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Comparisons of Isolates of *Heterodera avenae* using 2-D PAGE Protein Patterns and Ribosomal DNA[1]

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Abstract: Six geographic isolates of *Heterodera avenae,* including two isolates each from Sweden, Australia, and the United States, were compared on the basis of 2-D PAGE protein patterns and the complete DNA sequence for the two internal transcribed ribosomal DNA spacers (rDNA ITS1 and ITS2) and the 5.8S rRNA gene. The protein pattern data and rDNA ITS sequence data both indicated that the Swedish Gotland strain of *H. avenae* differed markedly from the rest of the isolates. Protein patterns for the Australia isolates differed more from a Swedish strict *H. avenae* isolate and isolates from Oregon and Idaho, than the two U.S. isolates and the Swedish strict *H. avenae* isolate differed from each other. Except for the Gotland strain isolate, the rDNA ITS sequences were highly conserved among all of the *H. avenae* isolates, just as we earlier found them to be conserved among species of the schachtii group of *Heterodera.*

Key words: Heterodera avenae, nematode, 2-D PAGE, protein pattern, ribosomal DNA, rDNA ITS1 and ITS2, 5.8S rRNA gene.

In a previous study (7) we compared protein patterns obtained by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of 10 isolates of *Heterodera avenae* Wollenweber from Sweden representing the two major groups of Swedish *H. avenae,* the strict *H. avenae* pathotype, and the Gotland strain pathotype. These two major groups of Swedish isolates are distinguished mainly by reactions of host plant differentials and by small morphological differences (7). In that study we found that patterns of all isolates were distinguished by at least some differences, although the differences were usually small and could not be correlated with pathotype. The protein patterns of two isolates of the Gotland strain of *H. avenae* differed markedly, however, from those of isolates of strict *H. avenae.* In the present study we compared 2-D PAGE protein patterns of six geographic isolates from three different continents: two from Sweden (one strict *H. avenae* and one Gotland strain isolate), two from Australia, and two from the United States. In addition, for all six isolates we compared a portion of the ribosomal DNA (rDNA), including the complete nucleotide sequence of both internal transcribed spacer regions (ITS1 and ITS2), and the 5.8S rRNA gene between them. Ribosomal gene sequences are known to be highly conserved, but the spacer regions are thought to be more variable (8,10,11). In a previous study (8) we found partial ITS sequence to be nearly identical among three species of the *H. schachtii* group, but to be 28% different from an isolate of *H. avenae.*

The objectives of the present study were to determine i) whether 2-D PAGE protein patterns differed enough among these geographic isolates to be consistently and readily recognizable without laborious procedures and calculations to compare positions of individual protein spots (1,2, 7); and ii) whether rDNA spacer sequence was more or less similar among these geographic isolates of a single species than it was between different species of the schachtii group of *Heterodera.* We also wished to determine whether the rDNA spacer sequences varied more among isolates than did the 5.8S gene sequence.

Materials and Methods

Sources of isolates: The Swedish isolates of *H. avenae* included a strict *H. avenae* isolate

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of pathotype Ha12 from Nässja and an isolate of the East Gotland pathotype from Alnarp (7). The Oregon isolate from an oat field in Union County, Oregon, was sent by Dr. Gene Newcomb. The Idaho isolate from an infested wheat field in Sugar City, Idaho, was sent by Dr. S. I. Hafez. The Australian isolates from wheat included one collected near Kadina and sent by Franky Green; and one from Rainbow sent by Dr. R. H. Brown. *Heterodera schachtti* from Michigan was a gift from Dr. L. I. Miller, and our rDNA data for it are included for comparison.

Nematode cultures: Following a 6-week period during which cysts were held at 4 C, all isolates of *H. avenae* were increased in plant growth chambers on barley cv. Varde (*Hordeum vulgare* L.) grown in 9- × 16-cm plastic pouches (12). The temperature of the seedling cultures was maintained at 10 C for the first 2 weeks and then 18 C for the remainder of the growth period. After 8–9 weeks, when young female nematodes were visible, 30 nematodes per sample were picked from the roots and rinsed in tap water for each protein analysis. Additional female nematodes were collected and frozen in 0.2 M sodium borate buffer, pH 9, for use in rDNA analysis.

