Molecular Ecology

WILEY

MOLECULAR ECOLOGY

ORIGINAL ARTICLE OPEN ACCESS

No Mate, No Problem: Molecular Mechanisms Involved in Parthenogenesis in the Cosmopolitan Earthworm *Aporrectodea trapezoides* (Annelida, Clitellata)

Irene de Sosa[1,2,3] | Marta Turon[1,4] | Sergi Taboada[1,5,6] | José Lorente-Sorolla[1,3] | Rosa Fernández[7] | Natasha Tilikj[3] | Alberto Piris[3] | Marta Novo[3] | Patricia Álvarez Campos[2] | Ana Riesgo[1,5,6]

[1]Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales (MNCN), CSIC, Madrid, Spain | [2]Departamento de Biología (Facultad de Ciencias) y Centro de Investigación en Biodiversidad y Cambio Global (CIBC), Universidad Autónoma de Madrid, Madrid, Spain | [3]Departamento de Biodiversidad, Ecología y Evolución, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, Madrid, Spain | [4]Departamento de Ecología Marina Centre d'Estudis Avançats de Blanes (CEAB-CSIC), Blanes, Spain | [5]Life Sciences Department, The Natural History Museum, London, UK | [6]Departamento de Ciencias de la Vida, EU-US Marine Biodiversity Group, Universidad de Alcalá, Alcalá de Henares, Spain | [7]Metazoa Phylogenomics Lab, Institute of Evolutionary Biology (CSIC-UPF), Barcelona, Spain

Correspondence: Irene de Sosa (irenedesosa91@gmail.com)

Received: 5 December 2024 | **Revised:** 27 May 2025 | **Accepted:** 16 June 2025

Handling Editor: Paula Arribas

Funding: I.S. was funded by the European Union-Next GenerationEU program through a Margarita Salas fellowship. M.T. was supported by a JdC (Juan de la Cierva Formación, 2020) personal grant (FJC2020-043677-I), MSCA Postdoctoral Fellowship (HORIZON-MSCA.2022-PF-01, Project 101105716). J.S. and A.P. were funded by the Spanish Ministry of Science, Innovation, and Universities through a predoctoral fellowship (JSPRE2020-095330 and PRE2022-103770, respectively). M.N. was supported by Grant PID2021-122243NB-I00 funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe". P.Á.C. was funded by MNCIN/AEI/10.13039/501100011033 and European Union "Next GenerationEU/PRTR" (CNS2023-145193). S.T. received funding from the grant PID2020-117115GA-I00 funded by MCIN/AEI/10.13039/501100011033 and from the grant CNS2023-144572 and by the Ramón y Cajal grant RYC2021-03152-I, funded by the MCIN/AEI/10.13039/501100011033 and the European Union «NextGenerationEU»/PRTR». A.R. acknowledges funding from three grants from the Spanish Ministry of Science and Innovation (RYC2018-024247-I, PID2019-105769GB-I00, and CNS2023-144571) in the framework of MCIN/AEI/10.13039/50110001103 and EI "FSE invierte en tu futuro" and an intramural grant from CSIC (PIE-202030E006). R.F. acknowledges support from the following sources of funding: the European Research Council (grant agreement no. 948281), the OSCARS project (funding from the European Commission's Horizon Europe Research and Innovation programme under grant agreement no. 101129751), grant CNS2023-144624 funded by MICIU/AEI/10.13039/501100011033, and by the "European Union NextGenerationEU/PRTR" and the Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement de la Generalitat de Catalunya (AGAUR 2021-SGR00420).

Keywords: microbiome | nGBS | reproduction | tetraploidy | transcriptomics

ABSTRACT

Approximately, 40% of earthworm species can reproduce by parthenogenesis. This is the case for the cosmopolitan species, *Aporrectodea trapezoides*, although sexual forms have been described sporadically. We analyse the genotypes and microbiomes of 30 individuals from four localities where both forms appear in order to understand the evolutionary mechanisms related to parthenogenesis. In all sites, heterozygosity values were approximately 30% higher in parthenogenetic individuals. However, we detected a stronger genomic structuring due to reproduction than to the geographical setting only in the Algerian population, underpinned by 195 loci that were related to gametogenesis, symbiont-like processes, and nitrate reduction. Similarly, statistical differences in the abundance of ZOTUs were only found between the Algerian sexual and parthenogenetic earthworms, with 754 ZOTUs that included the genus *Romboutsia*, which is involved in the production of nitric oxide, which enhances sperm motility. In summary, significant genomic and microbiome differences were found only between sexual and parthenogenetic lineages in a single locality. We hypothesise that obligate parthenogenesis evolved early, leaving traces at the genomic and microbiome levels

in the Algerian parthenogens that were the earliest splitting lineage. Such obligate parthenogenesis was lost secondarily and individuals in the Iberian sites were facultative parthenogens, with the potential to copulate and therefore erase the genomic and microbial traces of obligate parthenogenesis. Our results indicate a hybrid origin of parthenogenesis in *A. trapezoides* and shed light on the complex interplay between genomic, microbiome, and reproductive mechanisms in *A. trapezoides*.

1 | Introduction

Although the vast majority of animals reproduce sexually, the transition to parthenogenesis has been observed in a multitude of taxa across the Tree of Life (Bell 1982; van der Kooi et al. 2017; Liégeois et al. 2020). Nevertheless, the proportion of parthenogenetic species remains unknown, as there are only two quantitative estimates of the frequency of parthenogenesis, based on species lists (Vrijenhoek 1998; van der Kooi et al. 2017). Parthenogenetic development can proceed in different ways, depending on whether the meiosis of the oocyte is completed normally (automixis or meiotic parthenogenesis) or some of the stages have been omitted (apomixis or mitotic parthenogenesis). In meiotic parthenogenesis, meiotic divisions occur partially or completely, but the somatic ploidy level is maintained by different mechanisms (endoduplication, central and terminal fusion and gamete duplication). Depending on how recombination proceeds, some of these mechanisms are functionally equivalent to mitotic parthenogenesis, even if meiosis is fully maintained (Suomalainen et al. 1987; Engelstädter 2017). Automixis occurs in many parthenogenetic stick insects and some weevils (Simon et al. 2003). In functional mitotic parthenogenesis, no ploidy reduction takes place and the offspring are clones of their mother genotype. Apomixis is commonly found in invertebrates such as rotifers and arthropods (Simon et al. 2003). Despite no cross fertilisation being achieved during parthenogenesis, some parthenogenetic animals need sperm to stimulate egg development. This mechanism, called gynogenesis, can explain the existence of spermatophores in parthenogenetic specimens (Alavi et al. 2016; Foerster et al. 2021; Edwards and Arancon 2022; De Sosa et al. 2023).

Parthenogenetic lineages can arise from species with sexual reproduction in several ways, with hybridization as the most common process. Most parthenogenetic vertebrates have hybrid origin (Avise et al. 1992), and it also has been demonstrated in snails (Johnson and Leefe 1999), crustaceans (Dufresne and Hebert 1994), weevils (Tomiuk and Loeschcke 1992), stick insects (Mantovani 1998), and grasshoppers (Honeycutt and Wilkinson 1989). However, parthenogenesis can be acquired through other processes. Whether the microbiome has any possible effect on parthenogenesis is something that has never been proved, although it is known that many arthropods carry different endosymbiotic bacteria that can manipulate the reproduction of their hosts (Perlmutter and Bordenstein 2020). These bacteria can induce parthenogenesis causing cytoplasmic incompatibility between gametes, feminising males or even killing males during embryogenesis (Stouthamer et al. 1999; Werren et al. 2008). Examples of these bacteria inducing this behaviour in their hosts can be found in the genera *Wolbachia* Hertig, 1936, *Cardinium* Zchori-Fein and Perlman, 2004, *Rickettsia* Da Rocha-Lima, 1916, *Arsenophonus* Gherna et al., 1991 and *Spiroplasma* Saglio et al., 1973 (Saglio et al. 1973; Gherna et al. 1991; Zchori-Fein and Perlman 2004; Hagimori et al. 2006; Werren et al. 2008). Spontaneous loss of sex due to mutations in genes related to mating and fertilisation of eggs (Carson et al. 1982) or in genes involved in sexual forms (Simon et al. 2003) is another mechanism for the emergence of parthenogenesis. A small proportion of unfertilized eggs spontaneously develop into zygotes in many animal species such as ostracods (Turgeon and Hebert 1995), moths, aphids, and snails (Johnson and Leefe 1999). Parthenogenesis can also appear due to a contagious origin which alludes to an incomplete reproductive isolation between sexual individuals and pre-existing parthenogenetic lineages (Simon et al. 2003). Thus, it is the main pathway for polyploidy in parthenogenetic animals. This model has been validated in cladocerans (Hebert 1981), aphids (Rispe et al. 1998; Dedryver et al. 2001), and earthworms (Jaenike and Selander 1979).

Among the species considered parthenogenetic, some are classified as obligate parthenogenetic, indicating their inability to reproduce via other methods. However, other species are capable of reproducing sexually and switching to parthenogenesis when conditions are unfavourable (Booth et al. 2012; Neaves and Baumann 2011). Obligate parthenogenesis is predicted to have numerous impacts on the genome. The consequences are strongly influenced by how uniparental reproduction evolved from the sexual ancestor as well as by the cellular mechanisms underlying this reproduction mode. For example, heterozygosity and activity of transposable elements result in parthenogens with hybrid origin (Jaron et al. 2021). The presence of palindromes and a high abundance of horizontally acquired genes also can be found (Jaron et al. 2021).

Although sexual reproduction is the most common type of reproduction in earthworms (Annelida, Lumbricidae), 40% of the species can reproduce by parthenogenesis (Casellato 1987; Cosín et al. 2010). Parthenogenetic earthworms are automictic and thelytokous (i.e., in absence of gamete exchange, females produce offspring with the same ploidy as themselves) (Omodeo 1951; Muldal 1952; Omodeo 1952; Casellato and Rodighiero 1972). This implies a premeiotic doubling of chromosome number in the last oogonial division, followed by the formation of chiasmatic bivalent and regular meiosis with removal of the two polar bodies. By duplicating the chromosomes and mating with their identical copy, this mode of reproduction is perfectly compatible with any ploidy, even odd ones. It appears that this reproductive strategy is advantageous for earthworms, as the majority of parthenogenetic species are cosmopolitan and abundant on a global scale (e.g., *Aporrectodea rosea* (Savigny, 1826), *Eiseniella tetraedra* (Savigny, 1826), or *Aporrectodea trapezoides* (Dugès, 1828) (Cosín et al. 2010)). This can be attributed to numerous advantages such as high colonising capacity and high reproductive potential, rapidly counterbalancing mortalities (Hughes 1989). Also, several oligochaete clones did not exhibit signs of senescence after being maintained for many generations, which also indicates an advantage in the delay or prevention of senescence as somatic replicates are generated from undifferentiated somatic cells (Hughes 1989).

These characteristics make earthworms ideal organisms for the study of parthenogenesis. In fact, there are some species, like the dominant one in Mediterranean soils, *Aporrectodea trapezoides* with sexual individuals identified in France, Spain, and Algeria, which co-occur with parthenogenetic specimens (Bouché 1972; De Sosa et al. 2017). The presence of both types of reproduction in the same species makes *A. trapezoides* suitable to explore the biological factor driving the evolutionary history of both modes of reproduction by studying its gut microbiome and genomic composition and structure. Our prediction is that the imprint that parthenogenesis may leave on the genome in this species, as it does in other metazoans (Jaron et al. 2021), will be detected by using genomic approaches, comparing genotypes of both types of reproduction. We will also seek proof of whether the microbiome differs among the two reproductive strategies, by studying its composition and potential functionality and how that could affect the reproductive strategy. In this sense, our research scenario with individuals of *A. trapezoides* exhibiting both modes of reproduction and coexisting within the same locality facilitates the inference of associations between the microbiome composition and function and the reproductive strategy, in a natural setting. Our research opens a new avenue in the study of the reproductive microbiome of this ecologically important animal group.

