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Molecular diversity amongst *Radopholus similis* populations from Sri Lanka detected by RAPD analysis

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Summary – DNA extracted from fourteen populations of *Radopholus similis,* collected previously from roots of various hosts at different locations in Sri Lanka, and one new species of *Radopholus* from East Java was compared using the polymerase chain reaction based method of random amplified polymorphic DNA (RAPD). Four populations isolated from arecanut, tea, banana and citrus appeared to be particularly different in their RAPD profiles. Hierarchical cluster analysis was applied to the data obtained from RAPD patterns. This arranged most of the fifteen populations into three putative groups and revealed three more divergent isolates which are, thus far, monotypic. The results are discussed in the context of the genetic divergence and the existence of biotypes of *R. similis* in Sri Lanka.

Résumé – ***Diversité moléculaire des populations du nématode* Radopholus similis *au Sri Lanka.*** La réaction de polymérase en chaîne (RAPD) a été utilisée pour comparer l'ADN de quatorze populations de *Radopholus similis* provenant de différentes plantes hôtes et de régions variées du Sri Lanka, ainsi que l'ADN d'une nouvelle espèce de *Radopholus* originaire de l'est de Java. Quatre populations isolées d'aréquier, théier, bananier et citrus semblent très différentes d'après leur profil de RAPD. L'analyse hiérarchisée des groupes des données obtenus d'après les profils de RAPD a permis un essai de regroupement des quinze populations en trois groupes et a montré l'éloignement de trois isolats demeurant jusqu'à maintenant monotypiques. Les résultats sont examinés dans le contexte de l'existence de biotypes de *R. similis* et de leur divergence génétique au Sri Lanka.

Key-words : *Radopholus similis,* Random Amplified Polymorphic DNA, PCR, biotypes, host range, genetic diversity.

The endoparasitic nematode *Radopholus similis* (Cobb, 1893) Thorne, 1949 is an important, cosmopolitan pest of banana, plantains and other crops (Gowen & Quénéhervé, 1990). In parts of Africa crop losses caused by *R. similis* may be as much as 75 % (Sarah, 1989) and nematicide treatment to control *R. similis* has resulted in a maximum yield improvement of 267 % (Gowen & Quénéhervé, 1990).

Ever since the reported existence of three " physiological races " of *Radopholus similis* (Ducharme & Birchfield, 1956) there has been an increasing demand for more information concerning the degree of sub-specific variation of the burrowing nematode (Gowen, 1979; Pinochet, 1987; Kaplan & Gottwald, 1992). Based on differences in biology, biochemistry and karyotype between some populations of *R. similis,* the species was first split into two sibling species, *R. similis similis* and *R. similis citrophilus* (Huettel *et al.,* 1984 *b*), and more recently two separate subspecies, *R. similis* and *R. citrophilus,* have been established (Loof, 1991). However, field observations strongly suggest the existence of various biotypes within the species *R. similis sensu lato* (cf. Gowen & Quénéhervé, 1990), and in Sri Lanka the presence of two biotypes of *R. similis,* where the nematode is a major pest on tea, has been reported (Gnanapragasam *et al.,* 1991). The degree of genetic variation amongst populations of *R. similis* is not well understood, even though such information is essential for resistance breeding programmes and control schemes.

Recently the random amplified polymorphic DNA (RAPD) technique has been established as a powerful tool in the analysis of genetic variation in many organisms including insects and nematodes (Black *et al.,* 1992; Caswell-Chen *et al.,* 1992). By this method, arbitrary regions of the genome are amplified by random synthetic oligonucleotide primers. The DNA fragments obtained can then be compared by electrophoresis to reveal the degree of genetic similarity within or between populations.

In this study, RAPD markers were used to compare genetic variation in fifteen populations of *Radopholus.* Fourteen of these, identified by morphological charac-

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ters as being *R. similis*, were collected from various hosts and locations in Sri Lanka. The remaining population, isolated from citrus in East Java, is currently being described as a new species of *Radopholus* (J. Machon & J. Bridge, pers. comm.). The aim of this work was to estimate the degree of genetic diversity of *R. similis* populations occuring in Sri Lanka. It is expected that such information coupled with host range studies will improve the integrated management strategy currently used to control this nematode in Sri Lanka.

