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Earthworms, good indicators for palaeogeographical studies? Testing the genetic structure and demographic history in the peregrine earthworm *Aporrectodea trapezoides* (Dugès, 1828) in southern Europe

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ABSTRACT

It has been traditionally assumed that earthworms tend to remain in their areas of origin due to their low vagility, thus following the geological fate of the regions that they inhabit. Following this, many authors have correlated palaeogeographical events with earthworm distribution in several species to date diversification processes or to explain phylogenetic and phylogeographic patterns. Nonetheless, this correlation has been poorly tested, thus there is no scientific evidence supporting this assumption. This study aimed to test if widespread earthworm species are good indicators of geological changes by means of checking the population genetic structure at different levels (lineages, clades and populations). The results of the AMOVA supported the existence of strong population structure at the level of clades and populations. In some cases, substructure within populations was also observed. In addition, F_{ST} values indicated a lack of genetic flow between populations. Correlation between demographic history in the main Mediterranean clades and past Iberian palaeogeographical events was congruent, thus showing that it was a 'good' the candidate for this kind of studies.

1. Introduction

Earthworms have been traditionally thought to remain in their areas of origin due to their low vagility. Long before the use of molecular markers to study evolutionary processes, this idea was assumed by many authors that based their work on the correlation between the evolutionary history of earthworm species and the geological changes that occurred in their geographic range in order to shed light on evolutionary processes in this animal group (e.g., Omodeo and Rota, 2008). For instance, Michaelsen (1902, 1903) correlated endemicity of earthworms with glaciations and Černosvitov (1932) explained the phylogeny and geographic patterns of Czech earthworms on the basis of continental drift. Nonetheless, this approach has also been strongly criticised due to the lack of empirical evidence (e.g., Benham, 1922) and the existence of many peregrine earthworm species that expanded their distribution area by indirect human transport (e.g., Cameron et al., 2008; Decaëns et al., 2008) therefore violating the above-mentioned correlation between geological fate of their geographic range and their evolutionary history.

In the last few decades, molecular tools have been applied to different earthworm families and species, allowing the gathering of information about the evolutionary processes undergone by this animal group. However, non-polychaete groups within annelida such as lumbricid earthworms are still largely unknown regarding molecular phylogenetics, phylogeography and population genetics. This absence of knowledge is of special concern because this animal group plays a very important role in ecosystem functioning, including soil turnover, aeration and drainage, decomposition and nutrient cycling processes (Edwards, 2004; Lavelle and Spain, 2001). In addition, earthworms account for the main faunal biomass found on Earth (Bouché, 1984) and in soil ecosystems (Edwards and Bohlen, 1996). Both their crucial role in soils and their

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high biomass make this group vital not only to understand soil function, but also to design a suitable global soil management strategies. In this context, molecular studies have been biased towards phylogenetic studies (e.g., Buckley et al., 2011; Novo et al., 2011; Pérez-Losada et al., 2011; James and Davidson, 2012); for instance, just 5 pieces of work look at phylogeography (reviewed in Chang and James, 2011), and only a few about population genetics (e.g., Harper et al., 2006; Velavan et al., 2007; Novo et al., 2009, 2010; Dupont et al., 2011; Somers et al., 2011; Torres-Leguizamon et al., 2012). These and other phylogenetic studies have indicated the existence of genetic structure in different earthworm species (e.g., Pérez-Losada et al., 2009; Dupont et al., 2011; Fernández et al., 2012). In addition, a few recent studies have also used geological data as a reference point for dating, assuming low vagility of earthworms in geological time (Novo et al., 2011; Pérez-Losada et al., 2011). Nonetheless, until now population genetic structure has never been widely tested in widespread earthworm species, thus the role of earthworms in general as useful indicators of past palaeogeographical events remain untested.

Aporrectodea trapezoides is a medium-sized and parthenogenetic earthworm of Palaearctic origin but almost worldwide distribution. Although typically a Holarctic species, it has been introduced worldwide due to the spread of European agricultural practices and widespread use of this earthworm as fishing bait (Blakemore, 2006), thus it is considered a peregrine species *sensu* Michaelsen (1903). Smith (1917), Stephenson (1930) and Omodeo (1948) characterized it as the most commonly found earthworm. It is usually the dominant species in Mediterranean soils both in biomass and number of individuals, and sometimes even the only species present in this kind of soils due to its high adaptability to extreme environmental conditions (Fernández, 2011).

A recent phylogeographical study (Fernández et al., 2011), including individuals from nine different countries, found two well-supported and geographically-distinct lineages in *A. trapezoides* (lineages I and II) that were not recovered as different species following the 4X rule *sensu* Birky et al. (2005, 2010). Remarkably, one clone from lineage II (called 'clone 1') was shared by almost one third of the sampled individuals and was widely distributed. Earthworms from lineage I showed a Eurosiberian-like distribution and individuals from lineage II distributed mainly in Mediterranean areas (except clone 1). Both lineages were divided into clades of clones (marked as A–C in lineage I, and D–H in lineage II, Fig. 1).

