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Identification of Cyst Nematodes of Agronomic and Regulatory Concern with PCR-RFLP of ITS1[1]

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Abstract: The first internally transcribed spacer region (ITS1) from cyst nematode species (Heteroderidae) was compared by nucleotide sequencing and PCR-RFLP. European, Asian, and North American isolates of five heteroderid species were examined to assess intraspecific variation. PCR-RFLP patterns of amplified ITS1 DNA from pea cyst nematode, *Heterodera goettingiana,* from Northern Ireland were identical with patterns from Washington State. Sequencing demonstrated that ITS1 heterogeneity existed within individuals and between isolates, but did not result in different restriction patterns. Three Indian and two U.S. isolates of the corn cyst nematode, *Heterodera zeae,* were compared. Sequencing detected variation among ITS1 clones from the same individual, between individuals, and between isolates. PCR-RFLP detected several restriction site differences between Indian and U.S. isolates. The basis for the restriction site differences between isolates from India and the U.S. appeared to be the result of additional, variant ITS1 regions amplified from the U.S. isolates, which were not found in the three India isolates. PCR-RFLP from individuals of the U.S. isolates created a composite pattern derived from several ITS1 types. A second primer set was specifically designed to permit discrimination between soybean (*H. glycines*) and sugar beet (*H. schachtii*) cyst nematodes. *Fok* I digestion of amplified product from soybean cyst nematode isolates displayed a uniform pattern, readily discernible from the pattern of sugar beet and clover cyst nematode (*H. trifolii*).

Key words: clover cyst nematode, corn cyst nematode, diagnosis, *Heterodera glycines, Heterodera goettingiana, Heterodera schachtii, Heterodera trifolii, Heterodera zeae,* molecular nematology, nematode PCR-RFLP, pea cyst nematode, rDNA variation, regulatory nematology, soybean cyst nematode, sugar beet cyst nematode.

Expanded international trade will require increased awareness of nematodes of regulatory concern. Cyst nematode species, in particular, have a notorious history of global dispersal (Baldwin and Mundo-Ocampo, 1991; Barker, 1985). Their ability to withstand desiccation in the protective cyst stage greatly enhances their dispersal capabilities. Among the cyst species suspected to have been recently introduced into the United States are the corn cyst nematode, *Heterodera zeae,* and the pea cyst nematode, *H. goettingiana. Heterodera zeae* was first described from India by Koshy et al. (1971) where it was subsequently found to be widely distributed. It was later reported from Pakistan (Maqbool, 1981), the Nile Valley, Egypt, and Kent County, Maryland, USA (Ringer et al., 1987; Sardanelli et al., 1981). A second population in the United States has been identified in Virginia (Eisenback et al., 1993). This nematode had been considered a quarantinable species under the USDA Federal Domestic Quarantines: corn cyst nematode (CCN) 7CFR 301.90, which regulates the movement of soil, machinery, and root crops in the affected area (Anonymous, 1995). On 10 October 1996 U.S. federal *H. zeae* regulations were removed on the basis that it appeared that the infestation was contained by the two states affected. *Heterodera goettingiana* occurs throughout the world and is widespread in Europe (Di Vito and Greco, 1986). Occasionally it has been reported in the United States (Thorne, 1961), and Stone and Course (1974) suggested that U.S. populations are chance introductions. In 1992 an established field population was discovered on pea roots in western Washington (Handoo et al., 1994). Additional findings in western Washington indicate that the pea cyst is not confined to a single field (Inglis, pers. comm.).

The soybean cyst nematode (SCN), *H. glycines,* has been a concern to regulatory agen-

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cies in the United States since its discovery in North Carolina in 1954 (Winstead et al., 1955). In the central and western plains of the United States, discrimination between soybean cyst and sugar beet cyst nematode (SBCN), *H. schachtii,* is becoming more important as soybean and sugar beet production increasingly occurs in the same locations. Recently, both species have been identified from a single field in Michigan (G. Bird, pers. comm.). Proper nematode management in mixed-species situations will require accurate species identification.

PCR provides a rapid means for determining species identity from any nematode stage, including eggs. Samples can be collected, shipped, and stored in alcohol with no concern for accidental escape. Using PCR primers designed to amplify the first internal transcribed spacer region (ITS1), we investigated how species discrimination could be conducted quickly and efficiently for cyst species of agronomic and regulatory concern.

Materials and Methods

Nematode samples: Nematode samples used in this study were either collected from the field or sent live as dry cysts or as cysts preserved in 95% ethanol. Many soybean and sugar beet cyst samples were processed through the University of Nebraska Plant Disease Diagnostic Clinic. Pea cyst samples were obtained from Lewis and Skagit Counties, Washington, and from the Department of Agriculture of Northern Ireland. India corn cyst samples in ethanol were obtained from Hisar, Sonepat, and Ambla. U.S. corn cyst samples in ethanol were obtained from Maryland and Virginia.

DNA isolation, amplification and sequencing: DNA was isolated from individual cysts with a phenol-choloroform extraction technique similar to Taylor et al. (1996). Ethanol-precipitated DNA was resuspended in 50 µl Tris-EDTA (TE) (pH 7.5) and stored at –20 °C until use. Single juveniles or eggs were processed for PCR by placing them in a 15-µl drop of distilled water on a glass cover slip and manually disrupting them (Powers and Harris, 1993).