Nematode samples: The methods for protein and rDNA preparation from nematode samples have been described (7,8). The nematodes for protein analysis were homogenized over ice in a 0.2 M sodium borate buffer at pH 9, with a ground-glass homogenizer. The homogenate was centrifuged at 12,800 *g* for 5 minutes and the supernatant dialyzed against the borate buffer and then stored over liquid nitrogen. Proteins were labeled in vitro by reductive methylation with formaldehyde and sodium (^{3}H) borohydride (13). Urea sample buffer, which contained 9.5 M urea, 2% (v/v) Nonidet P-40, and 5% (v/v) β-mercaptoethanol, was added to each labeled, precipitated, and washed (with acetone-ether 1:3 v/v) protein sample, and the sample was stored at −80 C.

For each nematode sample for rDNA analysis, a single female nematode was crushed on a glass slide in 10 µl TE buffer (0.5 M tris-Cl, 100 mM EDTA, pH 8.0) and the body contents transferred to a 1.5-ml vial to which was added 20 µl of lysis solution consisting of 0.5 mM Tris-EDTA, 0.75% Triton-X-100, and 1.5% stock proteinase K solution (10 mg/ml). The nematode tissue was ground for 5 minutes with a hand-held homogenizer, the pestle rinsed twice with 50 µl of the same solution, and the homogenate heated at 50 C for 2 hours. The DNA was extracted from the homogenate with a GeneClean kit (Bio 101, San Diego, CA) and recovered in 30 µl sterile water. Two to 10 such preparations were made for each isolate.

2-D PAGE protein gels: O'Farrell's (15) methods were modified as described earlier (7). Briefly, proteins in a 25–50 µl sample were separated by isoelectric focusing in a tube gel using a Bio Rad model 155-gel electrophoresis cell. The anode electrode solution was 0.01 M H_3PO_4, and the cathode electrode solution was 0.02 M NaOH. Electrophoresis was carried out at 400 V for 18 hours and the power was then increased to 800 V for 1 hour to focus the proteins. In the second dimension each tube gel was placed on top of a sodium dodecyl sulfate 12% (w/v) polyacrylamide slab gel, 1.2 mm thick. Electrophoresis was in a Bio-Rad Protean dual vertical slab gel electrophoresis cell at 20 mA per gel for approximately 5 hours. Molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD), were run in the second dimension with the nematode proteins. Labeled proteins were located on the gels by fluorography with the EN^{3}HANCE procedure (New England Nuclear Research Products, Boston, MA). At least 10 gel patterns were obtained for each isolate, and proteins from each isolate were run in both dimensions in the same electrophoresis cell with proteins from other isolates to permit tracing small variations in protein positions. Several autoradiographs were made from each gel at a range of exposure times. The transparent autoradiographs were overlaid and compared directly. As is

customary for 2-D gels, we used internal "landmark" spots to align gels for comparison (3,16,19). Proteins with identical electrophoretic properties were assumed to be identical (1–3,15). A spot consistently pale on patterns of one isolate and dark on patterns of another isolate was scored as present in both isolates, and analysis was limited to spots reproducible in all gel patterns of a given isolate.

rDNA amplification, cloning, and sequencing: Methods for obtaining rDNA data were essentially as previously described (8). Amplification of a 1,100 bp region that spanned the two ITS regions and included the 5.8S gene was carried out using standard PCR (17) with reagents from Perkin Elmer (Norwalk, CT) and Promega (Madison, WI), and a Coy Tempcycler model 50. Primers were as described previously (8), and 15 µl of DNA template (prepared as described above) was used per 50 µl PCR reaction. The amplified rDNA product of all *H. avenae* isolates was cloned into the TA pCR 1000 cloning vector supplied by Invitrogen (San Diego, CA), and the rDNA of *H. glycines* was cloned into pGEM-3Z (Promega). Double-stranded sequencing was performed using Sequenase version 2.0 from U.S. Biochemical (Cleveland, OH). Primers to sequence from the vectors were as described (8). Additional primers were designed based on comparative study of internal sequence in order to

