TAXONOMIC DIFFERENTIATION OF THREE SPECIES OF *DITYLENCHUS* (NEMATODA) AND THEIR RACES USING RIBOSOMAL DNA ANALYSIS

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Title of Thesis/Project/Extended Essay

Taxonomic differentiation of three species of Ditylenchus and their races using ribosomal DNA analysis

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ABSTRACT

The nematode genus *Ditylenchus* has about 80 recognized species which are differentiated from each other on the basis of morphological characters and size. These characters are known to be influenced by environmental factors, and morphological techniques alone are insufficient to consistently identify all of the species in this genus. Intraspecific variation occurs even within *Ditylenchus dipsaci*, the stem nematode, in the form of morphologically indistinguishable races that parasitize different hosts. For practical reasons, such as crop rotation, a reliable method is needed to identify these biologically distinct populations.

Analysis of the DNA encoding ribosomes provides a powerful means of finding molecular markers that differentiate at both the species and the subspecific levels. This study examined the ribosomal cistron of populations of three species of *Ditylenchus* from different geographic locations to identify a region in the rDNA which contained specific sequences for delineating the species of *Ditylenchus* and a region in the rDNA which contains sequences for separating host races within *D. dipsaci*. The rDNA of populations of *D. dipsaci, D. destructor*, and *D. myceliophagus* was examined for sequence and length polymorphisms using restriction enzyme analysis. Southern hybridizations using the whole ribosomal cistron and the 18s ribosomal gene as probes, and restriction of the PCR-amplified internal transcribed spacer (ITS),

generated diagnostic restriction fragment length polymorphisms (RFLPs) among the populations of *Ditylenchus*.

The three species showed RFLPs in the three ribosomal regions examined, namely the ribosomal cistron, the 18s gene, and the ITS. Southern blot analysis using a 1.1 kb probe comprising the 18s gene differentiated *D. dipsaci* from the other two species and one of the five races of *D. dipsaci* from the others. A 7.5 kb ribosomal cistron probe differentiated between the three species and generated unique patterns for each of the host races of *D. dipsaci*.

The ITS, amplified by PCR, from *D. myceliophagus* and the host races of *D. dipsaci* was about 0.9 kb, while the ITS sizes in *D. destructor* populations were 1.0 kb and 1.2 kb. Digesting the PCR products with restriction enzymes consistently discriminated between the three species, and clearly differentiated between two biologically and geographically distinct populations of *D. destructor*. Some restriction enzymes showed also that the giant race of *D. dipsaci* had a unique RFLP pattern.

This study demonstrated for the first time that it was possible to differentiate between the host races of *D. dipsaci* by probing Southern blots with the whole ribosomal cistron. Analysis of rDNA in this thesis complements conventional morphological methods of nematode identification, and has unraveled some of the taxonomic problems associated with the nematode genus *Ditylenchus*.

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INTRODUCTION

Nematodes belonging to the genus *Ditylenchus* Filipjev, 1936, have historically been difficult to identify. The general morphology between the species is rather uniform. However, there is considerable intraspecific biological variation, as shown by the occurrence of host races in the stem nematode, *Ditylenchus dipsaci* Kuhn, 1857, which has made identification and control of this agronomic pest difficult.

Ditylenchus represents a large, adaptable, cosmopolitan and biologically diverse group of nematodes in the family Tylenchidae. Most of the species inhabit soil and feed on fungi, but some members of the genus occur in the frass of bark beetles, and others have evolved into specialized plant parasites (Sturhan and Brzeski, 1991).

Although most of the species of *Ditylenchus* have not been well studied, the biology of three of the most economically important species is well known. These are *D. myceliophagus* Goodey, 1958, *D. destructor* Thorne, 1945 and *D. dipsaci*. The stem nematode, *D. dipsaci*, was the third plant parasitic nematode ever to be described.

The three species each occupy a different ecological niche. The mushroom spawn nematode, *D. myceliophagus*, is a parasite of cultivated mushrooms. The potato rot nematode, *D. destructor*, is a fungal feeder which opportunistically attacks the roots, stolons, bulbs and

rhizomes of such plants as potatoes, irises and daffodils, and has been described more recently, on peanuts (De Waele *et al.*, 1989). There are strict international quarantine regulations against the potato rot nematode, and the presence of *D. destructor* in the field precludes the planting of potatoes in some countries (Esser and Smart, 1977).

D. dipsaci, the stem nematode, is a migratory endoparasite of the aerial parts of about 500 species of angiosperms, including agronomic crops, ornamentals and weeds. Heavy infestations of this species may occur and the loss of 60-80% of the crop is not unusual (Sturhan and Brzeski, 1991). The ability of fourth stage juveniles to resist desiccation and nematicides has made control costly. The main method of control of *D. dipsaci* is crop rotation. However, this is made difficult by the presence of morphologically indistinguishable races of *D. dipsaci* which have different host preferences. A practical technique for rapidly and reliably identifying these host races of *D. dipsaci* is not available.

In spite of the differences in biology, the morphological differences between the species of *Ditylenchus* are slight, and are based on the relative shape and size of the tail, relative length of the stylet and post-vulval sac, and the number of cuticular lateral lines (Fortuner, 1982). Measurements of these characters vary according to stage of nematode development, culture medium and temperature (Barraclough and Blackith, 1962). For example, culturing *D. destructor* on different hosts

produced a deviation in body length of 64% (Evans and Fisher, 1970). The conclusion from a recent taxonomical review of the genus *Ditylenchus* was "In effect identification is difficult and preparation of a workable key is almost impossible" (Brzeski, 1991). It is clear that a more sensitive and reliable technique is needed to distinguish *Ditylenchus* at the species level.

Although identification of *Ditylenchus* species may be difficult in general, the three species used in this study are morphologically distinct (Fortuner, 1982) and do not interbreed (Webster, 1967). These results, and those of biochemical analyses by Webster and Hooper (1968), Hussey (1979), and a recent molecular analysis by Palmer *et al.* (1991) confirm that *D. myceliophagus*, *D. destructor*, and *D. dipsaci* are distinct species. In order to clarify the taxonomy of the unknown species of this genus it is appropriate to analyse the DNA of these three confirmed species first.

In several species of plant-parasitic nematodes, difficulties arise at the subspecific level, with host races. Historically, there has been confusion between the designation of species and host races in *D. destructor* and, in particular, among populations of *D. dipsaci*.

Species are generally considered to be natural populations which are reproductively isolated from each other and which are following distinct and independent evolutionary paths (Mayr, 1969). Biological races are

genetically different forms of the same species that may be partially reproductively isolated and demonstrate definite differences in biology. Host races are regarded as biological races which have particular host preferences (Sturhan, 1971; Diehl and Bush, 1984). In nematology, host races are sometimes confused with host-associated sibling species (Sturhan, 1971; 1983; Viglierchio, 1971).

Sibling species morphologically resemble each other so closely that, though they are reproductively isolated, they can be recognized only after careful study of biochemical, cytological, or behavioral traits. The sibling species designation can be "established with certainty only in situations where the different populations occur sympatrically without substantial interbreeding or introgression" (Diehl and Bush, 1984).

Initially, several host races were observed in *D. destructor*. There was some question as to the validity of the races because although there were morphological differences among populations found on various hosts, all populations could interbreed successfully (Wu, 1960). Smart and Darling (1963) suggested that there were different physiological host races of *D. destructor* based on different host ranges of some of the Florida populations, but this argument was discarded and the situation clarified when *D. myceliophagus*, which they had considered to be a race of *D. destructor*, was defined as a separate species.

Recently, nematologists in South Africa were surprised to find that peanuts were being devastated by *D. destructor*, a nematode species not previously known to attack peanuts. This South African "peanut" population does not readily infect potatoes (De Waele *et al.*, 1991), and it reproduces at higher temperatures than do other populations of *D. destructor* (De Waele and Wilken, 1990). It has been suggested that the South African population is a biological race of *D. destructor*, with a more limited host range than that of other populations of the species (De Waele *et al.*, 1989).

In contrast to the potato rot nematode, the host races of the stem nematode have been well studied. No other nematode species has a comparable degree of intraspecific variation. The number of host races of *D. dipsaci* has grown from 11 (Seinhorst, 1957) to more than 20 (Sturhan, 1971). These host races are morphologically indistinguishable, except for a larger "giant" race, found on field beans (Hooper, 1983; Caubel and Leclerq, 1989). The host races differ mainly in host preference, but exhibit varying degrees of reproductive isolation such as partial or complete reproductive incompatibility (Sturhan, 1964; Eriksson, 1965; Webster, 1967; Ladygina and Barbarashova, 1982) and a wide range of chromosome numbers, from 2n=24 to 2n=56 (Barbarashova, 1986; Lamberti *et al.*, 1988).

Phytonematologists typically identify nematode "host races" by inoculating a series of host plants in a differential test. Seinhorst (1957) distinguished 11 races of *D. dipsaci* with nine host plants. The pathogenicity varied in different geographic populations of the same host race (Smith, 1951; Edwards and Taylor, 1963). When trying to screen crop varieties for resistance, plant breeders discovered that several populations of the same host race of stem nematode gave different reactions depending on which host plant varieties they used (Griffiths *et al.*, 1957; Whitehead *et al.* 1987, Cook and Evans, 1988). Some races are polyphagous and reproduce on a wide range of plant hosts, but other populations, such as the giant or alfalfa races, are confined to a more limited host range (Griffin, 1975). A workable host differential test for the identification of the races of the stem nematode has not been attained.

Karyotyping can be useful for identifying intra-specific forms of parasites, and is also indicative of populations undergoing speciation (Triantaphyllou and Hirshmann, 1980). Populations of *D. dipsaci* on cultivated plants have a karyotype of $2n=24$, (except for the polyploid giant race where $2n=56$), while *D. dipsaci* found on weeds has a much more heterogenous diploid karyotype, from $2n=36$ to $2n=56$ (Ladygina and Barabashova, 1982). In addition to host preference, geographical origin may affect chromosome number (D'Addabbo Gallo *et al.*, 1982).

All races presently combined in the collective species *D. dipsaci* appear to reproduce by obligate amphimixis, requiring males and females (Kostyuk, 1988). Consequently, cross-breeding experiments illustrate possible reproductive isolation. Cross-breeding experiments were accomplished between some host races using onions as the common host (Webster, 1967) or callus tissue (Eriksson, 1965; 1974) to help bridge the reproductive gap between host races. It was discovered that many of the races interbred and produced fertile progenies. The morphology of hybrid progeny often differed from that of the parents, and the host range of the hybrids was either the same as or intermediate between that of the parents.

Some inter-race crosses are not always successful and some races demonstrate partial or complete reproductive incompatibility. Specifically, stem nematode populations on cultivated plants do not interbreed with those found on weeds (Ladygina and Barabashova, 1982). The weed group of *D. dipsaci* is polyploid in origin and might represent an independent species (Barabashova, 1986).

Sturhan (1971) indicated that the polyploid giant race is a sibling species. The giant race of *D. dipsaci* infects only Faba bean, and occurs mainly in the Mediterranean region. It is more virulent and causes different symptoms in Faba bean than do other *D. dipsaci* races, such as the oat race, which also infect Faba beans (Hooper, 1983, Caubel and

Leclercq, 1989). The giant race is so called because it is slightly larger than the other races, with fourth stage juveniles being slightly over 1 mm, while the other races have juveniles under 1 mm (Hooper, 1983). Ladygina (1988) reported that viable F_1 progeny resulted when the giant race was cross bred with diploid races, but acknowledged that the giant race was regarded as a sibling species. Sturhan (1983) suggested that the progeny produced was "obviously infertile" and reiterated that the polyploid is a sibling species.

Even certain combinations of diploid host races from different crops were unsuccessful. Fertile offspring were produced in one combination but not in the reciprocal. In other combinations, such as the tulip/red clover race and the oat/tulip race, only infertile offspring were produced (Eriksson, 1974). This apparent reproductive isolation of certain races suggests that these subspecific populations are undergoing speciation.

Biologically distinct host races of *D. dipsaci* which were morphologically identical were recognized by Ritzema Bos in 1879 (Sturhan and Brzeski, 1991). As a consequence, species within *Ditylenchus* were among the first nematodes to be subjected to electrophoretic and biochemical analyses (Benton and Meyers, 1966).

