, on segment 15, in *Postandrilus* the male pores are located in the clitellum, at the beginning of the tubercula pubertatis, and at some distance from segment 15. The genus *Postandrilus* included three subgenera and six species, five from the Balearic island of Majorca and one from Galicia (NW Spain) (Qiu & Bouché, 1998a; Pérez-Losada *et al.*, 2011). However, this classification is not supported by the current phylogenetic findings, which clearly separate the Majorcan and Iberian *Postandrilus* clades. Thus, although *Postandrilus* is considered a valid lumbricid genus, it should be restricted to include only the five species from the Island of Majorca (*P. palmensis*, *P. lavellei*, *P. medoakus*, *P. sapkarevi* and *P. majorcanus*). We have previously proposed to reserve the taxonomic name *Postandrilus* only for the species occurring in Majorca and reassign *P. bertae* to a new genus other than *Cernosvitovia* (Pérez-Losada *et al.*, 2011). However, at that time, we did not have enough information to propose a taxonomic position and a new taxonomic name for *P. bertae*.

Our phylogenetic analyses also show that the Majorcan clade including the monophyletic *Postandrilus* clustered with another genetically very different (as denoted by the tree branch lengths), and also strongly supported, Iberian clade, including the other four species collected in this study (Fig. 1). In turn, the Iberian clade splits into another two wellsupported and phylogenetically distinct clades. The first includes the two earthworm species originally described as *C. bertae* and *A. morenoe*. The second includes the two new species described here, which also cluster with *Z. cyaneus*. This phylogenetic assemblages led us to the task of deciding on a taxonomic name for the new species, as this clade is clearly separate from all others in the lumbricid earthworms.

Zophoscolex cyaneus was originally described as Eophila cyanea on the basis of its external (clitellum, tubercula and position of the first dorsal pore) and internal anatomic features (calciferous glands, crop, gizzard, shape of typhlosole and lack of ovisacs) (Briones & Díaz Cosín, 1993). It was included in Eophila because this genus includes other similar species. However, other some important differences, such as the position of the gizzard and spermathecae and the structure of the seminal vesicles, may justify the establishment of a new genus (Briones & Díaz Cosín, 1993).

This species appears in the zoological taxonomic index Fauna Europaea as *Aporrectodea cyanea* (Briones & Díaz Cosín, 1993), with the taxonomic comment: Aporrectodea 'group Zophoscolex (Qiu & Bouche, 1998c)'.

The genus Zophoscolex was created by Qiu & Bouché (1998d) to group together 21 earthworm species from southern France, Spain and Portugal. Divided in four subgenera, it falls within the Zophoscolexini tribe, according to the taxonomic system proposed by Qiu & Bouché (1998a, b) for the Lumbricoidea. This genus includes medium-sized unpigmented lumbricids with epilobic prostomium, longitudinal furrows normally on the peristomium, closely paired or distant setae, nephridial pores aligned on a line in area B very close to line b, or in segments 3,5–3,6; spermathecal pores usually in C: segments 10, 11, simple, double or multiple; gizzard in segments 17–19 (20) (21); bifid, trifid

or multifid typhlosole, with one to three small longitudinal central lamellae, calciferous glands in segments 11–14 with pouches in segment 11; nephridial bladders sausage shaped with ampulla; two or three pairs of seminal vesicles in segments (9), 11 and 12 (Qiu & Bouché, 1998b, d).

As *Z. cyaneus* is morphologically different and genetically very different from the two new species, again demonstrated by the long length of the tree branch, the solution for the problem is to propose a new genus in which these species should be included.

Phylogenetic divergence (branch lengths) reported here among congeneric and conspecific Majorcan *Postandrilus* are similar to those described for other earthworms in the same taxonomic categories. Branch lengths in the taxa included in the Iberian clade are much longer than the average values estimated for most monophyletic genera within the family Lumbricidae (see Domínguez *et al.*, 2015; Pérez-Losada *et al.*, 2015).

These findings led us to propose two new genera for the Iberian specimens, *Galiciandrilus* gen. nov. for the originally described species *P. bertae* and *A. morenoe*, and *Compostelandrilus* gen. nov. for the two new species *C. bercianus* sp. nov. and *C. menciae* sp. nov.