The geological history of the Iberian Peninsula is especially interesting as it is the result of the fragmentation and fusion of several microplates with different origins that occurred at different geological moments. Thus, it is the perfect scenario to study demographic history parameters. Therefore, these analyses together with population structure studies of the above-mentioned clades of clones could give us some important hints to check the correlation between evolutionary history and past palaeogeographical events in this widespread earthworm species.

In this context, the aim of this study is to test if widespread earthworm species (e.g., *A. trapezoides*) are good indicators of past palaeogeographical events. For that purpose, we checked a) the existence of genetic structure in the peregrine earthworm *A. trapezoides* (that is, if it is a good candidate), and therefore b) if the evolutionary history of clades of clones from lineage II (D–H) can be correlated to well-recorded Iberian palaeogeographical events.

2. Materials and methods

2.1. Earthworm sampling

A total of 178 earthworms identified as *A. trapezoides* following the taxonomic key in Gates (1972) were collected by digging and manual sorting (Table 1) from Spain, France, Portugal, Italy, Greece, Turkey, Algeria, Egypt and Australia (see Fernández et al., 2011 for a map of the localities and further details; please note that the GenBank accession numbers in this previous work correspond to clones, thus the sequences of the separate individuals are original for this work).

Immediately after collection, the specimens were washed with distilled water, fixed in absolute ethanol or ethanol 96% and preserved at −20 °C. A tegument sample (±0.025 g) was collected; after carefully being cleaned under a stereomicroscope to remove

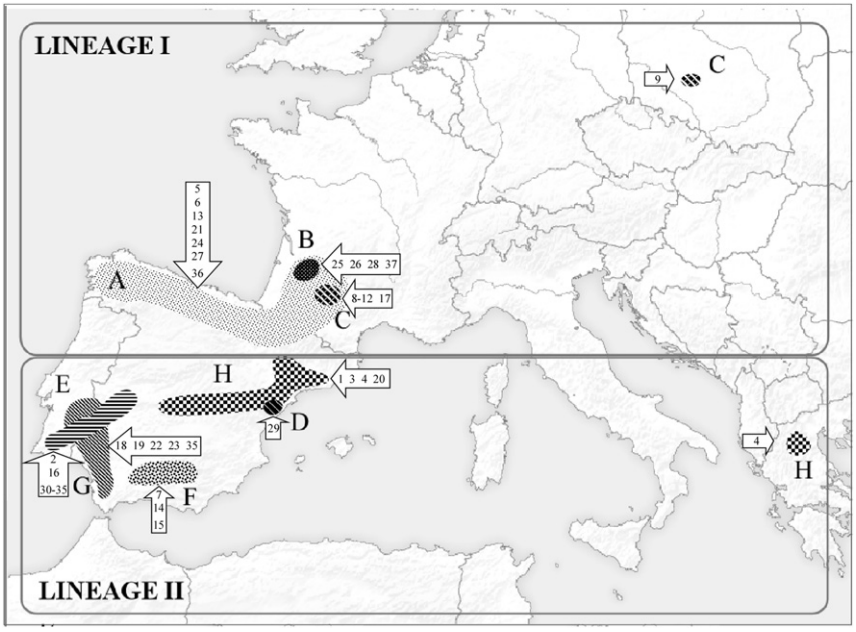

Fig. 1. Distribution of both lineages and clades of clones in *A. trapezoides* after Fernández et al., 2011. Clones clustering together in each clade are indicated within the arrows.

Table 1
Table of the sampled earthworms. Clone number, clade of clones in which the sample clustered sensu Fernández et al. (2011), localities, abbreviation code, number of sequenced individuals per locality (Ns) and GenBank accession numbers are included. Localities in italics correspond to sequences taken from GenBank (Pérez-Losada et al., 2009).

