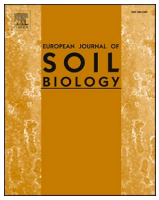
European Journal of Soil Biology 108 (2022) 103382

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: www.elsevier.com/locate/ejsobi



Molecular data confirms the existence of distinct lineages within *Lumbricus friendi* (Cognetti 1904) and related "friends"

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ARTICLE INFO

Handling Editor: S. Schrader

Keywords:
Earthworms
16S
COI bar Coding
AFLP
Genetic diversity
Lumbricus heracleus

ABSTRACT

Lumbricus friendi is a lumbricid earthworm mainly found in western Europe, considerably less well studied than its close relative *L. terrestris* and until now, has not been a subject of taxonomical debate. However, its disjointed geographical distribution suggesting striking similarities to the Celtic fringes and of the so-called "Lusitanian" species merits further investigation. Our aim was to examine the genetic variation of this species and other related taxa within the genus (*L. terrestris, L. rubellus, L. rubellus friendoides* and *L. friendi bouchei*) to test for the existence of cryptic lineages that might explain its current distribution. Using mitochondrial (16S and COI) and nuclear (Amplified Fragment Length Polymorphism, AFLP) markers, we provide the first genetic basis not only to support the recent claim that *L. friendi bouchei* should be elevated to species rank (*L. bouchei* Zicsi and Csuzdi 1999), but also to conclude that *L. rubellus friendoides* is a valid species too, deserving a new name (*L. heracleus* stat. nov.). In addition, the AFLP results indicated the existence of a high cryptic diversity within *L. friendi* populations, which lacked geographic structure, resulting in the French samples being genetically closer to those from Ireland and Wales than to the Spanish ones. Our findings further highlight the likelihood that *L. friendi* and *L. bouchei* might have been overlooked or confounded with *L. terrestris/L. herculeus* and question the reliability of *L. friendi* records reported in the literature and those deposited in museums and sequence libraries (we provide evidence that this seem to be the case with two examples). We therefore advocate for a better link between morphological diagnostic characters and molecular sequences and the taxonomical validation of museums' collections and sequence repositories.

1. Introduction

Lumbricus friendi (Cognetti 1904) is a lumbricid earthworm species predominantly found in western Europe [1]. Its limited distribution contrasts with that of its closely related species *Lumbricus terrestris* (Linnaeus 1758) having a much broader geographical range, from boreal climates to temperate areas [2]. However, both species can co-occur, for example in southwest Ireland [3] and in a few locations in northwest Spain (e.g. [4]). Both species exhibit the same ecological behaviour by building vertical burrows and feeding on the soil surface (i.e. anecic ecological group *sensu* [1]), suggesting that some kind of inter-specific competition might occur.

The native origin of *L. friendi* is believed to be Franco-Iberian [5] or, more specifically, Iberian-Aquitanian according to Bouché [1]. From there the species could have been transported by humans northwards reaching mainland Great Britain [3,6] and Ireland [7–9], southwards far-reaching north Africa [10–13] and eastwards to Germany [14–16], Italy [11,17], Austria [18], Switzerland [19,20] and Serbia [5]. In addition, *L. friendi* has also been introduced in North America (Baltimore: [21]; Maryland [22–26]), as well as in South America (Brazil [27]: and Uruguay [28]). Recently, a few specimens have been recorded in São Miguel Island in Azores [29], which reinforces the idea of current distribution being closely related to human transport. This disjointed geographical distribution of *L. friendi* has been noticed before [11], and outside its native range the presence of this species is highly localised, with the majority of the reported records representing few specimens

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https://doi.org/10.1016/j.ejsobi.2021.103382
Received 23 September 2021; Received in revised form 11 December 2021; Accepted 23 December 2021
Available online 29 December 2021

taken from a single location (e.g. [5,19]).

The distribution of *L. friendi* in Great Britain and Ireland is even more intriguing, being a species originally described from Ireland (as *Lumbricus papillosus* by Friend in 1893 [30], but renamed *Lumbricus friendi*, in honour of its discoverer, by Cognetti de Martiis [31] who collected it in the French Pyrenees). Because of the intense commercial trade with Great Britain, this was expected to be the foreseeable link for earthworms between the two isles. However, according to the published literature, it has only been recorded from a very limited number of locations in England [3,6,32], Wales and the Channel Islands [18]. If we accept the Franco-Iberian origin [1,5], the species could have made its way to Ireland (and from there perhaps to Great Britain) via accidental transport by prehistoric Iberian settlers arriving in boats, as many other wildlife species that have re-colonised Ireland after the ice retreated. Indeed, the intensive trading between Ireland and Spain is believed to be the undoubted origin of certain Irish species found only in northern Spain and Portugal such as the "Mediterranean'' heather *Erica erigena* [33] and the slug *Geomalacus maculosus* [34]. In relation to this, the term "Lusitanian" has been used to define the disjunct distribution of those species that have their origin centre in the Iberian Peninsula and an Atlantic-Mediterranean distribution, with the northernmost limits being Britain or Ireland. Among them there are many soil invertebrates, such as several species of gastropods, insects, woodlice, spiders and the earthworm *L. friendi* [35].

Because L. *friendi* is diploid and sex obligate (see [36] and references therein), its capabilities to invade new territories is expected to be low compared to those polyploid and parthenogenetic species with high clonal diversity, which tend to be 'peregrine' [37]. Therefore, the introduction events (single vs. multiple) and migration pathways (short vs. large distances) could have a strong influence on its genetic structure [38]. In relation to this, our previous molecular studies showed no genetic differences (COI and 16S genes) between the Irish specimens of *L. friendi* and those collected in NW Spain [36]. However, similar studies on populations from a wider geographical range (including Great Britain and other European areas) are lacking and would allow us to better understand the current sketchy distribution of *L. friendi* in western Europe as well as decipher its main spreading routes that permitted their successfully establishment in its present inhabited areas. Therefore, in this study, we examined the genetic structure of *L. friendi* by collecting specimens from their Atlantic-Mediterranean distribution origin (Spain, France, Great Britain and Ireland, including one of the type localities in Ireland where this species was first recorded), and compared it with two congeneric species (*L. terrestris* and *L. rubellus*) as well as with two problematic subspecies (*L. friendi bouchei* and *L. rubellus friendoides*) which have been described based on a few morphological differences to their respective nominal species [1]. Since both 16S rRNA and COI sequence fragments have a limited discriminatory value above the genus level, while being highly informative for solving intrageneric taxonomical uncertainties within Lumbricidae [36], genetic variation of the selected taxa was first derived from these two mtDNA genes to assess their interspecific relationships. Then we used amplified fragment length polymorphism (AFLP) markers to confirm these phylogenetic relationships and to examine the genetic structure of the *L. friendi* populations investigated here for further taxonomical discrimination.

