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Differentiation of Species and Populations of *Aphelenchoides* and of *Ditylenchus angustus* Using a Fragment of Ribosomal DNA

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Abstract: The polymerase chain reaction (PCR) was used to amplify a fragment of the ribosomal DNA (rDNA) from species and undescribed populations of *Aphelenchoides* and *Ditylenchus angustus*. The PCR primers used were based on conserved sequences in the 18S and 26S ribosomal RNA genes of *Caenorhabditis elegans*. In *C. elegans*, these primers amplify a 1,292 base pair (bp) fragment, which consists of the two internal transcribed spacers and the entire 5.8S gene. Amplification products from crude DNA preparations of 12 species and populations of *Aphelenchoides* and from *D. angustus* ranged in size from approximately 860–1,100bp. Southern blots probed with a cloned ribosomal repeat from *C. elegans* confirmed the identity of these amplified bands as ribosomal fragments. In addition to the differing sizes of the amplified rDNA fragments, the relative intensity of hybridization with the *C. elegans* probe indicated varying degrees of sequence divergence between species and populations. In some cases, amplified rDNA from the fungal host was evident. Storage of *A. composticola* at −45 C for 2 years did not affect the ability to obtain appropriate amplified products from crude DNA preparations. Amplified rDNA fragments were cut with six restriction enzymes, and the restriction fragments produced revealed useful diagnostic differences between species and some undescribed populations. These results were consistent with previous studies based on morphology and isoenzymes. Three undescribed populations of *Aphelenchoides* were found to be different from all the species examined and from each other.

Key words: *Aphelenchoides*, *Ditylenchus*, fungi, nematode, PCR, rDNA.

Aphelenchoides is an economically important nematode genus that includes fungivorous species, species associated with insects, and parasites of plants. The plant-parasitic species are not highly specialized, either in their morphology or in their feeding habits (11); they are able to attack the aerial parts of plants as well as the root systems of a wide range of hosts. *Aphelenchoides besseyi* is a seed-borne nematode that causes the disease known as "white tip" in most rice growing areas (22). However, there are inconsistent reports of the pathogenicity of *A. besseyi*, with marked variations of yield loss between different areas (4). For example, Buangsuwon et al. (5) reported that no symptoms of *A. besseyi* attack were observed on rice plantations in Thailand, despite widespread infection; by contrast, *A. besseyi* caused severe symptoms in rice crops in India (24). Recently, Hooper and Ibrahim (17) described two new species of *Aphelenchoides*, *A. nechaleos* and *A. paranechaleos*, from rice. *Aphelenchoides nechaleos* was extracted from infected rice samples from Sierra Leone, while *A. paranechaleos* was found with *Ditylenchus angustus* in infected rice from Vietnam. Preliminary experiments (Ibrahim and Perry, unpubl.) have shown differences between *A. besseyi*, *A. nechaleos*, and *A. paranechaleos* in their pathogenicity on rice, and it is important that the species are identified correctly. The identification of species of *Aphelenchoides* is very difficult because of their morphological similarity, although the enzyme profiles of *Aphelenchoides* species from different regions of the world are useful to identify species (21).

Other nematode species, such as *D. angustus*, may occur concurrently with *Aphelenchoides* species on rice. *Ditylenchus angustus* was first discovered in Bangladesh by Butler (7) as the causal organism of "Ufra" disease of rice, and it has been recognized as a serious pest of rice in several different countries (2,8,15,24,27).

The application of molecular biology techniques to the identification and sys-

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tematics of plant-parasitic nematodes is proving to be informative and of practical value (6,18). In recent years, the polymerase chain reaction (PCR) (25) has extended the use of nucleic acid-based taxonomy to include investigations in which very little biological material is available and(or) specific regions of nematode DNA are amplified for detailed analysis (3,9,12, 14,26). Many studies involving plant-parasitic nematodes have concentrated on the amplification and analysis of ribosomal DNA fragments (9,12,26). The rDNA is well suited for systematic studies in a variety of organisms (16). It is present in both prokaryotes and eukaryotes and is generally abundant in the genome. Furthermore, different parts of the tandem ribosomal repeats evolve at different rates, and this provides areas with varying degrees of sequence divergence for analysis.

