Mitochondrial genome diversity in dagger and needle nematodes (Nematoda: Longidoridae)

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Dagger and needle nematodes included in the family Longidoridae (viz. Longidorus, Paralongidorus, and Xiphinema) are highly polyphagous plant-parasitic nematodes in wild and cultivated plants and some of them are plant-virus vectors (nepovirus). The mitochondrial (mt) genomes of the dagger and needle nematodes, Xiphinema rivesi, Xiphinema pachtaicum, Longidorus vineacola and Paralongidorus litoralis were sequenced in this study. The four circular mt genomes have an estimated size of 12.6, 12.5, 13.5 and 12.7 kb, respectively. Up to date, the mt genome of X. pachtaicum is the smallest genome found in Nematoda. The four mt genomes contain 12 protein-coding genes (viz. cox1-3, nad1-6, nad4L, atp6 and cob) and two ribosomal RNA genes (rrnL and rrnS), but the atp8 gene was not detected. These mt genomes showed a gene arrangement very different within the Longidoridae species sequenced, with the exception of very closely related species (X. americanum and X. rivesi). The sizes of non-coding regions in the Longidoridae nematodes were very small and were present in a few places in the mt genome. Phylogenetic analysis of all coding genes showed a closer relationship between Longidorus and Paralongidorus and different phylogenetic possibilities for the three Xiphinema species.

The phylum Nematoda is one of the largest and most diverse groups of animal organisms, with a global distribution. Most species are found in oceanic, freshwater and soil ecosystems, and only a small number are pathogens of animals or plants. They cause reductions in agricultural productivity and disease in humans, and animals1. Plant-parasitic nematodes (PPNs) are distributed between Classes Chromadorea and Enoplea in only three orders viz. Rhabditida, Dorylaimida and Triplonchida2. The order Dorylaimida within Enoplea includes several genera of dagger and needle nematodes belonging to the family Longidoridae (viz. Australodorus, Longidoroides, Longidorus, Paralongidorus, Paraxiphidorus, Xiphidorus and Xiphinema)2. Longidoridae nematodes are highly polyphagous on wild and cultivated plants, and some are plant-virus vectors (nepovirus)3. Also some of the Longidoridae species are listed as A1 and A2 quarantine pests by the European and Mediterranean Plant Protection Organisation (EPPO, www.eppo.int/QUARANTINE/).

Mitochondrial (mt) genomes and sequences of individual mt genes are used to infer phylogenetic relationships among species at different taxonomic levels4-7. Animal mtDNAs are relatively constant in gene content and order, maternally inherited, and have a reduced recombination rate and high evolutionary rate8. There are fourteen mt genomes of PPNs sequenced to date, and only one of them was included in the class Enoplea (Xiphinema americanum) and fourteen in the class Chromadorea (Aphelelenchoides besseyi, Bursaphelenchus mucronatus, B. xylophilus, Globodera elliontanae, G. pallida, G. rostochiensis, Heteroderia glycines, Pratylenchus vulnus, Meloidogyne chitwoodi, M. floridensis, M. graminicola, M. hapla, M. incognita, Radopholus similis) and some of them display some unusual features8-21. Xiphinema americanum mt genome encodes genes in both strands and has short coding regions15. On the other hand, within PPN Chromadorean species, Globodera elliontanae, G. pallida and G. rostochiensis have multipartite mt genomes17,18,21, Meloidogyne spp. and Pratylenchus vulnus have a large non-coding region with tandem repeats and the control region19-21 and Radopholus similis has a unique genetic code, and uses the UAA codon for the aminoacid Tyr (Y) instead of a termination site22. Thus, there is a lack of information concerning the mt genomes for other genera within Longidoridae which is needed to derive insights into their taxonomy, phylogeny and possible molecular markers.

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The main objectives of this research were: (i) to determine the mitochondrial genomes in three different genera within important PPNs from Longidoridae (Xiphinema, Longidorus and Paralongidorus); and (ii) to associate their gene structures and protein-coding gene sequences with their phylogeny.

Results and Discussion

General features of the mitochondrial genomes of Longidorus vineacola, Paralongidorus litoralis, Xiphinema pachtaicuim and Xiphinema rivesi. A summary of the mt genomes in Longidoridae is shown in Table 1. The complete mt genomes of P. litoralis, L. vineacola, X. pachtaicum and X. rivesi were 12,763 bp (KU746819), 13,519 bp (KU746818), 12,489 bp (KU746821) and 12,624 bp (KU746820) in size, respectively (Fig. 1 and Table 1). The four new mt genomes showed a similar size and gene number complement to X. americanum (NC_005928) (12,626 bp). They are smaller in size than other Enopleans species such as Romanomermis culicivorax (26,194 bp) or Hexamermis agrotis (24,606 bp). The mt genome of X. pachtaicum represents the smallest mt Nematoda genome known so far (search done in GenBank, November 9, 2016).