2 | Materials and Methods

2.1 | Sample Collection

We collected 30 specimens of *A. trapezoides* between 2008 and 2012 in the three localities from which sexual (13 of sexual) and parthenogenetic (12 of parthenogenetic) individuals (Table S1) are known: Plasencia (Spain), Menorca (Spain), and Karkra (Algeria) (Figure 1). We also collected five sexual specimens from Collo (Algeria), where no parthenogenetic specimens were found (Figure 1, Table S1). All individuals were collected by manual sorting, washed in distilled water to remove soil rests, fixed in 96% ethanol and stored at −20°C in the earthworm collection of the Department of Biodiversity, Ecology and Evolution, Complutense University of Madrid (UCM-LT). The morphological study was developed in a previous study by De Sosa et al. (2017). Briefly, all the individuals were dissected to check the morphology and unequivocally assign them to *A. trapezoides* and also to check for parthenogenetic or sexual condition. This was performed following Gates (1972): in a sexual individual, spermathecae and male funnels are full of sperm, which are iridescent under a stereomicroscope; in parthenogens, spermathecae are invariably empty, as are the male funnels. The presence of spermatophores was also checked.

2.2 | DNA Extraction, Gene Amplification, and Sequencing

We analysed two different tissue parts for each of the specimens: a portion of the body wall for the phylogenetic analysis and normalised genotyping-by-sequencing (nGBS) analyses and the gut for the *16S* amplicon sequencing analysis.

We extracted total genomic DNA from approximately seven segments of the body wall using the Speedtools Tissue DNA Kit (Biotools), following the manufacturer's protocol. To ensure the reliability of the results, a phylogenetic analysis was conducted to test the monophyly of the *A. trapezoides* samples. Thus, a fragment of the cytochrome c oxidase subunit I (COI) was amplified for six individuals (TKRA1 and TKRA2, TKRB1 and TKRB2, and D3EL1 and D3EL2). The remaining samples were PCR negative. We used primers LepF1: 5′-ATTCAACCAATCATAAAGATATTGG-3′ and LepR1: 5′-TAAACTTCTGGATGTCCAAAAAATCA-3′ (Hebert et al. 2004), following the PCR program suggested in Marchán et al. (2021). All products were purified using ExoSAP-IT reagent (ThermoFisher Scientific), sequenced using the two primers mentioned above by Macrogen Spain Inc.

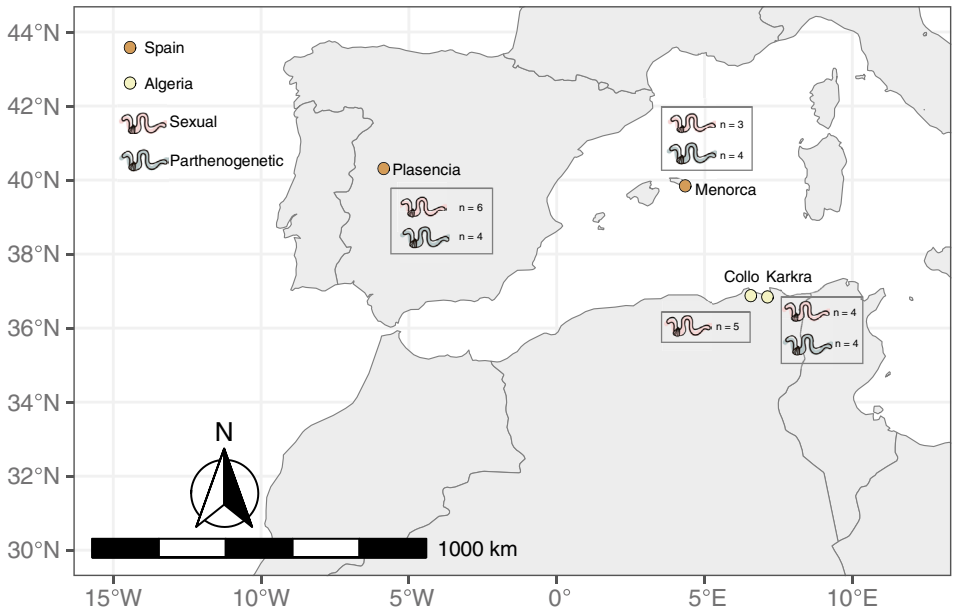

FIGURE 1 | Localities and number of sexual and parthenogenetic individuals sampled in Spain (Plasencia and Menorca) and Algeria (Collo and Karkra) for the studied species.

Sequences of *16S rRNA*, *28S* rRNA and H3 histone markers from each individual were retrieved from GenBank (Table S2).

We also extracted total genomic DNA from the post-clitellum gut tissue using soil content with DNeasy PowerSoil Kit (QIAGEN) according to the protocol supplied by the manufacturer. A fragment of the V4 regionf of the *16S* rRNA gene was amplified using the universal primers 515-F (Parada et al. 2016) and 806-R (Apprill et al. 2015), which amplify for both bacteria and archaea. DNA amplification was always performed in duplicate under the following conditions: 98°C for 30 s, followed by 32 cycles of 98°C for 9 s, 50°C for 1 min, 72°C for 1.5 min, and a final elongation at 72°C for 10 min. PCR products were purified with AgencourtAMPure XP Beads (Beckman Coulter Inc.), and libraries were prepared with the Nextera XT DNA Library Preparation Kit (Illumina Inc.). Next-generation, paired-end sequencing was performed on the Illumina MiSeq platform at the Genomic Unit of Complutense University of Madrid.

2.3 | Phylogenetic Analysis

Sequences were edited in Geneious Prime 2024.0.5, and multiple sequence alignments were run in MAFFT v.7 (Katoh and Standley 2013). Maximum likelihood (ML) partitioned analysis of the concatenated dataset was run in raxmlGUI v2.0.7 (Edler et al. 2021) using the SYM + I + G4m evolutionary model using ModelTest-NG (Darriba et al. 2020). Bootstrap support values were estimated using 1000 replicates. Sequences of *Aporrectodea rosea* (Savigny, 1826), *Aporrectodea tuberculata* (Eisen, 1874), *Carpetania elisae* (formerly *Hormogaster elisae* see Marchán et al., 2020), *Dendrobaena octaedra* (Savigny, 1826), *Eisenia lucens* (Vaga, 1857), *Eiseniella tetraedra* (Savigny, 1826), *Lumbricus terrestris* (Linneo, 1758), *Octodrilus complanatus* (Dugès, 1828), *Octolasion lacteum* (Orley, 1881), *Scherotheca gigas* (Bouché, 1972), and *Xana omodeoi* (Díaz Cosín, Trigo & Mato, 1989) were retrieved from GenBank and used as outgroups (Table S2).

2.4 | Genotype by Sequencing and Ploidy Analyses

2.4.1 | RAD Data Processing, SNP Calling and Filtering

nGBS libraries were generated using the DNA extracted from the body wall by LGC Biosearch Technologies (Berlin, Germany). 100–200 ng of DNA was digested with 200 Units of MslI (NEB), followed by adaptor ligation, purification with Agencourt XP beads and amplification using MyTaq (Bioline) and standard Illumina TrueSeq amplification primers (16 cycles). Libraries were pooled and normalisation was done using Trimmer Kit (Evrogen). The normalised library pool was reamplified using MyTaq (Bioline), size selected on a LMP-Agarose gel (250–500 bp), and sequenced in Illumina NovaSeq 6000 V1.5 platform.

We conducted quality filtering and locus assembly on raw data with *Stacks* v 2.55 (Catchen et al. 2013). RAD-tags were processed using the "*process_radtags*" function with the feature (*–r*) to recover minimally diverged barcodes and RAD-tags (*--barcode_dist* = 3; *--adapter_mm* = 2). The "*process_radtags*" trimming feature (*-t*) was used to trim the remaining reads to 135 bp, in order to increase confidence in SNP calling. Reads with ambiguous bases (*-c*) and low-quality scores (*-q*) were discarded for further analyses.

Parameters *-m*, *-M*, and *-n* in our dataset were the default ones (i.e., *-m* = 3, *-M* = 2, and *-n* = 1), which were implemented in the *Stacks* pipeline using all the individuals mentioned above. The "*populations*" function was applied to retain only loci with SNPs present in at least 80% of the individuals in a specific site (*-r* = 0.80). The dataset was then filtered by selecting the first SNP at each locus using the option *write-single-snp*, to reduce the probability of linkage disequilibrium among loci, and only SNPs with a minimum allele frequency of 0.05 (*-min-maf* > 0.05) (Roesti et al. 2012). We removed those loci that deviated from Hardy–Weinberg equilibrium (HWE; *p*-value = 0.05) present in at least two sites and also those SNPs showing an excess of heterozygosity (H_o > 0.5) (Hohenlohe et al. 2011). SNPs under selection were searched using *Arlequin* v.3.5.2.1 (Excoffier and Lischer 2010) and *Bayescan* v 2.1 (Foll and Gaggiotti 2008). *Arlequin* was ran using "no hierarchical island model", 100,000 simulations and 1000 demes per group; *p*-values were corrected using the "*p.adjust*" function in R with the "*fdr*" method, corresponding to the 'BH' in Benjamini and Hochberg (1995). *Bayescan* was run using a total of 10,000 output iterations and 100 prior odds; we considered outlier SNPs to be those with a FDR corrected *p*-value (*q*-value) > 0.05. No SNPs appeared to be under selection. The final neutral SNPs dataset consisted of 2916 SNPs in 28 individuals. Missing data values were calculated and visualised in https://bmedeiros.shinyapps.io/matrix_condenser/. We obtained a total of 16.20% of missing data but values per individual ranged from 24.75% to 6.33%. There was no correlation between missing data values and the type of reproduction.

2.4.2 | Population Differentiation and Structure

We used two different approaches to assess the population structure for *A. trapezoides*: *STRUCTURE* v 2.3.4 (Pritchard et al. 2000) and discriminant analysis of principal components (*DAPC*) as implemented in the R package *adegenet* v 2.2.10 (Jombart et al. 2010). We ran *STRUCTURE* with 100,000 MCMC iterations following a burn-in of 50,000 iterations, using the admixture ancestry model and no sampling location as a prior, setting a putative *K* from 1 to 6 in a total of 15 independent iterations for each run (Falush et al. 2003). We used *CLUMPAK* (https://tau.evolseq.net/clumpak/) to infer the most likely *K* value based on the highest posterior mean log-likelihood (mean LnP(K)) and to visualise the structure plots (Kopelman et al. 2015).

The population structure in *DAPC* was assessed by the function "*snapclust.choose.k*" used to infer the most likely number of clusters with the Akaike information criterion (AIC) and the Bayesian information criterion (BIC) using the *k*-means algorithm (*pop.ini* = "*kmeans*"), allowing a maximum *k* (*number of clusters*) of 8 (*max* = *8*) and a maximum number of iterations of 100 (*max.iter* = *100*) (Jombart et al. 2010). The optimal number of genetic clusters was identified as the one with the lowest AIC and BIC values (*k* = 3 in both cases). After defining the optimal number of clusters, the number of retained principal components (PCs) axes and eigen-values were chosen using the cross-validation "*xvalDapc*" function from the *adegenet* R package

with 1000 replicates (*n.rep = 1000*). "*xvalDapc*" identifies the lowest number of PCs retaining the maximum variance, which is associated with the lowest mean squared error. The *DAPC* function "*assignplot*" was used to plot the probabilities of assignment of the different individuals to the different clusters, and the function "*scatter.dapc*" was used to produce scatterplots of PCs with eigenvalues as the inset.

We calculated the observed heterozygosity (H_o) for each site using the "*populations*" module in *Stacks*.

2.4.3 | Ploidy Analysis

The ploidy of the individuals was estimated in accordance with the pipeline described in Viruel et al. (2023). As the genome of *A. trapezoides* was not available, the genome of a nearby species, *A. caliginosa* (Savigny, 1826) was retrieved from GenBank (GCA_020284085.1) and used to map the data. We estimated mean values of allele frequencies using nQuire (Weiß et al. 2018). In diploid samples, we expected a higher percentage of SNPs with allelic ratios < 2 and mean and median values below 2. On the contrary, polyploid samples showed allelic ratios > 2 (Viruel et al. 2023). To test the polyploidy of each specimen, we used a Gaussian mixture model (Viruel et al. 2019). When comparing the three fixed models (diploid, triploid, and tetraploid), the ploidy level that most accurately reflected the observed allelic frequency distribution corresponded to the lowest delta value (for more detail see https://github.com/clwgg/nQuire). However, it is not currently feasible to determine the precise ploidy level of tetraploids or higher. This is partly due to the inherent noise associated with sequencing errors generated by HTS platforms, which is achieved by removing low-frequency SNPs (Augusto Corrêa Dos Santos et al. 2017; Viruel et al. 2019). Thus, when tetraploidy is detected, we can not determine whether it corresponds to real tetraploidy or a higher ploidy (Viruel et al. 2019, 2023).