The two amplification primers, rDNA2 and rDNA1. 58S, are 21 and 20 nucleotides in length, respectively. The rDNA2 primer (5′-TTGATTACGTCCCTGCCCTTT-3′) has been described by Vrain et al. (1992), and rDNA1.58s (3′-GCCACCTAGTGAGCCGAGCA-5′) was designed by comparative sequence alignments of various nematode species. These primers amplify a 3′ portion of the 18S gene, entire ITS1 region, and a 5′ section of the 5.8S gene. The primer amplification set for SCN-SBCN discrimination, ITS1-F40 and ITS1-R380, are each 20 nucleotides in length and located within ITS1 (Fig. 1). The ITS1-F40 primer (5′-GTTGGGCTAGCGTTGGCACC-3′) and ITS1-R380 (5′-CCAGTCAGTGTGTTATGTGC-3′) were designed by comparative sequence alignments of SCN and SCBN sequences. Primers were synthesized by the University of Nebraska-Lincoln primer synthesis lab. PCR reactions were conducted in a reaction mixture consisting of 1.0 µl of target cyst DNA, 5.0 µl of 10× reaction buffer, 4.0 µl of dNTP mix (10 mM each of dATP, dTTP, dCTP, and dGTP), 1.0 µl (20 mM) of each primer, 2.5 units of *Taq* DNA polymerase, and deionized water to a total volume of 50.0 µl. Reaction buffer, dNTP, and *Taq* polymerase were obtained from a GeneAmp PCR reagent kit (Perkin Elmer Cetus, Norwalk, CT). The PCR amplification profile consisted of 40 cycles of 94 °C for 45 seconds, 55 °C for 1 minute, and 72 °C for 2 minutes. The product was stored at –20 °C.

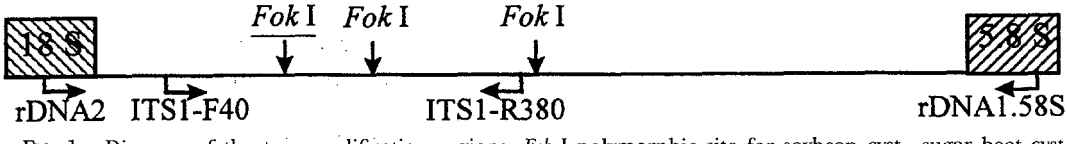

Fig. 1. Diagram of the two amplification regions. *Fok* I polymorphic site for soybean cyst—sugar beet cyst nematode discrimination is underlined. See Materials and Methods for primer designations.

DNA was amplified from a minimum of 20 individual cysts from each population.

Amplified DNA for DNA sequencing was purified with Geneclean II (Bio 101, Inc., Vista, CA). Sequences were obtained from amplification products cloned and sequenced in our laboratory following Sambrook et al. (1989), clones sequenced commercially by the University of Nebraska-Lincoln DNA Sequencing Lab (Lincoln, NE) with an LI-COR Model 4000 DNA Sequencer (LI-COR, Lincoln, NE) (Cherry et al., 1997) and PCR products directly sequenced by the Iowa State University DNA Sequencing and Synthesis Facility. Sequences were obtained for both DNA strands. The Genbank accession numbers for the rDNA ITS sequence for each species are given in Fig. 2. Sequences were aligned with GCG (Genetics Computer Group, Madison, WI) PILEUP program (with a gap-weight of 5.0 and a gaplengthweight of 0.3).

PCR-RFLP: Restriction sites were predicted from sequence data with Webcutter 2.0 (Heiman, 1997). Predicted diagnostic restriction sites were checked with restriction enzymes (New England Biolabs, Beverly, MA). Amplified ITS1 DNA was digested according to manufacturer's recommendations (New England Biolabs). The digestion reaction generally included 7.0 µl sterile double-distilled H_2O, 10.0 µl amplified DNA, 2.0 µl 10× restriction enzyme buffer, and 1.0 µl of restriction enzyme. Reactions were incubated at 37 °C for 6 to 24 hours.

For electrophoresis of digested DNA, the digestion mixture was added to 1.6 µl of gel dye and loaded on a 2.5% MetaPhor (FMC, Rockland, ME) agarose gel in 0.5× Tris-Borate-EDTA (TBE) buffer on a model H5 horizontal gel apparatus (BRL, Gaithersburg, MD). Ethidium bromide (0.015 mg) was added to the gel and electrophoresis buffer. Electrophoresis was conducted at a constant 100 volts for 4 hours. Gels were photographed under UV light with Polaroid film (ASA 3000).

The amplification products from all available isolates of corn and pea cyst nematode were evaluated by digestion with seven restriction enzymes selected on the basis of predicted digestion patterns inferred from nucleotide sequences. Restriction enzyme profiles produced by *Acc* I, *Alu* I, *Ava* I, *Hae* III, *Hha* I, *Hinf* I, and *Sau3A* I were compared with their predicted patterns (Table 1).

Results

Corn cyst and pea cyst nematodes: The ITS1 primer set amplified a single fragment from both cyst DNA extractions and single-egg or juvenile squashes. The ITS1 amplification product was 798 bp in length (including primers) for *H. zeae* and 811 bp in length for *H. goettingiana,* as determined by nucleotide sequencing (Fig. 2). The predicted ITS1 digestion pattern of the pea cyst isolates from Northern Ireland and Washington State were in agreement with their observed pattern on agarose gels, and no variation among isolates or individuals was observed with PCR-RFLP (Fig. 3). Nucleotide sequence variation was observed among different clones of the Northern Ireland isolate from a single amplification product, although these differences did not appear to affect the digestion pattern of the product. Ten nucleotide sites of the 811-bp product were observed to be polymorphic among ITS1 sequences of *H. goettinginana.* These included three substitutions observed among clones derived from a single amplification from a nematode representative of the Northern Ireland isolate, and seven differences between Northern Ireland and Washington isolates. A single difference observed in the conserved 18S gene of the Northern Ireland isolate may indicate the existence of nucleotide incorporation errors during the amplification process.

Corn cyst nematode displayed several intraspecific restriction site differences when all five isolates were compared. For example, *Ava* I digests of the U.S. isolates produce a three-fragment pattern compared to two-fragment pattern observed among isolates from India (Fig. 4) The *Alu* I restriction pattern of the U.S. isolates displays a 530-bp fragment not observed in the India isolates (Table 1). The *Hae* III digestion also produces two fragments, 424 and 118 bp in the

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                                10         20         30         40         50
H. goettingiana NI              TTGATTACGT CCCTGCCCTT TGTACACACC GCCCGTCGCT GCCCGGGACT
H. goettingiana USA             .......... .......... .......... .......... ..........
H. zeae India-A                 .......... .......... .......... .......... ..........
H. zeae India-H                 .......... .......... .......... .......... ..........
H. zeae India-S (cs)            .......... .......... .......... .......... ..........
H. zeae USA (b)                 .......... .......... .......... .......... ..........
H. zeae India-S (ds)            .......... .......... .......... .......... ..........
H. zeae USA (a)                 .......... .......... .......... .......... ..........

