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Ultrastructural and molecular insights into three populations of *Aporrectodea trapezoides* (Dugés, 1828) (Oligochaeta, Lumbricidae) with different reproductive modes

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ABSTRACT

Aporrectodea trapezoides is a widely distributed earthworm, consisting of sexual, pseudogamic and parthenogenetic forms. We sampled individuals from five differently reproducing populations: one sexual population (ROB), three parthenogenetic populations (MOL, ALC and SAN), and another population (SHB) in which earthworms had sperm-filled spermathecae (indicating copulation) but seminal vesicles empty of mature sperm (indicating inhibited sperm production). Ultrastructure of spermathecae of ROB, MOL and SHB showed a high number of functional spermatozoa in ROB, no presence of any sperm in MOL, but presence of sperm in SHB, however with morphological evidence for active degradation. Spermiogenesis was complete in ROB, but aberrant in MOL and SHB, which had less germ cells in the seminal vesicles and incomplete maturation of spermatozoa. SHB and MOL also showed a significantly higher amount of lipid droplets than ROB in spermathecae epithelium and seminal vesicles, indicating that the mechanisms involved in nurturing the sperm continues to be functional in SHB and MOL. Molecular analyses of mitochondrial cytochrome c oxidase I and nuclear histone H3 revealed a genetic proximity of the parthenogenetic populations MOL and ALZ to ROB, while SHB and SAN were more divergent and clustered together. Phylogenetic analyses indicated independent origin of uni-parental reproduction.

Introduction

Among lumbricids, not only parthenogens are commonly found, but also show a variety of different models of parthenogenesis: some forms are pseudogamic (Omodeo 1952; Suomalainen et al. 1987) while others are purely parthenogenetic (Omodeo 1952; Fernández et al. 2010a; Díaz Cosín et al. 2011). This variance can occur among and even within the species level, and suggests, a priori, a very labile mechanism of switching from sex to parthenogenesis and seems to point to different origins of uni-parental modes of reproduction within this group.

The existence of different reproductive modes can also be found in the hermaphroditic earthworm *Aporrectodea trapezoides*. Recent behavioural experiments (Fernández et al. 2010a) have shown the most abundant clone of *A. trapezoides* (Fernández et al. 2010b) to be purely parthenogenetic, thus eliminating the possibility of seasonal production of sperm. Sexual forms have also been discovered (Fernández et al. 2010c; Fernández 2011). One pseudogamic population was found in Italy (close to Naples) by Omodeo (1951a), with signs of copula (spermathecae containing sperm) and production of spermatozoa. In contrast, we found one population in France that exhibited full spermathecae – obviously a copulating form – but with empty male funnels (that is, no production of mature sperm). Thus, *A. trapezoides* is a suitable model system to study the transition from sex to parthenogenesis and its consequences at different levels, showing not only conspecific sexual and parthenogenetic forms, but also intermediate stages.

Therefore, the objectives of this study were: (1) to investigate the morphological differences in sperm production (seminal vesicles) and the presence of sperm in spermathecae indicating copula of several populations of *A. trapezoides* in a gradient sex-parthenogenesis, and (2) to compare the genetic consequences of each reproductive mode in the different populations.

To achieve these goals, we investigated in detail (i) the occurrence and fate of affiliated sperm in individuals from a population in which earthworms copulate but do not produce sperm by analyzing the structure and ultrastructure of spermathecae; and (ii) the maturation and functionality of sperm production in individuals from the above-mentioned population as well as from a fully

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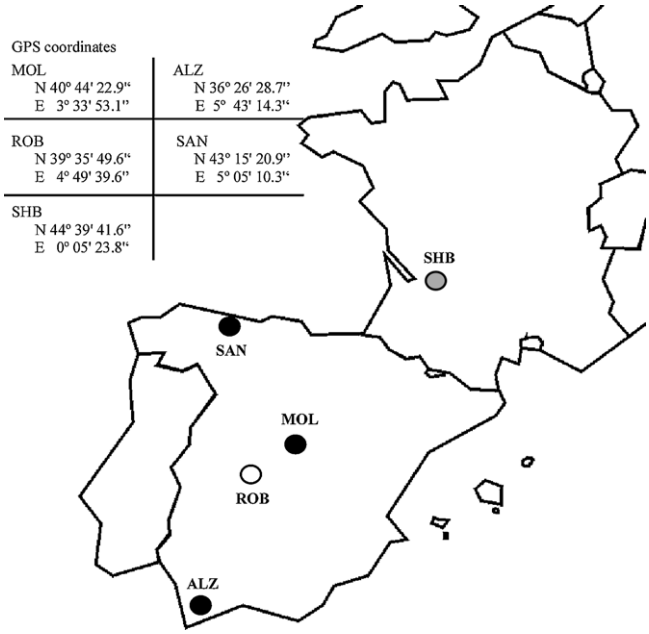

Fig. 1. Map of the locations of the studied populations: St. Hilaire-du-bois (SHB) (intermediate between sex and parthenogenesis, grey circle), Robledillo (ROB) (sexual, white circle), El Molar (MOL), San Román (SAN) and Alcalá de los Gazules (ALZ) (the last three parthenogenetic, black circle). GPS coordinates are shown.

parthenogenetic population and a fully sexual one by analyzing the ultrastructure of seminal vesicles. We also validated the genetic distinctness of the populations with molecular markers and analysed the phylogenetic relationships among them.

Materials and methods

Earthworm sampling

For electron microscopy, completely clitellate specimens of *A. trapezoides* were obtained in March 2010 by digging and hand sorting in three localities: El Molar (MOL, parthenogenetic form; province of Madrid, autonomous region of Madrid, Spain), Saint Hilaire-du-Bois (SHB, form with full spermathecae and seminal vesicles empty of mature sperm; region of Aquitaine, department of Gironde, France), and Robledillo (ROB, sexual form; province of Toledo, autonomous region of Castilla-La Mancha, Spain). For phylogenetic analysis, two more parthenogenetic populations (6 individuals from each population) were added: San Román (SAN, Asturias, Spain) and Alcalá de los Gazules (ALZ, Cádiz, Spain) (Fig. 1).

Mature earthworms were morphologically and anatomically identified as *A. trapezoides* following the taxonomic keys in Gates (1972) and Pérez Onteniente and Rodríguez Babío (2002). Before fixation, a portion of the tail was cut and preserved in absolute ethanol for molecular analysis. More samples from previous sampling campaigns held in 2008 and 2009 were added to our study for the molecular analysis for a total of 20 individuals per population.