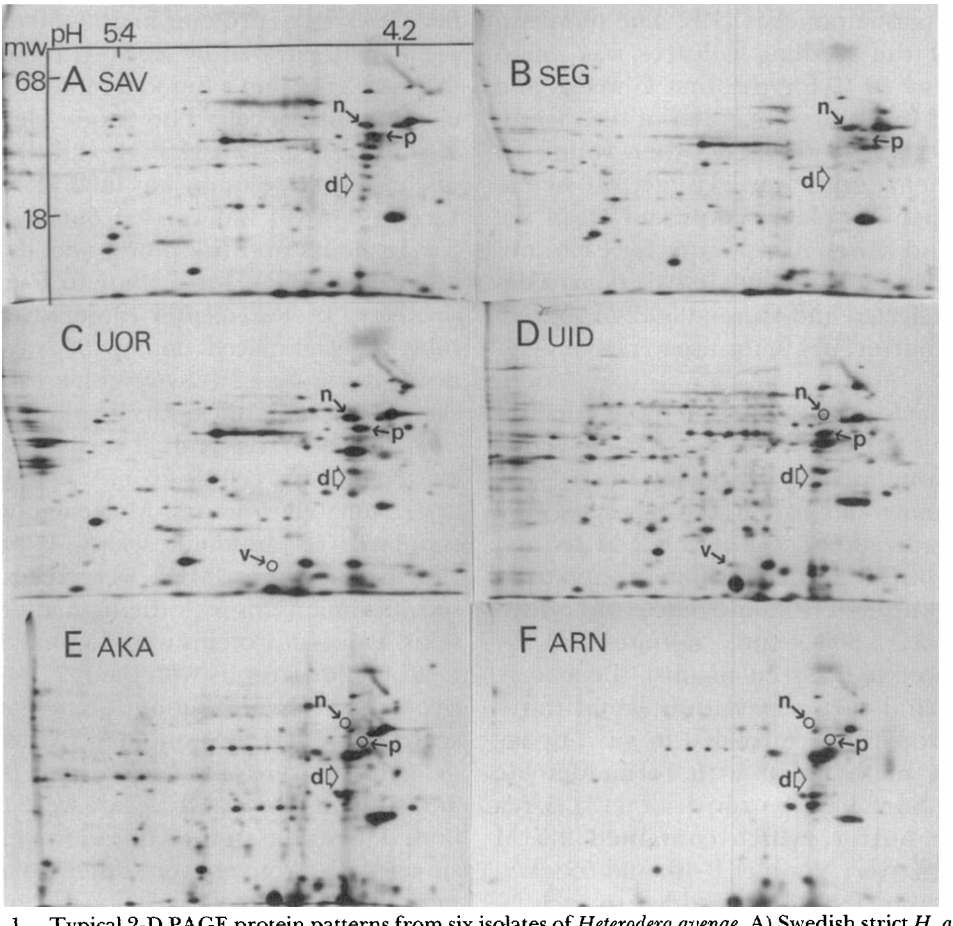

FIG. 1. Typical 2-D PAGE protein patterns from six isolates of *Heterodera avenae*. A) Swedish strict *H. avenae* isolate (SAV). B) Swedish East Gotland strain isolate (SEG). C) U.S. Oregon isolate (UOR). D) U.S. Idaho isolate (UID). E) Australia Kadina isolate (AKA). F) Australia Rainbow isolate (ARN). Protein spots d, n, p, and v are referred to in the text. The symbol O indicates the absence of a protein spot. Molecular weights are given in thousands.

sequence the entire amplified region in all clones. These primers were as follows: For the *H. avenae* isolates the forward internal sequencing primer was 5′-ATACTGACTCGTTGCTGAG-3′; for the *H. schachtii* isolate the forward internal sequencing primer was 5′-TATACCGCTCAGTGCTGCA-3′. The reverse internal sequencing primer was 5′-GTGCAATGGATGTAACTC-3′ for all isolates. Sequence data were from multiple clones and both strands. Each sequencing run for a given clone was repeated three to six times to ensure accuracy of the sequence.