The ribosomal RNA genes in most eukaryote nuclear genomes are typically arranged in tandem repeats of a basic transcription unit separated by a nontranscribed spacer (NTS). Each repeat consists of three rRNA genes; the 18S, 5.8S and 26S genes (or their equivalents), with two internal transcribed spacers (ITS) flanking the 5.8S gene and an external transcribed spacer at the 3′ end of the 18S gene. The rDNA is considered to be useful for phylogenic and diagnostic studies because different parts of the repeat evolve at different rates. Areas of the repeat can thus be selected for study depending on the systematic problems being addressed (16). Because the spacer regions are generally considered to be the most variable, they provide informative comparisons between closely related taxa.

The objective of this study was to use PCR to amplify a fragment of the rDNA array, comprising two internal transcribed spacers and the 5.8S gene, from species and populations of *Aphelenchoides* and from *D. angustus*. Restriction fragment length polymorphisms (RFLPs) in the rDNA fragment were used to compare and differentiate the nematode species and populations used.

Materials and Methods

Nematode material: The designations and sources of nematode species were as follows: two populations of *A. besseyi* from rice plants (*Oryza sativa*) from India and Sierra Leone; *A. bicaudatus* from a Pit-Pit plant (*Setaria palmaefolia*) originally from Papua New Guinea; *A. arachidis* from ground nuts (*Arachis hypogaea*) from Nigeria; *A. fragariae* from fern (*Asplenium nidus*) from California; *A. hamatus* from strawberries (*Fragaria X ananassa*) from Bristol, England; *A. nechaleos* from rice plants from Sierra Leone; *A. paranechaleos* from rice plants from Vietnam; *A. composticola* from mushroom (*Agaricus bisporus*) compost, England. Three populations of undescribed *Aphelenchoides* species were also included: one from leaves and moss from Surrey, England, one from mushroom compost, Hertfordshire, England, and one from rose roots, Guernsey. *Ditylenchus angustus* was originally received from Hau Giang Province, Vietnam, and cultured on rice (cv. IR-36) as previously described (19).

The populations of *Aphelenchoides* spp. were routinely maintained on the fungi *Botrytis cinerea* or *Rhizoctonia cerealis* growing on potato dextrose agar Petri dish cultures at 20–23 C. Possible effects of storage on subsequent amplification of DNA products was examined by including samples of *A. composticola* that had been extracted from *B. cinerea* or *R. cerealis* agar cultures and stored at −45 C for 2 years.

Nematode extraction: All the *Aphelenchoides* spp. were collected from culture plate lids and thoroughly washed with several changes of distilled water. Each species was transferred to separate 1.5-ml Eppendorf tubes and stored at −45 C. Suspensions of *D. angustus*, extracted as previously described (19), were filtered through a cotton wool milk filter to remove dead or inactive nematodes and then were poured into a Petri dish containing potato dextrose agar and left overnight. The nematodes moved up to the lid through the agar, leaving any contamination (agar and much of the fun-

gal host) behind and were collected and processed as above.

Extraction of nematode DNA: The nematode samples used were composed of mixed stages, approximately 90% J4 and adults. An aliquot of nematodes containing 10–30 individuals in 5 µl distilled water was transferred to a hand-held Eppendorf homogeniser (Biomedix Inc., UK) and crushed in 20 µl of lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Nonidet P-40 [Sigma, UK], and 100 µg/ml proteinase K [Sigma, UK]). The tube was capped and placed at 95 C for 3 minutes. The crude DNA extracts were stored at −45 C and were suitable for the PCR-mediated amplification of rDNA without further treatment.

Isolation of fungal DNA: Botrytis cinerea or *Rhizoctonia cerealis* were grown in Petri dishes on potato dextrose agar at 20–23 C. Fungal mycelia were collected by scraping the agar, and the DNA was extracted from aliquots of approximately 20 µl volume as described above for nematodes.

