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The rDNA Internal Transcribed Spacer Region as a Taxonomic Marker for Nematodes[1]

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Abstract: The ITS region from a wide taxonomic range of nematodes, including secernentean and adenophorean taxa, and free-living, entomopathogenic, and plant-parasitic species, was evaluated as a taxonomic marker. Size of the amplified product aided in the initial determination of group membership, and also suggested groups that may require taxonomic reevaluation. Congeneric species often displayed identically sized ITS regions, but genera such as *Pratylenchus* and *Tylenchorhynchus* had species with large differences in size. ITS heterogeneity in individuals and populations was identified in several nematode taxa. PCR-RFLP of ITS1 is advocated as a method of taxonomic analysis in genera such as *Helicotylenchus* that contain numerous species with few diagnostic morphological characteristics.
Key words: diagnosis, genetics, internal transcribed spacer region, ITS, molecular ecology, nematode, systematics, taxonomy.

The Internal Transcribed Spacer Region (ITS), located between the repeating array of nuclear 18S and 28S ribosomal DNA genes, is a versatile genetic marker. Among eukaryotes, including organisms as diverse as protozoa, plants, vertebrates, and fungi, ITS data have been used in constructing phylogenetic trees, estimating genetic population structures, evaluating population-level evolutionary processes, and determining taxonomic identity. The structure of the rDNA cistron contributes to its wide applicability. The rDNA cistron is divided into domains that evolve at different rates; thus, this region can be used to address diagnostic and evolutionary problems at different levels of divergence. The rDNA is a component of the middle repetitive family of the nuclear DNA genome, and the presence of multiple copies of these genes in the genome facilitate PCR amplification from single juvenile and adult nematodes. The ITS, intergenic spacer (IGS), and rDNA genes appear to display concerted evolution so that copies of these genes from a single individual tend to be similar to one another, although generally being distinct from those of other species (Elder and Turner, 1995). The application of the ITS to identification has received the most attention by nematologists (Campbell et al., 1995; Cherry et al., 1997; Chilton et al., 1995; Epe et al., 1996; Fallas et al., 1996; Ferris et al., 1993, 1994, 1995; Gasser and Hoste, 1995; Hoste et al., 1995; Ibrahim et al., 1994, 1997; Joyce et al., 1994; Kaplan, 1994; Nasmith et al., 1996; Orui, 1996; Reid, 1994; Stevenson et al., 1995; Szalanski et al., 1997; Thiery and Mugniery, 1996; Vrain et al., 1992; Vrain and McNamara, 1994; Wendt et al., 1995; Zijlstra et al., 1995, 1997). The majority of these studies have focused upon agriculturally important plant-parasitic species, animal parasites, or beneficial insect parasites. Yet, to the best of our knowledge, there is not a single nematode species that has failed to provide an amplification product of the ITS region when amplified with "universal" PCR primer sets. Universal amplification coupled with the ability to amplify ITS from individual nematodes suggests that any species, population, or ecological community of nematodes can be analyzed using a molecular approach based on the rDNA ITS region (Vrain and McNamara, 1994). A standardized taxonomic marker would be particularly useful when populations contain a

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large number of juvenile stages, when sexes are dimorphic, or when unfamiliar nematodes are encountered. In ecological studies, PCR-RFLP profiles of ITS from individual nematodes may be one method to assess nematode diversity in samples as well as provide critical taxonomic characters useful for species comparison and identification. In this study we have evaluated the diagnostic utility of the ITS region from a wide taxonomic range of nematodes, including representatives of both Secernentea and Adenophorea, among free-living, insect and plant-parasitic species. We demonstrate that the size of the amplified ITS product aids in the initial determination of group membership, and we investigate the occurrence of ITS heterogeneity in nematode populations and individuals and discuss its taxonomic implications.

Materials and Methods

Nematode samples: Cephalobid and rhabditid nematodes were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. *Steinernema* and *Heterorhabditis* spp. were maintained in the laboratory at the University of Nebraska.

PCR-RFLP: Amplification of the product including the entire ITS1, 5.8S rDNA gene, and ITS2 region was conducted using the primers described in Vrain et al. (1992). This primer set consists of one primer, designated as rDNA2 (5′-TTGATTACGTCCCTGCCCTTT-3′), located in the 3′ portion of 18 S, the small ribosomal subunit gene, approximately 190 bp from its junction with ITS1, the first internally transcribed spacer. The second primer, designated as rDNA2.144 (5′-GTAGGTGAACCTGCAGATGGAT-3′), is located in the 5′ portion of 28 S, the large ribosomal subunit gene, approximately 80 bp from the junction with ITS2, the second internal transcribed spacer. Between both spacers is the 5.8 S ribosomal gene that is generally around 155 bp in length. The ITS1 amplification procedure in this research used the 18 S primer of Vrain et al. (1992), rDNA2, together with a primer located in the first 20 bp of the 5.8 S gene flanking ITS1 (Cherry et al., 1997), designated as rDNA1.58s (5′-ACGAGCCGAGTGATCCACCG-3′).