Although analyses were done for a total of 22 individuals (Table S1), we decided to just plot one specimen per locality and type of reproduction.

2.5 | Transcriptome Assembly and Annotation

RNA extraction from different tissues of a single specimen of *A. trapezoides* brought to the laboratory for culturing (regenerated tail, pharynx, seminal vesicle, circulatory system, ganglia, typhlosole, and body wall) was obtained from flash-frozen dissected tissues using Tri-reagent (Ambion), following manufacturer's instructions. The tail fragment excised for RNA extraction was regenerated because a piece was previously used for COI barcoding. Then, mRNA was purified with the Dynabeads mRNA purification kit (Invitrogen, Carlsbad, CA). Concentration of all samples was assessed by Qubit RNA BR assay kit (Thermo Fisher Scientific). cDNA libraries were constructed in the Apollo 324 automated system using the PrepX mRNA kit (Wafergen). Library quality and size selection were checked in an Agilent 2100 Bioanalyzer (Agilent Technologies) with the HS DNA assay. The samples were run using the Illumina HiSeq 2500 platform with paired-end reads of 150 bp at the FAS Center for Systems Biology at Harvard University.

RNA-seq reads were trimmed using Cutadapt v2.9 (Martin 2011) and de novo assembled using Trinity v2.11.0 (Grabherr et al. 2011). Open reading frames (ORFs) were inferred with TransDecoder v.5.5.0 (https://github.com/sghignone/TransDecode). Isoforms in the protein mode were functionally annotated (i.e., Gene Ontology (GO) terms were assigned to each isoform) with both homology-based methods (eggNOG-mapper v2, Cantalapiedra et al. 2021) and natural language processing ones (FANTASIA, Martínez-Redondo et al. 2024).

2.6 | GO Enrichment of Loci Contributing to Separation of Sexual vs. Parthenogenetic Populations

In order to identify the loci contributing the most to the separation between sexual and parthenogenetic populations we used the *adegenet* package. To do this, we selected the SNPs in axis 1 that had a variable contribution ('var.contrib') threshold > 0.0010. This resulted in a total of 195 SNPs; the associated FASTA sequences of these SNPs were then extracted using the function *populations* in *Stacks*. These sequences were then mapped onto the transcriptome, resulting in a total of 105 sequences having a hit on annotated transcripts. Then, mapping the loci on the transcriptome built in 2.5 and then retrieving the GO terms associated to the loci from the FANTASIA dataset. We implemented these GO terms in REVIGO using default settings (Supek et al. 2011) and applied a dispensability value of 0.4 ("tiny") to obtain a treemap that was plotted in R.

2.7 | Microbiome Analysis

2.7.1 | Pipeline Analysis

Raw *16S* rRNA gene sequences were processed separately using the UPARSE pipeline (Edgar 2013). Briefly, primer sequences were removed and overlapping paired reads were merged using *-fastq_mergepairs*, allowing for a maximum of 15 bp of mismatches in the overlapping region. After quality check and de-replication, unique read sequences were detected using the *fastx_uniques* command. Denoising (error-correction) of amplicons was performed using *unoise3* (Edgar 2016), which removed chimeras, reads with sequencing errors, PhiX, and low complexity sequences due to Illumina artefacts, and generated ZOTUs ("zero-radius" Operational Taxonomic Units) with 100% identity sequences. Taxonomic assignment was done with SINA v1.2.11 (Quast et al. 2012) using the SILVA 138.1 database (https://www.arb-silva.de/documentation/release-1381/). Sequences with low alignment quality (< 75%) or unclassified as well as sequences identified as mitochondria or chloroplasts were removed from the analysis. The *initial* ZOTU table underwent rarefaction, with the minimum threshold being set at the sample containing the fewest reads. This rarefaction process was performed using the *rrarefy* function from the R package *vegan* v2.5-6 (Oksanen et al. 2013). The rarefied ZOTU table was subsequently employed for conducting alpha diversity analyses. Moreover, a relative abundance ZOTU table was utilised for characterising the microbial community and conducting beta diversity analyses.

Functional Annotation of Prokaryotic Taxa (FAPROTAX) database (http://www.zoology.ubc.ca/louca/FAPROTAX/) was used to predict the ecological functions of the microbial communities (Louca et al. 2016). The raw counts ZOTU table was used as input for the *collapse_table.py* function with the *-n columns_before_collapsing* option, which performs a TSS normalisation of the ZOTU table.

2.7.2 | Statistical Analysis

We conducted a distance-based multivariate analysis of the worms' microbial communities at the ZOTU level using the *vegan* v2.5-6 package (Oksanen et al. 2013) in R (R Core Team). We used a Bray–Curtis dissimilarity (BCD) distance matrix based on the log2-transformed relative abundance ZOTU table to visualise the patterns of microbial community structure between samples in a principal coordinates analysis (PCoA) using the *cmdscale* function of the *vegan* R package. A hierarchical clustering dendrogram based on the BCD matrix was used to summarise the relationship between samples. Homogeneity of dispersion between groups was checked using the *permutest* function of the *vegan* R package prior to conducting non-parametric permutational analysis of variance (PERMANOVA) using the *adonis* function using 999 permutations and a significance cut-off for *p*-values of 0.05. As factors, we first tested the effect of the sample site on the structure of microbial communities and then, we created separate subsets for each location to examine the influence of the reproductive mode on each subset. For each sample site, we calculated a Bray–Curtis dissimilarity distance matrix and visualised the ordinations through PCoAs, as described above. The effect of reproduction (parthenogenetic vs. sexual) and gut microbiomes of *A. trapezoides* was tested using the *adonis* function with the above mentioned parameters. Moreover, we performed generalised linear models using the raw counts table in the R package "EdgeR" v.3.26.8 (Robinson et al. 2010) to discern variations in the abundance of particular microbiome ZOTUs across the two different reproductive modes. For this analysis, we used a filtering threshold of minimum 0.05 of relative abundance in at least one sample and a significance cut-off value of $p = 0.05$. A rarefied dataset was used to compute alpha diversity indices, specifically Richness and Shannon, within the *vegan* package. To show the disparities in alpha diversity across localities and reproductive modes, we created boxplots employing *ggplot2*. Furthermore, we conducted an analysis of variance (ANOVA) followed by pairwise comparisons using TukeyHSD test to pinpoint significant differences in alpha diversity among localities and reproductive modes.

3 | Results

3.1 | Phylogenetic Analysis

Final alignments were composed of partial sequences of the mitochondrial *16S* rRNA (438 bp) and COI (445 bp) and the nuclear markers *28S* rRNA (802 bp) and H3 histone (292 bp) from 17 specimens. ML phylogenetic results showed that the *Aporrectodea trapezoides* samples belong to a well-supported monophyletic clade (Figure 2). However, some intraspecific diversity was observed, with three distinct clades. The Algerian individuals are divided

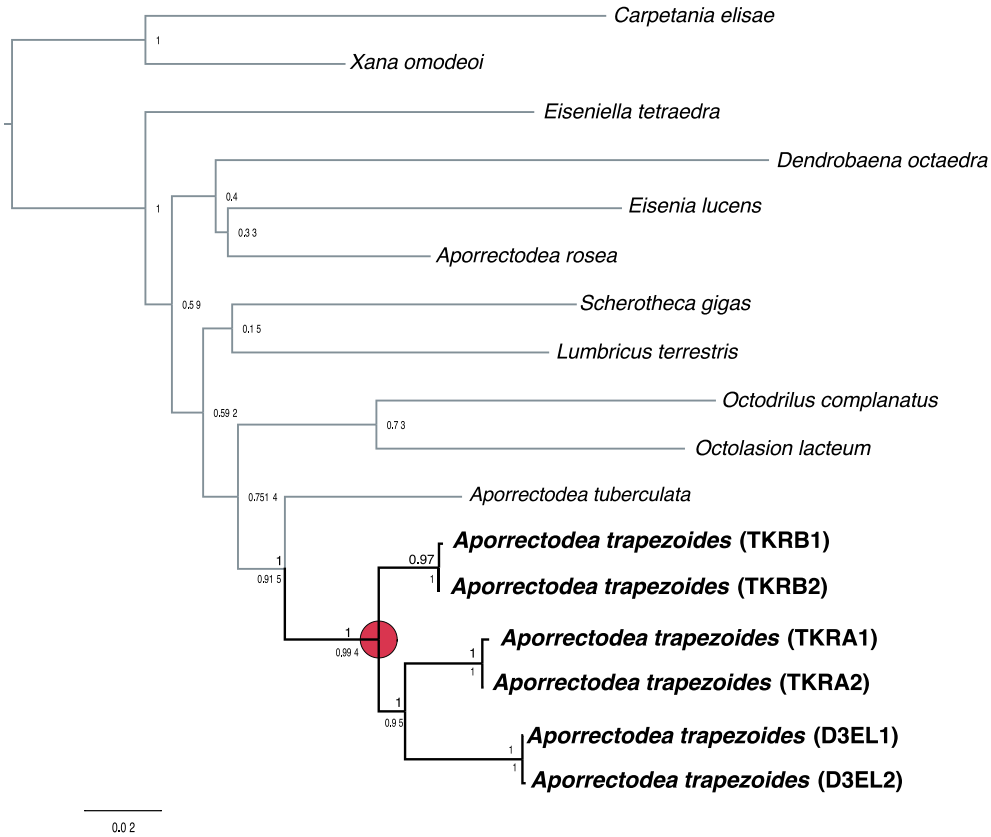

FIGURE 2 | ML phylogenetic hypothesis based on the concatenated sequences of the molecular markers COI, *16S* rRNA, *28S* rRNA and H3. Numbers above branches indicate bootstrap support values (only BS > 70% are indicated).

into two paraphyletic distinct clades (Figure 2), the parthenogenetic (TKRB) appearing as the first branching clade, and then the sexual clade (TKRA). Finally, the clade formed by the individuals collected in Plasencia (D3EL) was monophyletic and appeared as a sister group of the Algerian sexual clade (TKRA) (Figure 2).

3.2 | GBS and Transcriptome Analyses

STRUCTURE identified three different genetic clusters based on the best *K* identified by CLUMPAK (Figure 3A, Figure S1A): one grouping all individuals from Plasencia, regardless of their reproductive strategy, another one grouping samples from sexual localities of Algeria (TCOD and TKRA), and finally another group containing individuals from Menorca and parthenogenetic specimens from Algeria (TALQ and TKRB). Discriminant analysis of principal components (DAPC) showed similar results in three genetic clusters (Figure S1B,C), grouping the same individuals as in the STRUCTURE analysis (Figure 3B). Moreover, we studied the heterozygosity levels (observed heterozygosity) of all localities and types of reproduction and only found significant results between Algerian earthworms, where sexual individuals showed lower levels of heterozygosity (Figure 3C). Overall, the heterozygosity levels were higher for parthenogenetic forms when all populations were analysed together (Figure 3C).

Although no loci under selection were found, 195 loci were contributing the most to the differentiation between sexual and parthenogenetic individuals from Karkra. These loci were mapped against the annotated transcriptome of *A. trapezoides* using FANTASIA, finding annotations in 105 of them. We then plotted the Gene Ontology terms obtained in REVIGO for the category biological process (Figure 4, Table S3). We found several GO terms related to gamete production such as oocyte fate determination (e.g., TRINITY_DN73284_c0_g1_i1, TRINITY_DN51179_c0_g1_i1) and flagellated sperm mobility (e.g., TRINITY_DN108777_c0_g1_i1, TRINITY_DN12654_c1_g1_i5) (Figure 4). In addition, several other GO terms were involved in the processes mediating associations with other organisms including symbiont-mediated perturbation of host cell cycle G0/G1 transition checkpoint (e.g., TRINITY_DN44160_c0_g1_i1, TRINITY_DN884_c3_g1_i1) and actin-dependent intracellular transport of virus towards nucleus (e.g., TRINITY_DN23905_c0_g2_i1) (Figure 4).

All samples showed a wide range of genes with mean values of the allelic ratios greater than 2, indicating polyploidy (Figure 5). Moreover, among the three fixed models (diploid, triploid, and tetraploid), the ploidy level that best matched the observed distribution of allelic frequencies is tetraploid or more (see Section 2), as is the model with the lowest delta (Tables S1–S6) value (Table 1). Interestingly, all individuals from all sites showed a very similar ploidy level (Figure 5).

