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Adding complexity to the complex: New insights into the phylogeny, diversification and origin of parthenogenesis in the *Aporrectodea caliginosa* species complex (Oligochaeta, Lumbricidae)

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ABSTRACT

The importance of the *Aporrectodea caliginosa* species complex lies in the great abundance and wide distribution of the species which exist within it. For more than a century, chaos has surrounded this complex; morphological criteria has failed to solve the taxonomic status of these species. This present body of work aims to study the phylogeny of this complex by increasing the number of samples used in previous molecular works and by including morphologically-similar species that were never studied using molecular tools (*A. giardi, Nicodrilus monticola, N. carochensis* and *N. tetramammalis*). Two basal clades were obtained: one formed by *A. caliginosa* and *A. tuberculata* and the other by the rest of the species. This second clade was divided into two more: one with Eurosiberian and another with Mediterranean forms. *A. caliginosa* and *A. longa* were divided into two paraphyletic groups. Both *A. giardi* and *A. nocturna* showed characteristics consistent with monophyletic groups. Each of the two recovered lineages of *A. trapezoides* were phylogenetically related to different sexual species. While lineage I of *A. trapezoides* was monophyletic, lineage II resulted to be paraphyletic, as well as the three *Nicodrilus* 'species'. The diversification of the complex occurred during the Late Miocene–Early Pliocene (6.92–11.09 Mya). The parthenogenetic forms within the Mediterranean clade would have diversified before the ones in the Eurosiberian clade (3.13–4.64 Mya and 1.05–3.48 Mya, respectively), thus implying the existence not only of at least two different moments in which parthenogenesis arose within this complex of species, but also of two different and independent evolutionary lines. Neither the 4× rule nor the GMYC method for species delimitation were successful for distinguishing taxonomically-distinct species.

1. Introduction

Morphological stasis has traditionally been a problem in earthworm taxonomy. Michaelsen (1900) was one of the first authors to highlight this difficulty due to the shortage of discriminatory characters, thus defining this animal group as 'sine systemate chaos'. This pattern of limited morphological variation seems to be common in animals living in such a stable environment as soil, in which chemical signals play a more important role than visual ones in sexual selection (Lee and Frost, 2002). Moreover, some anatomical and morphological characters commonly used in earthworm taxonomy usually overlap among different taxa (Pop et al., 2003). These morphological limitations have led to the creation of species complexes, in which similar species that are thought to be phylogenetically closely related, but with an unknown status, are included (Bouché, 1972; Gates, 1972; Sims and Gerard, 1999; Briones, 1993, 1996).

As a consequence of this morphological stasis, recent molecular studies concerning earthworms have revealed a great cryptic diversity hidden under the level of morphospecies (Heethoff et al., 2004; Pérez-Losada et al., 2009; Novo et al., 2009, 2010; James et al., 2010; Buckley et al., 2011; Dupont et al., 2011), with molecular tools being the most appropriate ones to unravel the taxonomic problems of this animal group and to discover the real genetic variations not revealed as morphological differences. Recently, some methods for delimiting species based on molecular sequences have been developed to try to solve this problem, such as the 4× rule (Birky et al., 2005, 2010) or the GMYC method (Pons et al., 2006).

The importance of the *A. caliginosa* species complex lies in the great abundance and wide distribution of some of the species included in it. For example, Omodeo (1948) considered *A. trapezoides* as the most common earthworm in the world, and some of the

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other species of this complex are included in the category of peregrine earthworms (Blakemore, 2006). Due to the important role of earthworms as 'ecosystem engineers' (Lavelle et al., 1997; Decäens et al., 2003) and the possibility that different species have different roles in soil functioning, it is crucial to know not only the taxonomic status of the different species of the complex, but also the real genetic variability that hides beneath it, so that further ecological, systematic, evolutionary and conservation studies can be developed (Domínguez, 2004; King et al., 2008). In addition, the complex comprises both parthenogenetic (*A. trapezoides*) and sexual taxa (the remainder), thus being an appropriate model group for studying the evolutionary features of this kind of uni-parental reproduction.

Taxonomic chaos concerning this complex of species has lasted for more than a century; identification criteria failed to solve the taxonomic status of the species included in it. From 1900 onwards, several authors tried to solve the riddle using both morphological criteria and molecular tools. A good summary of the contributions of each author can be found in Pérez-Losada et al. (2009), which is the state-of the art study concerning the complex. The authors stated the existence of different species within the complex using an integrative approach for species delimitation: *Aporrectodea caliginosa* (Savigny, 1826), *Aporrectodea tuberculata* (Eisen, 1874), *Aporrectodea nocturna* (Evans, 1946), *Aporrectodea longa* (Ude, 1885) and *Aporrectodea trapezoides* (Dugès, 1828), mainly collected in Eurosiberian areas. The major contribution of this study is to unlink *A. trapezoides* and *A. caliginosa*, which were traditionally considered as subspecies. *A. trapezoides* were found to be phylogenetically more closely related to *A. longa* and *A. nocturna*. While *A. tuberculata*, *A. caliginosa* s.s., *A. nocturna* and *A. longa* were found to be monophyletic, *A. trapezoides* was paraphyletic. The authors argued that this fact was down to a possible lack of molecular evidence and marker resolution. Furthermore, Fernández et al. (2011a) found two highly divergent lineages in *A. trapezoides* (lineages I and II with Eurosiberian- and Mediterranean-like distribution, respectively), and their phylogenetic position respecting the rest of the species is unknown.

A second important result of the study of Pérez-Losada et al. (2009) is the existence of molecular evidence indicating that *A. longa* is closely related to some of the species of the complex, although it was traditionally not included in it. There are other species which are morphologically very similar to the ones in the complex but that were never studied on a molecular basis. That is the case of *Aporrectodea giardi* (Ribaucourt, 1901), *Nicodrilus carochensis*, *N. monticola* and *N. tetramammalis*. These three *Nicodrilus* species were recently analysed (Pérez Onteniente and Rodríguez Babío, 2002) and most of the earthworm taxonomists considered them as synonymous species of *A. caliginosa* (for example, Csuzdi and Zicsi, 2003; Blakemore, 2006) due to their morphological similarity and sexual reproduction and also because of the controversy surrounding the validity of this genus. In addition, these three species differ mainly in the number and position of their papillae, which has been stated to change in individuals and populations within a species in relation to their sexual activity. On the other hand, Pérez Onteniente and Rodríguez Babío (2002) stated that the main difference between them and *A. trapezoides* is the sexual condition of the first ones.

In a previous study, a first approach was made to try to shed light upon the origin of parthenogenetic forms in *A. trapezoides* (Fernández et al., 2011b); although phylogenetic analyses indicated an independent origin of uniparental reproduction, only one sexual, three parthenogenetic and one possibly pseudogamic populations were included in the analyses. Since strong lineage sorting was detected within this taxonomical species at a phylogeographical scale (Fernández et al., 2011a) and a paraphyletic status for *A. trapezoides* was recovered in previous molecular studies as stated before (Pérez-Losada et al., 2009), further research is necessary in order to gain stronger evidence regarding the origin of parthenogenesis and the phylogenetic position of the species in the context of the complex as a whole.