PCR-RFLP was performed according to previously described methods (Cherry et al., 1997; Powers and Harris, 1993).

Results and Discussion

ITS size variation: The phylum Nemata displays a wide range of ITS sizes. Figure 1 shows amplified product of the entire ITS1 and ITS2 region from representative rhabditid and cephalobid nematodes arranged according to size. The estimated size of the

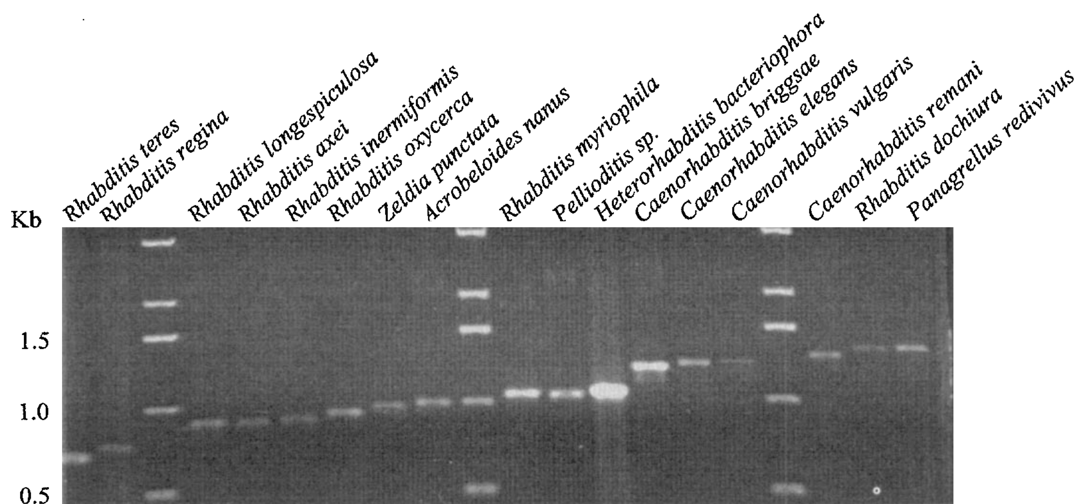

FIG. 1. Amplification of the ITS 1 and 2 regions of rhabditid and cephalobid nematodes.

product ranges from 0.7–1.3 kb. Assuming that the flanking 18S and 28S plus the internal 5.8S ribosomal gene sequences comprise approximately 425 bp of that amplified product, the combined size of ITS1 and ITS2 ranges from 275–875 bp. Slight size variation is observed among the four *Caenorhabditis* species; however, ITS size variation was not detected among seven species of *Heterorhabditis* (Joyce et al., 1994) or five species of *Steinernema* (T. Powers, unpublished). *Rhabditis* species include many ITS size classes (Fig. 1). The existence of size variation among rhabditid genera appears to be correlated with high levels of genetic divergence among members of this group. Nucleotide sequence variation of the 18S gene measured among some of the same rhabditid genera (Fig. 1) were estimated at eight times the level of divergence observed among tetrapod classes (Aves, Amphibia, Reptilia, Mammalia) (Fitch et al., 1995). Large genetic distances between *Caenorhabditis* spp. also were estimated from a comparison of the mitochondrial cytochrome oxidase subunit II and calmodulin genes (Thomas and Wilson, 1991). The high levels of 18S divergence were presumed to be due to the ancient origin of nematodes, elevated rates of molecular evolution, or a combination of both factors (Fitch et al., 1995). Like 18S nucleotide divergence, ITS size polymorphism (Fig. 1) appears to reflect a high level of genetic divergence among rhabditid and cephalobid nematodes, although the magnitude of the size difference does not necessarily correspond in a linear fashion to the degree of divergence based on 18S analysis.