2. Materials and methods

2.1. Earthworm samples and morphological data

Earthworms belonging to the species *Lumbricus friendi* were obtained from several locations in Spain (Galicia region, NW), Great Britain (Bangor, Wales), Ireland (including one type locality: Leeson Park, Dublin), and France (Midi-Pyrénées and Limousin regions), thanks to the efforts of several earthworm experts (Table 1). In addition, we also collected specimens of two closely related species, *L. terrestris* and *L. rubellus*, as well as of two problematic taxa described by Bouché [1]: *L. rubellus friendoides* (possibly a synonym of the nominal species) and *L. friendi* var. *lineatus* (later renamed *L. friendi bouchei* by Zicsi and Csuzdi [39] and recently elevated to species rank (*L. bouchei*) by Szederjesi et al. [40]) (Table 1). Morphological identification and taxonomic assignment were performed by the experts who collected the specimens at the selected sites and if necessary, further confirmation was obtained from specialised identification keys [1,18].

We opted for analysing fresh specimens instead of comparing the new sequences with all those stored in GenBank and BOLD systems to avoid introducing any genetic information that has not been properly

Table 1
Taxa analysed using molecular marker (COI, 16S and AFLPs).

Species	Location	Country	Coordinates	Collector
L. friendi	Glen of the Downs, County Wicklow, Dublin	Ireland	53° 8′ 21.013'' N, 6° 7′ 11.165'' W	Dr. Olaf Schmidt & Dr. M.J.I. Briones
L. friendi	Dun Laoghaire, Dublin	Ireland	53° 17′ 0.739'' N, 6° 9′ 20.684'' W	Dr. Olaf Schmidt & Dr. M.J.I. Briones
L. friendi	UCD campus, Dublin	Ireland	53° 18′ 29.753'' N, 6° 13′ 25.102'' W	Dr. Olaf Schmidt & Dr. M.J.I. Briones
L. friendi	Booterstown, Dublin	Ireland	53° 18′ 14.17" N, 6° 12′ 4.914" W	Dr. A. Keith
L. friendi	Dropping well, Dublin	Ireland	53° 18′ 28.75" N, 6° 15′ 18.295" W	Dr. A. Keith
L. friendi	Leeson Park, Dublin	Ireland	53° 19′ 42.823" N, 6° 15′ 6.828" W	Dr. A. Keith
L. friendi	Johnstown Castle, Wexford	Ireland	52° 17′ 35.844" N, 6° 30′ 8.388" W	Dr. Olaf Schmidt
L. friendi	Redes, A Coruña	Spain	43° 25′ 45.372" N, 8° 12′ 11.064" W	Dr. M.J.I. Briones
L. friendi	Monte Alto, A Coruña	Spain	43° 22′ 46.717" N, 8° 24′ 16.67" W	Dr. M.J.I. Briones & Dr. R. Schmelz
L. friendi	Parroquia de Castriz, Santa Comba, A Coruña	Spain	43° 5′ 11.333'' N, 8° 46′ 33.599'' W	Dr. M.J.I. Briones
L. friendi	Hospital, Tomiño, Pontevedra	Spain	42° 0′ 24.671" N, 8° 43′ 53.112" W	Dr. M.J.I. Briones & Dr. M.F.C. Lago
L. friendi	Tomiño, Pontevedra	Spain	42° 0′ 10.396" N, 8° 43′ 33.933" W	Dr. M.J.I. Briones & Dr. M.F.C. Lago
L. friendi	Salceda de Caselas, Pontevedra	Spain	42° 6′ 13.688'' N, 8° 33′ 40.011'' W	Dr. M.J.I. Briones
L. friendi	Isla de Miño, Rábade, Lugo	Spain	43° 7′ 49.85'' N, 7° 36′ 30.77'' W	Dr. M.J.I. Briones
L. friendi	Baamonde, Lugo	Spain	43° 10′ 34.781'' N, 7° 45′ 23.796'' W	Dr. M.J.I. Briones
L. friendi	A Derrasa, Ourense	Spain	42° 19′ 18.816" N, 7° 47′ 10.173" W	Dr. M.J.I. Briones
L. friendi	"Roman Camp", Bangor	United Kingdom	53° 14′ 0.564'' N, 4° 7′ 39.238'' W	Dr. M.J.I. Briones & Dr. A. Keith
L. friendi	"The poplars", Bangor	United Kingdom	53° 13′ 46.274'' N, 4° 7′ 47.954'' W	Dr. M.J.I. Briones & Dr. A. Keith
L. friendi	La Rochelle, Limousin Region	France	46° 4′ 40.084" N, 1° 5′ 41.794" W	Dr. Guénola Pérès
L. friendi	Midi-Pyrénées Region	France	43° 14′ 35.866" N, 1° 3′ 57.016" W	Dr. Guénola Pérès
L. friendi bouchei	Isla de Miño, Rábade, Lugo	Spain	43° 7′ 49.85'' N, 7° 36′ 30.77'' W	Dr. M.J.I. Briones
L. rubellus	Zas, A Coruña	Spain	43° 7′ 4.402'' N, 8° 54′ 8.857'' W	Dr. M.J.I. Briones
L. rubellus friendoides	Tomnafinnoge, County Wicklow, Dublin	Ireland	52° 58′ 47.5" N, 6° 2′ 53.746" W	Dr. Olaf Schmidt & Dr. M.J.I. Briones
L. terrestris	Redes, A Coruña	Spain	43° 25′ 45.372" N, 8° 12′ 11.064" W	Dr. M.J.I. Briones
L. terrestris	Lancaster	United Kingdom	54° 2′ 47.67'' N, 2° 48′ 2.663'' W	Dr. Trevor Piearce

validated by exhaustive taxonomical examination.

Morphological data of the examined specimens (body length and segment number) was also recorded as they have been proven to provide useful complementary information in previous discriminations between divergent lineages within *L. terrestris* [41]. The morphological examination included the freshly collected worms for this study, and also the preserved *L. friendi* specimens deposited by different donators at the Natural History Museum in London (UK), National Museum of Scotland in Edinburgh (UK), National Museum of Ireland (Natural History) in Dublin and the Museo Regionale di Scienze Naturali in Torino (Italy) (Table 2). However, the physical examination of the earthworm collection at the NM of Scotland from a published survey [42] revealed that all specimens labelled as *L. friendi*, had been misidentified and they were all either *L. terrestris* or immature *Lumbricus* spp. (Table 2) and therefore excluded from the morphological study. Analysis of Variance (ANOVA) was used to test for significant differences in body length and number of segments between populations, with separations of the means being conducted using Tukey's Honest Significant Difference (HSD) test ($\alpha = 0.05$). These statistical analyses were performed using SAS System Release 9.3 (SAS Institute Inc., Cary, NC).

2.2. DNA extraction, mtDNA sequencing and AFLP analysis

The specimens sampled for genetic analyses (Table 1) were dissected under a stereoscopic microscope and tissue samples of the body wall were preserved in absolute ethanol at 4 °C. Genomic DNA was isolated from a ~2 mm^2 piece of each tissue sample using a hexadecyltrimethylammonium bromide (CTAB) and chloroform extraction protocol following Galindo et al. [43] modified from Winnepenninckx et al. [44]. After ethanol precipitation, DNA quantity and quality were measured in a BioDrop μlite spectrophotometer (BioDrop). All DNA samples were normalised to 50 ng μl^{-1} prior to further genetic analyses.

