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Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes

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Summary – Partial sequences from internal transcribed spacer ribosomal DNA (ITS rDNA) obtained by polymerase chain reaction (PCR) from isolates of plant parasitic cyst nematodes, were compared with the published sequence for *Caenorhabditis elegans* and with each other. Data were from ITS1 and ITS2 of the rDNA array, and included also parts of the 18S and 28S genes adjacent to the spacer regions. The ITS data for cyst nematodes were highly dissimilar to those for *C. elegans*. The sequences for five geographic isolates of *Heterodera glycines* were very similar to each other, but showed nearly as many differences as were evident between this species and either *H. schachtii* or *H. trifolii*. More differences were observed in *H. carotae*, and many more in *H. avenae*. The findings confirm the utility of gene and spacer region rDNA for systematic inference among species and genera of cyst nematodes.

Résumé – ***Variabilité des régions intergéniques de l'ADN ribosomal des espèces de nématodes à kystes parasites des plantes*** – Des séquences partielles des régions intergéniques transcrites de l'ADN ribosomal (ITS rDNA) de souches de nématodes à kystes parasites de plantes ont été obtenues par une réaction de polymérisation en chaîne (PCR), puis comparées entre elles et avec les séquences publiées de *Caenorhabditis elegans*. Les données ont été obtenues des ITS1 et ITS2 du rDNA, mais elles incluaient également des parties des gènes 18S et 26S, adjacents aux régions intergéniques. Les données sur les ITS des nématodes à kystes sont très différentes de celles de *C. elegans*. Les séquences de cinq souches de *Heterodera glycines* provenant de régions différentes sont très proches les unes des autres, mais montrent à peu près autant de différences entre elles qu'avec *H. schachtii* ou avec *H. trifolii*. Il a été observé des différences plus prononcées avec *H. carotae*, et encore plus avec *H. avenae*. Ces résultats confirment l'utilité des gènes, et des régions intergéniques du rDNA, pour l'étude des relations systématiques entre espèces et genres du groupe de nématodes à kystes.

Key-words : ribosomal DNA, ITS-rDNA, spacer rDNA, *Heterodera*.

Members of the plant parasitic cyst nematodes of the genus *Heterodera* include some of the most important pests of agriculture worldwide (Hyman & Powers, 1991). Included among these are *H. schachtii*, the sugar beet cyst nematode (SBN), and *H. glycines*, the soybean cyst nematode (SCN). The latter is the most serious pest of soybeans in the USA, and is widely thought to have been imported to the USA from Japan in bulbs in the 1950's, then spreading rapidly throughout all soybean growing areas (Riggs, 1977; Norton, 1978). It is postulated to have arrived in southern Indiana about 1960 and spread quickly to northern Indiana within a few years. Following our observations that isolates from the northern half of Indiana are more virulent to many soybean cultivars than are isolates from farther south, we also found differences in protein patterns obtained by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), as well as morphological differences among isolates (Ferris *et al.*, 1985, 1986; Faghihi *et al.*, 1986*a*, *b*; Ferris *et al.*, 1987). These observations suggest that the Indiana isolates are not all part of one rapidly spreading population. An alternative view is that the recently discovered infestations are from indigenous local populations that moved from native weed species into cultivated soybeans as that crop became more intensively grown. Additional data on genetic similarity among cyst populations and species might help to resolve the issue.

Relationships among the species and genera of the cyst nematodes are controversial and are based on classical morphological data plus SEM and TEM (Wouts, 1985; Baldwin, 1992). The few existing molecular data for members of the group have been used mainly for diagnostics and identification (Hyman & Powers, 1991). Consistent 2-D PAGE protein patterns for all of the cyst nematode species and isolates discussed in this paper have been found (Ferris *et al.*, 1986; Bakker & Bouwman-Smits, 1988; Ferris & Ferris, 1989; Ferris *et al.*, 1989; unpubl. data, our laboratory), but the use of 2-D PAGE data to quantify differences among isolates or for phylogenetic inference is difficult and laborious (Bakker & Bouwman-Smits, 1988; Ferris & Ferris, 1988).

