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RAPD characterization of single females of the root-knot nematodes, *Meloidogyne* spp.

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Abstract

Random amplified polymorphic DNA fingerprinting was performed on single females of root-knot nematodes, *Meloidogyne* spp., using a new procedure for DNA isolation. One-fourth of the total DNA amount isolated from a single female proved to be sufficient as a template in a polymerase chain reaction. Electrophoretic patterns of the amplified fragments were reproducible between replicates from a single female or sister females from the same progeny, and identical to those obtained with genomic DNA purified from a large number of nematodes. Moreover, a comparative analysis over three successive generations showed stability of the amplification patterns, thus demonstrating the utility of this procedure for epidemiological and ecological studies on root-knot nematodes.

Abbreviations: PCR – polymerase chain reaction; RAPD – random amplified polymorphic DNA; RKN – root-knot nematode.

Introduction

Root-knot nematodes (RKNs) of the genus *Meloidogyne* are sedentary endoparasites whose life cycle is strictly dependent on the feeding site they induce in the tissues of their host plants (Jones, 1981). More than eighty RKN species have been described (Karssen and Van Hoenselaar, 1998). Some of them are responsible for considerable losses in world-wide crop production. Use of plant resistance is the most efficient way of controlling these pests, but rapid and accurate identification is needed for the design of successful management practices. The polymerase chain reaction (PCR) provides an attractive technique for developing procedures for *Meloidogyne* detection and characterization. Among such procedures, fingerprinting by random amplified polymorpic DNA (RAPD) analysis appeared promising, since no preliminary sequence information on the genome to be studied is needed (Welsh and McClelland, 1990; Williams et al., 1990). A number of studies have demonstrated the utility of such a technique for RKN specific and/or subspecific identification (Cenis, 1993; Baum et al., 1994; Castagnone-Sereno et al., 1994; Blok et al., 1997), but with the major inconvenience that DNA purification from a large pool of nematodes was required before amplification. As RKNs are obligate biotrophic parasites, multiplication on host plants is necessary to produce enough biological material for such tests. Such time- and space-consuming practices are incompatible with a routine diagnostic system. Until now, attempts to obtain reproducible RAPD patterns from single *Meloidogyne* individuals have been conducted on second-stage juveniles, and such experiements have failed (Cenis, 1993; Williamson et al., 1997). In this report, a RAPD-PCR method is presented, which enabled the amplification of reproducible markers from single females of *Meloidogyne* spp. Moreover, we also showed that the amplified patterns were stable over three successive generations, thus demonstrating the potential of RAPD for routine identification of single nematodes.

Materials and methods

Nematodes

Isolates belonging to the three main RKN species were used: *M. arenaria* (isolate from Pelotas, Brasil), *M. incognita* (isolate from Calissanne, France) and *M. javanica* (isolate from Mato Grosso do Sul, Brasil). The nematodes were maintained on tomato plants (cv. Saint Pierre) under greenhouse conditions. White females were hand-picked from the root tissues under a stereomicroscope and stored at −80 °C until used.

Sister females were obtained after inoculation of a tomato plant with a single egg-mass (i.e. juveniles from a single female) and completion of the nematode life-cycle. In order to obtain individuals from successive generations, nematodes were produced and stored as follows. From the original culture, a number of females (G1) were hand-picked from the roots each with its corresponding egg-mass. Each female was individually stored at −80 °C until use, while each egg-mass (i.e. each progeny) was individually inoculated on tomato plants. After two months, females (G2) were recovered and stored at −80 °C, and their egg-masses individually re-inoculated on tomato plants. The same procedure was repeated in order to collect females from successive generations.

DNA purification and RAPD reactions

Genomic DNA was purified from individual RKN females, according to a protocol used for cyst nematodes (Bekal et al., 1997) and modified as follows: each female was washed in sterile distilled water, transferred to a sterile Eppendorf tube and squashed with a tip-closed capillary Pasteur pipette in 90 µl of lysis buffer (0.1 M Tris-HCl, pH 8.0; 50 mM EDTA; 1% SDS; 0.17 M NaCl). Ten microlitre of proteinase K (5 µg µl^{-1}) were added to the tube, and the homogenate was incubated overnight at 37 °C. DNA was purified with phenol-chloroform-isoamyl alcohol (25 : 24 : 1) and precipitated with two volumes of cold absolute ethanol for 20 min at −80 °C. DNA was pelleted, washed twice with cold 70% ethanol, dried and resuspended in 20 µl of sterile distilled water. Five microlitre of the DNA solution from each individual specimen (i.e. one-fourth of the total DNA isolated from a single female) were used in RAPD-PCR experiments with conditions, cycling parameters, and primers as previously described (Castagnone-Sereno et al., 1994). Twenty random 10-mer primers, purchased from Eurogentec (Herstal, Belgium) were tested in a first set of experiments. Four of them gave reproducible amplification patterns and these were used: primer 2 (5′-ATGGATCCGC-3′), K01 (5′-CATTCGAGCC-3′), K06 (5′-CACCTTT-CCC-3′) and K14 (5′-CCCGCTACAC-3′). Each experiment was repeated at least three times.