                                60         70         80         90         100
H. goettingiana NI              GAGCCATTTC GAGAAACTCG GGGACGATTA GTGTGTCAGC TTCGGCTGGC
H. goettingiana USA             .......... .......... .......... .......... .........T.
H. zeae India-A                 .......... ......T... ......G... .......G.. .....TCTT.
H. zeae India-H                 .......... ......T... ......G... .......G.. .....TCTT.
H. zeae India-S (cs)            .......... ......T... ......G... .......G.. .....TCTT.
H. zeae USA (b)                 .......... ......T... ......G... .......G.. .....TCTT.
H. zeae India-S (ds)            .......... ......T... ......G... .......G.. .....TCTT.
H. zeae USA (a)                 .......... ......T... ......G... .......G.. .....TCTT.

                                110        120        130        140        150
H. goettingiana NI              GCGCAGATTG AAACCGATTT AATCGCAGTG GCTTGAACCG GGCAAAAGTC
H. goettingiana USA             .......... .......... .......... .......... ..........
H. zeae India-A                 A...G..C.. G......... .......... .......... ..........
H. zeae India-H                 A...G..C.. G......... .......... .......... ..........
H. zeae India-S (cs)            A...G..C.. G......... .......... .......... ..........
H. zeae USA (b)                 A...G..C.. G......... .......... .......... ..........
H. zeae India-S (ds)            A...G..C.. G......... .......... .......... ..........
H. zeae USA (a)                 A...G..C.. G......... .......... .......... ..........

                                160        170        180        190        200
H. goettingiana NI              GTAACAAGGT AGCTGTAGGT GAACCTGCTG CTGGATCATT ACCCAAGTGA
H. goettingiana USA             .......... .......... .......... .......... ..........
H. zeae India-A                 .......... .......... .......... .......... ..........
H. zeae India-H                 .......... .......... .......... .......... ..........
H. zeae India-S (cs)            .......... .......... .......... .......... ..........
H. zeae USA (b)                 .......... .......... .......... .......... ..........
H. zeae India-S (ds)            .......... .......... .......... .......... ..........
H. zeae USA (a)                 .......... .......... .......... .......... ..........
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FIG. 2. Alignment of corn and pea cyst rDNA ITS sequences. Restriction enzyme recognition sites are highlighted, 18S and 5.8S regions are italicized, and primer sequences are underlined. cs: cloned sequence; ds: direct sequence; a: haplotype a; b: haplotype b. NI: Northern Ireland; India-A: Ambla, India; India-H: Hisar, India; India-S: Sonepat, India; Genbank submission numbers: *H. goettingiana* USA—U89388; *H. goettingiana* NI—U89389; *H. zeae* India-A—U89394; *H. zeae* India-H—U89395; *H. zeae* India-S (cs)—U89393; *H. zeae* USA (b)—U89391; *H. zeae* India-S (ds)—U89392; *H. zeae* USA (a)—U89390.

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                          210        220        230        240        250
H. goettingiana NI        TTCCAATTCA CCACCTATCT GYGCTGTTCA TGTTGGCTTG TGCGGGCACC
H. goettingiana USA       .......... .......... .T........ .......... ..........
H. zeae India-A           ..-....... ...A...C.. .CCG.CCG.G A.GAAC.AGT GAT.......
H. zeae India-H           ..-....... ...A...C.. .CCG.CCG.G A.GAAC.AGT GAT..C....
H. zeae India-S (cs)      ..-....... ...A...C.. .CCG.CCG.G A.GAAC.AGT GAT..C....
H. zeae USA (b)           ..-....... ...A...C.. .CCG.CCG.G A.GAAC.AGT GAT.......
H. zeae India-S (ds)      ..-....... ...A...... .CCG.CCG.G A.GAAC.AGT GAT.......
H. zeae USA (a)           ..-....... ...A...C.. .CCGCCC..G A.GAAC.AGT G.T.......

                          260        270        280        290        300
H. goettingiana NI        ACCATATGCC ---TTCGCTT GCAGTAG--C GGRCTTAGAC CGTACAAGTT
H. goettingiana USA       .......... ---.......... .......--. ..A.......... ..........
H. zeae India-A           .......... CCG......C T.G.GC.GA. ..A.ACG... ...GTGGACC
H. zeae India-H           .......... CCG......C T.G.GC.GA. ..A.ACG... R..GTGGACC
H. zeae India-S (cs)      .......... CCG......C T.G.GC.GA. ..A.ACG... A..GTGGACC
H. zeae USA (b)           .......... CCG......C T.G.GC.GA. ..A.ACG... ...GTGGACC
H. zeae India-S (ds)      .......... CCG......C T.G.GC.GA. ..A.ACG... ...GTGGACC
H. zeae USA (a)           .T...G.... CCG......C T.G.GC.GA. ..A.ACGA.. ...GTGG..C

                          310        320        330        340        350
H. goettingiana NI        TRRYCTGACG CACTGGCTGG CTGACGCTAA TGRATGGCCG CCCTGT-GGC
H. goettingiana USA       .GGT...... ..-----..T. .......... ..A..A.... ......G...
H. zeae India-A           .GGT.C.TAT .....---.. GATT....GT ..GGC...T. .....CGA..
H. zeae India-H           .GGT.C.TAT .....---.. GATT....GT ..GGC...T. .....CGA..
H. zeae India-S (cs)      .GGT.C.TAT .....---.. GATT.....T ..GGC...T. .....CGA..
H. zeae USA (b)           .GGT.C.TAT .....---.. GATT....GT ..GGC...T. .....CGA..
H. zeae India-S (ds)      .GGT.C.TAT .....---.. GATT....GT ..GGC...T. .....CGA..
H. zeae USA (a)           .GGT.C.TAC .G...---.. GACT....GT ..G.C...T. .....CGA..