Light and transmission electron microscopy

Specimens (3 individuals in ROB, 6 in SHB and 2 in MOL) were sampled during their seasonal peak in reproductive activity, and were transferred into and kept submersed until death in ice-cold Karnovsky's fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde diluted in 0.05 HEPES buffer at pH 7.56 with 4% saccharose and 0.01 mM $MgSO_4$. After this, earthworms were dissected in ice-cold Karnovsky's fixative; the seminal vesicles and spermathecae were removed for processing. During the dissection, iridiscence in male funnels (a sign of presence of sperm) was also checked. The samples were dehydrated in a graded ethanol–acetone series. After dehydration, samples were gradually infiltrated and embedded in epoxy resin. Polymerisation was conducted in an oven at 60 °C for 72 h.

Semi-thin (0.5 μm) and ultrathin (50 nm) sections were cut using a Reichert Ultracut microtome and diamond knives. Ultrathin sections were post-stained with uranyl acetate in ethanol 50% followed by aqueous lead citrate. TEM was conducted both on a Siemens Elmiskop 1A (Siemens, Germany) and on a FEI Tecnai 10 transmission electron microscope (FEI, Netherlands) at 80 kV.

To assess the relative amounts of lipid droplets found in the spermathecae and seminal vesicles tissues and of germ cells in the seminal vesicles (measured as occupied surface in the photographs), we checked 10 micrographs (1750X) taken randomly in 1 mm^2 per grid and in 5 grids per individual to be sure that the sampling was done in three dimensions and to check that the distribution of lipid droplets was homogeneous. The program ImageJ 1.43 was used for the measurements. Statistical analyses were performed using the program Statistica 6.0.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted using the DNAeasy Tissue kit (Qiagen, Germany). Polymerase chain reaction was performed using an Eppendorf Mastercycler epgradient (Eppendorf, Germany) thermal cycler. Twenty individuals per population were analysed in SHB, MOL and ROB, and 6 in SAN and ALC.

A fragment of the mitochondrial gene citochrome oxidase I (COI, 458 bp) was amplified using a specific pair of primers designed by Rosa Fernández for the *Aporrectodea caliginosa* species complex: forward primer LCO_AT (5′-CATTYGTRATAATYTTCTTT-3′) and reverse primer HCO_AT (5′-GCTGATATARAATAGGGTCTC-3′). Amplification conditions were: initial denaturation at 95 °C for 15 min, followed by 35 cycles of 94 °C (30 s) denaturation, 50 °C (70 s) annealing and 72 °C (90 s) extension, and terminated at 72 °C (10 min) final extension.

A fragment of the nuclear gene histone H3 (311 bp) was amplified using the forward primer H3aF (5′-ATG GCTCGTACCAAGCAGACVGC-3′) and the reverse primer H3aR (5′-ATATCCTTRGGCATRATRGTGAC-3′) (Colgan et al. 1998). Amplification conditions were: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C (30 s) denaturation, 50 °C (30 s) annealing and 72 °C (60 s) extension, and terminated at 72 °C (7 min) final extension.

PCR in both cases was performed with the HotStar Taq (Qiagen, Germany); the total reaction volume of 20 ml contained 1.5 mM $MgCl_2$, 200 mM of each dNTP, 100 pmol of each primer, 2.5 units of Taq polymerase and 1 μl DNA template, regardless of extraction yield.

All PCRs were specific and resolved by 1% agarose gel electrophoresis, visualized by ethidium bromide fluorescence under UV-light, and products were purified using a the Speedtools PCR clean-up kit (Biotools, Spain); non-specific PCRs were not further processed. Sequencing in both directions was conducted by Macrogen Inc. (Korea).

All PCR products gave unequivocal nucleotide chromatograms. Sequences were compared with known earthworm sequences in GenBank using the BLAST search algorithm (Altschul et al. 1997), and aligned with CLUSTAL X (Thompson et al. 1997). All alignments were unambiguous; all sequences were of the same length. The DNA sequences were deposited in GenBank under the Accession Numbers JF313475–JF313618 (to be provided).