Material and methods

Cultures of nematodes

Populations of *R. similis* were cultured on excised maize roots. The maize seeds (cv. Baron) were surface sterilized by rinsing them in Industrial Methylated Spirit for 1 min, then soaked in 0.1 % (w/v) mercuric chloride for 30 min and finally rinsed 10 times in sterile distilled water. The seeds were placed aseptically on standard Murashige and Skoog's (1962) medium (Sigma Chemical Co.), *p*H 5.7, in 9 cm diameter Petri dishes. The medium was supplemented with 2 % (w/v) sucrose and solidified with 1 % (w/v) agar. Plates were incubated at 25 °C and after two weeks or when there was 10-15 cm of root growth the germinated seed and the shoot were excised and discarded. The roots were then inoculated with either surface sterilised nematodes (Hooper, 1986) or nematode infected tissue subcultured from an existing plate. Plates were incubated at 25 °C for 6-8 weeks.

To extract the nematodes, Petri dishes were opened under sterile conditions and sterile distilled water (10 ml) was added. The plates were returned to the culture room, inverted and left at 25 °C for 24 h. Nematodes of all stages moved into the lids and this suspension was then poured onto a 20 mm diameter sieve (75 µm aperture). The sieve, which had been placed in a cell culture plate (Sterilin Ltd.) filled with sterile distilled water (10 ml), was left until the nematodes had migrated through it. They were pelleted in a microcentrifuge and the pellet was either used for immediate extraction of DNA or stored in sterile distilled water at - 80 °C. Up to 7000 nematodes could be obtained from a single plate.

Radopholus new species (Ra ns) was cultured on carrot discs (modified from Moody *et al.*, 1973). After removal of tops and fine roots carrots were soaked in sodium hypochlorite [1 % (v/v) available Cl_2] for 5 min, peeled as described and sliced into 3-5 mm thick discs. The discs were soaked in sodium hypochlorite for 3 min and finally rinsed 10 times in sterile distilled water. To drain excess liquid, the carrot discs were transferred to a dry, sterilized Petri dish and left in a laminar flow cabinet for 30 min. Discs were then placed in Petri dishes (9 cm diameter) containing 1 % (w/v) water agar. Nematodes were added and plates incubated as described above.

Table 1. *Original hosts and locations of* Radopholus *populations used in this study.*

Population	Host	Location
Ra 5	Tea	Morawakka Estate, Deniyaya
Ra 6	Arecanut *(Areca catechu)*	Imboolpitya Estate, Kandy
Ra 7	Tea	Imboolpitya Estate, Kandy
Ra 8	Coffee	Hantane, Kandy
Ra 9	Banana	Hantane, Kandy
Ra 10	Tea	Imboolpitya Estate, Kandy
Ra 11	Banana	Hantane, Kandy
Ra 14	Guatemala grass *(Tripsacum fasciculatum)*	Montecristo Estate, Kandy
Ra 15	*Anthurium*	Baranagalla Estate, Kandy
Ra 20	Tea	Imboolpitya Estate, Kandy
Ra 21	Clove	Baranagalla Estate, Kandy
Ra 30	Banana	St. Coombs, Talawakele
Ra 32	Plantain cv. ashplantain	Gannoruwa, Peredeniya
Ra 33	Banana	Talgampola, Galle
Ra ns*	Citrus	East Java, Indonesia

* To be described as a new species of *Radopholus* (pers. comm. J. Machon and J. Bridge, CABI).

Fifteen populations of *Radopholus* from different origins were used in this study (Table 1).

DNA extraction

Lysis buffer was prepared as described by Black *et al.* (1992). Mixed nematode stages were washed twice in sterile distilled water, pelleted in a microcentrifuge and a standard 3 µl-packed pellet of nematodes (a mixture of approximately 4000 juveniles and adults) were transferred to a micro-homogenizer tube (BioMedix). Lysis buffer (7 µl) was added to the tube and the nematodes homogenized on ice for 30 s. After centrifugation at 13 000 *g* for 15 s the supernatant was transferred to a sterile 500 µl Eppendorf tube. The pellet was resuspended in 10 µl lysis buffer and further homogenized for 20 s. After centrifugation both extracts and the pellet were mixed, the tube capped and placed in a 95 °C heating block for 3 min. Samples were then centrifuged for 10 min at 13 000 *g* and the supernatant (approximately 17 µl) stored at – 43 °C.

