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A vagrant clone in a peregrine species: Phylogeography, high clonal diversity and geographical distribution in the earthworm *Aporrectodea trapezoides* (Dugès, 1828)

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ABSTRACT

The peregrine lumbricid *Aporrectodea trapezoides* is originally from the Palaearctic region but is distributed worldwide. Little is known about its clonal diversity or the existence of a pattern of biogeographical diversification of clones. This study aimed to explore the evolutionary history of *A. trapezoides* by analysing the mitochondrial (COI and COII) and nuclear (28S rRNA and histone H3) DNA sequences of individuals collected in 11 different countries. High clonal diversity was found for this species, with thirty-seven clones clearly divided into two distinct lineages (I and II). The marked biogeographical boundary between these lineages corresponds to the line separating the Eurosiberian and Mediterranean climates in North Spain. Clone 1 was shared by one-third of the earthworms. While this clone was found in most of the sampled localities, the rest of the clones showed geographically-restricted distributions. This clone may have originated in the Mediterranean area of Central Spain. As it was obviously introduced in Australia and it was found in locations occupied by members of both lineages, we hypothesise that it may have also been introduced in other countries and that it could be a general-purpose genotype able to adapt to a wide range of niches.

1. Introduction

Earthworms are of particular phylogenetic and biogeographical interest because they are an ancient group with little mobility and are mostly confined to their areas of origin. Due to their restriction to small areas at all stages of their life cycle and their susceptibility to extreme environmental conditions (i.e., desiccation), their distributions can reflect past palaeogeographic events.

In contrast, in relatively recent times a few earthworm species have become widespread due to two main factors. First, human movement across the globe has caused their unintentional transportation in connection with agriculture and commerce. Secondly, in recent decades, the phenomenon of biological improvement in soils via the stimulation or introduction of earthworms has started to attract the attention of scientific research. During this period, many studies have shed light on the importance of these species in pedogenesis and their effect on the recycling of nutrients, the maintenance of soil fertility, soil structure and plant productivity (i.e., Brun et al., 1987; Edwards and Bohlen, 1996), leading to several introduction projects. For example, Huhta (1979) introduced *Aporrectodea caliginosa* into coniferous forests in Finland with encouraging results. Several other instances of earthworm introduction in Dutch polders (i.e., Van Rhee, 1969; Hoogerkamp et al., 1983; Hoogerkamp, 1984), peat areas in Ireland (Curry and Bolger, 1984; Curry and Boyle, 1987), American mine spoil sites (Dunger, 1969; Vimmerstedt and Finney, 1973; Hamilton and Vimmerstedt, 1980) or New Zealand pastures (Stockdill, 1982) were reported to have yielded very positive results.

Peregrine species are those distributed far from their regions of origin. The term was first used with regard to earthworms by Michaelsen (1903), who described the wide distribution of some earthworm species and their presence in geographically remote localities. Peregrine species are also anthropochorus and allochthonous. Most peregrine earthworms are supposed to share several characteristics: small size, parthenogenetic reproduction often accompanied by polyploidy (Gates, 1972), high fecundity, resistant cocoons, wide environmental or feeding tolerances and rapid rates of spread (Lee, 1985, 1987).

Aporrectodea trapezoides is categorised as a peregrine earthworm. It is a very widely distributed species. It is also one of the five species commonly sold and used as fishing bait in North America (Blakemore, 2006). It is usually the dominant species in

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Mediterranean soils, both in terms of biomass and in terms of the number of individuals, and it is often the only species present because it can adapt to a wide range of extreme environmental conditions. Both its wide geographical distribution and its high reproductive rate (Fernández et al., 2010) make *A. trapezoides* a key species in global soil management.

Like *A. trapezoides*, some other peregrine species are also parthenogenetic, and several studies have examined their genetic diversity. It has emerged that some species have low clonal diversity, with a low number of clones (i.e., only one or two clones found in the case of *Octolasion cyaneum* and *Octolasion tyrtaeum*; Terhivuo and Saura, 2003; Heethoff et al., 2004), whereas other species have a very large number (i.e., 20 clones out of 50 earthworms in the case of *Aporrectodea rosea*, Terhivuo and Saura, 2006).

The present study examined the genetic variation (mtDNA COI and COII and the nuclear 28S rRNA and H3 regions) and phylogeographic relationships of several populations of *A. trapezoides* collected worldwide. The aim of this study was to ascertain i) the clonal variability within the species, and ii) whether distinct lineages exist and, if so, how different they are.

