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Short communication

Structural and evolutionary analysis of the ribosomal genes of the parasitic nematode *Meloidogyne artiellia* suggests its ancient origin☆

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Root-knot nematodes belonging to the genus *Meloidogyne* constitute one of the major groups of plant pathogens of outstanding economic importance. They are facultative or obligate parthenogens, so a single individual can establish a population, a useful attribute in species that exploits disturbed ecosystems such as the agro-ecosystem [1]. The group of obligatorily mitotic parthenogenetic species, (apomictic species), includes three of the agronomically most important species *Meloidogyne incognita*, *M. javanica* and *M. arenaria*. Another *Meloidogyne* species characterized by extensive geographical distribution and host range is *M. hapla* in which both mitotic and meiotic parthenogenetic races have been reported [1]. Finally *M. artiellia*, and *M. chitwoodi* are reported as facultatively sexual species [2,3]. Recently, a number of laboratories have undertaken molecular studies of root knot nematode ribosomal genes both to elucidate their phylogenetic relationships and to develop DNA-based methods for their identification [4,5].

Ribosomal genes have long been recognized as a useful tool for studies on molecular evolution and phylogenetic inferences [6]. In nematodes, the 18S, 5.8S and 26S genes for rRNA appear in multiple copies organized in tandem arrays separated by non transcribed spacers, as well as in other eukaryotes. The three rRNA genes are separated by a spacer called ITS1, (internal transcribed spacer 1, located between the 18S and the 5.8S gene), and by another spacer called ITS2, (internal transcribed spacer 2, between the 5.8 and the 26S gene). The IGS (intergenic spacer) separates the different repeating units. The coding regions are highly conserved even between distantly related species. As a matter of fact, the sequences of the small subunit of rRNA genes from different taxa sampled across the entire *nematoda* phylum has provided the most comprehensive molecular phylogeny of nematodes [7]. On the other hand, the ITS spacers show genetic variability both between and even within species [8,9].

Another ribosomal gene, a ubiquitous component of large ribosomal subunits in eukaryotic cells, is 5S ribosomal RNA. The gene encoding 5S rRNA is highly conserved both in size and in sequence but shows different localization in different eukaryotic groups and in general it is not linked to rRNA repeating units. Surprisingly, the localization of 5S rRNA linked to ribosomal gene repeated units has been described in the three apomictic species of *Meloidogyne*, *M. arenaria*, *M. javanica*, *M. incognita* as well as in *M. hapla* and *M. mayaguensis*, within the Tylenchida order [10,11]. On the other hand, the 5S rRNA gene is linked to the trans-

Abbreviations: AN, accession number; IGS, Intergenic Spacer; ITS, Internal transcribed spacer; ML, maximum likelihood; GTR, general time reversible; TSL, *Trans*-spliced leader.

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™. The nucleotide sequence of all the ribosomal DNA genes and spacers of *Meloidogyne artiellia* (8274 bp) under the accession number AF248477; that of 5S RNA and spacers under the accession number AF248479; and that of the ITS1, 5.8S RNA and ITS2 from *M. artiellia* from Syria under the accession number AF248478.

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spliced leader repeated (TSL) unit in *Caenorhabditis elegans*, and in other Rhabditida spp. [12,13]. In another Tylenchida species (the plant parasitic *Globodera pallida*) 5S rRNA is linked to the TSL as in *C. elegans*, but on the opposite strand [12]. The TSL genes code for the 22 nt long primary transcript which is trans-spliced onto a variety of mRNAs [13,14].

From a genomic library of the plant parasitic nematode *M. artiellia* we have isolated a clone spanning the entire ribosomal repeated unit, the intergenic spacers and portions of the flanking repeated units. In this fragment, the gene for 5S rRNA was not found. Therefore, in order to study the localization of the 5S rRNA gene on *M. artiellia* genome, we decided to use PCR amplification on DNA from *M. artiellia*, by using primers designed on the conserved part of 5S rRNA and on the 22 nt trans-spliced sequences, on both orientations. The results reported in Fig. 1 clearly demonstrate that the 5S rRNA gene is linked to the tandemly repeated units of the trans-spliced leader in *M. artiellia*. Moreover, the localization and the orientation are the same in *M. artiellia* and *C. elegans*, although the size of the spacer is different.

