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Winterfell survivor: The European phylogeography of a riparian earthworm (Annelida, Clitellata)

Irene de Sosa[1] | **Daniel F. Marchán**[1] | **Marta Novo**[1] | **Christer Erséus**[2] | **Emmanuel Lapied**[3] | **Mišel Jelić**[4] | **Aleksandra Jabłońska**[5] | **Tímea Szederjesi**[6] | **Ana Almodóvar**[1] | **Darío J. Díaz Cosín**[1]

[1]Biodiversity, Ecology and Evolution Department, Faculty of Biology, Complutense University of Madrid, Madrid, Spain

[2]Department of Biological and Environmental Sciences, University of Gothenburg, Göteborg, Sweden

[3]Isotope Laboratory, Norwegian University of Life Sciences (NMBU), Ås, Norway

[4]Department of Natural Sciences, Varaždin City Museum, Varaždin, Croatia

[5]Department of Invertebrate Zoology and Hydrobiology, University of Lodz, Łódź, Poland

[6]Department of Zoology, Eszterházy Károly Catholic University, Eger, Hungary

Correspondence

Irene de Sosa, Biodiversity, Ecology and Evolution Department, Faculty of Biology, Complutense University of Madrid, C/José Antonio Nováis 12, 28040 Madrid, Spain.
Email: irenedesosa91@gmail.com

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Abstract

Although some earthworms can survive in permafrost and tolerate cold conditions, it is generally believed that most earthworms were eradicated from northern latitudes during the Last Glacial Maximum. The main goal of this study is to test this hypothesis using a cosmopolitan earthworm as model, *Eiseniella tetraedra*. We collected 1640 specimens in 294 localities from 19 different countries. We examined three molecular markers (COI, 16S and 28S) and their morphology. Furthermore, we investigated their potential niches through Species Distribution Models. Eleven genetic lineages were found, nested in five clades. Clade I was more prevalent in cold biogeographical regions such as the Continental, the Atlantic or even the Arctic, while clade II was prevalent in Mediterranean regions. Potential niches agreed with the distribution trends. The presence of restricted clades in the Iberian and Scandinavian peninsulas, as well as in Eastern Europe, suggests that these three regions served as refugia during the Last Glacial Maximum. Thus, nunatak and *tabula rasa* hypotheses were necessary to explain the actual distribution of this riparian earthworm.

KEYWORDS

biogeography, diversity, earthworm, Ecological niche modelling, Glacial Maximum, last refugia

1 | INTRODUCTION

Nowadays, earthworms include ca 5738 species/subspecies described belonging to 23 families (Misirlioğlu et al., 2023). The family Lumbricidae (Rafinesque-Schmaltz, 1815) includes about 300 species (Csuzdi, 2012) and originated in the Lower Cretaceous in the Holarctic region (Dominguez et al., 2015). Earthworms belonging to this family are the most abundant invertebrates in the soil of temperate regions and account for 90% of invertebrate biomass (Edwards, 2004). Since earthworms are not able to live in permafrost for long periods of time (Holmstrup et al., 1991), it is generally assumed that they were eradicated from northern latitudes during the Last Glacial Maximum (LGM) (Tiunov et al., 2006). Thus, species now found in northern European areas must have recently recolonized from historical refugia such as the southern European peninsulas. This basic expansion-contraction model is known as the "*tabula rasa*" hypothesis. In contrast, the nunatak hypothesis suggests that some ice-free refuge existed in northern Europe, such as mountains rising above inland ice or coastal ice-free refuges, where the biota could survive. When the ice melted, plants and animals recolonized previously ice-covered areas from these northern refugia. There is now strong geological evidence suggesting that some nunataks and ice-free coastal shelves existed within the maximum limits of the last glacial period 25,000–10,000 years ago (Hansen et al., 2006). Earthworms are part of the soil fauna and given their ecological requirements for rainfall, habitat cover, temperature, soil carbon and pH (Phillips et al., 2020; Rutgers et al., 2009; Rutgers et al., 2016), they do not appear to be good candidates for survival in these nunataks. However, this hypothesis has been proposed for Fennoscandia (Fridolin, 1936; Stöp-Bowitz, 1969) and Greenland (Hansen et al., 2006), where it would seem that certain robust species of Lumbricidae, such as *Dendrobaena octaedra* (Savigny, 1826), were able to survive the last glacial period in ice-free refugia, in association with some arctic plants (Julin, 1950; Stöp-Bowitz, 1969). Individuals of *D. octaedra* (Savigny, 1826) overwinter in frozen ground either as adults or in cocoons. In Greenland, it exhibited high genetic diversity, which, combined with its high frost tolerance, suggests its survival in ice-refuge in this area (Hansen et al., 2006), following the nunatak hypothesis. Interestingly, certain earthworm species rely on cocoons as crucial survival structures during environmental stress. For instance, some lumbricids and megascolecids can survive freezing temperatures by dehydrating their cocoons, which prevents cellular damage in the embryos (Holmstrup & Westh, 1994). Görres et al. (2016) demonstrated that earthworm cocoons can withstand air temperatures as low as −24°C in the field. Despite the potential of species with such cocoons to withstand low temperatures, no study has yet been conducted to test whether they could have survived in nunatak refuges.