Two fragments of the mitochondrial COI and 16S rRNA genes were amplified using the primer combinations LCO1490 and HCO2198 [45], 16L29 [46] and 16SBr [47], respectively. Amplifications were carried out for each gene in a 50 μL final volume containing 100 ng of DNA, 50 μM of each dNTP (Thermo Fisher), 50 μM of each primer, 15 μM of $MgCl_2$ and 0.5 U of JumpStart™ *Taq* DNA Polymerase (Sigma) in 1X PCR buffer (Sigma). A total of 35 amplification cycles at 95 °C, 48 °C and 72 °C, 30 s each were employed. PCR products were then purified with ExoSAP-IT (Thermo Fisher) and sequenced in both directions with the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Thermo Fisher), following manufacturer's instructions. The sequences were analysed on an ABI PRISM 3130 Genetic Analyzer (Thermo Fisher) at the genomics facility at CACTI (Centro de Apoyo Científico y Tecnológico a la Investigación, Universdade de Vigo, Spain). Sequences were visualized, edited and aligned with MUSCLE v.3.8.31 [48] set to default parameters (see also Table S1).

The AFLP analysis was carried out following the protocol described by Galindo et al. [49] although different combinations of primers with selective nucleotides were used at the selective PCR step. In brief, for each specimen, 100 ng of DNA undergone enzymatic digestion with 4 U of *Eco*RI and 2 U of *Mse*I (New England Biolabs) for 3.5 h at 37 °C. Then a ligation reaction was carried out by adding to the digestion 3 μl of a solution with 5 pmol of *Eco*RI and *Mse*I adaptors and 0.25 U of T4 DNA ligase (Roche) in 1X T4 DNA ligase buffer containing ATP. The ligation was incubated for 16 h at 16 °C. Preselective PCR reactions were performed in 10 μl volumes containing 24 μl of 1:4 diluted ligation products, 2 mM of $MgCl_2$, 0.3 mM dNTPs, 5 pmol of *Eco*RI-A and *Mse*I-C preselective primers and 0.3 U of *Taq* polymerase in 1X PCR buffer (Bioline). Then, selective PCR reactions (10 μl final volume) were conducted with 1 μl of 1:4 diluted preselective, 2 mM of $MgCl_2$, 0.3 mM dNTPs, 4 pmol of FAM labelled Eco selective primer (2.5 pmol in the case of NED labelled Eco primers) and 5 pmol of unlabelled Mse primer and 0.3 U of *Taq* polymerase (Bioline) in 1X PCR buffer. Four different selective PCR amplifications were performed: Eco-ACT (FAM labelled) + Mse-CAC, Eco-AAG (NED) + Mse-CAC, Eco-ACT (FAM) + Mse-CTA, Eco-AAG (NED) + Mse-CTA. PCR conditions, adaptor and primer sequences are described in Galindo et al. [43]. PCR products of the first two selective amplifications (FAM, NED) were analysed simultaneously with a GeneScan 500 ROX size standard (Thermo Fisher) on an ABI PRISM 3130 Genetic Analyzer (Thermo Fisher) at the genomics facility at CACTI. A second run on the sequencer was performed with the last two selective primer combinations. The analysis of the electropherograms was carried out in PeakScanner v.2.0 (Applied Biosystems) in order to create the combined table needed for RawGeno v2.0 [50], where bins for loci were assigned by visual inspection of all the samples simultaneously overlapping and a table of peak heights was generated for the selected bins (loci) for all individuals. Then the replicated samples (30% of the total) were used to estimate the error rate in the R package [51] AFLPtools (https://github.com/genevalab/AFLPTools) based on the method described by AFLPScore [52] and that estimate the number of mismatched loci between replicates divided by the total number of comparisons (i.e. all loci across replicated comparisons). The final genotypes were also generated with AFLPtools. We replicated 30% of the analysed samples across all the AFLP steps and all primer combinations in order to estimate the genotyping error rate, which was ~5% on average. Finally, a total of 598 polymorphic AFLP loci for 107 individuals (Table S2) were used in subsequent genetic analyses.

2.3. Genetic analyses

Maximum-likelihood (ML) phylogenetic analysis (with 500 bootstrap replicates) was performed using PHYML [53] implemented using Geneious v.2021.0.3 (https://www.geneious.com) independently for COI (561 bp) and 16S (477 bp) mtDNA genes. TN93 + G was selected as the best-fit nucleotide substitution model employing JModelTest2 [54] for both fragments. Thereafter intra and inter-group K2P genetic distances were estimated using MEGA X [55], as it is the most effective model when genetic distances are low [56].

AFLP-SURV v.1.0 [57] was used to calculate the percentage of polymorphic loci (5%; loci with allele frequencies comprised between 0.05 and 0.95) and the expected heterozygosity as a measure of genetic diversity within population or species. This AFLP dataset (598 polymorphic loci) was used to perform a Discriminant Analysis of Principal Components (DAPC) with Adegenet v.2.1.4 [58] in R [51], with only the two first principal components (PCs) and two discriminant functions being retained. Pairwise Nei's genetic distances were calculated with StAMPP v.1.6.2 [59].

Finally, a Bayesian clustering analysis was performed on the AFLP profiles of *L. friendi* individuals to determine the genetic structure of these samples (i.e. number of genetic clusters). This analysis was performed using STRUCTURE v.2.3.4 [60] from K = 1 until K = 5, which is the number of geographical regions (Ireland, Wales, France, and Spain) plus one. Five replicate runs of 250,000 iterations (burn-in 50000) were performed for each K-value. These analyses were done assuming an admixture model, correlated allele frequencies and no prior population information. STRUCTURE HARVESTER [61] was then run to estimate the Delta K (ΔK) [62], which was used as the most probable number of clusters or best K.

3. Results

3.1. Genetic diversity within L. friendi and closely-related taxa

Fig. 1 shows the maximum likelihood (ML) trees for the 16S rRNA and COI for the earthworm samples corresponding to the species listed in Table 1. In both trees, the basal branches show a clear separation (as measured by the bootstrap support) between the two congeneric species (*L. terrestris* and *L. rubellus*), the two problematic subspecies (*L. rubellus friendoides* and *L. friendi bouchei*) and the rest of specimens assigned to the nominal species *L. friendi*. Furthermore, both trees also show that

Table 2
Examined specimens labelled as "*Lumbricus friendi*" which have been deposited at the relevant Museums.