The PCR product obtained on *M. artiellia* DNA was extracted and sequenced. The sequence confirm the authenticity of the 5S rRNA, by comparison with other nematode 5S rRNA sequences [10,13]. Furthermore, the sequence obtained indicate the existence of canonical termination signals required by RNA polymerase III consisting of one or two oligo (dT) > 3 clusters at the 3′ end of the gene [15]. RT-PCR experiments revealed the existence of specific transcript with at least 64 nucleotide extension at the 3′ end of 5S rRNA. The much lower concentration of the shorter transcript, with a 27 nucleotide extension, suggests possible different stages of 5S rRNA precursor processing (results not shown).

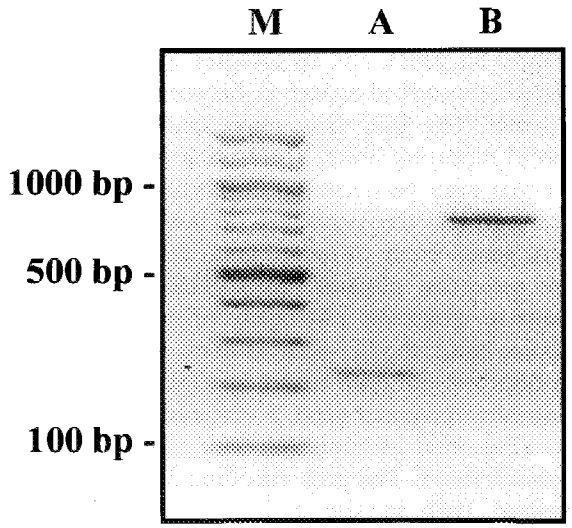

Fig. 1. Agarose gel electrophoresis of PCR amplified spacer region between 5S RNA and TSL sequence in *M. artiellia* (A) and in *C. elegans* (B). Molecular weight marker (M). In both cases the primers used were 5S2 and SL1. PCR primers were designed on the conserved region of 5S rRNA and on the spliced leader sequence on both DNA strands in order to assess the orientation of 5S rRNA. 5S1:5′-TTAACTTGCCAGATCGGACG-3′; 5S2: 5′-CGTCCGATCTGGCAAGTTAA-3′; SL1: 5′-GGTTTAATTACCCAAGTTTGAG-3′. SL2RC: 5′-CTCAAACTTGGGTAATTAAAC-3′.

The results reported here demonstrate that within the order Tylenchida, *M. artiellia* 5S rRNA has a different location, with respect to other *Meloidogyne* species, but similar to that observed in the distantly related *C. elegans*, order Rhabditida. Studies on the localization of 5S rRNA have indicated that it has undergone frequent changes during evolution. A possible explanation of the phylogenetic distribution of 5S rRNA gene linkage among nematodes is that the 5S rRNA gene was linked to the TSL unit in the common ancestor which gave rise to the Rhabditida and Thylenchida lineages and that such linkage has been subsequently inverted or lost in some species [16].

The conserved synteny, or the similarity in gene linkage seems to be a symplesiomorphic (primitive) character, still present in both *M. artiellia* and *C. elegans*. Therefore, the peculiarities presented by *M. artiellia* in comparison with the other members of *Meloidogyne* raise questions as to the relationships within these lineages, their dispersal and when they differentiated.

The availability of the complete sequence of the *M. artiellia* repeated ribosomal unit, allowed us to address phylogenetic relationships within the genus *Meloidogyne* [5].

One of the problems in the use of the rRNA genes for studying evolutionary relationships, is the existence of polymorphisms among repeat units, which may results in extensive variability even within a single individual. Most studies on *Meloidogyne* eggs DNA tend to show little genetic variation within ITS regions of a variety of *Meloidogyne* species [4,5,17], However, the work by Hugall et al. [3] revealed high diversity in the ITS sequences of *Meloidogyne*, by cloning the DNA from single-egg mass cultures, and thus amplifying any possible ribosomal target, irrespective of their abundance and functional character. In order to assess the intra-individual variability in *M. artiellia*, we sequenced different portions of this fragment obtained by PCR amplification on total genomic DNA, using different DNA preparations. Although only portions of the genes were sequenced, no evidence of sequence divergence was obtained from the PCR amplified fragments. Moreover, in the course of our studies, we have amplified and sequenced the entire region spanning from the 3′ end of 18S RNA gene to the 5′ end of the 26S RNA gene of other *M. artiellia*, coming from a different geographic origin, from Syria. No different nucleotides were detected in several clones of three different amplified products. Therefore, the sequencing of pooled indivi-

duals from two different populations strongly indicates that the ITSs containing region does not show nucleotide sequence differences in *M. artiellia*. Although it cannot be ruled out that minor variability might still exist, it seems that in the case of *M. artiellia*, concerted evolution has been able to drive the tandem array virtually to homogeneity. In addition, this homogeneity represents further support for the use of rDNA sequences for diagnostic identification of *M. artiellia*. In contrast with the homogeneity of the internal spacers (ITS), the intergenic regions (IGS) appear to be variable in length.