                          360        370        380        390        400
H. goettingiana NI        AACTCTCGTT GGGGTGTTTA CGCCTACGGT TGGAGCTGGT ATACCAGCCA
H. goettingiana USA       .......... .......... .......... .......... ..........
H. zeae India-A           ...G..T... ......C..T TC.T.--T.. ...T..... ..G.T....T
H. zeae India-H           ...G..T... ......C..T TC.T.--T.. ...T..... ..G.T....T
H. zeae India-S (cs)      ...G..T... ......C..T TC.T.--T.. ...T..... ..G.T....T
H. zeae USA (b)           ...G..T... ......C..T TC.T.--T.. ...T..... ..G.T....T
H. zeae India-S (ds)      ...G..T... ......C..T TC.T.--T.. ...T..... ..G.T....T
H. zeae USA (a)           ...G..T... ......C..T .C.T.--T.. ...CA..... ..G.T....T
```

FIG. 2. Continued

U.S. samples not seen in the India isolate. Two *Hae* III fragments of 300 and 240 bp were observed in the India Hisar and Sonepat isolates and were not seen in other isolates (data not shown). These additional fragments appear to be evidence of heterogeneity in the ITS1 region within individual nematodes. Extended digestion periods, mixing experiments with control DNA, and repeated amplifications suggest the observed heterogeneity is not the result of partial DNA digestion. Sequences derived from cloning and direct sequencing of PCR products from the Maryland isolates revealed that PCR-RFLP patterns are comprised of at least two ITS1 variants (Fig. 2, MD-A, MD-B). No differences were observed between PCR-RFLP profiles of amplified DNA generated

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                                410        420        430        440        450
H. goettingiana NI      CTGCTTACAC GCATGAAAGC CTGAGGTTTG GCTGCGTCTA GCTGTGTGCG
H. goettingiana USA     .......... .......... .......... .......... ..--......
H. zeae India-A         ..T.G.CGG. ..TG..---- ---------- ---------- ----C.AAT.
H. zeae India-H         ..T.G.CGG. ..TG..---- ---------- ---------- ----C.AAT.
H. zeae India-S (cs)    ..T.G.CGG. ..TG..---- ---------- ---------- ----C.AAT.
H. zeae USA (b)         ..T.G.CGG. ..TG..---- ---------- ---------- ----C.AAT.
H. zeae India-S (ds)    ..T.G.CGG. ...G..---- ---------- ---------- ----C.AAT.
H. zeae USA (a)         ..T.G.CAG. ..TG..---- ---------- ---------- ----C.AAT.

                                460        470        480        490        500
H. goettingiana NI      CTGGTGGTGA GTGGGCTGCT CTGCGGTCCT TCTCGCTGCA CCGACCTTGG
H. goettingiana USA     .......... .......... .........C .......... ..........
H. zeae India-A         ..........---- .CT.A.C..C T...T.GTTG GT.T....TG .T...T.A..
H. zeae India-H         ..........---- .CK.A.C..C T...T.GTTG GT.T....TG .T...T.A..
H. zeae India-S (cs)    ..........---- .C..A.C... T...T.GTTG GT.T....TG .T...T.A..
H. zeae USA (b)         ..........---- .CT.A.C..C T...T.GTTG GT.T....TG .T...T.A..
H. zeae India-S (ds)    ..........---- .C..A.C..C T...T.GTTG GT.T....TG .T...T.AA.
H. zeae USA (a)         ..........---- .C....C..C T...T.GTTG GT.T....TG .TT..T.G..

                                510        520        530        540        550
H. goettingiana NI      GTACGGACAC GCCCGCASA- -CACTAACGG CTGTGCTGGC GTCTAAGCGT
H. goettingiana USA     .......... .......G.A CA..........  .......... ..........
H. zeae India-A         .A.---G.T. ..T..TGGGG CAC.......  -..C.....T ....GT....
H. zeae India-H         .A.---G.T. ..T..TGGGG CAC.......  -..C.....T ....GT....
H. zeae India-S (cs)    .A.---G.T. ..T..TGGGG CAC.......  -..C.....T ....GT....
H. zeae USA (b)         .A.---G.T. ..T..TGGGG CAC.A.....  -..C.....T ....GT....
H. zeae India-S (ds)    .A.---G.T. ..T..TGGGG CAC......C  -..C.....T ....GT....
H. zeae USA (a)         .G.----TT. ..T..TGGGG CAC......C  -..C.....T ....GT..C.

                                560        570        580        590        600
H. goettingiana NI      TGTTGAGTGG TTGTTGTGCC ARGC---GTA TGCATGCTGG CTTTG----G
H. goettingiana USA     .......... .......... .G..---... .......... .....----.
H. zeae India-A         C.......C.. .......... TT..ACTCA. ....GA.... .CG..GACT.
H. zeae India-H         C.......C.. .......... TT..ACTCA. ....GA.... .CG..GACT.
H. zeae India-S (cs)    C.......C.. .......... TT..ACTCA. ....GA.... .CG..GACT.
H. zeae USA (b)         C.......C.. .......... TT..ACACA. ....GA.... .CG..GACT.
H. zeae India-S (ds)    CC...G.C.. .......... TT..ACACA. .....-A.... .CG..GACT.
H. zeae USA (a)         C-....AC.. .......... TT..ACACA. ....ACTG.C .CG..GACT.
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FIG. 2. Continued

from individual eggs vs. individual cysts. Overall sequencing of *H. zeae* isolates revealed 49 polymorphic nucleotide sites for the 798-bp amplification product. Twenty-six of these polymorphic sites were found only in the cloned MD-B variant. Eleven of the polymorphic sites were shared by more than one isolate.

Soybean, sugar beet, and clover cyst nematodes: Inferred restriction site polymorphism from nucleotide sequence data indicated the existence of polymorphic *Fok* I site that allowed the discrimination of soybean and sugar beet cyst nematodes. Digestion with *Fok* I, however, resulted in indistinct gel patterns. On the assumption that two invariant *Fok* I sites near the polymorphic site were interfering with restriction enzyme digestion, a second set of primers was designed to eliminate interference of the invariant *Fok* I

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                          610        620        630        640        650
H. goettingiana NI        AAGTGGTTCT TC--CTTAGT CTTACGTACC GTAACTAGCG GTGTGTGTGC
H. goettingiana USA       .......... ..--.....C .......... .......... ..........
H. zeae India-A           ..T....CTG ..CT..CG.C .....AC... .......... ..C..C...T
H. zeae India-H           ..T....CTG ..CT..CG.C .....AC... .......... ..C..C...T
H. zeae India-S (cs)      ..T....CTG ..CT..CG.C .....AC... .......... ..C..C...T
H. zeae USA (b)           ..T....CTG ..CT...G.C .....AC... .......... ..C..C...T
H. zeae India-S (ds)      ..T....CTG ..CT...G.C .....AC... .......A.C ..C..C...T
H. zeae USA (a)           ..T....CTG ..CT...G.C .....AC... .......... ..T..C...T

                          660        670        680        690        700
H. goettingiana NI        GCTTGTGTGC TACGTCCGTG GCCACGATGA GACACCGTGT TAGGGC-CCG
H. goettingiana USA       .......... .......... .......... .......... ......-...
H. zeae India-A           .......... ..........  ...GT..... ...GA..C.G ......TG.A
H. zeae India-H           .......... ..........  ...GT..... ...GA..C.G ......TG.A
H. zeae India-S (cs)      .......... ..........  ...GT..... ...GA..C.G ......TG.A
H. zeae USA (b)           .......... ..........  ...GT..... ...GA..C.G ......TG.A
H. zeae India-S (ds)      .......... ..........  ...GT..... ...GA..C.G ......TG.A
H. zeae USA (a)           .......... ..........  ...GT..... ...GA..C.G ......TG.A

                          710        720        730        740        750
H. goettingiana NI        TGCTCGCTGC AGGCACGTGG CTTAAGACTT AATGAGTGCA CAGGGATTGC
H. goettingiana USA       .......... .......... .......... .......... .--.......
H. zeae India-A           ...GT.GCAT TA...T.....  .......... G.........T T.--.C....
H. zeae India-H           ...GT.GCAT TA...T.....  .......... G.........T T.--.C....
H. zeae India-S (cs)      ...GT.GCAT TA...T.....  .......... G.........T T.--.C....
H. zeae USA (b)           ...GT.GC.T TC...T.....  .......... G.........T T.--.C....
H. zeae India-S (ds)      ...GT.GCAT TA...T.....  .......... G.........T T.--.C....
H. zeae USA (a)           ...GT.GC.T TA...T.....  .......... G.........T T.--.C....

                          760        770        780        790        800
H. goettingiana NI        ACCGCCAGC- -TTTATTATT TATTTATTTT TTTACATTCT ATGAATGGAC
H. goettingiana USA       .........- -.......... .......... .......... .......A..
H. zeae India-A           .........A C...T.C..C ..CAC.AA.. ...TA.ACT. CC..G..A.G
H. zeae India-H           .........A C...T.C..C ..CAC.AA.. ...TA.ACT. CC..G..A.G
H. zeae India-S (cs)      .........A C...T.C..C ..CAC.AA.. ...TA.ACT. CC..G..A.G
H. zeae USA (b)           .........A T...T.C..C ..CA.TAA.. ...TA.ACT. CC..G..A.G
H. zeae India-S (ds)      .........A T...T.C.... ..CAC.A... ...TA.ACT. CC..G..A.G
H. zeae USA (a)           .........A T...T.C.... ..CAC..... ...TA.ACT. CC..G..A.G

                          810        820        830        840 842
H. goettingiana NI        AA---GTATA TTCTAGCCTT ATCGGTGGAT CACTCGGCTC GT
H. goettingiana USA       ..---..... .......... .......... .......... ..
H. zeae India-A           T.ACG--.G. ......T... .......... .......... ..
H. zeae India-H           T.ACG--.G. ......T... .......... .......... ..
H. zeae India-S (cs)      T.ACG--.G. ......T... .......... .......... ..
H. zeae USA (b)           T.ACGAA.G. ......T... .......... .......... ..
H. zeae India-S (ds)      T.ACG-A.G. ......T... .......... .......... ..
H. zeae USA (a)           T.ACG-A.G. ......T... .......... .......... ..
```