PHYLOGEOGRAPHY OF THE NEW GENERA GALICIANDRILUS AND COMPOSTELANDRILUS

While the five species of the monophyletic Postandrilus are endemic to the Island of Majorca (western Mediterranean, Spain), the other earthworms included in the Iberian clade have been found only in a small area of northwestern Spain (Fig. 2). This restricted and disjunct geographical distribution is puzzling, considering the geographical distance separating both regions and the geological history of the Iberian Peninsula. However, it may be the result of a vicariant event occurring in the Late Oligocene (30-28 Mya) that separated the Balearic Islands-Kabylies microplate from the proto-Iberian Peninsula (Pérez-Losada et al., 2011). For most of the Miocene and until the Messinian salinity crisis (MSC, 5.96-5.33 Mya), an event characterized by dramatic drying and salinity increase of the Mediterranean Sea due to isolation from the Atlantic Ocean (Krijgsman et al., 1999; Krijgsman, 2002; Garcia-Castellanos et al., 2009), the Balearic Islands remained isolated from the continent. During the MSC, the western Mediterranean water level decreased by more than 1000 m, allowing reconnection of the Balearic Islands to eastern Spain for a short period of time. Subsequent Pleistocene glaciations (1.8–0.01 Mya) also caused important drops in sea level (of up to 150 m), which allowed episodic contacts among the Balearic Islands, but not between the islands and mainland (Waelbroeck et al., 2002; Dawson, 2013).

Our molecular estimates indicate that the split between Majorcan Postandrilus and the related Iberian clade took place 29.9 Mya. Our tree and time estimates also indicate that the proto-Iberian ancestor subsequently speciated into Galiciandrilus and the clade Compostelandrilus + Z. cyaneus 26.5 Mya, while the Majorcan Postandrilus experienced a period of apparent evolutionary stasis, followed by a period of cladogenesis starting 6.2 Mya when many new lineages radiated simultaneously. The rapid diversification of the Majorcan Postandrilus matched the onset of the MSC (5.96-5.33 Mya), and we believe that this estimate actually reflects the impact of the MSC on an already established insular lineage. The drop in sea level that occurred during the MSC may have given rise to new terrestrial habitats on the island of Majorca. The subsequent reconnection of Mediterranean and Atlantic basins and rapid refill (a few years) of the Mediterranean (Garcia-Castellanos et al., 2009) may have contributed to the isolation of the previously expanded earthworm populations and thus to their speciation (Pérez-Losada et al., 2011).

The split between the two Iberian clades took place 26.5 (23.6–29.2) Mya. Within the Iberian clade, *G. bertae* would have diverged from *G. morenoe* 19.2 (15.3–23.5) Mya; the Iberian *Z. cyaneus* would have diverged from the two new species 21.7 (18.1–25.0) Mya; and the two new species, *C. bercianus* and *C. menciae*, would have diverged 14.9 (11.0–18.6) Mya.

The Iberian clade includes two sister clades: one is found in Galicia, with *G. bertae* occurring in the west, very close to the Atlantic Ocean, and restricted to a small geographic area of less than 2 km², and *G. morenoe* occurring in eastern Galicia. The second sister clade includes the new species, which occur in 'El Bierzo', an area in the west of the province of León, in northwestern Spain. The two new species, *C. bercianus* and *C. menciae*, are confined to a small area of < 40 km² and cluster with *Z. cyaneus*, located ~100 km east in another very small area of < 2 km² (Fig. 2). These restricted distributions are typical of all the relict species included in the Iberian clade.

Zophoscolex may represent the nexus between the eastern and western populations of these ancient lumbricids. In order to clarify their radiation, future systematic work should concentrate on the study of the 21 species included in the genus Zophoscolex (Qiu & Bouché, 1989d). Our current sampling efforts are focussed on those earthworm species.

TAXONOMIC IMPLICATIONS: DISAGREEMENT BETWEEN MORPHOLOGICAL AND MOLECULAR EVIDENCE

The results of this study are a good example of the anatomical plasticity of the morphological features commonly used in earthworm alpha taxonomy. Although these characters (Table 1) are useful for identifying Lumbricidae species, they seem unreliable for reconstructing Lumbricidae evolutionary relationships or delimiting supra-specific taxa, given their disagreement with our molecular phylogenetic trees (Figs 1, 4; Table 1; Domínguez *et al.*, 2015).