The present work aims (i) to review the phylogeny of the *A. caliginosa* species complex by including morphologically similar species that could be potentially closely related to the ones included in the complex, and by increasing both the number of samples and localities with regard to previous studies, (ii) to shed light upon the diversification of the complex in the evolutionary time with special regard to the origin of the parthenogenetic forms, and (iii) to check the species status of the studied taxa by means of two state-of-the-art methods for species delimitation based on molecular information.

2. Material and methods

2.1. Earthworm sampling

A total of 212 earthworms belonging to the *A. caliginosa* species complex or being morphologically very similar and that can potentially be included in it (*A. caliginosa, A. trapezoides, A. nocturna, A. longa, A. giardi, N. carochensis, N. monticola* and *N. tetramammalis*) were collected by digging and hand sorting from nine different countries: Spain, France, Portugal, Italy, Greece, Turkey, Algeria, Egypt and Australia (Fig. 1). Most part of the individuals ($n = 178$) belonged to *A. trapezoides* and were previously sequenced in order to perform a phylogeographical study of this species (Fernández et al., 2011a). The rest of the sequences generated in this study represent a new contribution. All *A. trapezoides* earthworms were identified following the taxonomic key in Gates (1972), the *Nicodrilus* individuals following the key in Pérez Onteniente and Rodríguez Babío (2002) and the rest of the *Aporrectodea* specimens following the taxonomic keys in Bouché (1972) and Blakemore (2006).

In addition, sequences belonging to 28 individuals (*A. caliginosa, A.tuberculata, A. trapezoides, A nocturna and A. longa*) collected in Spain, France, Finland, Denmark, Germany, United Kingdom, Poland and Serbia were retrieved from GenBank (COII and 28S rRNA). Thus, a total of 240 individuals from 15 different countries were included in this study.

After collection, specimens were washed in distilled water and kept in ethanol 96° at −20 °C. A portion of the body wall (±0.025 g) was extracted and carefully cleaned under a stereomicroscope in order to eliminate soil particles and macroscopic parasites. Tissue samples were kept in ethanol 96° at −20 °C until digestion prior to extraction of DNA. All the sampled individuals are deposited at the earthworm criocollection of the Departamento de Zoología y Antropología Física, Universidad Complutense de Madrid (Spain).

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted using the DNAeasy Tissue kit (Qiagen). Polymerase chain reactions (PCR) were performed using a Perkin Elmer 9700 thermal cycler.

A fragment of the cytochrome oxidase subunit I (COI) mitochondrial gene was amplified using the forward primer LCO_AT (5′-CATTYGTRATAATYTTCTTT-3′) and the reverse primer HCO_AT (5′-GCTGATATARAATAGGGTCTC-3′) (Fernández et al., 2011b). 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 μM of each primer and 1.5 units of Taq polymerase.

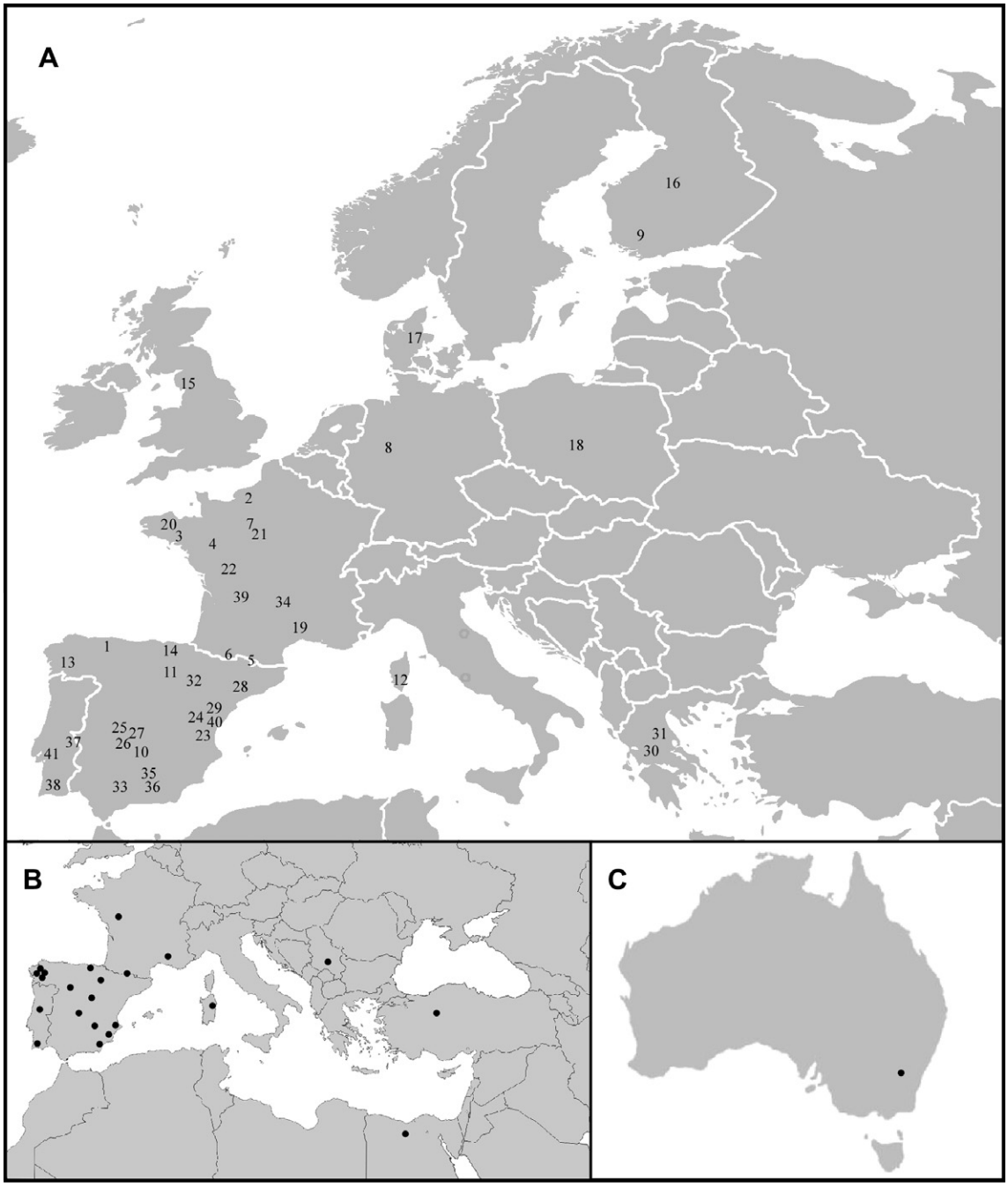

Fig. 1. (a) Geographic situation of the sampled localities included in the phylogenetic studies. (b and c) Approximate situation of the localities were clone 1 of *A. trapezoides* was found. For further information about these localities, see Fernández et al. (2011a).