PCR amplification: Nematode rDNA fragments were amplified using PCR primers (26) based on conserved sequences in the 18S and 26S ribosomal RNA genes of *Caenorhabditis elegans* (13). The primer sequences were as follows:

18S primer—
5′ TTGATTACGTCCCTCCCTTT 3′.
(Bases 2503–2523 *C. elegans*)

26S primer—
3′ GGAATCATTGCCGCTACTTT 5′.
(Bases 3774–3794 *C. elegans*)

PCR amplifications were performed in duplicate in a Hybaid OmniGene Thermal Cycler. Each reaction was prepared on ice and consisted of 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM dNTPs, 30 pM each primer, 1 µl (1–50 ng) of nematode template DNA, 2.5 units of Taq DNA polymerase and sterile distilled water to a final volume of 25 µl. A control without nematode DNA template was always included. Reactions were overlaid with one drop of light mineral oil and subjected to temperature cycling conditions as follows: 1 cycle of 3 minutes at 94 C, 1 minute at 55 C, 1 minute at 72 C; 38 cycles of 1 minute at 94 C, 1 minute at 55 C, 1 minute at 72 C; finally, 1 cycle of 1 minute at 94 C, 1 minute at 55 C, 5 minutes at 72 C.

Agarose gel electrophoresis: Amplification products were analyzed by electrophoresis through 1.2% agarose gels buffered in 0.5X TBE, which contained ethidium bromide at a final concentration of 0.02 µg ml^{-1}. DNA was visualized on a UV transilluminator and photographed using Polaroid type 667 film.

Restriction endonuclease digestion: Six restriction enzymes (BRL Life Technologies, Inc., UK) were used to digest the amplified rDNA fragments. The enzymes used were: *Alu*I, *Eco*RI, *Hae*III, *Mbo*II, *Rsa*I, and *Sau*96. Restriction digests were performed according to the suppliers' recommendations. Each digest contained 5 µl of an appropriate amplification reaction in a total volume of 20 µl. Restriction digests were incubated for at least 4 hours at 37 C and then analyzed by agarose gel electrophoresis as described above.

Southern blotting and DNA hybridization: Amplified rDNA fragments separated by agarose gel electrophoresis were transferred by capillary blotting to a nylon membrane (Hybond-N, Amersham Int., UK) and probed with a cloned ribosomal repeat from *C. elegans* (13). Southern blots were prehybridized for 1–2 hours at 65 C in prehybridization solution containing 5X SSPE, 5X Denhardt's solution, 0.5% SDS and 100 µg ml^{-1} sheared and denatured salmon DNA. Sufficient prehybridization solution was used to provide 1–2 ml cm^{-2} of nylon membrane. Hybridization was carried out for 12–16 hours at 65 C in prehybridization solution containing 20 ng ml^{-1} of cloned ribosomal repeat from *C. elegans* that had been ^{32}P-labeled using random oligonucleotide priming (Multiprime, Amersham Int., UK).

After hybridization the membranes were washed three times for 20 minutes each in 1X SSC, 0.1% SDS at 60 C fol-

lowed by two washes for 20 minutes each at higher stringency in 0.1X SSC, 0.5% SDS at 65 C. Autoradiography was done at −80 C for 12 hours to 10 days.

Results

Amplification of the 5.8S gene and the bordering internal transcribed spacers (ITS) from crude DNA preparations of 12 species and populations of *Aphelenchoides* and from one population of *D. angustus* yielded products ranging in size from approximately 860–1,100 base pairs (Fig. 1A). The negative controls, without template DNA, gave no amplification products. Long-term storage (2 years) of nematodes at −45 C did not affect the ability to amplify an appropriate product from the extracted crude DNA samples (Fig. 1A, samples 9, 10, and 11).