Sequence data for the ITS1, the ITS2, and the 5.8S gene were compared and aligned with those for each of the other isolates using the computer program GAP in the Sequence Analysis Software Package of the Genetics Computer Group (4). GAP uses the algorithm of Needleman and Wunch (14) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. In these analyses gap weight = 5.0 and gap length = 3.0. Multiple alignments were done initially using the Multiple Alignment program in the same computer package, followed by manual adjustments to improve the fit (18).

Results

Protein patterns

Protein pattern differences between strict *H. avenae* and a Gotland strain isolate can be seen by inspection of typical autoradiographs (Fig. 1A,B). Obvious differences between them include the vertical row of spots (Fig. 1, arrow d), which was largely missing in the Gotland strain isolate (Fig. 1B). Though less striking, several of

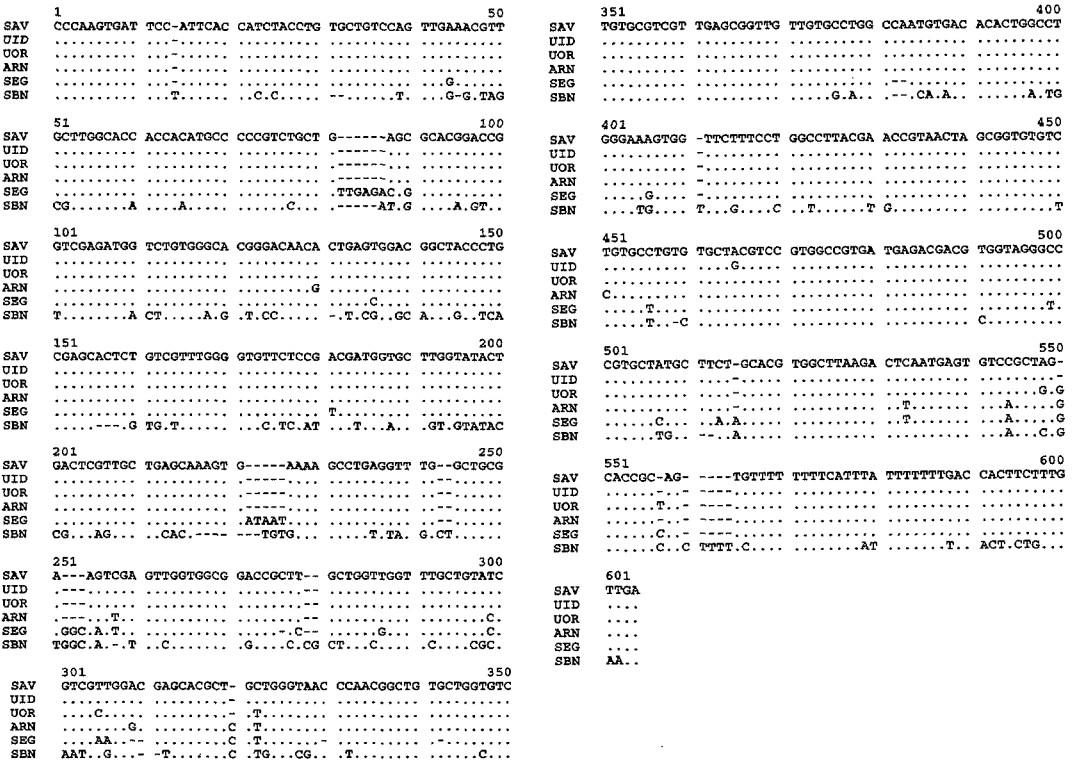

FIG. 2. Alignment of rDNA ITS1 sequences for Swedish strict *Heterodera avenae* isolate (SAV), Swedish East Gotland strain isolate (SEG), U.S. Oregon isolate (UOR), U.S. Idaho isolate (UID), Australia Rainbow isolate (ARN), and *H. schachtii* (SBN). All base notations are for the nontranscribed strand. Numbering is based on sequence for SAV, and the isolates are listed in the order of increasing dissimilarity to SAV. Sequence differences are uncorrected for multiple changes at a site.

```
          1                                                  50
SAV   TCATAAAACG ACTGCTGTGC CTGTTATGTT GGTGAGATCA TGTCGGCATG
UID   .......... .......... .......... .......... ..........
UOR   .......... .......... .......... .......... .........-
ARN   .......... .......... .......... .......... .......T..
SEG   .......... .......... .......... .......... ....T...G.
SBN   C.......T. CACTGCT..T GC...-GC.. C...G..... ...ACTTG.-

          51                                                 100
SAV   ACGTGTTCTT GCGCTATTCT TGAAAATGCT CGGCCGTGGA GTGTGGTTGT
UID   .......... .......... .......... .......... ..........
UOR   .......... .......... .......... .......... ..........
ARN   .......... .......... .......... .......... ..........
SEG   .......... .......... .......... .......... ..........
SBN   .......... A..T..C.TG CTC.---... ....T....G ..T.T.G...

          101                                                150