Museum source	Labelled species	Museum code	Country	Location	Collected by	Date	Determined by	Maturity	Additional comments
Natural Science Museum of Torino	*Lumbricus friendi*	MZUT OI98OA	France	Ria, Eastern Pyrenees	A. Borelli	1904	L. Cognetti de Martiis	Juvenile	
	Lumbricus friendi	MZUT OL981	France	Bain de la Prejte, Eastern Pyrenees	A. Borelli	1904	L. Cognetti de Martiis	Juvenile	
	Lumbricus friendi	MZUT OI983	France	Mont Louis, Eastern Pyrenees	A. Borelli	1904	L. Cognetti de Martiis	Mature Juvenile Juvenile	
	Lumbricus friendi	MZUT OI982	France	Gavarnie, High Pyrenees	A. Borelli	1904	L. Cognetti de Martiis	Mature Juvenile Mature Mature Mature Mature Mature Juvenile Mature Mature Mature Mature	Fragmented specimen Fragmented specimen Fragmented specimen Clitellum extending over 6 segments instead of 5
	Lumbricus friendi	MZUT OI982b	France	Gavarnie, High Pyrenees	A. Borelli	1904	L. Cognetti de Martiis	Mature Mature Juvenile Juvenile Juvenile Mature Juvenile Juvenile	Fragmented specimen Fragmented specimen
NMHN Dublin	***Lumbricus friendi***	**NMNH: 1892.186.18**	**Ireland**	**Glasnevin, Co. Dublin**		**1892**	**H. Friend**	**Mature**	**Holotype: mounted on a glass plate and dissected**
	Lumbricus papillosus	NC:zool.jar.507	Ireland	Dundrum		February 18, 1903		Mature	
	Lumbricus papillosus [ilegible label]		Ireland	Kilmashogue		February 11, 1906		Mature Mature	
	Lumbricus friendi			Front Lawn		March 08, 1906		Mature	
	Lumbricus friendi		France	Pyrenees, Eaux-Bonnes		Oct. 1892		Mature Sub-adult	R. F. Scharff collection. Three individuals, but third specimen probably not *L. friendi*
	Lumbricus friendi	NMNH: 1892.186:1906.299	Ireland	Glencar, near hotel, Co. Kerry		June 26, 1906		Mature Mature Mature	
	Lumbricus friendi	NMNH: 1906.299.2	Ireland	Co. Kerry	J. N. Hackett			Mature Mature	
	Lumbricus friendi	NMNH: 1977.191.1	Ireland	Johnstown Castle Co., Wexford	D.C.F. Cotton	February 22, 1977	D.C.F. Cotton	Mature Mature	
NHM London	*Lumbricus friendi*	1983:54:53–54	United Kingdom	Bangor, North Wales	T.G. Piearce		C. Webster	Mature Sub-adult	

(*continued on next page*)

Table 2 (*continued*)

Museum source	Labelled species	Museum code	Country	Location	Collected by	Date	Determined by	Maturity	Additional comments
	Lumbricus friendi	1982:2:28–30	Spain	Ciudad Universitaria, Madrid	A.G. Moreno & D.J. Diaz-Cosin	1978	A.G. Moreno & D.J. Diaz-Cosin	Mature Mature Mature	
	Lumbricus friendi	1976:14:36–38	United Kingdom	Bangor, North Wales	T.G. Piearce	March 13, 1974	T.G. Piearce	Mature Mature Sub-adult	
	Lumbricus friendi	1980:1:10–20	Ireland	Johnstown Castle Co., Wexford	D.C.F. Cotton	February 22, 1979	D.C.F. Cotton	Mature Mature Mature Mature Sub-adult Sub-adult Sub-adult Sub-adult Sub-adult Sub-adult	
NM Scotland[a]	*Lumbricus friendi*	F100	United Kingdom	Wigtownshire, Dumfries	Boag et al. [42]	October 02, 1991	Boag et al. [42]	Juvenile Juvenile Juvenile Mature Mature	*Lumbricus* spp. *Lumbricus* spp. *Lumbricus* spp. *Lumbricus terrestris* *Lumbricus terrestris*
	Lumbricus friendi	F045	United Kingdom	Duns, Borders	Boag et al. [42]	September 24, 1991	Boag et al. [42]	Sub-adult Mature Mature	*Lumbricus terrestris* *Lumbricus terrestris* *Lumbricus terrestris*
	Lumbricus friendi	F077	United Kingdom	Stow, Borders	Boag et al. [42]	September 27, 1991	Boag et al. [42]	Sub-adult Mature Mature	*Lumbricus terrestris* *Lumbricus terrestris* *Lumbricus terrestris*
	Lumbricus friendi	F048	United Kingdom	Thurso, Sutherland	Boag et al. [42]	April 08, 1992	Boag et al. [42]	Juvenile	*Lumbricus* spp.
	Lumbricus friendi	F003	United Kingdom	Ellon, Grampian	Boag et al. [42]	October 31, 1991	Boag et al. [42]	Sub-adult	*Lumbricus terrestris*
	Lumbricus friendi	F087	United Kingdom	Amulree, Tayside	Boag et al. [42]	October 11, 1991	Boag et al. [42]	Mature	*Lumbricus terrestris*
	Lumbricus friendi	F063	United Kingdom	Maryculter, Grampian	Boag et al. [42]	October 25, 1991	Boag et al. [42]	Juvenile Juvenile	*Lumbricus* spp. *Lumbricus* spp.
	Lumbricus friendi	F022	United Kingdom	Angus, Tayside	Boag et al. [42]	November 13, 1991	Boag et al. [42]	Juvenile Juvenile	*Lumbricus* spp. *Lumbricus* spp.
	Lumbricus friendi	F044	United Kingdom	Duns, Borders	Boag et al. [42]	September 24, 1991	Boag et al. [42]	Juvenile Mature	*Lumbricus* spp. *Lumbricus terrestris*
	Lumbricus friendi	F076	United Kingdom	Maryhill, Glasgow	Boag et al. [42]	November 06, 1991	Boag et al. [42]	Mature	*Lumbricus terrestris*
	Lumbricus friendi	F050	United Kingdom	Lockerbie, Dumfries	Boag et al. [42]	September 20, 1991	Boag et al. [42]	Mature Mature	*Lumbricus terrestris* *Lumbricus terrestris*
	Lumbricus friendi	F070	United Kingdom	Glencaple, Dumfries and Galloway	Boag et al. [42]	October 22, 1991	Boag et al. [42]	Juvenile Mature Mature Mature	*Lumbricus* spp. *Lumbricus terrestris* *Lumbricus terrestris* *Lumbricus terrestris*
	Lumbricus friendi	F037	United Kingdom	Maybole, Strathclyde	Boag et al. [42]	October 01, 1991	Boag et al. [42]	Mature	*Lumbricus terrestris*
	Lumbricus friendi	F082	United Kingdom	Clovenfords, Borders	Boag et al. [42]	September 27, 1991	Boag et al. [42]	Juvenile Juvenile Juvenile	*Lumbricus* spp. *Lumbricus* spp. *Lumbricus* spp.
		F004						Juvenile	*Lumbricus* spp.