Four of the nematode samples, *A. besseyi* from India, *Aphelenchoides* mushroom isolate, *A. arachidis,* and *Aphelenchoides* rose isolate, gave two amplification products with these rDNA primers (Fig. 1A, samples 2, 3, 4, and 14, respectively). Amplification of the 5.8S/ITS region from the fungi *B. cinerea* and *R. cerealis* (Fig. 1C, samples 1 and 5) indicated that in three out of the four nematode samples giving two bands (Fig. 1, samples 2, 3, and 14), the lower band was of a size suggesting fungal origin and reflected the particular host (*B. cinerea*) on which these nematodes were cultured. However, this was not the case for *A. arachidis* (Fig. 1, sample 4), which consistently gave two PCR amplification products, neither of which appeared to be derived from the fungal host.

To verify that the amplification products obtained from these nematode species and populations were of ribosomal DNA origin, a Southern blot of PCR reaction products was probed with a cloned ribosomal repeat from *C. elegans* (Fig. 1B). Under stringent washing conditions, all of the amplification products showed hybridization with the ribosomal probe, but the relative intensity of the bands indicated varying levels of sequence divergence in this region of the ribosomal repeat. The most extreme example of this was with the species *A. nechaleos* (Fig. 1B, sample 16) which showed no band on the autoradiograph after 10 hours exposure and only became visible after 24 hours (results not shown).

Amplified rDNA fragments were digested with six restriction enzymes and showed numerous differences in the length and number of the restriction fragments derived from the 12 nematode species and isolates (e.g., Figs. 2,3). For identification purposes, digestion of the rDNA fragments with *Alu* I (Fig. 2) was the most successful because, with the exception of the two populations of *A. besseyi* (Fig. 2, lanes 2 and 7), all of the nematode species could be differentiated. The rDNA fragments amplified from *A. besseyi* from India and *A. besseyi* from Sierra Leone are apparently the same size and, based on the restriction enzymes used in this study, they are also of similar sequence. Only one of the enzymes, *Hae* III (Fig. 3), separated these two isolates.

The two recently described species from rice, *A. nechaleos* and *A. paranechaleos* (17), could be separated by five of the six restriction enzymes; *Alu* I (Fig. 2), *Hae* III (Fig. 3), *Mbo* II, *Sau* 96, and *Eco*R I (data not shown), despite having rDNA fragments of very similar size. This result, which indicates that *A. nechaleos* and *A. paranechaleos* show marked sequence divergence in this region of the rDNA, is consistent with the DNA hybridization using the *C. elegans* ribosomal repeat (see above). This probe showed little hybridization with the amplified rDNA fragment of *A. nechaleos* but had comparatively more homology with *A. paranechaleos.*

Three populations of undescribed *Aphelenchoides* species from mushroom compost, rose roots, and moss (samples 3, 14, and 15, respectively) were readily differentiated from each other (e.g., enzymes *Alu* I and *Hae* III, Figs. 2 and 7) and from the other described species.