Protein electrophoresis enabled the separation of species of *Ditylenchus* (Dickson *et al.*, 1971; Palmer, 1989), but attempts to separate races of *D. dipsaci* using esterase or catalase profiles were

inconclusive (Eriksson and Granberg, 1969; Hussey and Krusberg, 1971). Only single band differences between specific enzymes were detected from electrophoregrams between nematodes cultured on different hosts, and cluster analysis failed to divide the races into groups (Palmer, 1989). Moreover, it appears that protein analysis does not give clear results with mixed stages (Hussey, 1979).

Polyclonal antibodies developed against the surface antigens of *D. dipsaci* races (Gibbons and Grandison, 1968; Webster and Hooper, 1968) were insufficiently sensitive to distinguish these subspecific populations. In one instance, replicate samples of the red clover race harvested during the same season, but at different field sites showed marked differences (Gibbons and Grandison, 1968). However, Palmer (1989) used monoclonal antibodies against the oat race to screen six races by ELISA. Cluster analysis of the results suggested an intraspecific division into two groups: (1) giant, oat and alfalfa races and (2) narcissus and red and white clover races.

The technique of protein analysis was successfully exploited in species separation primarily because the protein differences reflected differences in the nucleotide sequences that make up a gene. Nevertheless, gene expression can be influenced by the environment and this could obscure some taxonomic differences. Recent developments in

molecular biology offer the distinct advantage of facilitating studies on the genetic make-up, rather than on gene products.

Analysis of DNA is a more direct measure of molecular variability than is protein analysis. Only 15-20% of the nematode's genome translates into a protein product of gene expression (Burrows, 1990). Besides, not all proteins may be expressed at all stages of the lifecycle, whereas DNA remains constant through all stages of development (Burrows, 1990).

Molecular analysis recently has provided precise and reliable identification of species of other nematode genera. Initially this was done for different species of the free-living nematode *Caenorhabditis* (Emmons *et al.*, 1979; Snutch and Baillie, 1984), and subsequently for species of the plant parasitic root-knot nematode *Meloidogyne* (Curran *et al.*, 1986; Curran and Webster, 1987; Hyman, 1990). Unique patterns generated by the repeated DNA from total genomic DNA showed some promise for differentiating the races of four species of *Meloidogyne* (Curran *et al.*, 1986), the races within the soybean cyst nematode, *Heterodera glycines* (Kalinsky and Huettel, 1988) and some geographic isolates of the insect-parasitic nematode *Heterorhabitis* (Curran and Webster, 1989; Smits *et al.*, 1991).

In these early experiments, total genomic DNA was digested with restriction enzymes, stained with ethidium bromide and size fractionated

on agarose gels. The patterns of bands, restriction fragment length polymorphisms (RFLPs), represent repeating sequences of DNA. However, for practical purposes the faint bands in the digested nematode DNA "smear" generated by this technique are often difficult to see, so researchers have progressed to Southern hybridization experiments, in which the digested genomic DNA is transfered to a filter, and hybridized to specific radioactively labeled DNA fragments, or probes, which allow the RFLPs to be seen clearly when exposed to X-ray film.

Castagnone-Sereno *et al.* (1991) have used random probes to differentiate geographic isolates of *Meloidogyne incognita* and Carpenter *et al.* (1992) differentiated three South Carolina populations of *M. arenaria* race 2 with the same technique. Southern hybridizations using a mitochondrial probe showed three different mitochondrial genome sizes within one field population of the mosquito parasite *Romanomermis culicivorax* (Powers *et al.*, 1986). Geographic subspecific populations of the pinewood nematode, *Bursaphalenchus xylophilus* were differentiated by total genomic probes (Bolla *et al.*, 1988), a ribosomal probe (Webster *et al.*, 1990), a *C. elegans* muscle protein gene (Abad *et al.*, 1991), and two random repetitive DNA probes (Tares *et al.*, 1992).

The two main factors to consider when initiating DNA analysis of a group of organisms are, firstly, what region in the genome is most

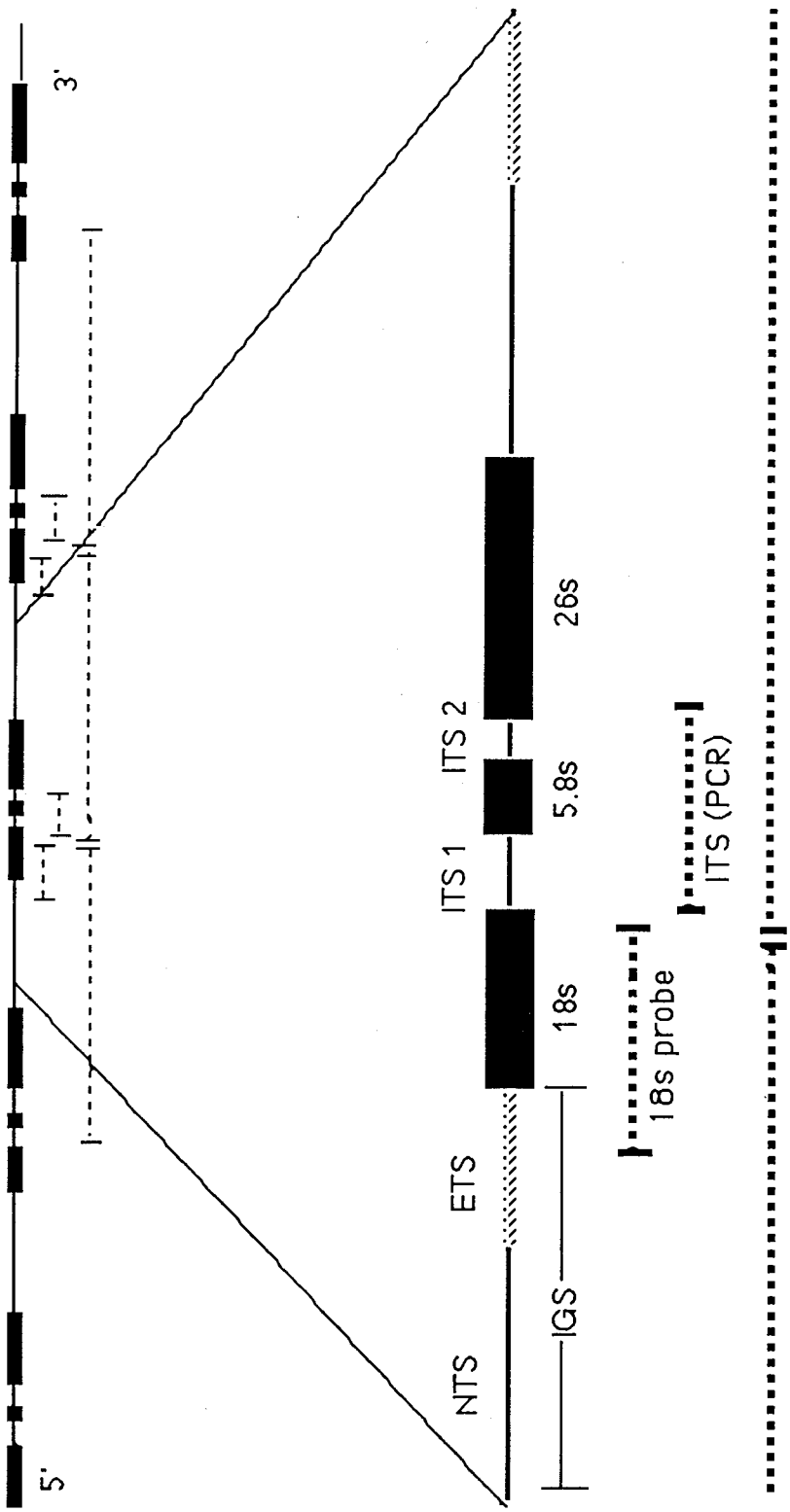
appropriate for the particular study, and secondly, which molecular techniques should be used to survey the chosen region in order to answer the particular question.

Ribosomal DNA has proved to be a particularly interesting and useful region for investigating reproductively isolated populations at all levels of taxa. Each ribosomal gene encodes for the 18s, 5.8s, and 26s ribosomal RNA's, separated by the internal transcribed spacer (ITS), ITS 1 and ITS 2 (Figure 1). The ribosomal cistrons, arranged 5' end to 3' end, repeated head to tail, are separated from each other by the intergenic spacer (IGS), which is itself composed of the external transcribed spacer (ETS) and the nontranscribed spacer (NTS) (Gerbi, 1985; Rogers and Bendich, 1987).

Ribosomal DNA has been well studied in many organisms. The DNA sequences of the ribosomal encoding 18s, 5.8s, and 26s genes are necessarily more conserved than the ITS or IGS introns. Thus, depending on the organism, regions which differentiate a range of taxa from the level of genera to subspecific populations can be found (Allard and Honeycutt, 1991).

In attempts to define species of plants, animals, fungi and protists using rDNA analysis, the consensus is that the IGS is most variable followed by the ITS, then the 18s and 26s genes (Rollinson *et al.*, 1986; Ellis and Bumstead, 1990; Allard and Honeycutt, 1991; Gardes *et al.*,

FIGURE 1 Schematic diagram of the general organization of ribosomal coding and noncoding regions within the ribosomal cistron. The regions that were probed and PCR amplified are shown.


5'
3'
NTS
ETS
ITS 1
ITS 2
18s
5.8s
26s
IGS
18s probe
ITS (PCR)
whole rDNA cistron probe

1990). With nematodes, the IGS has been employed to generate species specific probes for the pinewood nematode *Bursaphelenchus xylophilus* (Webster *et al.*, 1990), and restriction analysis of the ITS has facilitated species differentiation of the dagger nematode, *Xiphenema* spp. (Vrain *et al.*, 1992).

In contrast to species separation, the identification of subspecific populations by analyzing the ribosomal cistron presents a much less clearcut story. Many investigators have distinguished among intraspecific isolates by using Southern blot analysis of the whole ribosomal gene, coding and noncoding regions, as a probe. RFLPs between various isolates and geographic populations usually have been attributed to insertions and deletions in the IGS (Rollinson *et al.*, 1986; Gardes *et al.*, 1991), but even when the probe lacked the IGS, high intraspecific variation has been reported such as in the fungus *Rhizoctonia solani* (Jacabaji-Hare *et al.*, 1990). The other spacers, the ITS 1 and 2, do not appear to differentiate amongst fungal isolates of *Chondrostereum* (Shamoun *et al.*, 1991).

In some instances, RFLP analysis in the IGS reflects even greater variation than at the population level. Sequence and length differences in the IGS have been detected between individuals, particularly in plants (Arnold *et al.*, 1990; Furnier *et al.*, 1990; van den Ven *et al.*, 1990; Molnar and McKay, 1991), and also in animals (Barbieri *et al.*, 1990).

In phytopathogenic nematodes, a homologous 26s ribosomal DNA gene probe could differentiate several geographic isolates of the root-knot nematode, *Meloidogyne arenaria* race 2 (Hiatt *et al.*, 1991). Two groupings of populations of the pinewood nematode were determined by dot blots using a homologous IGS fragment as a probe (Webster *et al.*, 1990). Despite some exceptions, the available information indicates that the IGS region of the ribosomal gene generally contains sufficient, if not too much, sequence and length variation to differentiate subspecific populations.

Interestingly, the ability to detect species specific rDNA regions varies from one study to the next, depending on whether the length of the spacer region or the base pair sequence is measured (Allard and Honeycutt, 1991). For example, in rats, length variation of the IGS is a major source of variation amongst genera, while length of the ITS enables differentiation down to the species level. The situation is completely reversed when nucleotide sequence, rather than fragment length, is analyzed. In this instance, the ITS sequence information reveals more variation amongst rat genera, and the IGS is unique to species.

So, even if the appropriate rDNA region is selected, the sensitivity of the technique employed to detect variation can greatly affect the success or failure in defining the specific region of a species or population.

Southern transfer of digested DNA to a nylon membrane, and the subsequent hybridization with one, or several, rDNA gene probes has been used to successfully detect sequence and length variation between species or subspecific groupings of organisms (Curran, 1991). Although this is a reasonably sensitive method for surveying the fine and gross organization of the ribosomal gene, Southern hybridization analysis can require a large amount of DNA and typically, though not necessarily, calls for the use of radioactive techniques for visualization. This latter feature is undesirable for practical field purposes.