2. Materials and methods

2.1. Earthworm sampling

A total of 178 specimens of *A. trapezoides* were collected by digging and manual sorting in 47 different locations (Fig. 1, Table 1) from Spain, France, Portugal, Italy, Greece, Turkey, Algeria, Egypt

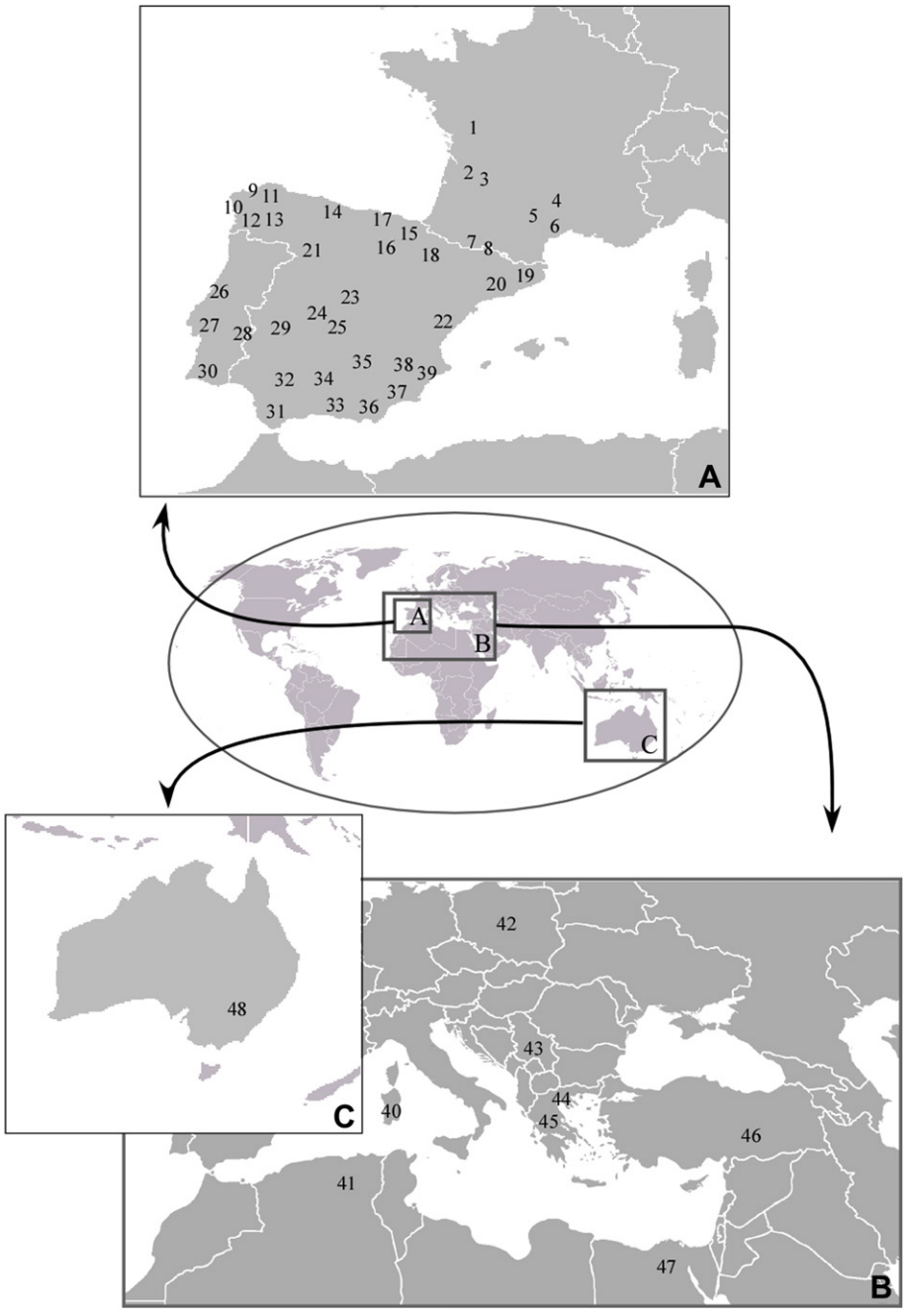

Fig. 1. Map of the sampled populations. See Table 1 for details.

Table 1
Table of correspondence of clones and populations. Number of sampled individuals per clone per population, GPS coordinates and identifying number of each population corresponding to Fig. 1 are specified. Populations in italics were taken from GenBank (COII and 28S rRNA sequences; between parenthesis, code identifying each sequence in GenBank starting by MPL; Pérez-Losada et al., 2009). Between parenthesis after the number of each clone, number of individuals per clone. When different clones were found within a population, GPS coordinates and number of population in Fig. 1 are only specified the first time the population is mentioned.