PCR protocol

PCR was done in a total reaction volume of 25 µl. PCR reactions were prepared on ice and each consisted

of 10 mM Tris HCl *p*H 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % (v/v) Triton X-100, 200 µM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 32-34 pM primer and 1 unit of Taq DNA Polymerase (Northumbria Biologicals Ltd.).

Finally *Radopholus* genomic DNA (0.1 µl) was added to each tube and the reactions were overlaid with one drop of light mineral oil. Reactions were always performed in duplicate.

Amplification was done in a Hybaid Omni Gene Thermo Cycler using block control. PCR cycle conditions were as follows : 2 min at 85 °C – 41 cycles of 1 min at 92 °C, 1 min at 35 °C, 1 min at 72 °C with the final cycle remaining at 72 °C for 7 min. When using decameric primers the fastest available transitions between each temperature were employed, however with the longer primers (20-mers, 24 mers) the annealing step was ramped at 4 s/°C from 35 °C to 72 °C. The primers used are listed in Table 2.

Table 2. *Primers used in this study.*

Primer sequence (5′ to 3′)	Source/Reference
CCCAAGGTCC	OPERON KIT[1] E code OPE-01
TCAGGGAGGT	OPERON KIT E code OPE-05
AAGACCCCTC	OPERON KIT E code OPE-06
TCACCACGGT	OPERON KIT E code OPE-08
CTTCACCCGA	OPERON KIT E code OPE-09
AACGGTGACC	OPERON KIT E code OPE-20
CAGGCCCTTC	OPERON KIT A code OPA-01
TGCCGAGCTG	OPERON KIT A code OPA-02
AGTCAGCCAC	OPERON KIT A code OPA-03
AATCGGGCTG	OPERON KIT A code OPA-04
AGGGGTCTTG	OPERON KIT A code OPA-05
GAAACGGGTG	OPERON KIT A code OPA-07
GGGTAACGCC	OPERON KIT A code OPA-09
GTGATCGCAG	OPERON KIT A code OPA-10
GGGAAGACAGGGTTGATATCATCG	3′ PFKB[2]
CAACTCCTCTTCTCTTCTCC	PFKB END[2]
ATGAATTCGC	P-Eco; Black *et al.*, 1992
ATGGATCCGC	P-Bam; Black *et al.*, 1992

[1] Operon Instruments Alameda, CA 94501, USA.
[2] Originally designed for other laboratory purposes.

Agarose gel electrophoresis

PCR products were resolved by electrophoresis through a 1.3 % agarose gel buffered in 1× TAE and the DNA was stained with ethidium bromide. DNA fragments of *Hind III* digestion of Lambda DNA (cI 857 Sam7) and 123 base pair ladder (BRL) were used as size markers. DNA was visualised on a UV Transilluminator and photographed with Polaroid Type 667 film.

Cluster analysis

Cluster analysis on all data obtained by RAPD was done using " Genstat " statistical software (Payne *et al.*, 1987).

In this study the simple matching coefficient (Sneath & Sokal, 1973) was used to calculate a genetic similarity matrix. The matrix was used to perform hierarchical cluster analysis based on the unweighted pair-group method using arithmetic averages (UPGMA) (Sneath & Sokal, 1973).

Results

Throughout this study RAPD reactions were always duplicated and care was taken to ensure consistency in DNA banding profiles between replicates and between separate experimental runs. Primers that produced inconsistent results or minor DNA bands that appeared to come and go between identical reactions were not used in the analysis and are not included in the list of the primers used in this study (Table 2). In general, RAPD profiles between samples and runs were reproducible.