Polymerase chain reaction (PCR) amplification of specific DNA fragments is an elegant technique for examining specified gene regions using negligible amounts of the original DNA (Mullis and Faloona, 1987). A DNA region between two convergent primers is enzymatically amplified without using conventional cloning procedures. The PCR amplified fragment can then be restricted with enzymes and the resulting RFLPs are seen after ethidium staining of agarose gels.

Harris *et al.* (1990) were able to amplify the mitochondrial DNA of single juveniles from *Meloidogyne* populations using PCR. Depending on the enzyme used, RFLPs separated four species of *Meloidogyne*, but no host race-unique RFLPs were discovered. Harris *et al.* suggested that the *Meloidogyne* population patterns corresponded to isogenic female lines of these parthenogenetic plant-parasitic nematodes. Beckenbach *et*

al. (1992) used a sequence analysis from a PCR amplified heat shock fragment to show the relationships between geographic populations of the amphimictic pinewood nematode, *B. xylophilus*. The origin of the domestic isolates of the pig parasite, *Trichinella* spp., were investigated by successfully developing PCR primers from a repeated sequence (Dick *et al.*, 1992). A major limitation to the use of PCR is the selection of primers to amplify the gene region of choice. Fortunately, some of these primers can be used "universally" on many different organisms because they are located in conserved regions of the genome (Vrain *et al.*, 1992).

In *Ditylenchus*, the genetic heterogeneity represented by the races is disguised by their morphological similarity. Separation of these genetically distinct groups is critical for any further study of their biology.

Ditylenchus comprises populations whose taxonomy is subject to debate, and populations or races that have not been completely identified or accurately described. For example, it was suggested that the *D. dipsaci* giant race is a sibling species (Sturhan, 1983), and that the South African peanut population of *D. destructor* is a separate biological race (De Waele *et al.*, 1989). DNA analysis of this genus would complement the previous taxonomic work, and could provide conclusive new evidence in some instances.

The objective of this study was to use some of the homologous and heterologous regions of the ribosomal gene cluster, such as the

complete rDNA cistron, the 18s gene and the ITS, to separate three species of *Ditylenchus* and several of their races.

MATERIALS AND METHODS

Origin and culture of Ditylenchus populations

The nematode isolates used in this study originated from North America, Europe and Africa from cultivated and noncultivated plant hosts (Table 1). Some populations were derived directly from the field, and others had been in culture for a number of years. David Hooper (Rothamsted Experimental Station, Harpenden, Herts, England) provided cultures of *D. myceliophagus*, *D. destructor* and the *D. dipsaci* giant and teasel races. Dr. Fred Gommers (Wageningen Agricultural University, Wageningen, The Netherlands) supplied populations of the *D. dipsaci* tulip and potato races. Dr. Richard Peaden (USDA-ARS, Prosser, Washington, U.S.A.) and Dr. K. Bengt Eriksson (Agricultural University, Sweden) both supplied populations of *D. dipsaci* alfalfa race. Dr. George Caubel (INRA, Le Rheu, France) sent me populations of the *D. dipsaci* beet, red clover, and Faba bean races. Dr. Ann MacGuidwin (University of Wisconsin, Madison U.S.A.) and Cheryl Venter (Grain Crops Research Institute, Potchefstroom, South Africa) both provided *D. destructor* cultures.

Most nematodes were subjected to DNA extraction within a month of arrival, but the *D. myceliophagus* and two of the *D. destructor* populations were maintained in culture for the duration of this project.

TABLE 1 Origin of *Ditylenchus* populations used in this study

	CODE	HOST	ORIGIN	SOURCE
D. myceliophagus	MYC	mushrooms	Germany	fungal culture plates
D. destructor	UK	potatoes	Ireland	fungal culture plates
	WIS	potatoes	U.S.A. (Wisconsin)	excised roots
	SA	peanuts	South Africa	dried peanut hulls
D. dipsaci				
giant	GIA	faba beans	England (Hertford-shire)	dried zuchini marrows
teasel	TEA	teasel	England (Somerset)	dried teasel stems
tulip	TUL	tulips	Netherlands	dried tulip bulbs
potato	POT	potatoes	Netherlands	dried potatoes
alfalfa	ALF	alfalfa	U.S.A. (Washington)	greenhouse alfalfa plants
alfalfa	FAL	alfalfa	France	alfalfa callus
red clover	RCL	red clover	France (Domagne)	greenhouse red clover plants
giant	FGI	faba bean	France (Le Rheu)	greenhouse faba beans
beet	BET	beets	France (Alsace)	greenhouse faba beans

Both the *D. myceliophagus* and *D. destructor* cultures from the U.K. were maintained on *Rhizoctonia cerealis* cultures on potato dextrose agar (Difco specifications) in 30 X 15 mm Petri dishes at 22^{0}C in the dark. The nematodes were transferred to fresh plates every 3-6 months by rinsing them off the lids, where they had congregated, onto fresh fungal plates using a small volume of sterile water. Initially, the Wisconsin isolate of *D. destructor* was cultured on sterile excised corn roots (Huettel and Rebois, 1985). After one year this *D. destructor* culture was successfully transferred to fungal culture for ease of maintenance. All the cultures were maintained in a dark incubator at 22-24^0 C.

Extraction of genomic DNA

Nematode infested, dried or fresh plant material was cut into small pieces and placed in plastic Petri dishes (30 X 15 mm) containing distilled water. Nematodes were allowed to migrate from the plant material at room temperature for up to 10 days, but often after about 5 days many free-living nematodes had appeared. In this case, the non-plant-parasitic nematodes were removed from the water solution using fine forceps or a Pasteur pipette under a dissecting microscope. To insure a living population of *Ditylenchus*, the nematodes were collected daily in a 500 mesh sieve, stored at 4^{0}C and supplied periodically with fresh water.

Nematodes raised on fungal cultures were rinsed from the Petri dish lids with a small volume of distilled water and concentrated using a 500 mesh sieve. The aqueous suspension of nematodes was transferred to 10 ml plastic Falcon test tubes, cooled at -20 ^{0}C for 20 minutes to sedate vigorously moving nematodes and centrifuged for 10 min at 1000 rpm.

To extract DNA, the pelleted, centrifuged nematodes were resuspended in six volumes of proteinase K buffer [0.1 M Tris (pH 8.5), 0.05 M EDTA, 0.2 M NaCl, 1% SDS] and 1.0 mg/ml proteinase K and then instantly frozen in liquid nitrogen. The frozen nematodes were ground in a mortar and pestle and the powder was warmed gradually up to 65^{0}C. The nematode sample was transferred again to 10 ml phenol resistant Falcon tubes, and extracted twice with 1:1 phenol:SEVAG [chloroform/isoamyl alcohol 24:1 (v/v)]. The DNA was precipitated for 1 h, or overnight in two volumes of 95% ethyl alcohol at -20^{0}C, pelleted, washed in two volumes of cold 70% ethyl alcohol, repelleted and allowed to air dry under a sterile laminar flow hood. The pellet was resuspended in 1 X TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and stored at 4^{0}C. When appropriate, the DNA was treated with one-twentieth volume boiled RNAse I (1 mg/ml) for 30 min at 37^{0}C.

Gel electrophoresis and restriction digests

DNA samples were size fractionated in horizontal agarose gels (Maniatis *et al.*, 1982) of 0.7% to 1.5% concentration depending on the

size of the fragments. Nuclear or PCR amplified DNA samples (1.0 to 2.0 μg) were digested with 10 to 20 units of restriction endonucleases with four, five, or six-base recognition sites, using the manufacturers' recommended procedures [Bethesda Research Laboratories (BRL), Boehringer Manheim, Pharmacia]. Fragment distances were measured with a ruler and their size determined using a regression line generated by the GEL program.

Southern transfers and hybridizations

Initially, Southern blots of digested *Ditylenchus* DNA were probed with the whole ribosomal cistron. There were two Southern hybridization experiments: one compared three species of *Ditylenchus* with *Bursaphelenchus*; the other compared five host races of *D. dipsaci*. The 7.5 kb ribosomal probe was then washed off the blots, and the blots were reprobed with a portion of the ribosomal gene, the 1.1 kb 18s probe.

There was not enough DNA from some *D. dipsaci* and *D. destructor* samples to perform Southern hybridization analysis with all populations.

From 1.0 to 2.0 μg of genomic DNA were digested with *Rsa-1*, and the fragments were separated by electrophoresis on 0.7% agarose gels in 1 X TBE buffer (0.089 M Tris,0.089 M Boric Acid,0.0025 M EDTA) for 12 h at 20 volts. DNA was transferred unidirectionally to nylon

membrane (Genescreen, Dupont), after 8-10 min of hydrolysis in 0.25 M HCl, using an alkaline transfer method and a vacuum blotting unit (LKB Vacugene). The gel was soaked for 1 h in 1.5 M NaCl/1.5 M NaOH at 40 cm.H_20 vacuum. The membranes were rinsed in 2 X SSPE solution [1XSSPE = 0.18 M NaCl, 10 mM sodium phosphate, 1 mM disodium EDTA (pH 7.4)] to remove residual agarose, crosslinked at 254 nm for 40 s in a Stratagene crosslinker, and then baked for 30 min at 80^0C. The membranes were stored at -20 C.

For the 18s Southern hybridization experiments, the nylon filters were stripped of the rDNA probe and subsequently reprobed. The filters were washed twice for 5 min in a solution of 1.5M NaCl and 0.5M NaOH at room temperature and neutralized for 30-60 min in a solution of 1M ammonium acetate and 0.02M NaOH.

Nylon filters were soaked prior to hybridization for at least 12 h at 62^0C in 5 X SSPE, 5 X Denhardts [50XDH = 1% of the following: 0.02% bovine serum albumin (w/v), Ficoll (molecular weight 400,000) and polyvinyl pyrrolidone] and 0.3% SDS, using 1.0 to 4.0 ml hybridization solution per 100 cm^3 of filter. Hybridization conditions were identical, except for the addition of denatured probe labelled with ATPs-^{32}P(800-3000ci/mmol). The filters were washed four times at 45^0C in 2 X SSPE and 0.2% SDS. If the Geiger counter indicated that the filter had much background radioactivity after the second wash, the wash temperature

was raised to 62^0 C. The damp filter was then sealed in plastic and exposed to preflashed Kodak X-Omat K X-ray film at -70°C with an intensifying screen (Dupont Lightning Plus) for not less than 4 h.

Source of probes

Two probes, developed earlier in this laboratory by K. Beckenbach (Webster *et al.*, 1990), were used in these experiments. The 7.5 kb ribosomal gene fragment (pBx2) from the Japanese isolate of *Bursaphalenchus xylophilus* Ibaraki (Webster *et al.*, 1990) comprises the ribosomal cistron from the 5' end of the 18s coding unit to the 3' end of the 28s coding unit. The 18s probe (pBm3), from *B. mucronatus*, is a 1.1 kb *Hae III* fragment in the *Sma I* site of plasmid pVZ1 (Webster *et al.*, 1990). The probe contains the entire 18s subunit coding region.

Preparation of probes

The inserts were removed from the plasmid by digestion with *EcoRI* and isolated by gel purification of DNA (Maniatis *et al.*, 1982). The DNA fragments were recovered from agarose pieces by placing the bands in dialysis tubing with 500 μl 1XTE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and sealed using removable clips (Maniatis *et al.*, 1982). The tubing containing the agarose pieces was then electrophoresed at 20-70 volts for 2-12 h. After the DNA had moved into the buffer, the electrode charge

was briefly reversed for 30 s to dislodge the DNA from the membrane. The buffer was collected, centrifuged for 15 min to pellet the residual agarose and transferred to a fresh tube. The DNA fragments were precipitated in 95% ethanol and one-tenth volume 8M ammonium acetate at -20^{0}C, washed in cold 70% ethanol, vacuum desiccated, and resuspended in 1XTE buffer.

DNA probes used for Southerns were synthesized using random oligonucleotides labeled with ATP-^{32}P (Feinberg and Vogelstein, 1983). Unincorporated nucleotides were removed by chromatography through Sephadex-50 spun columns (Maniatis *et al.*, 1982).