The nucleotide composition of the mtDNA genomes studied showed an A + T content similar among dagger nematode species (66.50%, 68.50% and 68.86% for X. americanum, X. rivesi and X. pachtaicum, respectively), but slightly lower in needle nematode species (63.64% and 63.89%, respectively) (Table 1). These levels were lower than that for other members of the class Enoplea such as Romanomermis culicivorax (79.34%) or Hexamermis agrotis (78.42%). The GT-rich sequences in Xiphinema species were 54.00% and 56.63%.
Table 2. Comparison of mitochondrial and rRNA genes within Longidoridae. *Partially overlap a tRNA. "Partially overlap a gene. In same strand sense overlapping. In opposite strand sense overlapping.

(G + T) in one of the strands for X. rivesi (GT rich strand not containing the cox1 gene) and X. pachtaicum (strand containing the cox1 gene), respectively; while, for Longidorus and Paralongidorus these differences were minimal (50.51% and 50.75%, respectively). These differences influence the coding genes, rRNA and tRNA distribution in the genome. In X. rivesi, the GT rich strand had sense sequences of 8 protein coding genes (PCGs), 12 tRNAs and the 2 rRNA genes whereas for X. pachtaicum 10 PCGs, 12 tRNAs and the 2 rRNA genes were detected. These differences were minimal for Longidorus and Paralongidorus between AC-rich vs GT-rich strands with 6 vs 6 PCGs, 10 vs 11 tRNAs and 1 rRNA gene in each of the strands in the case of P. littoralis (GT-rich strand containing the cox1 gene) and 5 vs 7 PCGs proteins, 14 vs 8 tRNAs, and the 2 vs 0 rRNA genes in the case of L. vineacola (GT-rich strand containing the cox1 gene).

The mtDNA genomes of L. vineacola, P. littoralis, X. pachtaicum and X. rivesi contained 12 PCGs, viz. cox1-3, nad1-6, nad4L, atp6 and cob; and two ribosomal RNA genes (rrnL and rrnS) but the atp8 gene was not detected (Fig. 1; Table 2). The gene arrangement within Longidoridae was very different within dagger and needle nematode species (Fig. 1), with the exception of X. americanum and X. rivesi, in which it was identical. This is in concordance with the very high degree of variation in mtDNA genome gene arrangements across the Metazoa5. A comparison of closely related species with different gene orders suggests that there are several types of “elementary” rearrangement events5: inversions, transpositions, inverse transpositions (i.e. a transposition in which the re-inserted fragment is inverted), and tandem duplications followed by the random loss of one of the copied genes.

Protein encoding genes and codon usage. Protein encoding genes were transcribed from both strands in the four mt genomes sequenced. Genes nad4L and nad3 were always together and separated by a non-coding region in all the studied species (Fig. 1). The gene order of two genes in nad5-nad6, atp6-nad4 and nad1-cox1 were conserved between X. americanum/X. rivesi vs X. pachtaicum. On the other hand, the gene order between Longidorus and Paralongidorus species was kept in two regions: cob-nad4L-nad3-cox1 and cox2-cox3-nad2. Only the gene associations of nad5-nad6 (inverted gene sense in L. vineacola) and atp6-nad4 were kept between Paralongidorus and Xiphinema species. We could not find coincidences for arrangement of PCGs between the genus Longidorus and Xiphinema. For instance, the association between nad5-nad6 and atp6-nad4 seem derived from an inversion between Longidorus and Paralongidorus.

All PCGs shared an ATA start codon. The PCGs all terminated with a potential TAA stop codon with the exception of cox3 and nad3 for X. rivesi, cox1, cox3, nad4, nad4L, nad6 and cob for X. pachtaicum, and nad1 and nad3 for P. littoralis (Table 2). Some genes in all the nematode species studied partially overlapped and probably terminated with T/TA (Table 2). These features were not conserved in these genes among the studied species. Only the cox2 termination codon was conserved in X. americanum, X. rivesi, X. pachtaicum and L. vineacola. However, they were partially conserved between X. americanum and X. rivesi for cox1 and atp6 genes. Additionally to these termination codons, some genes overlap several tRNA codons and a few bases (1 or 2) with other genes (Table 2). Coding gene overlapping seems to be a common feature in Longidoridae, since the five sequenced species showed this feature in their genomes. Overlaps were detected in the same or in the opposite direction. However, gene overlap in the same sense strand was not detected in P. littoralis. In X. americanum, X. rivesi and X. pachtaicum a 1 bp overlap was detected between nad2 and cox2 in the same sense strand, X. pachtaicum has a 4 bp overlap between nad4 and cox3, and L. vineacola has a 1 bp overlap between cox2 and cox3 in the same sense strand in both cases. In the case of overlapping coding genes in humans (atp8/atp6 and nad4L/nad4) both
Table 3. Codon usage of 12 protein coding genes of *Xiphinema americanum* (Xa), *Xiphinema rivesi* (Xr), *Xiphinema pachtaicum* (Xp), *Paralongidorus litoralis* and *Longidorus vineacola* (Lv) mtDNAs.