Eiseniella tetraedra (Savigny, 1826) is a parthenogenetic and tetraploid (Casellato, 1987) earthworm with a riparian lifestyle that inhabits margins of freshwater. It has a worldwide distribution and is therefore referred as a cosmopolitan earthworm (Blakemore, 2006). *E. tetraedra* has been found to the north of the 65 parallel (Haraldsen & Engelstad, 1998), and has been detected as far as the northern coast of the Scandinavian Peninsula (Terhivuo, 1988). It is also present in Iceland (Blakemore, 2007). According to these occurrences, *E. tetraedra* seems to be a cold tolerant earthworm. However, de Sosa et al. (2022) suggested that *E. tetraedra* adults from the centre of the Iberian Peninsula are not suited to withstand freezing conditions. They found downregulated a variety of genes involved in the respiratory chain. This suggests that *E. tetraedra* is poorly adapted to freezing conditions, as those with a high capacity for ATP production under cold conditions also demonstrate good tolerance to cold stress (Lu et al., 2017; Wang et al., 2014). It is possible that populations situated in more northerly locations have evolved greater resilience to these more extreme conditions. Moreover, the resistance of its cocoons remains to be tested.

Eiseniella tetraedra showed a high intra-specific genetic diversity in the Iberian Peninsula, with eight divergent phylogenetic lineages nested in two clades (de Sosa et al., 2023). Javidkar et al. (2021) found six lineages in Iran, probably introduced by human activity, while de Sosa et al. (2017) found only one lineage in Scandinavia, which could be due to a limited number of samples. Genetic diversity of *E. tetraedra* in the Iberian Peninsula was distributed according to three environmental factors: temperature, precipitations and pH (de Sosa et al., 2023). Si-Moussi (2020) identified soil texture as the most significant factor influencing the distribution of this species using Ecological Niche Modelling (ENM). However, the macroecological preferences of the clades and lineages phylogenetically identified within this species remain undetermined. ENM has facilitated ecological inference in soil due to its high power when only presence data are included (Phillips et al., 2006). It has been implemented in several groups such as termites (Maynard et al., 2015), beetles (Crawford & Hoagland, 2010), millipedes (Marek et al., 2012) and earthworms (Marchán et al., 2015; Marchán et al., 2016).

The present study investigated the genetic variation and phylogeographic relationships of a large number of populations of *E. tetraedra* collected from 19 countries in Europe. The main objective of this study was to test the *tabula rasa* and nunatak hypothesis in earthworms using a cosmopolitan earthworm species as a model. Phylogeographic studies can help us determine the history of diversification and dispersal of a species, and earthworms with high genetic diversity are good models for these studies (de Sosa et al., 2023; Fernández et al., 2013; Shekhovtsov et al., 2020). A phylogeographical study covering the whole Europe, might therefore throw light on these hypotheses. If we find greater diversity in southern Europe than in northern Europe, as well as clades or lineages restricted to that area, we could assume that the *tabula rasa* hypothesis explains the history of that species. A contrary pattern, would support the nunatak hypothesis. Three secondary objectives were also identified: (i) to examine the genetic diversity of *E. tetraedra* in Europe; (ii) to infer its phylogeography in this continent and (iii) to test macroecological preferences for clades.

2 | MATERIALS AND METHODS

2.1 | Earthworm sampling and morphological analyses

We collected 1640 specimens in 294 localities from 19 different countries: Belgium, Bulgaria, Czech Republic, Finland, France, Germany, Greece, Israel, Italy, Netherlands, Norway, Poland, Portugal, Russia, Slovakia, Spain, Sweden, Turkey and United Kingdom (Tables S1, S2, S3, S4 and S5, including samples from de Sosa et al., 2017 and de Sosa et al., 2023). According to European Environmental Agency, 11 biogeographical regions in Europe could be defined: Alpine, Anatolian, Atlantic, Artic, Black Sea, Boreal, Continental, Macaronesian, Mediterranean, Pannonian and Steppic. In this study, we sampled *E. tetraedra* in all regions except four (Black Sea, Anatolian, Macaronesian and Steppic).

Earthworms were collected by hand-sorting and fixed in 96% ethanol and stored at −20°C. Individuals were identified to the level of the species according to Bouché, 1972. Whenever possible, we selected 10 individuals per locality, and a portion of the posterior body section was excised and carefully cleaned under a stereomicroscope to remove gut and soil particles. Tegument samples were stored in ethanol and preserved at −20°C until DNA extraction. Morphological characters were studied on a subset of 1193 specimens, which were complete (not broken or missing parts) and therefore suitable for morphological assessment. We focused on: length, dry weight, number of segments, position of clitellum and *tubercula pubertatis*, position of male pores, number and position of seminal vesicles, spermathecae and spermiducal funnels.