Clone	Population	GPS coordinates	No. of population in Fig. 1
1 (78)	*Adé (France, MPL86)*	*N 43° 09′ 59.4″ W 00° 00′ 20.6″*	7
	Constantine (Algeria)	N 36° 33′ E 06° 21′	41
	Archena (Spain)	N 38° 07′ 05.7″ W 01° 16′ 44.8″	37
	Harden (Australia)	S 34° 33′ E 148° 21′	48
	Carnota (Spain)	N 42° 49′ 26.01″ W 09° 06′ 09.60″	9
	El Cairo (Egypt)	N 30° 01′ E 31° 12′	47
	El Molar (Spain)	N 40° 44′ 22.9″ W 03° 33′ 53.1″	24
	Fátima (Portugal)	N 39° 37′ 33.5″ W 08° 40′ 07.0″	26
	Lugo (Spain, MPL109)	*N 43° 11′ 34.2″ W 07° 13′ 46.2″*	11
	Mojácar (Spain)	N 37° 08′ 48.0″ W 01° 52′ 15.7″	36
	Molinicos (Spain)	N 38° 28′ 31.5″ W 02° 11′ 19.1″	35
	Navarra (Spain, MPL100)	*N 42° 01′ 33.1″ W 01° 39′ 22.9″*	15
	Navarrés (Spain)	N 39° 06′ 26.1″ W 00° 42′ 32.8″	38
	Ourense (Spain, MPL48)	*N 42° 08′ 13.5″ W 08° 02′ 52.5″*	13
	St. Teodoro (Italy)	N 40° 47′ 05.9″ E 09° 40′ 05.5″	40
	St. Gély-du-fesc (France)	N 43° 42′ 19.0″ E 03° 48′ 03.7″	6
	Sta. Cristina de la Polvorosa (Spain)	N 41° 59′ 56.10″ W 05° 41′ 53.96″	21
	Kragujevac (Serbia, MPL119)	*N 44° 00′ E 20° 59′*	43
	Soudan (France, MPL85)	*N 46° 25′ 11.5″ W 00° 04′ 09.2″*	1
	Diyabakir (Turkey)	N 37° 54′ W 40° 13′	46
	Bilbao (Spain, MPL103)	N 43° 17′ 54.10″ W 03° 02′ 32.30″	17
	Vigo (Spain, MPL312)	*N 42° 10′ 01.9″ W 08° 41′ 03.5″*	12
	Vitoria (Spain, MPL104)	*N 42° 55′ 35.3″ W 02° 43′ 46.0″*	
2 (5)	Alcalá de los Gazules (Spain)	N 36° 26′ 28.7″ W 05° 43′ 14.3″	31
3 (1)	El Brull (Spain)	N 41° 48′ 00.0″ E 02° 21′ 00.0″	20
4 (21)	El Brull (Spain)		
	Puerto Querol (Spain)	N 40° 33′ 49.1″ W 00° 01′ 03.5″	22
	Robledillo (Spain)	N 39° 35′ 49.6″ W 04° 49′ 39.6″	25
	Sitagri (Greece)	N 41° 07′ E 24° 01′	44
	Maara (Greece)	N 41° 09′ E 24° 07′	45
	Villavelayo (Spain)	N 42° 06′ 23.63″ W 02° 55′ 41.06″	16
5 (11)	Corzos (Spain)	N 42° 39′ 33.02″ W 09° 01′ 27.75″	10
	Lanne (France)	N 43° 09′ 57.2″ E 00° 00′ 18.6″	8
	Laubert (France)	N 44° 35′ 15.2″ E 03° 39′ 45.6″	4
	San Román (Spain)	N 43° 15′ 20.9″ W 05° 05′ 10.3″	14
	Vitoria (Spain, MPL156)	*N 42° 55′ 35.3″ W 02° 43′ 46.0″*	17

(*continued on next page*)

Table 1 (*continued*)

Clone	Population	GPS coordinates	No. of population in Fig. 1
6 (17)	Corzos (Spain)		10
	Peña Oroel (Spain)	N 41° 23′ 27.3″ E 01° 49′ 39.3″	18
	Saint Hilaire-du-Bois (France)	N 44° 39′ 41.6″ W 00° 05′ 23.8″	2
	San Román (Spain)	N 43° 15′ 20.9″ W 05° 05′ 10.3″	
	Sant Joan de les Abadesses (Spain)	N 42° 13′ 30.0″ E 02° 14′ 57.5″	19
7 (5)	Écija (Spain)	N 38° 44′ 38.1″ W 02° 35′ 02.7″	32
8 (2), 10–13 (1 each)	Laubert (France)		
9 (3)	Laubert (France)		
	Lomianki (Poland, MPL94)	*N 52° 20′* *E 20° 53′*	42
14 (3), 15 (6)	Linares (Spain)	N 38° 05′ 17.7″ W 03° 39′ 00.8″	34
	Píñar (Spain)	N 37° 24′ 39.2″ W 03° 29′ 47.0″	33
16 (5)	Marvão (Portugal)	N 39° 22′ 06.0″ W 07° 20′ 12.9″	28
17 (5)	Mende (France)	N 44° 29′ 43.6″ E 03° 27′ 41.9″	5
18 (3), 19 (1)	Monchique (Portugal)	N 37° 12′ 14.9″ W 08° 32′ 07.5″	30
20 (1)	Robledillo (Spain)		
21 (1)	Laubert (France)		
22 (3), 23 (2)	Romangordo (Spain)	N 39° 44′ 10.6″ W 05° 42′ 53.7″	29
24 (1)	San Román (Spain)		
25 (4), 26 to 28 (1 each)	Saint Hilaire-du-Bois (France)		
29 (2)	Vall d'Uixó (Spain)	N 39° 50′ 25.2″ W 00° 15′ 59.7″	39
30–34 (1 each)	Vendas Novas (Portugal)	N 38° 40′ 13.7″ W 08° 26′ 35.8″	27
35 (1)	*Toledo (Spain, MPL57)*	*N 39° 51′ 23.36″* *W 04° 06′ 21.42″*	24
36 (1)	*Adé (France, MPL87)*		
37 (2)	*Monségur (France, MPL89, MPL92)*	*N 44° 39′ 19.27″* *E 00° 04′ 50.54″*	3

and Australia. All individuals were morphologically and anatomically identified as *A. trapezoides* following the taxonomic key in Gates (1972). The specific characteristics that were taken into account were the positioning of setal genital tumescence (in segments 9, 10 and 11), the location of the clitellum and tubercula pubertatis (undivided in segments 31–33) and male sterility (empty spermathecae and male funnels, both in segments 10 and 11).