SAV   GTTGGCGCGA AACTGTCAGG TTCTTTCGCG TTTTACGGTC CGTAACTAGG
UID   .......... .......... .......... .......... ..........
UOR   .......... .......... .......... .......... ..........
ARN   .......... .......... .......... .......... ..........
SEG   .......... .......... .......... .......... ..........
SBN   CG........ .CT...GGTT C.AA..... ........A. .......C..

          151                                                200
SAV   ACGCAACTGC TCGCCATGTG -TGCTGGGTG GAACGTCGCT GGTAGGCATT
UID   .......... .......... -.......... .......... ..........
UOR   .......... ...T...... -.......... .......... ..........
ARN   .......... .......... -.......... .......... ..........
SEG   .......... ..T....... -.......... .......... ..........
SBN   G....C.AAT G.TTTGCA.. C..TG.C.GA CTG.C.G.A. TACT......

          201       213
SAV   CCGTGTTTGA ATT
UID   .......... ...
UOR   .......... ...
ARN   .......... ...
SEG   .......... ...
SBN   ..TGC..... T..
```

FIG. 3. Alignment of rDNA ITS2 sequences for Swedish strict *Heterodera avenae* isolate (SAV), Swedish East Gotland strain isolate (SEG), U.S. Oregon isolate (UOR), U.S. Idaho isolate (UID), Australia Rainbow isolate (ARN), and *H. schachtii* (SBN). All base notations are for the nontranscribed strand. Numbering is based on sequence for SAV, and the isolates are listed in the same order as in Figure 2. Sequence differences are uncorrected for multiple changes at a site.

these spots are missing also in the other isolates, especially those from Australia. In addition, a dense protein spot (Fig. 1, arrow n) is present in the strict *H. avenae* isolate from Sweden (Fig. 1A), the Gotland strain (Fig. 1B), and the Oregon isolate (Fig. 1C), but is absent from the Idaho isolate (Fig. 1D) and from both isolates from Australia (Fig. 1E,F). Another dense protein spot nearby (Fig. 1, arrow p) is present in the isolates from Sweden and the United States, but is missing from both isolates from Australia. A small protein spot

TABLE 1. Pairwise percentage nucleotide dissimilarities in rDNA ITS1 for *Heterodera avenae* isolates; data for *H. schachtii* (SBN) are included for comparison.

	SAV	SEG	UOR	UID	ARN	SBN
SAV	—	5.1	1.4	0.2	1.4	26.5
SEG		—	4.7	5.3	3.0	22.0
UOR			—	1.6	1.6	25.7
UID				—	1.6	26.6
ARN					—	27.4

TABLE 2. Pairwise percentage nucleotide dissimilarities in rDNA ITS2 for *Heterodera avenae* isolates; data for *H. schachtii* (SBN) are included for comparison.