A fragment of the cytochrome oxidase subunit II (COII) mitochondrial gene 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 40 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 μM of each primer and 1 unit of Taq polymerase.

A fragment of the nuclear histone H3 (H3) gene 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 μM of each primer and 1.5 units of Taq polymerase.

A fragment of the nuclear ribosomal 28S rRNA gene 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 ml included 1.5 μM $MgCl_2$, 10 mM of each dNTP, 10 μM of each primer, DMSO 1 μl and 1 unit 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). Non-specific PCRs were not further processed. Automated sequences were generated 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 JQ763461–JQ763596. 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. The rest of the sequences of *A. trapezoides* (Fernández et al., 2011a) are deposited in GenBank under Accession Numbers JF92325–JF92512. *Hormogaster elisae, H. castillana*, *Lumbricus terrestris* and *Aporrectodea rosea* were used as outgroups; the sequences were also retrieved from GenBank (accession numbers HQ621986, HQ622028, HQ621960, EF653878.1, HQ622019, GQ409653.1; NC_001673.1, AF185262.1 and AF185195.1) or generated in this study (JQ771761-JQ771764).

2.3. Gene diversity and phylogenetic relationships

Estimates of variability were computed using ARLEQUIN v 3.5 (Excoffier and Lischer, 2010) and DnaSP v5 (Librado and Rozas, 2009). Sequences of each individual gene were aligned in CLUSTALX v. 2.0.12 (Thompson et al., 1997) using default settings. Models of sequence evolution and their respective parameters were calculated with jMODELTEST v. 0.1.1 (Posada, 2008) following the Akaike Information Criteria (AIC). The models that best explained the evolution of each gene were used in the different phylogenetic analyses.

Saturation in the third codon position for the protein-coding genes was checked with DAMBE (Xia and Xie, 2001). Transitions were more saturated than transversions. Thus, phylogenetic analyses were done with and without the third codon positions. Nevertheless, the results were the same.

The phylogenetic analyses were performed using a reduced dataset. All the samples excluded belonged to *A. trapezoides*; the included individuals (Fig. 1, Table 1) represented all the major clades found in the previous phylogeographic study (Fernández et al., 2011a). Bayesian phylogeny estimation (BI) was performed using the program MRBAYES v.3.1.2 (Ronquist and Huelsenbeck, 2003). The parameters in MRBAYES were set to four million generations, and 4000 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 (1000 trees) were discarded as 'burn in'. The posterior probabilities (pP) for individual clades obtained from separate analyses were compared for congruence and then combined and summarised using a 50% majority-rule consensus tree. Maximum likelihood (ML) analyses were performed using RAxML v. 7.0.4 (Stamakis, 2006). Clade support was assessed using non-parametric bootstrapping with 1000 pseudoreplicates. Bootstrap analysis (Felsenstein, 1985) with 1000 replicates was used to estimate support for the resulting topologies. In both BI and ML the dataset was partitioned into genes, and protein-coding genes (mitochondrial and H3 genes) were also partitioned into codons.

Gene regions were analysed in two different matrixes: a first one just with COII and 28S rRNA sequences (as the number of samples for these two genes is the highest) ($n = 82$) and a second multiple partitioned supermatrix (COI, COII, 28S rRNA and H3) ($n = 62$). This last matrix was performed without the sequences retrieved from GenBank with the code MPL, as COI and H3 sequences for these individuals were unknown. Nevertheless, the sequences from *A. tuberculata* ($n = 6$) and *A. nocturna* ($n = 2$) retrieved from GenBank were maintained in the different analyses performed with the supermatrix as the phylogenetic signal of COII and 28S rRNA genes alone was strong enough to place this taxon in the same position as in the rest of matrix combinations. 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).

Alternative topologies were tested using parametric bootstrapping procedures outlined by Huelsenbeck et al. (1996). We created alternative hypotheses with topological constraints consistent with each hypothesis (i.e., monophyly of each taxon) using Mesquite 2.75 (Maddison and Maddison, 2011). We simulated 1000 data matrices equal in size to the original matrix using the Genesis module in Mesquite v. 2.75. Afterwards, two parsimony searches for each data matrix (either constraint to the hypothesis being tested or unconstraint) were performed in PAUP*. Differences in tree length for constrained and unconstrained searches for each of the simulated matrices were calculated and plotted as histograms using Mesquite v. 2.75. so as to build a null distribution of tree length differences between two potential topologies. If the difference between constrained and unconstrained topologies in the original data set fell outside the 95% confidence interval of this distribution, then the hypothesis that the constraint tree constitutes the true evolutionary history would be rejected in favor of the shorter unconstrained topology.

2.4. Delimiting species: the 4× rule and the GMYC method

An ultrametric tree was inferred from both single, unlinked mitochondrial genes (COI and COII) ($n = 62$ and $n = 82$, respectively) in BEAST v.1.4.8. (Drummond and Rambaut, 2007). A Bayesian inference analysis implementing an uncorrelated log-normal relaxed clock was performed. BEAST was run for 50 million generations, saving trees at every 5000th generation. The results were visualised in Tracer v.1.5 and the "burn in" was set to 2000 in TreeAnnotator v.1.6.1. (Drummond and Rambaut, 2007). Each gene was analysed under the best-fit model of sequence evolution. A coalescence (constant size) prior was used for the tree prior. Once the ultrametric tree was obtained, the GMYC method developed by Pons et al. (2006) was applied. The GMYC method was implemented in R v.2.8.0. (R Development Core Team, 2008) with the extension ape v.2.2-2 (Paradis et al., 2004). The number of recovered lineages was represented opposite to time and a limit between speciation and coalescence processes was established. Both a single (Pons et al., 2006) and a multiple threshold (Monaghan et al., 2009) were used and the results were compared with a chi-squared test.

As an alternative method for delimiting species, the 4× rule (Birky et al., 2005; Birky et al., 2010) was used, which intended to delimit independent populations by comparing the genetic diversity within and between reciprocally-monophyletic clades.

2.5. Estimates of divergence times

Estimation of divergence times was performed to shed light upon the diversification processes in this complex of species and also to study the origin and antiquity of the parthenogenetic forms of *A. trapezoides*.

Multilocus estimation of divergence times was conducted in BEAST v.1.4.8 (Drummond and Rambaut, 2007) under the same conditions described above. For this analysis, we included one individual representing each species delimited under the GMYC model (see above). A Yule speciation prior was then used for the tree prior. Each partition was analysed again under the best-fit model of sequence evolution. For both mitochondrial genes, we used a calibration based on the work of Chang et al. (2008) for *Metaphire* (Oligochaeta, Megascolecidae) (2.4% substitutions/My^{-1}). For the

Table 1
Sampled localities for each taxon. GPS coordinates are shown for each locality, as well as the code used in the phylogenetic trees. The four genes (COI, COII, H3 and 28S rRNA) were sequenced in all the individuals new to this study ($n = 34$). Samples in italics were retrieved from GenBank; the ones marked with the code MPL were taken from Pérez-Losada et al. (2009), and the rest of them from Fernández et al. (2011a). Samples from clone 1 of *A. trapezoides* (At1) were collected in 22 localities, as shown in Fig. 1b. For further information on all the localities and exact GPS coordinates, see Fernández et al. (2011a).