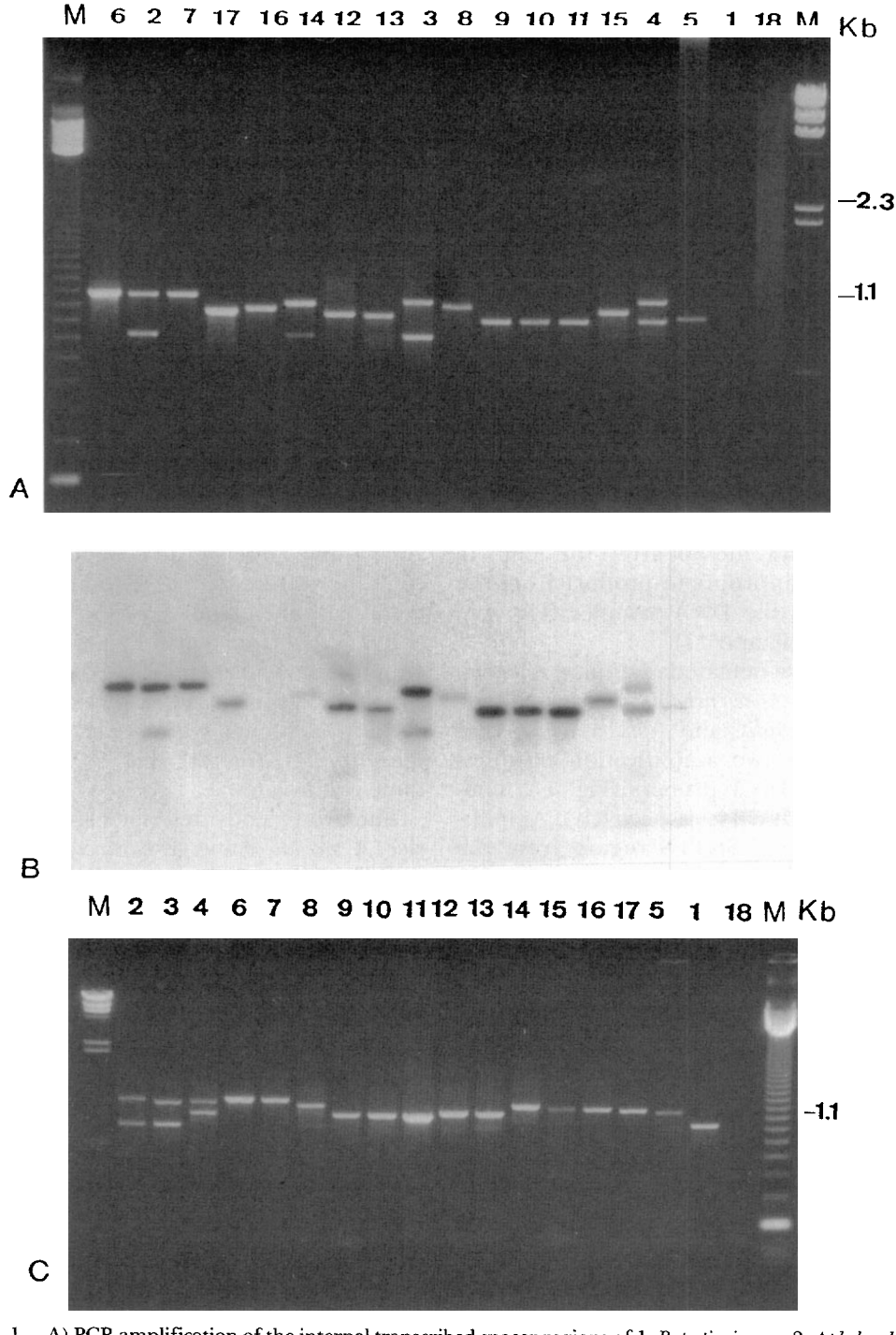


FIG. 1. A) PCR amplification of the internal transcribed spacer regions of 1: *Botrytis cinerea;* 2: *Aphelenchoides besseyi* from India; 3: *Aphelenchoides* sp. from mushroom compost; 4: *A. arachidis;* 5: *Rhizoctonia cerealis;* 6: *Ditylenchus angustus;* 7: *A. besseyi* from Sierra Leone; 8: *A. hamatus;* 9: *A. composticola* from *B. cinerea* cultures (fresh extracts); 10: *A. composticola* from *B. cinerea* cultures (stored at −45 C for 2 years); 11: *A. composticola* from *R. cerealis* cultures (stored at −45 C for 2 years); 12: *A. bicaudatus;* 13: *A. fragariae;* 14: *Aphelenchoides* sp. from rose roots; 15: *Aphelenchoides* from moss; 16: *A. nechaleos;* 17: *A. paranechaleos;* 18: negative control. B) The same gel transferred to nitrocellulose and probed with a ^{32}P labelled *C. elegans* specific probe. C) Further aliquots of the same samples loaded on a gel in order of fragment size. M = Lambda phage/*Hin*d III molecular weight marker (kb).