| AA | Codon | No. | % | Xa | Xr | Xp | Pl | Lv | No. | % |
|----|-------|-----|---|----|----|----|----|----|-----|---|
| Ala | GCG | 17 | 0.33 | 0.31 | 0.57 | 0.51 | Lys | AAG | 22 | 0.95 | 0.95 | 0.85 | 0.66 |
| Ala | GCA | 17 | 0.95 | 0.55 | 0.88 | 1.46 | Lys | AAA | 78 | 1.37 | 1.49 | 1.87 | 1.81 | 1.85 |
| Ala | GCC | 17 | 2.05 | 0.18 | 1.84 | 2.21 | Met | ATG | 48 | 1.91 | 1.40 | 1.65 | 1.57 | 1.43 |
| Arg | CGG | 8 | 0.53 | 0.89 | 0.12 | 0.24 | Phe | TTC | 32 | 2.44 | 2.33 | 1.53 | 1.96 | 2.95 |
| Arg | CGA | 8 | 0.04 | 0.27 | 0.02 | 0.06 | Pro | CCG | 19 | 0.66 | 0.48 | 0.21 | 0.21 | 0.21 |
| Arg | CGC | 8 | 0.47 | 0.12 | 0.94 | 0.49 | Ser | AGG | 56 | 1.82 | 1.06 | 1.02 | 1.02 | 1.02 |
| Cys | TGC | 20 | 0.01 | 0.10 | 0.76 | 0.49 | Ser | AGC | 29 | 1.00 | 0.93 | 0.91 | 0.91 | 0.91 |
| Gly | GGC | 20 | 1.10 | 1.16 | 1.80 | 2.32 | Thr | AGG | 56 | 0.67 | 0.69 | 0.70 | 0.70 | 0.70 |
| Gly | GGG | 20 | 1.07 | 1.00 | 1.00 | 1.00 | Tyr | CAT | 34 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Gly | GGA | 20 | 0.50 | 0.69 | 0.50 | 0.50 | Val | GTG | 48 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Gly | GGT | 20 | 1.04 | 1.04 | 1.04 | 1.04 | Val | GGT | 121 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| His | GAG | 20 | 1.04 | 1.04 | 1.04 | 1.04 | Thr | AGC | 29 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| His | GAT | 20 | 0.50 | 0.50 | 0.50 | 0.50 | Thr | ACT | 120 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Ile | ATC | 40 | 1.13 | 1.19 | 1.19 | 1.13 | Trp | TGG | 34 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Ile | ATT | 40 | 0.57 | 0.70 | 0.79 | 0.74 | Trp | TGA | 70 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Ile | ATG | 40 | 5.54 | 6.14 | 6.14 | 6.14 | Tyr | CAT | 71 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Leu | TTG | 25 | 1.49 | 1.30 | 1.60 | 2.03 | Tyr | TAC | 25 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Leu | TTA | 25 | 0.10 | 0.14 | 0.22 | 0.35 | Val | GTG | 54 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Leu | CTG | 25 | 0.86 | 0.76 | 0.65 | 0.10 | Val | GTT | 98 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Leu | CTA | 75 | 2.95 | 2.74 | 2.29 | 2.48 | Val | GTG | 23 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Leu | TTC | 75 | 2.95 | 2.74 | 2.29 | 2.48 | Val | GTC | 23 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |

**Note:** As in other published nematode mtDNAs, the mtPCGs of our sequenced species were not biased toward using amino acids encoded by T-rich codons. The higher frequency of amino acids encoded by T-rich codons, and unequal synonymous codon usage with bias against C-rich codons is consistent with the high percentage of A + T content in the nucleotide composition of PCGs as in other mt genomes for nematodes.23.
Table 4. Secondary structures of predicted mt-RNAs in selected Enoplea species. Up-dated with the species sequenced in this study from Jühling et al.27. The first row enumerates all 20 amino acids (A = alanine; C = cysteine; D = aspartic acid; E = glutamic acid; F = phenylalanine; G = glycine; H = histidine; I = isoleucine; K = lysine; L1 = leucine 1; L2 = leucine 2; M = methionine; N = asparagine; P = proline; Q = glutamine; R = arginine; S1 = serine 1; S2 = serine 2; T = threonine; V = valine; W = tryptophan; Y = tyrosine. As mitochondrial genomes encode two distinct tRNA\(^{\text{Lys}}\) and tRNA\(^{\text{Ser}}\) genes, both are listed twice as L1/L2 and S1/S2, respectively. Typical nematode tRNAs with a D-arm but no T-arm are indicated by (\(\cdot\)), if they retained their T-arm and lack the D-arm are indicated by (\(\cdot\)). Structures lacking both the T-arm and the D-arm are denoted by (\(\cdot\)). The intact clover-leaf structures are shown as (\(\cdot\)). nf: not found.