Hillis and Davis (1986) showed that ribosomal DNA (rDNA) could be used for phylogenetic analysis of frog species in the genus *Rana*. For nematodes, thought to be an ancient group (Ferris *et al.*, 1976), data from highly conserved (gene) regions might be useful to infer rela-

tionships among genera or higher taxa, whereas data from spacer regions might be used to infer relationships among species and subspecific groupings (Hyman & Powers, 1991). In a recent review of the uses of rDNA for phylogenetic inference, Hillis and Dixon (1991) noted that spacer regions heretofore have been infrequently used for phylogenetic studies and emphasized a need for investigations of the usefulness of comparative studies of rDNA spacers for closely related species and populations.

The objective of our study was to determine whether data from the two internal transcribed spacer regions of rDNA (rDNA ITS1 and ITS2) might be useful for phylogenetic inference in the cyst nematodes at either the population or species level. We have collected sequence data for rDNA ITS1 and ITS2 following amplification using polymerase chain reaction (PCR). PCR amplification of DNA is of particular utility for plant parasitic nematodes, where the problem of obtaining large quantities of DNA from many isolates for analytical procedures can quickly become insurmountable. For evaluation of the usefulness of rDNA ITS sequence for study of intra-specific variability, we used five geographic populations of SCN; and for comparison of inter-specific variability we used two additional members of the *schachtii* group, *Heterodera schachtii* and *H. trifolii*, plus *H. carotae* and *H. avenae*.

Material and methods

Nematodes

We collected the five isolates of SCN, *Heterodera glycines*, from five Indiana counties as a part of earlier investigations to assess similarities and differences among geographic isolates of SCN (Ferris *et al.*, 1986). From south to north the counties are Posey, Vigo, Tippecanoe, White and Pulaski. We also collected the clover cyst nematode (CCN), *H. trifolii*, from Tippecanoe County, Indiana. SBN, *H. schachtii*, from Michigan was a gift from Dr. L. I. Miller; the carrot cyst nematode, *H. carotae*, from Michigan was a gift from L. Grainey and Dr. G. Bird; and the cereal cyst nematode, *H. avenae*, from Australia was a gift from F. Green.

rDNA amplification, cloning and sequencing

A single mature female cyst nematode was cut open in 10 µl of TE buffer (0.5 M tris-Cl, 100 mM EDTA, pH 8.0) on a glass slide under a dissecting microscope, the body contents extruded and transferred by pipet to a 1.5 ml vial, the area rinsed with 8 µl additional buffer, and the preparation stored at – 20 °C. Prior to amplification, 20 µl of a solution comprised of 0.5 mM Tris-EDTA, 0.75 % Triton-X-100, and 1.5 % stock proteinase K solution (10 mg/ml) was added to the tube and the nematode tissue ground with a hand-held homogenizer for 5 min. The pestle was rinsed twice with 50 µl of the same solution and the homogenate heated at 50 °C for 2 h (Sulston & Hodgkin, 1988; B. C. Hyman, pers. comm.). The DNA was extracted from the homogenate with a GeneClean kit (Bio 101, San Diego, CA) and recovered in 30 µl of sterile water. Ten µl of the DNA suspension were used for each amplification. One to ten additional such preparations were made for all isolates with sequence that differed more than 1 % from that of other population isolates or species in the study.

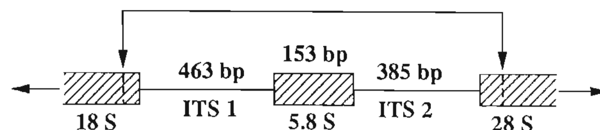

Fig. 1. Diagram with arrows indicating our amplified region of rDNA, with base pair (bp) numbers based on published data for *Caenorhabditis elegans* (Ellis *et al.*, 1986). ITS = internal transcribed spacer. 18S, 5.8S and 28S are rDNA genes.

Amplification was carried out using PCR (Saiki *et al.*, 1988; Saiki 1990) with reagents and recommended protocols from Perkin Elmer, and the Coy Tempcycler model 50. The 1100 bp amplified region began near the end of the 18S gene and terminated a short distance into the 28S gene, and included the ITS regions between them (Fig. 1). The forward primer, 5'-CGTAACAAGGTAGCTGTAG-3' was derived from the 18L primer of Hamby *et al.* (1988), modified to agree with the published rDNA sequence for *C. elegans* (Ellis *et al.*, 1986). The reverse primer, 5'-TCCTCCGCTAAATGATATG-3' was similarly modified from the ITS4 primer of White *et al.* (1990).