Taxon	Code	Locality	N. locality	GPS
A. caliginosa	AcSpSAN1,2	San Román (Spain)	1	N 43°15′20.9″ W 05°05′10.3″
	AcFrYVE2	Yvetot (France)	2	N 49°37′ W00°35′
	AcFrBRE1,2	Bretagne (France)	3	N 48°11′ W02°55′
	AcMPL61	*Soudan* (*France*)	4	N 46°25′11.5″ W 00°04′09.2″
	AcMPL119	*Antignac* (*France*)	5	N 42°49′21.4″ E 00°36′16.6″
	AcMPL121	*Adé* (*France*)	6	N 43°07′55.8″ W 00°02′15.4″
	AcMPL123	*Antignac* (*France*)		
	AcMPL133	*París* (*France*)	7	N 48°43′14.91″ E 02°57′15.42″
	AcMPL138	*Göttingen* (*Germany*)	8	N 51°11′40.26″ E 10°16′23.02″
	AcMPL139	*Jokioinen* (*Finland*)	9	N 60°48′02.82″ E 23°27′39.77″
	AcMPL141	*Quinto Real* (*Spain*)	10	N 42°46′02.8″ W 01°49′56.9″
	AcMPL143	*Echarri* (*Spain*)	11	N 43°05′46.4″ W 01°39′22.9″
	AcMPL147	*Bains-de-Taccana* (*Corsica*, *France*)	12	N 41°50′02.4″ E 08°57′45.9″
	AcMPL150	*Orense* (*Spain*)	13	N 42°07′55.26″ W 08°03′04.59″
	AcMPL154	*Bilbao* (*Spain*)	14	N 43°17′54.1″ W 03°02′32.3″
A. tuberculata	*AtubMPL56*	*Lancaster* (*United Kingdom*)	15	N 54°02′ W 02°45′
	AtubMPL62	*Jokioinen* (*Finland*)		E 23°27′37.54″ N 60°48′02.34″
	AtubMPL68	*Jyväskylä* (*Finland*)	16	N 62°14′44.75″ E 25°41′27.49″
	AtubMPL73	*Silkeborg* (*Denmark*)	17	N 56°12′ W 09°30′
	AtubMPL74	*Silkeborg* (*Denmark*)		
	AtubMPL96	*Lomianki* (*Poland*)	18	N 52° 20′ E 20° 53′
A. nocturna	AnFrBRE1,2	Bretagne (France)		
	AnMPL306	*Avignon* (*France*)	19	N 43°54′43.6″ E 004°53′07.7″
	AnMPL307			
A. longa	AlFrADRI1,2	Saint-Adrien (France)	20	N 48°29′ W 03°07′
	AlFrBRU1,2	Brunoy (France)	21	N 48°41′ W 02°30′
	AlFrYVE1,2	Yvetot (France)		
	AlMPL47	*París* (*France*)		
	AlMPL52	*Lancaster* (*Reino Unido*)		
	AlMPL60	*Soudan* (*France*)		
	AlMPL79	*Soudan* (*France*)		
	AlMPL82	*Marnay* (*France*)	22	N 46°23′51.0″ E 00°21′47.2″
	AlMPL93	*Soudan* (*France*)		
	AlMPL309	*Vigo* (*Spain*)		
	AlMPL311	*Vigo* (*Spain*)		
A. giardi	AgFrBRU1,2,4,11	Brunoy (France)		
N. carochensis	NcSpMOG1,2,4	Mogente (Spain)	23	N 38°54′39.5″ W 00°49′48.1″
	NcSpBUN5	Buñol (Spain)	24	N 39°23′56.2″ W 00°50′40.6″
N. monticola	NmSpROB1,2,3,4	Robledillo (Spain)	25	N 39°35′49.6″ W 04°49′39.6″
	NmSpRMA5	Robledo del Mazo (Spain)	26	N 39°36′43.7″ W 04°54′30.2″
	NmSpRCRU2,3,5	Cruce a Robledo del Mazo (Spain)	27	N 39°35′49.2″ W 04°52′11.2″
N. tetramammalis	NtSpROB1,4,5	Robledillo (Spain)		

Table 1 (*continued*)

Taxon	Code	Locality	N. locality	GPS
	NtSpRCRU2,3,5	Cruce a Robledo del Mazo (Spain)		
A. trapezoides	*At1*	*22 populations*[*]		
	At4	*El Brull* (*Spain*)	28	N 41°48′ 00.0″ E 02°21′ 00.0″
		Puerto Querol (*Spain*)	29	N 40°33′ 49.1″ W 00°01′ 03.5″
		Robledillo (*Spain*)		
		Maara (*Greece*)	30	N 41°15′ E 23°14′
		Drama (*Greece*)	31	N 41°09′ E 23°32′
		Villavelayo (*Spain*)	32	N 42°11′ 28.1″ W 02°59′ 03.9″
	At7	*Écija* (*Spain*)	33	N 38°44′ 38.1″ W 02°35′ 02.7″
	At13	*Laubert* (*France*)	34	N 44°35′ 15.2″ E 03°39′ 45.6″
	At14	*Linares* (*Spain*)	35	N 38°05′ 17.7″ W 03°39′ 00.8″
	At15			
		Píñar (*Spain*)	36	N 37°24′ 39.2″ W 03°29′ 47.0″
	At16	*Marvão* (*Portugal*)	37	N 39°22′ 06.0″ W 07°20′ 12.9″
	At18	*Monchique* (*Portugal*)	38	N 37°12′ 14.9″ W 08°32′ 07.5″
	At19			
	At21	*Laubert* (*France*)		
	At24	*San Román* (*Spain*)		
	At25	*Saint Hilaire-du-Bois* (*France*)	39	N 44°39′ 41.6″ E 00°05′ 23.8″
	At26			
	At27			
	At28			
	At29	*Vall d'Uixó* (*Spain*)	40	N 39°50′ 25.2″ W 00°15′ 59.7″
	At30	*Vendas Novas* (*Portugal*)	41	N 38°40′ 13.7″ W 08°26′ 35.8″

ribosomal gene, we used the rate calculated for *Postandrilus* (Oligochaeta, Lumbricidae)(0.063 s/My^{-1}) (Pérez-Losada et al., 2011). Although different substitution rates can be found in the literature for different earthworm families and species (i.e., Novo et al., 2011), we selected these ones because those of *Postandrilus* fell within the 95% of confidence intervals of the rate priors that these authors estimated using the sequences in Chang et al. (2008). Furthermore, they were similar to those reported in other invertebrates including annelids (Pérez-Losada et al., 2011), whilst the ones calculated for *Hormogaster* (Oligochaeta, Hormogastridae) were reported to be quite different and even to change between clades within the family (Novo, 2010; Novo et al., in press). As no rate was ever calculated for the nuclear gene H3, firstly, we estimated the root-to-tip ML tree length in TreeStat v1.6.1 (part of the BEAST package) and then used the median of those lengths to generate a lognormal prior distributions, as described in Pérez-Losada et al. (2011). The confidence interval was set to 95%.