An equally large range of size variation is observed among the plant-parasitic nematodes. The ITS1 amplification products

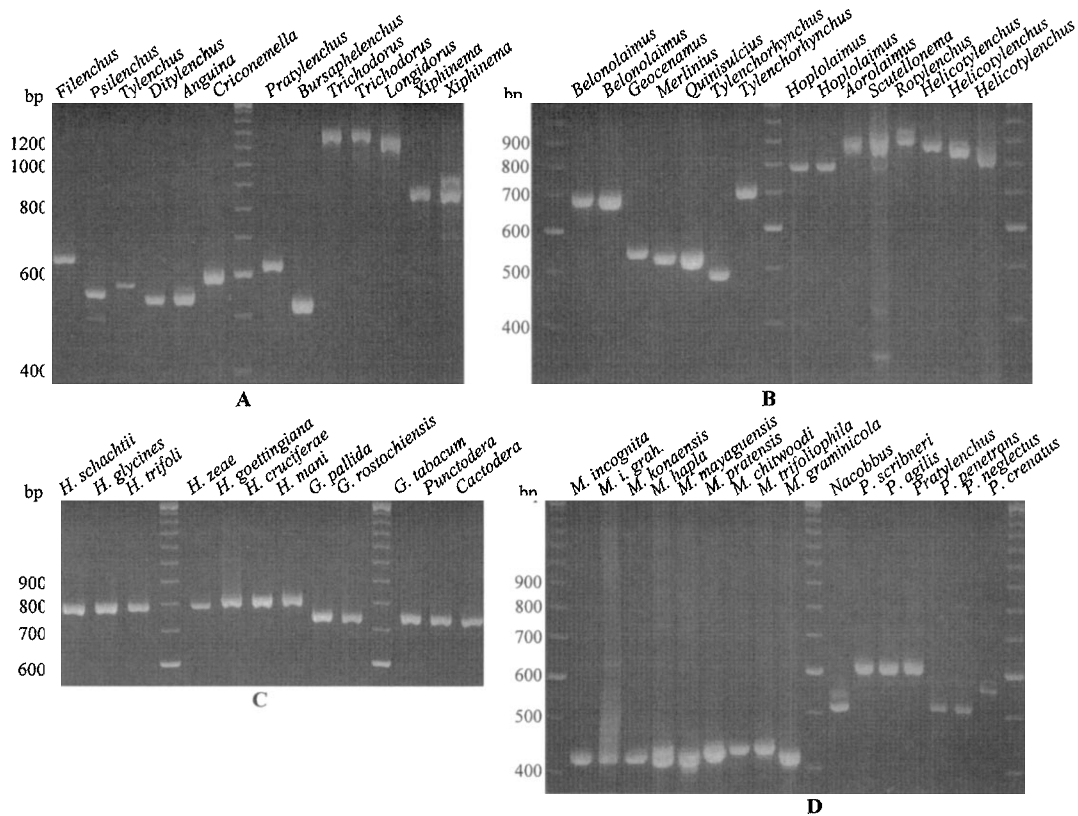

FIG. 2. A) ITS1 size variation among various tylenchid and dorylaimid nematodes. B) ITS1 size variation within Belonolaimidae and Hoplolaimidae. C) ITS1 size variation within Heteroderidae. D) ITS1 size variation among *Meloidogyne, Nacobbus,* and *Pratylenchus* species.

```
               1                                                          60
M. arenaria    TTTGATGGAA ACCAATTTAA TCGCAGTGGC TTGAACCGGG CAAAAGTCGT AACAAGGTAG
M. incognita   .......... .......... .......... .......... .......... ..........
M. javanica    .......... .......... .......... .......... .......... ..........
M. chitwoodi   .......... .......... .......... .......... .......... ..........
M. hapla       .......... .......... .......... .......... .......... ..........
               61                                                        120
M. arenaria    CTGTAGGTGA ACCTGCTGCT GGATCATTAC --TTTATGTG ATGTTC--AA ATTTGAATT-
M. incognita   .......... .......... .......... --........ ......--.. .........-
M. javanica    .......... .......... .......... --........ ......--.. .........-
M. chitwoodi   .......... .......... .........C --T....... ......AA.T T........A
M. hapla       .......... .......... .........C --T..-.... ......--.. ...C...-.A
               121                                                       180
M. arenaria    --CGCAA-TG AAATGAT--C GTTGTGAAAC GGCTGTCGCT GGTGTCTAAG TGTTGCTGAT
M. incognita   --.....-.. .......--. .......... .......... .......... ..........
M. javanica    --.....-.. .......--. .......... .....C.... .......... ..........
M. chitwoodi   TA....C--- ..T.------ -G....T... .....C.... ........G. ..........
M. hapla       GT.T...--- CGT.T--AT. ......-... .......... ........G. ..........
               181                                                       240
M. arenaria    ACGGTTGTGA ACGTCCGTGG CTGTATATGT GGTGACATGT TAGGACTCT- -------AAT
M. incognita   .......... .......... .......... .......... .........- -------...
M. javanica    .......... .......... .......... .......... .........- -------...
M. chitwoodi   T.A....... G........A ...A.....A .......... .....T..CT TTATAAG...
M. hapla       T.R.C...C. .......... ...A.....A .......... .....--.CT T------...
               241                                                       300