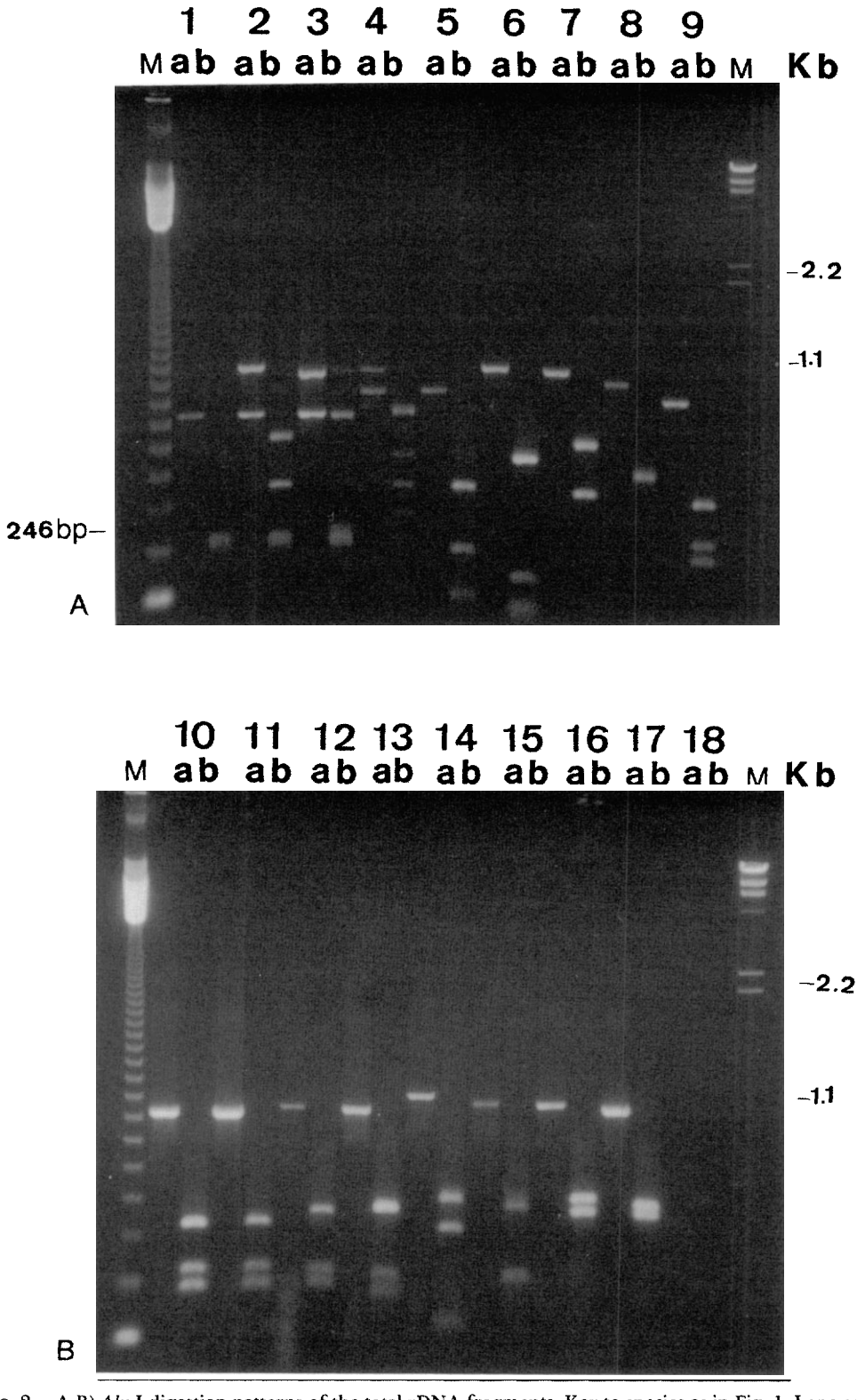
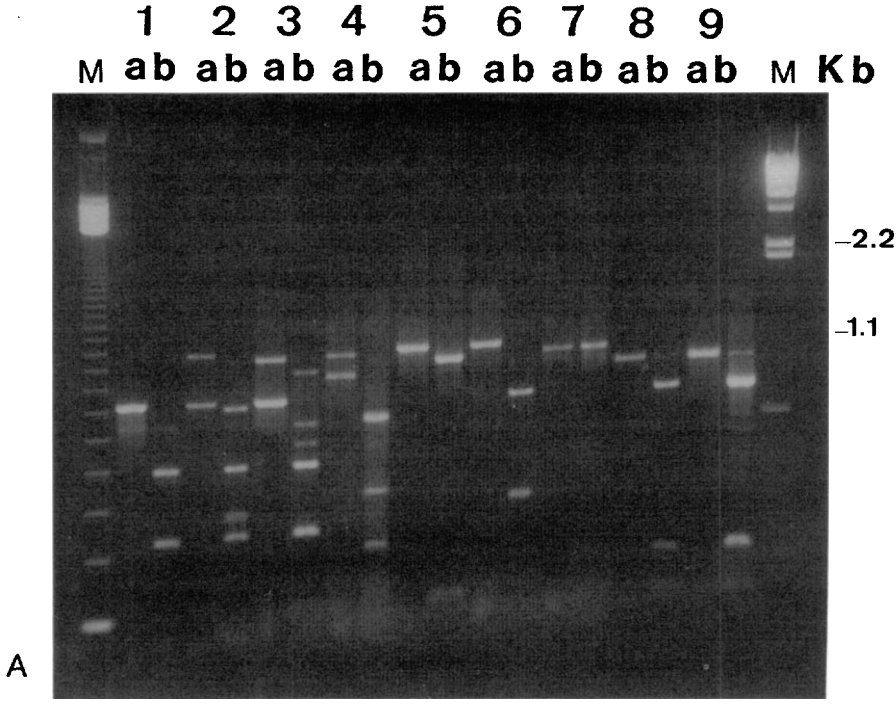