After collection, the specimens were washed with distilled water and preserved in absolute ethanol or ethanol 96° at −20 °C. A portion of the bodywall (±0.025 g) was collected and carefully cleaned under a stereomicroscope to remove soil particles and macroscopic parasites. Tegument samples were kept in ethanol and preserved at −20 °C until DNA extraction.

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted using the DNAeasy Tissue kit (Qiagen). A polymerase chain reaction (PCR) was performed using a Perkin Elmer 9700 thermal cycler.

The length of final alignment and other parameters for each gene are shown in Table 2.

The 28S rRNA fragment was amplified using the primers from Whiting et al. (1997, F1 and R1). The PCR involved an initial denaturation step at 95 °C for 3 min and 40 amplification cycles (94 °C for 45 s, 54.5 °C for 60 s, 72 °C for 90 s); it ended with a 72 °C (5 min) final extension. The total reaction volume of 26 μl included 1.5 mM $MgCl_2$, 10 mM of each dNTP, 10 μM of each primer, DMSO 1 μl and 1 unit of Taq polymerase.

Table 2
Sequence parameters of the studied genes (COI, COII, H3 and 28S rRNA).

	COI	COII	H3	28S rRNA
Length of sequence alignment	456	551	307	810
Total number of mutations	209	211	118	68
No. Polymorphic sites	153	165	89	61
No. Parsimony informative sites	151	165	71	35
G + C content	0.441	0.369	0.639	0.623
Haplotype diversity	0.853 ± 0.022	0.853 ± 0.024	0.819 ± 0.045	0.479 ± 0.057
Nucleotide diversity	0.096 ± 0.004	0.085 ± 0.004	0.034 ± 0.007	0.005 ± 0.001
Mean number of pairwise differences	43.687 ± 19.415	47.673 ± 18.875	9.256 ± 3.337	1.998 ± 0.587

The COII fragment was amplified using the primers from Pérez-Losada et al. (2009) (COII-LumbF1 and COII-LumR2). The PCR included an initial denaturation step at 95 °C for 3 min and forty cycles of 95 °C (60 s) denaturation, 48 °C (70 s) annealing and 72 °C (75 s) extension. The PCR ended with a 72 °C (5 min) final extension. The total reaction volume of 22 μl included 1.5 mM $MgCl_2$, 10 mM of each dNTP, 10 μmol of each primer and 1 unit of Taq polymerase.

The COI fragment was amplified using the forward primer LCO_AT (5′-CATTYGTRATAATYTTCTTT-3′) and the reverse primer HCO_AT (5′-GCTGATATARAATAGGGTCTC-3′) (Fernández et al., in press). The amplification process included an initial denaturation step at 95 °C for 15 min; 35 cycles of 94 °C (30 s) denaturation, 50 °C (70 s) annealing and 72 °C (90 s) extension; and a 72 °C (10 min) final extension. The total reaction volume of 50 μl included 1.5 mM $MgCl_2$, 10 mM of each dNTP, 20 μmol of each primer and 1.5 units of Taq polymerase.

The H3 fragment was amplified using the forward primer H3aF and the reverse primer H3aR (Colgan et al., 1998). The amplification process included initial denaturation time of 2 min at 94 °C, followed by 35 cycles of 94 °C (30 s) denaturation, 50 °C (30 s) annealing and 72 °C (60 s) extension; the last step was a final extension at 72 °C (7 min). The total reaction volume of 50 μl included 1.5 mM $MgCl_2$, 10 mM of each dNTP, 100 μmol of each primer and 1.5 units of Taq polymerase.

All PCRs were specific and resolved via 1.5% agarose gel electrophoresis; they were visualised using ethidium bromide, and all products were purified using a Speedtools PCR clean-up kit (Biotools, Madrid, Spain). Non-specific PCRs were not further processed. Automated sequences were generated in both directions by the Genomic Unit, Scientific Park of Madrid (Spain) and Macrogen Inc. (Korea).

All PCR products yielded unequivocal nucleotide chromatograms. The sequences were compared with known earthworm sequences in GenBank using the BLAST search algorithm (Altschul et al., 1997). The DNA sequences were deposited in GenBank under Accession Numbers JF918579–JF918719. Eighteen additional sequences (COII and 28S rRNA) of *A. trapezoides* from different locations in Spain, France, Serbia and Poland that were available in GenBank (Pérez-Losada et al., 2009) were added to our analysis (Table 1). Sequences of COI, COII, H3 and 28S rRNA from *Hormogaster elisae*, *H. abatissae*, *H. ireguana*, *H. castillana* and *H. huescana* were also taken from GenBank and/or generated in this study to be used as outgroups and external groups (Accession Numbers JF918613–JF918617).