(*continued on next page*)

Table 2 (*continued*)

Museum source	Labelled species	Museum code	Country	Location	Collected by	Date	Determined by	Maturity	Additional comments
	Lumbricus friendi		United Kingdom	Inverurie, Grampian	Boag et al. [42]	November 05, 1991	Boag et al. [42]		
	Lumbricus friendi	F094	United Kingdom	Glenoran, Highland	Boag et al. [42]	December 12, 1991	Boag et al. [42]	Mature	*Lumbricus terrestris*
	Lumbricus friendi	F043	United Kingdom	Ruthven, Borders	Boag et al. [42]	September 25, 1991	Boag et al. [42]	Sub-adult	*Lumbricus terrestris*
	Lumbricus friendi	F026	United Kingdom	Oban, Strathclyde	Boag et al. [42]	December 16, 1991	Boag et al. [42]	Juvenile Juvenile Juvenile	*Lumbricus* spp. *Lumbricus* spp. *Lumbricus* spp.
	Lumbricus friendi	F052	United Kingdom	Thornhill, Dumfries	Boag et al. [42]	September 18, 1991	Boag et al. [42]	Mature Juvenile	*Lumbricus terrestris* *Lumbricus* spp.
	Lumbricus friendi	F053	United Kingdom	Alexandria, Strathclyde	Boag et al. [42]	December 05, 1991	Boag et al. [42]	Juvenile Mature	*Lumbricus* spp. *Lumbricus terrestris*
	Lumbricus friendi	F078	United Kingdom	Dalkeith, Lothian	Boag et al. [42]	September 26, 1991	Boag et al. [42]	Juvenile Juvenile	*Lumbricus* spp. *Lumbricus* spp.
	Lumbricus friendi	F010	United Kingdom	Methlick, Grampian	Boag et al. [42]	October 29, 1991	Boag et al. [42]	Juvenile Mature Mature Mature	*Lumbricus* spp. *Lumbricus terrestris* *Lumbricus terrestris* *Lumbricus terrestris*

[a] Since no museum code was available each vial is linked to the 'Farm code' where the specimens were collected (and contained all '*L. friendi*' specimens from that farm).

both *L. rubellus* and *L. rubellus friendoides* form one well-supported clade (93% and 100% BS for 16S and COI, respectively), although this latter grouping most likely consists of two genetically isolated taxa (>93% BS; Fig. 1). The K2P interspecific distances for COI and 16S show that the divergence between these two taxa is 13.9% and 3.3%, respectively (Table 3), which was very similar to the calculated pairwise distances between the other *Lumbricus* species examined here (e.g., 15.5% and 4.1%, respectively between *L. friendi* and *L. terrestris* from Great Britain; Table 3).

In addition, both trees also placed *L. friendi bouchei* as a sister taxa of *L. friendi* with good support (100% BS; Fig. 1), which reinforces the notion that this subspecies might deserve the species rank. In this case, the divergence between these two taxa ranged between 16.4 and 17% for COI and around 4% for 16S (Table 3), which are in line with the interspecific variation observed between *L. friendi* and *L. terrestris* but lower than between any of these two species and *L. rubellus* (Table 3).

According to the most conservative marker (16S), all *L. friendi* samples conformed to a monophyletic group (98% BS in Fig. 1a and KP2 interspecific distance <0.55% according to Table 3), despite the distinct geographical distribution of the specimens included. However, the more variable gene (COI), albeit confirming that all specimens were very closely related (81% BS; Fig. 1b), also revealed some intraspecific variability. For example, the two French samples from the Midi-Pyrénées (62% BS), two Galician samples (from Redes in A Coruña and Hospital in Tomiño) and the Dun Laoghaire (Dublin) specimens appeared to exhibit some genetic differences when compared to the rest of the populations (Fig. 1b). The mean intraspecific variation for COI within *L. friendi* (Table 3) was around 4%, which could indicate that some cryptic diversity exists within the nominal species.

The comparative analyses of the body size of these earthworms

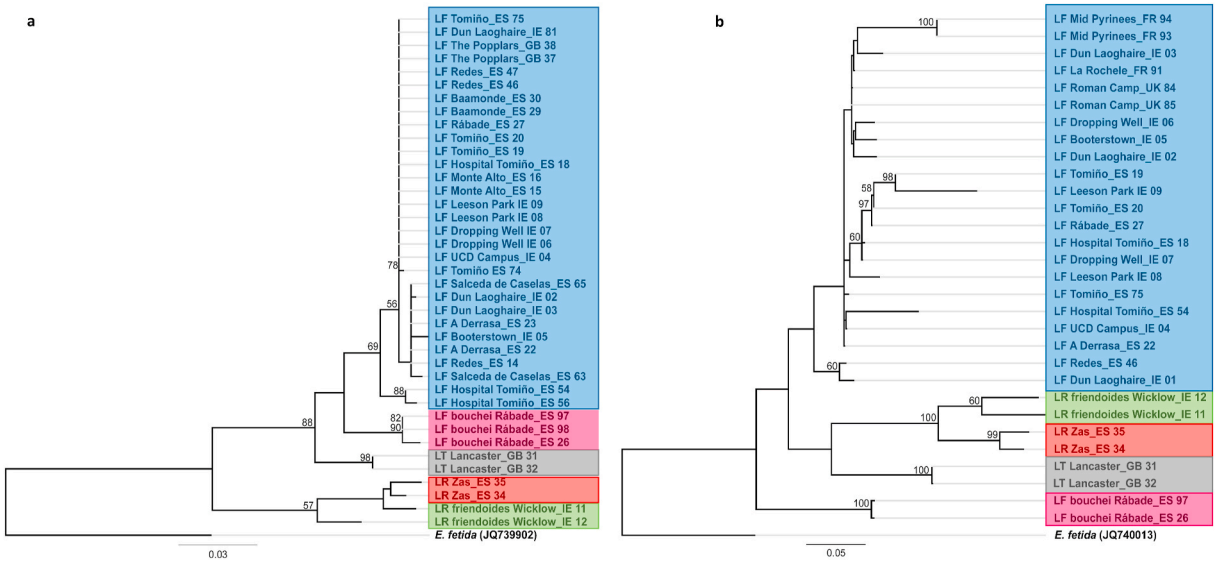

Fig. 1. Maximum-likelihood (ML) phylogenetic trees for the 16S (a) and COI (b) mtDNA genes of the *Lumbricus* taxa examined in this study (see Table 1). Numbers above branches are bootstrap values (only values above 50% are shown). The scale bar indicates the number of expected substitutions per site. ISO 3166-1 alpha-2 codes for countries are used: ES-Spain, FR-France, IE-Ireland, GB-Great Britain.

Table 3
Geographic differentiation between *Lumbricus* spp. populations estimated by pairwise evolutionary divergence.

	L. rubellus Spain	*L. rubellus friendoides* Ireland	*L. friendi* Spain	*L. friendi* Ireland	*L. friendi* Great Britain	*L. friendi* France	*L. friendi bouchei* Spain	*L. terrestris* Great Britain
COI K2P % (n = 30)								
L. rubellus Spain	4.80							
L. rubellus friendoides Ireland	13.92	10.22						
L. friendi Spain	19.24	20.65	5.20					
L. friendi Ireland	19.80	21.39	6.41	7.35				
L. friendi Great Britain	18.34	19.63	3.98	4.15	0.18			
L. friendi France	19.75	20.71	7.04	6.78	3.97	7.39		
L. friendi bouchei Spain	23.04	19.46	16.59	17.00	15.96	16.40	0.72	
L. terrestris Great Britain	19.53	18.14	14.93	16.67	15.52	15.95	17.15	0.18
16S K2P % (n = 39)								
L. rubellus Spain	1.48							
L. rubellus friendoides Ireland	3.29	4.35						
L. friendi Spain	9.50	9.08	0.55					
L. friendi Ireland	9.57	9.05	0.49	0.44				
L. friendi Great Britain	9.69	9.20	0.32	0.26	0.00			
L. friendi bouchei Spain	8.14	8.61	3.97	4.00	3.89		0.42	
L. terrestris Great Britain	8.69	8.81	4.26	4.38	4.10		4.56	0.00
AFLPs Nei's Distance (n = 107)								
L. rubellus Spain								
L. rubellus friendoides Ireland	0.049							
L. friendi Spain	0.060	0.055						
L. friendi Ireland	0.057	0.056	0.017					
L. friendi Great Britain	0.064	0.064	0.023	0.013				
L. friendi France	0.054	0.055	0.022	0.023	0.030			
L. friendi bouchei Spain	0.083	0.074	0.065	0.066	0.072	0.061		
L. terrestris Great Britain	0.073	0.064	0.071	0.069	0.074	0.070	0.083	
L. terrestris Spain	0.099	0.089	0.091	0.084	0.095	0.083	0.104	0.075