2.2 | DNA extraction, gene amplification and sequencing

Total genomic DNA was extracted using the Speedtools Tissue DNA Kit (Biotools). We amplified a fragment of cytochrome *c* oxidase subunit I (COI) in all specimens, and we chose two specimens per site and lineage (see Results) for amplification of a fragment containing 16S rRNA + tRNAs Leu, Ala and Ser (16S), and a fragment of 28S rRNA (28S) (see Table S6). For 174 localities (out of 294) localities we were unable to obtain 16S and 28S data: all localities from Scandinavia, Genga (Italy), Bavaria (Germany), Porquerolles island and Port Cros (France), Cap Lardier (France), Blanska (Czech Republic), Derbyshire (United Kingdom), Antalya (Turkey), Jan Soors (Belgium), and Rossum (The Netherlands) (see Tables S2, S3, S4, S5). For COI (632 bp) primer sequences and polymerase chain reactions (PCR) followed Pop et al. (2003). For 16S-tRNAs (775 bp) and 28S (804 bp) primer sequences and PCR conditions followed Fernández et al. (2015). All PCRs were resolved via 1% agarose gel electrophoresis and were visualized with GelRed stain (Biotium). PCR products were purified using ExoSAP-IT reagent (ThermoFisher Scientific) and sequenced in both directions by Macrogen Spain Inc.

2.3 | Phylogenetic analyses and genetic variability

Forward and reverse sequences were merged in BioEdit v7.0.9 (Hall, 1999). Sequences of each marker were aligned in MAFFT v.7 (Katoh and Standley, 2013) using default settings and concatenated with BioEdit v7.0.9 (Hall, 1999). Phylogenetic analyses with the concatenated sequence of the three markers (2213 bp) included Bayesian inference (BI) using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) and Maximum Likelihood (ML) with RAxML v.7.03 software (Stamatakis, 2006) both implemented in Cipres Science Gateway v.3.3 (Miller et al., 2010). Phylogenetic trees obtained were visualized in FigTree v1.3.1 (Morariu et al., 2008). The best-fitting substitution model selected by jModelTest2 (Darriba et al., 2012) for each partition was GTR + Γ + I. The Bayesian analyses consisted of two parallel runs of 10 million of generations, sampling 10,000 trees every 1000th generation, starting the analysis with a random tree. 20% of the trees were discarded as burn-in. ML analysis with rapid bootstrapping was performed with 1000 replicates. Sequences of *Lumbricus rubellus* Hoffmeister, 1843, *Dendrobaena byblica* Rosa, 1893, *Eiseniona oliveirae* (Rosa, 1894), *Prosellodrilus biauriculatus* Bouché, 1972 and *Carpetania matritensis* Marchán et al., 2020, were retrieved from GenBank and used as outgroups (Table S6). All nucleotide sequences generated in this study were deposited in the GenBank (Table S6).

Uncorrected pairwise distances for COI were calculated with Arlequin v.3.5 (Excoffier & Lischer, 2010) within and between main clades. We also examined haplotype and nucleotide diversity for clades and for the three peninsulas sampled: Iberian, Italian and Scandinavian using DNAsp v.6 (Rozas et al., 2017). Due to lack of data for 16S and “8S markers in many localities and the bias this could introduce, the genetic diversity values associated with these markers were not analysed.

Species delimitation was performed with COI marker using the Assemble Species by Automatic Partitioning (ASAP) method, which uses a hierarchical clustering algorithm (Puillandre et al., 2021).

2.4 | Ecological niche modelling

Presence data were aggregated according to the two principal clades (I and II) recovered in the phylogenetic analyses (see Results). Clades III, IV and V were not analysed (see Results) due to the small number of samples. The large-scale variables mentioned below were chosen as predictor variables since they are the same (or equivalent) as the found as the most influential for the distribution of *Eiseniella tetraedra* by Si-oussi (2020).

- *Bioclimatic variables* (downloaded from Worldclim—http://www.worldclim.org/ accessed 01/12/2020). Min Temperature of Coldest Month (BIO6); Temperature Annual Range (BIO7); Precipitation of Wettest Month (BIO13).

- *Soil variables*: Lithology (PARMA), represented by the PAR-MATDOM2 (Second level code for the dominant parent material of the STU) layer obtained from the European Soil Database Raster Library 1 km _ 1 km (http://eusoils.jrc.ec.europa.eu/ESDB_Archive/ESDB_data_1k_raster_intro/ESDB_1k_raster_data_intro.html accessed 01/12/2020).

Soil crusting class (CRUST) layer obtained from the European Soil Database Raster Library 1 km _ 1 km (http://eusoils.jrc.ec.europa.eu/ESDB_Archive/ESDB_data_1k_raster_intro/ESDB_1k_raster_data_intro.html accessed 01/12/2020).

Topsoil available water capacity (AWC) layer obtained from the European Soil Database Raster Library 1 km _ 1 km (http://eusoils.jrc.ec.europa.eu/ESDB_Archive/ESDB_data_1k_raster_intro/ESDB_1k_raster_data_intro.html accessed 01/12/2020).

- *Biotic variables*: Vegetation and dominant land use (CLC) were represented by the CORINE 2018 Land Cover layer (version v.2020_20u1: https://land.copernicus.eu/pan-european/corine-land-cover/clc2018?tab=download).

