A FRAGMENT OF THE LARGE SUBUNIT OF THE tRNA GENE AMPLIFIED BY POLYMERASE CHAIN REACTION IN INDIVIDUAL NEMATODES

by

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Summary. The Polymerase Chain Reaction (PCR) technique has been used for the amplification of a small portion at the 5' end of the large subunit of tRNA gene in different genera of plant parasitic nematodes. Furthermore, by using several different species of Xiphinema, a technique has been developed by which the amplification of the same fragment in a single individual is achieved. Studies on the amplified fragments strongly suggest that their sequences and their organization in the genome are species-specific.

Nematode systematics on a biochemical or molecular biology basis, represent useful approaches that are complementary to the traditional methods. However, the availability of large quantities of parasitic nematodes can be a serious limitation to the broad applicability of biochemical techniques. Moreover, the possibility of investigating characters that are not homogeneous within the population makes biochemical techniques less attractive. Because of recent advances stemming from the polymerase chain reaction, it is now possible to overcome the problem of quantity.

The Polymerase Chain Reaction (PCR) is a new and powerful technology which has been widely used in forensic sciences, in genetics, and evolutionary biology. Furthermore, the diagnostic potential for several isolates of the genus Meloidogyne has recently been demonstrated (Harris et al., 1990).

The PCR is an enzymatic amplification of a specific target DNA segment. It is made possible by the highly specific annealing of oligonucleotides complementary to the sequences flanking the segment. Each oligonucleotide acts as primer in the synthesis of a new DNA strand. The amplification is achieved because each newly made DNA molecule can be used as a template for further duplication.

In this paper we present data obtained by using the PCR with nematodes from different genera. Also a method is described which allows the amplification of a DNA fragment from a single nematode. The mapping of few restriction endonuclease sites allowed us to recognize particular features correlated with the different nematode species.

Material and methods

Total DNA was extracted as reported previously by Curran et al., (1986) with minor modifications. The nematode pellet was frozen in liquid nitrogen and ground in a mortar and pestle. The resulting fine powder was dissolved in a small volume of 2 µg/ml buffered solution of proteinase K (Boehringer) (100 mM Tris pH 8, 200 mM NaCl, 50 mM EDTA, 1% sodium dodecyl sulfate) at 65 °C for 1-1.5 hours. The phenol extraction and the ethanol precipitation were carried out as described by Maniatis et al., (1982).

In order to isolate DNA from a single specimen, individual juveniles or adults, were handpicked into a 1.5 ml microcentrifuge containing 10 µl of distilled sterile water and 500 µl of the extraction buffer (10 mM Tris pH 8, 2 mM EDTA PH 8, 10 mM NaCl, 1% SDS, 8 mg/ml DTT and 0.4 mg/ml proteinase K) were added. The suspension was incubated at 37 °C for 1-1.5 hours with constant gentle mixing. The resulting solution was extracted once with an equal volume of equilibrated phenol and once with an equal volume of chloroform/isoamyl alcohol (24:1), centrifuged for 5 min in a microcentrifuge and the aqueous layer transferred to another 1.5 ml microcentrifuge tube. The cellular DNA was precipitated by adding 1 µl of tRNA (10 µg/ml) 2.5 volumes of 95% ethanol and 0.3 M NaAc (f. c.) at -80 °C. The precipitated DNA was pelleted and resuspended in 20 µl of distilled sterile water.

The PCR amplification was performed in 100 µl reactions containing 12.5 µM each of dGTP, dATP, dCTP, dITP; 50 pmols of each primer; 2.5 units of Taq polymerase (Boehringer); and 1/10 of the volume of 10x Taq buffer (Boehringer). When total DNA extracted from pooled individuals was used, it was added in various amounts (1-400 ng). When the DNA was isolated from a single individual, the entire preparation was used. Each cycle of the polymerase chain reaction consisted of 30 cycles of denaturation at 94 °C for 1 min., annealing at 52 °C for 1 min., and extension at 72 °C for 2 min.
Double stranded DNA was purified by electrophoresis in 1% low-melting-temperature agarose. The DNA band was cut out of the gel and then extracted by 

**Results and discussion**

In this study we have amplified a region of the large subunit of rRNA gene, using as primers two oligonucleotides designed on the sequence of the large subunit of the rRNA gene of *Fusarium* (Guadet et al., 1989). DNA extracted from the free-living nematode *Caenorhabditis elegans* (Maupus) Dougherty, whose rDNA sequence is known (Ellis et al., 1986), was used as a control.

In Fig. 1 the fragment of the rRNA gene obtained by PCR amplification on different nematode genera is presented. Total DNA was extracted from *Meloidogyne arcellia* Franklin, *M. incognita* (Kofoid et White) Chitw., *X. index* Thorne et Allen, and *C. elegans*, as previously described. As can be seen, all the samples gave an amplification product, thus demonstrating that the same region which has been shown to be conserved in the genus *Fusarium* is also conserved in the Phylum Nematoda.