In addition to the fourteen populations of *R. similis* collected from Sri Lanka, one population of a new species of *Radopholus* found on citrus in East Java (Ra ns) was included in the analysis as an outgroup. This new species was very different from the *R. similis* populations in all eighteen RAPD profiles. Four of these RAPD profiles obtained from the fifteen populations tested are shown in Figure 1. Primer OPA-07 (Fig. 1 D), for example, yielded DNA fragments of approximately 0.94 kilobases (Kb), 0.74 Kb, 0.67 Kb and 051 Kb specific to Ra ns whereas all populations from Sri Lanka were found to yield fragments of approximately 1.20 Kb and 0.92 Kb. Interestingly, with this same primer the isolate collected from tea (Ra 7) in Imboolpitya (Fig. 1 D) revealed a major DNA fragment of approximately 0.76 Kb not found in any of the other populations.

A more complex pattern was produced by primer 3'PFKB (Fig. 1 A). Here, the population from banana in Hantane (Ra 9) has an additional fragment of approximately 0.9 Kb, while the population from arecanut in Imboolpitya (Ra 6) displays a completely different pattern to the other *Radopholus* isolates. A similar result was revealed by primer OPA-05 (Fig. 1 B). Again, the populations Ra 6, Ra 7, Ra 9 and Ra ns appeared to be the most divergent.