Ecological niche models were obtained using the R package ‘SSDM’ (Schmitt et al., 2017). Presence (localities where the target clade was found) and absence (localities were the other clade but not the target clade- was found) data were used as input. Ensemble species distribution models (ESDMs) were built by combining the algorithms (‘MAXENT’, ‘GLM’, ‘CTA’ and ‘MARS’) producing kappa values greater than 0.5, with three repetitions for each algorithm and default parameters. AUC was chosen as metric to include models in the ensemble prediction, with a threshold of 0.7. Cross-validation was performed using the holdout method with a fraction of 0.7 representing the training set.

3 | RESULTS

3.1 | Phylogenetic analysis

Bayesian and ML approaches yielded trees with congruent topology for *E. tetraedra* (Figure 1). All sequences were nested in five different and well supported clades (labelled I–V). Clades were defined based on tree topology. Clades I and II included four different lineages (B-C-D-G, A-H-E-F, respectively), whereas clades III to V comprised only one each (I, J and K, respectively).

3.2 | Clades and lineages distribution

The distribution of genetic lineages in Europe is shown in Figures 2 and 3. Clades I and II were widespread in Europe. However, clade I was clearly dominant in cold biogeographical regions such as the Alpine, Arctic, Atlantic, Boreal, Continental and Pannonian regions. Clade II was predominant in Mediterranean regions, but also occurred in Alpine, Atlantic, Boreal, Continental and Pannonian regions. In contrast, clades III, IV and V had a restricted distribution, with clade III occurring mainly in Eastern Europe and clades IV and V found mainly in Scandinavian Peninsula. In the Iberian Peninsula, the northern area was more diverse than the southern one, with lineages D and H restricted to the northern area. (Figures 2 and 3). Lineages G and I were also restricted to some localities in Europe. All lineages (except for those restricted ones D, G, H, I) were found in the Scandinavian Peninsula (Figure 2). Lineage B was clearly predominant in all Scandinavia and lineage J and K occurred mostly in

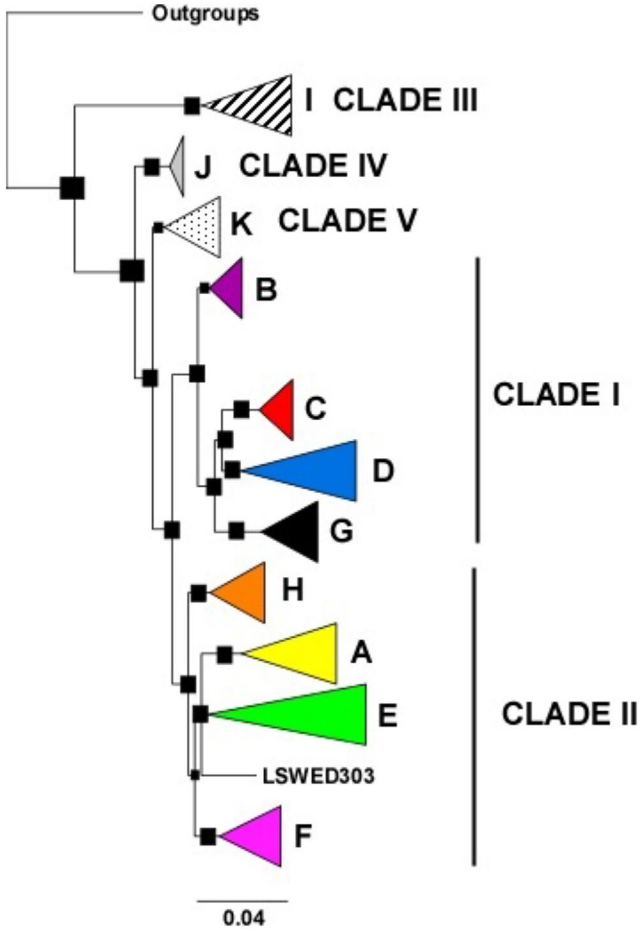

FIGURE 1 Bayesian inference (BI) of the phylogenetic tree based on concatenated sequences of COI (1038), 16S (262) and 28S (378). Posterior probability/bootstrap support values (of Maximum Likelihood Analysis, ML) are shown as black squares when higher than 0.9/0.7 (BI/ML). The scale bar represents 0.04 substitutions per position. Colours and names of lineages (a–h) and number of clades (I and II) are the same as in de Sosa et al., 2023 and in Figures 2 and 3. LSWED303 correspond to a mature specimen from Frösslundabäcken Stream, Öland, Sweden.

this area. The Italian Peninsula was one of the least diverse areas (Figure 2), with only lineages from the clade II (A, E and F), although north–south differences were observed in this region. Lineage A predominated in the northern area, while lineage F was more prevalent in the central and southern areas. The two locations of United Kingdom showed high diversity, with the presence of seven lineages (Figure 2). Only lineages A, E and F were represented at Central Europe with the exception of two individuals of lineages G in the Netherlands and J in Slovakia (Figure 2). The eastern regions of Europe were less represented and lineages A, F and J were found (Figure 2). In northern France, lineage B was predominant, while southern France showed greater diversity, especially in the Pyrenees, showing six lineages in one location.