2.3. Genetic variability and phylogenetic analysis

Models for sequence evolution and corresponding parameters were estimated using jMODELTEST v. 0.1.1 (Posada, 2008). The best-fitting models were used in the phylogeny estimation for the ML and BI analyses.

Saturation in the third positions in COI and COII was checked using DAMBE (Xia and Xie, 2001). As expected, higher saturation was observed in transitions. Trees were generated with and without these positions, but the phylogenetic relationships among and within the clades were exactly the same.

Maximum likelihood (ML) analyses were performed using Phyml v. 2.4.4 (Guindon and Gascuel, 2003), including α and I values provided by jMODELTEST. Clade support was assessed using bootstrapping with 1000 pseudoreplicates. This ML approach included heuristic searches involving tree bisection and reconnection (TBR) branch swapping with 10 random stepwise additions of taxa. Bootstrap analysis (Felsenstein, 1985) with 1000 replicates was used to estimate support for the resulting topologies.

Maximum parsimony (MP) trees were generated using TNT v. 1.1 (Goloboff et al., 2003). Heuristic searches with 1000 rounds of random addition of taxa holding 10 trees per round were performed up to a total of 10,000 trees. The best trees were then submitted to an extra round of TBR swapping. Clade support was assessed using bootstrap with 1000 pseudoreplicates.

Bayesian phylogeny estimation was performed using the program MRBAYES v.3.1.2 (Ronquist and Huelsenbeck, 2003). The parameters in MRBAYES were set to one million generations, and 1000 trees were sampled for every 1000th generation, using the default random tree option to initiate the analysis. All sample points prior to the plateau phase (200 trees) were discarded as 'burn in'. The posterior probabilities (p*P*) for individual clades obtained from separate analyses were compared for congruence and then combined and summarised using a 50% majority-rule consensus tree.

Gene regions were analysed both in combination using a single data set and as multiple concatenated partitions using a partitioned supermatrix (COI, COII, 28S rRNA and H3). To test for conflict across data sets, the incongruence length difference test (ILD) (Farris et al., 1994) was performed as implemented in WINCLADA v.1.00.08 (Nixon, 2002).

As a measure of neutral difference in mitochondrial coding regions, the uncorrected percentage difference at fourfold degenerate sites (D4) was calculated using the Kumar method (Nei and Kumar, 2000). This distance is slower to saturate and less sensitive to transition–transversion bias than are the differences at twofold and threefold degenerate sites (Li, 1993; Mark Welch and Meselson, 2000). Fourfold degenerate sites were identified in DnaSP v. 5.0 (Librado and Rozas, 2009). Genetic distances were calculated in MEGA 4.0.

2.4. Other statistical analyses

Mean sequence diversities (π) within and between clusters were calculated for COII using the complete deletion option, as it was the mitochondrial gene from which more samples were available. Diversity values were used to test whether sister-pairs of clusters conform to the 4X rule (Birky et al., 2005, 2010), which delineates independent populations by comparing diversity within and between reciprocally monophyletic clusters, thus checking for complete lineage sorting. This rule was originally created to study speciation processes in parthenogenetic organisms (Birky et al., 2005, 2010) and is widely used in studies with parthenogenetic animals (Birky and Barraclough, 2009; Bode et al., 2010) to delimit species.

3. Results

3.1. High clonal diversity and genetic variability

Our results reveal high clonal variation within *A. trapezoides*. Two main lineages (lineage I and II) are clearly distinguished. Not only is clonal diversity high, but it is strongly structured into distinct groups of related sequences that are separated by short or medium-length internal branches. More clones were found in lineage II than in lineage I.

The distribution of sampled individuals per clone is shown in Fig. 2. The graphic showed that most of the clones were shared by few individuals (less than 6) excepting two clones (clones 1 and 4) that were shared by a high number of them (21 and 78 earthworms, respectively).

The percentage of fourfold degenerate sites (D4) ranged from 16% to 19% in the analysed coding regions in both lineages. The measurement of percentage distances in D4 sites is a highly suitable method of nonparametric distance estimation for nucleotide sequences; these sites do not affect the protein sequence and are

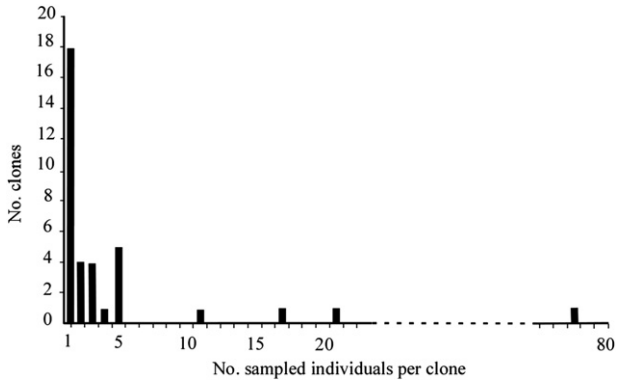

Fig. 2. Graphical representation of the number of clones containing the same number of sampled individuals.

assumed to exhibit slower saturation and less sensitivity to transition–transversion bias (Li, 1993). In our data, half of the D4 sites were variable, and the mean pairwise distances were 30% in COI and 41% in COII; these distances do not indicate an early split by these lineages.