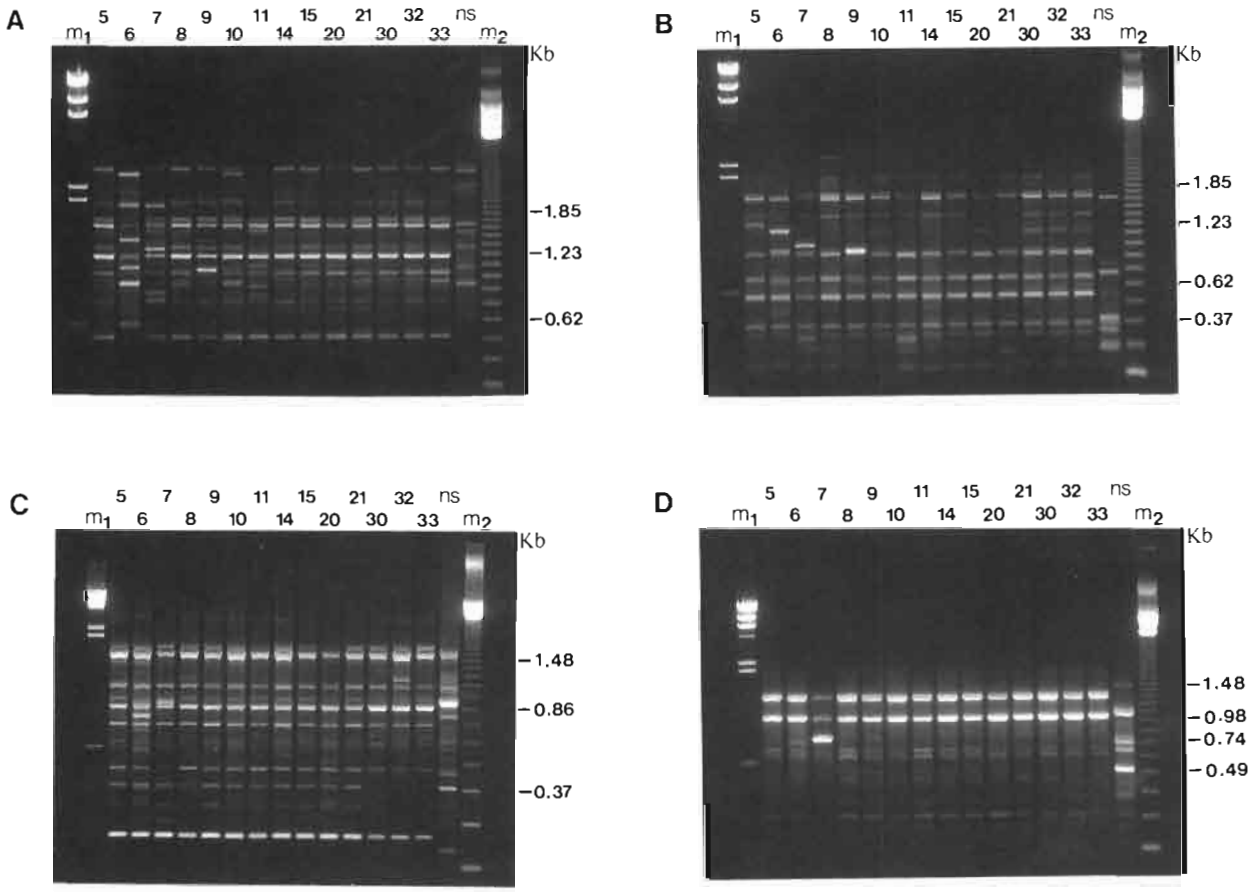

Fig. 1. *RAPD profiles from fifteen populations of* Radopholus. *Banding patterns yielded by four different primers : (A) 3' PFKB, (B) OPA-05, (C) OPA-01 and (D) OPA-07. Lanes numbered according to population numbers given in Table 1. Molecular mass values given in kilobases (Kb) were estimated from lambda phage DNA cut with* Hind III *(m_1) and 123 bp ladder molecular weight marker (m_2).*

Less obvious banding differences were detected amongst the other *R. similis* populations, for example, the additional fragment of approximately 1.15 Kb in the isolate from ashplantain in Gannoruwa (Ra 32) in Figure 1 C.

The generation of RAPD profiles from the fifteen *Radopholus* populations by eighteen different primers produced a total of 167 DNA bands that could be scored.

Hierarchical cluster analysis was performed on these data and it arranged some of the fourteen populations from Sri Lanka into three putative groups of genetic similarity. It also separated three isolates (Ra 6, Ra 7 and Ra 9) that were dissimilar from the other populations and each is, thus far, monotypic. The dendrogram, generated from the genetic similarity coefficient matrix (Fig. 2), was based on the principle of UPGMA and is shown in Figure 3.

The first putative group is formed by isolates from tea in Morawakka Estate (Ra 5), from Guatemala grass in Montecristo Estate (Ra 14), from *Anthurium* in Baranagalla Estate (Ra 15) and from coffee in Hantane (Ra 8). The last two populations were indistinguishable by RAPD in this study. The second group contains isolates from tea in Imboolpitya Estate (Ra 20), from clove in Baranagalla Estate (Ra 21) and from tea in Imboolpitya Estate (Ra 10). Populations from banana in Talawakele (Ra 30) and from banana in Talgampola (Ra 33) form the third putative group. At the level of 95 % similarity these three groups, together with isolates from banana in Hantane (Ra 11) and from ashplantain in Gannoruwa (Ra 32), have merged. The three remaining populations from Sri Lanka, isolates from arecanut and tea in Imboolpitya Estate (Ra 6 and Ra 7) and the population from banana in Hantane (Ra 9), appear to be more genetically divergent.

Although Ra 7, Ra 10 and Ra 20 were all collected from tea roots in Imboolpitya Estate, Ra 7 was distinct in its RAPD profiles from the other two populations.

5	-														
6	76.6	-													
7	90.4	75.4	-												
8	98.8	77.8	91.6	-											
9	95.2	77.8	90.4	96.4	-										
10	95.8	77.2	89.8	97.0	95.8	-									
11	95.8	74.9	89.8	97.0	94.6	97.6	-								
14	98.2	77.2	91.0	99.4	95.8	97.6	97.6	-							
15	98.8	77.8	91.6	100.0	96.4	97.0	97.0	99.4	-						
20	96.4	76.6	89.2	97.6	94.0	98.2	97.0	98.2	97.6	-					
21	96.4	76.6	89.2	97.6	94.0	98.2	97.0	98.2	97.6	98.8	-				
30	95.2	77.8	88.0	96.4	92.8	95.8	94.6	97.0	96.4	97.6	96.4	-			
32	95.2	77.2	88.6	97.0	93.4	95.2	94.0	96.4	97.0	97.0	95.8	97.0	-		
33	94.6	76.0	87.4	95.8	92.2	96.4	95.2	96.4	95.8	98.2	97.0	98.2	95.2	-	
ns	33.5	26.9	32.3	33.5	32.3	34.1	36.5	34.1	33.5	33.5	33.5	33.5	32.9	32.9	-
	5	6	7	8	9	10	11	14	15	20	21	30	32	33	ns

Fig. 2. *Genetic similarity coefficient matrix of fifteen populations of* Radopholus. *Matrix calculated from a total of 167 DNA bands yielded by eighteen different primers. Simple matching coefficients given in percentage similarity. Populations represented by population numbers as given in Table 1.*