FIG. 2 Continued

TABLE 1. Corn and pea cyst restriction fragment sizes.

Restriction enzyme	Population[a]	Position of recognition site	Predicted size of resultant fragment (bp)
Acc I	*H. g.* WA1	378	378 433
	H. g. NI	378	378 433
Alu I	*H. g.* WA1	89 162 373 429 732	89 73 211 56 303 79
	H. g. NI	89 162 373 429 732	89 73 211 56 303 79
	H. z. INs	162 466 701	162 304 235 98
	H. z. MDa[b]	162 694	162 532 98
	H. z. MDb	162 466 701	162 304 235 98
	H. z. VA		
Ava I	*H. z.* INs	42 269	42 227 530
	H. z. MDa	42 226 268	42 184 42 530
	H. z. MDb	42 269	42 227 530
	H. z. VA		
Hae III	*H. g.* WA1	326 646 670	326 320 24 141
	H. g. NI	326 646 670	326 320 24 141
	H. z. INs	245 585 615 665	245 310 30 50 170
	H. z. MDa	585 615 665	555 30 50 170
	H. z. MDb	585 615 665	555 30 50 170
	H. z. VA		
Hha I	*H. g.* WA1	101 438 626	101 337 188 185
	H. g. NI	101 438 626	101 337 188 185
	H. z. INs	405 493	405 88 306
	H. z. MDab	405 493	405 88 306
Hinf I	*H. g.* WA1	199	199 612
	H. g. NI	199	199 612
	H. z. INs	199 317 763	199 118 446 36
	H. z. MDab	199 317 763	199 118 446 36
	H. z. VA		
Sau3A I	*H. g.* WA1	183 796	183 613 15
	H. g. NI	183 796	183 613 15
	H. z. INs	183 782	183 599 17
	H. z. MDab	183 782	183 599 17