Results

Amplifications from single RKN females

Using primers 2, K01, K06 and K14, reproducible fragments were amplified for each of the three RKN species tested, with different patterns obtained for each species. As shown in Figure 1, for primer K14, each set of four replicate reactions for *M. arenaria*, *M. incognita* and *M. javanica*, produced identical banding patterns in the average range of 200–2000 base pairs.

Comparative RAPD-PCR of sister females (i.e. individuals belonging to the same progeny) was then carried out, in order to test the reliability of the patterns. As exemplified in Figure 2 with *M. arenaria* and *M. incognita* samples, no variation in the number and

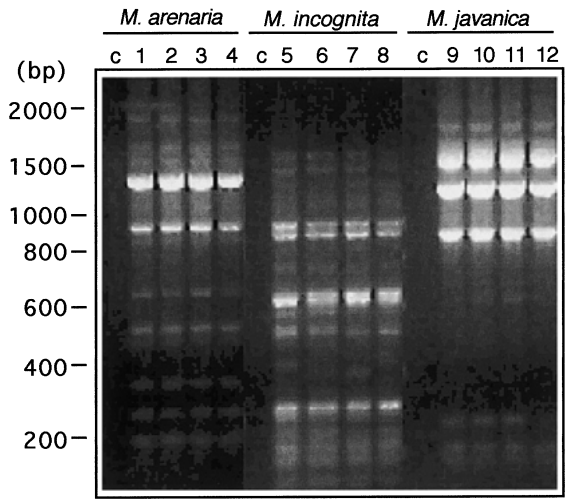

Figure 1. RAPD patterns from single females of *M. arenaria*, *M. incognita* and *M. javanica*. For each species, four replicate reactions from the same female were performed, with one-fourth of the DNA purified from the female used as template in each reaction. The patterns were obtained with primer K14. c = negative control (water). Molecular weights are given in base pairs (bp).

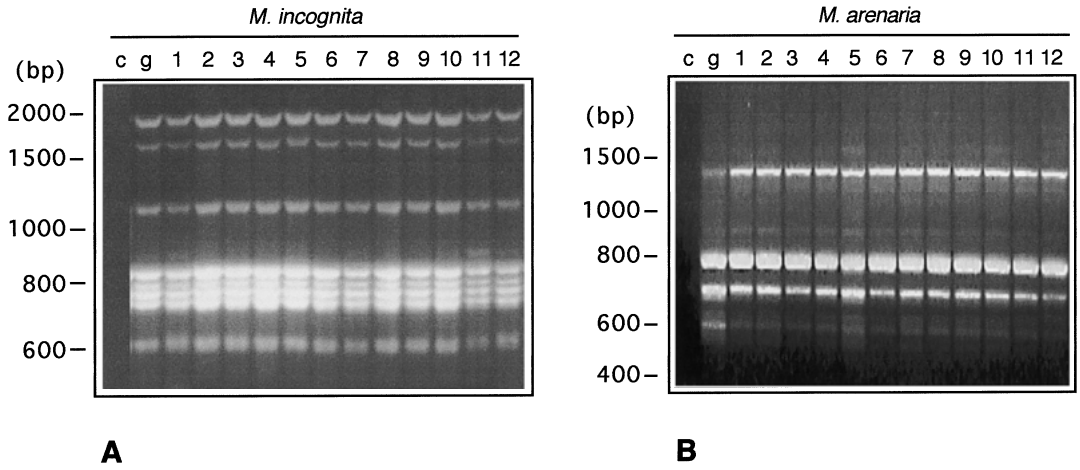

Figure 2. RAPD patterns from *Meloidogyne* spp. females. A = *M. incognita* females; B = *M. arenaria* females. In each of lanes 1–12, one-fourth of the DNA purified from a single female was used as template in the reaction. The patterns in panels A and B were obtained with primers 2 and K06, respectively. c = negative control (water); g = positive control (genomic DNA purified from a large number of nematodes). Molecular weights are given in base pairs (bp).