Discussion

The development of RAPD markers, used originally to construct genetic maps in a variety of species (Williams *et al.,* 1990), has been used in this study to investigate DNA polymorphisms amongst populations of *R. similis* collected from different hosts and locations in Sri Lanka. As reviewed recently (Hadrys *et al.,* 1992) RAPD profiles can serve as diagnostic molecular characters at different taxonomic levels; genus specific markers, species specific markers and even clonal specific markers have been identified (Smith *et al.,* 1992). The analysis of genetic variation using RAPDs is well suited for use in population genetics and studies of biodiversity (Waugh & Powell, 1992) and offers the advantages of being sensitive and independent of stage in the life-cycle.

The biodiversity of *R. similis* in Sri Lanka is extremely important when planning management programmes that employ resistant or non-host crops. In this respect, the more divergent *Radopholus* populations Ra 6, Ra 7 and Ra 9 are very interesting, as it is possible that their apparent genetic divergence is paralleled by important changes in pathogenicity and/or host range.

Populations of *R. similis* that differ in biochemical markers, host range or morphology have been variously described in the literature as races, physiological races, biotypes and pathotypes (Ducharme & Birchfield, 1956; Pinochet, 1979; Huettel & Dickson, 1981; Pinochet, 1988; Gnanapragasam *et al.,* 1991).

Attempts have been made to clarify this inconsistant usage in the species *R. similis* (Huettel *et al.,* 1984 *a*) as well as amongst phytonematodes in general (Dropkin, 1988). Dropkin defined biotype, based on its usage in zoology and plant pathology, as a distinctive population with uniform genetics. Furthermore, if this distinction consists of the ability to reproduce on a host resistant to other isolates it should be termed a resistance-breaking biotype. Using Dropkin's criteria, we could consider Ra 6, Ra 7 and Ra 9, with their generally distinctive RADP profiles, as biotypes of *R. similis*. Host range studies on these populations will reveal, whether they should be regarded as resistance-breaking biotypes.

Although the populations Ra 7, Ra 10 and Ra 20 were all collected from the same host and location (from tea in Imboolpitya Estate), our results indicate that genetically divergent isolates might occur in the same area. The significance of this apparent diversity to local agriculture has yet to be determined.

The burrowing nematode was first detected in Sri Lanka in 1967 (Sivapalan, 1968). However, the genetic diversity shown amongst these populations indicates that its occurrence there is unlikely to have resulted merely from one or two introductions this century. The apparent level of genetic similarity between populations can, in common with many investigations of this kind, be influenced by founder effects : sampling in the field and subsequent culturing in the laboratory may select a non-representative proportion of the natural gene pool. Further experiments are planned to estimate the influence of such founder effects on these populations. In this context, the question of whether or not field populations of *R. similis* are truly amphimictic is of considerable