Regarding the position of the male pores, all species included in this study except two, Z. cyaneus and G. morenoe, are 'postandrilus' (in the etymological sense of the word, with the male pores in a posterior location, on the clitellum at the beginning of the tubercula pubertatis). The male pores in Z. cyaneus and G. morenoe occur on segment 15, the common and usual position (at some distance from the tuberculo-clitellum system) in earthworms of the family Lumbricidae. In the family Hormogastridae and in the most primitive lumbricids such as Prosellodrilus, male pores are located in segment 15, and this seems probably to be the ancestral condition of the family Lumbricidae. From that ancestor, taxa with the male pore displaced to posterior positions evolved multiple times (Cernosvitovia, Octodriloides, Fitzingeria and Postandrilus), indicating that the position of the male pore is not a reliable character for earthworm systematics and taxonomy. Thus, the species Z. cyaneus and G. morenoe are puzzlingly placed in separate and wellsupported clades in the Iberian clade. The anterior or posterior position of the male pores and, more importantly, their position inside or outside of the clitellum are not only a numerical or positional question but also implies important differences in earthworm mating patterns. Further comparative studies are needed to interpret the variation of the male pore in lumbricids and the relationship between this position and different mating strategies.

The two species of the *Galiciandrilus* clade do not share any morphological characters except several plesiomorphies (e.g. the setal and nephridial pore arrangement and the positions of the oesophaegal hearts, female pore, testes, seminal funnels, female funnels, ovaries and ovisacs) and differ in the remaining characters (Table 1). In fact, in view of their similar morphology, we would expect *G. bertae* to be sister taxon to *C. menciae* and *C. bercianus*.

The two new species of the *Compostelandrilus* clade also share several plesiomorphic characters, such as the setal and nephridial pore arrangement, the shape of the typhlosole and nephridial bladders and the positions of the oesophaegal hearts, female pore, testes, seminal funnels, female funnels and ovaries (Table 1).

The two new genera are not defined by any morphological synapomorphies. In the absence of phylogenetic information, we could be tempted to relate these new species to the very few lumbricid genera with the male pore far from segment 15 (*Cernosvitovia*, *Octodriloides*, *Fitzingeria* and *Postandrilus*). This was the argument that, considering the different mode of reproduction, led Qiu & Bouché to propose a new Lumbricidae subfamily, Postandrilinae, to include *Postandrilus* gen. nov., *Cernosvitovia* Omodeo, 1956 and *Fitzingeria* Zicsi, 1978 (Qiu & Bouche, 1998b). As *G. bertae* is not closely related to *Postandrilus*, the intuitive subfamily Postandrilinae is not supported by the phylogenetic findings and the four genera with male pores in a posterior position are included in different and distant clades in the phylogeny of the Lumbricidae (Domínguez *et al.*, 2015).

Considering that the two species included in the new genus *Galiciandrilus* are morphologically very different from each other and that their separation occurred several Mya, they could, in fact, represent two different earthworm genera. Further sampling and phylogenetic analyses of earthworms from this geographic area may confirm this hypothesis, but at this point, we opt for a more conservative approach, including both taxa in the genus *Galiciandrilus*.

CONCLUSIONS

This study presents a well-supported phylogeny of a basal clade within the Lumbricidae tree including the genus *Postandrilus* and its relatives. Our phylogenetic analyses led to the relocation of some lumbricids, the proposition of two new genera (Galiciandrilus gen. nov. and Compostelandrilus gen. nov.) and the identification of two new species (C. menciae sp. nov. and C. bercianus sp. nov.). The comparative study of the external and internal morphological characters commonly used in earthworm alpha taxonomy did not reveal synapomorphies matching the phylogenetic assemblages depicted in our molecular phylogenetic analyses. This confirms the anatomical plasticity of Lumbricidae earthworms and the need for further revision of the Lumbricidae taxonomy. Finally, given the restricted geographical distribution ($< 50 \text{ km}^2$) of the earthworm species described here and their basal phylogenetic position in the Lumbricidae tree, future conservation efforts should be initiated to preserve this unique biological diversity and characterize their ecological and habitat requirements.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Lumbricidae Bayesian 50% majority-rule consensus molecular tree including all specimens. Bayesian posterior probabilities are shown above the branches as percentages. The species considered in the present study are highlighted in green. Code numbers are provided for each specimen.