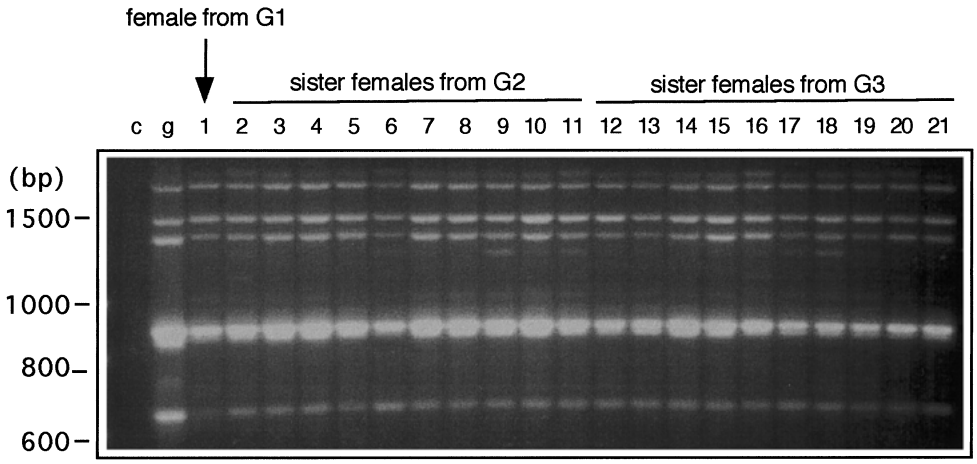

Figure 3. RAPD patterns from *M. incognita* females over three successive generations. In each of lanes 1–21, one-fourth of the DNA purified from a single female was used as template in the reaction. The patterns were obtained with primer K01. c, g = negative and positive controls, respectively, as described in the legend of Figure 2. Molecular weights are given in base pairs (bp).

size of the amplified DNA fragments was observed between individuals. Amplification patterns were generally identical to the one produced with genomic DNA of the same nematode isolate purified from a large pool of nematodes, which clearly demonstrates the informative value of the fragments amplified from one-fourth of the DNA from a single female (Figure 2). In a few instances, some differences from the positive control were observed (i.e. variable intensity or absence of minor bands), presumably due to insufficient template.

Comparative analysis over successive generations

In order to further test the stability of the RAPD patterns produced with one-fourth of the DNA purified from a single female, comparative analyses were performed over successive generations. Figure 3 shows a banding pattern obtained with *M. incognita* females belonging to three successive generations: one female from G1, sister females from G2 (originating from the egg-mass

produced by the female from G1), and sister females from G3 (originating from the egg-mass produced by one female from G2). The RAPD bands obtained were nearly identical for all generations and to those produced from DNA purified from a large pool of nematodes. As observed previously, a few minor differences could be noted in the patterns, although the main fragments were reproducibly amplified. This result indicates that the general RAPD profile is stable between sister females and between females from successive generations. Similar results were obtained with combinations of the other *Meloidogyne* species and primers tested (data not shown).

Discussion

A number of studies have shown that RAPD markers are suitable to differentiate *Meloidogyne* species and isolates using purified genomic DNA from pooled nematodes as a template (Cenis, 1993; Baum et al., 1994; Castagnone-Sereno et al., 1994; Blok et al., 1997). However, to our knowledge, this is the first report of the use of such markers to produce accurate and reproductive fingerprints from a single female of *Meloidogyne* spp. PCR methods for RKN identification have been previously developed that work on single RKN individuals, based on the specific amplification of repetitive sequences, for example, mitochondrial DNA (Powers and Harris, 1993), ribosomal DNA (Petersen et al., 1997), or satellite DNA (Castagnone-Sereno et al., 1995). However, these methods all produced banding patterns useful for diagnosis at the species level only. Moreover, in the case of mitochondrial DNA, digestion of the amplified products with restriction endonucleases was needed to discriminate among species (Powers and Harris, 1993).

The observation that amplification patterns from single female RKNs are stable over three successive generations could be related to the mitotic parthenogenetic mode of reproduction of this nematode, theoretically leading to clonal populations (Triantaphyllou, 1981). However, to assess the risk of genetic drift, which is of outstanding importance in the case of laboratory cultures multiplied over years from the progeny of a single female, periodical checks should be the rule in the case of these parthenogenetic nematodes.

The major advantage of the method described here is that it avoids the preliminary space- and time-consuming step of nematode production on host plants (eight weeks on average) heretofore needed for RAPD analysis of *Meloidogyne* spp. In fact, our entire procedure can be performed in one day for many samples, and could be a convenient and reliable tool for epidemiological and ecological studies of root-knot nematodes.

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