Polymerase chain reaction

The ITS PCR product, which includes ITS 1, ITS 2 and the 5.8s subunit, was amplified for all populations using 100-200 ng of genomic DNA. Amplification kits were used following manufacturers' recommendations (Perkin-Elmer Cetus, Promega). The reaction mixture consisted of 3-6 μl of diluted template (1:100 in sterile, double-distilled water) template, 8 μl dideoxynucleotidetriphosphate (dNTP) mix (2mM each nucleotide), 0.2 μl *Taq* polymerase (2.5 units), 10 μl 10X buffer [10X: 30mM mgCl, 500 mM KCl, 100 mM Tris (pH 8.3), 0.1% gelatin], 0.25 μl (1.2 mg/ml) 10X desalted primers made up to 50 μl total volume with double-distilled sterile water. This was overlaid with 50 μl mineral oil.

Negative controls, consisting of the mixture less template DNA but brought to the same volume, were run with each amplification. Reaction profiles were (denature 1 min 30 s at 96°C, anneal 30 s at 50°C, extension 4 min at 72°C), 40 cycles of (denature 45 s at 96°C, anneal 30 s at 50°C, extension 4 min at 72°C) and a final cycle (denature 45 s at 96°C, anneal 30 s at 50°C, extension 10 min at 72°C). PCR products from several amplification runs were pooled for digests.

These universal ITS primers were developed from *Xiphenema bricolensis* (Vrain *et al.*, 1992) and are each 21 base pairs long. One primer is situated 171 base pairs from the 3' end of the 18s ribosomal subunit, and the other primer is 80 base pairs into the 5' end of the 26s gene. PCR primers were supplied by Dr. Thierry C. Vrain (Agriculture Canada, Vancouver, B.C., Canada). Fourteen restriction enzymes, which recognize four, five or six base pair sites, were employed to differentiate the three species. Not all gels were shown, but all fragment sizes were tabulated in the Appendix. Examples of enzymes that generated RFLPs which were likely to separate host races, or that produced common bands between species, were shown in this study.

RESULTS

I. DIFFERENTIATION OF SPECIES WITHIN THE GENUS *DITYLENCHUS*

Southern hybridizations probed with the complete ribosomal cistron

The *B. xylophilus* rDNA probe hybridized strongly to the positive control *B. mucronatus*. The rDNA probe showed four bands in *D. myceliophagus*, six bands in *D. destructor*, and seven bands in *D. dipsaci* teasel (Figure 2; Table 2). All four nematode species showed RFLPs.

D. destructor shared three bands (Figure 2, see arrows) with *D. myceliophagus*. *D. destructor* shared one band at 0.50 kb with *D. dipsaci* and all four nematode species had a common band of 0.35 kb.

Some of the species generated fainter RFLPs than others because less DNA was used for these species. In order to show these faint patterns more clearly in Figure 2, a longer exposure of the radioactive blot to the film was shown for *D. dipsaci* (84 h exposure) and *D. myceliophagus* (12 h exposure) than the exposure for *B. mucronatus* and *D. destructor* (4 h exposure). *D. destructor* had two bands of larger molecular weight than the other nematodes because the DNA was probably only partially digested.

FIGURE 2 Autoradiograph of a Southern transfer of *Rsa-1* digested genomic DNA from three species of *Ditylenchus* and *Bursaphalenchus mucronatus* probed with the whole 7.5 kb rDNA cistron (isolated from *B. xylophilus*) under moderately stringent conditions (compilation of film exposures, from 4 hrs to 18 hrs)

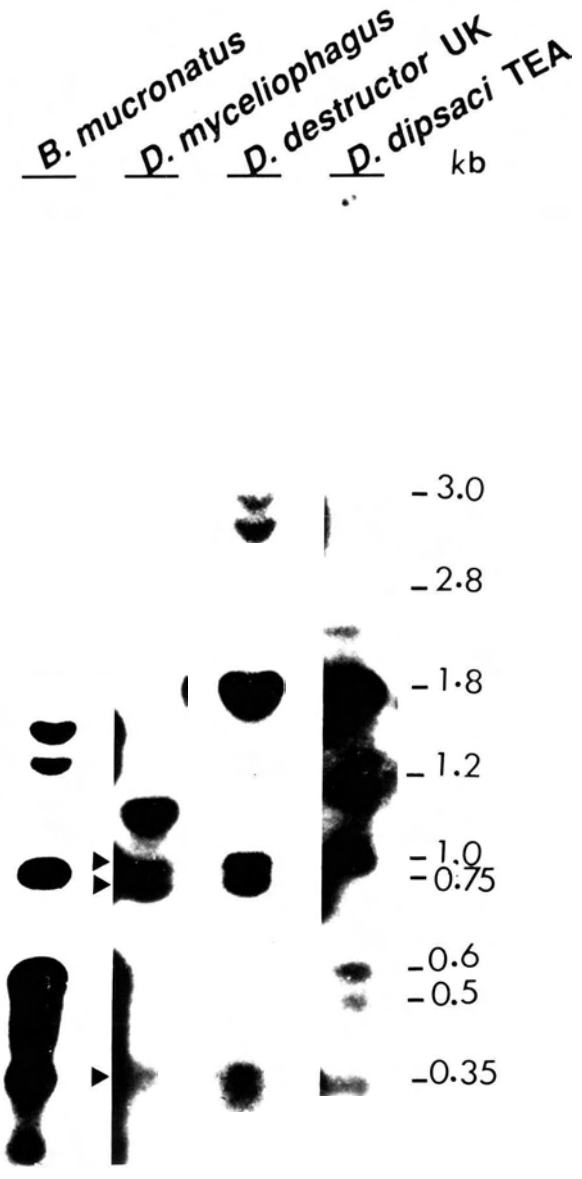

B. mucronatus
D. myceliophagus
D. destructor UK
D. dipsaci TEA
kb
3.0
2.8
1.8
1.2
1.0
0.75
0.6
0.5
0.35

TABLE 2 Fragment sizes (kb) resulting from a Southern transfer of *Rsa-1* digested *Ditylenchus* spp. and *Bursaphelenchus mucronatus* genomic DNA probed with the whole ribosomal cistron. Bands illuminated by both the 18s probe (see Figure 3) and the rDNA cistron (see Figure 2) are denoted by an asterisk (*).

B. mucronatus	*D. myceliophagus*	*D. destructor* UK	*D. dipsaci* TEA
		3.3	
		3.0	
			2.8
			2.4
		1.8	1.8
1.5*			
1.25*			
			1.2
	1.1		
1.0*			1.0*
	0.85*	0.85*	
	0.75	0.75	
			0.60
0.50*			0.50*
0.45			
	0.35*	0.35*	0.35
0.30*			
0.25*			

Southern hybridizations probed with the 18s ribosomal gene

The bound rDNA probe was washed away. The described Southern transfer of the digested DNA of *D. myceliophagus* (UK), *D. destructor* UK, *D. dipsaci* teasel (UK), and *B. mucronatus* was reprobed with a fragment from *B. mucronatus* containing a portion of the rDNA comprising most of the 18s rDNA gene (Figure 3; Table 2). In this experiment, the presence of faint bands were disregarded because they could represent residue from the previous ribosomal probe.

The control *B. mucronatus* exhibited six bands. The 18s gene also hybridized to two fragments in *D. myceliophagus*, *D. destructor* and *D. dipsaci*. *D. myceliophagus* and *D. destructor* shared a 0.85 kb fragment and a 0.35 kb fragment (Figure 3, see arrows). *D. dipsaci* showed two bands of 1.0 kb and 0.50 kb.

D. dipsaci and the control *B. mucronatus* showed unique RFLPs but the 18s probe generated identical patterns for *D. myceliophagus* and *D. destructor* so these species could not be distinguished using this probe.

PCR amplification of the ITS fragment and restriction analysis

Amplification of *D. myceliophagus* and all populations of *D. dipsaci* generated a single ITS fragment of 0.9 kb (Figure 4) . The PCR product of *D. destructor* isolates was either 1.0 kb or 1.2 kb (Figure 4).

FIGURE 3 Autoradiograph of the described Southern transfer of *Rsa-1* digested genomic DNA from three species of *Ditylenchus* and *Bursaphalenchus mucronatus* stripped and reprobed with the 1.1 kb 18s rDNA gene fragment (isolated from *B. mucronatus)* under moderately stringent conditions (72 h exposure).

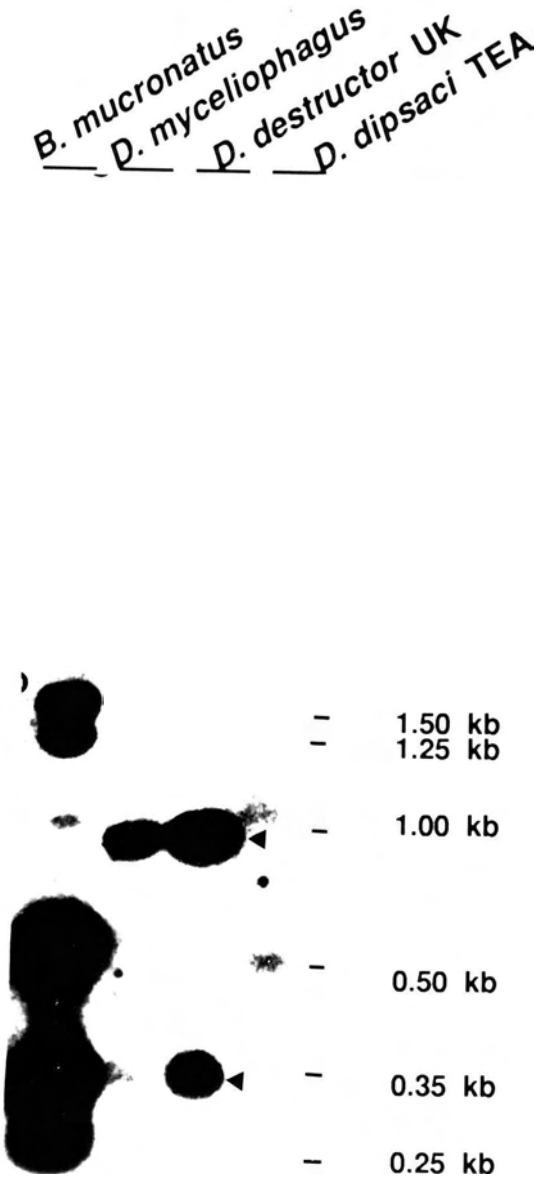
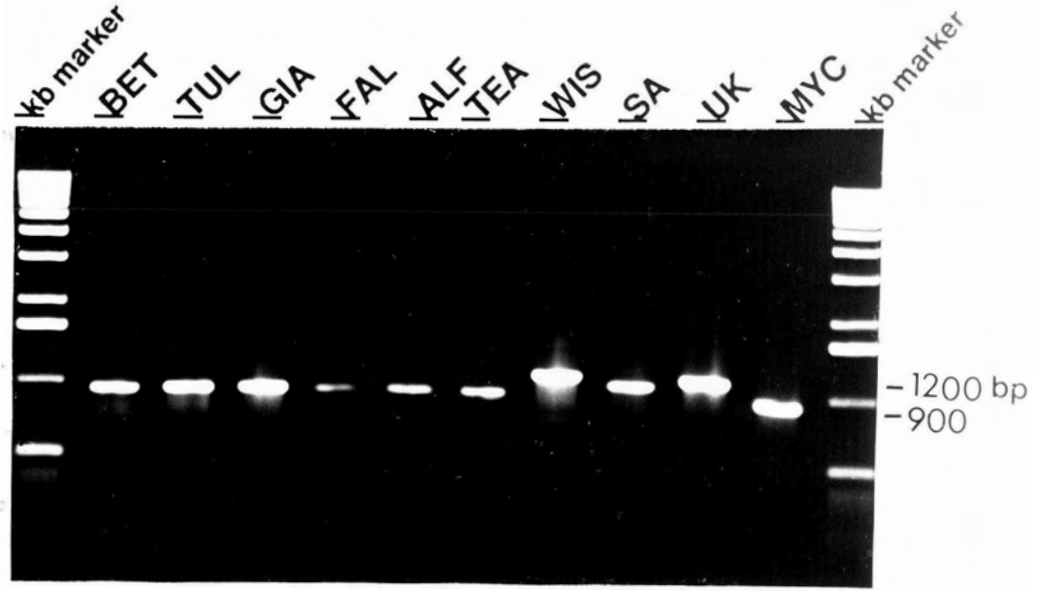

B. mucronatus
D. myceliophagus
D. destructor UK
D. dipsaci TEA
1.50 kb
1.25 kb
1.00 kb
0.50 kb
0.35 kb
0.25 kb

FIGURE 4 1.5% agarose gel of the PCR amplified internal transcribed spacer (ITS) of *Ditylenchus myceliophagus*, *D. destructor* and host races within *D. dipsaci*

undigested


λkb marker
λBET
λTUL
λGIA
λFAL
λALF
λTEA
λWIS
λSA
λUK
λMYC
λkb marker
1200 bp
900

All enzymes which restricted the DNA provided different patterns for each of the three species of *Ditylenchus* (Figures 5 to 10; Appendix). If the enzymes did not recognize any restriction sites, such as *Acc-1* (Appendix), then *D. myceliophagus* was indistinguishable from *D. dipsaci*, because the undigested ITS is the same size in those two species. The different genus, *A. rhyntium,* was digested as a control.