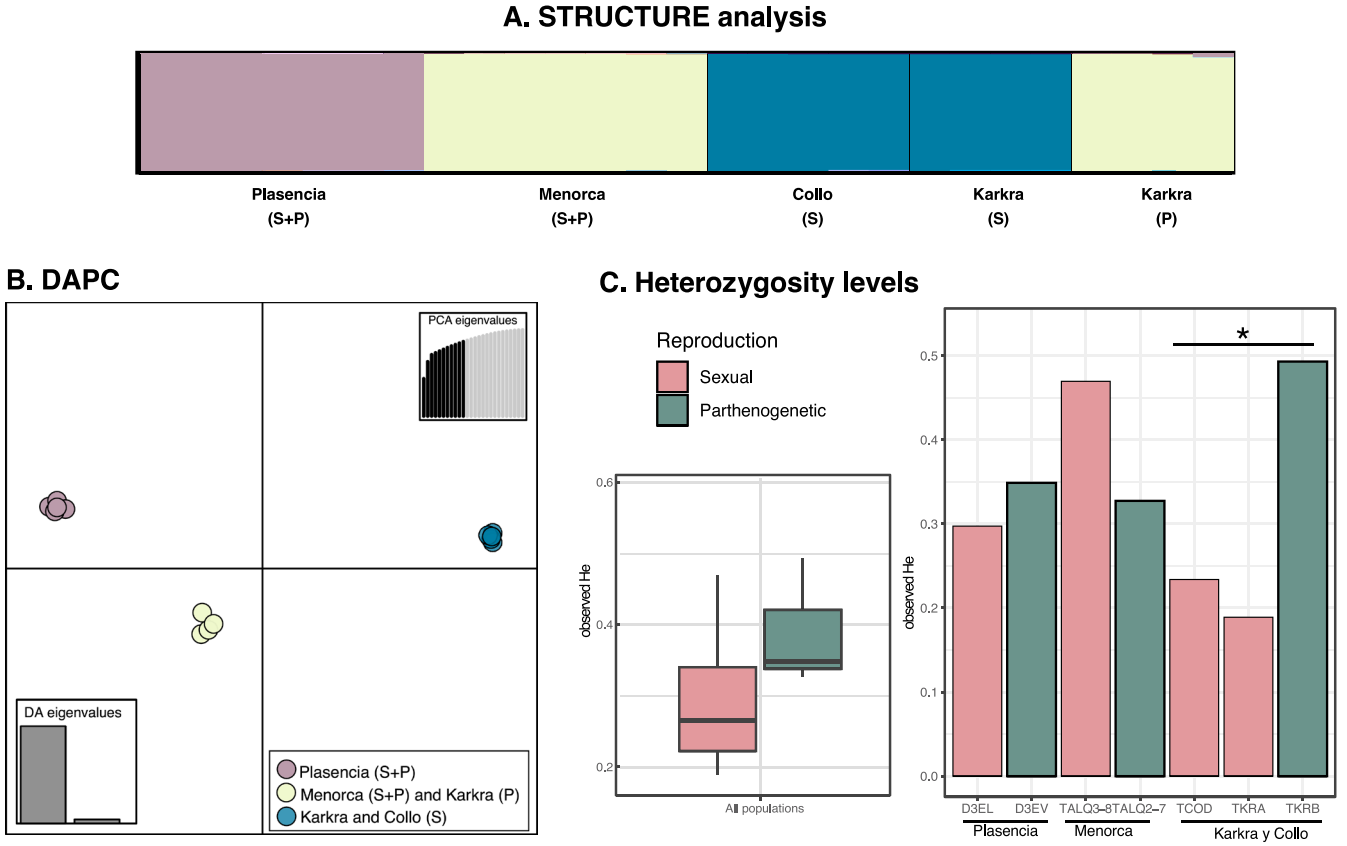

FIGURE 3 | Population structure and differentiation analyses based on the SNPs dataset. (A) STRUCTURE results with $K = 3$. (B) DAPC results: Representation of the first (horizontal axis) and the second (vertical axis) PCA eigenvalues. (C) Observed heterozygosity (H_e) levels for sexual (pink) and parthenogenetic (green) individuals from each sample site. *Significant differences.

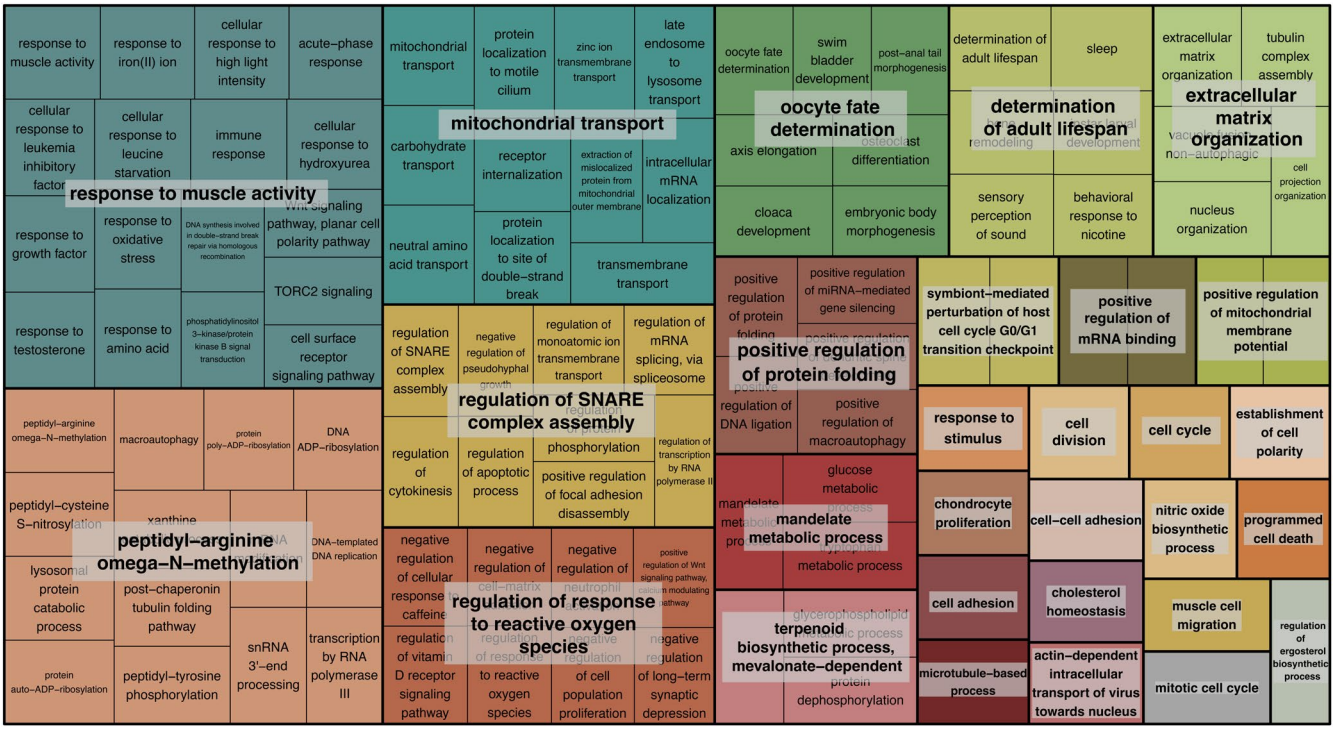

FIGURE 4 | Gene Ontology treemap related to the category Biological Process found in REVIGO, for annotated differentially expressed loci between both types of reproduction in Karkra (Algeria).

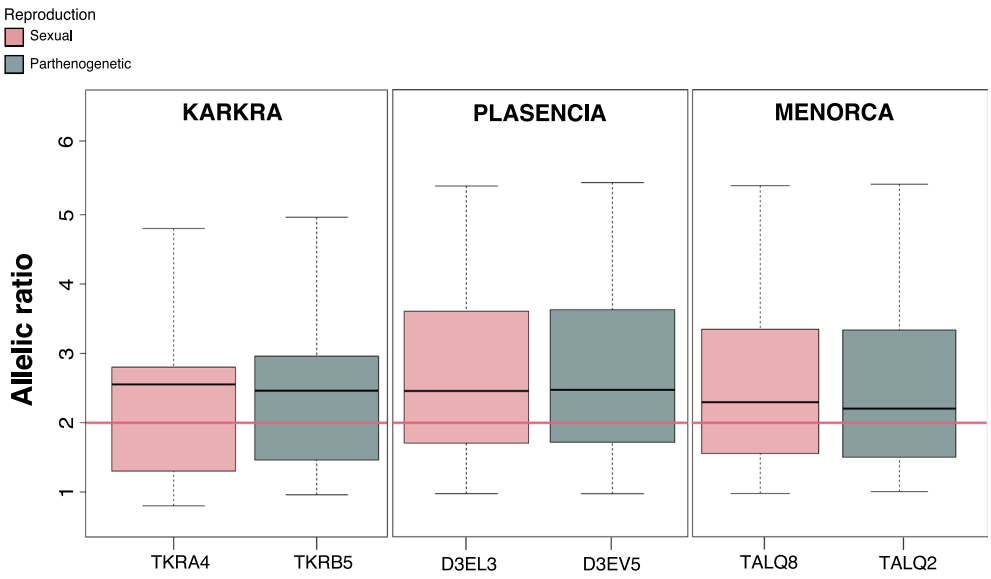

FIGURE 5 | Box plot of ploidy analysis of the different localities sampled in sexual (pink) and parthenogenetic (green) specimens.

3.3 | Microbiome Analysis

3.3.1 | Overall Microbial Composition and Microbiome Diversity and Structure

A total of 5297 ZOTUs (zero-radius Operational Taxonomic Unit) were identified within the 23 samples of *A. trapezoides* sequenced. Among them, we identified a total of 16 prokaryotic phyla: *Actinobacteriota* was the most abundant phylum in our samples (42.47%), followed by *Firmicutes* (29.05%) and *Proteobacteria* (12.56%). At the class level, 22 different classes were identified, with *Actinobacteria* (29.27%), *Bacilli* (21.24%), and *Thermoleophilia* (9.84%) as the most dominant classes. No significant differences were observed between sample sites or reproduction types at these taxonomic levels (Figure 6A).

Alpha diversity values of *A. trapezoides* differed among sites. Significant differences were found in Shannon ($p < 0.05$) and Richness indexes ($p < 0.01$). The Tukey pairwise test showed significant differences between Menorca and Karkra ($p < 0.05$) for the Shannon index and between Menorca and Karkra ($p < 0.05$) and Plasencia and Karkra ($p < 0.01$) for the Richness index.

TABLE 1 | Statistical results of Gaussian mixture model.

	Fee	Dip	Trip	Tet	d_dip	d_trip	d_tet
Sample							
D3EL3	97,063.18	23,472.95	66,980.94	92,124.02	73,592.22	30,082.23	2939.16
D3EV5	98,582.38	25,478.31	69,343.77	95,394.47	73,104.06	29,238.60	3187.90
TALQ2	314,540.00	19,493.20	31,252.76	34,604.30	295,046.79	283,287.23	279,935.69
TALQ8	82,783.52	32,629.28	64,849.51	77,693.90	50,154.24	17,934.12	5089.62
TKRA4	255,539.17	79,156.76	122,764.70	222,722.61	185,382.40	132,774.46	32,816.56
TRKB5	464,078.95	132,057.64	240,255.96	410,694.74	332,021.31	223,822.99	53,384.20

Note: d_dip, diploid delta log-likelihood; d_tet, tetraploid delta log-likelihood; d_tri, triploid delta log-likelihood; dip, diploid fixed model maximised log-likelihood; Fee, free model maximised log-likelihood; tet, tetraploid fixed model maximised log-likelihood; tri, triploid fixed model maxi-mized log-likelihood.

The Richness index suggested that the microbial community of earthworms from Karkra was the most diverse ($p < 0.05$). In each site subset, significant differences between parthenogenetic and sexual earthworms were only observed in Karkra for the Richness index (Figure 6B, Data S1).