Transfer RNA (tRNA) and ribosomal RNA genes. The mt genome of metazoans typically encodes 22 tRNAs. However, only L. vineacola should show the typical 22 tRNAs, whereas 21 tRNAs were identified in X. rivesi and in P. litoralis in which we could not identify the mt-tRNA\(^{\text{Lys}}\) gene, and 19 tRNAs were identified in X. pachtaicum in which we could not identify the mt-tRNA\(^{\text{Lys}}\), mt-tRNA\(^{\text{A}}\) and mt-tRNA\(^{\text{B}}\) genes. The position predicted for mt-tRNA\(^{\text{B}}\) in mt genomes of X. rivesi and P. litoralis based on sequence similarity in the genes predicted by Jühling et al. was deep inside the l-rRNA annotation and in the same sense strand27. The annotation of our l-rRNA was based on similarity to sequences deposited in GenBank and mainly with the X. americanum l-rRNA annotation done by He et al. using mRNA sequencing8. The same situation was also found in X. pachtaicum for mt-tRNA\(^{\text{A}}\) where additionally, the other two mt-RNAs, mt-tRNA\(^{\text{B}}\) and mt-tRNA\(^{\text{B}}\), were not found. These tRNAs would therefore need to be imported from the nucleus, implying that a mechanism of this sort exists in nematodes. Import of tRNA from the nucleus to the mitochondrion has been demonstrated in marsupials28 and in a protozoan (Trypanosoma brucei)29. Another possibility is that the tRNA detection methods used in this study (Mitos-MITFI and Arwen v1.2) could not identify them.

The tRNA structures detected in the four studied mtDNA genomes shared some features with those of other metazoans30 including a 5 bp anticodon stem, a 7 base anticonodon loop with a T always preceding an anticondon as well as a purine always following an anticondon (Figs S1–S3). Four secondary structures have been found in Longidoridae (Table 4; Figs S1–S3): (i) typical nematode tRNAs structure with a D-arm but no T-arm; (ii) structure retaining their T-arm and lacking the D-arm; (iii) structures lacking both the T-arm and the D-arm; and (iv) the intact clover-leaf structure is also present. The conventional cloverleaf structure it is also present in the mt-tRNAs of Trichuris ovis, Trichuris discolor30 and Trichinella spiralis31. All of the tRNAs with a clover-leaf structure found in the species included in this study were coincident with those found in T. spiralis and only the tRNAs which showed a clover-leaf structure in X. rivesi, does not appear with this secondary structure in T. spiralis. This diversity of structures in our mt tRNAs support the hypothesis expressed by Jühling et al., that mt-tRNA not only evolve rapidly at the sequence level but also exhibit a variety of deviations from the common clover-leaf structure32.

Non-coding regions. The sizes of the non-coding regions in Longidoridae nematodes in this study were very small and in few places in the mt genome (Table 5). In the mt genome of X. americanum, the longest non-coding region was just 96 bp in length between the nad3 and the nad4L genes with an A + T content of 72%, and inverted or direct repeats were not present32. A sequence motif (5’-GAGACCTGAGCCCAAGATA-3’) was present in this 96-bp noncoding region for X. americanum32 that was similar to the conserved promoter element sequence (5’-CA(G)ACC(G)CC(A)AAAGATA-3’) around the transcription start site in the D-loop region of the human mt genome33. We could not find a clear similarity in our non-coding sequences to this promoter element sequence. However, the position of a non-coding region and a stable gene arrangement between

| Organism                 | A | C | D | E | F | G | H | I | K | L1 | L2 | M | N | P | Q | R | S1 | S2 | T | V | W | Y |
|-------------------------|---|---|---|---|---|---|---|---|---|----|----|---|---|---|---|---|----|----|---|---|---|---|
| Dorylaimida             |   |   |   |   |+ |   |   |   |   |    |    |   |   |   |+ |   |   |   |   |   |+ |
| Xiphinema americanum    |   |+ |+ |+ |+ |+ |+ |+ |+ |    |    |   |   |   |+ |+ |   |   |   |   |   |+ |
| Xiphinema rivesi        |   |+ |   |+ |  |  |+ |  |+ |    |    |   |   |   |+ |  |   |+ |   |   |   |+ |
| Xiphinema pachtaicum    | nf |+ |+ |+ |   |+ |    |    |   |    |+ |    |    |+ |    |    |    |   |   |   |   |   |
| Paralongidorus litoralis|   |+ |+ |+ |   |+ |   |   |+ |+ |    |+ |    |    |   |+ |    |+ |   |+ |   |+ |
| Longidorus vineacola    |   |+ |+ |   |   |+ |+ |   |   |+ |    |    |+ |    |    |+ |    |+ |   |   |+ |   |+ |
| Mermithida              |   |+ |+ |+ |+ |   |+ |   |+ |   |   |   |+ |+ |+ |   |   |+ |   |+ |   |   |+ |
| Agameris sp BH-2006     |   |+ |+ |+ |+ |   |   |   |   |+ |    |    |   |+ |   |+ |   |   |   |   |   |+ |
| Hexameris agrotis       |   |+ |+ |+ |+ |   |   |   |   |   |+ |    |    |+ |    |+ |   |+ |   |   |   |+ |
| Romanomermis calcivorax |   |+ |   |+ |+ |    |   |+ |+ |   |   |    |    |   |   |+ |+ |+ |   |   |   |+ |
| Romanomermis iyengari   |   |+ |+ |   |+ |+ |   |   |+ |   |   |    |    |+ |    |   |   |+ |+ |   |   |+ |
| Romanomermis nielseni    |   |+ |+ |+ |+ |    |   |   |+ |   |   |    |+ |   |   |+ |   |   |   |   |+ |   |+ |
| Strikelaimermis spiculatus|   |+ |   |+ |   |+ |+ |   |   |+ |   |   |   |+ |+ |   |+ |   |   |   |+ |
| Thauamermis coevrsei     |   |+ |   |+ |+ |   |+ |   |+ |   |   |   |   |   |+ |+ |+ |   |   |   |   |+ |
| Trichocephalida          |   |+ |   |+ |   |   |   |   |   |   |   |   |   |   |   |+ |+ |   |   |+ |   |   |+ |
| Trichinella spiralis     |   |+ |+ |   |+ |   |   |   |   |   |   |   |   |+ |   |+ |   |   |   |   |+ |