FIG. 2. A,B) *Alu* I digestion patterns of the total rDNA fragments. Key to species as in Fig. 1. Lane a: total rDNA fragment (uncut); b: same sample digested with restriction enzyme.



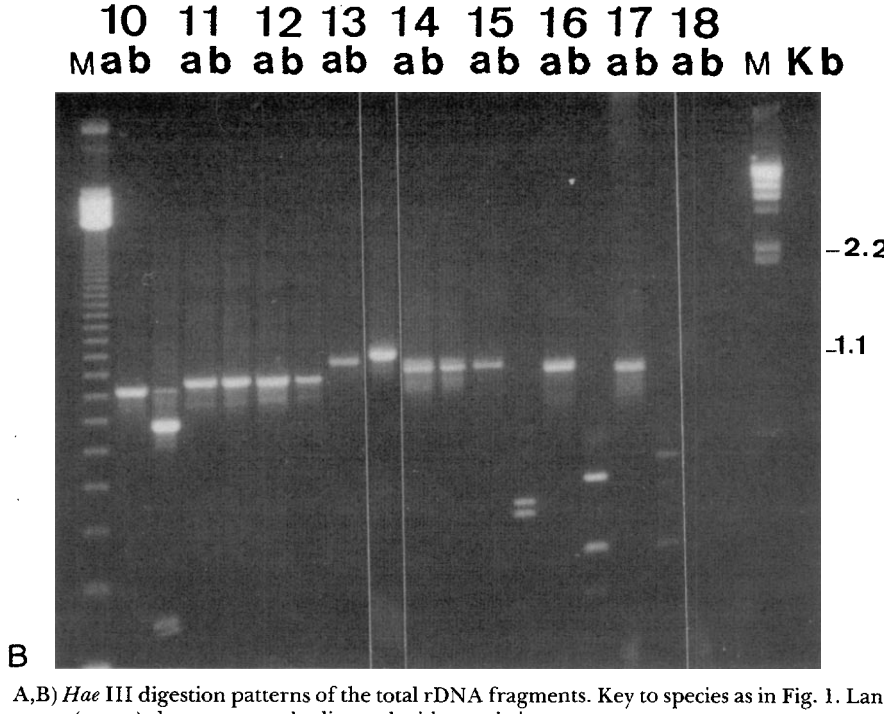


FIG. 3. A,B) *Hae* III digestion patterns of the total rDNA fragments. Key to species as in Fig. 1. Lane a: total rDNA fragment (uncut); b: same sample digested with restriction enzyme.