Clone	Clade of clones	Locality	Code	Ns	GenBank accession number
1	H	*Adé (France)*	ADE	1	FJ967786
		Constantine (Algeria)	ALG	5	JQ309978–JQ309982
		Archena (Spain)	ARC	5	JQ309983–JQ309987
		Harden (Australia)	AUS	5	JQ309988–JQ309992
		Carnota (Spain)	CAR	5	JQ309993–JQ309997
		El Cairo (Egypt)	EGY	5	JQ309998–JQ310002
		El Molar (Spain)	MOL	6	JQ310003–JQ310009
		Fátima (Portugal)	FAT	4	JQ310010–JQ310013
		Lugo (Spain)	LUG	2	FJ967733–FJ967734
		Mojácar (Spain)	MOJ	5	JQ310014–JQ310018
		Molinicos (Spain)	MOAB	5	JQ310019–JQ310023
		Navarra (Spain)	NAR	2	FJ967727–FJ967728
		Navarrés (Spain)	NAV	6	JQ310024–JQ310029
		Ourense (Spain)	OUR	1	FJ967766
		St. Teodoro (Italy)	STEO	5	JQ310030–JQ310034
		St. Gély-du-fesc (France)	SGEL	1	JQ310035
		Sta. Cristina de la Polvorosa (Spain)	CRI	5	JQ310036–JQ310040
		Kragujevac (Serbia)	SER	1	FJ967755
		Soudan (France)	SOU	4	FJ967785
		Diyarbakir (Turkey)	TUR	1	JQ310041–JQ310044
		Bilbao (Spain)	BIL	1	FJ967729
		Vigo (Spain)	VIG	1	FJ967762
		Vitoria (Spain)	VIT	1	FJ967730 FJ967730
2	E	Alcalá de los Gazules (Spain)	ALZ	5	JQ310045–JQ310049
3	H	El Brull (Spain)	BRU	1	JQ310050
4	H	El Brull (Spain)		4	JQ310051–JQ310054
		Puerto Querol (Spain)	QUE	5	JQ310055–JQ310059
		Robledillo (Spain)	ROB	2	JQ310060–JQ310061
		Maara (Greece)	MAA	3	JQ310062–JQ310064
		Drama (Greece)	DRA	2	JQ310065–JQ310066
		Villavelayo (Spain)	VIL	5	JQ310067–JQ310071
5	A	Corzos (Spain)	COR	3	JQ310072–JQ310074
		Lanne (France)	LAN	3	JQ310075–JQ310077
		Laubert (France)	LAU	3	JQ310078–JQ310080
		San Román (Spain)	SAN	1	JQ310081
		Vitoria (Spain)		1	FJ967754
6	A	Corzos (Spain)		2	JQ310082–JQ310084
		Peña Oroel (Spain)	ORO	3	JQ310085–JQ310087
		Saint Hilaire-du-Bois (France)	SHB	2	JQ310088–JQ310089
		San Román (Spain)		5	JQ310090–JQ310094
		Sant Joan de les Abadesses (Spain)	SJOA	5	JQ310095–JQ310099
7	F	Écija (Spain)	ECI	5	JQ310100–JQ310104
8	C	Laubert (France)		2	JQ310105–JQ310106
9	C	Laubert (France)		2	JQ310107–JQ310108
		Lomianki (Poland)	POL	1	FJ967791
10	C	Laubert (France)		1	JQ310109
11	C	Laubert (France)		1	JQ310110
12	C	Laubert (France)		1	JQ310111
13	A	Laubert (France)		1	JQ310112
14	F	Linares (Spain)	LIN	1	JQ310113
		Píñar (Spain)	PIN	2	JQ310114–JQ310115
15	F	Linares (Spain)		3	JQ310116–JQ310118
		Píñar (Spain)		3	JQ31019–JQ310121
16	E	Marvão (Portugal)	MAR	5	JQ310122–JQ310126
17	C	Mende (France)	MEN	5	JQ310127–JQ310131
18	G	Monchique (Portugal)	MON	3	JQ310132–JQ310134
19	G	Monchique (Portugal)		1	JQ310135
20	H	Robledillo (Spain)		1	JQ310136
21	A	Laubert (France)		1	JQ310137
22	G	Romangordo (Spain)	ROM	3	JQ310138–JQ310140
23	G	Romangordo (Spain)		2	JQ310141–JQ310142
24	A	San Román (Spain)		1	JQ310143
25	B	Saint Hilaire-du-Bois (France)		4	JQ310144–JQ310147
26	B	Saint Hilaire-du-Bois (France)		1	JQ310148
27	A	Saint Hilaire-du-Bois (France)		1	JQ310149
28	B	Saint Hilaire-du-Bois (France)		1	JQ310150
29	D	Vall d'Uixó (Spain)	VAL	2	JQ310152
30	E	Vendas Novas (Portugal)	VEN	1	JQ310153
31	E	Vendas Novas (Portugal)		1	JQ310154
32	E	Vendas Novas (Portugal)		1	JQ310155
33	E	Vendas Novas (Portugal)		1	JQ310156
34	E	Vendas Novas (Portugal)		1	JQ310157
35	G	*Toledo (Spain)*	TOL	1	FJ967771
36	A	*Adé (France)*		1	FJ967787
37	B	*Monségur (France)*	MSG	2	FJ967788–FJ967789

soil particles and macroscopic parasites, the 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). Polymerase chain reaction (PCR) was performed using a Perkin–Elmer 9700 thermal cycler.

A fragment of the cytochrome *c* oxidase II (COII hereafter) (551 bp) was amplified using the primers from Pérez-Losada et al. (2009) (COII-LumbF1and COII-LumR2). This gene was chosen because further COII sequences from the same species are available in GenBank, therefore increasing the number of samples. PCR conditions were similar to the ones described in Fernández et al. (2011).

All PCRs were specific and resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide. Automated sequences were generated by the Genomic Unit, Scientific Park of Madrid (Spain) and Macrogen Inc., Korea. The DNA sequences were deposited in GenBank (accession numbers in Table 1). Additional COII sequences of *A. trapezoides* from different locations in Spain, France, Serbia and Poland which available in GenBank were added to our analysis (Table 1), thus a total of 196 individuals from 49 localities and 11 different countries were included in the analyses.