3.2. Phylogenetic analyses and biogeographical distribution of the lineages

Hierarchical likelihood ratio tests, the Akaike information criterion (AIC) and the Bayesian information criterion (BIC) were used to generate the general time-reversible model with a gamma distribution and invariable sites (GTR + I + G) as the best-fitted model for the data in COII and 28S rRNA and the Hasegawa–Kishino–Yano model with a gamma distribution (HKY + G) in COI and H3.

No major differences between the ML, MP and Bayesian topologies were identified. The few topological differences observed were always at the terminal level and mainly the result of a larger number of polytomies in the Bayesian analysis. However, exactly the same terminal clusters were recovered in the different analyses. All of the trees generated with different partitions (data not shown) and the concatenated data set (Fig. 3) produced congruent topologies. Moreover, the ILD test did not indicate significant incongruence among them ($P > 0.05$).

Two major lineages (called lineages I and II) were found in *A. trapezoides*; they were represented by two deep clades and supported by high posterior probability and bootstrap values (Fig. 3). Each lineage was formed by groups of closely related clades, as well-supported by Bayesian, parsimony and ML analyses.

The distribution of the number of individuals belonging to each clone is shown in Table 1. Clone 1 was shared by one-third of the sampled earthworms and located phylogenetically in lineage II. This clone was present in most parts of the sampled countries, exhibiting a very wide distribution area (Fig. 4b). The analysis of the biogeographical distribution without taking into account clone 1 shows that the two lineages are clearly separated. Lineage I exhibits a North European distribution (North of Spain, France and Poland), whereas lineage II is distributed mainly in South Europe, the Mediterranean countries and Australia (Fig. 4a and b). In some populations from both lineages, clone 1 individuals were mixed with earthworms of other clones.

Sister-pairs of clusters were checked for the 4X rule. However, most of them were not found to be consistent with that rule; in all of these cases, the mean distance between the clusters was less than four times the larger of the mean distances within the clusters. Only the singletons 36 and 37 (belonging to lineage I) were consistent with it.

4. Discussion

4.1. Clonal diversity

Our results reveal high genetic variation within *A. trapezoides*. These findings are consistent with the allozyme diversity previously reported in Italian (Cobolli-Sbordoni et al., 1987) and Australian populations (Dyer et al., 1998), in which the mean values of genetic similarity between populations varied from 64.92% to 86.82% and from 80.4% to 89%, respectively. On the other hand, an earlier analysis of mitochondrial and nuclear sequence data (Pérez-Losada et al., 2009) did not show great genetic variation within the species. However, the number of sampled populations was low, and most of the samples belonged to clone 1. In any case, both studies were based on relatively geographically-restricted sampling of the species' distribution or on a low number of individuals.

4.2. Lineage sorting

The 4X rule proposed by Birky et al. (2005, 2010) allows us to test whether clusters have been evolving independently long enough to become reciprocally monophyletic because they are adapted to different niches or are allopatric. Under diversifying selection, the gaps separate populations that have been evolving independently long enough to become reciprocally monophyletic, i.e., to complete speciation.

In our data, none of the clusters analysed followed this rule. Thus, it can be assumed that the ratio between/within divergence in *A. trapezoides* is not big enough to consider both lineages (or any clusters within them) as differentiated evolutionary lines sensu Birky and Barraclough (2009). The D4 distance values found between lineages also supports this hypothesis. Then, the time since lineages I and II geographically split seems to have not been enough to make of them different species according to this rule, while an underlying speciation event seems to be occurring.

4.3. Phylo- and biogeographical implications

The overall findings for this study indicate that the analysed individuals of *A. trapezoides* represent two separate, well-supported lineages called lineages I and II (Fig. 4). Another immediate result is the presence of high clonal diversity and one dominating clone (clone 1) shared by one-third of the analysed earthworms and present worldwide. The clone 1 individuals are clustered together with some locally restricted clones only present in Central Spain. Thus, we hypothesise that it originated in this area. If clone 1 is excluded, there is evidence of strong geographical sorting by both lineages. Lineage I is distributed mainly in Eurosiberian areas (North of Spain, France and Poland), whereas lineage II seems to have a Mediterranean-focused distribution. Several hypotheses could be postulated as explanations for this lineage sorting. On one hand, the phylogenetic tree seems to indicate different origins of parthenogenesis in the two lineages, as the dichotomy is very basal and well-supported. On the other hand, the role of quaternary glaciations in explaining the actual distribution of geographically sorted lineages has been postulated several times (Hewitt, 2001). In particular, the Mediterranean peninsulas (mainly the Iberian, Italian and Balkan peninsulas) were an important refuge for both animals and plants during these glaciations (Huntley and Birks, 1983; Taberlet et al., 1998; Hewitt, 2001). However, we do not have sufficient data to support either suggestion. More samples from the Italian and Balkan peninsulas and further research on the origin of the parthenogenetic forms will shed light on these points.