Amplification was carried out in two phases for each isolate in order to enhance the target product and to decrease background products (White & Blake, 1991). Phase 1 consisted of 30 cycles with denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and extension at 72 °C for 1 min. The amplified DNA band was monitored by electrophoresis in SeaPlaque agarose (FMC Corporation), excised, and diluted in 1 ml sterile water. Then 1 ng of the diluted DNA was amplified for 30 cycles as before, except that annealing was at 53 °C. The product from this amplification was passed through a Centricon 100 filter (Amicon Corporation). Following filtering, the DNA was either treated with Klenow fragment for blunt-end cloning (Ausubel *et al.*, 1992) into the plasmid pGEM-3Z (Promega), or it was cloned directly into the TA pCR1000 cloning vector supplied by Invitrogen Corporation. Double-stranded sequencing was performed using Sequenase version 2.0 from U.S. Biochemical Corporation. Sequencing primers (from Promega) were SP6 and T7 for pGEM-3Z, and T7 and pUC/M13 Forward Sequencing Primer for the pCR1000 vector. For most isolates, sequence data were

from multiple clones and both strands. Each sequencing run was repeated three to six times to assure accuracy of the sequence.

Sequence data for 640 bp for each isolate were aligned with those for SCN-Vigo, using the computer program GAP in the Sequence Analysis Software Package of the Genetics Computer Group (Devereaux *et al.*, 1984). GAP uses the algorithm of Needleman and Wunsch (1970) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. Based on these pairs of alignments, in which gap weight = 5.0 and gap length = 0.3, the multiple alignments were done manually (Swofford & Olsen, 1990).

Results

The sequence alignments include 278 bp from ITS1 plus the adjacent 42 bp from the 18S gene (Fig. 2), and 261 bp from ITS2 plus the adjacent 59 bp from the 28S gene (Fig. 3). The sequence for the Vigo isolate of SCN is listed first and is the basis for the numbering. The other four geographic isolates of SCN are listed in the order they occur in Indiana from south to north. The other *Heterodera* species are added in an order that corresponds to their presumed evolutionary proximity to SCN, based on classical data (Ferris, 1979; Mindell, 1991). For comparative purposes, the published sequence for *C. elegans* (Ellis *et al.*, 1986) is also listed. The degree of dissimilarity in ITS1 and ITS2 for both *H. avenae* and *C. elegans* makes their alignments in these areas problematical (Hillis & Dixon, 1991).

ITS1 region

Four geographic isolates of SCN plus *H. carotae* shared a difference in position 197 (G to A) from the Vigo isolate, whereas SBN and CCN were the same as SCN-Vigo at this position. The Posey and White isolates of SCN shared a difference in position 102 (A to C) that was also shared by *H. carotae* and *H. avenae*. SCN-White also had a unique difference in position 175 (G to A).

SBN and CCN were identical to each other in ITS1 and the adjacent 18S region except for one difference in position 299 (G to T). These two species shared two differences from SCN-Vigo. One of these was a difference in position 26 of the 18S gene area (C to T) and the second a difference in position 67 (T to C). *Heterodera carotae* had four bp differences from SCN-Vigo, including one unique difference (position 320), and in addition, had an 11-bp gap in sequence, which was unique for this group of taxa (Fig. 2). For *H. avenae* we found 104 differences from SCN-Vigo, all in ITS1, with only one (in the alignment shown, Fig. 2) shared with other *Heterodera* isolates (position 315). In this alignment, *H. avenae* shared 15 differences with *C. elegans*.

ITS2 region

Three of the geographic isolates of SCN, plus CCN and *H. carotae*, shared a difference from SCN-Vigo at position 229 (C to G). CCN had one additional difference, A to C at position 237 (Fig. 3). *Heterodera carotae* had three additional differences, position 228 (A to T), and positions 269 (T to C) and 294 (A to G) in 28S (Fig. 3). *Heterodera avenae* had 86 differences, with two of them, positions 284 (A to G) and 285 (C to T), in 28S. SBN had no differences from SCN-Vigo (Fig. 3).

Phenetic and phylogenetic analysis

The phenetic similarity of the pairs of sequences aligned by GAP varied from 99 % + for some of the *Heterodera* isolates/taxa to 46 % for the SCN-Vigo/*C. elegans* comparison. We calculated the sequence dissimilarity for each pair of the *Heterodera* isolates (Table 1). Most of the SCN, SBN and CCN comparisons show less than 1 % dissimilarity, with slightly more dissimilarity (1.36-2.63 %) between *H. carotae* and the *schachtii* group; and more than 28 % dissimilarity between *H. avenae* and the rest of the taxa.