![Fig. 1 - Electrophoresis of the amplification products from DNA isolated from different nematode genera. The DNA was extracted from pooled individuals. The sequences of the primers used were: 5'-GCATATCAATAAGCGAGGAAG and 5'-GGTCCGTGTTTCAAGACG. The electrophoresis was in 2% agarose. With the exception of the sample N 5, all the experiments were carried out in duplicate. 1-2, *M. arcellia*; 3-4, *M. incognita*; 5, *X. index*; 6-7, *C. elegans*; M, Molecular weight markers.](image1.png)

![Fig. 2 - Restriction fragment pattern of DNA fragments amplified from *C. elegans* (A) and *X. italicae* (B). The restriction fragments used were: 1, None; 2, Alu I; 3, Clf I; 4, Hae III; 5, Sau 3A; 6, Taq I; The sizes of the marker fragments (M) were: 1.6311, 517, 396, 344, 298 and 220 nt.](image2.png)
The *C. elegans* amplification product appears to be slightly smaller than those obtained from plant parasitic nematodes. It has been shown (Ellis et al., 1986) that this region corresponds to the 5' end of the 26S rDNA and is 643 nucleotide (nt) long. The amplified fragment has been partially sequenced by us and it coincides with the published sequence. The products obtained with *X. index* and *Melodogynae* were estimated to be 800 and 700 nt long respectively. These size differences in the same highly conserved region are not surprising, since it has been reported that it is 622 nt long in *Saccharomices cerevisiae* and 601 nt long in *Neurospora crassa* (Guadet et al., 1989). However, only when the complete sequences of this region are available can it be established which nucleotides of the shorter molecules have not been conserved.

All the samples amplified in plant parasitic nematodes have been sequenced for a short region (100-200 nt) at their 5' end, and the sequences compared with that of the *C. elegans* gene. The observation that the sequences we obtained correlate very well with that of the *C. elegans* (unpublished) is a strong indication that the region we amplified corresponds with the same portion of the large subunit of the rRNA gene.

In order to provide a versatile and powerful taxonomic indicator, it is necessary to set up a procedure allowing the study of the indicator extracted from a single individual.

Harris *et al.*, (1990), have already reported the amplification of a mtDNA fragment from a single individual, and they also reported the failure of amplification in some cases. We were unable to obtain amplification of the rDNA fragment by using the published method, when few *Xiphinema* individuals (from 1 to 5) were used. We set up a different method which was suitable with few *Xiphinema* individuals and even with a single individual.

In order to test whether the procedure of lysis using one individual nematode was appropriate, we amplified the same product from *X. index*, *X. italae* (Meyl), *X. pachtaicum* (Tulaganov) Kirjanova and *X. diversicaudatum* (Micoletzky) Thorne.

*X. index* and *X. italae* gave a single band. Both fragments have been partially sequenced and the sequence compared with the *C. elegans* gene.

The results indicate that the fragment we amplified from a single individual correspond to the rDNA, thus confirming the results obtained when DNA extracted from pooled individuals was used. Furthermore, when the sequence comparison is carried out between *X. index* and *X. italae* the homology is very high, but the two sequences are not identical, thus suggesting that the base substitution analysis can be used for establishing precise phylogenetic relationships.

Restriction fragment sizes have been evaluated as possible indicators of taxonomic differences. The restriction patterns of the fragments obtained from *C. elegans* and *X. italae* are presented in Fig. 2. Although the agarose gel does not resolve perfectly all the restriction fragments, the *C. elegans* fragment sizes have been measured exactly by counting the nucleotides on the published sequence. In particular, the digestion with Cfo I gave one big fragment 566 nt long clearly visible on the gel. The two fragments obtained with Hae III were 475 and 168 nt long, those obtained with Sau 3A were 445 and 198 and with Taq I the fragments visible on the gel were 348, 192 and 83 nt long (a smaller fragment of 20 nt was not detectable). The differences between *C. elegans* and *X. italae* appear evident particularly when using Cfo I and Hae III restriction enzymes. The Cfo I digestion of *X. italae* gave only fragments of low molecular weight clearly distinguishable from the one produced in the *C. elegans*. As far as the Hae III enzyme is concerned, in the case of *X. italae* the most visible fragments are 220, 160, 140 nt long, while in the case of *X. index* fragments of 420 and 250 were produced (results not shown). Therefore, the size differences in the amplified product (643, about 700 and about 800) and the difference observed with the Hae III restriction enzyme can indicate taxonomic peculiarity.

In the case of *X. diversicaudatum* and *X. pachtaicum*, two bands whose sizes were estimated to be 800 and 700 nt, were present in the amplification. They were always present even if the relative amount was variable from expe-
riment to experiment. From Fig. 3 it can be seen that in the four *X. pachtaicum* individuals the two bands are always present even though in the first sample the lower band is very faint and in the last sample the higher band is hardly detectable.

These results indicate that in several plant parasitic nematodes this region is 700 nt long, in others 800, and in others both regions are present in the genome.

Conclusions

The technique described in this paper has been used for successful amplification and sequencing of a portion of rRNA gene from a single nematode. The ability to compare individual nematodes in this way can have an important impact on the identification of nematodes. Furthermore, since the PCR has also been used for archeological remains (Paabo et al., 1988) and museum specimens (Thomas et al., 1989), it should be extremely useful in the case of non-viable nematodes.

The fragment we amplified is a portion of the 5' end of the large subunit of the rRNA gene. As previously discussed, the sequence of this fragment amplified from the *C. elegans*, exactly matches the published sequence, thus demonstrating that the amplified product is what we expected. The authenticity of the rDNA fragments obtained in different genera of nematodes has been verified by their degree of similarity to the *C. elegans* gene and to the partial sequences of the closely related species.

The sizes of the amplified fragment, together with the specific restriction fragment sizes, can be regarded as distinctive characteristics. In the two species, *X. diversicaudatum* and *X. pachtaicum*, the amplification product is represented by two clearly distinguishable bands. Obviously, only the complete nucleotide sequence of both fragments can answer the question about the variation within individuals. However, the observation that in all the experiments the two species gave the two fragments indicates that this particular organization is present in both species.

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Literature cited