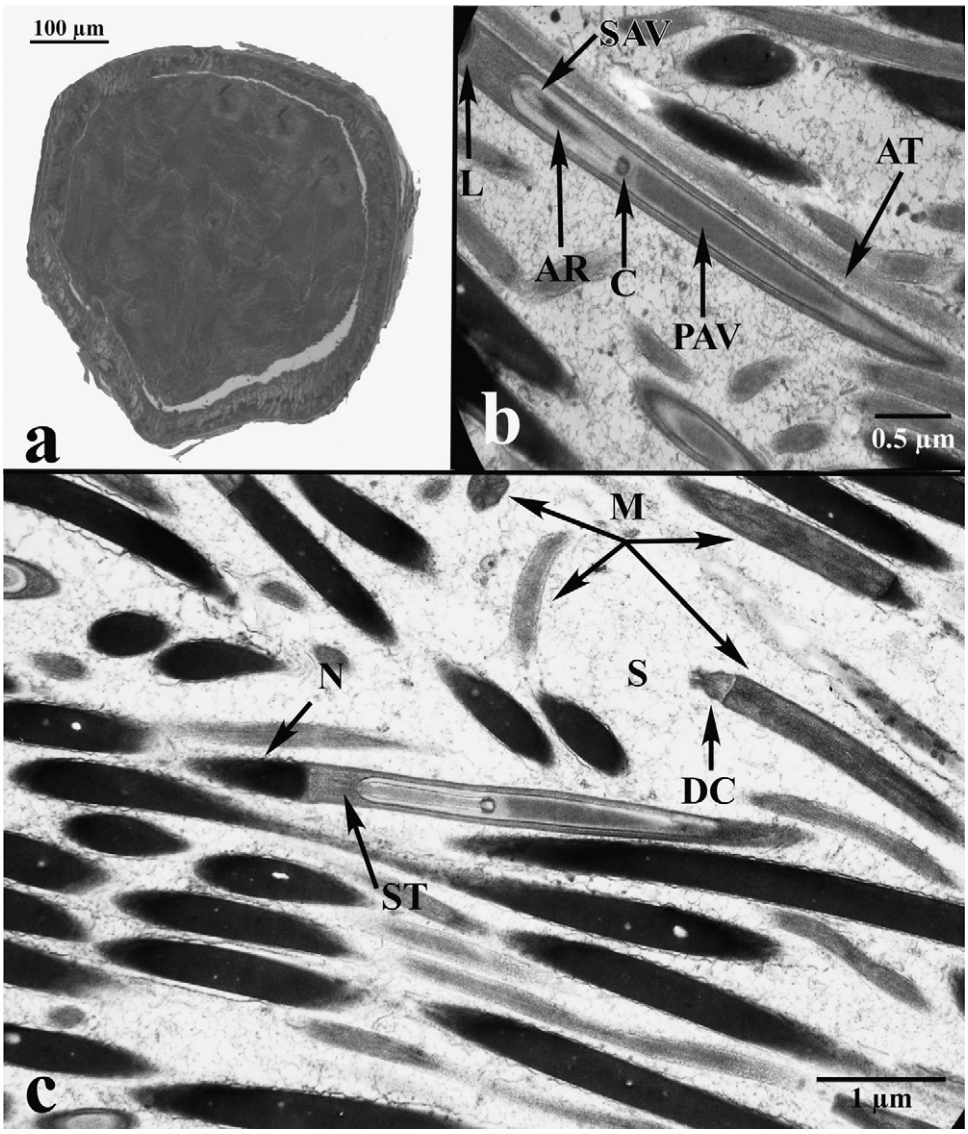

Fig. 2. (a) Semithin section of spermathecae in the sexual population (Robledillo, ROB). The lumen is full of sperm. (b and c) Ultrathin sections showing the lumen content. (b) Detail of mature sperm. AT, acrosomal tube; PAV, primary acrosome vesicle; SAV, secondary acrosome vesicle; C, capitulum; AR, axial rod; L, limen; M, midpiece in which the six mitochondria are around a central axis; N, nucleus; ST, secondary tube; DC, distal centriole; S, Secreted substances forming the matrix of the lumen.

Phylogenetic analyses

Mean genetic distances based on pairwise differences per site (p-distance) among and within populations were estimated using Arlequin version 2.000 (Schneider et al. 2000).

Models for sequence evolution and corresponding parameters were estimated with jMODELTEST v. 0.1.1 (Posada 2008). Hierarchical likelihood ratio tests, Akaike information criterion (AIC) and Bayesian information criterion (BIC) were used to choose the model Hasegawa–Kishino–Yano (Hasegawa et al. 1985), with a gamma distribution and invariable sites (HKY + I + G) as the best-fitted model for the data in COI, and F81 + I + G (Felsenstein 1981) for H3. These models were used in the phylogeny estimation for the ML and BI analyses.

Maximum likelihood (ML) analyses were performed with Phyml v. 2.4.4 (Guindon and Gascuel 2003) including α and I values provided by jMODELTEST. Clade support was assessed using bootstrap 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 100 replicates was used to estimate support for the resulting topologies.

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

Bayesian phylogeny estimation was performed using the program MRBAYES v.3.1.2 (Ronquist and Huelsenbeck 2003). Parameters in MRBAYES were set to one million generations and 1000 trees were sampled for every 100th 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 remaining trees were combined to find the maximum a posteriori probability estimate of phylogeny.

Phylogenetic analyses were performed with COI and with the concatenated data set (COI 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).

A haplotype network for both genes was constructed using the statistical parsimony procedure (Templeton et al. 1992; Crandall

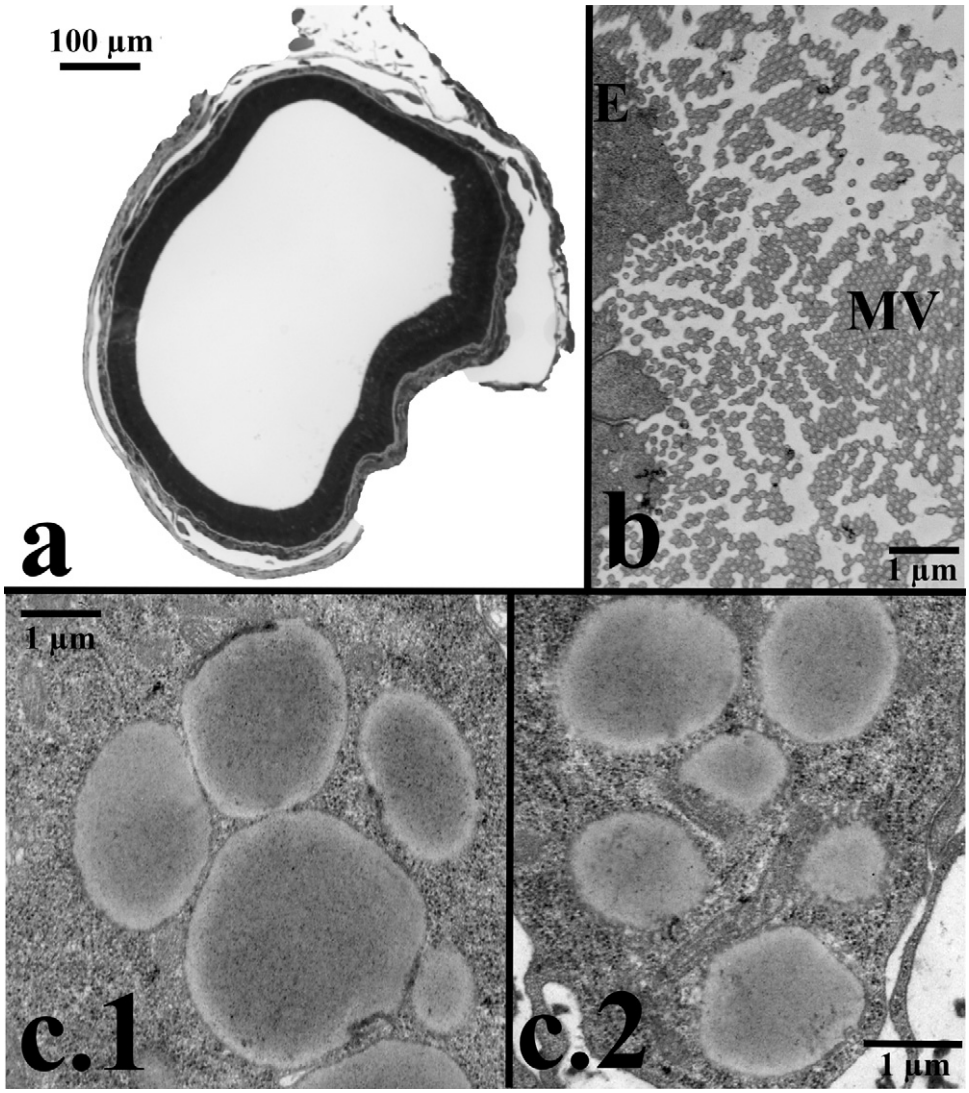

Fig. 3. (a) Semithin section of spermathecae in the parthenogenetic population (El Molar, MOL). The epithelium is slim and the lumen is completely empty. (b and c) Ultrathin sections showing the ephitelium. (b) Detail of the microvilli (MV). (c) Detail of the lipidic droplets.