Discussion

RFLPs in the rDNA have been used previously to differentiate species and populations of plant-parasitic nematodes (9,26, 28). Ferris et al. (12) compared DNA sequence of the ITS regions of some cyst nematodes. Although optimistic about this general approach for phylogenetic analysis, they concluded that this region of rDNA was conserved in cyst nematodes (despite *Heterodera avenae* being clearly divergent), especially among the few species of the *H. schachtii* group that were studied. The low level of sequence divergence in the ITS regions of *H. schachtii* and *H. glycines* is not consistent with other biochemical and molecular studies that have indicated genetic dissimilarity between these species (1,20,23). By contrast, RFLPs in the amplified 5.8S/ITS region readily differentiated populations and species of *Xiphinema* (26). Clearly, the utility of this region of rDNA for taxonomic investigations varies according to the particular nematode group studied. In the case of *Aphelenchoides,* the 5.8S/ITS region has accumulated much sequence variation and, thus, was capable of readily differentiating species. The technique may be useful for finding new species; the three undescribed populations of *Aphelenchoides* were clearly differentiated from each other and from the named species by the numbers and positions of the restriction fragments. These results reinforce the proposed separate taxonomic status of these populations.

The relative degree of length heterogeneity seen in the amplified rDNA fragments from *Aphelenchoides* was surprising when compared to *Xiphinema* in which the same amplified rDNA fragments was consistent at 1.5 kb in all 16 species and populations studied (26). Most of the length heterogeneity seen here is most likely to result from sequence rearrangements, such as insertions or deletions, within the ribosomal repeat rather than from an accumulation of point mutations. This is because the length heterogeneity observed in the amplified rDNA fragment and the differential hybridization of the *C. elegans* probe indicate extensive sequence divergence that would be difficult to explain solely by an accumulation of changes at single bases during the evolution of the genus *Aphelenchoides.*

The isolate of *A. arachidis* used in this study consistently gave two PCR amplification products. Southern blot analysis indicated that both of these products were of ribosomal origin. Within this population (or possibly the species), there would appear to be two distinct sizes for the 5.8S/ ITS region. It is not clear from this work whether both size variants observed can occur together in the same individual or whether different sizes are found in different subpopulations. De Giorgi et al. (9) observed a similar phenomenon in *X. diversicaudatum* and *X. pachtaicum,* both of which yielded two amplified products (700 bp and 800 bp), with primers directed to the large ribosomal subunit. In this particular case, the 5′ end of the large rRNA gene was amplified from individual worms, and both size variants were present in the same genome.

The results of the present study confirm those of Ibrahim et al. (21), where nonspecific esterase banding patterns effectively separated species of *Aphelenchoides.* Ibrahim et al. (21) reported a minor difference, based on the presence or absence of a weak band, between two populations of *A. besseyi* from different locations. In the present study, a difference was also observed between populations of *A. besseyi* from India and Sierra Leone when *Hae* III was used. Intraspecific differences have been detected with species of *Meloidogyne* collected from various geographical regions (10).

The specific status of *A. nechaleos* and *A. paranechaleos* (17) is supported by the present work and by Ibrahim et al. (21). Both species were originally from infected rice, and their appearance is very similar to *A. besseyi,* an economically important nematode pest of rice. Recent observations on the biology of *A. nechaleos* and *A. paranechaleos* indicate a marked difference

in their pathogenicity to rice (Ibrahim and Perry, unpubl.), and inconsistencies in the damage to rice reportedly caused by *A. besseyi* may be due to incorrect nematode identification. Accurate identification of nematodes is central to all efficient control programs, and this is increasingly true on rice, where morphologically similar species of differing pathogenicity from the genera *Aphelenchoides* and *Ditylenchus* can be found together. Classical descriptions of nematode morphology are being supported and extended by biochemical and molecular biology techniques. Rapid, reliable diagnostic approaches are required, and PCR clearly has great potential for development as the basis of such an approach and for providing information about phylogenetic relationships.

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