2.3. Overall genetic diversity and genetic structure analysis

In order to assess the genetic differences between the clades of clones (A–H, Fig. 1), estimates of variability expressed as haplotype diversity (H), nucleotide diversity (π) and number of segregating sites (S) were calculated with Arlequin v. 3.5 (Excoffier and Lischer, 2010). Mean genetic differentiation between and within populations was estimated using uncorrected p-distances. F_{ST} values were also calculated with Arlequin v. 3.5.

Hierarchical population structure was evaluated in Arlequin via an Analysis of the Molecular Variance (AMOVA) with 10,000 permutations for statistical confidence. In order to check if population genetic structure existed at different levels, the analysis was performed following a hierarchical structure at different levels: first lineages and clades of clones, second lineages and populations and third clades of clones and populations. When considering the population level, the analyses were performed without including the worldwide-distributed clone 1 (Fernández et al., 2011; see

discussion below). In addition, this last AMOVA was performed both considering all the samples from the same population together and subdividing populations whose individuals clustered in different clades of clones (Saint Hilaire-du-Bois, SHB, and Laubert, LAU) into two sub-populations, each of them clustering in a different clade (SHB clustering in A and B, LAU in A and C; see Discussion).

A haplotype network was constructed using the statistical parsimony procedure (Templeton et al., 1992; Crandall and Templeton, 1993) as implemented in TCS version 1.21 (Clement et al., 2000); they show reticulate relationships at the population level not represented in traditional phylogenetic trees (Posada and Crandall, 2001). As described by Pfenniger and Posada (2002), the assumptions derived from coalescent theory were used to resolve the loops in the statistical parsimony network.

2.4. Demographic analysis

In the phylogenetically-recovered clades of clones (A–H), Tajima's *D* and Fu's *FS* were calculated in Arlequin based on 10,000 replicates. DnaSP v5 (Librado and Rozas, 2009) was used to calculate Fu and Li's F^*, Fu and Li's D^* and R_2 statistic (Ramos-Onsis and Rozas, 2002) given the observed number of segregating sites.

Population demography was detected by examination of frequency distribution of pairwise differences of sequences (mismatch distributions) between all sampled haplotypes within clades of clones given the null model of sudden population expansion as described by Rogers and Harpending (1992). As conventionally done, it was initially determined whether these statistics were significantly different from what is expected under a standard neutral model by performing coalescent simulations; concordance of the data with this expected distribution was evaluated by the sum of squared deviation statistic (SSD). Raggedness statistic (Harpending, 1994) of the mismatch distribution was also calculated with Arlequin based on 10,000 replicates. To estimate the mode of the mismatch distribution, the parameter τ was estimated for distributions that did not differ significantly from the expectations under a sudden-expansion scenario (Schneider et al., 2000).

3. Results

3.1. Overall genetic diversity

Mean number of pairwise differences, haplotype and nucleotide diversity were calculated separately for each lineage and for the different clades of clones (Table 2). The values in both lineages were similar except for a higher haplotype diversity found in lineage I. While this parameter was lower in H when compared to the remainder clades of clones, nucleotidic diversity was higher in B, E and G and lower in A, C, F and H, showing again this clade of clones the lowest value recorded.

Genetic divergence values are shown in Table 3. Within clade values were up to 5.91% and genetic distances between clades from the same lineage were lower in clades from lineage I (A–C) than in those of lineage II (D–H). The values increased in general when comparing clades of clones from different lineages with a few exceptions (e.g., 11.64% of genetic divergence between clades B and D).

3.2. Population genetic structure

Hierarchical population structure in *A. trapezoides* was studied through an AMOVA approach to check for structure at the level of lineages, clade of clones and populations. As clone 1 (belonging

Table 2
Sequence parameters of the COII sequences in both lineages and in the clades of clones in *A. trapezoides* (A–C lineage I, D–H lineage II).

	Lineage I	Lineage II	A	B	C	D	E	F	G	H
No. Samples	54	142 (64)	33	8	13	2	15	14	10	101 (23)
No. haplotypes	17	20	7	4	6	1	7	3	5	4
Haplotypic diversity	0.874 ± 0.029	0.673 ± 0.002	0.630 ± 0.062	0.750 ± 0.139	0.818 ± 0.096	0	0.809 ± 0.075	0.692 ± 0.065	0.844 ± 0.079	0.355 ± 0.050
No. polymorphic sites	183	143	53	31	23	0	72	7	80	3
Nucleotidic diversity	0.231 ± 0.112	0.209 ± 0.102	0.007 ± 0.004	0.024 ± 0.013	0.011 ± 0.006	0	0.059 ± 0.030	0.006 ± 0.004	0.058 ± 0.032	0.001 ± 0.001
Mean no. pairwise differences	46.613 ± 20.481	42.211 ± 18.552	3.813 ± 1.968	13.036 ± 6.579	6.030 ± 3.091	0	22.552 ± 10.054	5.527 ± 1.909	22.222 ± 10.387	3.678 ± 0.523

Table 3
Measures of population differentiation for clades of clones in *A. trapezoides* obtained from COII data. Above the diagonal, pairwise genetic divergence (uncorrected *p*-distances) between clades of clones of *A. trapezoides* (in percentage of changes). In the diagonal. within-clades divergence (italics). Below the diagonal, F_{ST} values.