M. arenaria    -GAG-TTAAG ACCTAATGAG CCTCTTAAGT GAGGCCGCCA GCAACCTTTT TTT-TCTCTA
M. incognita   -...-..... .......... .......... .......... ......-... ...-......
M. javanica    -...-..... .......... .......... .......... ......-... ...-......
M. chitwoodi   C.----.... ..T....... .......... ....A..... ...C..A... ...CA-AA..
M. hapla       C.G.T..... ..T....... .......... ....A..... ....TA.... ...CA-A...
               301                                                       360
M. arenaria    CA---TTTTA AAAAAAAA-- ---ACTAAAA TTCTACCCTT ATCGGTGGAT CACTAGGCTC
M. incognita   ..---..... ........-- ---....... .......... .......... ..........
M. javanica    ..---..... ........-- ---....... .....T.... .......... ..........
M. chitwoodi   A.TTT....T T.TTC...-- ---.AAT... ..T..G.... .C........ ....C..T..
M. hapla       ---TT....T ..---...-- ---..G.... ..T..T.... .......... ....C.....
               361                                                       420
M. arenaria    GTGGATCGAT GAAGAACGCA GCAAACTGCG ATAATTATTG CGAACTGCAG AAGTATTGAG
M. incognita   .......... .......... .......... .......... .......... ..........
M. javanica    .......... .......... .......... .......... .......... ..........
M. chitwoodi   .......... .......... ..T....... ......TA.. T......... ..A.C.....
M. hapla       .......C.. .......... ..T....... ......TG.. .......... ..AC......
               421                                                       480
M. arenaria    CACAAAAGTT TTGAACGCAA ATGGCCGCAT TGAGGTCAAA CTCTTTGCAA CGTCTGGTTC
M. incognita   .......... .......... .......... .......... .......... ..........
M. javanica    .......... .......... .......... .......... .......... ..........
M. chitwoodi   ..T....... .........T ..T..G.... ..G....... .C.....GC. ..........
M. hapla       ..T....... .....T.... .CT..G...C ..G...AG.. .C......C. ..........
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FIG. 3. Alignment of 18S, ITS1, 5.8S, ITS2, and 28S sequences among five *Meloidogyne* species. Sequence identity is indicated by ".", deletions by "–", and coding regions are italicized.

from 56 nematode species were examined (Fig. 2A–D). The 1.2-kb product of *Trichodorus* is three times the size of the 0.4-kb *Meloidogyne* product (Figs. 2B,2D). The ITS1 size of *Meloidogyne*, exclusive of the flanking 18 S and 5.8 S ribosomal gene sequences, is approximately 215 bp (Fig. 3). This is among the shortest ITS1 sequences known for any eukaryotic organism.

Congeneric species often display the same