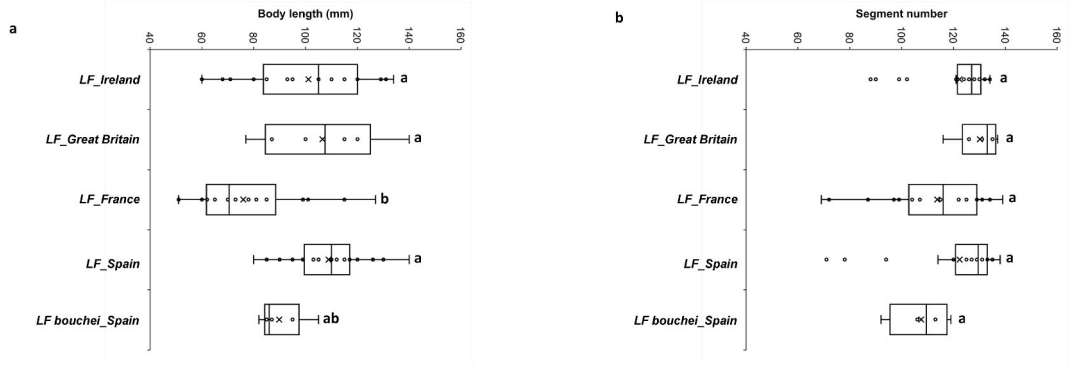

Fig. 2. Boxplots of morphological features for *L. friendi* specimens (including fresh collected samples and those preserved and deposited at the Natural History Museum of London, National Museum of Ireland in Dublin and the Museo Regionale di Scienze Naturali in Torino; see also Table 2) and *L. friendi bouchei*: a) body length, b) segment number. Each boxplot represents the median and the lower and upper quartiles. Different letters indicate significant differences between populations.

(including those adult specimens correctly identified and deposited at the different museums; Table 2) indicate that there is a high degree of overlap in relation to their body length and segment number across *L. friendi* s.l. (Fig. 2a and b). However, the French samples contained a significantly higher number of smaller specimens ($p < 0.0001$), with the majority being the specimens deposited in 1904 at the Museo Regionale di Scienze Naturali of Torino (measuring 67 mm on average). Furthermore, *L. friendi bouchei* showed some morphological differences, albeit not significant, when compared with the nominal species in relation to body length (90 mm vs. 97 mm, respectively; Fig. 2a), and segment number (107 vs. 120, respectively; Fig. 2b), but not in body mass (1.8 g vs 1.7 g; results not shown). However, due to the paucity of the data and the small number of specimens analysed, these results will require further confirmation.

Nonetheless, the analyses of the AFLP nuclear markers obtained from these samples (Fig. 3) provided further insights at the population level and allowed for the identification of different lineages within *L. friendi*. Indeed, and despite the mean Nei's genetic distances between populations of *L. friendi* being low (ranging from 0.013 to 0.104; Table 3), these results show that the Galician samples form a genetically well-consolidated group (Nei's intergroup genetic distances was 0.02 on average; Table 3) and are well differentiated from the rest of specimens collected in the other three countries (Fig. 3).

The great overlap between the Irish and Welsh AFLP profiles in the

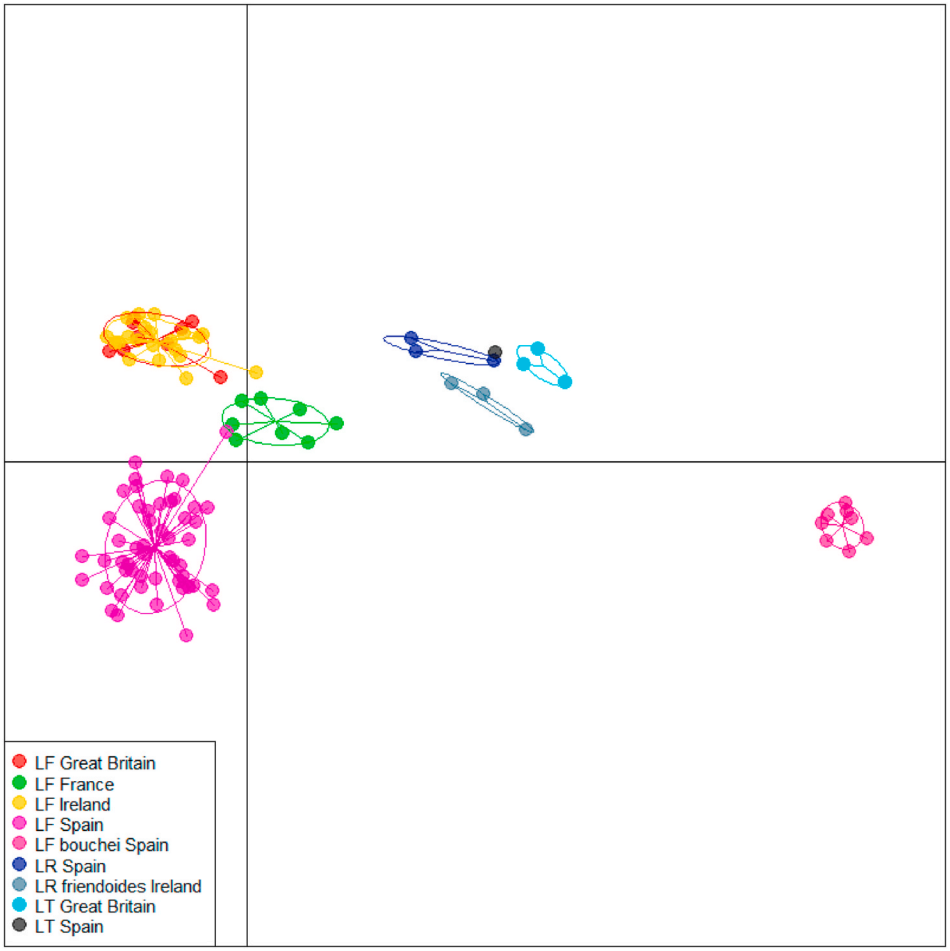

Fig. 3. Discriminant Analysis of Principal Components (DAPC) of AFLP loci for the three *Lumbricus* species investigated: *Lumbricus terrestris*, *L. rubellus*, *L. friendi* and two related taxa (*L. friendi bouchei* and *L. rubellus friendoides*) collected from Great Britain, Ireland, France and Spain. Discriminant functions 1 and 2 accounted for 7.9% and 4.7% explained variance, respectively.