In general, there were few shared bands between the species, and the three species could be distinguished quite readily from each other by the RFLPs.

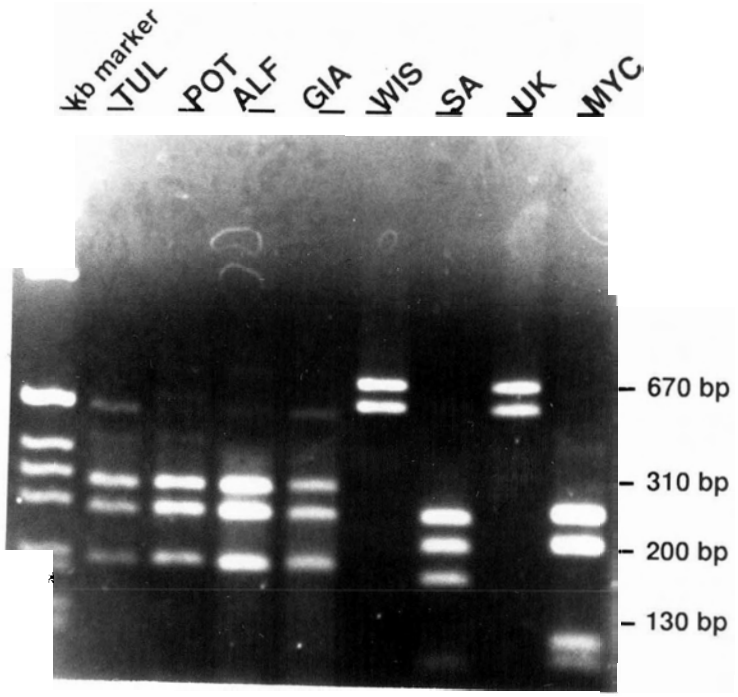
Fragments less than 0.10 kb were excluded from the Appendix because they are difficult to resolve using 1.5% agarose gels stained with ethidium bromide. Consequently, the sum of the fragment sizes did not always add up to the total 1 kb ITS fragment. Partial digests were easily detected if the sum of fragments exceeded 1 kb.

A PCR amplification of a mixed sample of *D. dipsaci* and *D. destructor* DNA generated two bands of 0.9 kb and 1.2 kb, corresponding to these respective species (Figure 11a). Also, PCR amplification showed a mixture of *D. dipsaci* (ALF) and *Aphelenchoides* spp. (APH) by producing two bands corresponding to each of these genera (Figure 11b).

FIGURE 5 1.5% agarose gel of the PCR amplified internal transcribed spacer (ITS) of *Ditylenchus myceliophagus*, *D. destructor* and host races within *D. dipsaci* digested with the restriction enzyme *Dde-1*

FIGURE 6 1.5% agarose gel of the PCR amplified internal transcribed spacer (ITS) of *Ditylenchus myceliophagus*, *D. destructor* and host races within *D. dipsaci* digested with the restriction enzyme *Rsa-1.* Faint bands ie. TEA, POT and GIA occured because there was only a small amount of DNA digested

Dde-1 **digested**



Rsa-1 **digested**

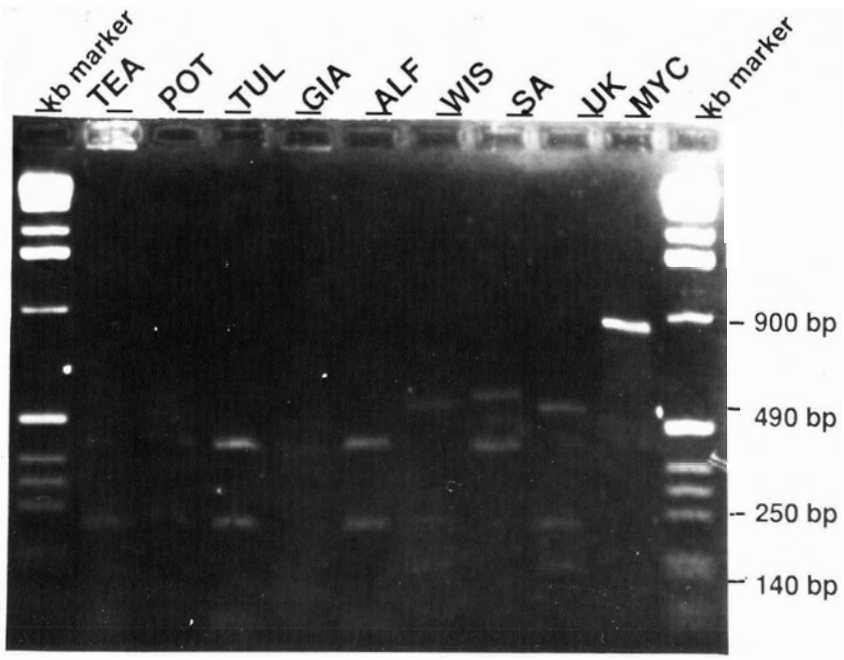
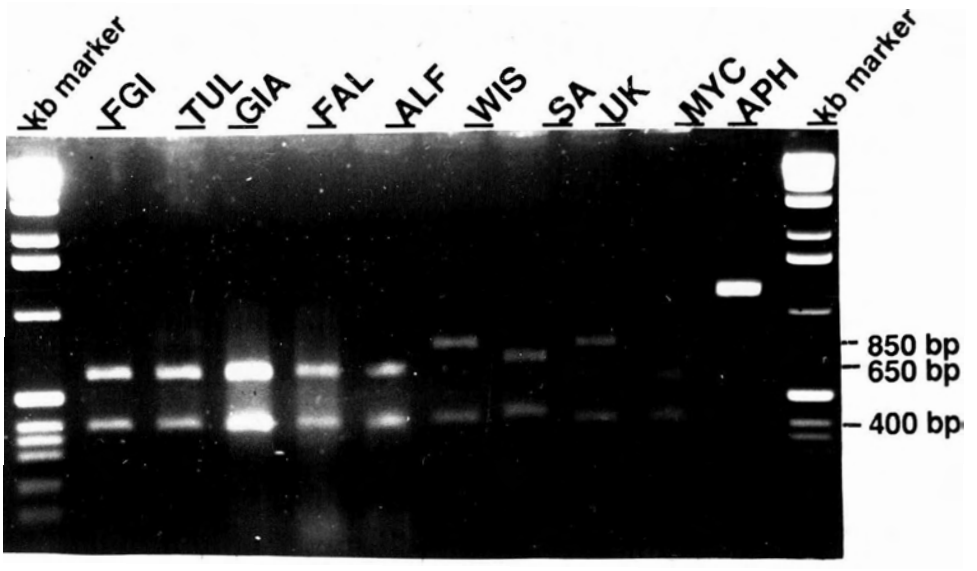


FIGURE 7 1.5% agarose gel of the PCR amplified internal transcribed spacer (ITS) of *Ditylenchus myceliophagus*, *D. destructor*, host races within *D. dipsaci* and *Aphelenchoides* (APH) digested with the restriction enzyme *Pst-1*. Faint bands occured because of small amounts of DNA ie. MYC.

FIGURE 8 1.5% agarose gel of the PCR amplified internal transcribed spacer (ITS) of *Ditylenchus myceliophagus*, *D. destructor*, host races within *D. dipsaci* and *Aphelenchoides* (APH) digested with the restriction enzyme *Hae-3*. The 1.0 kb fragment in GIA is a consequence of a partial digest.

Pst-1 **digested**



Hae-3 **digested**

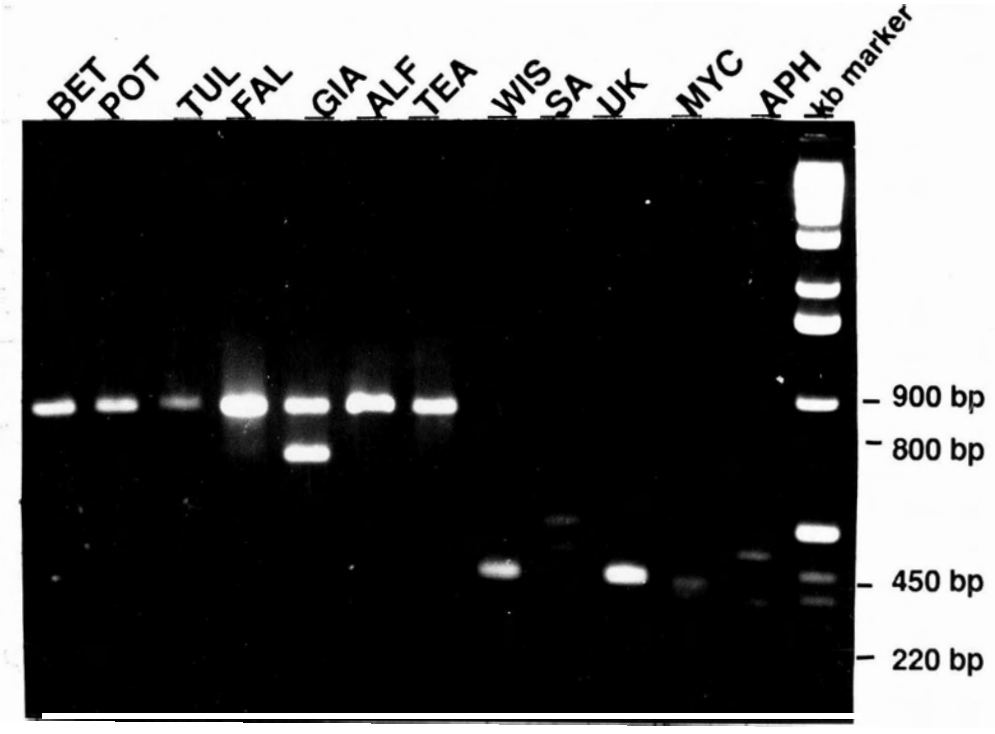
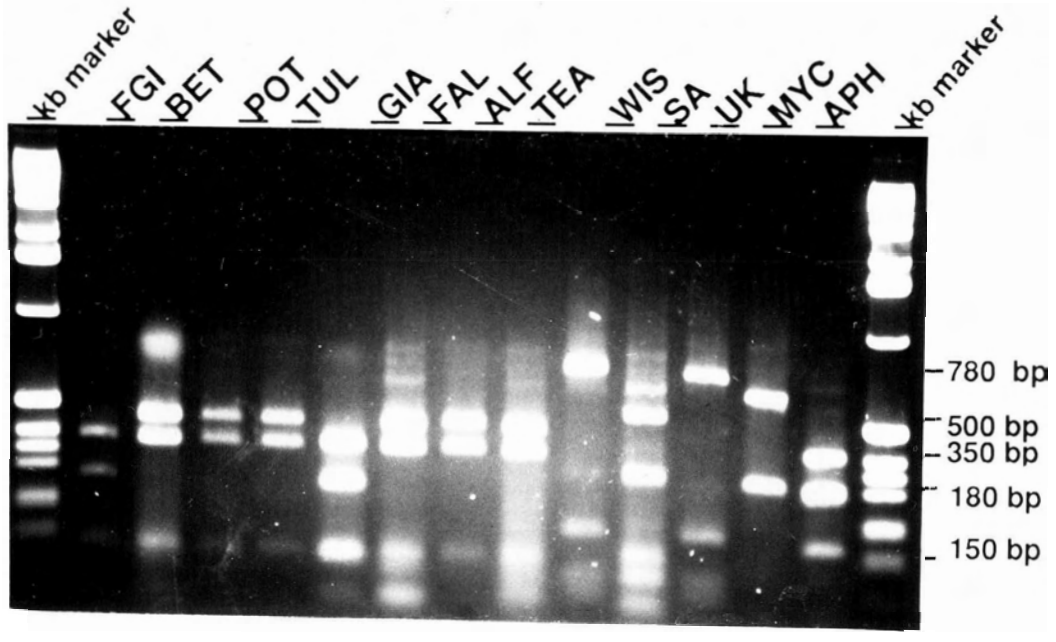


FIGURE 9 1.5% agarose gel of the PCR amplified internal transcribed spacer (ITS) of *Ditylenchus myceliophagus*, *D. destructor*, host races within *D. dipsaci* and *Aphelenchoides* (APH) digested with the restriction enzyme *Hinf-1*. The 0.9 kb fragment in BET is the result of a partial digest.