The comparison of ITSs containing regions sequenced in *M. artiellia*, with the same region from other *Meloidogyne* sequences could indicate the level of its diversity with co-generic species. Therefore, we calculated the genetic distances by the GTR method [18,19], using the sequence of *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*, and *M. chitwoodi* [5] spanning the entire region containing the ITS1, the 5.8S RNA and the ITS2 sequences and the available portions of the 18S and 26S rRNA genes. The *C. elegans* sequence was used as an outgroup.

The evolutionary analysis was carried out on unambiguous positions of a multiple alignment spanning a total of 358 nucleotides. The results of the genetic distances (not shown here), clearly indicate that *M. artiellia* is more distantly related to the other *Meloidogyne* than they are to each other. In order to verify the existence of a Molecular Clock, a likelihood ratio test was performed under the GTR assumption of sequence evolution. The observation that the trees constructed with and without the Molecular Clock constraint had the same topology and likelihood values not significantly different ($2\Delta = 2(\log L_{\text{clock}} = 1109.6 - \log L_{\text{n clock}} = 1107.4) = 4.4$; $\chi^2_{(5\text{DF},\ 0.05)} = 11.0$, $P = 0.48$) allowed us to conclude that the sequence data under analysis were consistent with the Molecular Clock hypothesis. We then constructed the phylogenetic tree using ML/GTR enforcing the Molecular Clock hypothesis. From this rooted tree the time of divergence between species have been estimated, fixing the time of divergence between *M. hapla* and *M. incognita* at 43 million years ago [20]. The phylogenetic tree presented in Fig. 2 shows that while the establishment of different *Meloidogyne* species was a relatively recent event, *M. artiellia* and the other *Meloidogyne* species separated from a common ancestor around 78 million years ago.

The independent evolutionary story of *M. artiellia* may reflect the existence of niche separation. In contrast with the apomictic *Meloidogyne* species largely dispersed throughout the world, the presence of *M. artiellia* has only been detected sporadically [21]. Moreover *M. artiellia* is a facultative parthenogen and thus involving meiotic type of parthenogenesis combined with amphimixis, is less successful biologically when

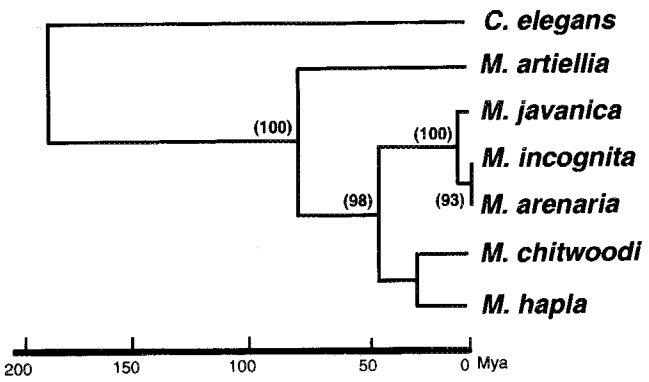

Fig. 2. Phylogenetic tree calculated from genetic distances by ML and GTR with and without the assumption of the Molecular Clock. Branch lengths are proportional to the measured genetic distances. Numbers indicate bootstrap percentage. The divergence time scale reported below the tree is calibrated assuming the separation between *M. hapla* and *M. incognita* to be 43 Mya. Evolutionary analysis was carried out using the programs implemented in the PAUP* package. Genetic distances were calculated by using the stationary Markov Model [19] also known as the GTR model [18].

compared with the apomictic species, which have an extensive host range and whose rapid population expansion over a large geographic area has been favored by the special environment of crop lands. *M. artiellia* displays several characteristics, presumably present in the common ancestor, whose traces are still present in members of the different order Rhabditida, such as *C. elegans*, in spite of the extremely fast evolution of nematodes. As a matter of fact it is already known that the order Rhabditida has radiated into one of the most diverse and abundant groups of nematodes in which the level of molecular divergence in the same family is about eight times as great as distances between tetrapod class [8].

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