	SAV	SEG	UOR	UID	ARN	SBN
SAV	—	1.4	0.5	0	0.5	37.7
SEG		—	1.9	1.4	1.9	37.2
UOR			—	0.5	0.9	37.2
UID				—	0.5	37.7
ARN					—	37.7

reported previously (7) as being present only in another Swedish isolate (the Växtorp isolate) is present also in the Idaho isolate (Fig. 1D, arrow v) but not in the Oregon isolate (Fig. 1C).

Taken as a whole, the patterns of both U.S. isolates resemble closely, but not exactly, the pattern of the strict *H. avenae* isolate from Sweden. The patterns for the two Australian isolates differ more from the others, but they seem to be nearly identical to each other.

rDNA sequence data

ITS1 and ITS2: Sequence data for the two isolates from Australia were identical for all rDNA sequenced, so data are presented for only the Rainbow isolate (ARN). Figures 2 and 3 show the sequence alignment for the complete ITS1 and ITS2 respectively, and Tables 1 and 2 show pairwise percentage nucleotide dissimilarities among all isolates. In addition to the data for the *H. avenae* isolates, our data for *H. schachtii,* sugar beet cyst nematode (SBN), are included for comparison.

For ITS1, the least dissimilarity was 0.2% between the strict *H. avenae* isolate from Sweden (SAV) and the Idaho isolate (UID). The Australian isolates (ARN) and Oregon isolate (UOR) were 1.4% dissimilar to the Swedish strict *H. avenae* (Fig. 2, Table 1). The Oregon and Idaho isolates were both 1.6% dissimilar to the Australian isolates. Among the *H. avenae* isolates, the Gotland strain isolate (SEG) was the most dissimilar, with 5.3% dissimilarity to the Idaho isolate, 5.1% dissimilarity to the Swedish strict *H. avenae* isolate, 4.7% dis-

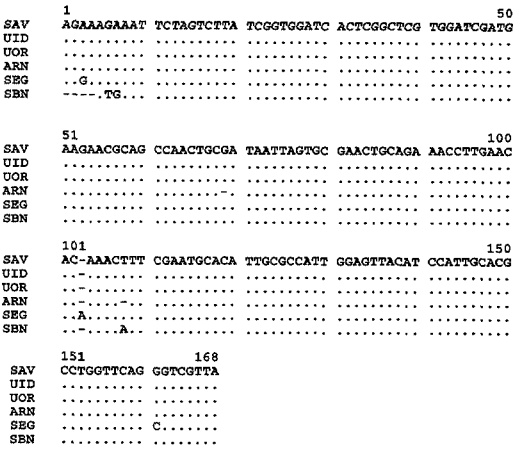

FIG. 4. Alignment of rRNA 5.8S gene sequences for Swedish strict *Heterodera avenae* isolate (SAV), Swedish East Gotland strain isolate (SEG), U.S. Oregon isolate (UOR), U.S. Idaho isolate (UID), Australia Rainbow isolate (ARN), and *H. schachtii* (SBN). All base notations are for the nontranscribed strand. Numbering is based on sequence for SAV, and the isolates are listed in the same order as in Figure 2. Sequence differences are uncorrected for multiple changes at a site.

similarity to the Oregon isolate, and 3.0% dissimilarity to the Australian isolates. The ITS1 dissimilarity to SBN was least for the Gotland strain (22%) and ranged between 25.7% (Oregon) and 27.4% (Australia) for the other isolates (Fig. 2, Table 1).

The sequence data for ITS2 (Fig. 3, Table 2) were less dissimilar (and therefore more similar) than those for ITS1 among the *H. avenae* isolates. The sequence for the Swedish strict *H. avenae* isolate was identical to that of the Idaho isolate, and differed from the Oregon and Australian isolates by only 0.5%. The Idaho and Oregon isolates differed from the Australian isolates by 0.5 and 0.9% respectively. The Gotland strain was 1.4% dissimilar to the Swedish strict *H. avenae* and Idaho isolates and was 1.9% dissimilar to the Oregon and Australian isolates. The dissimilarity between SBN and the group of *H. avenae* isolates ranged from 37.2% for the Gotland strain and Oregon isolates to 37.7% for the rest.