Table 1. Pairwise percent nucleotide dissimilarities among *Heterodera* isolates. Calculations based on formulas of Swofford and Olsen (1991). $D = (1 - M/L)100$; $L = M + U + W_g G$; M = number of alignment positions with synonymous residues; L = an effective sequence length; U = number of alignment positions with nonsynonymous residues; W_g = weight given to gaps (= 0.5); G = number of alignment positions with a gap in one sequence juxtaposed with a residue in other sequence.

	S-VIG	S-POS	S-TIP	S-WHT	S-PUL	SBN	CCN	CAR	AVEN
S-VIG		0.48	0.32	0.56	0.48	0.32	0.80	2.15	28.40
S-POS			0.16	0.40	0.32	0.80	0.80	1.36	28.25
S-TIP				0.56	0.16	0.64	0.80	1.83	28.40
S-WHT					0.72	0.88	1.35	2.08	28.44
S-PUL						0.80	0.96	1.67	28.25
SBN							0.48	2.47	28.72
CCN								2.63	28.72
CAR									28.17

```
        1                                                                              80
S-VIG   CGTAACAAGGTAGCTGTAGGTGAACCTGCTGCTGGATCATTACCCAAGTGATTCCTATTCACCACCTACCTG--CTGTCC
S-POS   ........................................................................--......
S-TIP   ........................................................................--......
S-WHT   ........................................................................--......
S-PUL   ........................................................................--......
SBN     .........................T........................................C.....--......
CCN     .........................T........................................C.....--......
CAR     ........................................................................--......
AVEN    .......................................................-.....T..........TG......
CEL     .............................A..........CG...CC.....AT.C.CAA...TTAAGATGC--TC.A.T
                                                    |
                                              GTGCCTCTGTG

        81                                                                            160
S-VIG   TGTTG-GGCTAGCGTTGGCACAACCAAATGCCCCCGTCCGCTGATGGGCACAGGTCGTTCGAGATGACTTGTGGACGCTG
S-POS   .....-...............C..........................................................
S-TIP   .....-..........................................................................
S-WHT   .....-...............C..........................................................
S-PUL   .....-..........................................................................
SBN     .....-..........................................................................
CCN     .....-..........................................................................
CAR     .....-...............C..........................................................
AVEN    A...AAA.GTTGC........C....C...........T.....--C....G.AC..G........GTC.....G.A.G.
CEL     G.C.T-CA.GGT.AG.T.AGTGT.G.....T.AA.....AG.TGA.ATGC..CAA.AACT.GC.A..G.A...ACT..C.
                                            |                                        |
                                            T                                        T

        161                                                                           240
S-VIG   CCCAAC-ATTACGGGGCAGCTGCCTCACGAGC---CGTGCTTTTGGGGTGCTTCCATACGTTGGAGCTGTGGTATACCGC
S-POS   ......-.........................---.A...........................................
S-TIP   ......-.........................---.A...........................................
S-WHT   ......-.......A.................---.A...........................................
S-PUL   ......-.........................---.A...........................................
SBN     ......-.........................---.............................................
CCN     ......-.........................---.............................................
CAR     ......-.........................---.A...........................................
AVEN    GA....GC.G.GT..A.G...A..CTG.....ACT.TGT...........T.CT.CG...A...T...TG.TATACTGA.
CEL     A..A.T-GGA...T.....T.ATTC..AA.CT---AC.TT.GTGTCA.G....AATG.T.AGT..AAA..-..T.G.T.T

        241                                                                           320
S-VIG   TCAGTGCTGCACATGTGAAAGCCTGTGTATGGCTGCTGCGTGGCAATGTGTCGGTGGCGGGCCGCCTCGCTTGGCTGGTT
S-POS   ................................................................................
S-TIP   ................................................................................
S-WHT   ................................................................................
S-PUL   ..........................................................................T.....
SBN     ................................................................................
CCN     ..........................................................T.....................
CAR     ...................-----------............................................T....A
AVEN    ..GT....AG..AAGTG..AAGCC..AGGTTTG.......AA.--T..A..T........A.GTTGCT.G....T..C.G
CEL     ..T.CAG.AG..G.TGTTC.CGAGTC..C.CAACA.AA.CGCTATG...C..CTG.TG.CTATATGCGT..A....TC..
```