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Within all the studied Longidoridae nematodes pointed out the importance of this region in the viability of the nematode mitochondria. This feature, the strong secondary structure (Figs 2 and S4) and the location of the Control Region (CR) for \textit{X. americanum} were the basis for annotating this sequence as CR. The CRs were of different size and composition in comparison to the A+T rich sequence found in \textit{X. americanum}.

| Position | size | Genes/tRNA | Position | size | Genes/tRNA | Position | size | Genes/tRNA | position | size | Genes/tRNA |
|----------|------|------------|----------|------|------------|----------|------|------------|----------|------|------------|
| 3943–4038* | 96  | \textit{nad4L-nad3} (+) | 8396–8433 | 37  | \textit{Pro-Gln} (−) | 114–148 | 34  | \textit{Val-atp6} (+) | 2839–3204 | 365 | Ser2-Asn (+) |
| 12437–12472 | 36  | \textit{Pro-Gln} (−) | 12569–12624* | 96  | \textit{nad4L-nad3} (+) | 821–858 | 37  | \textit{Gly-Tyr} (+/−) | 3261–3391 | 130 | Asn-atp6 (+) |
| 12529–12575 | 47  | \textit{Gln-Phe} (−) | 2887–2928 | 41  | \textit{Gln-Pro} (+) | 4140–4263 | 123 | \textit{Phe-Ala} (+/−) | 7040–7136 | 96  | \textit{nad4-Ser1} (−) |
| 7254–7393* | 140 | \textit{Asp-nad1} (−/+ +) | 8314–8354 | 40  | \textit{Pro-Gln} (−) | 9824–9860 | 36  | \textit{nadZ-nad6} (−) |
| 9235–9274 | 39  | \textit{nad5-nad6} | 11455–11511 | 56  | \textit{Lys-cob} (−/+) | 9807–9848 | 41  | \textit{Ile-coxII} (+) | 12929–13021* | 93  | \textit{nad4L-nad3} (−) |

Table 5. Non-coding regions longer than 30 bp found in the Longidoridae mitochondrial genomes.

*Annotation position starts from \textit{atp6}. In brackets is shown the sense in which the fragment is inserted: (+) sense strand; (−) antisense strand; (+/−) starts in sense and finished in antisense; (−/+ +) starts with antisense and finished with sense strand. CR: Replication Control Region.