DAPC plot (Fig. 3) also indicates that they have a high number of alleles in common and hence, the two populations are genetically indistinguishable (Nei's genetic distance = 0.013; Table 3). Interestingly, the AFLP genotypes of French samples show greater similarities to those of the Irish-Welsh group than to the Galician profiles (Fig. 3), which was also corroborated by the Bayesian clustering analysis with STRUCTURE that showed a value of K = 2 as the most probable number of genetic clusters within the *L. friendi* samples: Irish, Welsh and French samples forming one cluster and Galician a different cluster. This suggests that the latter population had experienced geographical segregation for a period of time long enough for genetic divergence to develop.

The distinct cluster formed by *L. friendi bouchei* samples along the first component of the DAPC analysis (Fig. 3) validates the results derived from the mtDNA markers (Fig. 1) and these specimens are genetically very different to *L. friendi.* According to our mtDNA data, the mean divergence between these two lineages was in the range of 16–17% and the Nei's genetic distance was greater than 0.061 (Table 3).

3.2. Taxonomical assignments

Because both the DNA sequences for two fragments of the mitochondrial COI and 16S genes and nuclear AFLP markers accurately discriminated between *L. friendi* and *L. friendi bouchei*, we can confirm that they are indeed different species, and *L. bouchei* Zicsi and Csuzdi 1999 is a valid species. The small morphological differences between these two very similar species are not as unequivocal as the genetic differences and requires expertise knowledge to enable the correct identification of these two taxa in future studies.

A more complicated nomenclature dilemma is the subspecies of *L. rubellus*, i.e. *L. rubellus friendoides*, which was also found to be genetically different from the nominal species according to the molecular markers used in this study. This is a large worm: our Irish specimens had a body length of 112.7 ± 6.7 mm and therefore, overlapping in size with the *L. friendi* specimens investigated here (Fig. 2a). Both the clitellum (extending from 1/n 26 to 1/n 33) and the tubercula pubertatis locations (28–31) are coincidental with those of *L. rubellus* and its main distinctive characteristic is the shape of the tubercula pubertatis forming two distinct dimples in 28–29 and 30–31 similar to those observed in *L. friendi*, as originally described by Bouché (1972). In his description it is also stated that "the general appearance is comparable to that of *L. herculeus*".

In view of this information and since the epithet derived from "*friendoides*" cannot be used, we propose *L. heracleus* as the objective nominal taxon for elevating this subspecies to species rank. The reasoning behind this etymological choice is that Heracles was known for his extraordinary strength, courage and cleverness, and the greatest divine hero in Greek mythology (adopted by the Romans who added some anecdotal details and renamed it as Hercules). By doing this, we reinstate Heracles' primacy and provide a new species name that refers to a morphological characteristic of this species, but it is now assigned to a unique sequence tag to enable future identifications/confirmations of new or past collected specimens. Since the Museum National d'Histoire Naturelle of Paris confirmed they are in the process of getting the whole collection of earthworms of Dr Marcel Bouché (pers. comm., August 25,

2021), a holotype could be designated when the specimens are finally transferred.

In the case of the divergent AFLP profile of *L. friendi* from Galicia, the low values observed for the Nei's genetic distance between this lineage and those of the populations from Wales, Ireland and France (0.017–0.022; Table 3) prevent any further taxonomical discrimination and further research is needed to estimate the true number of lineages present in this species.

4. Discussion

Phylogenetic ML analyses of mitochondrial COI and 16S mitochondrial genes showed the existence of interspecific genetic variation among three common European *Lumbricus* species (*L. terrestris*, *L. friendi* and *L. rubellus*), resulting in five distinct clusters: *L. terrestris, L. rubellus, L. rubellus friendoides, L. friendi bouchei* and *L. friendi*. The average COI sequence divergences between these five lineages ranged between 13.9 and 19.8%, in line with previous observations for interspecific divergence within this genus on these markers [41].

While . *L.terrestris*, *L. friendi* and *L. rubellus* are accepted as valid taxa, *L. rubellus friendoides* is considered to be an unassessed and uncertain taxon in some biodiversity databases (e.g. WORMS, EOL), a synonym of the nominal species (DriloBASE Taxo), not included in others (ITIS, Fauna Europaea), but reported as valid subspecies in Edaphobase (based on the records from a single project report by Sommer et al. [63]), with the latter data being transferred and accepted in GBIF. Besides the original description provided by Bouché [1], we have only found two research papers mentioning this subspecies, which provided evidence of its presence in west Germany [14] and of its anecic behaviour under laboratory conditions, being more comparable to that of *L. terrestris* than to *Aporrectodea longa* [64]. In addition, the French national long-term monitoring programme involving more than 250 trained observers has reported this subspecies as endemic from Alsace (EcoBioSoil; http://ecobiosoil.univ-rennes1.fr/). Morphologically, it is very similar to *L. rubellus rubellus*, except for its bigger size (140–180 mm vs. 60–130 mm) and the shape of the tubercula pubertatis according to Bouché [1], who recorded this subspecies in the north of Alsace and east of Lorraine areas in France. Our Irish specimens are smaller (113 mm on average) than those reported from France and hence, falling into the upper end of the body range given for *L. rubellus* (60–130 mm; Sims and Gerard [18]: 104), which suggests that these two subspecies can be easily mistaken. The size difference can also be seen in Ehrmann et al. ([65]: 77) who showed individual live biomasses of *L. rubellus* and *L. rubellus friendoides* from unidentified German long-term observation sites, ranging from about 0.7 to 1.6 g for *L. rubellus* (25 sites) and from about 2.2 to 7.1 g for *L. rubellus friendoides* (7 sites).

Furthermore, *L. rubellus* is considered to be a highly polymorphic species, and several divergent lineages have been described [66–73], although some of them do not appear to be reproductively isolated [70]. According to our analyses, both mitochondrial DNA and AFLP data evidenced that, even within the relatively small number of samples taken, *L. rubellus friendoides* can be elevated to species rank and given a different name: *L. heracleus* stat. nov. (*Lumbricus rubellus friendoides* Bouché [1]: 372–373). Although we acknowledge that a much larger genetic study (using multiple different markers on a larger sample size from different localities/countries) coupled with an exhaustive anatomical revision is needed in the future, we decided to provide a new taxonomic name upon the consideration of "divergent lineage" to comply with recent petitions that DNA sequences need to be connected to updated species names [74]. The observed COI lineage divergence (13.9%) is comparable to that measured among other *L. rubellus* lineages reported in the literature (11.5–16% [67,70,71]), and other cryptic lumbricid species (13–15% [66]), but slightly lower than the interspecific variation observed for the *Lumbricus* genus (14.8–23.7% [41]). According to our results, the mean COI K2P divergence within *L. rubellus friendoides* was 10%, a higher value than that observed within *L. rubellus* (4.8%) and could be indicative of the existence of some cryptic diversity in the former taxa that would warrant further investigation.