FIGURE 10 1.5% agarose gel of the PCR amplified internal transcribed spacer (ITS) of *Ditylenchus myceliophagus*, *D. destructor*, host races within *D. dipsaci* and *Aphelenchoides* (APH) digested with the restriction enzyme *Hpa-2*. The 0.9 kb fragment in GIA is the result of a partial digest.

Hinf-1 digested



Hpa-2 digested

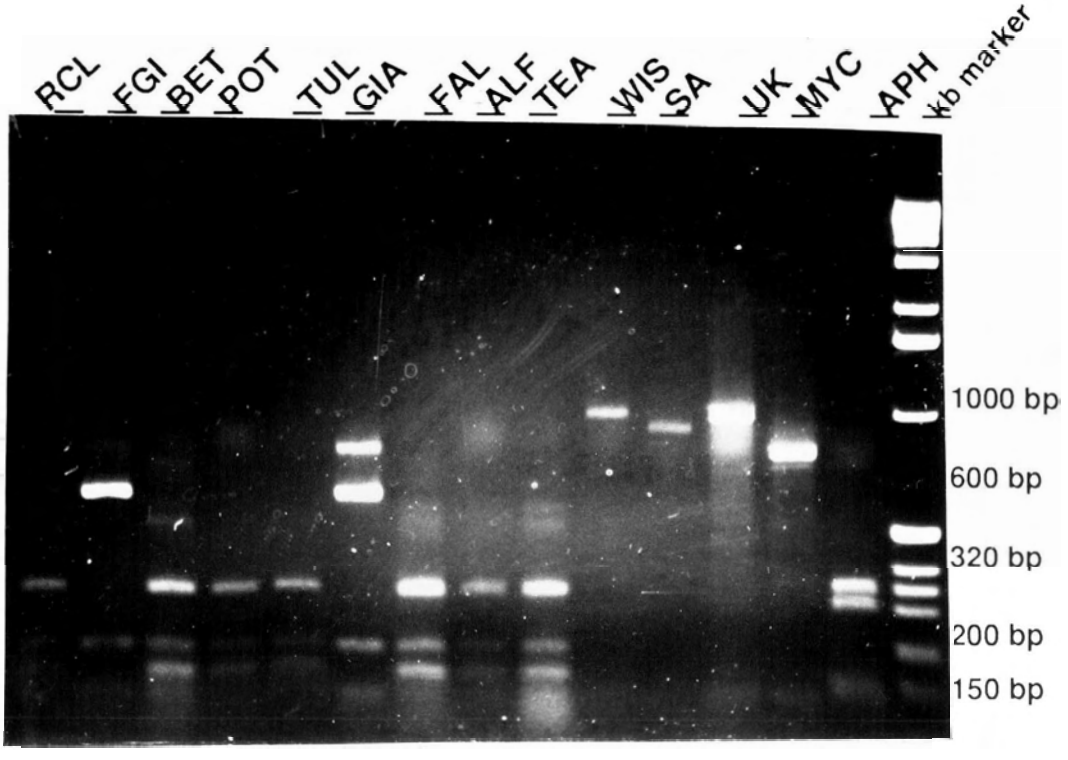
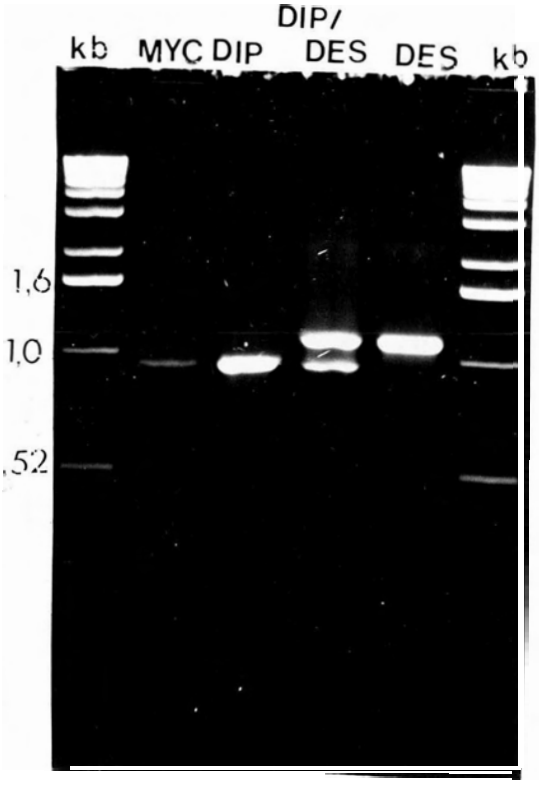
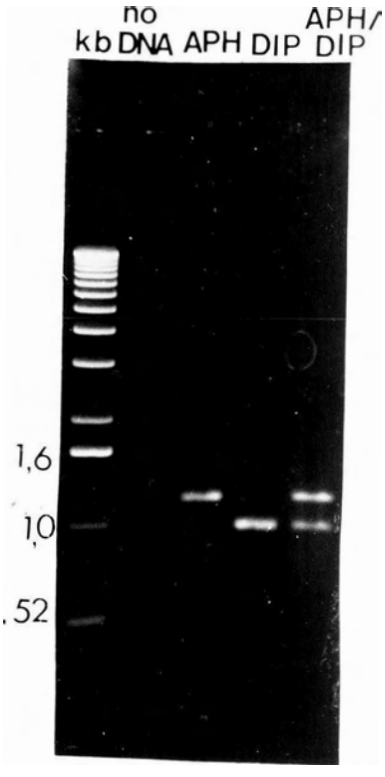


FIGURE 11 Photograph of a 0.7% agarose gel showing the ITS fragments resulting from the PCR amplification of (a) *Ditylenchus myceliophagus* (MYC), *D. dipsaci* (DIP) alone, *D. dipsaci* and *D. destructor* (DES UK) DNA mixed together and *D. destructor* (DES UK) alone; and (b) water (no DNA), *Aphelenchoides* spp. (APH) alone, *D. dipsaci* (DIP) alfalfa alone, and *Aphelenhoides* and *D. dipsaci* mixed.

a.


kb
MYC
DIP
DIP/
DES
DES
kb
1,6
1,0
,52

b.


kb
no
DNA
APH
DIP
APH/
DIP
1,6
1,0
,52

II. DISTINGUISHING BETWEEN POPULATIONS OF THE POTATO ROT NEMATODE, *D. DESTRUCTOR*

PCR amplification and restriction analysis of the ITS fragment

PCR amplification generated a 1.2 kb fragment from the WIS and UK isolates of *D. destructor*. The SA isolate of *D. destructor* produced a slightly smaller fragment of 1.0 kb (Figure 4).

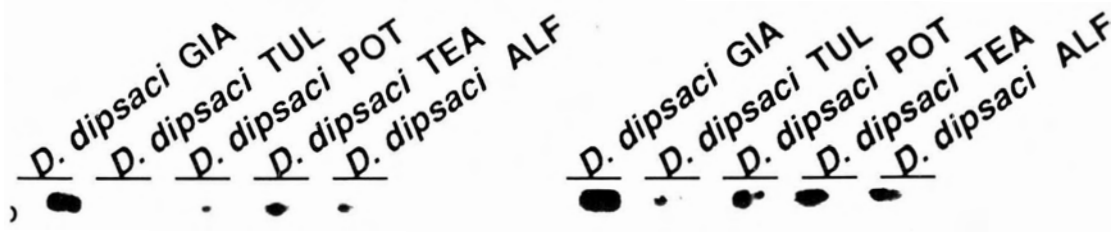
The UK and WIS isolates of *D. destructor* consistantly showed similar patterns to each other, while the SA population consistently produced a different pattern from that of the other two *D. destructor* isolates (Figures 5 to 10; Appendix). Not a single fragment was shared between the SA isolate and either the WIS or the UK isolates after digestion with seven different restriction enzymes.

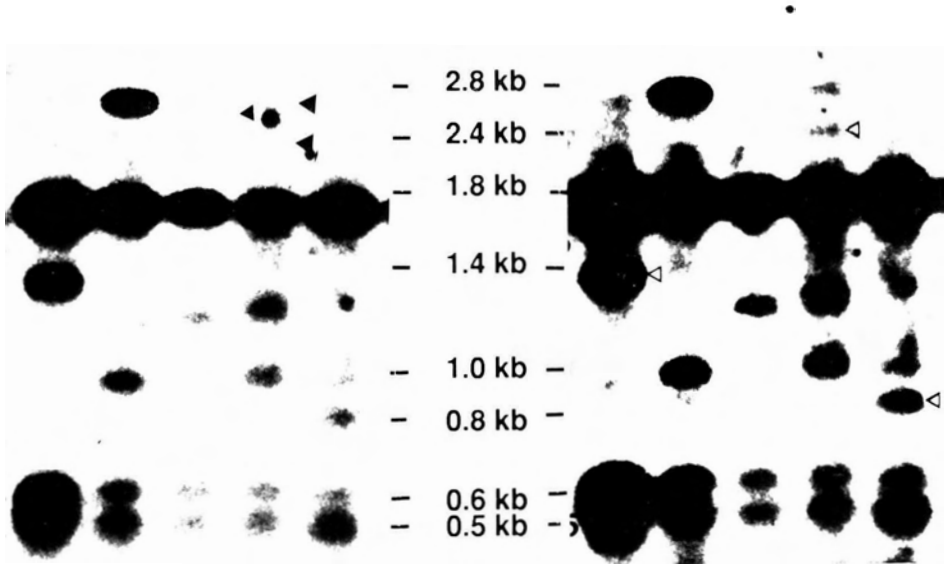
III. DISTINGUISHING BETWEEN POPULATIONS OF THE STEM NEMATODE, *D. DIPSACI*

Southern hybridizations probed with the complete ribosomal cistron

Southern hybridizations of five *D. dipsaci* host races probed with the 7.5 kb whole ribosomal cistron produced RFLPs of strong and faint bands. The heterologous probe bound strongly to fragments which contained conserved sequence, and the faint bands represented fragments with smaller amounts of conserved gene sequence. The faint bands could be seen more clearly in the longer (up to 84 h) exposures

FIGURE 12 Autoradiograph of a Southern transfer of *Rsa-1* digested genomic DNA from five host races of *Ditylenchus dipsaci* probed with the 7.5 kb rDNA cistron (from *Bursaphelenchus xylophilus*) under moderately stringent conditions. **12a** Film exposed to the Southern blot for 12 h; **12b** Film exposed for 84 h





a) 12 h exposure

b) 84 h exposure

as compared with the shorter (12 h) of the X-ray film (compare arrowed bands in Figures 12a and 12b).