The associations between microbiome samples were represented in a cluster dendrogram (Figure 6A) and in a principal coordinate analysis (PCoA) plot (Figure 6C), both based on the Bray–Curtis dissimilarity matrix of the microbial ZOTUs. Both representations revealed that the majority of the samples grouped by the site, which was the factor explaining most of the variation between samples (Data S1, Permanova: R^2: 0.43, p-value < 0.001). However, we detected differences in dispersion between sampling sites using the *permutest* function (p-value < 0.05). As shown in the cluster dendrogram, two samples from Plasencia were highly differentiated from the others, which can explain the differences in dispersion (Figure 6A). Population and reproduction, as well as the interaction of both factors, significantly explained the variation between samples (Data S1). However, as population was the factor explaining most of the variation (> 40%) we chose to analyse each location separately to test the effect of reproduction. Significant differences between parthenogenetic and sexual individuals were only detected in Karka earthworms, for which the reproductive mode explained almost 50% of the microbiome variation (Permanova: R^2: 0.49, p-value < 0.05, Data S1). In the cluster dendrogram, differentiation between sexual and parthenogenetic forms in Karka exceeded 40% (Figure 6A), and in the PCoA for this locality, both reproductive modes appeared clearly separated along the first PCoA axis (Figure S3).

3.3.2 | Differential Abundance Analysis

Differential abundance analysis was conducted to identify specific ZOTUs associated to each type of reproduction in the different sites. Significant results were only found in the population from Karkra, with a total of 754 differentially abundant (DA) ZOTUs between parthenogenetic and sexual worms (Table S4). From these DA ZOTUs, 314 ZOTUs were significantly more abundant in sexual earthworms, whereas 440 ZOTUs were significantly more abundant in parthenogenetic earthworms (Table S4). For representative purposes, we used a filtered dataset with a minimum abundance of 0.25% in at least two samples (Figure 7A, Table S5).

According to this filtered dataset, in sexual earthworms the DA ZOTUs belonged to five different phyla (*Actinobacteriota*, *Crenarchaeota*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobiota*), which encompassed eight different classes (*Actinobaceria*, *Thermoleophilia*, *Rubrobacteria*, *Nitrososphaeria*, *Bacilli*, *Alphaproteobacteria*, and *Verrucomicrobiae*) (Figure 7A). We found 19 different orders (e.g., *Solirubrobacterales*, *Rhizobiales*, and *Elsterales*), 15 families (e.g., *Acidothermaceae*, *Bacillaceae*, and *Xanthobacteraceae*), and 14 different genera (e.g., *Romboutsia*, *Acidothermus*, and *Actinoallomurus*) that were more abundant in sexual individuals (Table S5).

In contrast, in parthenogenetic individuals, and according to the filtered data set, the DA ZOTUs were included in five different phyla (*Actinobacteriota*, *Chloroflexi*, *Crenarchaeota*, *Firmicutes*, and *Proteobacteria*) which comprised six different classes (*Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, *KD4–96*, *Nitrososphaeria*, *and Thermoleophilia*) (Figure 7A). We observed 10 different orders (e.g., *Bacillales*, *Nitrososphaerales*, and *Corynebacteriales*), 12 families (e.g., *Bacillaceae*, *Nitrososphaeraceae*, and *Methyloligellaceae*), and 7 different identified genera (e.g., *Gaiella*, *Mycobacterium*, and *Nocardiopsis*) (Table S5).

To perform the functional prediction of the microbial taxa, only the annotated taxa could be used, which represented between 15% and 60% of relative abundance depending on the sample (Figure S4). Relevant functional differences between sexual and parthenogenetic individuals were only detected for Karkra (Figure S2). In parthenogenetic individuals, taxa performing dark oxidation of sulfur compounds, nitrate ammonification, methanotrophy, oxygenic photoautotrophy, and photosynthetic cyanobacteria were more abundant (Figure 7B, Figure S2). In contrast, taxa performing nitrate reduction were more abundant in sexual individuals consistently (Figure 7B, Figure S2), including *Rubrobacter*, *Haemophilus*, *Hamadea*, and *Aeromonas* (Table S6), and 3 out of 4 samples of sexual worms contained more taxa involved cellulolysis, including *Acidothermus* and *Sorangium* (Table S6).

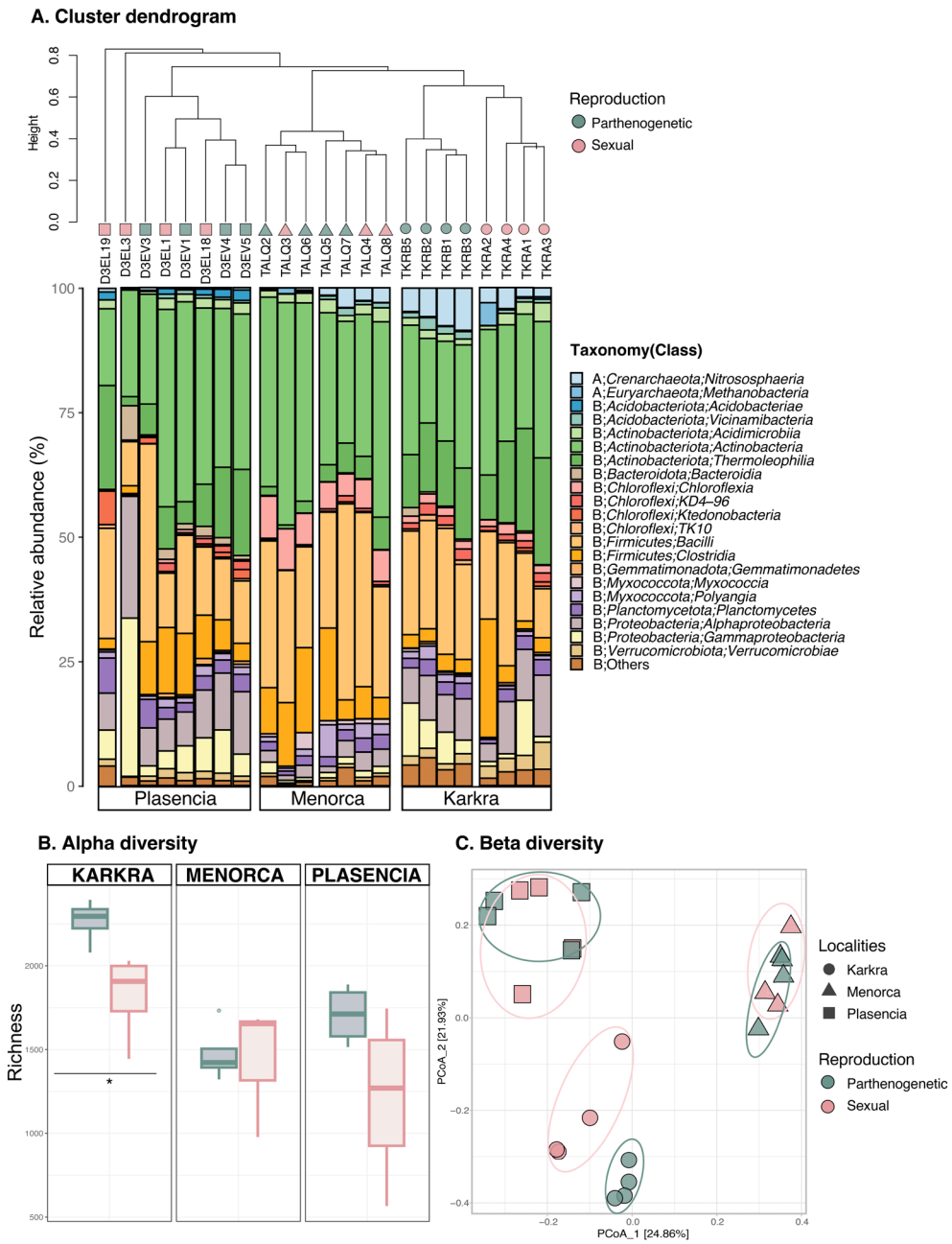

FIGURE 6 | Microbiome diversity in each locality and reproduction mode. (A) Cluster dendogram based on Bray–Curtis dissimilarity matrices of microbial communities in the localities sampled. Each colour represents a reproductive mode and prokaryotic taxonomic composition at class level in the localities sampled for *A. trapezoides*. A: Archaea; B: Bacteria. (B) Box plot showing alpha diversity measures of localities sampled of *A. trapezoides* using Richness diversity index. (C) Principal coordinate analysis (PCoA) plot based on Bray–Curtis dissimilarity matrix of the microbial composition across the different localities sampled of *A. trapezoides* (indicated by different shapes). Colours on the figure legend correspond to different reproductive modes. Variation explained by the first two axes is indicated as %. Statistical results are shown in Appendix S1.

4 | Discussion

Our results demonstrate that different sexual forms (sexual and parthenogenetic) of the earthworm *Aporrectodea trapezoides* host divergent microbiomes, potentially selected by fine-tuning of the immune system at the genomic level, involving genes mediating symbiont perturbations of host cell cycle. The population differentiation also shows genes contributing to the divergence of sexual and parthenogenetic populations within Algeria, including important genes related to gametogenic processes such as sperm capacitation and oocyte fate determination. The following discussion places our results in a broader evolutionary context that would help understand the emergence and maintenance of parthenogenetic systems in earthworms.

Parthenogenetic earthworm species are typically polyploid (Suomalainen et al. 1987). Indeed, a single morphological

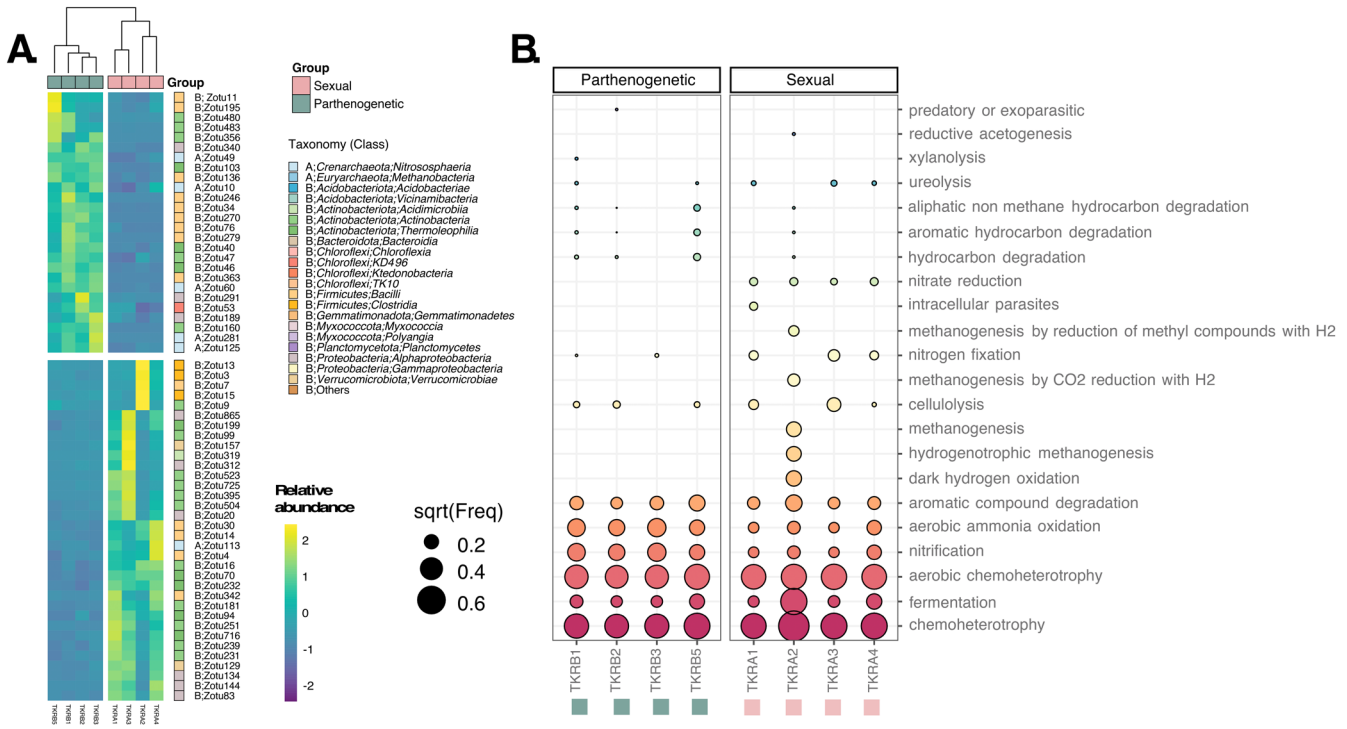

FIGURE 7 | (A) Heatmap of the filtered DA ZOTUs of sexual and parthenogenetic earthworms from Karkra. (B) Predicted functionality of the prokaryotic taxa of Karka samples.

species may exhibit a range of ploidy levels. For instance, *Aporrectodea rosea* (Savigny, 1826) exhibits a range of forms with varying degrees of ploidy, from 3n to 10n (Omodeo 1952). Some of these forms are sexual, while others are parthenogenetic. Our ploidy analysis determined that the studied individuals of *A. trapezoides* were polyploid, potentially tetraploid or with a higher ploidy (as the analysis is not able to distinguish between both). While the most common form of this species is triploid in other populations, both diploid (Onteniente and Rodríguez Babío 2002) and tetraploid (Omodeo 1952; Casellato and Rodighiero 1972) individuals have been identified in the past. According to our results (Figure 5, Table 1) and since there are no known cases of ploidy greater than 4n in *A. trapezoides* (Omodeo 1952; Casellato and Rodighiero 1972; Onteniente and Rodríguez Babío 2002), we assume that the individuals included in this study are tetraploids. This may provide a framework for an explanation as to why significant differences between sexual and parthenogenetic samples are only identified in all applied analyses between the Algerian earthworms. An even ploidy level enables facultative parthenogenesis, and recent studies have demonstrated the occurrence of rare or cryptic sex in several species previously considered to be obligately parthenogenetic (Boyer et al. 2021; Freitas et al. 2023; Kuhn et al. 2021; Laine et al. 2022). It is plausible that individuals from Spain are facultative parthenogens. The absence of differences between the two reproductive types in these localities may be attributed to secondary sexual contacts between these individuals. Conversely, the Algerian earthworms would be obligate parthenogens, without sexual contact, which would emphasise their genomic and microbiome differences.