Fig. 2. Alignment of 18S-ITS1 rDNA sequences for taxa used; SCN = soybean cyst nematode, S-VIG = SCN-Vigo, S-POS = SCN-Posey, S-TIP = SCN-Tippecanoe, S-WHT = SCN-White, S-PUL = SCN-Pulaski, SBN = sugar beet cyst nematode, CCN = clover cyst nematode, CAR = *Heterodera carotae,* AVEN = *Heterodera avenae,* CEL = *Caenorhabditis elegans.* Data for *C. elegans* are from Ellis *et al.* (1986), and all base notations are for the non-transcribed strand. Numbering is based on sequence for SCN-Vigo. Italicized bases are in the 18S gene. Sequence differences are uncorrected for multiple changes at a site.

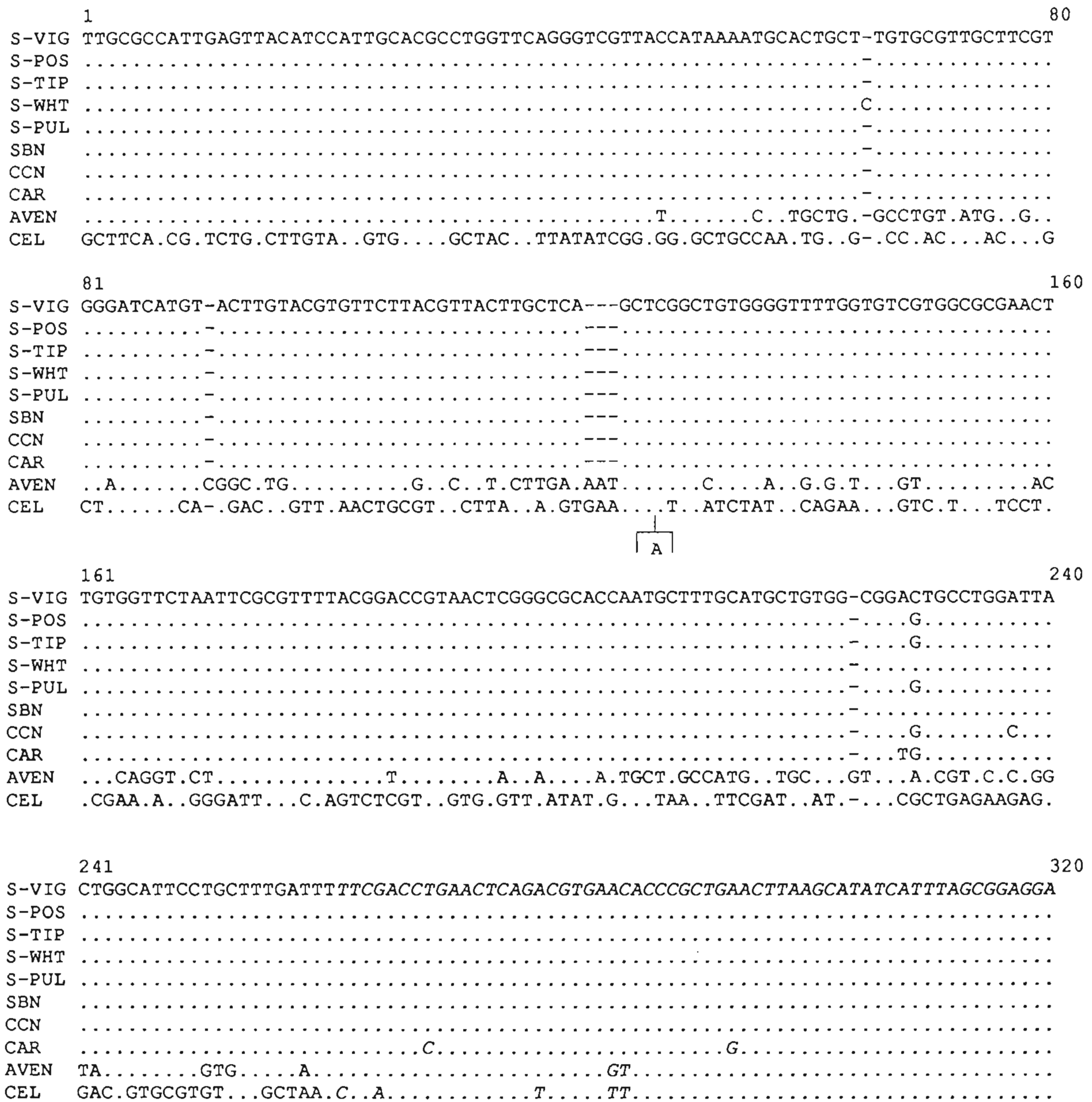

Fig. 3. Alignment of ITS-28S rDNA sequences for taxa used. SCN = Soybean cyst nematode, S-VIG = SCN =Vigo, S-POS = SCN-Posey, S-TIP = SCN-Tippecanoe, S-WHT = SCN-White, S-PUL = SCN-Pulaski, SBN = sugar beet cyst nematode, CCN = clover cyst nematode, CAR = *Heterodora carotae,* AVEN = *Heterodera avenae,* CEL = *Caenorhabditis elegans.* Data for *C. elegans* are from Ellis *et al.* (1986), and all base notations are for the non-transcribed strand. Numbering is based on sequence for SCN-Vigo. Italicized bases are in the 28S gene. Sequence differences are uncorrected for multiple changes at a site.

Only five base pair differences were shared by two or more isolates of the *Heterodera* species, and these plus two unique differences that distinguished species from each other (Table 2) were analyzed for the *schachtii* group using the computer program PAUP (Swofford, 1991). Because of the uncertainty about the manual alignment within the ITS sequences (discussed above), *H. avenae* was excluded from the analysis. In addition, we excluded *H. carotae* because many of the base pair differences between *H. carotae* and the *schachtii* group of species were unique for *H. carotae* and therefore were not phylogenetically informative for this group of species. We used both *H. avenae* and *H. carotae* to make polarity decisions for those PAUP computations that required the designation of a hypothetical ancestor.