1996) as implemented in TCS version 1.21 (Clement et al. 2000). As described by Pfenniger and Posada (2002), the assumptions derived from coalescent theory were used to resolve the loops in the statistical parsimony network.

Results

Light and transmission electron microscopy

Spermathecae

The spermathecae of the three studied populations showed a highly different structure and ultrastructure. In both the sexual (ROB) and the parthenogenetic (MOL) populations, the lumen occupied a great part of the total volume (Figs. 2a and 3a), surrounded by a slim epithelium of elongated, cylindrical cells bearing numerous microvilli, enclosed by a sheath of connective tissue. The tissue around the lumen looked similar under light microscopy, the only difference being an apparently lighter staining and stronger cytoplasmal differentiation of ROB as compared to MOL. In ROB, the lumen was occupied by a dense mass of mature sperm embedded in a fibrous substance, whereas in MOL it was completely empty. In the population with full spermathecae and empty male funnels (SHB) (Fig. 4a), the lumen was reduced due to the spermathecae epithelium being much thicker than in the other samples. Epithelial cells were approximately twice as wide and several times longer than in the above cases and showed conspicuous signs of cytoplasmal differentiation, with a lighter staining, vacuolated basal part and a darker staining apical portion. The lumen content looked also different, with scarce sperm and an overall lighter staining.

Regarding its ultrastructure, there were also clear differences. Spermatozoa were heavily packed in ROB (Fig. 2c). They showed the same structure found in other lumbricid species: the acrosome implanted on the tip of the elongating and condensing nucleus, the mitochondria of the midpiece (the so-called nebenkern) which abut on the distal end of the nucleus, the centriolar complex and the flagellum, constituted by the axoneme with its surrounding cytoplasm and membrane (Fig. 2b and c). Lipid droplets of small size were found homogeneously distributed in the spermathecal wall (a mean of 1.5% of total tissue surface). In SHB, the lumen content contained degraded spermatozoa and cell debris (Fig. 4b). The ephitelium showed numerous spermatozoa apparently taken up by phagocytosis singularly or in groups, and being degraded within endocytotic vesicles throughout the epithelial cytoplasm (Fig. 4c). The presence of lipid droplets of different size (a mean of 4.9% of total tissue surface) was common in the epithelial cytoplasm. In MOL, neither spermatozoa nor cell debris could be found in the

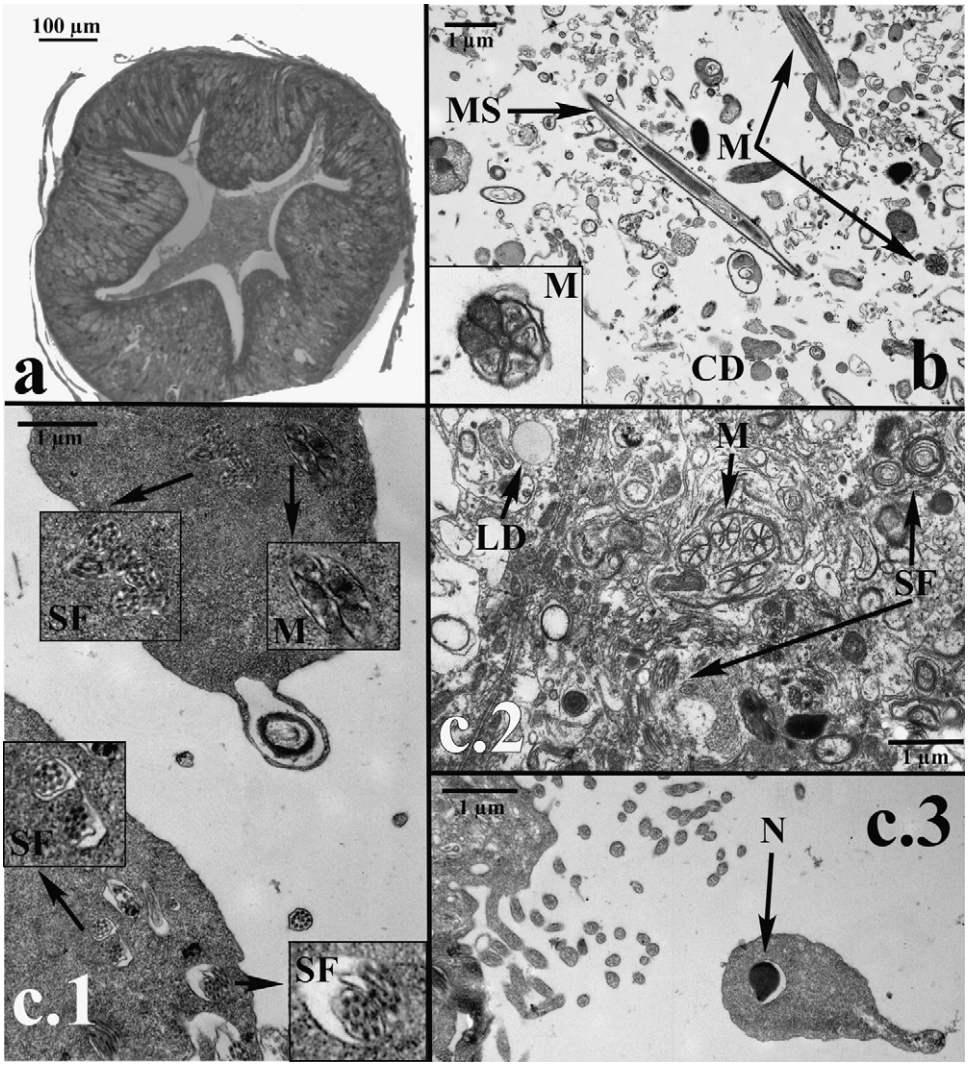

Fig. 4. (a) Semithin section of spermathecae in the intermediate population between sex and parthenogenesis (Saint-Hilaire-du-bois, SHB). The epithelium is very thick and the lumen is reduced. (b) Ultrathin sections showing the lumen content. The matrix contains cell debris (CD) and mature sperm (MS) in process of degradation. (c) Epithelium of spermathecae. Active degradation of mature sperm. (c.1 and c.3) Detail of phagocytosis of mature sperm. M midpiece in which the six mitochondria are around a central axis; the abnormal appearance of the membrane of the mitochondria denotes degradation. SF, spermatozoon flagellum; N, spermatozoon nucleus; LD, lipidic droplet.

lumen (Fig. 3a and b). In addition, the amount of lipid droplets in the tissue was higher than in the other samples (a mean of 6.4% of total surface) (ANOVA, $F_{(7,7)} = 4.454$; $P < 0.05$), but was only significantly higher when compared to the sexual population (t-test$_{(8)} = -11.339$; $P < 0.05$). SHB and ROB also differed significantly in the amount of lipid droplets (t-test$_{(8)} = -9.997$; $P < 0.05$).