According to both the 12 h and an 84 h autoradiograph exposures, all five populations produced unique RFLPs when hybridized to a heterologous rDNA cistron (Figures 12a and 12b; Table 3). Three bands at 1.8 kb, 0.6 kb and 0.5 kb characterized all *D. dipsaci* populations (Figure 12a, see white arrows). Bands of sizes 2.8 kb, 1.2 kb and 1.0 kb were shared between some populations and not others (Table 3). Unique bands, which were not shared with any other *D. dipsaci* isolate examined, were generated at 1.4 kb for the giant race, and at 0.8 kb for the alfalfa race (Figure 12b, see outlined arrows).

Southern hybridizations probed with the 18s ribosomal gene

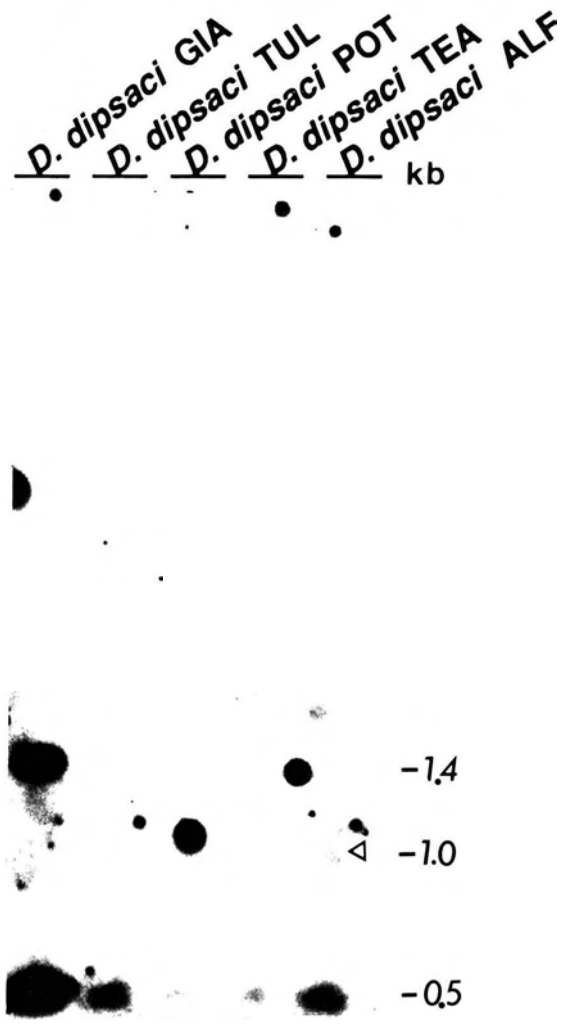
When the above Southern blot was stripped of the rDNA probe and reprobed with the 18s probe, all host races of *D. dipsaci* produced a strong 0.5 kb band (Figure 13). The four races, other than the giant race, produced a second faint band at 1.0 kb (Figure 13, see arrow). The giant race could be distinguished from the other races by a strong 1.4 kb band (Figure 13, see arrow).

By superimposing the rDNA blots for the species and races over the 18s probed patterns, it was possible to determine which bands in the

TABLE 3 Fragment sizes (kb) resolved from a Southern transfer of *Rsa-1* digested genomic DNA from five host races of *Ditylenchus dipsaci* probed with the 18s rDNA gene and the whole ribosomal cistron. Bands illuminated by both the 18s gene and the whole ribosomal cistron are denoted by an asterisk (*).

GIANT	TULIP	POTATO	TEASEL	ALFALFA
2.8	2.8	2.8	2.8	2.8
2.4			2.4	
1.8	1.8	1.8	1.8	1.8
1.4*				
		1.2	1.2	1.2
1.0	1.0*	1.0*	1.0*	1.0*
				0.8
0.6	0.6	0.6	0.6	0.6
0.5*	0.5*	0.5*	0.5*	0.5*

FIGURE 13 Autoradiograph of the described Southern transfer of *Rsa-1* digested genomic DNA from five host races of *Ditylenchus dipsaci* stripped and reprobed with the 1.1 kb 18s rDNA gene probe (from *Bursaphelenchus mucronatus*). Exposed for 72 h.


D. dipsaci GIA
D. dipsaci TUL
D. dipsaci POT
D. dipsaci TEA
D. dipsaci ALF
kb
-1.4
-1.0
-0.5

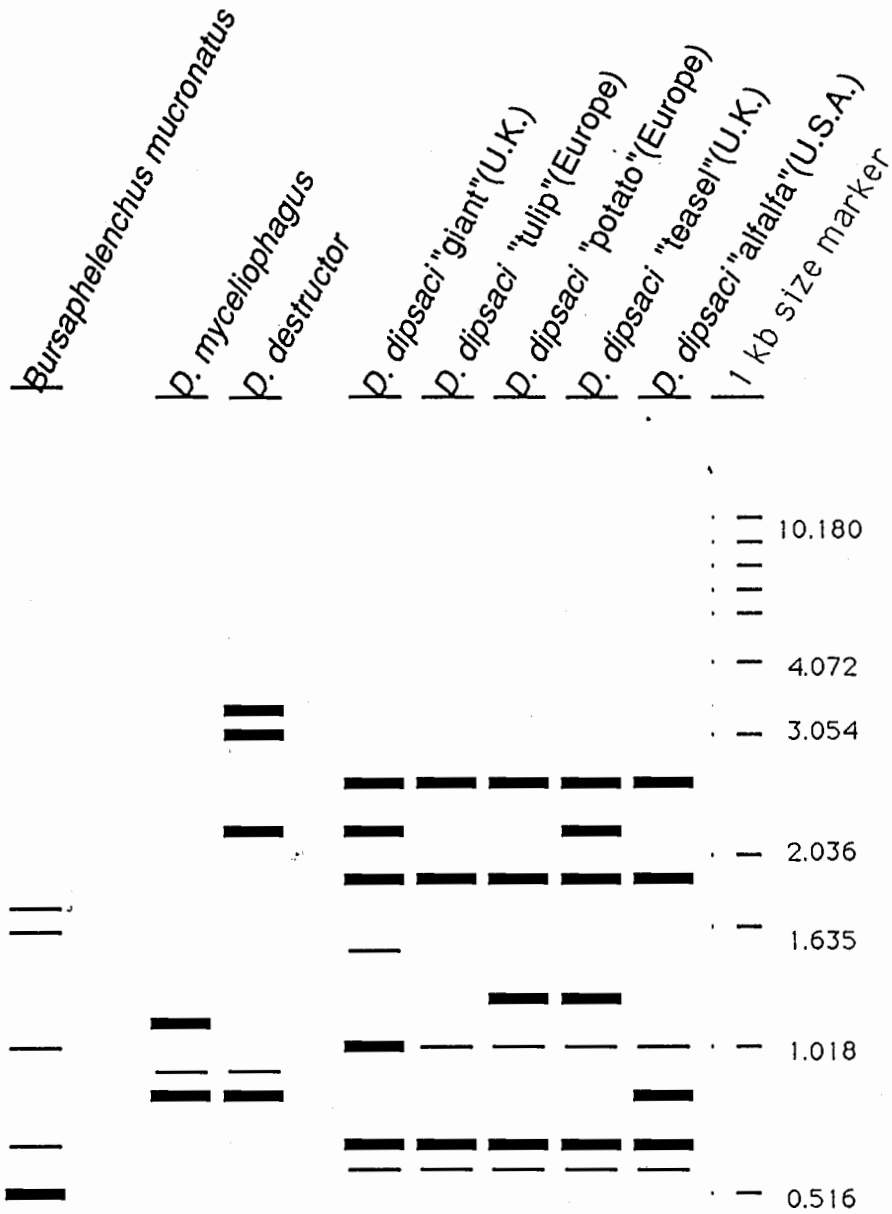
rDNA Southern hybridization were the result of the 18s probe. The results from the four Southern hybridization experiments with the species and *D. dipsaci* races are summarized in a line diagram (Figure 14).

PCR amplification and restriction analysis of the ITS fragment

All host races of *D. dipsaci* amplified a single 0.9 kb ITS fragment (Figures 4). In order to sample the variability in this ribosomal spacer region, intitially 14 enzymes were applied to the ITS fragment of two *D. dipsaci* races, namely TEA and ALF. The results of the restriction analysis demonstrated the similar banding patterns between these two races (Appendix). Subsequently, other populations were digested with seven of the enzymes that recognized more restriction sites than the other enzymes. Digestion of the ITS of all races, except the two giant races, produced patterns identical with those of the teasel and alfalfa races. The ITS of the giant race was digested with seven enzymes. Four of the seven enzymes tested (*Hae-3*, *Hpa-2, Hinf-1* and *Rsa-1*) generated patterns unique to this giant race, and different from those of all other populations of *D. dipsaci*, as well as from the other two *Ditylenchus* species tested (Figures 6, 8, 9, 10). Not all enzymes, namely *Pst-1* and *Dde-1* [Figure 5, 7) showed differences between the giant race and the other races. Sometimes the RFLP of the giant race was indistinguishable from that of the other races.

FIGURE 14 Line diagram summarizing Southern hybridization experiments (Figures 2, 3, 12 and 13) on the species and races of *Ditylenchus* using the 18s rDNA gene and the rDNA gene as probes. Bands smaller than 0.5 kb were omitted because they were present on some, but not all, of the blots.

hybridizes only with the rDNA cistron probe
hybridizes with both the 18s gene and the rDNA cistron probe



DISCUSSION

Traditional morphological methods of species identification among populations of *Ditylenchus* are not always completely satisfactory. Moreover, the unequivocal differentiation of subspecific forms that have apparent biological differences has not been possible. This study has demonstrated that a molecular approach can effectively complement traditional methods for identifying nematode species and host races. Restriction analysis of the rDNA identified three of the economically important species of *Ditylenchus* and distinguished between some of the subspecific populations eg. the host races of *D. dipsaci* and the geographic populations of *D. destructor*. In the latter case, the molecular evidence strongly supports the creation of a new species for the SA isolate, rather than confirming subspecific status on all isolates.

This investigation confirms and expands on an earlier study (Palmer *et al.*, 1991) of the use of molecular techniques to assist in *Ditylenchus* taxonomy. Palmer *et al.* (1991) provided the first DNA analysis of *Ditylenchus* species. In that case, Southern hybridizations were probed with random DNA fragments isolated from two libraries, one from *D. myceliophagus* and the other from *D. dipsaci* oat race. Although these probes successfully separated the two species from each other, such dot blot experiments do not elucidate the molecular taxonomy of unknown

Ditylenchus species. The two probes would show only that the unknown population is, or is not, *D. myceliophagus* or *D. dipsaci*.

In this study, rDNA is used as a tool for finding species and population markers in the genus *Ditylenchus*. The discrimination of subspecific populations proved more challenging because the subspecific populations are evolutionarily much more closely related than are the species, and not all techniques were sensitive enough to discriminate host races in *D. dipsaci*.

Employing the 7.5 kb whole rDNA probe in the initial Southern blot experiments demonstrated that there is sequence variation between each species of *Ditylenchus* and between host races of *D. dipsaci* within the ribosomal cistron. The three species of *Ditylenchus* generated RFLPs different from each other and from those of a different genus, *Bursaphelenchus* (Figure 2) and even five host races of *D. dipsaci* showed distinctly different RFLPs (Figure 12, 14). Restriction patterns from the ribosomal cistron obtained with the enzyme *Rsa-1* suggested that this gene cluster would be useful as a specific or subspecific marker for *Ditylenchus*.

The ribosomal cistron comprises an array of conserved genes and nonconserved spacer regions (see Figure 1; reviewed in Hillis and Dixon, 1991). Where, precisely, in the ribosomal gene cluster, are the sequence differences between species and races of *Ditylenchus*?

To further evaluate the nature of the sequence variation in the ribosomal cistron, the Southern blots were stripped of the rDNA probe and reprobed with a fragment composed of the 18s ribosomal subunit. The 18s gene probe did not differentiate between all three *Ditylenchus* species, but did delineate *D. dipsaci* from *D. destructor* and *D. myceliophagus* (Figure 3). *D. myceliophagus* and *D. destructor* had identical RFLPs, which suggests that these two fungal feeding species are more closely related to each other than both of them to the stem nematode, *D. dipsaci*.