[a] *H. g.* = *Heterodera goettingiana; H. z.* = *Heterodera zeae.*
[b] MDa is the sequence from direct sequencing and MDb is from cloned DNA.

sites (Fig. 1). The relocation of the primer set to eliminate one of the sites produced a 342-bp amplicon (Fig. 5). All *H. glycines* isolates tested, including samples from China, Japan, North America, and Colombia, contained a single *Fok* I site resulting in 252-bp and 89-bp fragments (Fig. 6). All *H. schactii* isolates tested had two *Fok* I sites resulting in 181-, 89- and 58-bp fragments (Figs. 6,7). *Fok* I digestion of *H. trifolii* ITS1 amplification products was characterized by a fragment of approximately 150 bp not observed in the other cyst nematode amplifications. However, only three clover cyst isolates were examined by this method, and one sample from Australia differed from the Arkansas isolates by loss of a 350-bp fragment (Fig. 6).

During the course of this study, an opportunity arose to test SCN/SBCN discrimination when a shipment of infested soil arrived at the University of Nebraska Diagnostic Clinic from Montana to be tested for soybean cyst nematode. Since SCN had not been previously identified from that state, eggs and juveniles were examined by PCR-RFLP (Fig. 7). All of the specimens tested produced the characteristic fragments of sugar beet cyst nematode, and it was subsequently learned that the agency submitting the sample had mislabeled the soil bag regarding the crop and suspected problem.

DISCUSSION

The ITS1 region has been demonstrated to be a useful genetic marker for nematode species. This study and others (Campbell et al., 1995; Chilton et al., 1995; Ferris et al., 1993; Ferris et al., 1994; Ferris et al., 1995;

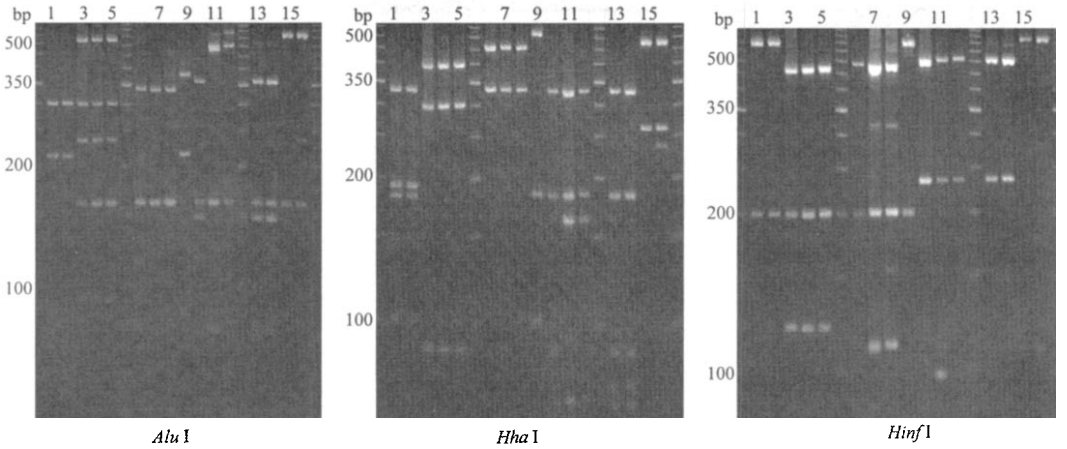

FIG. 3. *Alu* I, *Hha* I, and *Hinf* I restriction enzyme digestion patterns of various cyst nematodes for the rDNA1.58S—rDNA2 amplicon. Lanes: 1–2) *H. goettingiana;* 3–5) *H. zeae;* 6) *H. glycines;* 7) *H. schachtii;* 8) *H. trifolii;* 9) *H. cruciferae;* 10–11) *Globodera rostochiensis;* 12) *G. tabacum;* 13–14) *Punctodera* sp.; 15–16) *Cactodera* sp. Four lanes of 50-bp-size markers are included on each gel.