\textit{nad4l} within all the studied Longidoridae nematodes pointed out the importance of this region in the viability of the nematode mitochondria. This feature, the strong secondary structure (Figs 2 and S4) and the location of the Control Region (CR) for \textit{X. americanum} were the basis for annotating this sequence as CR. The CRs were of different size and composition in comparison to the A + T rich sequence found in \textit{X. americanum}. Sizes of...
Mitochondrial phylogeny of Enoplea nematodes. We conducted BI and ML phylogenetic analyses of an amino acid sequence dataset (13 protein-coding genes) for 25 nematode species. Phylogenetic analysis separated four different clades and subclades including Trichinellidae, Trichuridae, Mermithidae and Longidoridae (Figs 3 and 4). In our analysis, Trichinellidae and Trichuridae formed a well-supported group which is sister to Mermithidae, and all these three groups formed a clade weakly supported in both analysis (BI and ML). This phylogenetic position is mainly consistent with recent reports based on mt genome analysis\(^{11,35}\), but those were only based on one sequence for Longidoridae (viz. *X. rivesi*). Meanwhile, Longidoridae clade, with 5 representatives in our analysis, was a sister clade with the other groups in the Bayesian inference (BI) and Maximum likelihood (ML) analyses in the amino acid dataset (Figs 3 and 4). In Longidoridae, two well-supported subclades were formed, one of them with *Paralongidorus*, *L. vineacola* and *X. rivesi* and the other with *P. litoralis* and *X. americanum*. A different position has strong effects on replication and transcription. Lewis et al. found that mtDNA synthesis in the *C. elegans* gonad produces branched-circular lariat structures with multimeric DNA tails; they were able to detect multimers up to four mtDNA genome unit lengths\(^{34}\). These authors have raised the possibility that the rolling circle mtDNA replication mechanism may be an ancestral trait among metazoa. However, *C. elegans* has two well-defined non-coding regions and with our data only one was detected and in a similar position in the studied Longidoridae species. Many species of Arthropoda, Nematoda, Mollusca and Annelida harbor palindromes and inverted repeats in their CRs\(^{35}\). Length of the CR in the nematode mtDNA genome could be variable because of the presence of repeated sequences\(^{22}\). We only found a partially conserved palindrome sequence in the CR of *P. littoralis* and *X. pachtaicum* applying the procedure explained in Arunkumar and Nagaraju\(^{35}\). It is possible that these regions function as splicing recognition sites during processing of the transcripts. Mitochondrial genomes in Longidoridae have the important trait of genome size economy and in having fewer non-coding regions. These features were retained even with the important differences in gene arrangements between the studied sequences.
related phylogenetically and this is shown with the strong support of their relationship in both analyses (100% bootstrap support (BS) and 1.00 BPP), while the position of *X. pachtaicum* was well resolved in this clade by BI (1.00 BPP), but not by ML (65% BS). Nucleotide phylogenetic analysis showed a similar pattern of well-supported phylogenetic clades as in the aminoacid dataset forming two sister clades, one with Trichinellidae and Trichuridae and another with Mermithidae and Longidoridae in BI and ML analysis (Figs S7 and S8). However, this phylogenetic position with Longidoridae as a sister clade to Mermithidae was well-supported in BI and weakly supported in ML analysis. Longidoridae clade was formed by two subclades, one for *Paralongidorus* and *Longidorus* and the other for *Xiphinema* species. This clade was strongly supported in the BI analysis and weakly supported in the ML analysis. *Xiphinema americanum* and *X. rivesi* were closely related phylogenetically as in the case of amino acid dataset (100% BS and 1.00 BPP). This different position of the Longidoridae clade was shown by other researchers with only one sequence from Longidoridae (*X. americanum*)11,36. The results of Kim et al.36 were similar to our phylogenetic analysis using a BI approach and using amino acid and nucleotides datasets, while the phylogenetic approach used by Humphreys-Pereira and Elling11 was similar in the amino acid dataset and different in the nucleotide dataset excluding the third codon position. In order to assess whether the phylogenetic relationships recovered by the mt genome sequences were influenced by the use of an entirely maternal marker, we assessed the phylogeny of the Enoplea using an available nuclear marker, partial 18S rRNA. Enoplea partial 18S (Fig. S9) showed two main well-supported clades (100% BPP) for Enoplia and Dorylaimia. Longidoridae species were closely related phylogenetically and forming a well-supported clade (100% BPP) with Nordiidae, Qudsianematidae, Dorylaimidae and Aporcelaimidae. However, no member of these families has a complete mt genome currently available. The Longidoridae clade was formed by two subclades, the superior subclade is well-supported (BPP = 0.99) comprising four different genera, *Longidorus*, *Paralongidorus*, *Xiphinema americanum* group and *Xiphidorus*. *Longidorus* species were phylogenetically related with *Paralongidorus* species forming a well-supported clade (BPP = 1.00) and the *Xiphinema americanum* group which formed a sister-clade with *Xiphidorus*, however the BPP values for this sister-clade is moderately supported (BPP = 0.95). The second subclade is well-supported (BI = 1.00) and was formed by *Xiphinema* non-*americanum* group species. The relationships between these three groups were not well-defined in these analyses or in other phylogenetic analysis using other phylogenetic markers with more Longidoridae species57. Unfortunately, we could not obtain a complete mt genome sequence for a *Xiphinema* non-*americanum* group species, so this point could not be resolved. Taking into account the sequence evolution in both clades (*Longidorus-Paralongidorus*) and (*Xiphinema-Xiphidorus*) neither of these clades seems more ancient than the others. But, clearly there are parallels in gene arrangements, phylogenetic relationships and non-coding regions between these three groups and *Longidorus* and *Paralongidorus*. Additionally, *L. vineacola* has the longest non-coding regions followed by *P. litoralis*. For this reason, we hypothesized a less evolved mt genome or different selection pressure in the evolution of these species. Another observation found in recent phylogenetic analysis, is the extreme diversity of some regions (cox1 gene) with *Longidorus orientalis* showing incongruence of phylogenies inferred from ITS1 rRNA and cox1 genes36 while other species show low differences within the populations sampled (i.e. *X. pachtaicum* and *X. index*)56. The high variation observed in the cox1 priming sites in *L. orientalis* can adversely affect the certainty of the nematode identification by barcoding36 and thus integrative taxonomical approaches are needed for an accurate identification of these and other plant-parasitic nematodes40-42. Our complete mt sequences for *Xiphinema, Longidorus* and *Paralongidorus* genera could help in resolving these problems by comparing sequences for primer design.