The inability of the 18s probe to distinguish between the three species is not unexpected, since the 18s subunit is one of the most conserved regions in the ribosomal gene cluster (reviewed in Hillis and Dixon, 1991; Gerbi, 1985). The restriction pattern differences in the ribosomal cistron (Figure 2) result from sequence variation in another part of the ribosomal gene cluster.

The species of *Ditylenchus* could readily be identified by PCR amplification of the intragenic spacer (ITS), including the 5.8s gene, and restricting it with an appropriate enzyme (Figures 5 to 10; Appendix).

RFLPs of the ITS fragment produced five groups: (1) *D. myceliophagus* (MYC), (2) the U.S. and U.K. populations of *D. destructor* (WIS and UK), (3) the South African population of *D. destructor* (SA), (4) the giant race of *D. dipsaci* and (5) the other diploid races of *D. dipsaci*.

These results show that the ITS is at least partially responsible for rDNA RFLP variation shown between between species in Figure 2.

In addition to the sequence differences in the ITS detected by restriction analysis, the size of the ITS region alone distinguished genera and some species from the others. *Aphelenchoides rhyntium* had a larger ITS than any *Ditylenchus* examined (Figure 11). All three *D. destructor* populations examined had a larger ITS than the other two *Ditylenchus* species (Figure 4). The size of the ITS in the UK and WIS *D. destructor* populations was larger than that of the SA *D. destructor* (Figure 4), even though each of these populations presently belong in the same species.

The South African *D. destructor* population consistently showed RFLPs different from those of any other *Ditylenchus* population. Restriction analysis of the ITS suggested that the ITS sequence of the South African population of *D. destructor* was as different from the other two populations of *D. destructor* as it was from the other two species, *D. dipsaci* and *D. myceliophagus* (Figures 5 to 10; summarized in Appendix). Moreover, just as the two species *D. destructor* and *D. dipsaci* were differentiated by the size of the ITS (Figures 4, 11), so the South African *D. destructor* was separated from the other two *D. destructor* populations (Figure 4).

The morphological data indicate that these peanut parasitizing nematodes from South Africa look like *D. destructor* (De Waele *et al.*, 1989). However, the SA *D. destructor* behaves differently from other potato rot nematode populations, so it has been suggested that it is a host race of *D. destructor* (De Waele *et al.*, 1991). Restriction analysis of the ITS confirms the biological data that the South African *D. destructor* is very different, and even provides strong evidence that the difference could be at the species level rather than subspecific. This suggests that a more in-depth morphological study should be undertaken and the results used in combination with the above molecular data in the species description.

This diagnostic method may be of great importance for quarantine purposes, since strict guidelines are enforced for the movement, storage and sale of potatoes grown in *D. destructor* infested land (Esser, 1985). The South African isolate of *D. destructor* is biologically different from other populations of *D. destructor* (De Waele and Wilken, 1990; De Waele *et al.*, 1991) but morphologically similar (De Waele *et al.*, 1989); thus DNA analysis provides so far the only practical means of diagnosis.

Probing with the rDNA cistron allows the subspecific host races of *D. dipsaci* tested to be distinguished (Figure 12; summarized in Figure 14). The five tested host races of *D. dipsaci* exhibited a strong pair of bands at 0.5 kb and 0.6 kb signifying that they belong to *D. dipsaci*. Some of

the *D. dipsaci* host races examined (alfalfa and giant races) showed unique fragments which were not shown in the other races.

In a different study the host races were differentiated using monoclonal antibodies (Palmer, 1989), which generated two groups of host races: the giant, oat and alfalfa races, and the narcissus, white and red clover races. The patterns generated between the host races of *D. dipsaci* using rDNA do not group these populations, excluding the giant race, into any obvious geographic or host race divisions.

In this study, when the blot was stripped and reprobed with the 18s gene, the *D. dipsaci* host races, except for the giant race, demonstrated identical patterns (Figure 13, 14). The similarity suggests that the rDNA sequence variation between the races is probably not located in the 18s ribosomal gene. This suggests that the host races of *D. dipsaci* are closely related, which confirms other serological and interbreeding studies, and the 18s gene would not have evolved differently between these subspecific populations. The 18s probe was, therefore, not useful as a host race marker and did not differentiate between four of the five races of *D. dipsaci*. The different RFLP pattern generated by the 18s probe in the giant race may be due to the polyploid nature of this race.

In contrast to the differences detected between the species, restriction analysis of the ITS amplified by PCR was not sensitive enough to show the sequence differences in the ITS of most of the host races of

D. dipsaci. The host races have been found to be closely related in another DNA analysis study of the stem nematode, where Palmer (1989) was unable to differentiate the host races using a *D. dipsaci* oat race probe in dot blot experiments. The sequence variation shown by the whole ribosomal gene cluster between the races of the stem nematode (Figure 12) were not a result of sequence variation in the 18s (Figure 13) or the ITS (Figures 5 to 10; summarized in Appendix).

Restriction digests of the ITS gave rise to identical restriction patterns between all but one of the races examined. The notable exception was the polyploid giant race (compare Figures 5 and 7 with 6, 8, 9 and 10). The giant race showed RFLPs different from the other races of *D. dipsaci* in four (*Rsa-1, Hpa-2, Hae-3, Hinf-1*) out of six enzymes shown. Sturhan (1971; 1983) suggested that the giant race is not a mere host race, but should be considered a sibling species because it did not produce viable offspring when crossed with another race. Host races can be distinguished from sibling species "on the degree of reproductive isolation and restriction of gene flow" (Diehl and Bush, 1984). Sturhan (1983) indicated that crossbred progeny between the giant and diploid races were infertile, although in another study fertile F_1 progeny was observed in crosses (Ladygina, 1988). Polyploidy is suspected of playing an important role in the cytogenic evolution of some amphimictic groups of nematodes (Triantaphyllou, 1984) and this lends credence to

the idea that the giant race is a sibling species. A new species of *Meloidogyne* was recently described based primarily on karyotype (Triantaphyllou, 1990). The rDNA sequence heterogeneity presented here with restriction analysis of both the 18s probe (Figure 13) and the ITS (summarized in Appendix) supports the observation that the giant race should be considered a sibling species.

Distinguishing the host races of *D. dipsaci* required a more sensitive method than that used to identify the species. Southern hybridizations were more sensitive than restriction of PCR amplified fragments, and they were able to detect subtle differences in the host races. The Southern blot experiments used radioactive labeling, which is more readily detected by film than ethidium stained DNA, and the rDNA probe was composed of very heterogeneous regions of rDNA.

In addition to the content of the probe, the size of the DNA fragment to be used as a probe was important. Presumably, a larger probe will sample a larger DNA sequence, and will detect more subtle sequence changes between closely related groups. This was true in the Southern blots probed with the 7.5 kb ribosomal cistron, compared with the results from the smaller 1.1 kb 18s ribosomal gene probe.

Although a Southern hybridization proved to be a sensitive diagnostic technique, it was not an ideal method for routine sampling because it was a relatively time consuming process, it required more DNA than can

be extracted from the few nematodes which may be available, and it employed radioisotopes. There was not enough DNA available from all populations to perform Southern hybridizations, however. The second method of generating RFLPs involved the PCR amplification of the internal transcribed spacer (ITS) of the ribosomal cistron, and cutting this spacer with restriction enzymes. PCR is practical because the technique does not require an unwieldy radioactive probe, and the DNA from only a few nematode cells is sufficient for amplification, unlike Southern hybridizations where much more DNA is required.

The ITS length variation between some of the species and different genera was very useful for detecting mixes of nematodes. Amplification of the ITS by PCR was sensitive enough to detect a mixture of species and genera (Figure 11). It is quite common in alfalfa fields to find a mixture of *D. dipsaci* and *Aphelenchoides ritzema-bosi*, the leaf and bud nematode. All alfalfa samples collected in Wyoming which showed typical damage from the alfalfa stem nematode contained a mixture of these two nematode species (F. Gray, personal communication), and they are relatively similar to one another under low power magnification. When the ITS of *A. rhyntium*, a closely related aphelenchid nematode, was amplified and restricted, it showed a different size and RFLP from that of either *D. dipsaci* or *D. destructor* (Figure 11). The method described here could confirm morphological diagnosis for potentially

mixed (Figure 11) or pure infestations of nematodes in lab cultures or in the field.

The RFLPs produced from the three species *D. myceliophagus*, *D. destructor* and *D. dipsaci*, by restriction analysis of the ITS are not confused by morphological similarities between species or geographic populations, or by host and temperature induced morphological differences within a species. In addition, taxonomic features currently obscured by fixative techniques will not confound the diagnosis of preserved museum specimens, as PCR amplification has been used successfully on preserved organisms (Thomas *et al.*, 1990).

Analysis of the ribosomal cistron by restriction analysis of Southern hybridizations and fragments amplified by PCR indicates that *Dityenchus* species and the host races of *D. dipsaci* can be identified using DNA techniques. The use of the ribosomal cistron in this thesis provides a powerful tool for systematics of unknown species, as well as for the identification of known species and host races in the nematode genus *Ditylenchus*.

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APPENDIX Fragment sizes (kb) resulting from digestion of the ITS of *D. myceliophagus*, *D. destructor*, and *D. dipsaci* species and races *Ditylenchus* with various restriction enzymes.

Diploid *D. dipsaci* populations which had identical RFLPs were the teasel, alfalfa (two populations, from France and the U.S.), tulip, potato, beet, and red clover races. Two populations of the *D. dipsaci* giant race were examined (from England and France). The two *D. destructor* populations with the same RFLP originated in the U.S. (WIS) and England (U.K.). The other *D. destructor* population originated in South Africa (SA). Fragments smaller than 0.10 kb were not included in this table.

APPENDIX: RFLP fragments (kb) of the ITS of *Ditylenchus*					
ENZ-YME	*D. dipsaci*		*D. destructor*		*D.myc-celio-phagus*
	HOST RACES	**GIANT RACES**	**UK/WIS**	**SA**	
Acc-1	0.90	-	1.20	-	0.90
Alu-1	0.90	-	0.37 0.29	-	0.90
BamHI	0.34 0.22 0.18	-	1.0	-	0.90

APPENDIX: RFLP fragments (kb) of the ITS of *Ditylenchus*					
ENZ-YME	*D. dipsaci*		*D. destructor*		*D.myc-celio-phagus*
	HOST RACES	**GIANT RACES**	**UK/WIS**	**SA**	
Dde-1	0.31 0.29 0.20	0.31 0.29 0.20	0.67 0.57	0.29 0.25 0.19	0.30 0.25 0.13
Dra-1	0.34 0.25	-	1.2	-	0.90
Hae-3	0.90	0.80 0.20	0.45 0.17	0.65 0.54	0.40 0.33 0.25

APPENDIX: RFLP fragments (kb) of the ITS of *Ditylenchus*

ENZ-YME	*D. dipsaci*		*D. destructor*		*D.myc-celio-phagus*
	HOST RACES	**GIANT RACES**	**UK/WIS**	**SA**	
Hinc-2	0.80	0.80	0.90 0.25	1.0	0.90
Hinf-1	0.44 0.35 0.15	0.35 0.15	0.78 0.18 0.13	0.50 0.31 0.15 0.10	0.63 0.31
Hpa-2	0.32 0.20 0.18	0.60 0.20 0.15	1.0	.95	.90
Nsi-1	0.90	-	1.2	-	0.90

APPENDIX: RFLP fragments (kb) of the ITS of *Ditylenchus*

ENZYME	*D. dipsaci*		*D. destructor*		*D.myc-celio-phagus*
	HOST RACES	**GIANT RACES**	**UK/WIS**	**SA**	
Pst-1	0.65 0.37	0.65 0.37	0.85 0.40	0.70 0.42	0.62 0.40
Rsa-1	0.45 0.25 0.14	0.49 0.45	0.60 0.25 0.17	0.69 0.45	0.90
Sau3A	0.34 0.26 0.20 0.11 0.10	-	0.54 0.40 0.18		0.44 0.10

APPENDIX: RFLP fragments (kb) of the ITS of *Ditylenchus*

ENZ-YME	*D. dipsaci*		*D. destructor*		*D.myc-celio-phagus*
	HOST RACES	**GIANT RACES**	**UK/WIS**	**SA**	
Taq-1	0.34 0.23 0.13	-	0.64 0.20 0.15	-	0.32 0.26 0.16