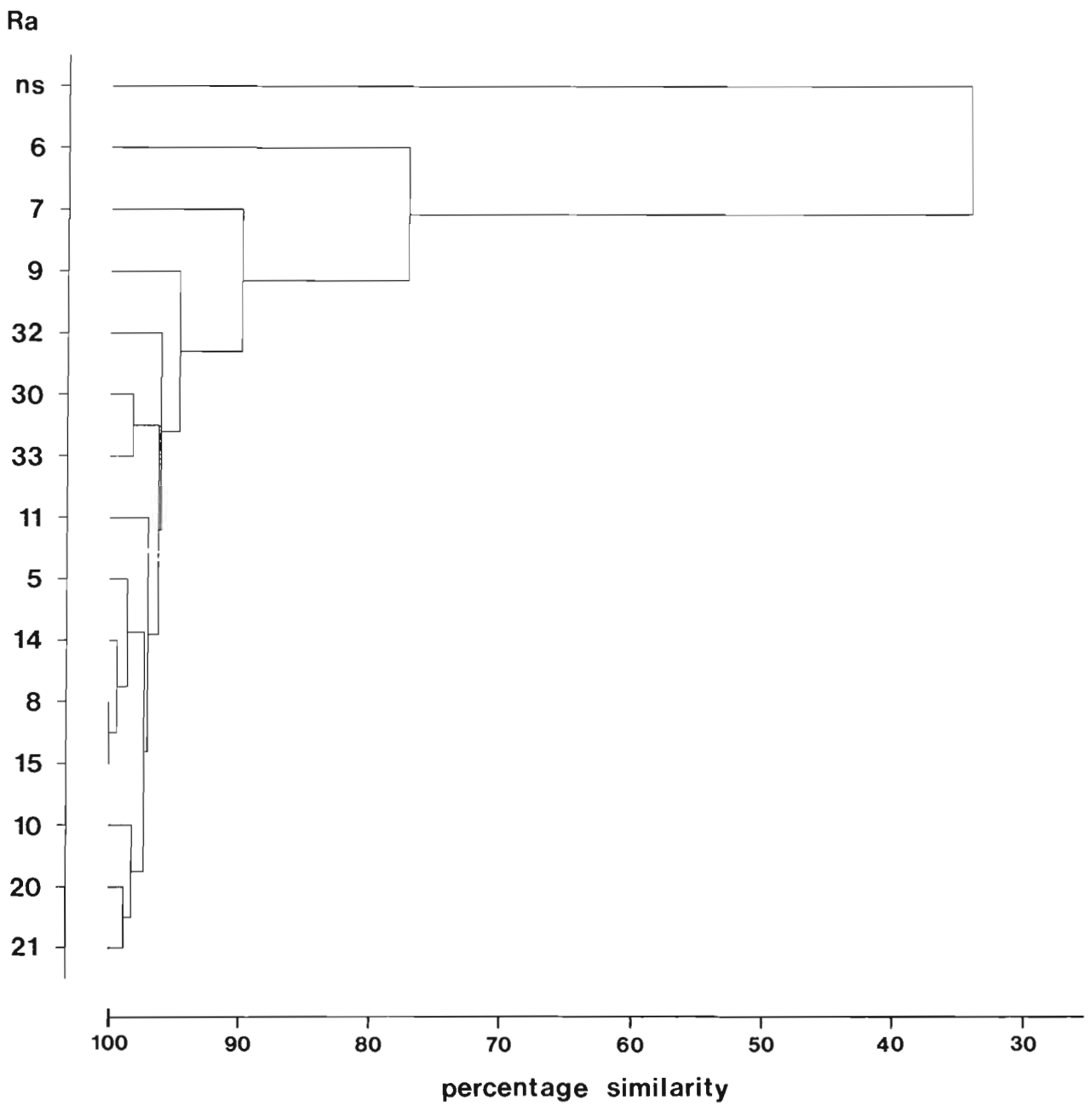

Fig. 3. *Relationships of fifteen populations of* Radopholus. *The dendrogram was generated from genetic similarity coefficients (see Fig. 2A) obtained from presence or absence of a total of 167 RAPD markers and is based on the "unweighted pair-group method using arithmetic averages" (UPGMA).*

importance in the likely diversity within natural populations. Parthenogenic or clonal populations are less diverse and under laboratory conditions both, *R. similis* and *R. citrophilus,* have been reported to reproduce parthenogenetically (Huettel & Dickson, 1981).

The new species of *Radopholus* was used here in order to compare the degree of sub-specific genetic variation between the isolates of *R. similis* with that which may be expected between separate *Radopholus* species. Although on the basis of molecular markers (RAPD profiles) the populations Ra 6, Ra 7 and Ra 9 are considered separate biotypes of *R. similis,* it is not yet possible to

conclude whether or not they are sufficiently different to be regarded as sub-species or even new species. Clearly, for the latter two classifications, much will depend on the relative genetic divergence of the species used as an outgroup; a comparison including *R. citrophilus,* which is regarded as a sibling species of *R. similis,* would be valuable. Further investigations are planned to study the host range, biology, morphology and karyotype of these isolates to clarify their taxonomic status.

This work is part of a larger ongoing research programme funded by the Natural Resources Institute (NRI) and is carried out within the CABI-AFRC Research Group on Nematodes of Subtropical and Tropical Agriculture and at Imperial College Silwood Park. The objectives of this research are to assess the biochemical diversity, host range morphology and pathogenicity of *Radopholus similis* populations from around the world. The work presented here complements the research carried out in Sri Lanka.

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