2.6. Character mapping of ancestral states: parthenogenesis vs. sexual reproduction

We used Mesquite v. 2.75 to reconstruct the parthenogenetic state of ancestral node by using a likelihood reconstruction method, assigning to each node the state that maximises the probability of arriving at the observed states in the terminal taxa, given the model of evolution, and allowing the states at all other nodes to vary (Schluter et al., 1997; Pagel, 1999).

3. Results

3.1. Gene diversity and phylogenetic relationships

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

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	186	405	57	38
No. polymorphic sites	117	254	43	37
No. parsimony informative sites	109	217	35	25
G + C content	0.435	0.373	0.632	0.659
Haplotype diversity	0.991 ± 0.006	0.992 ± 0.003	0.804 ± 0.008	0.948 ± 0.009
Nucleotide diversity	0.125 ± 0.004	0.127 ± 0.003	0.022 ± 0.008	0.007 ± 0.0004
Mean number of pairwise differences	39.129 ± 17.21	70.177 ± 30.44	6.837 ± 3.265	5.487 ± 2.659
% divergence	8.58 ± 3.77	12.74 ± 5.52	2.23 ± 1.06	0.677 ± 0.328

The multiple sequence alignment of the concatenated sequence data was 2124 bp in length. Likelihood ratio tests performed with jModelTest following the Akaike Information Criterion (AIC, Posada and Buckley, 2004) indicated that the best-fit model of sequence evolution is GTR + I + G for the concatenated data set and for the individual partitions excepting the 28S rRNA gene, which adjusted to a HKY + I model. ILD test was non-significant ($P > 0.05$).

The trees generated by the different phylogenetic analyses (BI, ML) showed the same topology in the different analysed partitions and in the concatenated dataset. The few topological differences

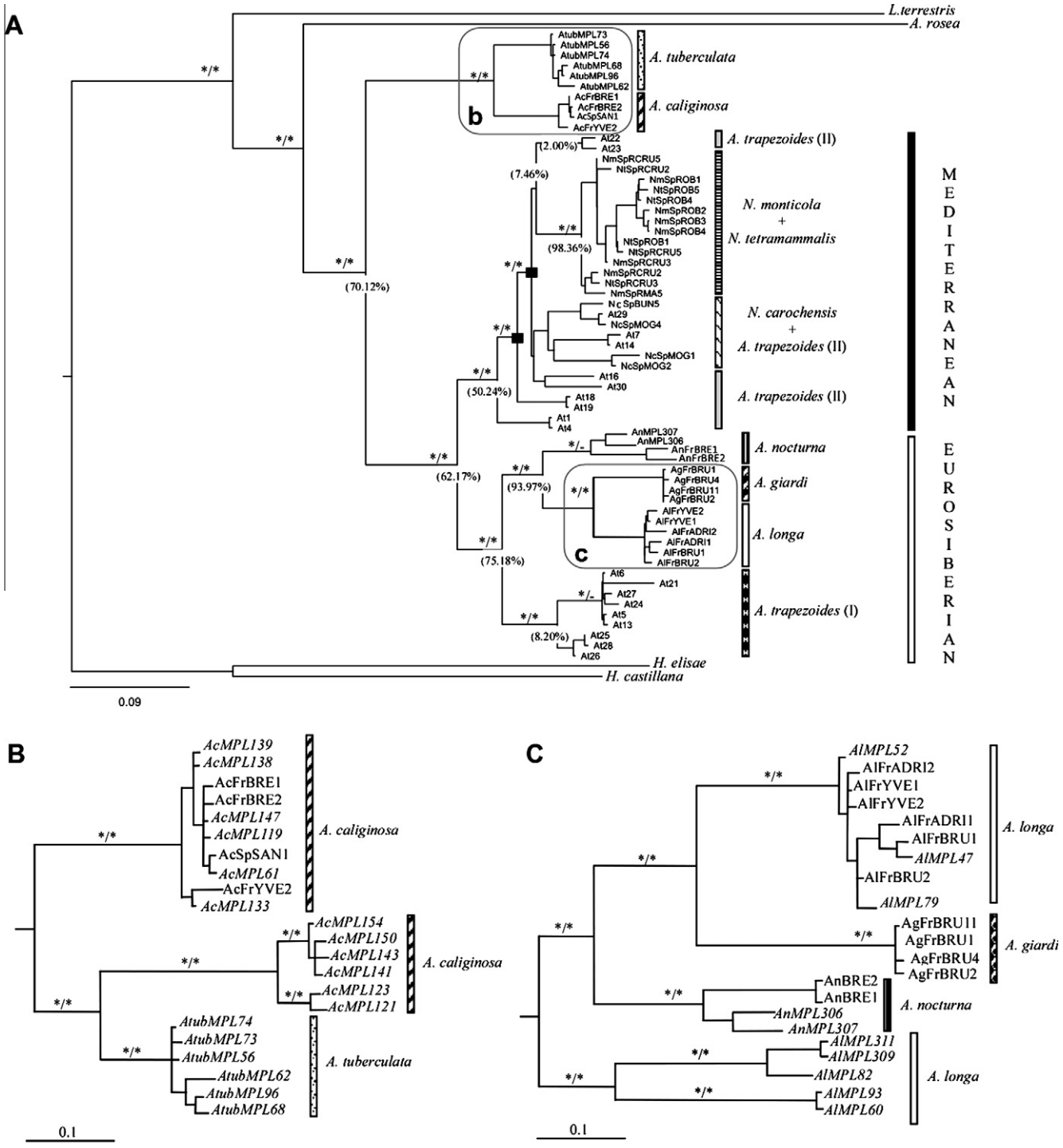

Fig. 2. (a) Maximum likelihood tree (ML) based on the four gene fragments amplified for the *A. caligonosa* species complex (COI, COII, H3 and 28S rRNA, $n = 62$). Branch lengths are shown proportional to the amount of change along the branches. Posterior probability (BI) and bootstrap values of coincident associations are represented above branches with an asterisk (*) when >0.70/70 (BI/ML). A black square represents a node that showed a polytomy in one of the performed analyses. The probability of sexual reproduction in the ancestral nodes is indicated. (b and c) Detail of the Maximum likelihood tree (ML) based on two gene fragments (COII and 28S rRNA); *A. caligonosa* and *A. longa* became paraphyletic when sequences retrieved from GenBank were added to the phylogenetic analyses.

observed were always observed at a terminal level.The main phylogenetic relationships were recovered in all the analyses with a good statistical support (Fig. 2).