Parthenogenesis is predicted to have many consequences for the evolution of the genome, resulting in high heterozygosity, high abundance of horizontally acquired genes, low transposable element load, or the presence of palindromes (Jaron et al. 2021). This is true for *A. trapezoides* in Algeria, where sexual and parthenogenetic specimens belonged to different genomic clusters (Figure 3A,B). This strong separation without shared ancestry may indicate a hybrid origin of the parthenogenetic forms (with unsampled parental lineage). Moreover, parthenogenetic individuals also present higher levels of heterozygosity than sexual ones in Algeria. This difference may also indicate that parthenogenesis in *A. trapezoides* is of hybrid origin. Hybridisation-induced parthenogenesis can result in the generation of a highly heterozygous genome, contingent upon the divergence between the parental sexual species prior to the hybridisation event (Jaron et al. 2021).

Some of the biological processes of the earthworms contributing more to the differentiation between the two types of reproduction were clearly related to potential differences between parthenogenetic and sexual forms of reproduction. For example, oocyte fate determination or sperm motility and capacitation. Indeed, nitric oxide (NO) has been shown to promote human sperm motility via activation of the cyclic GMP/protein kinase G signalling pathway (Miraglia et al. 2011). In parthenogenesis, despite the presence of sperm in seminal vesicles, they do not participate in the reproductive process, as there is no exchange of gametes. It is plausible that the levels of NO are insufficient to promote motility in the sperm of parthenogenetic individuals, hampering the fertilisation process. We also detected virus-related processes in

A. trapezoides. Interestingly, virus vaccinations are known to trigger the process of parthenogenesis in birds (Ramachandran and McDaniel 2018). Whether the virome may play a role in parthenogenesis in *A. trapezoides* remains unknown, something that should be investigated in further studies.

Regarding the bacterial phyla found in the samples, *Actinobacteriota* and *Proteobacteria* are two of the most common phyla found in the gut of worms (Yang et al. 2024; Aira et al. 2022), while members of the genus *Firmicutes* often vary between species (Aira et al. 2022).

The hypothesis that the intestinal microbiome of earthworms is species-specific, with a strong phylogenetic component, has been supported in the past (Thakuria et al. 2009; Nechitaylo et al. 2010; Gómez-Brandón et al. 2011, 2012; Aira and Domínguez 2014). Other authors, though, have proposed that the gut microbiota is predominantly derived from the microbiota found in the soil in which they live (Barois and Lavelle 1986; Zeibich et al. 2019; Medina-Sauza et al. 2019). Our results demonstrate that both hypotheses can be compatible. When examining generic taxonomic ranks, such as phylum or class (Figure 6A), no significant differences were observed at the locality level. This suggests that the gut microbiome possesses a robust phylogenetic component and is species-specific, regardless of the soil where it is found. However, this is not the case when we examine more specific taxonomic ranks specifically at the level of ZOTUs (Figure 7). Here, we observed that microbial diversity is organised according to the sample site, thereby supporting the hypothesis that the gut microbiome originates from the soil in which they reside. It seems probable that, on a large scale, there is a phylogenetic component in which each species selects the same group of microorganisms. However, in each locality, the specific microorganisms are selectively incorporated to each of the phylogenetic groups from the soil where they live. In addition, we provide evidence of fine tuning of the microbiome that diverges across lineages living within the same site and is associated with the reproductive strategy. This has been reported previously in the human gut microbiome (Davenport 2016; Kolde et al. 2018; Spor et al. 2011), mouse lines (Benson et al. 2011), and plant genotypes (Bouffaud et al. 2014; Qian et al. 2018). Recent findings in the earthworm *Eisenia andrei* reveal the presence of antimicrobial peptides encoded in the genome with demonstrated activity against bacteria, supporting the idea that earthworms may actively shape their gut microbial communities via immune modulation (Wu et al. 2023).

It is possible that this strong genomic and microbiome structuring in Algerian earthworms is due to ecological factors. Although both reproductive types coexist at the same sampling site, they may occupy different microniches with different ecological requirements and a different microbial composition in the soil (Vos et al. 2013; Medina-Sauza et al. 2023). However, although this possibility cannot be discarded, we collected individuals of both types of reproduction within a few square meters, suggesting they inhabit an ecosystem with very similar biotic and abiotic factors.

The considerable taxonomic, genetic, and metabolic diversity of the microbiome presents a significant challenge to the task of elucidating the mechanisms through which the microbiome affects the host (Kuziel and Rakoff-Nahoum 2022). In our study, following the pattern found at the genomic level, the only earthworms with a microbiome that differed according to whether they were parthenogenetic or sexual were the Algerian individuals. However, it is unclear whether a combination of microbial taxa was behind the promotion of sexuality in earthworms or only specific taxa were determinant for it. In fact, we did not identify any endosymbiotic bacteria known to influence the reproductive behaviour in other species, typically arthropods (e.g., Perlmutter and Bordenstein 2020; Aguin-Pombo et al. 2021; Verhulst et al. 2023). In turn, we detected different taxa that could be associated to the parthenogenetic and sexual strategies. For instance, sexual earthworms showed more abundance of bacteria from the genus *Romboutsia*, which are known to prevent male infertility in rats (Sheng et al. 2023) and also play a protective function in male infertility pathogenesis in humans (Fu et al. 2023). Also, as said before, high levels of nitric oxide could be beneficial for sperm quality (Miraglia et al. 2011), and the fact nitrate reduction microbes were only found in sexual individuals of Algeria, might be linked to more production of NO and therefore motile sperm in these sexual forms. In contrast, the *Methyloligellaceae* family, differentially more abundant in parthenogenetic individuals, promotes oestrogen (estrone and estradiol) production through the steroid hormone biosynthesis pathway in rats (Wang et al. 2024). In this sense, further experimental (including microbial transplantation) and functional studies should be conducted in order to test whether there is a direct link between the reproductive mode and specific microbial taxa in *A. trapezoides*.

5 | Conclusions

This study reveals the different molecular mechanisms involved in parthenogenesis in the earthworm *Aporrectodea trapezoides*. We hypothesize that facultative parthenogenesis is occurring in the Spanish localities, which would explain the lack of differences between genomic and microbiome-level data between these separated localities. In contrast, obligate parthenogenesis is postulated to occur only in the Algerian earthworm population, since we find evidences of the biological drivers related to each type of reproduction at the genomic and microbiome levels. At the genomic level, we show strong genomic differentiation between sexual and parthenogens and also high levels of heterozygosity in the last ones, supporting the hypothesis of a hybrid origin of parthenogenesis in *A. trapezoides*. We also showed evidence that suggests that the microbiome has been subject to fine-tuning due to divergence in the sexual and parthenogenetic individuals at the genomic level, resulting in divergence in composition and function across lineages inhabiting the same site. This highlights the intriguing relationship between the microbiome and the reproductive process that occurs across metazoans. However, further experimental studies, including antibiotic treatments or transplantation experiments with living specimens, may help elucidate the role of the microbiome in the reproduction of earthworms. Additionally, directly quantifying microbial products, such as nitric oxide or hormone-like compounds, could help to validate functional predictions and clarify the mechanisms underlying host–microbiome interactions.

Author Contributions

I.S., P.Á.C., and A.R. conceived the project. I.S., N.T., and A.P. performed laboratory work. I.S., M.T., S.T., J.L.-S., R.F., M.N., P.Á.C., and A.R. analysed the data. I.S., M.T., and S.T. wrote the manuscript with assistance from all coauthors.

Acknowledgements

We are grateful to the team of the Soil Zoology Group of Complutense University of Madrid (UCM) and Kamel-eddine Bazri for sampling work. We want to thank Maria Conejero for earthworms' illustrations. I.S. was funded by the European Union-Next GenerationEU program through a Margarita Salas fellowship. M.T. was supported by a JdC (Juan de la Cierva Formación, 2020) personal grant (FJC2020-043677-I), MSCA Postdoctoral Fellowship (HORIZON-MSCA.2022-PF-01, Project 101105716). J.S. and A.P. were funded by the Spanish Ministry of Science, Innovation, and Universities through a predoctoral fellowship (JSPRE2020-095330 and PRE2022-103770, respectively). M.N. was supported by Grant PID2021-122243NB-I00 funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe". P.Á.C. was funded by MNCIN/AEI/10.13039/501100011033 and European Union "Next GenerationEU/PRTR" (CNS2023-145193). S.T. received funding from the grant PID2020-117115GA-I00 funded by MCIN/AEI/10.13039/501100011033 and from the grant CNS2023-144572 and by the Ramón y Cajal grant RYC2021-03152-I, funded by the MCIN/AEI/10.13039/501100011033 and the European Union «NextGenerationEU»/PRTR». A.R. acknowledges funding from three grants from the Spanish Ministry of Science and Innovation (RYC2018-024247-I, PID2019-105769GB-I00, and CNS2023-144571) in the framework of MCIN/AEI/10.13039/50110001103 and EI "FSE invierte en tu futuro" and an intramural grant from CSIC (PIE-202030E006). R.F. acknowledges support from the following sources of funding: the European Research Council (grant agreement no. 948281), the OSCARS project (funding from the European Commission's Horizon Europe Research and Innovation programme under grant agreement no. 101129751), grant CNS2023-144624 funded by MICIU/AEI/10.13039/501100011033, and by the "European Union NextGenerationEU/PRTR" and the Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement de la Generalitat de Catalunya (AGAUR 2021-SGR00420).

Disclosure

Benefit-Sharing Statement: Algerian specimens were collected under existing permits of the University of Constantine (Algeria) and are deposited in the UCM-LT collection (voucher nos. 01273-01288); no additional benefit sharing applies.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/. Transcriptome assemblies were deposited in Zenodo under the DOI: https://doi.org/10.5281/zenodo.15600607. Reference numbers of molecular markers are available in Table S1. Raw sequence reads and metadata are deposited in the SRA under project PRJNA1194614. We also provide the code used as Data S3.

References

Aguin-Pombo, D., M. C. Rodrigues, B. Voetdijk, and J. A. Breeuwer. 2021. "Parthenogenesis and Sex-Ratio Distorting Bacteria in *Empoasca* (*Hemiptera: Cicadellidae*) Leafhoppers." *Annals of the Entomological Society of America* 114, no. 6: 738–749.

Aira, M., and J. Domínguez. 2014. "Changes in Nutrient Pools, Microbial Biomass, and Microbial Activity in Soils After Transit Through the Gut of Three Endogeic Earthworm Species of the Genus *Postandrilus*." *Journal of Soils and Sediments* 14, no. 8: 1335–1340.

Aira, M., M. Pérez-Losada, K. A. Crandall, and J. Domínguez. 2022. "Host Taxonomy Determines the Composition, Structure, and Diversity of the Earthworm Cast Microbiome Under Homogenous Feeding Conditions." *FEMS Microbiology Ecology* 98, no. 9: fiac093.