While mapping the gene arrangement with the species in this study, we found an important variability in Enoplea (Figs 3 and 4). Some genera such as *Trichinella* and *Trichuris* were homogenous with their PCG.
Xiphinema pachtaicum
IAS
Córdoba, Córdoba province, Spain
37°51’37.28”N, −4°17’3.27”W
cultivated olive
5′-GGACACGGAAATATTATGTTGCG-3′
5′-GAGGATTAACCTGGATTTTATT-3′
KU746821

Xiphinema rivesi
Isla
Castillo de Lociubin, Jaén province, Spain
37°32’36.49”N, −5°37’22.58”W
cherry tree
5′-GTTTACGCCGAAATCATAGACAG-3′
5′-TTAGCTTCTTTTAGGGGAGAGG-3′
KU746820

Longidorus vineacola
AR31
Tarifa, Cádiz province, Spain
36°03’49.5”N, −5°40’18.2”W
wild olive
5′-GCTGTCTTCATACGACAAATCTCG-3′
5′-TCTCAACAGGATTAAGTTTGA-3′
KU746818

Paralongidorus litoralis
Zahara
Zahara de los Atunes, Cádiz province, Spain
36°06’27.79”N, −5°49’32.94”W
Pistacia lentiscus L.
5′-TTTAAAGGTACCATCGCTTTG-3′
5′-AATGCGCTACCTTTCCCTACT-3′
KU746819

Table 6. Longidoridae nematodes studied for their mitochondrial genome. Species identifications were based on morphology and barcoding using D2-D3 expansion segments of 28S rDNA. *For species identification see37,39,44,45.

arrangements, however, the genus Romanomeris showed an important variability in its arrangement in a similar way to Longidoridae species. We could not find similarities in the PCG arrangement between the species studied, with the exception of very closely related species (X. americanum and X. rivesi).

Material and Methods
Samples and nematode extraction. Soil samples from which nematodes were extracted were collected in 2015 in Spain from several crops and wild habitats (Table 6). Soil samples were collected with a shovel discarding the upper 5-cm topsoil profile, from a depth of 5- to 40-cm, in the close vicinity of active roots. Nematodes from the soil were extracted from a 500-cm³ sub-sample using the magnesium sulphate centrifugal-flotation method43. Xiphinema pachtaicum, X. rivesi, L. vineacola, and P. litoralis were identified using integrative taxonomy as described in previous studies37,42,44,45. Only live and individual nematodes were used for DNA extraction. No pure populations were multiplied in pots in greenhouse and nematodes were extracted from original sampling points.

Mitochondrial DNA extraction and amplification. For the molecular analyses, in order to avoid complications from mixed populations in the same sample, at least two live nematodes from each sample were temporarily mounted in a drop of 1 M NaCl containing glass beads (to avoid crushing the nematode) and diagnostic morphological characters were observed and measurements were taken to confirm the species identity. The slides were then dismantled and DNA extracted. Nematode DNA was extracted from single individuals and PCR assays were conducted as described by Subbotin et al.38. A portion of the cox1 gene was amplified as described by Lazarova et al. using primers COIF (5′-GATTGGTGGAAAGCGG-3′) and COIR (5′-GATGGTGGAAAGCGGCGG-3′) and PCR cycling conditions as described by He et al.32. PCR products were purified using ExoSAP-IT (Affymetrix, USB products) and used for direct sequencing in both directions. The resulting products were run on a DNA multipilayer sequencer (Model 3130XL genetic analyzer; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA), at the Stab Vida sequencing facilities (Caparica, Portugal).

For all mt DNA amplification, the DNA extraction protocol was similar to that described in Subbotin et al.38 with the exception that several live nematodes previously identified under microscope were used for each extraction and the proteinase K digestion was performed at 50°C. Primers were designed using the cox1 sequences for each species. The primer design was performed using Primer346 (Table 6) with the correct sense for long-range PCR which was carried out using Advantage® 2 PCR Kit (Clontech, Takara Biotechnology, Japan). Each reaction contained 0.3 μM primer, 1X BD Advantage 2 PCR buffer and 2 μl of DNA nematode extraction in a final PCR volume of 25μl. Long-range PCR conditions were as follows: initial denaturation at 94°C for 3 min followed by 40 cycles of 94°C for 15 s, annealing between 55 and 57°C depending on the primer (Table 6) for 30 s and extension at 68°C for 1 min during the first 10 cycles and after 15 min + 15 s/cycle. The product was visualized using an agarose gel; gel purified using Cut & Spin (Griip, Portugal) and quantified using a Nanodrop spectrophotometer.