Three main monophyletic clades were recovered: a first one with *A. caliginosa* and *A. tuberculata*, a second one clustering together *A. longa, A. nocturna, A. giardi* and the lineage I of *A. trapezoides*, and a third one formed by the lineage II of *A. trapezoides*, *N. carochensis, N. monticola* and *N. tetramammalis*. This last clade showed in general more polytomies than the rest.

A. caliginosa resulted in two paraphyletic clades (Fig. 2b), one of them being closer to *A. tuberculata* than to the other one. *A. longa* was also divided into two paraphyletic groups (Fig. 2c), one of them very close to *A. nocturna*. Both *A. giardi* and *A. nocturna* were recovered as monophyletic groups. While the lineage I of *A. trapezoides* resulted to be monophyletic, the lineage II was paraphyletic, as well as the three *Nicodrilus* species. *N. carochensis* appeared mixed with some samples from the lineage II of *A. trapezoides*. *N. monticola* and *N. tetramammalis* clustered together and mixed in the same clade.

3.2. Delimiting species: 4× rule and GMYC model

The 4× rule for species delimitation (Birky et al., 2005, 2010) was applied to all the clades in the complex by comparing the genetic diversity within and between reciprocally-monophyletic clades. In general, genetic diversity within clades showed a high value. Only one clade of *A. caliginosa* (the one not clustering together with *A. tuberculata*), one clade of the lineage I of *A. trapezoides* and *A. giardi* were consistent with the rule; in these clades the mean distance between clades was at least four times bigger than the distance within clades. This rule could not be applied to the clade of Mediterranean forms due to the presence of polytomies.

The GMYC model delimited 16 and 19 different species (COI and COII, respectively), considering both a single or a multiple threshold (Fig. 3). The three more species recovered in the COII analyses corresponded to clades of sequences retrieved from GenBank. A chi-square test did not show significant differences between both analyses (COI: $\chi^2 = 3.80$; $P = 0.70$; COII: $\chi^2 = 2.52$; $P = 0.86$). This method indicated the existence of one species of *A. nocturna*, two (COI) and three (COII) of *A. longa*, two within the lineage I of *A. trapezoides*, one of *A. giardi*, three within the clade *N. tetramammalis/N. monticola*, two of *N. carochensis*, three of the lineage II of *A. trapezoides*, two (COI) and three (COII) of *A. caliginosa* and one of *A. tuberculata*. Clones 1 and 4 of *A. trapezoides* (the most common ones, Fernández et al., 2011a) resulted in the same species under this model. One species of *A. carochensis* resulted from the union with a clone of *A. trapezoides*. Following this method, *A. giardi* was the only taxon recovered as a species that included all the individuals taxonomically identified with this name, but only under the single threshold approach.

3.3. Estimates of divergence times and evolution of parthenogenesis

Divergence times are shown in Fig. 4. The complex started to diverge between 6.92 and 11.09 Mya. The differentiation of the clade clustering the lineage II of *A. trapezoides* and the *Nicodrilus* species (clade of Mediterranean forms from now on) occurred in a similar period to that of the clade clustering the lineage I of *A. trapezoides* with *A. longa, A. nocturna* and *A. giardi* (clade of Eurosiberian forms hereafter) (3.13–4.64 Mya and 3.85–6.41 Mya, respectively).

The common ancestor of all the parthenogenetic forms within the Mediterranean clade is older than that of the Eurosiberian clade (3.13–4.64 Mya and 1.05–3.48 Mya, respectively), thus implying the existence of at least two different moments in which parthenogenesis arose within this complex of species. In the Eurosiberian clade, parthenogenetic forms are monophyletic, while in the clade of Mediterranean forms they appeared to be mixed with the different *Nicodrilus* species. This could indicate different origins of parthenogenesis within this Mediterranean clade, however further studies are needed to confirm this hypothesis. The diversification of two of the sexual forms in the Mediterranean clade (*N. monticola* and *N. tetramammalis*) occurred between 2.60–4.05 Mya. This fact is of special interest as the three populations included in this clade are located between only 5 and 10 km apart.

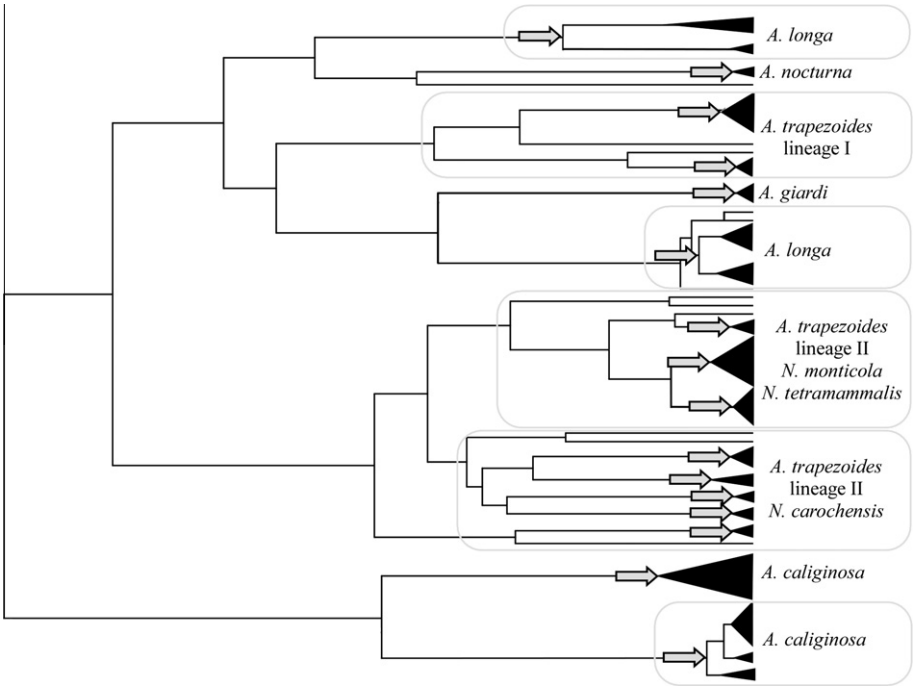

Fig. 3. Ultrametric tree showing the species delimited under the GMYC model for species delimitation, after analysing the fragment of the mitochondrial gene COI ($n = 62$). Grey arrows indicate each delimitated species.