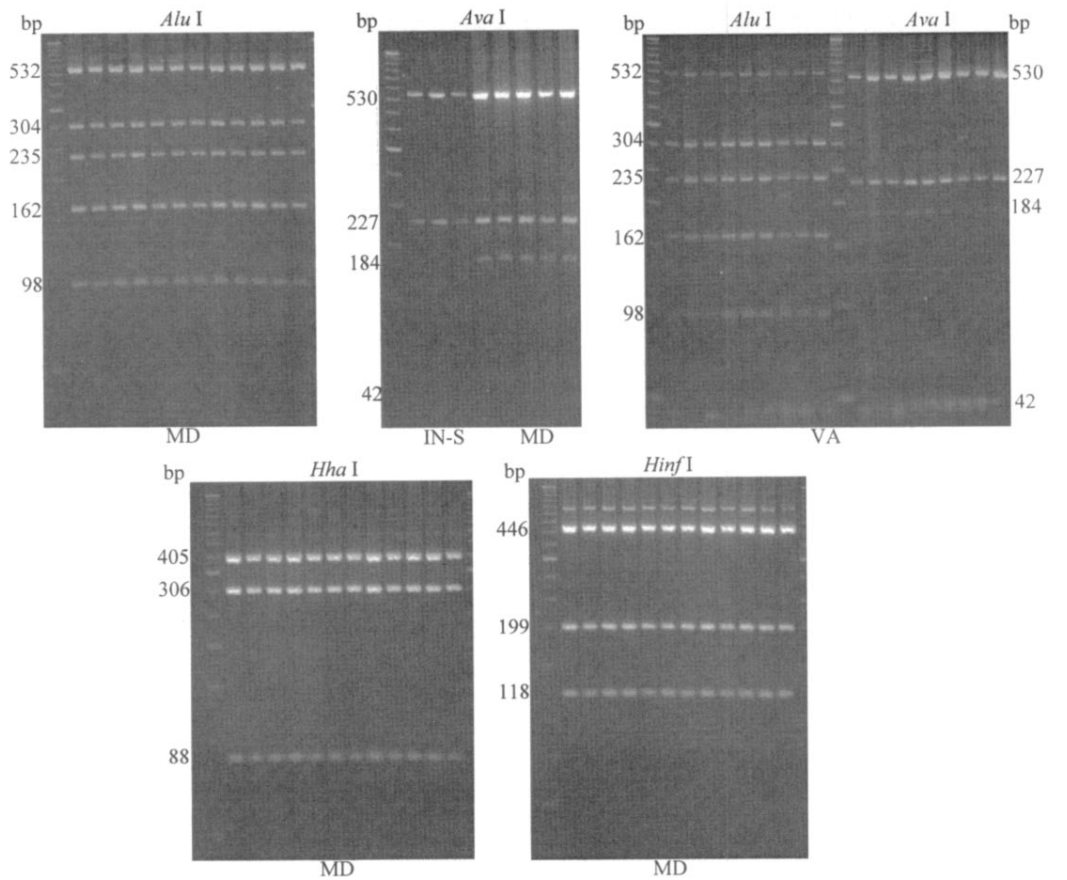

FIG. 4. *Alu* I, *Ava* I, *Hha* I, and *Hinf* I restriction enzyme digests of corn cyst rDNA1.58S-rDNA2 amplicon. IN-S: Sonepat, India, isolate; MD: Maryland isolate; VA: Virginia isolate.

```
                1                                                    50
H. glycines GTTGGGCTAG CGTTGGCACC ACCAAATGCC CCCGTCCGCT GATGGGCACA
H. schactii GTTGGGCTAG CGTTGGCACC ACCAAATGCC CCCGTCCGCT GACGGGCACA
               ITS1-F40
               51                                                   100
H. glycines GGTCGTTCGA GATGACTTGT GGACGTCTGC CCAACATTAC GGGGCAGCTG
H. schactii GGTCGTTCGA GATGACCTGT GGACG.CTGC CCAACATTAC GGGGTAGCTG
               101                                                  150
H. glycines CCTCACGTGC C.ATGCTTTT GGGGTGCTTC CATACGTTGG AGCTGTGGTA
H. schactii CCTCACGAGC CTATGCTTTT GGGGTGCTTC CATACGTTGG AGC..TGGTA
               151                                                  200
H. glycines TACCGCTCAG TGCTGCACAT GTGAAAGCCT GTGTATGGCT GCTGCGTGGC
H. schactii TACCGCTCAG TGCTGCACAT GTGAAAGCCT GTGGATGGCT GCTGTGTGGC
                                                  Fok I
               201                                                  250
H. glycines AATGTGTCGG TGGCGGGCCG CTCGCTTGGC TCGGTTCGCT GCGCCAATGT
H. schactii AATGTGTCGG TGGCGGGCCG CTCGCTTGG. TTGGTTCGCT GCGCCAACGG
               251                                                  300
H. glycines GGGATGCACG CTCGTGGGGC GACCTAACGG CTGTGCTGGC GTCTGTGCGT
H. schactii GGGATGCACG CTCGTGGGGC GACCTAACGG CTGTGCTGGC GTCTGTGCGT
               Fok I
               301                                                  342
H. glycines CGTTGAGCGG TTGTTGTGGC AGGCACATAA CACACTGACT GG
H. schactii CGTTGAGCGG TTGTTGTGGC AGGCACATAA CACACTGACT GG
                                                  ITS1-R380
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FIG. 5. Alignment of *H. schachtii* and *H. glycines* sequences amplified with an internal ITS1 amplification primer set. *Fok* I restriction enzyme recognition sites are bold-faced and italicized, primer sequences are underlined.

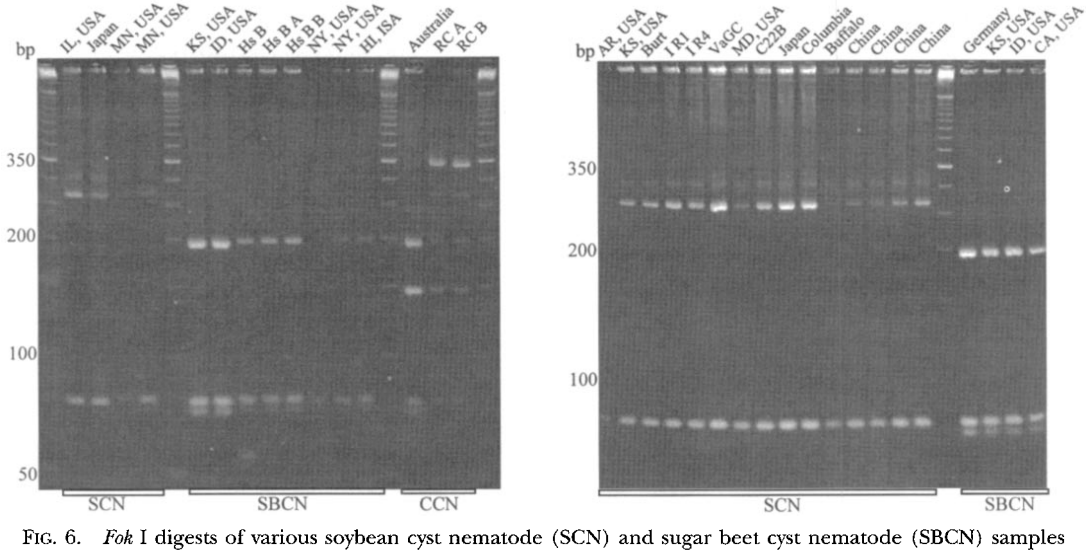

FIG. 6. *Fok* I digests of various soybean cyst nematode (SCN) and sugar beet cyst nematode (SBCN) samples from the ITS1-F40-ITS1-R380 amplicon. SCN isolates; IL, Illinois; MN, Minnesota; KS, Kansas; AR, Arkansas; Burt, Burt County, Nebraska; IR1, race 1 from Illinois; IR4, race 4 from Illinois; VaGC, Virginia; MD, Maryland; C22B, China; Colombia, Colombia, South America; Buffalo, Buffalo Center, Iowa. SBCN isolates: KS, Kansas; ID, Idaho; HsB, HsBA, HsBB, Bonn, Germany; NY, New York; HI, Hawaii; CA, California. Clover cyst nematode (CCN) isolates RCA and RCB: Arkansas. Unlabeled lanes are checks with 50-bp-size markers.

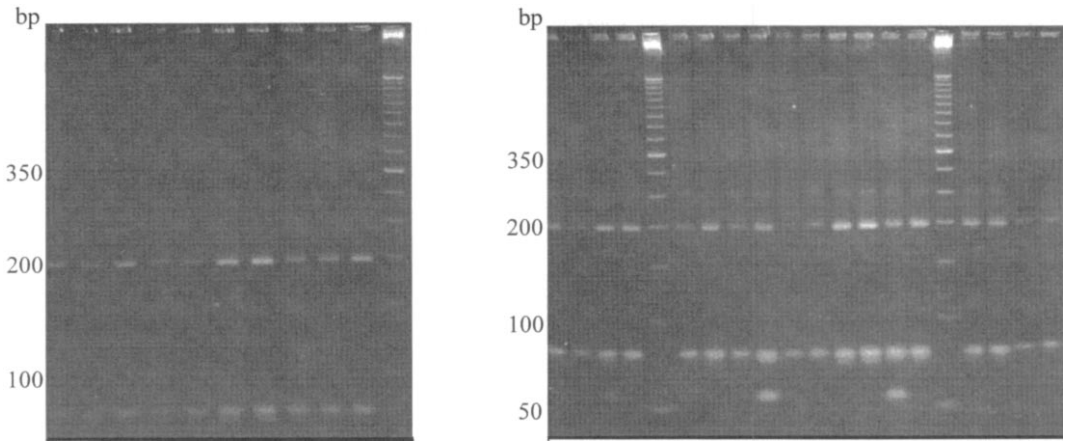

FIG. 7. *Fok* I digests of Montana sugar beet cyst nematode egg and juvenile samples from the ITS1-F40-ITS1-R380 amplicon. Each lane represents digestion of the amplification product from individual eggs or juveniles processed for egg counts from a field sample submitted to the University of Nebraska-Lincoln Plant Disease Clinic. Unlabeled lanes are checks with 50-bp-size markers.