3.3 | Genetic diversity and divergence

A total of 485 haplotypes were found among 999 sequences for COI, 153 haplotypes within 262 sequences for 16S and 187 haplotypes within 378 sequences for 28S. Values of haplotype and nucleotide diversity of COI for each clade are shown in Table 1. Clade IV showed the lowest COI haplotypic diversity with only three different haplotypes, while clade II was the most diverse with 35.76% of the total haplotypes. Haplotype diversity (H) and nucleotide diversity (π) based on COI including all the specimens within the study were 0.97 and 0.059 respectively. H and π for 16S-tRNas were 0.88 and 0.022. Finally, genetic diversity parameters for 28S were 0.33 and 0.003. Moreover, the COI diversity (H/π) parameters in the three peninsulas studied were: 0.82/0.055 for the Iberian Peninsula, 0.91/0.06 for the Italian Peninsula and 0.65/0.06 for the Scandinavian Peninsula.

Genetic distances within clades based on COI (Table 2), ranged from 0% to 5% and showed little variability. In contrast, inter-clade distances were higher, 6.55–15.22%. The clade III showed the greatest genetic distance with other clades.

We obtained 10 different species delimitation hypothesis. The range of proposed species is between 2 and 122. The best ASAP score (1.5) suggested two putative species (Figure S2).

3.4 | Ecological niche modelling

The ecological niche models obtained for the Clade I and Clade II localities displayed different predictive power, with higher AUC and kappa values (0.71 vs. 0.79 and 0.41 vs. 0.57 respectively), higher sensitivity and specificity (0.72 vs. 0.79 and 0.71 vs. 0.80 respectively) and lower omission rates (0.28–0.21) for the Clade II model (Table S7).

The geographical representation of the predicted suitability values is shown in Figure 4. For Clade I, highly suitable areas corresponded to countries in the same latitude as Britain and higher, being especially widespread in Scandinavia. Highly suitable areas were more widespread for Clade II, covering most of the Mediterranean countries and Britain while being scarce in Scandinavia and other northern countries.

The relative contributions of the predictor variables to each model are shown in Table S7. BIO7, BIO6, BIO13, AWC and CLC were the most influential variables for Clade I, while BIO6, PARMA, AWC and CRUST were the most influential variables for Clade II (Table S7).