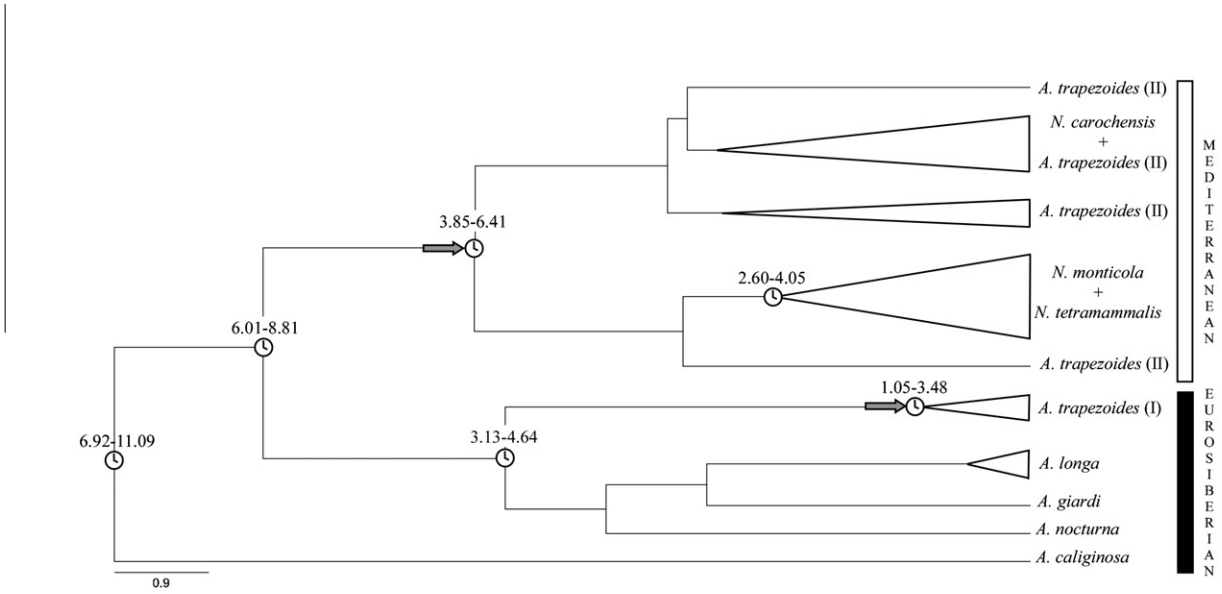

Fig. 4. Estimate of divergence times in the main clades under a Yule speciation process, in millions of years (95% probability range). Grey arrows indicate the origin of parthenogenetic clades.

The estimates of the ancestral probability of a common ancestor to be sexual are shown in Fig. 2. The probabilities ranged from 2% in common ancestors of parthenogenetic terminal data and 97% in the case of sexual taxa, and decreasing as we go back in time to more ancestral nodes.

4. Discussion

4.1. Taxonomic and phylogenetic implications

All the phylogenetic analyses revealed the existence of two main clades, one including *A. caliginosa* and *A. tuberculata*, and the other clustering together the remaining taxa. This result is congruent with that presented by Pérez-Losada et al. (2009) who showed that *A. trapezoides* is not phylogenetically closer to *A. caliginosa* but to other species such as *A. longa*, not included in the former complex of species. Similarly, in the present study there is enough molecular evidence to include *A. giardi, N. monticola, N. tetramammalis* and *N. carochensis* in the complex of species. Thus, it seems that the phylogenetic relationships between all these morphologically-similar species are much more complex than traditionally thought, and that the complex is composed by different lineages that are phylogenetically closely-related. In addition, the phylogenetic position does not usually correspond with the taxonomic expectations as most of the taxonomic species are not recovered as monophyletic groups. The taxonomic implications are different in each clade, as discussed in detail below.

A. longa appears as a polyphyletic taxon, with one of its clades closely related to *A. giardi* and the other one with *A. nocturna*. The relationship between *A. longa* and *A. giardi* has been traditionally considered as a close one by some taxonomists. *A. giardi* is a synonymous of *A. terrestris*, which was frequently subdivided in two subspecies: *A. terrestris typica* (synonymous of *A. giardi*) and *A. terrestris longa* (synonymous of *A. longa*). In addition, there are several authors that support the existence of a taxonomic similarity between *A. giardi, A. longa* and *A. nocturna* (Gates, 1972; Blakemore, 2006). The morphological relationships of the group *A.giardi/A. longa/A. nocturna* cannot be solved in this study, possibly due to the scarce number of collected samples and their geographical distribution, so this division should be revaluated with molecular tools by using a larger number of samples.

The taxonomic implications of this study are more striking in the case of *A. trapezoides*. If both lineages are considered as *A. trapezoides*, thus should also be the rest of the taxa that share the same common ancestor as them, which would include *A. giardi, A. longa, A. nocturna* and the three *Nicodrilus* species described by Pérez Onteniente and Rodríguez Babio (2002). Thus, linages I and II of *A. trapezoides* could be considered as different evolutionary entities due to their ancient diversification, and also a different name for each should be coined. The holotype of the nominal species *A. trapezoides* was sampled in Montpellier (France), but was subsequently lost (Gates, 1972). The ideal condition would be to sample more individuals close to this locality in order to establish a neotype as similar as possible to the original holotype. Due to the geographical situation of this area, the neotype would presumably belong to the lineage I (Fernández et al., 2011a), thus this lineage would conserve the name of *A. trapezoides*. Nevertheless, it is also possible that these samples belonged to the widely-distributed clone 1 (which is included in the lineage II). Further research on this topic will help in taking a decision on this issue.

Lineage II of *A. trapezoides* appears as polyphyletic and mixed with the three *Nicodrilus* species. This study highlights the phylogenetic relationship of these three species with the rest of the species of the *A. caliginosa* complex, indicating that they should be included in the same genus and be nominated as *A. monticola, A. tetramammalis* and *A. carochensis*. This implies the necessity of a further revision in the genus *Nicodrilus*. Moreover, the genus *Aporrectodea* seems to be polyphyletic (Pérez-Losada et al., 2011) and also needs a thorough revision. Thus, the phylogenetic position of the species studied here should be reviewed after further sampling and analysing efforts. *A. monticola* and *A. tetramammalis* appeared mixed in the same clade but clearly divided in localities, which does not support their differentiation as distinct taxa. *A. carochensis* is also mixed with a clone of lineage II of *A. trapezoides*, which was sampled in a close area. The main difference between the three

previously named *Nicodrilus* species lies within the number and position of the anterior papillae, and between them and *A. trapezoides* mainly their sexual condition. A priori it seems that their status as species is not justified under both morphology and sequence information. It is not only their position in the phylogenetic trees that gives the clue, but also the low genetic divergence between them and also with the lineage II of *A. trapezoides* (Fernández, 2011). Therefore, after the results of this study, they could be interpreted as sexual forms of *A. trapezoides*. Thus, this taxon would include both sexual and parthenogenetic forms, as will be discussed elsewhere.

The shape and position of the tubercula pubertatis has been traditionally an important character in earthworm taxonomy. It has been especially important in the differentiation of the species in the *A. caliginosa* species complex: some species present the tubercula pubertatis placed in the segments 31, 32 and 33 in a linear band (both lineages of *A. trapezoides, A. carochensis, A. monticola* and *A. tetramammalis*) or in ridge-like shape (*A. caliginosa, A. tuberculata, A. nocturna*), and others in the segments 32, 33 and 34 also in a linear band (*A. longa*) or in ridge-like shape (*A. giardi*). After the results of this study, this character does not seem to be adequate for species identification.