The AFLP data also corroborated the outcome from the mtDNA analyses and revealed an unexpected high cryptic diversity within *L. friendi* but with a high genetic identity, which is in agreement with previous observations for other earthworm species [75–79]. However, no clear link between geographical pattern and population distribution was observed, with the French samples being genetically closer to those from the Ireland and Wales than to the Spanish ones. This apparent lack of geographic structure is either the reflection of a non-panmictic mating system [79] and limited dispersal capability (even if it is human-mediated), not acting as effective barriers to gene flow [80], or the result of admixture due to extensive transport [75]. However, this explanation cannot be applied to accommodate the peripheral and north and western distribution of this species, which shows some similarities to the Celtic fringes observed for humans and small mammals [81]. The fact that this species has a Franco-Iberian origin [5] explains why the French populations were grouped with the Irish-British samples in the Bayesian clustering analysis and suggests that France was the most likely source for the introduction of this species to Ireland and Britain, but not why this species is well-established in southern Ireland and not in Great Britain. Although a more comprehensive sampling effort is needed to detect the presence of *L. friendi* in mainland Great Britain, the available literature indicates that this is a very rare species in Great Britain (except for Wales). The discoverer of this species, Hilderic Friend, stated in one of his publications: *"For twenty years I have sought it in vain in England, and as the search has been carried out in almost every part of the country, there seems good reason to believe that it is not English at all"* [82]. Therefore, it is possible to suggest that after *L. friendi* first colonised Great Britain, its small and dispersed distribution made it prone to be replaced by better adapted genotypes, as it has been proposed for small mammals [81], and with the obvious candidate being *L. terrestris* thought to also be native to Western Europe and now an invasive species (Global Invasive Species Database: http://www.iucngisd.org/gisd/species.php?sc=1555).

By contrast, the samples collected in NW Spain conformed a homogenous and well-differentiated group from the populations sampled in Ireland, Wales and France. Previous biogeographical studies of earthworms in the Iberian Peninsula concluded that Galicia and Asturias regions, west of León and northwest of Zamora provinces, together with the north Portugal (above Mondego River) conform a different biogeographical unit from the rest of the Iberian Peninsula in terms of their earthworm species assemblages [83]. This is favoured by an altitudinal gradient marked by the Cantabrian Mountains, León Mountains, and the highest mountain range in Portugal (Serra da Estrela). Indeed, rapid changes in topography and land cover within short distances have seen to favour endemism and speciation rates in vertebrates [84]. The sandy, highly organic and acidic soils that are dominant in this area could represent ecological constraints to the colonisation by other less acid-tolerant earthworm species (including *L. terrestris*), and has been advanced as the main argument to explain the divergence of *L. badensis* from its sister taxa *L. friendi* in the Black Forest of Germany between 8000 and 6000 years ago [85]. Furthermore, previous life history studies [86] have also shown that *L. friendi* produces smaller cocoons, but three times more, than *L. terrestris*, which is an indication that the former species is more adapted to unstable environments with extreme temperature regimes (such as many southern European regions).

Importantly, our mtDNA and AFLP data provided the first genetic basis to support the recent claim that *L. friendi bouchei* should be elevated to species rank [40] on the basis of morphological differences with the nominal species and their sympatric distribution. This taxon was originally described by Bouché [1] as *L. friendi* var. *lineatus* in reference to its linear, straight band-like tubercula pubertatis extending on segments 34–36, whereas in the nominal species *L. friendi* (or *L. friendi friendi*) they form two large clitellar papillae on segments 34 and 36 (and hence, its original designation as *L. papillosus* by Friend in

1893 [30]). Because names of varieties described after 1960 are no longer valid names, according to the International Code of Zoological Nomenclature (ICZN), Zicsi and Csuzdi [39] renamed this taxon as subspecies *Lumbricus friendi bouchei* in honour of the author who first described this taxon. The fact that our genetic analyses showed that COI K2P divergence between the two taxa is comparable to the mean interspecific divergence between the *L. terrestris* and *L. herculeus* lineages (17.5% [41]) and the genetic differentiation observed in the DAPC plot of the AFLP dataset allows us to conclude that *L. bouchei* Zicsi and Csuzdi 1999 is a valid species.

The material examined by Szederjesi et al. [40] was collected in the Midi-Pyrénées and in the list of locations by Bouché [1] the majority of the records are also from the Pyrénées and nearby regions (e.g. Aquitaine, Occitaine). Therefore, our specimens represent the most western location ever recorded for this species. However, Csuzdi and Szlávecz [21] indicated that *L. friendi* might have been overlooked in North America and confounded with *L. terrestris*. This possibility is expected to be even more likely when trying to identify specimens with linear or band-shaped tubercula as in the case of *L. bouchei.* Indeed, when we compared our genetic sequence of *L. friendi bouchei* Spain against the two main public repositories of DNA barcode sequences, BOLD and GenBank, we found a matching sequence in the latter database (96% similarity: MK731228), which corresponds to an individual captured in North America (Minnesota) and identified as *L. terrestris* [87]. Therefore, the results of our study adds more doubts about *L. friendi* records reported in the literature, as was the case in previous molecular work on *L. terrestris* [41]. As a case in point, our own work for the present study has revealed that the 47 earthworms sampled from Scottish farms and deposited as *L. friendi* at the NM Scotland [42] were identified incorrectly and were in fact *L. terrestris* (see Table 2).

Accurate taxonomic identification is imperative and some quality checks on the sequences submitted to these public repositories are required to verify their reliability. In relation to this, Meiklejohn et al. [88] assessed the performance of the two genomic repositories available for species assignation (GenBank and BOLD) and concluded that, although GenBank outperformed BOLD, for species-level identification of insects, plants and macro-fungi taxa, both produced ambiguous correct matches. Two potential causes for these misidentifications were postulated: cross-contamination with non-target specimens and lack of taxonomical validation of the DNA sequences stored in these two platforms [89]. If the sequences stored in DNA databases are incorrectly assigned to their putative species, errors can be transferred to subsequent analyses and research applications. Therefore, a better integration of taxonomical and molecular genetics expertise is needed to not only ensure the identity of the genetic material deposited in the sequence libraries, but also to find more reliable diagnostic criteria besides morphology and DNA barcoding [36,90].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are extremely grateful to Emma Sherlock (NHM London), Susan Chambers and Sankurie Pye (National Museums Collection Centre, NM Scotland), and Amy Geraghty (NM Ireland) for providing access to the specimens deposited in their collections. We would also like to express our great appreciation to Luca Ghiraldi and Roberta Tota (Museo Regionale di Scienze Naturali, Torino) for providing information and taking the measurements of the *L. friendi* specimens on which Cognetti based his publication in 1904. Thanks are also due to all the scientists who provided specimens. MJIB also wants to thank Mrs. J. Browne (UK CEH) for acquiring all the old references reporting *L. friendi*. D. Garcia-Souto was supported by a postdoctoral contract from Xunta de Galicia (Ref. ED481B-2018/091) and J. Galindo was funded by MICIU research grant (Ref. RTI2018-101274-J-I00). Authors also acknowledge funding for open access charge from Universidade de Vigo/CISUG.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejsobi.2021.103382.

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