Clades	A	B	C	D	E	F	G	H
A	*0.69*	10.59	13.02	14.41	13.93	15.70	15.18	14.64
B	0.902	*2.37*	12.63	11.64	13.06	13.43	12.66	12.45
C	0.935	0.873	*1.09*	13.64	13.50	12.94	13.17	13.22
D	0.954	0.828	0.928	*0.00*	8.19	9.50	8.02	8.24
E	0.825	0.651	0.724	0.412	*5.91*	10.39	9.33	8.97
F	0.957	0.906	0.934	0.938	0.678	*3.08*	9.48	9.29
G	0.869	0.663	0.751	0.420	0.370	0.695	*5.85*	8.88
H	0.982	0.977	0.983	0.985	0.880	0.980	0.911	*1.2*

phylogenetically to lineage II) showed a worldwide distribution and sometimes was found in the same population as other clones, it was excluded from the AMOVA when applied at the population level (Table 4). In the first AMOVA performed (checking for structure in clades within lineages), most of the genetic variation could be explained by differences between clades within lineages. In the second AMOVA (testing genetic structure in populations within lineages), the percentage of genetic divergence explained between lineages and among populations within lineages was similar. In the third AMOVA (that checked for structure in populations within clades) most part of the genetic variation was due to the differences between clades of clones both when subdividing SHB and LAU and when not dividing these populations, although the values differed considerably (81.86% and 48.56%, respectively). The percentage of genetic variation explained by differences within clades and within populations varied in both analyses (within clades 37.5 and 15.43%, within populations 13.54 and 2.71% in the undivided and subdivided analyses, respectively). In any case, the results suggest the existence of genetic structure at the level of clades and populations. F_{ST} values were high in all the comparisons ranging from 0.37 to 0.98, suggesting a lack of gene flow between all pairs of clades and populations (Table 3), further supporting the genetic population structure indicated by the AMOVA (Table 4).

Table 4
Results of hierarchical AMOVA analyses for *A. trapezoides*. Results are shown with or without including the worldwide-distributed clone 1 belonging to the lineage II, and dividing or not the populations of Saint Hilaire-du-Bois (SHB) and Laubert (LAU). d.f.: degrees of freedom.

Source of variation	d.f.	Sum of squares	Variance components	% of variation
With clone 1				
Between lineages	1	1781.094	11.892 Va	**31.69**
Among clades within lineages	7	2160.301	22.574 Vb	**60.16**
Within clades	188	569.167	3.060 Vc	**8.15**
Total	196	4510.532	37.5264	100
Without clone 1				
Between lineages	1	110.966	17.205 Va	**44.29**
Among populations within lineages	26	1955.071	17.243 Vb	**44.39**
Within populations	91	395.071	4.399 Vc	**11.33**
Total	118	3462.025	38.848	100
SHB and LAU undivided				
Between clades	7	2040.426	15.78344 Va	**48.53**
Among populations within clades	20	1025.610	12.32056 Vb	**37.90**
Within populations	91	395.989	4.39988 Vc	**13.54**
Total	118	3462.025	32.50387	100
SHB and LAU subdivided				
Between clades	7	2921.558	28.12202 Va	**81.86**
Among populations within clades	22	458.593	5.30132 Vb	**15.43**
Within populations	89	81.874	0.93038 Vc	**2.71**
Total	118	3462.025	34.35372	100

The parsimony haplotype network (Fig. 2) of the two lineages, constructed to better visualize the relationships between them, recovered several unconnected haplotype networks at a confidence level of 95% that in all the cases corresponded to the different clades. However, more basal haplotypes in some clades following the phylogeographic study by Fernández et al. (2011) appeared unconnected to the remainder at this confidence level, most of the times in clades of clones from lineage I (A–C). Although most of the networks were formed by a central haplotype connected to a few derived ones, some of the subnetworks showed a star-like shape (e.g., H), whilst others seemed to be reticulated networks (e.g., A and E).

3.3. Demographic analysis

In order to check if the demographic history of the clades of clones could be correlated to past Iberian palaeogeographical events, demographic analyses were performed with the samples belonging to the clades of lineage II. Clades of lineage I were not included as the geological history of Northern Iberia did not suffer strong episodes of change (e.g., Andeweg, 2002; Rosenbaum et al., 2002; see Discussion for further details). Clade D was also excluded as it is only formed by two samples that share the same haplotype. Thus, analyses were performed in clades E to H (in this last one, the Turkish population – Diyarbakir was excluded as it was not located in the Iberian Peninsula; see Discussion).