Seminal vesicles and male funnels

In the seminal vesicles, spermatogonia arrive from the testis and undergo spermiogenesis passing through the stages of primary and secondary spermatocyte and developing into spermatids that finally mature into mature spermatozoa. All the different stages were observed in the sexual population (Fig. 5), whereas mature spermatozoa were not found in SHB and MOL. The number of germ cells observed among the three populations was significantly different, much less abundant in MOL and SHB than in ROB (ANOVA, $F_{(7,7)} = 5.874$; $P = 0.037$). In MOL and SHB, individual cases of germ cells reaching the different stages of spermatocyte development but not maturation, could be observed (Figs. 6 and 7). The most advanced stage of spermiogenesis observed was the production of spermatids. In both MOL and SHB, morphological differences in the functional germ cells in ROB, such as spermatid nuclei with an irregular shape (Fig. 6b) instead of the normal round ones, and presence of less organelles were observed. Another main difference was the percentage of volume (referring to the total seminal vesicle surface) occupied by lipid droplets (a mean of 1.2%, 5.7% and 6.7% of the total surface in ROB, SHB and MOL, respectively). The size of lipid droplets was highly variable, from less than a micrometer to more than 5 µm. The size in the small ones was round and in the big ones mainly oval. As observed in the spermathecal epithelium, the amount of lipid droplets in the tissue was similar in MOL and SHB (t-test$_{(8)} = -1.500$; $P = 0.1720$), and significantly higher than in ROB (ANOVA, $F_{(7,7)} = 4.884$; $P = 0.028$). In MOL and SHB, most of the mitochondria found in the cytoplasm was either around or close to the lipid droplets (Fig. 7c), and their inner membranes appeared very dark. The male funnels were iridescent in ROB and non-iridescent in SHB and MOL.

Phylogenetic analyses

Genetic divergence values are shown in Table 1. Divergence values with *A. caliginosa* and *Hormogaster castillana* are included to provide a reference point. Corrected divergence values of *A.*

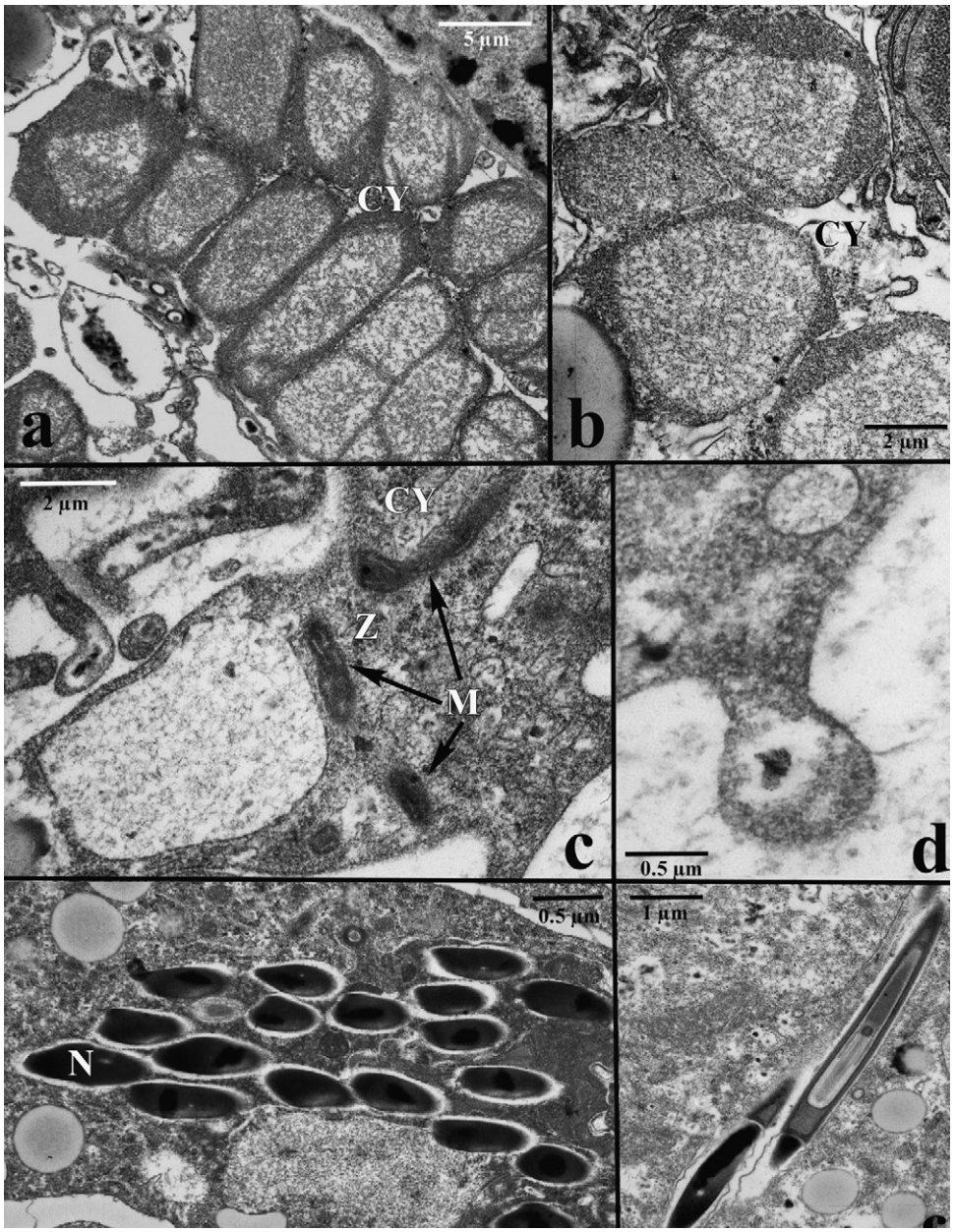

Fig. 5. (a–f) Seminal vesicles in the sexual population (ROB). (a and b) Early spermatids in a morula. CY, Cytophore. (c) Spermatid attached to the cytophore. Note the mitochondria migrating through the zonula collaris (Z). (d) Detail of mature spermatid separating from the morula. (e) Cross section of immature spermatozoa at the nucleus (N) level. (f) Longitudinal section of mature spermatozoon.

trapezoides among populations were generally medium-high (from 10.24 to 13.70% in COI, from 0.31 to 2.84% in H3), and low (from 0 to 3.69% in COI) within populations.