```
               481                                                               540
M. arenaria    AGGGTCATTT TCTCTTATAG CGGAAGCTTT AATTTCTATA A-TGATGTTG T---T---GC
M. incognita   .......... .......... .......... .......... .-........ .---.---..
M. javanica    .......... .......... .......... .......... .-........ .---.---..
M. chitwoodi   .......... ...TAC.A.. ..A....... T.A..T.... T-..T-A..- ATTG.ATAC.
M. hapla       .......... .TCTA..A.. TAT..--A.. TTA..T...T T-..CCA..- -------G..
               541                                                               600
M. arenaria    TTTATA---- --TTTTAAAA -GGATT---- --TTTGTTTA ----TT--CA TGTATTAAAT
M. incognita   ......---- --........ -.....---- --........ ----..--.. ..........
M. javanica    ......---- --........ -.....---- --........ ----..--.. ..........
M. chitwoodi   ......A--- ---...-TTC -T.T..TGA- TGCAAT.... ---------T ..A..-....
M. hapla       AC....A--- AC......TG TT.G.ACGCA GCGA.T.G.. --------A. ..A..-..C.
               601                                                               660
M. arenaria    CTAACTGTGA AAATCAAACA A---TTTTGA CCTGAACTCA GTCGAGAGCA CCCGCTGAAC
M. incognita   .......... .......... .---...... .......... .......... ..........
M. javanica    .......... .......... .---...... .......... .......... ..........
M. chitwoodi   A.TTG.A.C- ....ATGCTT TATT...... .......... .......T.. ..........
M. hapla       ..TT---.CG CTG...C.TT TA-T...... .....G.... .......T.. ..........
               661                                         704
M. arenaria    TTAAGCATAT CAGTAAGCGG AGGAAAAGAA ACTAAATAGG ATTC
M. incognita   .......... .......... .......... .......... ....
M. javanica    .......... .......... .......... .......... ....
M. chitwoodi   .......... .......... .......... ......C... ....
M. hapla       .......... .......... .......... .......... ....
```

GenBank Accession Numbers: *M. arenaria* U96301, *M. incognita* U96304, *M. javanica* U96305, *M. chitwoodi* U96302, *M. hapla* U96303.

FIG. 3. Continued

size amplification product. Three *Heterodera* spp. (Fig. 3) show a consistent ITS1 size that is slightly larger than that of *Globodera, Punctodera,* and *Cactodera.* Little or no size variation was observed among congeneric species of the following genera: *Heterodera, Globodera, Meloidogyne, Hoplolaimus, Ditylenchus, Belonolaimus, Trichodorus,* and *Xiphinema.* Both *Tylenchorhynchus* and *Pratylenchus* have species displaying extreme ITS1 size variation (Figs. 2B,2D). ITS size variants have been observed among *Aphelenchoides* (Ibrahim et al., 1994) and *Pratylenchus* (Orui, 1996) spp. When large size variation does occur in a genus, it may signal the need for taxonomic action. ITS size alone, however, is insufficient evidence upon which to base taxonomic reevaluation.

ITS restriction site variation: Restriction analyses of PCR-amplified rDNA ITS products have been used in the diagnosis of many species of nematodes that are difficult to identify morphologically. *Meloidogyne hapla* and *M. chitwoodi* can be distinguished by digestion of their ITS products with a number of restriction enzymes (Zijlstra et al., 1995). *Meloidogyne fallax,* a species closely related to *M. chitwoodi,* can be identified by a small length variation in digestion products (Zijlstra et al., 1995). Differentiation among *M. arenaria, M. javanica,* and *M. incognita* by means of PCR-RFLP profiles has not been achieved (Xue et al., 1993; Zijlstra et al., 1995), and identical copies of the ITS region have been cloned and sequenced from these three *Meloidogyne* species (Figure 3). This sequence identity among the three major mitotic parthenogenetic species is in sharp contrast to sequence divergence observed when those species are compared with *M. hapla* or *M. chitwoodi.* Approximately 16% sequence divergence is found in these