Patterns of genetic variation were characterized by calculating several common summary statistics of the within-population allele frequency distribution in the different clades of clones, including Tajima's *D*, Fu and Li's D^*, Fu and Li's F^* and Fu's *FS* (Table 5).

The analyses of each clade of clones within lineage II yielded different results (Table 5). Parameters in clades E and G pointed towards stability in the populations (e.g., non-significant values in any of the parameters). On the contrary, clade H seemed to have undergone a recent expansion, as indicated by values that significantly differed from the neutral Wright–Fisher model. In the case of clade F, some parameters were consistent with population stability, while others indicated a demographic expansion (Fu and Li's F^* and SSD). The mismatch distribution's null hypothesis of sudden population expansion was rejected in clades E and G based on the sum of squared deviation ($P < 0.05$), but accepted in clade H and partially accepted in clade F (i.e., a significant value of raggedness index but not in SSD). The mismatch distributions of these clone groups were also consistent with these results: bimodal in clades E and G, unimodal in H and a transition pattern between unimodal and bimodal in clade F (Fig. 3). Thus, τ was calculated for H and F, although the raggedness index in this last clade was not significant. τ value was higher in clade F (13.647) than in clade H (2.272), suggesting an older expansion.

4. Discussion

4.1. A. trapezoides, good candidate to correlate with palaeogeographical events

Traditionally, it has been widely assumed that earthworms show a low vagility. However, its magnitude at different geographic and/or temporal scales has been poorly tested, thus the risk of making this assumption is high.

Several studies have tried to shed light on the dispersal rate in some species. For instance, *Aporrectodea longa* (Ude, 1885) was reported to have a colonization rate in earthworm-free soils of 5–8 m year^{-1}, and *Lumbricus terrestris* (Linnaeus, 1758) of

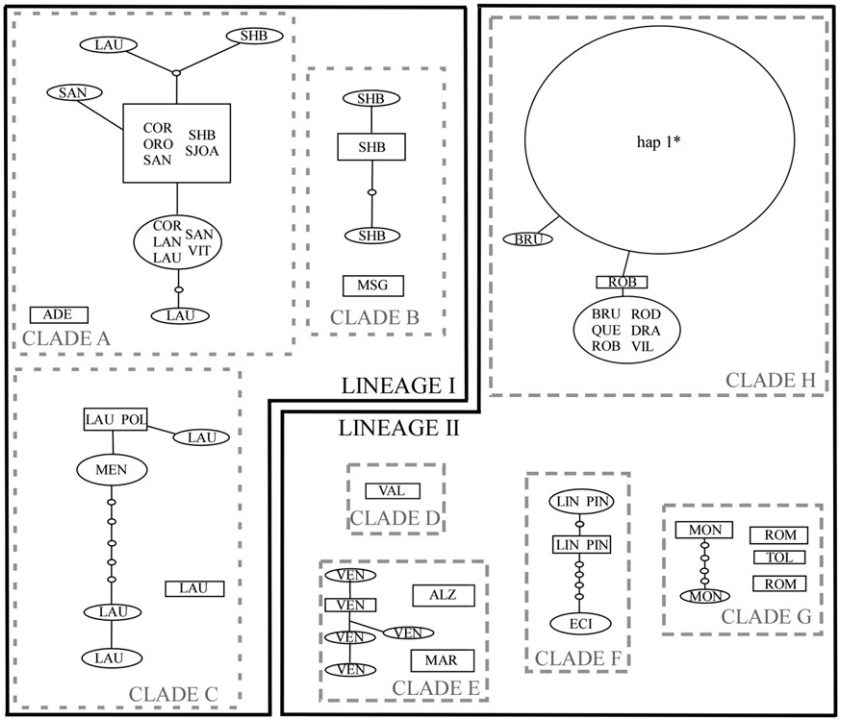

Fig. 2. Haplotype networks recovered for COII in *A. trapezoides* (95% confidence). Empty circles represent unsampled haplotypes. Both lineages and the several clades of clones are remarked.

1.5–4 m year^{-1}(Eijsackers, 2011). In *Aporrectodea giardi* (Ribaucourt, 1901), the dispersal velocity in artificial microcosms was between 0.6 ± 0.3 and 4.5 ± 2.8 cm min^{-1} (Caro et al., 2012). The estimated dispersal rates in these earthworm species can be indeed considered low, however the results of this study make them potentially inconsistent with the concept of low vagility at a geological time scale. Let's consider a mean distance of approximately 700 km from the middle point of clade F to the one of clade H and a dispersal rate of 8 m year^{-1} which is the maximum rate estimated in field conditions from the examples previously given; this rate was estimated in an experiment with *A. longa*, which is anecic as *A. trapezoides* and have also a similar size. Thus, 87,500 years would be needed to go from one point to the other, considering a continuous dispersal. If we take into account that the mean divergence between both lineages is 9.29%, then the mutation rate would be 106 substitutions My^{-1} (that is, 19.24% subs. My^{-1} in the studied fragment of 551 bp from the COII gene), which is much higher than the estimated rates for mitochondrial genes (e.g., 2.4% subs. My^{-1} in mitochondrial genes, Chang et al., 2008; Pérez-Losada et al., 2011; between 0.6 and 2.9 subs. My^{-1} in hormogastrid earthworms, Novo et al., 2011).