5.8S gene: In the 5.8S gene, few nucleotide sequence differences were evident among any of the isolates (Fig. 4, Table 3). We found identical sequence for strict *H. avenae* from Sweden and the Oregon and Idaho isolates, and small differences in the Australian isolates (Fig. 4) not reflected in the computed dissimilarities (Table 3). The 5.8S sequence for the Gotland strain was 0.6% dissimilar to that of the Swedish strict *H. avenae* and to the Oregon and Idaho isolates, and 1.2% dissimilar to the Australian isolates. It was more dissimilar (2.5%) to the SBN sequence, which was 1.8% dissimilar to the Swedish strict *H. avenae,* Oregon and Idaho isolates, and 1.2% dissimilar to the Australian isolates.

TABLE 3. Pairwise percentage nucleotide dissimilarities in the 5.8S rRNA gene for *Heterodera avenae* isolates; data for *H. schachtii* (SBN) are included for comparison.

	SAV	SEG	UOR	UID	ARN	SBN
SAV	—	0.6	0	0	0	1.8
SEG		—	0.6	0.6	1.2	2.5
UOR			—	0	0	1.8
UID				—	0	1.8
ARN					—	1.2

DISCUSSION

Except for the Gotland strain, the percentage dissimilarity in rDNA ITS sequences among these *H. avenae* isolates from Sweden, Oregon, Idaho, and Australia was comparable to that we found earlier among the different species of the schachtii group of *Heterodera,* with more differences in ITS1 than in ITS2 (8). The ITS dissimilarities between the Gotland strain of *H. avenae* and the rest of the *H. avenae* isolates greatly exceeded those we found earlier between the schachtii group and *H. carotae* (8). It is interesting to note that although dissimilarities in ITS1 were greater than in ITS2 within groups (e.g., the schachtii group or the *H. avenae* group), in the comparison between SBN and the *H. avenae* isolates, the dissimilarities were greater for ITS2, despite the fact that ITS1 is longer than ITS2. We expected to find little dissimilarity in 5.8S gene sequence within this group of isolates, but were surprised to find that in some instances the dissimilarity in ITS2

was only slightly greater, despite the widely held perception that spacer regions are less conserved than gene regions. However, it must be emphasized that the 5.8S gene sequence was highly conserved also between SBN and the *H. avenae* group, despite large differences in both ITS regions.

We found an interesting difference between the *H. avenae* isolates and the species of the schachtii group we have studied (8) in the groupings of the thymine nucleotide bases (represented by the letter T in the DNA sequence) near the end of the ITS1 (Fig. 2). In the schachtii species, the Ts are arranged in a 5-8-2-9 order (Fig. 2, SBN), whereas for the *H. avenae* isolates (including the Gotland strain isolate) the groups are 8-3-7 (Fig. 2). We have not been able to locate "T" groups in the published ITS1 sequence for *Caenorhabditis elegans* (5). In published sequence for house mouse (9) a 5-9 grouping of Ts occurs in ITS1 before the start of the 5.8S gene, which suggests that groups of T nucleotides in ITS1 are ancestral in nature.

The 2-D PAGE protein patterns and the rDNA data both indicated that the Gotland strain of *H avenae* is markedly different from the rest. The protein patterns for the Australian isolates differed more from the Swedish strict avenae isolate and the isolates from Oregon and Idaho than the latter three isolates differed from each other. On the basis of these protein patterns, we expected to find more rDNA differences than we did between the Australian isolates and the rest. Such 2-D PAGE protein patterns are useful to indicate whether isolates differ overall greatly or very little, but the protein pattern data are more difficult to quantify than are DNA data, and will, therefore, be less useful for phylogenetic analysis (2,6). For such analysis it will be necessary to evaluate candidate DNA sequences to determine the taxonomic level at which they will be useful. Despite the similarities we found earlier (8) among species of the schachtii group of *Heterodera*, our results here show that rDNA ITS data will be useful for evaluating relationships among species and some geographic isolates of species, and for closely related genera. For a study of differences between very similar isolates, some less conserved DNA sequence will be more useful.

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