Ion-torrent Sequencing and read processing. Ion-torrent sequencing platform was performed at Stab Vida sequencing facilities (Caparica, Portugal). The 200–300 bp insert size library was constructed using enzymatic fragmentation of amplified DNA, Ion-torrent specific adapter ligation, size selection and amplification. The concentration and size distribution of library DNA fragments was determined using Qubit® fluorometer 2.0 (Invitrogen) and Bioanalyzer 2100 (Agilent Technologies). The numbers of reads obtained were 351,698; 665,110; 339,777; and 596,021 for X. rivesi, X. pachtaicum, L. vineacola and P. litoralis, respectively. Raw data obtained were analyzed and trimmed using CLC Genomics Workbench 7.5.1 (Qiagen) following standard procedures described by the manufacturer in Stab Vida facilities.

Mitochondrial genome assembly and annotation. Filtered data was de novo assembled using CLC Genomics Workbench 7.5.1 (Qiagen). Prediction of protein-coding genes and rRNA genes was done by using...
a combination of BLAST searches in Artemis v. 16.0.0\textsuperscript{49}, and MITOS online software\textsuperscript{50}. Putative tRNA (transfer RNA) genes were identified in the MITOS online software\textsuperscript{50}, which uses the strategy presented in Jühling \textit{et al.}\textsuperscript{27}. Additionally, these tRNA predictions were checked using the program Arwens v1.2\textsuperscript{12}. The assembled genomes were annotated using Artemis v. 16.0.0\textsuperscript{49}. Annotated sequences were submitted to GenBank with the accession numbers KU746820, KU746821, KU746818 and KU746819 for \textit{X. rivesi}, \textit{X. pachtaicum}, \textit{L. vineacola} and \textit{P. litoralis}, respectively. Codon usage was studied on-line using the server \url{http://www.bioinformatics.org/sms2/codon_usage.html}.

**Phylogenetic analyses.**

Phylogenetic analyses were performed on amino acid (AA) and nucleotide data sets and the nuclear partial 18S rRNA. The newly obtained and published sequences for mt coding genes in complete Enoplea mt genomes were used for phylogenetic reconstruction. \textit{Lithobius forficatus} (NC002629) and \textit{Limulus polyphemus} (NC003057) were used as outgroups, according to previous studies\textsuperscript{11,36}. For multiple alignments of AA sequences, the nucleotide sequences of each of the protein coding genes (PCG) were initially translated into AA with MEGAl\textsuperscript{62} using the invertebrate mt genetic code setting. The amino acid sequences for each PCG were aligned individually using ClustaW\textsuperscript{53}, implemented in MEGA6 under default settings. Conserved regions in the alignments of the 13 PCGs were selected using the Gblocks v 0.91b server set at the “less stringent” parameters. Each individual gene alignment was tested for the best-fit substitution model using ProtTest 2.4\textsuperscript{55} based on the Akaike information criterion (AIC). All AA alignments of the 13 PCGs were concatenated into a single alignment using Mesquite v3.04\textsuperscript{66}. The final alignment included 2468 out of 3926 AA, representing 63% of the original sequence alignment. Similarly, aligned nucleotide sequences from the PCG using the aligned amino acid sequences were trimmed, concatenated in a similar procedure as for AA dataset. The best fitted model of DNA evolution for each individual gene alignment was obtained using \textit{jModelTest} v. 2.1.7\textsuperscript{57} with AIC. Model used in both datasets are shown in Table S1. Selected models from the available programs in MrBayes 3.1.2 and RAxML 8.2.2 which best fit our dataset from the ranked models in Protest 3.2 and jModelTest 2.1.7. were used in the phylogenetic analysis.

The partial 18S rRNA data set consisted of 88 Enoplea sequences from GenBank and was 1666 bp in length, and comprised representatives of the main families. \textit{Lithobius forficatus} (EU024571) and \textit{Limulus polyphemus} (HQ588741). Sequences for partial 18S were aligned using MAFFT v. 7.205\textsuperscript{58}. Conserved regions in the alignments of the 13 PCGs were trimmed, concatenated in a similar procedure as for AA dataset. The best fitted model of DNA evolution was obtained using \textit{jModelTest} v. 2.1.7\textsuperscript{57} with AIC.

Phylogenetic analyses of the AA data sets were performed based on maximum likelihood using the rapid bootstrap algorithm in RAxML v. 8.2.2\textsuperscript{59} with 200 bootstrap replicates. BI was performed using MrBayes 3.1.2\textsuperscript{60}. In both cases, the models were included in the analysis. BI was performed including the model in a partition setting with the model for each PCG and for nucleotides and was run under a general time reversible of invariant sites and a gamma-shaped distribution (GTR+I+\text{G}) model with four chains for 1 × 10\textsuperscript{6} generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority rule consensus tree. Trees were visualized using TreeView\textsuperscript{61} and FigTree v1.4.2 (\url{http://tree.bio.ed.ac.uk/software/figtree/}).

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