Table 5
Demographic parameters and standard deviations for the clades of clones from lineage II *sensu* Fernández et al. (2011) in *Aporrectodea trapezoides*. Significant values are indicated in bold.

	Lineage II			
	E	F	G	H
Tajima's *D*	2.049	**2.223**	0.688	**−1.581**
Fu's *F*	9.328	4.602	8.570	9.511
Fu and Li's *D**	1.334	1.331	−0.030	**1.638**
Fu and Li's *F**	0.767	**1.791**	0.169	**0.730**
R_2 statistic	0.222	0.252	0.201	**0.077**
Test of goodness of fit:				
SSD	**0.139**	0.147	**0.117**	0.040
Raggedness index	**0.213**	**0.445**	**0.157**	0.451
τ	–	13.647	–	2.272
θ_0	–	0.690	–	0.011
θ_1	–	18029	–	34207

On the other hand, this study supports the low vagility in a geological time scale in widespread earthworm species such as *A. trapezoides* due to the strong genetic structure found at the level of localities. As a cosmopolitan earthworm, *A. trapezoides* is assumed to have a wide tolerance to different environmental conditions (Blakemore, 2006); it also has a high reproductive rate (Fernández et al., 2010) and it reproduces mainly parthenogenetically. All these features are advantageous and facilitate the colonization and survival in soils. However, they tend to remain in their areas of origin, with the exception of clone 1. The reasons of this curious phenomenon are scientifically very interesting and should be deeply studied.

One plausible hypothesis could be that forms are locally adapted thus occupying a narrow range of ecological niches, and when dispersal forms (cocoons or juveniles) reach a different area they may be outcompeted by local races or lineages. An example for this could be the case of clades B and C. These clades appeared in the area of distribution of clade A (except the population of Poland that belongs to clade C). Nonetheless, they are genetically very different, which explains the differences in the third AMOVA (that aimed to check for structure in populations within clades) when subdividing or not subdividing individuals from Saint Hilaire-du-Bois and Laubert. Thus, a local race could have surged in this localities previously occupied by clade A, or the opposite (i.e., individuals from clade A moved into areas occupied by those of clades B and C) and now both kind of individuals can be present at the same place. Other possible causes explaining this hypothesis could be the indirect transport of earthworms or cocoons by human actions (by this mechanism, forms that are genetically very distant could be found in the same localities) or ecological preferences (e.g., different habitat preferences). The existence of different sub-

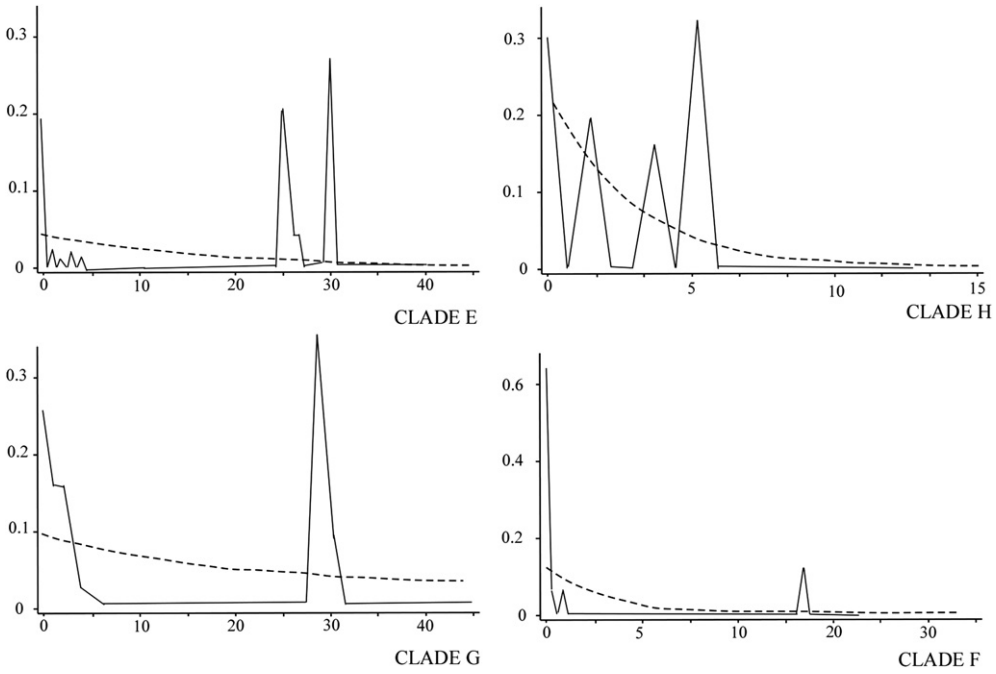

Fig. 3. Mismatch distributions for clades of clones in *A. trapezoides* (lineage II), based on a 551-bp segment of COII gene. The abscissa represents number of pairwise differences and the ordinate represents the number of observations. The smoothed line represents the observed distribution of mismatches and the discontinuous line the expected distribution under the sudden-expansion model of Rogers (1995) as modified by Schneider and Excoffier (1999).