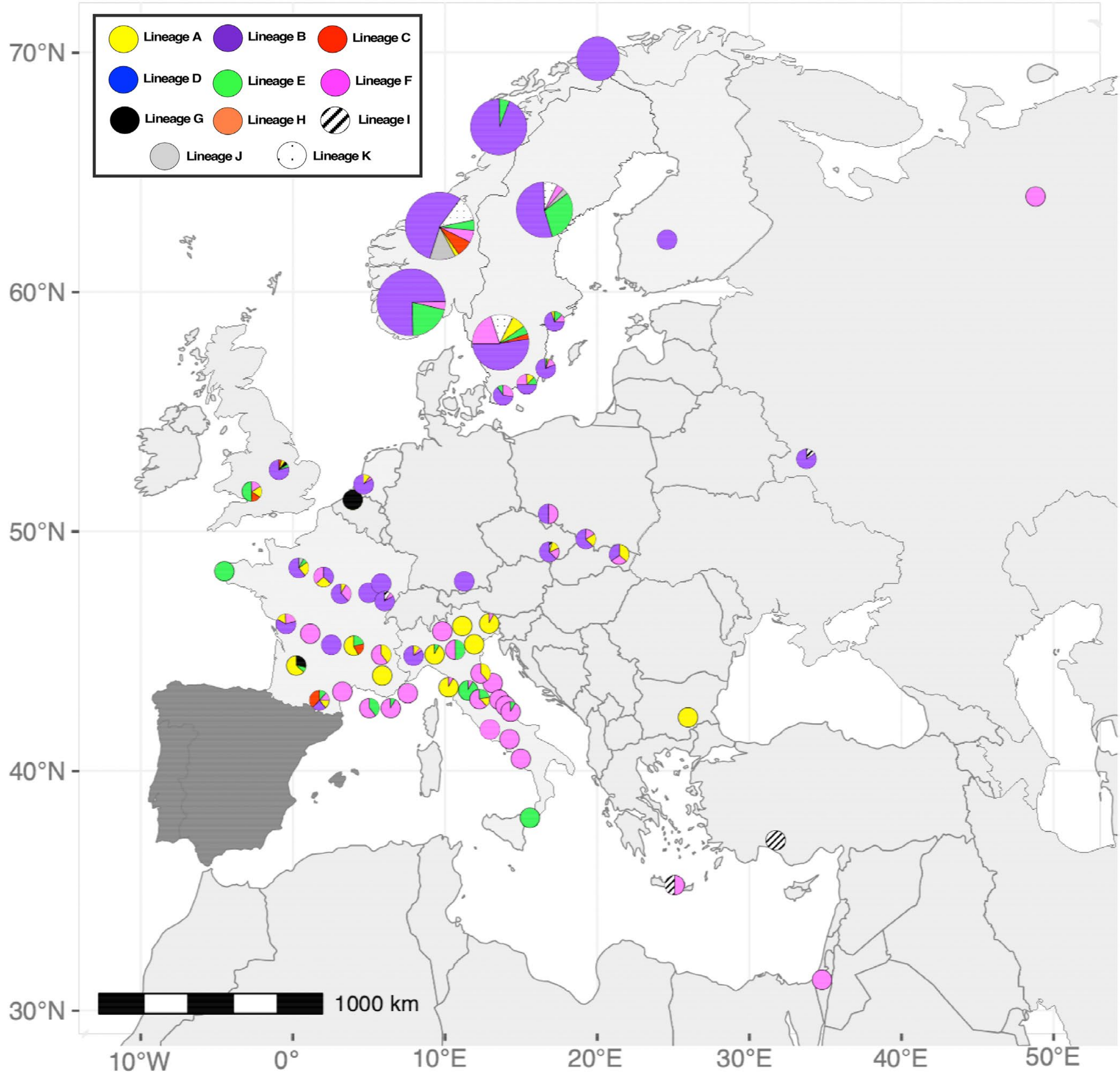

FIGURE 2 Lineages distribution in Europe. Proportion of individuals from each genetic lineage in each locality is represented in pie charts. Many of the lineage pie charts show information from several adjacent populations. The size of each circle is proportional to the number of localities it includes. Colours used are the same as in Figure 1. Details of the dark grey area (Iberian Peninsula) are showed in Figure 3.

3.5 | Morphological data

Morphological data is shown in Table S8. The sexual characters belonging to the male apparatus were the most diverse in relation to its presence/absence (seminal vesicles and spermathecae) and position (male pore) and no presence of sperm was found in any specimen. However, 37 earthworms had spermatophores, but empty. These individuals are nested in the same lineages or even haplotypes with other specimens that do not have spermatophores. Thus, no phylogenetic basis for the presence of this trait can be assumed. No association was found between the most variable traits (those related to the male reproductive system) and genetic diversity.

4 | DISCUSSION

The model of glacial refugia as core areas for the survival of thermophilic and/or temperate animal and plant species during unfavourable Pleistocene environmental

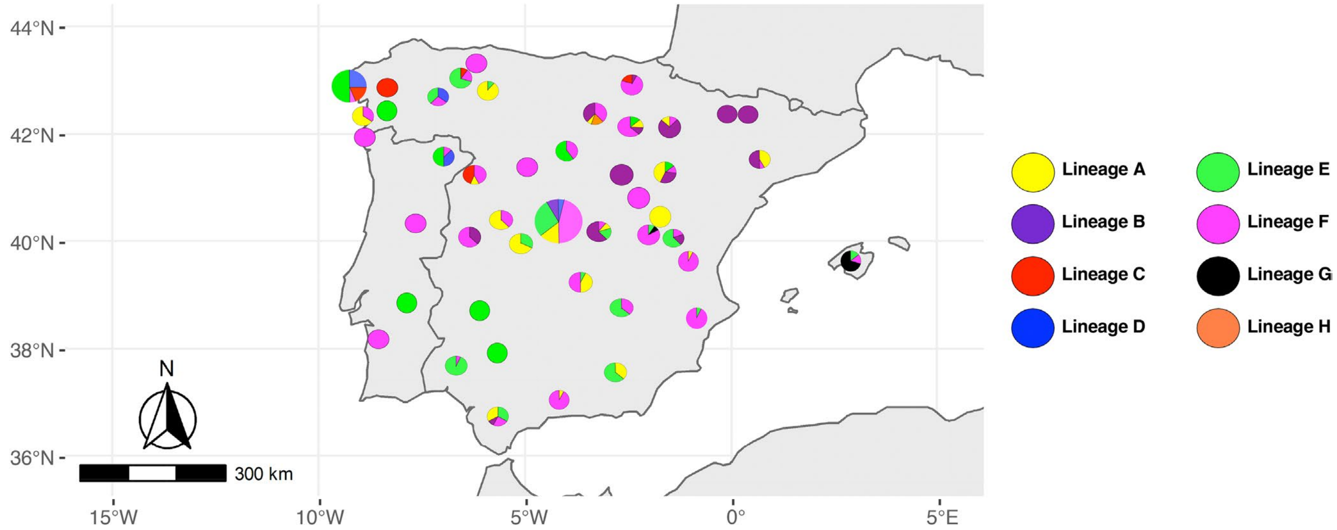

FIGURE 3 Lineages distribution in the Iberian Peninsula (modified from de Sosa et al., 2023). The size of each circle is proportional to the number of localities it includes. Colours used are the same as in Figure 1.

TABLE 1 Genetic diversity parameters of each clade based on the COI gene.

	Number of COI sequences	Number of COI haplotypes	H	π
CLADE I	415	193	0.93	0.027
CLADE II	553	281	0.97	0.035
CLADE III	5	4	0.9	0.038
CLADE IV	8	1	0	0
CLADE V	18	6	0.67	0.006

Abbreviations: H, haplotype diversity; π, nucleotide diversity.