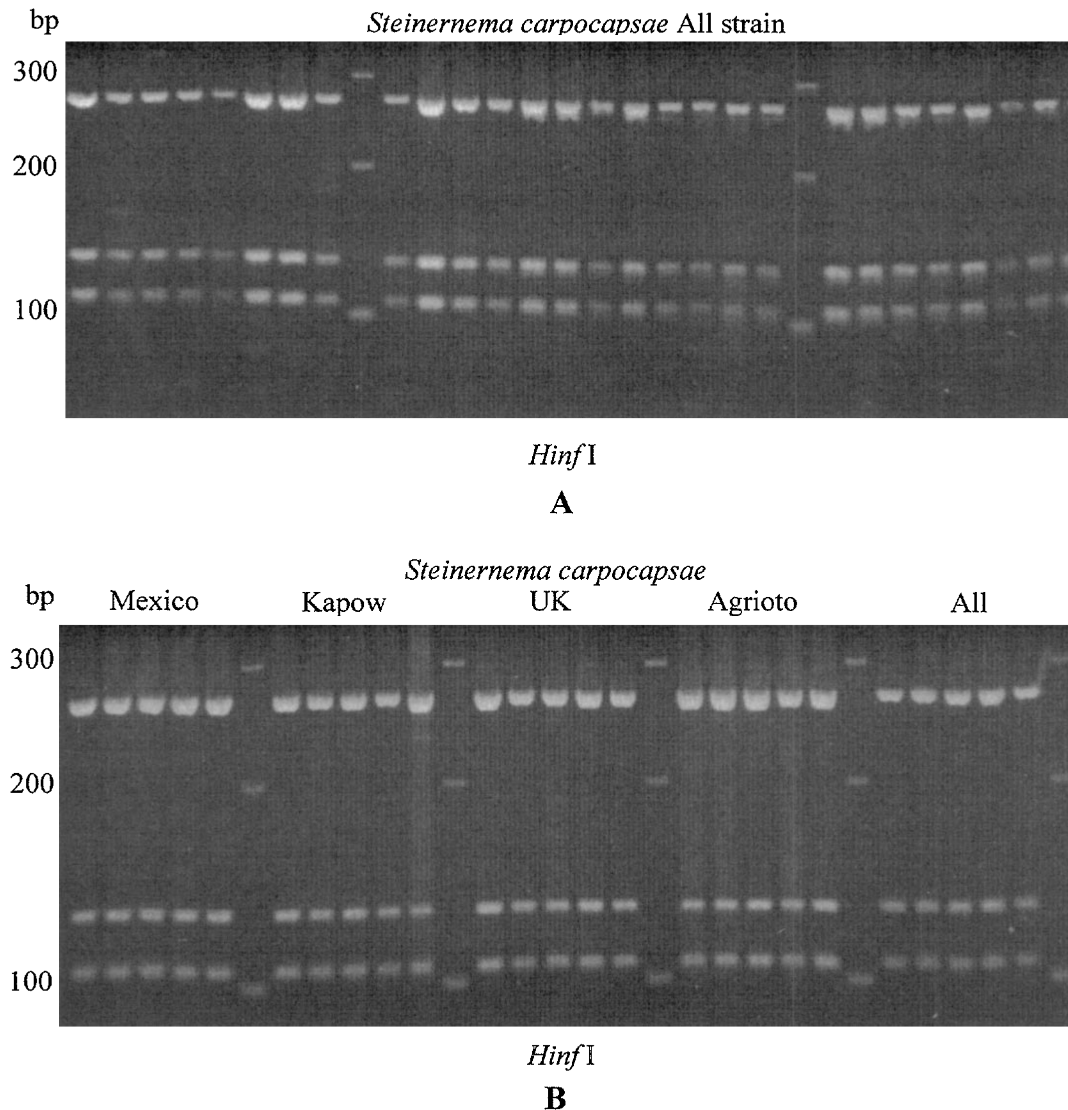

FIG. 4. A) Hinf I digest of ITS1 amplicon from *Steinernema carpocapsae* All strain. B) Hinf I digest of ITS1 amplicon from *Steinernema carpocapsae* Mexico, Kapow, UK, Agrioto, and All strains.

comparisons, underscoring the large number of discriminating restriction sites available for identification of those species by PCR-RFLP.

Globodera and *Heterodera* species have been examined by PCR-RFLP and nucleotide sequencing (Ferris et al., 1993, 1994, 1995; Thiery and Mugniery, 1996; Szalanski et al., 1997). The relatively low level of ITS sequence divergence between *H. glycines* and *H. schachtii,* reported as less than 1.0% dissimilarity (Ferris et al., 1993), is still of sufficient magnitude to allow discrimination by the restriction enzyme Fok I (Szalanski et al., 1997). *Globodera pallida* and *G. rostochiensis* ITS can be separated by several restriction enzymes; however, identification is complicated if *G. "mexicana"* and *G. tabacum* isolates are included in comparisons (Thiery and Mugniery, 1996). French, U.S., and Mexican isolates of *G. tabacum* produced ITS profiles very similar to those of *G. rostochiensis,* whereas isolates identified as *G. "mexicana"* were similar to *G. pallida.* Each of the four taxa could be discriminated by at least one restriction pattern (Thiery and Mugni-

ery, 1996). Laboratory cross-mating between *G. pallida* and *G. rostochiensis* has demonstrated that composite ITS restriction patterns can be produced in hybrid offspring, further complicating interpretation of species status based on ITS restriction patterns alone (Thiery and Mugniery, 1996).

Interspecific ITS comparisons in *Radopholus* have been conducted in *R. similis, R. citrophilus,* and *R. bridgei* (Fallas et al., 1996; Kaplan, 1994). Limited variation was observed using six restriction enzymes in a survey of *R. similis* isolates from banana-growing regions around the world, but *R. similis* was readily separated from *R. bridgei* by PCR-RFLP (Fallas et al., 1996). Kaplan (1994) sequenced the ITS region of representative isolates of *R. similis* and *R. citrophilus* and considered the region too highly conserved for the development of diagnostic assays. However, among the seven nucleotide differences recorded between those species in the ITS1 region, one substitution is predicted to result in a restriction site polymorphism; further evaluation of the ITS1 and ITS2 regions of *Radopholus* could produce a taxonomic marker of the desired specificity.