populations genetically differentiated was also observed in *Allolobophora chlorotica*, in which some individuals from the same localities were separated by more than 15 mutational steps (Dupont et al., 2011); the authors concluded that it may be due to an important genetic structure at small scales due to limited gene flow between sub-populations or to a recent mixing of different populations due to human activities. This last cause would seem to explain the case of clades C and H, as two populations from Greece clustered together in clade H and one from Poland in clade C; in these cases, no individuals from a different clade were found, although the scarce number of samples ($n = 1$, 3 and 2, respectively in Poland, Maara and Drama) should be highlighted. Indirect transport by human actions could also explain the case of clone 1 in *A. trapezoides*, found in many localities and sharing the niches occupied by local and geographically-restricted clones. It could be possible that this clone shows a general purpose genotype, thus being able to adapt to a wide range of environmental conditions. Further research is necessary in order to discover the truth underlying this interesting fact that could be important to understand biological invasions.

4.2. Testing how 'good' the candidate is: correlation between demographic history in A. trapezoides and Iberian palaeogeographical events

The direction of Tajima's *D*, Fu and Li's D^*, Fu and Li's F^* and Fu's *FS* is potentially informative about the evolutionary and demographic forces that a population has experienced. For example, Tajima's *D* measures departures from neutrality that are reflected in the difference between low-frequency and intermediate frequency alleles; a positive Tajima's *D* value indicates a decrease in population size and/or that purifying selection is operating. The R_2 test is based on the difference between the number of singleton mutations and the average number of nucleotide differences and detects recent severe population growth, indicated by low R_2 value. In general, the demographic parameters in the different clades yielded consistent estimations of demographic processes. The only exception is Fu's *FS* in the clade H, with a non-significant value. Fu's *FS* statistic compares the observed number of haplotypes with that expected from neutrality and showed significant signals. In clade H it indicated a pattern of haplotype diversity that did not deviate from expectation under neutrality. Although non-significant, the positive direction of the value indicates a deficiency of alleles, as would be expected from a recent population bottleneck or from overdominant selection.

The geological history of the Iberian Peninsula (especially the Mediterranean area) is the result of the fragmentation and fusion of several microplates with a different origin. It has been deeply studied and is well-documented, which makes it a good scenario to study demographic history parameters. As previously shown, even widespread earthworms show a low vagility in the geological time, thus tending to remain in the same areas during long periods of time. Thus, the analyses of the demographic history of each clade of clones could give us some important hints to contrast with past palaeogeographical events, therefore gathering information to understand their diversification and evolution. As stated above, we focused on the clades of clones from lineage II, where the palaeogeographical events were more drastic and well-documented.

At late Eocene (36 Mya), Iberia was attached to Aquitania (France) and the Baetic–Rifan system was a large island separated from the Peninsula by a sea loch (Omodeo and Rota, 2008). The distribution area of the Atlantic clone group was attached to the proto-Iberian Peninsula, while the area where the Northern Mediterranean group is distributed was placed inside the Peninsula but partially surrounded by an arm of the sea formed by a shallow marine area (Andeweg, 2002; Rosenbaum et al., 2002; Omodeo and Rota, 2008). In the late Oligocene (25 Myr), the internal parts of the Baetic–Rif system (Spain and Morocco, respectively), which were previously an island, collapsed with the Hercynian belt, and the shallow internal sea loch occupying the central and Eastern part of Iberia disappeared (Andeweg, 2002; Rosenbaum et al., 2002;

Omodeo and Rota, 2008). These geological events correspond with the demographic history of the clades F and H, which showed signs of population expansion, while clades E and G (distributed in the Hercynian belt) indicated population stability: all the statistics were non-significant and positive, reinforcing the hypothesis of stable populations. Moreover, the results of this study indicated an old expansion and actual transition towards stability in the clade F, while all the statistics showed an event of recent expansion in clade H. Genetic diversity values were also higher in the clades E and G than in the remainder and it also showed a higher number of haplotypes, indicating a more ancient origin for that group.

The results of this study demonstrate that evolutionary history of widespread earthworms (at least in *A. trapezoides*) could be potentially correlated with past palaeogeographical events. It was not the aim of this study to check the extent and power of the correlation, but to test its congruence with well-documented geological events. In the last few decades an intensive effort has been done in order to reconstruct the history, vegetation and climate of the Iberian Peninsula; one of the most powerful tools has been the botanical approach through palaeopalynological studies, which have deeply contributed to achieve this target (e.g., Bessedik, 1984; Suc et al., 1995; Fauquette et al., 2006; Jiménez-Moreno et al., 2010). Here we present a study indicating that earthworms can also be good indicators for these kinds of studies, not only endemic species, but also widespread ones due to the strong population genetic structure that they exhibit.

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