TABLE 2 Percentage of uncorrected pairwise genetic distances based on COI retrieved for *E. tetraedra* in Europe.

	CLADE I	CLADE II	CLADE III	CLADE IV	CLADE V
CLADE I	2.39	8.57	13.8	7.47	8.22
CLADE II		5	15.11	8.8	9.33
CLADE III			3.79	14.77	15.22
CLADE IV				0	6.55
CLADE V					0.58

conditions and as sources of postglacial recolonization processes is widely accepted in biogeography (Hewitt, 2000; Willis & Whittaker, 2000). It is generally believed that the main hypothesis of the recolonization of Europe after the LGM for earthworms is the "*tabula rasa*" hypothesis. It states that earthworms became extinct in northern latitudes and recolonized these areas after the ice melted from refugia. The most prominent refugia were the three southern European peninsulas: the Iberian, Italian, and Balkan (Feliner, 2011). However, other areas may have acted as a shelter and source of colonization during the LGM. Thus, the nunatak hypothesis suggests that there were some ice-free refugia in northern Europe, such as mountains rising above the ice sheet or ice-free refugia on the coast, where the biota could survive. According to our results, *E. tetraedra* could follow both patterns. Although the high haplotype diversity of Italian Peninsula, 0.96, could be due to its role as a refugia during the LGM, the number of individuals from Italy was lower than on other sampled peninsulas. de Sosa et al. (2017) reported higher haplotype diversity in their microscale study focused on the northwestern and central regions of the Iberian Peninsula compared to de Sosa et al. (2023), who found significantly lower diversity despite covering

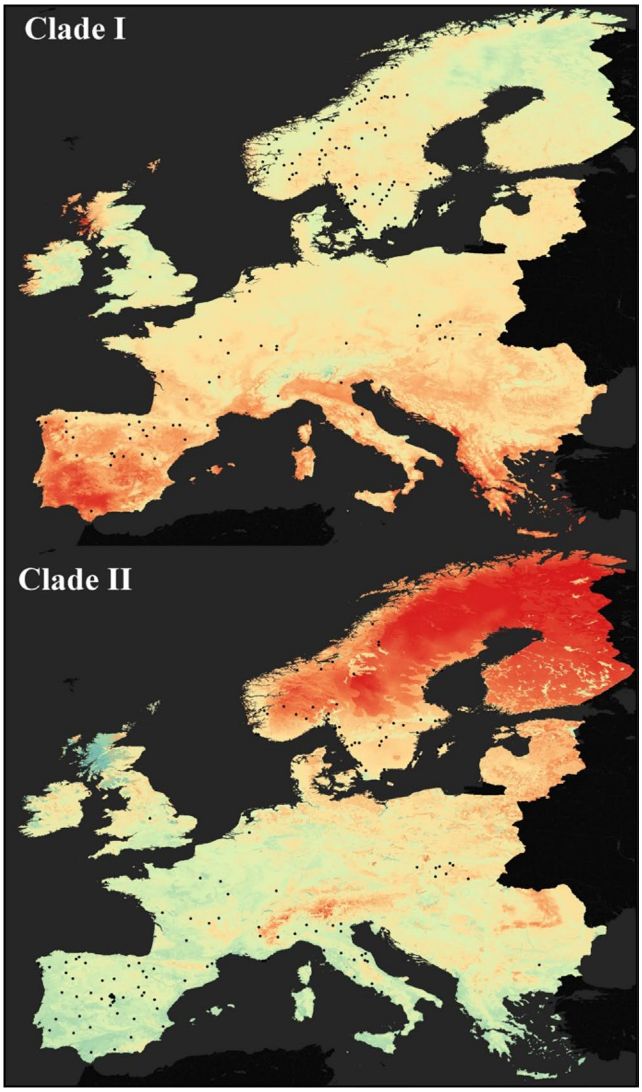

FIGURE 4 Ecological niche models obtained for the Clade I and Clade II localities (shown as black dots). Red colour indicates the lowest estimated suitability and blue-green colour indicate the maximum suitability.

the entire Iberian Peninsula in their study. Therefore, we think that this high haplotype diversity could be explained by the smaller number of samples, as it appears to show an asymptotic haplotypic increase as a function of the number of individuals sampled. The presence of restricted lineages at Iberian Peninsula and even clades IV and V at Scandinavian Peninsula and clade III at Eastern Europe suggests that the three areas served as refugia during the LGM, bringing *E. tetraedra* to the rest of Europe after the ice melted. The Iberian Peninsula was one of the most important glacial refugia of the Pleistocene in Europe (Hewitt, 1999; Hewitt, 2001) and served as a species repository for northern countries (Beebee & Rowe, 2000; Vernesi et al., 2002). Eastern Europe was also an important refugia during the LGM (Sommer & Zachos, 2009) and biota, including earthworms, survived in some ice-free refugia in northern Europe (Hansen et al., 2006).