All generated phylogenetic trees (Bayesian inference and Maximum likelihood) produced congruent topologies. The ILD test did not find significant incongruence among partitions in the matrix with the concatenated data ($P > 0.05$). The Bayesian inference tree of the combined data set is shown in Fig. 8. The five populations appeared clearly differentiated, with supporting bootstrap and posterior probability values, whereas some terminal relationships within populations differed among analyses. SHB appeared in a more basal position than MOL and ROB. The internal branches in the tree were long, while the terminal ones were very short, thus highlighting the genetic distinctiveness of the populations.

The parsimony haplotype networks of COI (Fig. 9) constructed using statistical parsimony as implemented in TCS, recovered the different populations as different lineages. To obtain a complete connection of the network, a connection limit of 65 steps was defined. The COI sequences of *A. trapezoides* consisted of 24 haplotypes. In the H3 network, only three different sequences were found. The same sequence was shared by all the individuals in SHB and SAN. Two different ones were found in ROB – one of them shared by all the samples in MOL and ALZ. The sequences among the populations differed in one to 9 substitutions.

Discussion

The main focus of the present work lies in the comparison, within the same species, of a sexual population, several parthenogenetic ones and another one that showed signs of copulation but not of sperm production using both morphological and molecular approaches.

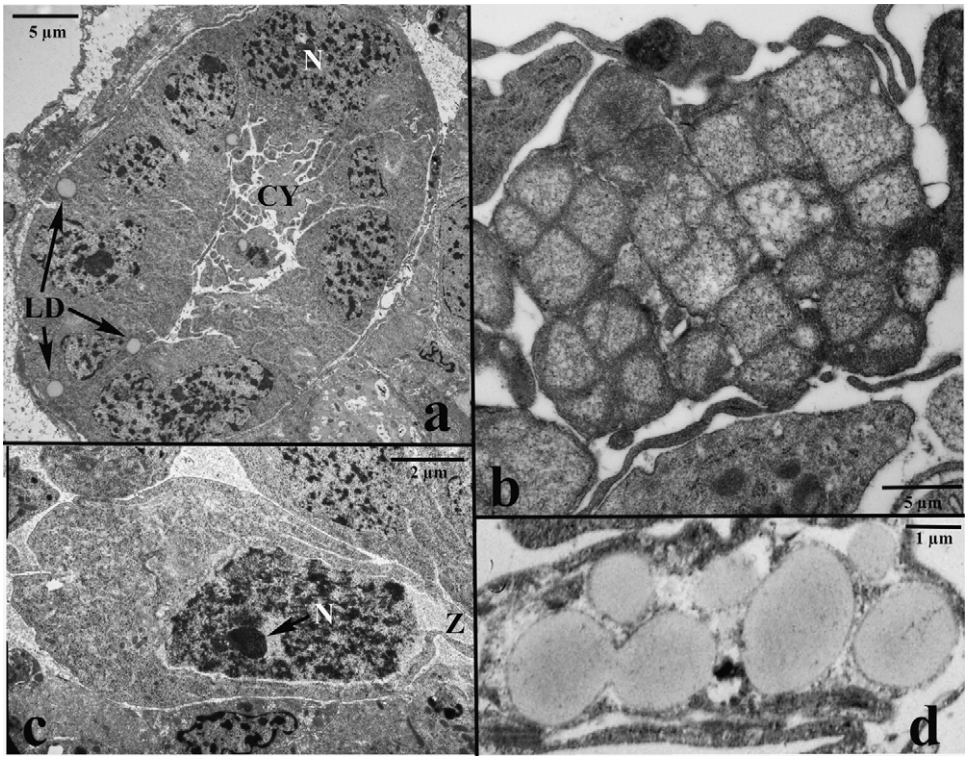

Fig. 6. (a–d) Seminal vesicles in the pseudogamic population (SHB). (a) Spermatogonia in interphase attach to a cytophore (CY). LD, lipidic droplets. (b) spermatids in a morula. (c) Detail of primary spermatocyte attached to a cytophore through the zonula collaris (Z). (d) Detail of lipidic droplets.

Although an exhaustive investigation of spermiogenesis in *A. trapezoides* was not the purpose of this study, we did obtain enough data to suggest that spermiogenesis in the sexual population (ROB) is similar to that described for other lumbricids (Jamieson 1981). Spermatogenic stages, first spherical and later elongated, develop around an anucleate cytophore from which they separate as mature spermatozoa. At the end of the maturation process in the spermatids, only six mitochondria migrate to the region of the midpiece (the nebenkern), before having undergone a modification in their form and intracellular distribution. The mitochondria elongate and their crests become irregular. This detail, clearly shown in this study (Figs. 5c and 7c), is not frequently described in literature for oligochaetes, as suggested by Rolando et al. (2007). Mature spermatozoa also showed the same morphological features as other lumbricid species (acrosomal complex, nucleus, capitulum, midpiece and flagellum, for example) (Rolando et al. 2007; Anderson et al. 1967; Jamieson 1981).

The presence of immature stages of spermatogenesis (spermatogonia, spermatocytes and spermatids) in seminal vesicles in the uniparental populations suggests the production of male germ cells in the testes. Why the testes continue producing them is unknown. The results of this study correspond to the ones of Omodeo (1952) for this same species, who stated that in some populations nearly all the germ cells degenerated before reaching

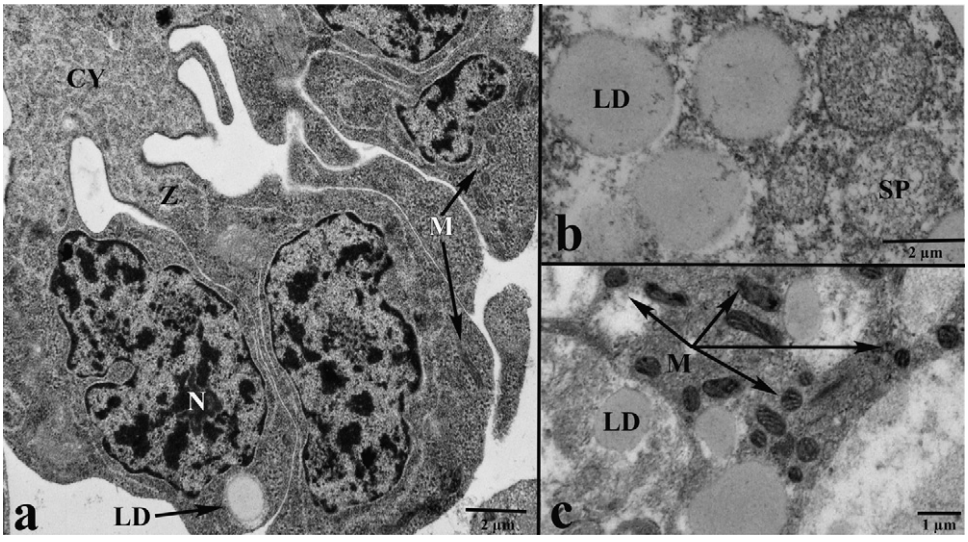

Fig. 7. (a–c) Seminal vesicles in the parthenogenetic population (MOL). (a) Spermatogonia in interphase appear attached to a cytophore (CY) through a zonula collaris (Z). M, mitochondria; N, nucleus. (b) Example of early spermatids (SP) close to lipidic droplets (LD). (c) Detail of high mitochondrial activity (M) close to lipidic droplets (LD).