Alavi, Y., M. A. Elgar, and T. M. Jones. 2016. "Male Mating Success and the Effect of Mating History on Ejaculate Traits in a Facultatively Parthenogenic Insect (*Extatosoma tiaratum*)." *Ethology* 122, no. 7: 523–530.

Apprill, A., S. McNally, R. Parsons, and L. Weber. 2015. "Minor Revision to V4 Region SSU rRNA 806R Gene Primer Greatly Increases Detection of SAR11 Bacterioplankton." *Aquatic Microbial Ecology* 75, no. 2: 129–137.

Augusto Corrêa Dos Santos, R., G. H. Goldman, and D. M. Riaño-Pachón. 2017. "ploidyNGS: Visually Exploring Ploidy With Next-Generation Sequencing Data." *Bioinformatics* 33, no. 16: 2575–2576.

Avise, J. C., J. M. Quattro, and R. C. Vrijenhoek. 1992. "Molecular Clones Within Organismal Clones: Mitochondrial DNA Phylogenies and the Evolutionary Histories of Unisexual Vertebrates." In *Evolutionary Biology*, vol. 26, 225–246. Springer US.

Barois, I., and P. Lavelle. 1986. "Changes in Respiration Rate and Some Physicochemical Properties of a Tropical Soil During Transit Through *Pontoscolex corethrurus* (*Glossoscolecidae*, *Oligochaeta*)." *Soil Biology and Biochemistry* 18, no. 5: 539–541.

Bell, G. 1982. *The Masterpiece of Nature: The Evolution and Genetics of Sexuality*. University of California Press.

Benjamini, Y., and Y. Hochberg. 1995. "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing." *Journal of the Royal Statistical Society, Series B: Statistical Methodology* 57, no. 1: 289–300.

Benson, J. M., D. Peritt, B. J. Scallon, et al. 2011. "Discovery and Mechanism of Ustekinumab: A Human Monoclonal Antibody Targeting Interleukin-12 and Interleukin-23 for Treatment of Immune-Mediated Disorders." *MAbs* 3, no. 6: 535–545.

Booth, W., C. F. Smith, P. H. Eskridge, S. K. Hoss, J. R. Mendelson III, and G. W. Schuett. 2012. "Facultative Parthenogenesis Discovered in Wild Vertebrates." *Biology Letters* 8, no. 6: 983–985.

Bouché, M. B. 1972. *Lombriciens de France. Ecologie et Systématique*. Vol. 72 (No. HS, pp. 671). INRA Editions.

Bouffaud, M. L., M. A. Poirier, D. Muller, and Y. Moënne-Loccoz. 2014. "Root Microbiome Relates to Plant Host Evolution in Maize and Other P Oaceae." *Environmental Microbiology* 16, no. 9: 2804–2814.

Boyer, L., R. Jabbour-Zahab, M. Mosna, C. R. Haag, and T. Lenormand. 2021. "Not So Clonal Asexuals: Unraveling the Secret Sex Life of *Artemia parthenogenetica*." *Evolution Letters* 5, no. 2: 164–174.

Cantalapiedra, C. P., A. Hernández-Plaza, I. Letunic, P. Bork, and J. Huerta-Cepas. 2021. "eggNOG-Mapper v2: Functional Annotation, Orthology Assignments, and Domain Prediction at the Metagenomic Scale." *Molecular Biology and Evolution* 38, no. 12: 5825–5829.

Carson, H. L., L. S. Chang, and T. W. Lyttle. 1982. "Decay of Female Sexual Behavior Under Parthenogenesis." *Science* 218, no. 4567: 68–70.

Casellato, S. 1987. "On Polyploidy in Oligochaetes, With Particular Reference to Lumbricids." In *On Earthworms*, 75–87. Mucchi Editore.

Casellato, S., and R. Rodighiero. 1972. "Karyology of Lumbricidae. III Contribution." *Caryologia* 25: 513–524.

Catchen, J., P. A. Hohenlohe, S. Bassham, A. Amores, and W. A. Cresko. 2013. "Stacks: An Analysis Tool Set for Population Genomics." *Molecular Ecology* 22, no. 11: 3124–3140.

Cosín, D. J. D., M. Novo, and R. Fernández. 2010. "Reproduction of Earthworms: Sexual Selection and Parthenogenesis." In *Biology of Earthworms*, edited by M. Novo, 69–86. Springer Berlin Heidelberg.

Da Rocha-Lima, H. 1916. "Zur aetiologie des fleckfiebers." *DMW-Deutsche Medizinische Wochenschrift* 42, no. 44: 1353–1354.

Darriba, D., D. Posada, A. M. Kozlov, A. Stamatakis, B. Morel, and T. Flouri. 2020. "ModelTest-NG: A New and Scalable Tool for the Selection of DNA and Protein Evolutionary Models." *Molecular Biology and Evolution* 37: 291–294.

Davenport, E. R. 2016. "Elucidating the Role of the Host Genome in Shaping Microbiome Composition." *Gut Microbes* 7, no. 2: 178–184.

De Sosa, I., D. F. Marchán, M. Novo, A. Almodóvar, and D. J. Díaz Cosin. 2023. "Phylogeography of a Riparian Earthworm Shows Environmental Factors Influence Genetic Structure." *Journal of Biogeography* 50, no. 1: 156–168.

De Sosa, I., D. F. Marchán, M. Novo, D. J. D. Cosín, G. Giribet, and R. Fernández. 2017. "Insights Into the Origin of Parthenogenesis in Oligochaetes: Strong Genetic Structure in a Cosmopolitan Earthworm Is Not Related to Reproductive Mode." *European Journal of Soil Biology* 81: 31–38.

Dedryver, C. A., M. Hullé, J. F. Le Gallic, M. C. Caillaud, and J. C. Simon. 2001. "Coexistence in Space and Time of Sexual and Asexual Populations of the Cereal Aphid *Sitobion avenae*." *Oecologia* 128: 379–388.

Dufresne, F., and P. D. Hebert. 1994. "Hybridization and Origins of Polyploidy." *Proceedings of the Royal Society of London, Series B: Biological Sciences* 258, no. 1352: 141–146.

Edgar, R. C. 2013. "UPARSE: Highly Accurate OTU Sequences From Microbial Amplicon Reads." *Nature Methods* 10, no. 10: 996–998.

Edgar, R. C. 2016. "UNOISE2: Improved Error-Correction for Illumina 16S and ITS Amplicon Sequencing." *bioRxiv*. https://doi.org/10.1101/081257.

Edler, D., J. Klein, A. Antonelli, and D. Silvestro. 2021. "raxmlGUI 2.0: A Graphical Interface and Toolkit for Phylogenetic Analyses Using RAxML." *Methods in Ecology and Evolution* 12, no. 2: 373–377.

Edwards, C. A., and N. Q. Arancon. 2022. "Earthworm Life Histories and Biology." In *Biology and Ecology of Earthworms*, edited by C. A. Edwards and N. Q. Arancon, 81–108. Springer US.

Engelstädter, J. 2017. "Asexual but Not Clonal: Evolutionary Processes in Automictic Populations." *Genetics* 206, no. 2: 993–1009.

Excoffier, L., and H. E. Lischer. 2010. "Arlequin Suite Ver 3.5: A New Series of Programs to Perform Population Genetics Analyses Under Linux and Windows." *Molecular Ecology Resources* 10, no. 3: 564–567.

Falush, D., M. Stephens, and J. K. Pritchard. 2003. "Inference of Population Structure Using Multilocus Genotype Data: Linked Loci and Correlated Allele Frequencies." *Genetics* 164, no. 4: 1567–1587.

Foerster, S. I. A., W. Dionisio-da-Silva, A. B. dos Santos, C. M. R. de Albuquerque, and A. F. Araújo Lira. 2021. "Notes on the Courtship Behavior of the Parthenogenetic Scorpion *Tityus stigmurus*." *Acta Arachnologica* 70, no. 2: 83–86.

Foll, M., and O. Gaggiotti. 2008. "A Genome-Scan Method to Identify Selected Loci Appropriate for Both Dominant and Codominant Markers: A Bayesian Perspective." *Genetics* 180, no. 2: 977–993.

Freitas, S., D. J. Parker, M. Labédan, Z. Dumas, and T. Schwander. 2023. "Evidence for Cryptic Gene Flow in Parthenogenetic Stick Insects of the Genus Timema." *bioRxiv*. https://doi.org/10.1101/2023-02.

Fu, Z. D., Y. Wang, and H. L. Yan. 2023. "Male Infertility Risk and Gut Microbiota: A Mendelian Randomization Study." *Frontiers in Microbiology* 14: 1228693.

Gates, G. E. 1972. "Contributions to North American Earthworms (Annelida: Oligochaeta): No. 3." In *Toward a Revision of the Earthworm Family Lumbricidae; IV. The Trapezoides Species Group*. Tall Timbers Research Station.

Gherna, R. L., J. H. Werren, W. Weisburg, et al. 1991. "*Arsenophonus nasoniae* Gen. Nov., sp. Nov., the Causative Agent of the Son-Killer Trait in the Parasitic Wasp *Nasonia vitripennis*." *International Journal of Systematic and Evolutionary Microbiology* 41, no. 4: 563–565.

Gómez-Brandón, M., M. Aira, M. Lores, and J. Domínguez. 2011. "Epigeic Earthworms Exert a Bottleneck Effect on Microbial Communities Through Gut-Associated Processes." *PLoS One* 6, no. 10: e24786.

Gómez-Brandón, M., M. Lores, and J. Domínguez. 2012. "Species-Specific Effects of Epigeic Earthworms on Microbial Community Structure During First Stages of Decomposition of Organic Matter." *PLoS One* 7, no. 2: e31895.

Grabherr, M. G., B. J. Haas, M. Yassour, et al. 2011. "Full-Length Transcriptome Assembly From RNA-Seq Data Without a Reference Genome." *Nature Biotechnology* 29, no. 7: 644–652.

Hagimori, T., Y. Abe, S. Date, and K. Miura. 2006. "The First Finding of a *Rickettsia* Bacterium Associated With Parthenogenesis Induction Among Insects." *Current Microbiology* 52: 97–101.

Hebert, P. D. 1981. "Obligate Asexuality in *Daphnia*." *American Naturalist* 117, no. 5: 784–789.

Hebert, P. D., E. H. Penton, J. M. Burns, D. H. Janzen, and W. Hallwachs. 2004. "Ten Species in One: DNA Barcoding Reveals Cryptic Species in the Neotropical Skipper Butterfly *Astraptes fulgerator*." *Proceedings of the National Academy of Sciences of the United States of America* 101, no. 41: 14812–14817.

Hertig, M. 1936. "The Rickettsia, *Wolbachia pipientis* (Gen. Et sp. n.) and Associated Inclusions of the Mosquito, *Culex pipiens*." *Parasitology* 28, no. 4: 453–486.

Hohenlohe, P. A., S. J. Amish, J. M. Catchen, F. W. Allendorf, and G. Luikart. 2011. "Next-Generation RAD Sequencing Identifies Thousands of SNPs for Assessing Hybridization Between Rainbow and Westslope Cutthroat Trout." *Molecular Ecology Resources* 11: 117–122.

Honeycutt, R. L., and P. Wilkinson. 1989. "Electrophoretic Variation in the Parthenogenetic Grasshopper *Warramaba virgo* and Its Sexual Relatives." *Evolution* 43, no. 5: 1027–1044.

Hughes, R. N. 1989. *Functional Biology of Clonal Animals*. Springer Science & Business Media.

Jaenike, J., and R. K. Selander. 1979. "Evolution and Ecology of Parthenogenesis in Earthworms." *American Zoologist* 19, no. 3: 729–737.

Jaron, K. S., J. Bast, R. W. Nowell, T. R. Ranallo-Benavidez, M. Robinson-Rechavi, and T. Schwander. 2021. "Genomic Features of Parthenogenetic Animals." *Journal of Heredity* 112, no. 1: 19–33.

Johnson, S. G., and W. R. Leefe. 1999. "Evolution and Ecological Correlates of Uniparental Reproduction in Freshwater Snails." *Journal of Evolutionary Biology* 12: 1056–1068.

Jombart, T., S. Devillard, and F. Balloux. 2010. "Discriminant Analysis of Principal Components: A New Method for the Analysis of Genetically Structured Populations." *BMC Genetics* 11: 1–94.