Temperature and precipitation were the most important factors affecting the distribution of genetic diversity of *E. tetraedra* in Iberian Peninsula (de Sosa et al., 2023). It seems that these variables remain important in Europe as well according to our results. Thus, we found that Clade I predominates in the colder areas with more precipitation, while Clade II in temperate ones. Similar patterns were found for the parthenogenetic and cosmopolitan earthworm species *Aporrectodea trapezoides* Dugès, 1828 and *Aporrectodea rosea* Dugès, 1828 each of which appears to be divided into two distinct clades: one present in the Eurosiberian region and the other in the Mediterranean region. (Fernández et al., 2012; Fernández et al., 2015). Although clades I and II of *E. tetraedra* were not restricted to one region; the trend seems similar to those species. While paleographic events seem to be of great importance for the earthworm's present-day distribution due to its low vagility (Fernández et al., 2013; Novo et al., 2011), the ability of *E. tetraedra* to disperse by hydrochory, possibly also by zoochory (Terhivuo & Saura, 2006) and by anthropochory (Gates, 1977; Javidkar et al., 2021) may contribute to the underlying processes being hidden in a confusing phylogeography due to lack of distribution boundaries.

Several subspecies had been defined for *E. tetraedra* based on the position of the male pores but no phylogenetic basis for this trait has been found (de Sosa et al., 2017). The tremendous variability related to the male reproductive system suggests that individuals with this variation are not under selection pressure, which is to be expected in a parthenogenetic earthworm. Thus, the fact that these traits do or do not occur, or where they occur, has no a priori effect on the fitness of the individuals that exhibit these modifications, since they are traits that are of no use to them. Although the presence of spermatophores in animals that do not have gamete exchange may be contradictory, we found evidence in the literature for the presence of these structures in other parthenogenetic earthworms (Jaenike & Selander, 1979; Muldal, 1952). Apparently, the exchange of empty spermatophores would be necessary to activate parthenogenesis (Jaenike & Selander, 1979; Muldal, 1952). It is possible that this action is an intermediate step prior to pure parthenogenesis, and based on the number of individuals found with these structures (just 37); this would be a process that is being lost in *E. tetraedra*.

The predominant presence of clade III in Eastern Europe, its position on the phylogenetic tree, and its high genetic distance from the other clades of *E. tetraedra* indicate the ancestry of the clade and the possible origin of *E. tetraedra* in Eastern Europe, where most of the sexually related species of the genus were found.

The genus *Eiseniella* Michaelsen, 1900 includes two quite distinct groups (Omodeo & Rota, 1991). One includes only the parthenogenetic and cosmopolitan species *Eiseniella tetraedra* and the other includes sexual species such as *Eiseniella lacustris* Cernosvitov, 1931 and *Eiseniella ochridana* Cernosvitov, 1931, most of which occur only in Eastern Europe. Parthenogenetic lineages can arise from sexual species in a variety of ways: bacterial infections such as *Wolbachia* or *Rickettsia* (Huigens et al., 2000), spontaneous loss of sex due to mutations in genes related to mating and fertilization of eggs (Carson et al., 1982), or in genes involved in sexual forms (Simon et al., 2003) and contagious origin, with incomplete reproductive isolation between sexual individuals and preexisting parthenogenetic lineages (Simon et al., 2003) and hybridization origin between individuals of the same or closely related species (Lorenzo-Carballa & Cordero-Rivera, 2009). The biogeographic pattern in this genus may indicate the existence of geographic parthenogenesis (Butlin, 2002), in which sexual forms are restricted to areas around the Mediterranean Sea, while parthenogens spread to the rest of the world, presumably due to a greater capacity for colonization.

5 | CONCLUSIONS

Eiseniella tetraedra has been found to have a high genetic diversity in Europe. This diversity was classified into 11 lineages nested in five clades. Clades I and II were widely distributed in Europe, while the others had a limited distribution. Clade I was more represented in cold biogeographical regions such as the Continental, the Atlantic or even the Arctic, while clade II was prevalent in Mediterranean regions. Potential niches were also consistent with distribution trends. This is consistent with the phylogeographic patterns of other cosmopolitan and parthenogenetic earthworms. The clade III is largely restricted to Eastern Europe and appears to be the original clade. Clades IV and V were mostly present in Scandinavian Peninsula. The presence of restricted clades in the Iberian and Scandinavian Peninsula and Eastern Europe, suggests that the three acted as refugia during the LGM. Thus, both the "*tabula rasa*" and nunatak hypotheses could apply to *E. tetraedra* in Europe.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/. Reference numbers are available in Supplementary Table 6.

ORCID

Irene de Sosa https://orcid.org/0000-0002-4020-6375
Daniel F. Marchán https://orcid.org/0000-0001-7971-4329
Marta Novo https://orcid.org/0000-0001-7902-3819
Christer Erséus https://orcid.org/0000-0002-9251-4046
Emmanuel Lapied https://orcid.org/0000-0001-7242-6703
Mišel Jelić https://orcid.org/0000-0003-4119-3948
Aleksandra Jabłońska https://orcid.org/0000-0001-6178-8506
Tímea Szederjesi https://orcid.org/0000-0001-7695-1468
Ana Almodóvar https://orcid.org/0000-0003-1465-3857
Darío J. Díaz Cosín https://orcid.org/0000-0001-8415-3001

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