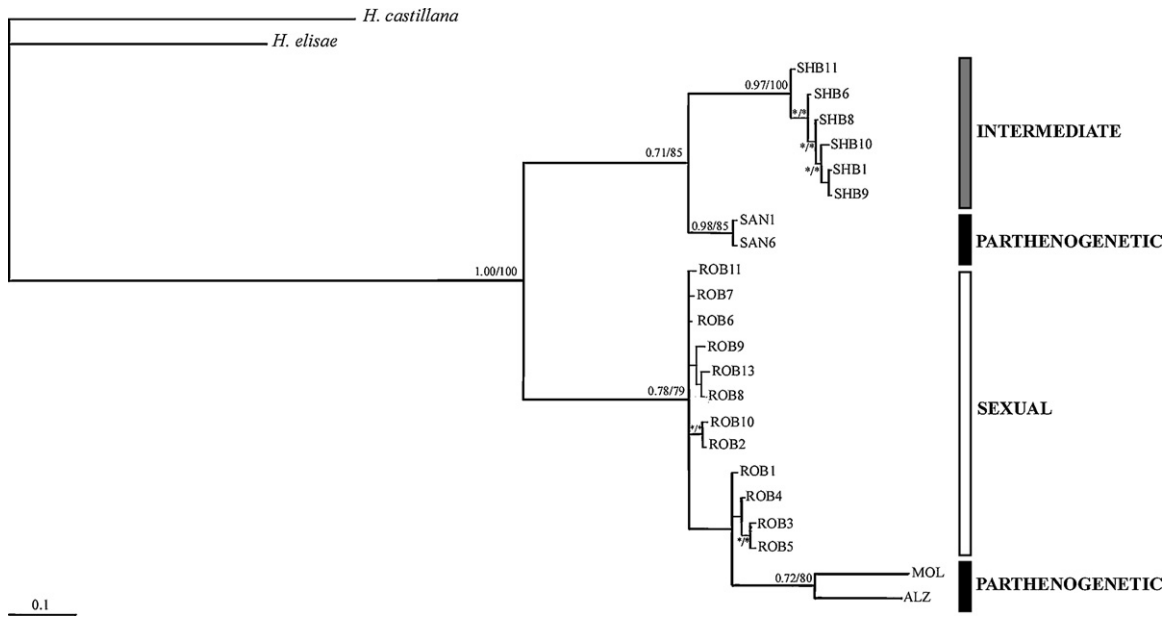

Fig. 8. Bayesian inference (BI) tree based on the concatenated analysis of a fragment of COI and H3 genes in *Aporrectodea trapezoides*. Posterior probability and bootstrap values of coincident associations, given by Maximun Likelihood (ML), are shown above branches (BI/ML). Asterisks above terminal branches represent posterior probability and bootstrap values >0.80/80.

the seminal vesicles, while in others they mature until the stage of spermatid, which are subsequently phagocytized. What is clear is that these germ cells suffer an aberrant maturation process in MOL and SHB, already described by Omodeo (1951a) for the parthenogenetic forms of *A. trapezoides*, although this author did not describe a common pattern of degradation, just the accumulation of aberrant structures during the maturation process. The most advanced stage of maturation found in these populations was spermatids in morula, thus not reaching the stage of immature spermatozoon. Nevertheless, the presence of sperm in the spermathecae in SHB

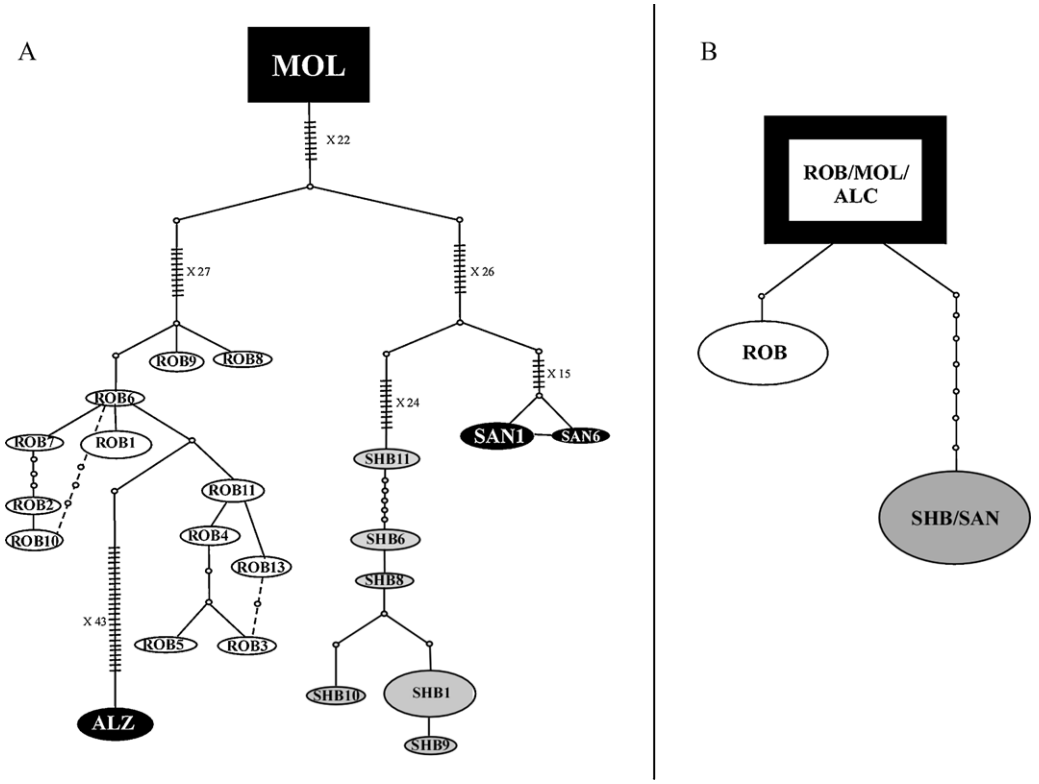

Fig. 9. TCS haplotype networks for the nested clade scheme of sampled populations of *A. trapezoides*. Grey shaded circles belong to the population with an intermediate mode of reproduction between sex and parthenogenesis, black circles to the parthenogenetic populations and white ones to the sexual one. Connection limit in COI network: 65 steps. Oval size depends on haplotype frequency. Internal nodes represent intermediate haplotypes (unsampled). Equally parsimonious connections are indicated by dashed lines. In the COI network, each branch represents one mutational step except for the cases in which the number of steps exceeds 13 (parsimony level of 90%). In these cases, the number of steps is shown. Branch length is meaningless.