Other plant-parasitic nematodes that have been successfully differentiated by PCR-RFLP of ITS include five species of *Ditylenchus* and eight described species of *Aphelenchoides* (Ibrahim et al., 1994; Wendt et al., 1995). Among insect-parasitic genera, both *Steinernema* (Nasmith et al., 1996; Reid, 1994) and *Heterorhabditis* (Joyce et al., 1994; Nasmith et al., 1996) species display species-specific digestion profiles. At subspecific levels, remarkable consistency is observed within isolates of one of these genera as demonstrated by a Hinf I digestion of ITS1 amplified from 53 infective juveniles representing five strains of *S. carpocapsae* (Fig. 4A,B). This same enzyme differentiates many of the described species of *Steinernema* (T. Powers, unpublished). The consistency in patterns among isolates of *Steinernema* and *Heterorhabditis* species may be due, in part, to homogeneity resulting from founder effects associated with the infection process, inbreeding by sexual stages in the infected cadaver, or, in the case of *Heterorhabditis,* hermaphroditic reproduction by first-generation adult females.

Animal-parasitic nematodes for which ITS has shown to be a useful diagnostic tool include *Dictyocaulus* (Epe et al., 1996), *Hypodontus* (Chilton et al., 1995), *Trichostrongylus* (Gasser and Hoste, 1995; Hoste et al., 1995), *Strongylus* (Campbell et al., 1995), and *Haemonchus* (Stevenson et al., 1995).

ITS profiles may be particularly useful in large genera in which taxonomic characters are difficult to interpret. *Helicotylenchus* contained 184 described species in 1991 (Ebsary, 1991), with 34 North American species (Nematode Geographical Distribution Committee, 1984). The morphological variability and small distinctions among *Helicotylenchus* spp. have practically eliminated dichotomous keys as an option for species identification and have led to the development of a probability-based computer identification system called NEMISYS (Fortuner, 1993). In our survey of *Helicotylenchus* isolates from the Great Plains region of North America, we have found ITS1 patterns correlated with plant hosts such as Hinf I patterns from eight individual nematodes representative of populations from agronomic and native hosts (Fig. 5A). Patterns A1 and A2 have been observed in more than 30 *Helicotylenchus* isolates from corn, soybeans, sorghum, and bluegrass lawns throughout eastern Kansas, Nebraska, and South Dakota. In northeast Nebraska and South Dakota counties bordering Nebraska, five corn fields were found with mixtures of patterns A1-A2 and B. Pattern C has been observed only from corn in western Kansas, whereas patterns D1 and D2 have been recorded from native and agronomic hosts from Florida to Hawaii. Both of these patterns, together with pattern E, are the predominant HinfI patterns found on the remnant, native, tallgrass prairies in the Great Plains of North America. D1 was the only pattern observed in samples taken from St. Augustine grass throughout Florida. D1 also has been recorded from saguaro cactus in Arizona, in culture on jade plant at Cornell University, and in pineapple fields in Hawaii. Pattern F has been observed from *Helicotylenchus* spp.

from bluegrass lawns in Brookings and Sioux Falls, South Dakota.

Species names can be placed only tentatively on the *Helicotylenchus* specimens from which these patterns were derived. For example, pattern F in Figure 5A has been exclusively associated with a *Helicotylenchus* species that conforms morphologically to *H. digonicus* Perry, 1959 and has been collected for molecular studies from localities previously identified by Thorne and Malek (1968). Similarly, many of the cornfield samples contained species that could be morphologically identified as *H. dihystera* (Cobb, 1893) Sher, 1961. Yet, from the PCR-RFLP patterns, it is clear that many populations identified as *H. dihystera* exist as mixtures of genotypes. Since many *Helicotylenchus* species are collections of parthenogenetic lineages, we believe application of species names is ill-advised before a detailed study that links morphological variation with molecular patterns is conducted.