The most parsimonious trees were obtained when SBN and CCN were entered as a monophyletic outgroup to the SCN isolates. The four trees of equal length varied somewhat in the placement of the various SCN isolates, with SCN-Vigo always separated from the other four (Fig. 4).

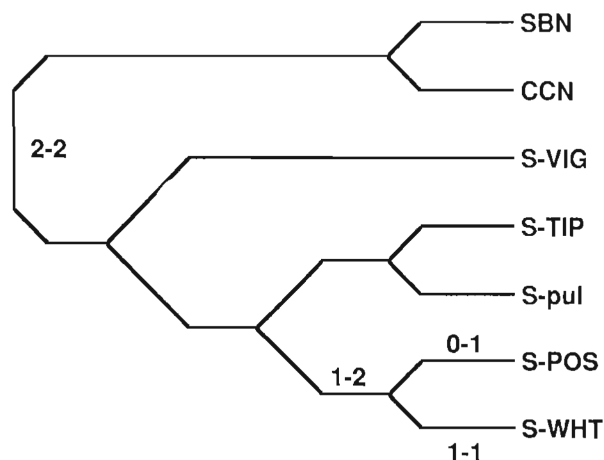

Fig. 4. One of four most parsimonious trees with total length of 9 steps using the global search option of PAUP. SBN = sugar beet cyst nematode; CCN = clover cyst nematode; SCN = soybean cyst nematode; S-VIG = SCN-Vigo; S-TIP = SCN-Tippecanoe; S-PUL = SCN-Pulaski; S-POS = SCN-Posey; S-WHT = SCN-White. Overall consistency index is 0.778, or 0.750 when uninformative characters are excluded. Branch lengths between internal nodes are given and include minimum and maximum number of steps over all equally parsimonious reconstructions. The number of informative characters was too limited for meaningful bootstrapping (Felsenstein, 1985).

Discussion

Within the genus *Heterodera* SCN, SBN and CCN are considered to belong to the *schachtii* group and to be phylogenetically related to the other taxa as is shown in Fig. 5 (Ferris, 1979; Hyman & Powers, 1991). These members of the *schachtii* group have distinctive 2-D PAGE protein patterns as noted above. SBN and SCN were shown to differ in 59 % of their protein/polypeptides in 2-D patterns (Bakker & Bouwman-Smits, 1988) and, based on RFLP data, to have a 14 % difference in their mtDNA (Radice *et al.*, 1988). The extreme similarity we found in the ITS sequences of SCN, SBN and CCN was, therefore, a surprise. The phenetic dissimilarity between several pairs of the SCN geographic isolates was greater than that between SCN-Vigo and SBN (Table 1). In ITS2, SBN had no differences at all from SCN-Vigo.