Table 1
Mean corrected genetic divergence (p-distance, in percentage of changes) among studied populations of *A. trapezoides*. Diagonal shows genetic divergence withineach lineage (in italics). Reference species (*A. caliginosa* and *H. castillana*) are included.

COI	ROB	MOL	SHB	SAN	ALZ	*A. caliginosa*
ROB	*1.11*					
MOL	10.24	*0*				
SHB	13.31	12.02	*3.69*			
SAN	13.20	13.21	6.89	*0.21*		
ALZ	10.04	11.32	13.52	13.70	*0*	
A. caliginosa	18.55	17.21	17.01	18.08	16.33	–
H. castillana	20.47	19.17	19.88	20.26	20.69	19.17

H3	ROB1/MOL/ALC	ROB2	SHB/SAN	*A. caliginosa*
ROB1/MOL/ALC	*0*			
ROB2	0.31	*0*		
SHB/SAN	2.84	1.62	*0*	
A. caliginosa	7.07	6.75	6.87	–
H. castillana	9.77	8.47	8.14	9.99

opens the interesting question of who the donor of the sperm could be. As mentioned in the introduction, Omodeo (1952) found one pseudogamic population of *A. trapezoides* in Italy in which he described an aberrant spermiogenetic process, but a few mature spermatozoa were also observed. Given this fact, it seems plausible that individuals of both conditions coexist and copulate. In contrast, it could also be hypothesized that the donor might be an individual of a closely related species or their polyploid forms belonging to the *Aporrectodea caliginosa* species complex (Gates 1972; Pérez-Losada et al. 2009) into which *A. trapezoides* should be included. It is also remarkable that both primary and secondary sexual characters (i.e., clitellum, tubercula pubertatis and papillae) in the parthenogenetic form are very conspicuous although copula is not necessary, suggesting that these organs are more resistant to mutations or that male sterility appeared recently (Gates 1972). Further research on this topic is needed to clarify this interesting point.

In lumbricid earthworms, pseudogamy and parthenogenesis seem to be related to polyploidy (Omodeo 1951b, 1953a,b). In *A. trapezoides*, triploid and tetraploid forms have been found (Omodeo 1952, 1955; Casellato 1987; Cobolli-Sbordoni et al. 1987), being the triploid form the dominant one, while only individuals from two populations were found to be tetraploid. In both strains, Omodeo (1951a, 1952, 1955) suggested that abortive spermatogenesis is due to inefficiency of the mitotic mechanism, but found that a small percentage of germ cells successfully produced mature sperm in the tetraploid (pseudogamic) strain (Omodeo 1955). In the same strain, the author also observed the penetration of the spermatozoa in the ovule and the expulsion of two polocytes. In SHB, however, spermatogenesis was completely aborted and sperm stored in the spermathecae was digested; thus, it seems improbable that this population is pseudogamic; otherwise, sperm should be normally stored in the spermathecae in order to fertilize the ovule. Nevertheless, cytological evidence is needed to check if it is pseudogamic or the individuals just copulated accidentally. It also remains unclear if the sperm found in the spermathecae comes from the same species/population (that is, some individuals produce sperm while others do not), or from a different one. The triploid strain studied by Omodeo (1951a, 1952, 1955) were found to be parthenogenetic. In the present study, we do not have any data about the ploidy level of the individuals. Therefore, further research on the topic will clarify the correspondence between the ploidy level and the mode of reproduction in our samples.

Lipid droplets have been reported in spermathecae of other animal groups, such as salamanders, in which they serve as an endogenous source of energy (Zalisko and Larsen 1990) and are usually associated with mitochondria and endoplasmic reticulum, suggesting a role in endogenous energy production (such as in triglyceride metabolism) (Sever and Kloepfer, 2003). In *A. trapezoides*, the differences in lipid amount stored in droplets, significantly bigger in SHB and MOL compared to ROB, suggests that there is a supply of nutritional resources. These nutritional resources accumulate since, perhaps, energy is not needed to feed mature sperm in the spermathecae nor to produce them in the seminal vesicles. This could suggest that despite parthenogenesis or other stages leading to it without production of sperm, the mechanisms involved in nurturing the sperm have still not been suppressed. Thus, besides the lack of sperm, seminal vesicles would retain their functional organization, and could be a sign of recent parthenogenesis. This abundant resource of non-used energy could be a possible hypothesis explaining the high parasitic load often found in seminal vesicles in this species (Díaz Cosín and Fernández, pers. obs.).

High genetic divergence among populations of the same species seems to be common in earthworms (Heethoff et al. 2004; Field et al. 2007; Novo et al. 2009, 2010). The divergence values found among populations on this study are compatible with their grouping in the same species (Hebert et al. 2003; Novo 2010). Regarding COI, two of the parthenogenetic populations (MOL and ALZ) diverged less from the sexual one (10.24% and 10.04%, respectively) than the third parthenogenetic one, SAN (13.20%). The value of divergence of SHB with respect to the sexual population (13.31%) was similar to the one found in SAN. In addition, one of the two sequences of the nuclear gene H3 was shared by MOL and ALZ and ROB, while SHB and SAN had a different one with a high value of divergence from the ROB population (2.84%). These results support the position of SHB and SAN in the phylogenetic and the haplotype networks, as these populations always appear clustered together more distant from other populations.

Phylogenetic analyses also showed an independent origin of the uniparental populations (SHB, SAN, MOL and ALZ), as all the parthenogenetic populations did not cluster together Thus, the origin of parthenogenesis in this species seems to be multiple. The interior clade in the COI haplotype network is the parthenogenetic population MOL. In the H3 network, the interior clade is formed both by the sexual and the parthenogenetic populations MOL and ALZ. It is improbable that a parthenogenetic form is the most ancient one from which the other ones stem, thus finding the parthenogenetic populations in the interior clade could be due to the inclusion of only five populations in this analysis and also because all the individuals shared the same haplotype, giving it more weight as an ancient and stable population. Regardless, further studies with a higher number of populations are needed to confirm these hypotheses.

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