4.2. Diversification of the complex: origin and evolution of parthenogenesis in A. trapezoides

Previous evidence indicating a multiple origin of parthenogenesis in *A. trapezoides* already existed (Fernández et al., 2011b). However, due to the scarce number of populations included in this previous study, this statement was rather premature. Here we confirm that parthenogenesis seems to have arisen at least in two different moments and, in addition, that despite their morphological convergence, both lineages of *A. trapezoides* clustered together with different sexual species. In addition, the diversification of both lineages of *A. trapezoides* is even older than the one of other species from the complex (i.e., *A. giardi* and *A. longa*), which reinforces the idea of their consideration as different evolutionary entities. Different degrees of degradation in reproductive structures can be found in parthenogenetic lumbricids. There are species such as *Dendrobaena pantaleonis* in which secondary sexual organs such as spermathecae and tubercula pubertatis are completely absent, while in others these organs are maintained, becoming a real phylogenetic burden. Gates (1972) proposed two hypotheses to explain this last fact: either these organs were highly resistant to change through accumulation of mutations, or parthenogenesis was recent. It could also be considered that they represent different steps in an evolutionary process tending to get rid of these energetically expensive structures, thus parthenogenesis could be hypothetically older in species showing higher levels of degradation in their sexual organs. Thus, this points towards a recent parthenogenesis in *A. trapezoides*. Again, deeper research is necessary to shed light upon the issue.

The divergence time estimation analysis in BEAST indicated that the split between theMediterranean and Eurosiberian clades occurred in the Late Miocene-Early Pliocene. Palaeobotanical studies indicate that by this time, the Iberian Peninsula already experienced a warm and dry climate in the southern part and a warm and wetter climate in its northern part that originated in the Middle Miocene and showed a latitudinal gradient in temperature and precipitation (Jiménez-Moreno et al., 2010). Jiménez-Moreno and Suc (2007) also inferred the existence of a gradient of seasonality related not to a strong change in temperature, but to the existence of a drought period from Southern Iberia to North Iberia/Southern Europe. Thus, the split of the complex into clades formed only by Mediterranean or Eurosiberian taxa could have been driven by adaptations to different environmental conditions (i.e., drought resistance). Indeed, there is evidence to support the influence of actual climate conditions in the distribution range of earthworms. Trigo et al. (1988) reported two major biogeographical areas for earthworms in western Iberia (Galicia in Northern Spain and Portugal) which they referred to as Galaico-Lusitanian and Atlantic provinces, divided by the river Mondego in Serra da Estrela. Rodríguez et al. (1997) also found that the limit between them was further south than conventionally accepted, regarding vegetation-type classification (Rivas-Martínez, 1987) and that the transition areas between both major regions corresponded with a mean annual precipitation within the 700–1000 mm range. Following this, actual environmental conditions seem to have an influence in earthworm distribution not by means of being the direct cause, but possibly as a modelling agent that only allows adapted forms to exist in an area under certain climatic conditions.

The origin of parthenogenetic forms occurred in a different period in both main clades. In the clade of Mediterranean forms, parthenogenesis originated during the Late Miocene–Pliocene epochs. The most widespread clones of lineage II (At1 and At4; Fernández et al., 2011b) would have diversified earlier than the remaining parthenogenetic or sexual forms belonging to the Mediterranean clade. Indeed, the rest of the parthenogenetic clones from this clade show a restricted distribution and are sometimes more related to sexual forms than to other parthenogens (i.e., At29 and NcMOG or NcBUN), which could also be an evidence of multiple and recent origin of parthenogenesis from local sexual forms. The earlier origin of parthenogenesis in the Mediterranean clade could also be due to the harsher environmental conditions and drought periods, as parthenogenesis could mean a faster way of colonising new habitats; parthenogenetic animals are common in environments that are hospitable for only a relatively short time frame, because mating would imply an important loss of time and these animals would need to produce large numbers of offspring to compensate for the low survival rate of the hatchlings (Fernández et al., 2010). On the other hand, the origin of parthenogenesis in the clade of Eurosiberian forms occurred during the Pleistocene epoch. This period also corresponds with harsh environmental conditions (Pleistocenic glaciations) that could have also triggered this kind of uniparental reproduction. Obviously, the common ancestors of all the parthenogenetic forms were estimated to have some ancestral probability for reproducing parthenogenetically, which reached values of 30% in the basal node of the whole complex and values of 93% and 20% for the more basal common ancestor of the clades of Mediterranean and Eurosiberian forms respectively. In some cases, there was a strong change in the probability from two common ancestors within the same clade, as the case of the common ancestor of all the *A. monticola/A. tetramammalis* forms (2%) and the common ancestor of all the Mediterranean forms (93%). The implications of these facts lie on a strong evolutionary potential to changing from sex to parthenogenesis (or viceversa) in all the forms of the complex.

4.3. Earthworm species delimitation under the 4× rule and the GMYC method

Both methods to delimit species (the 4× rule and GMYC) yielded completely different results. While almost no clade fulfilled the 4× rule, 16 (COI) and 19 (COII) species were recovered under the GMYC method. This method did not recover morphologically-distinct species in earthworms. The scarce mobility of these animals results in a reproductive isolation and high genetic divergence whilst maintaining a morphological constancy. Due to this fact, it is probable that in this animal group, speciation phenomena work at a slower rhythm. For instance, two phylogenetic species of hormogastrids whose divergence in COI is almost 20% can be considered as the same morphological species (Novo et al., 2010).

Earthworm populations tend to remain in the same areas during longer periods of time due to their physiological aptitudes, their ecological restrictions and their low dispersal ability, thus following the fate of their habitats and geographical areas (Omodeo and Rota, 2008). Moreover, it has been suggested that genetic changes with repercussion in an evolutionary level occur very slowly in earthworms (Cobolli-Sbordoni et al., 1992; Omodeo, 2000), thus events of allopatric speciation in this animal group should occur during millions of years (that is to say, in the same time scale as geological events). As a good example for this, the three sites where *A. monticola* and *A. tetramammalis* were collected (Robledillo, Robledo del Mazo and a sampling point in the middle of this transect, Fig. 1) was only 5–10 km apart and they would have diversified 2.60–4.05 Mya. The 4× rule yielded results more compatible with the taxonomic delimitation of the species considered in this study. Genetic diversity within clades resulted to be very high, presumably as a consequence of reproductive isolation processes that lead to high genetic divergences, thus being very difficult that two reciprocally-monophyletic groups fulfil the rule.

The results of this work have important implications for the taxonomy of this species complex, evidencing that morphological characters alone (at least the ones usually considered in traditional taxonomy), fail to mirror not only the high molecular diversity, but also the underlying evolutionary events. Additional sampling efforts and a deeper study of the phylogeography of each of the species would be necessary to shed light upon the phylogeny of the whole complex with special regard to the morphological stasis and deceitful characters that it displays.

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