ITS heterogeneity: In addition to size variation between species and genera and restriction site variation between genera, species, and populations, nematodes also appear to commonly possess ITS heterogeneity within individuals. Heterogeneous individuals, defined as nematodes containing more than one ITS pattern in their genome, have been reported in *Meloidogyne* (Zijlstra et al., 1995, 1997), *Heterodera* (Szalanski et al., 1997), *Belonolaimus* (Cherry et al., 1997), and have been demonstrated experimentally in hybridization studies between *G. pallida* and *G. rostochiensis* (Thiery et al., 1996). Amplification of ITS with two size classes within an individual could be inferred from two-banded amplification patterns of *Aphelenchoides besseyi* and *A. arachidis* (Ibrahim et al., 1994). Heterogeneity also may be inferred from digestion experiments in which digestion profiles exhibit "extra" fragments relative to a standard profile, without the expected alteration of another fragment in the

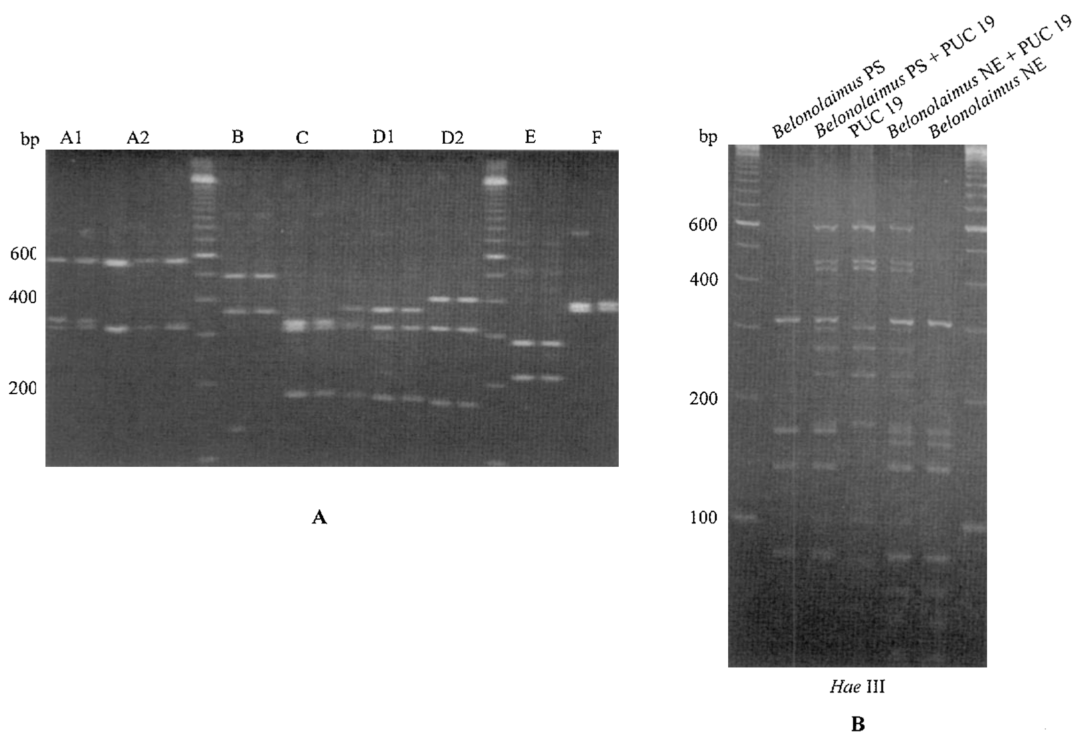

FIG. 5. A) Eight Hinf I patterns from individual *Helicotylenchus* nematodes representative of populations from agronomic and native hosts. B) Hae III digestion of *Belonolaimus* ITS1 amplified from individuals from Nebraska (NE) and Palm Springs, CA (PS) isolates. Nematode DNA was mixed with PUC 19 vector DNA prior to digestion to evaluate completeness of restriction digestion.

putative heterogeneous profile. These extra fragments also result in inconsistencies between the sum of the estimated size of digestion products and the size of the undigested amplification product, as in two ITS1 amplifications from *Belonolaimus* individuals (Fig. 5B): the Hae III profile of a nematode from Palm Springs, Florida (Lane 2), and the Hae III profile of a nematode from Nebraska (Lane 6). The Nebraska isolate is characterized by extra fragments of approximately 170, 70, 60, and 50 bp. Digestion of plasmid DNA mixed with the ITS1 amplification product (Fig. 5B, lanes 3,5) demonstrated that the additional products were not the result of incomplete restriction digestion. When plasmid DNA was mixed with either *Belonolaimus* isolate, a pattern that represents a mixture of each of the individual DNA digestions was produced. Digestion patterns with extra fragments also can be observed in comparisons of *Radopholus similis* isolates (Fallas et al., 1996). As a taxonomic marker in PCR-RFLP studies, heterogeneity provides added genetic resolution and may contribute information in studies of population structure and gene flow. However, ITS heterogeneity will complicate phylogenetic analyses unless questions of DNA sequence homology can be addressed (Baldwin et al., 1995).

Overall, the ITS region may become an important taxonomic feature in future nematode diagnoses. ITS versatility, specificity, ease of experimental manipulation, and growing ITS databases should accelerate its application in nematology. Its usefulness, however, will hinge on a careful evaluation of the relationship between ITS genetic variation and traditional taxonomic characters.

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