Katoh, K., and D. M. Standley. 2013. "MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability." *Molecular Biology and Evolution* 30, no. 4: 772–780.

Kolde, R., E. A. Franzosa, G. Rahnavard, et al. 2018. "Host Genetic Variation and Its Microbiome Interactions Within the Human Microbiome Project." *Genome Medicine* 10: 1–13.

Kopelman, N. M., J. Mayzel, M. Jakobsson, N. A. Rosenberg, and I. Mayrose. 2015. "Clumpak: A Program for Identifying Clustering Modes and Packaging Population Structure Inferences Across K." *Molecular Ecology Resources* 15, no. 5: 1179–1191.

Kuhn, A., S. Aron, and O. J. Hardy. 2021. "Detection of Cryptic Sex in Automictic Populations: Theoretical Expectations and a Case Study in *Cataglyphis* Desert Ants." *Frontiers in Ecology and Evolution* 9: 688903.

Kuziel, G. A., and S. Rakoff-Nahoum. 2022. "The Gut Microbiome." *Current Biology* 32, no. 6: R257–R264.

Laine, V. N., T. B. Sackton, and M. Meselson. 2022. "Genomic Signature of Sexual Reproduction in the Bdelloid Rotifer *Macrotrachella quadricornifera*." *Genetics* 220, no. 2: iyab221.

Liégeois, M., M. Sartori, and T. Schwander. 2020. "Extremely Widespread Parthenogenesis and a Trade-Off Between Alternative Forms of Reproduction in Mayflies (*Ephemeroptera*)." *Journal of Heredity* 112, no. 1: 45–57.

Louca, S., L. W. Parfrey, and M. Doebeli. 2016. "Decoupling Function and Taxonomy in the Global Ocean Microbiome." *Science* 353, no. 6305: 1272–1277.

Mantovani, B. 1998. "Satellite Sequence Turnover in Parthenogenetic Systems: The Apomictic Triploid Hybrid *Bacillus lynceorum* (*Insecta*, *Phasmatodea*)." *Molecular Biology and Evolution* 15, no. 10: 1288–1297.

Marchán, D. F., C. Csuzdi, T. Decaëns, T. Szederjesi, V. Pizl, and J. Domínguez. 2021. "The Disjunct Distribution of Relict Earthworm Genera Clarifies the Early Historical Biogeography of the Lumbricidae (*Crassiclitellata*, *Annelida*)." *Journal of Zoological Systematics and Evolutionary Research* 59, no. 8: 1703–1717.

Marchán, D. F., R. Fernández, J. Domínguez, D. J. Díaz Cosín, and M. Novo. 2020. "Genome-Informed Integrative Taxonomic Description of Three Cryptic Species in the Earthworm Genus *Carpetania* (Oligochaeta, Hormogastridae)." *Systematics and Biodiversity* 18, no. 3: 203–215.

Martin, M. 2011. "Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads." *EMBnet.journal* 17, no. 1: 10–12.

Martínez-Redondo, G. I., I. Barrios-Núñez, M. Vázquez-Valls, A. M. Rojas, and R. Fernández. 2024. "Illuminating the Functional Landscape of the Dark Proteome Across the Animal Tree of Life Through Natural Language Processing Models." *bioRxiv*. https://doi.org/10.1101/2024-02.

Medina-Sauza, R. M., M. Álvarez-Jiménez, A. Delhal, et al. 2019. "Earthworms Building Up Soil Microbiota: A Review." *Frontiers in Environmental Science* 7: 81.

Medina-Sauza, R. M., I. A. Solís-García, M. Blouin, et al. 2023. "Microniches Harbor Distinct Bacterial Communities at the Soil-Plant-Earthworm Interface." *European Journal of Soil Biology* 118: 103531.

Miraglia, E., F. De Angelis, E. Gazzano, et al. 2011. "Nitric Oxide Stimulates Human Sperm Motility via Activation of the Cyclic GMP/Protein Kinase G Signaling Pathway." *Reproduction* 141, no. 1: 47–54.

Muldal, S. 1952. "The Chromosomes of the Earthworms. I. The Evolution of Polyploidy." *Heredity* 6, no. 1: 55–76.

Neaves, W. B., and P. Baumann. 2011. "Unisexual Reproduction Among Vertebrates." *Trends in Genetics* 27, no. 3: 81–88.

Nechitaylo, T. Y., M. M. Yakimov, M. Godinho, and J. M. Tiedje. 2010. "Effect of the Earthworms *Lumbricus terrestris* and *Aporrectodea caliginosa* on Bacterial Diversity in Soil." *Microbial Ecology* 59, no. 4: 574–587.

Oksanen, J., F. G. Blanchet, R. Kindt, et al. 2013. "Package 'Vegan': Community Ecology Package (Version 2.9)."

Omodeo, P. 1951. "Corredo cromosomico e spermatogenesi aberrante in *Allolobophora caliginosa trapezoides*." *Estratto Dal Bollettino di Zoologia* 18: 27–39.

Omodeo, P. 1952. "Cariologia dei Lumbricidae." *Caryologia* 4: 173–275.

Onteniente, A. P., and C. Rodríguez Babío. 2002. "Three New Species of Earthworm (*Annelida: Oligochaeta: Lumbricidae*) From the Valencian Community, Spain." *Journal of Natural History* 36, no. 5: 515–530.

Parada, A. E., D. M. Needham, and J. A. Fuhrman. 2016. "Every Base Matters: Assessing Small Subunit rRNA Primers for Marine Microbiomes With Mock Communities, Time Series, and Global Field Samples." *Environmental Microbiology* 18, no. 5: 1403–1414.

Perlmutter, J. I., and S. R. Bordenstein. 2020. "Microorganisms in the Reproductive Tissues of Arthropods." *Nature Reviews Microbiology* 18, no. 2: 97–111.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. "Inference of Population Structure Using Multilocus Genotype Data." *Genetics* 155: 945–959.

Qian, H., T. Deng, J. Beck, et al. 2018. "Incomplete Species Lists Derived From Global and Regional Specimen-Record Databases Affect Macroecological Analyses: A Case Study on the Vascular Plants of China." *Journal of Biogeography* 45, no. 12: 2718–2729.

Quast, C., E. Pruesse, P. Yilmaz, et al. 2012. "The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools." *Nucleic Acids Research* 41, no. D1: D590–D596.

Ramachandran, R., and C. D. McDaniel. 2018. "Parthenogenesis in Birds: A Review." *Reproduction* 155, no. 6: R245–R257.

Rispe, C., J. S. Pierre, J. C. Simon, and P. H. Gouyon. 1998. "Models of Sexual and Asexual Coexistence in Aphids Based on Constraints." *Journal of Evolutionary Biology* 11, no. 6: 685–701.

Robinson, M. D., D. J. McCarthy, and G. K. Smyth. 2010. "edgeR: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data." *Bioinformatics* 26, no. 1: 139–140.

Roesti, M., W. Salzburger, and D. Berner. 2012. "Uninformative Polymorphisms Bias Genome Scans for Signatures of Selection." *BMC Evolutionary Biology* 12: 1–7.

Saglio, P., M. Lhospital, D. Lafleche, et al. 1973. "*Spiroplasma citri* Gen. and sp. n.: A mycoplasma-Like Organism Associated With "Stubborn" Disease of citrus." *International Journal of Systematic and Evolutionary Microbiology* 23, no. 3: 191–204.

Sheng, W., W. Xu, J. Ding, et al. 2023. "Guijiajiao (*Colla carapacis et Plastri*, CCP) Prevents Male Infertility via Gut Microbiota Modulation." *Chinese Journal of Natural Medicines* 21, no. 6: 403–410.

Simon, J. C., F. Delmotte, C. Rispe, and T. Crease. 2003. "Phylogenetic Relationships Between Parthenogens and Their Sexual Relatives: The Possible Routes to Parthenogenesis in Animals." *Biological Journal of the Linnean Society* 79, no. 1: 151–163.

Spor, A., O. Koren, and R. Ley. 2011. "Unravelling the Effects of the Environment and Host Genotype on the Gut Microbiome." *Nature Reviews Microbiology* 9, no. 4: 279–290.

Stouthamer, R., J. A. Breeuwer, and G. D. Hurst. 1999. "*Wolbachia pipientis*: Microbial Manipulator of Arthropod Reproduction." *Annual Review of Microbiology* 53, no. 1: 71–102.

Suomalainen, E., A. Saura, and J. Lokki. 1987. *Cytology and Evolution in Parthenogenesis*. CRC Press.

Supek, F., M. Bošnjak, N. Škunca, and T. Šmuc. 2011. "REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms." *PLoS One* 6, no. 7: e21800.

Thakuria, D., O. Schmidt, D. Finan, and D. Egan. 2009. "Gut Wall Bacteria of Earthworms: A Natural Selection Process." *ISME Journal* 4, no. 4: 357–366.

Tomiuk, J., and V. Loeschcke. 1992. "Evolution of Parthenogenesis in the *Otiorhynchus scaber* Complex." *Heredity* 68, no. 5: 391–397.

Turgeon, J., and P. D. N. Hebert. 1995. "Genetic Characterization of Breeding Systems, Ploidy Levels and Species Boundaries in *Cypricercus* (*Ostracoda*)." *Heredity* 75, no. 6: 561–570.

Van der Kooi, C. J., C. Matthey-Doret, and T. Schwander. 2017. "Evolution and Comparative Ecology of Parthenogenesis in Haplodiploid Arthropods." *Evolution Letters* 1, no. 6: 304–316.

Verhulst, E. C., B. A. Pannebakker, and E. Geuverink. 2023. "Variation in Sex Determination Mechanisms May Constrain Parthenogenesis Induction by Endosymbionts in Haplodiploid Systems." *Current Opinion in Insect Science* 56: 101023.

Viruel, J., M. Conejero, O. Hidalgo, et al. 2019. "A Target Capture-Based Method to Estimate Ploidy From Herbarium Specimens." *Frontiers in Plant Science* 10: 937.

Viruel, J., O. Hidalgo, L. Pokorny, et al. 2023. "A Bioinformatic Pipeline to Estimate Ploidy Level From Target Capture Sequence Data Obtained From Herbarium Specimens." In *Plant Cytogenetics and Cytogenomics: Methods and Protocols*, 115–126. Springer US.

Vos, M., A. B. Wolf, S. J. Jennings, and G. A. Kowalchuk. 2013. "Micro-Scale Determinants of Bacterial Diversity in Soil." *FEMS Microbiology Reviews* 37, no. 6: 936–954.

Vrijenhoek, R. C. 1998. "Animal Clones and Diversity: Are Natural Clones Generalists or Specialists?" *Bioscience* 48, no. 8: 617–628.

Wang, Q., B. Liu, X. Li, et al. 2024. "Calcium-Fortified Fresh Milk Ameliorates Postmenopausal Osteoporosis via Regulation of Bone Metabolism and Gut Microbiota in Ovariectomized Rats." *Food Science and Human Wellness* 13, no. 3: 1258–1270.

Weiß, C. L., M. Pais, L. M. Cano, S. Kamoun, and H. A. Burbano. 2018. "nQuire: A Statistical Framework for Ploidy Estimation Using Next-Generation Sequencing." *BMC Bioinformatics* 19: 1–8.

Werren, J. H., L. Baldo, and M. E. Clark. 2008. "*Wolbachia*: Master Manipulators of Invertebrate Biology." *Nature Reviews Microbiology* 6, no. 10: 741–751.

Wu, Y., S. Deng, X. Wang, M. Thunders, J. Qiu, and Y. Li. 2023. "Discovery and Mechanism of Action of a Novel Antimicrobial Peptide From an Earthworm." *Microbiology Spectrum* 11, no. 1: e03206-22.

Yang, J., S. Schrader, and C. C. Tebbe. 2024. "Legacy Effects of Earthworms on Soil Microbial Abundance, Diversity, and Community Dynamics." *Soil Biology and Biochemistry* 190: 109294.

Zchori-Fein, E., and S. J. Perlman. 2004. "Distribution of the Bacterial Symbiont *Cardinium* in Arthropods." *Molecular Ecology* 13, no. 7: 2009–2016.

Zeibich, L., O. Schmidt, and H. L. Drake. 2019. "Fermenters in the Earthworm Gut: Do Transients Matter?" *FEMS Microbiology Ecology* 95, no. 2: fiy221.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.