4.4. A vagrant clone in a peregrine species

Most clusters of clones showed a restricted geographical distribution (Fig. 3b), suggesting low dispersal and/or colonising ability. On the other hand, clone 1 was found in a high percentage of the sampled earthworms and localities, and it was the only one found in the distribution area of both lineages. However, clones 4 and 9 (including populations from Spain and Greece, and France and Serbia, respectively) also exhibited a broad distribution, as they were found in geographically distant countries. Clone 4 was found also in a high number of earthworms (21), but clone 9 only in 3. Nonetheless, clone 9 was only found in the distribution area of lineage I, and clone 4 in the area of lineage II. In addition, we did not have a number of samples from northern Europe or eastern

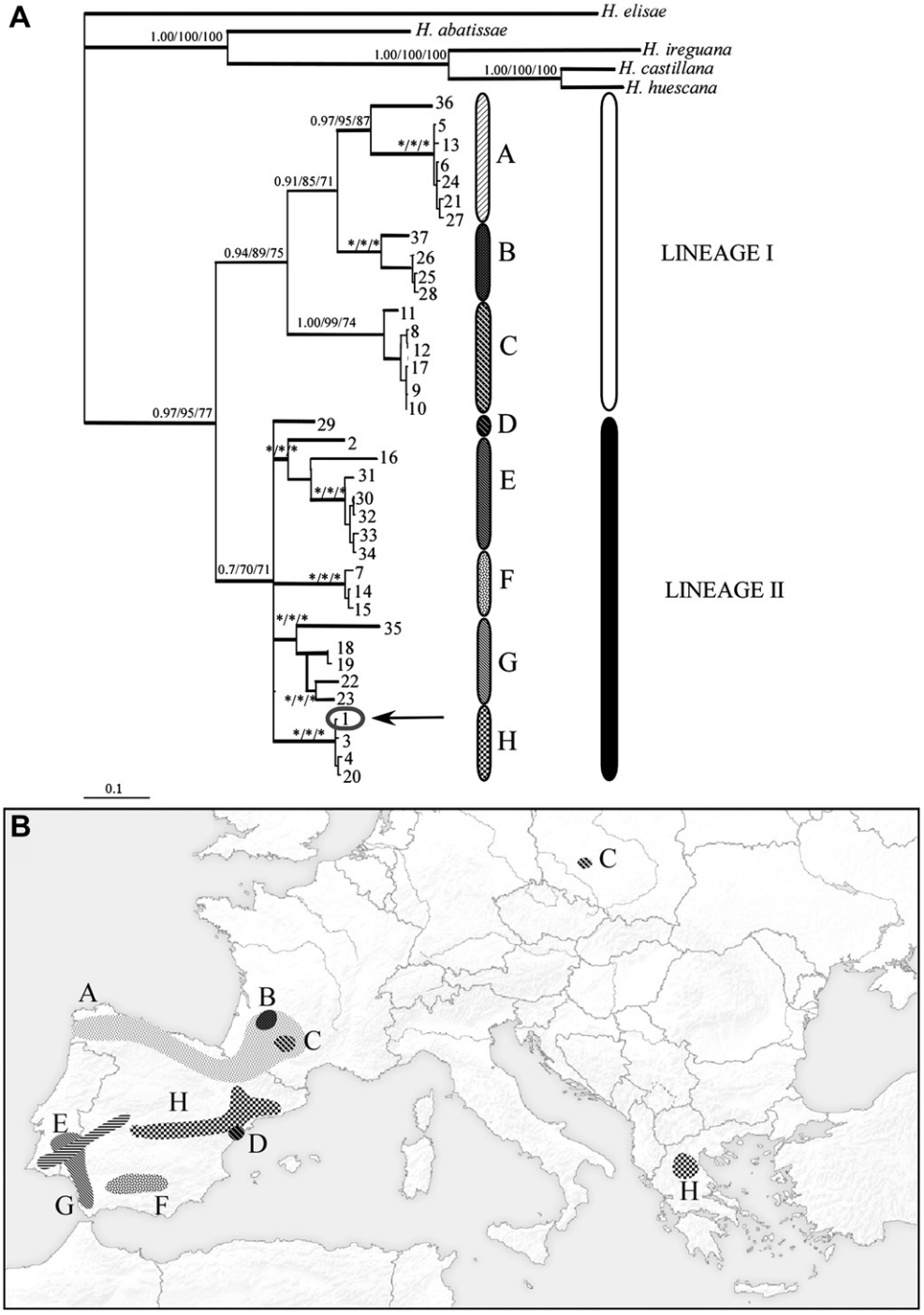

Fig. 3. (a). Majority-rule consensus phylogram based on Bayesian Inference (BI) of the concatenated database. Posterior probability and bootstrap values of coincident associations given by Maximum Likelihood (ML) and Parsimony (P) are shown above branches when >0.70/70/70 (BI/ML/P). An asterisk indicates a terminal branch having significant support (>0.70/70/70). The scale bar represents 0.1 substitutions per site. Both recovered lineages are shown. Within them, clades were assigned a letter (A–C in lineage I, D–H in lineage II) for a better geographical visualization. (b). Geographical representation of the clades named in the tree (A–H). Each area contains the clones of each cluster with a representative purpose; it does not predict whether the same clones should be found outside the sampled points.