Hoste et al., 1995; Joyce et al., 1994; Nasmith et al., 1996; Stevenson et al., 1995; Thiery and Mugniery, 1996; Vrain et al., 1992; Wendt et al., 1995; Zijlstra et al., 1995, 1997) have shown that nematode species, typically recognized by subtle morphological or host differences, are generally readily distinguished by restriction digestion patterns of the ITS region. In the case of soybean cyst-sugar beet cyst nematode discrimination, the design of specific primer sets based on the ITS1 region permits species identification in spite of relatively low levels of nucleotide divergence between species. This study demonstrated that PCR-RFLP patterns were consistent among globally distributed isolates of soybean, sugar beet, and pea cyst nematodes. The U.S. isolates of the corn cyst nematode provided patterns that were distinct from those of the three Indian isolates. Cloning and sequencing of the ITS1 region showed that the Maryland restriction pattern is a combination of ITS1 variants, possibly within an individual nematode. ITS heterogeneity has been reported within individual nematodes of *Meloidogyne* (Zijlstra et al., 1995, 1997) and *Belonolaimus* (Cherry et al., 1997), and has been experimentally demonstrated in progeny of matings between *G. pallida* and *G. rostochiensis* (Thiery et al., 1996). This heterogeneity could compromise phylogenetic studies if nonhomologous ITS variants were included in comparisons. It also complicates efforts to measure genetic distance among isolates. For example, sequencing of the MD-B variant of ITS1 from the Maryland *H. zeae* isolate and comparison with any of the three *H. zeae* isolates from India might lead to the conclusion that the Maryland isolate is a different species. Conversely, nucleotide sequence of the MD-A variant from Maryland is quite

TABLE 2. Morphological identification of *Heterodera zeae* and related common species.[a]

Species[b]	Bullae	J2 stylet length (μm)	J2 tail length (μm)	J2 stylet knob shape	Hosts
H. schachtii	scattered	25–27	48–55	anchor	wide
H. glycines	scattered	22–25	40–49	subventral rounded	wide
H. trifolii	scattered	>27	>55	anchor	wide
H. zeae	four distinct "fingers"	<22	40–49	concave on top	Poaceae

[a] Adapted from Baldwin and Mundo-Ocampo (1991).
[b] Species of the *H. schachtii* group: Bullae and underbridge well developed; vulva slit >35 μm; ambifenestrate.

TABLE 3. Morphological identification of *Heterodera goettingiana* and related species.[a]

Species[b]	Underbridge	Egg mass	J2 hyaline tail terminus (μm)	Hosts
H. goettingiana	slender, present	small	>34	legumes
H. cruciferae	slender, present	small	<26	crucifers
H. carotae	very thin; rarely persists in cysts	large, often size of female	26–32	carrots

[a] Adapted from Baldwin and Mundo-Ocampo (1991).
[b] Species of the *H. goettingiana* group: No bullae, underbridge poorly developed or absent; vulva slit >30 μm; ambifenestrate or bifenestrate.

similar to the ITS1 sequences from India isolates. Clearly, caution should be used in evolutionary inference with the ITS region.

For diagnostic purposes, the ITS1 variants contribute to the complexity of the restriction digestion pattern and serve as highly specific genetic markers (Vogler and DeSalle, 1994). The "extra" fragments generated in *Ava* I, *Alu* I, and *Hae* III digestions with ITS1 DNA from the U.S. *H. zeae* isolates could assist the monitoring of these populations. These characteristics, together with key morphological data (Tables 2,3) and photographic images, could make high-resolution identification much easier, especially when presented in an online, World Wide Web (WWW)-style format.

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