Table 2. Sequence data used for phylogenetic analysis of Figure 4. Data include the five base pairs shared by two or more isolates in the *schachtii* group, plus two unique differences (Figs 2, 3).

States for each character	1	2	3	4	5	6	7
SBN	T	C	A	G	C	C	A
CCN	T	C	A	G	C	G	C
S-TIP	C	T	A	A	C	G	A
S-PUL	C	T	A	A	T	G	A
S-POS	C	T	C	A	C	G	A
S-WHT	C	T	C	A	C	C	A
S-VIG	C	T	A	G	C	C	A

The data for *H. carotae* showed a number of differences from the *schachtii* group (Figs 2, 3; Table 1), but these were unique in our study because no other species closely related to *H. carotae* were included (Fig. 5). If we had done so, it is likely that some of the unique changes in *H. carotae* would have become shared changes, thus expanding the informative data base for phylogenetic analysis. The few ITS sequence differences we found

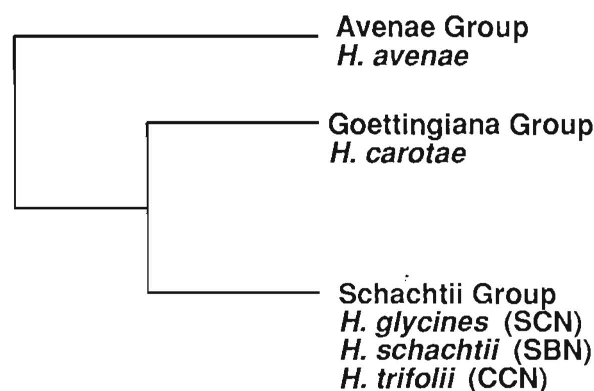

Fig. 5. Diagram of groupings of the *Heterodera* species of this study based on classical data (Ferris, 1979).

among these species, especially within the *schachtii* group, relative to those found with other kinds of molecular data, suggest that ITS1 and ITS2 are highly conserved among cyst nematode species.

The data for *H. avenae* ITS, however, were surprising for the opposite reason, i.e., the many differences we found for this species. The systematic relationship of *H. avenae* to the rest of the *Heterodera* species has been debated for several years. Krall and Krall (1978) proposed the generic name *Bidera* for the cereal cyst nematodes (i.e., the *H. avenae* group of Fig. 5) on the basis of host relationships plus morphological differences that they considered to differentiate them from the rest of the cyst nematodes of the genus *Heterodera*. This proposal was not accepted by most of the leaders of the international community of cyst nematologists, who were unable to establish clear differentiation between this group and the other *Heterodera* species (Mulvey & Golden, 1983; Luc *et al.*, 1988). The rDNA data suggest that *H. avenae* is well differentiated from the other *Heterodera* species in our study.

The rDNA ITS data for these cyst nematodes underscore the importance of surveying a range of taxa before deciding the utility of a particular kind of DNA sequence for phylogenetic investigation. If we had confined our study to just the three species of the *schachtii* group, we might have concluded that the ITS regions are too conserved to be of much use for phylogenetic analysis of nematodes. If we had compared only SCN, *H. avenae* and *C. elegans*, we would have concluded that the ITS regions are too variable to be useful. Based on the data for all of the species we examined, however, we conclude that the two ITS regions under study will be useful for analysis of evolutionary relationships among many of the plant parasitic cyst nematodes, which comprise many putative species and genera. The phenetic distances (Table 1) indicate that ITS data for more taxa, including more species in the *goettingiana* group of *H. carotae* and more bridging taxa between *H. avenae* and the others (Fig. 5), would improve the phylogenetic analysis. Even the few phylogenetically useful base pair differences available for the *schachtii* group when used in a cladistic analysis with PAUP (Swofford, 1991), separated the SCN isolates as a group from CCN and SBN (Fig. 4). The ITS sequence conservatism that is evident among this group of species suggests that even a few differences might be more useful than they would be for less conserved sequences. Because of the likely antiquity of many nematode taxa (Ferris *et al.*, 1976), rDNA ITS sequences will probably be useful for sorting out relationships of relatively closely related species and genera, though probably not for most putative intraspecific relationships or even for some groups of species like SCN, SBN and CCN. For these, sequence from some other, less conserved, DNA will be necessary to sort out phylogenetic relationships with any certainty. For comparisons of distantly related taxa, data from selected rDNA gene regions may be more useful (Hillis & Dixon, 1991).

Acknowledgments

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