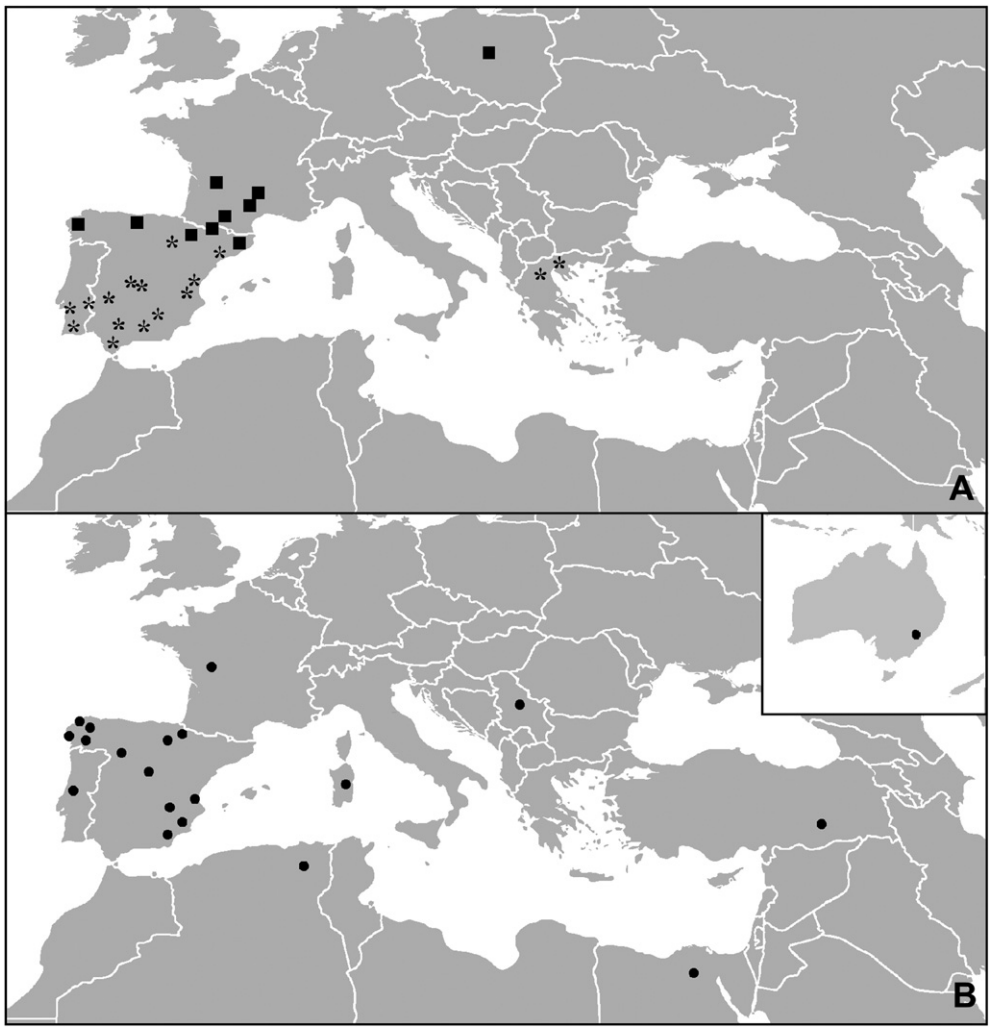

Fig. 4. (a) Distribution of both lineages found in *A. trapezoides* (black squares: clones of lineage I; black stars: clones of lineage II) excluding clone 1. (b). Distribution of clone 1 (black circles).

Mediterranean countries, as would have been necessary to verify their distributions and possible vagrant character.

These two patterns of geographically-restricted versus widely distributed clones seem to present contradictory explanations for the evolutionary traits of this species. Due to its Palaearctic origin, *A. trapezoides* seems certainly to have been introduced in Australia. However, its presence in a large number of localities, especially where only clones from lineage I should be found according to the overall pattern found in this study, makes it very probable that it was also introduced in many other places. This clone is obviously able to adapt to a wide range of environmental conditions. In addition, Fernández et al. (2010) studied the life cycle of this clone; it was relatively short (reaching maturity at day 150) and its reproductive fitness high (105 cocoons per year), which could explain its wide distribution and prevalence worldwide.

Almost none of the clusters satisfied the 4X rule; therefore, the clustering may be interpreted as strong spatial population structure within the species. Only singlets 36 and 37 were consistent with this rule and thus could be considered two different species based on the evolutionary and general lineage species concept (EG species; Birky et al., 2005, 2010). However, both are singlets and thus formed by only one clone, and it remains possible that future additions to the tree will cluster together with these singlets. This could increase the nucleotide diversity within the clade, thus making the groups inconsistent with the rule, which on the other hand is the general pattern found in the phylogenetic tree.

5. Conclusions

To our knowledge, this research is the first to attempt the phylogeographical study of a parthenogenetic earthworm on a worldwide scale. We found two geographically-distinct lineages, one with a Eurosiberian and the other with a Mediterranean distribution. Of the 37 clones that were found, one was shared by one-third of the sampled individuals and was found worldwide, whereas the others were geographically-restricted and were shared only by a few earthworms. This generalistic clone probably originated in Central Spain. Its ability to adapt to a wide range of environmental conditions and its high level of reproductive success could explain its worldwide distribution and high occurrence.

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