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Intraspecific Variation in Ribosomal DNA in Populations of the Potato Cyst Nematode *Globodera pallida*

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Abstract: The relationships among a number of populations of *Globodera pallida* from Britian, the Netherlands, Germany, Switzerland, and South America were examined using PCR amplification of the ribosomal cistron between the 18S and 28S genes that include the two intergenic spacer regions (ITS1 and ITS2) and the 5.8S gene. Amplifications produced a similar-sized product of 1150 bp from all populations. Digestion of the amplified fragment with a number of restriction enzymes showed differences among the populations. The restriction enzyme RsaI distinguished the most populations. The RFLP patterns revealed by this enzyme were complex and could have arisen from heterogeneity between individuals within populations and from differences between the repeats of an individual. Sequence analysis from six of the populations, together with RFLP analysis of PCR products, shows that there is intraspecific variation in the rDNA of *G. pallida*.

Key words: *Globodera* spp, PCR, potato-cyst-nematode, rDNA.

The potato cyst nematodes (PCN), *Globodera rostochiensis* (Wollenweber) Behrens and *G. pallida* (Stone) Behrens, are major pests of potato (*Solanum tuberosum* L.) that were introduced into Europe from South America (Evans et al., 1975). Determining whether there have been more than one introduction of each species has practical importance in relation to the management of this pest, as the genetic basis of virulence may differ with distinct introductions. Biochemical and molecular characterization of PCN populations are being conducted to determine the range of genetic diversity in relation to the virulence of these populations (Blok and Phillips, 1995; Blok et al., 1997: Phillips et al., 1992).

Different regions of the ribosomal DNA (rDNA) cistron have been used to examine phylogenetic relationships between and within species in a wide range of organisms in the plant, animal, and bacterial kingdoms, and recently these techniques have been applied to nematodes. Studies of rDNA using polymerase chain reaction (PCR) amplification are of particular utility where the quantities of DNA are limited. Ferris et al. (1993, 1994, 1995) compared sequence data from the internal transcribed spacer (ITS) rDNA of the cyst nematodes *Heterodera glycines, H. schachtii, H. trifolii, H. carotae, H. avenae,* and *Globodera* spp. following PCR amplification, and found that they have characteristic intra- and interspecific variation. In other studies, Vrain et al. (1992) used restriction fragment length polymorphism (RFLP) in the PCR product from the ITS regions and 5.8S gene to examine relationships in the *Xiphinema americanum* group. Wendt et al. (1993) and Ibrahim et al. (1994) differentiated species and populations of *Aphelenchoides* and *Ditylenchus angustus,* and Zijlstra et al. (1995, 1997) differentiated isolates of *Meloidogyne hapla* and *M. chitwoodi* with restriction site differences in this region. In contrast, no inter- or intraspecific variation was observed in the ITS1 and ITS2 regions of *M. incognita, M. arenaria, M. javanica,* and *M. hapla* with sequence or restriction enzyme analysis by Xue et al. (1993).

In this study of intraspecific variation within *G. pallida,* in relation to the original introductions into Europe, PCR was used to amplify a region of the tandemly repeated ribosomal cistron including the ITS1 and ITS2 regions, the 5.8S gene, and short lengths of the 3′ end of the 18S gene, and the 5′ end of the 28S gene. Nematode popu-

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lations from Europe and South America were examined for RFLP and sequence differences in the rDNA and the results interpreted in relation to the complexities of rDNA evolution.

Materials and Methods

Nematode populations: Eighteen populations of *G. pallida* from Europe and South America were used. Details of their origin, virulence, and pathotype designations are given in Table 1. Virulence was assessed in a glass house pot test where the nematode cyst production on a susceptible host was compared with reproduction on a range of partially resistant potato genotypes (Phillips and Trudgill, in press).

DNA extraction: DNA was extracted from bulked second-stage juveniles or dry cysts as described by Phillips et al. (1992). Single juvenile nematodes were ground in a 1-ml glass homogenizer (Burkard Scientific, Uxbridge, UK) in 5 µl of water, after which the extract was removed to a 0.5-ml Eppendorf tube. The homogenizer was washed with an additional 5 µl of water, which was combined with the previous 5 µl of extract.

PCR reaction: The primers used for PCR amplification were as described by Vrain et al. (1992). PCR reactions (100 µl) consisted of 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 10 µM each dNTP, 0.2 µM of each primer, 10 ng template DNA or 10 µl of extract from a single nematode, 2.5 units Taq polymerase (Boehringer, Mannheim, Germany). A negative control with no template DNA and a positive control with *Caenorhabditis elegans* DNA was included in each experiment. The cycling parameters used were 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 90 seconds for 35 cycles followed by 72 °C for 5 minutes. Amplification was performed in a thermocycler (Techne PHC-3).

The amplified product was further purified using phenol-chloroform and chloroform, then ethanol-precipitated at −20 °C for at least 1 hour (Sambrook et al., 1989). This step was required to give clear digestion patterns following electrophoresis of the products on polyacrylamide gels. Following centrifugation and resuspension in TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA), the DNA samples were divided into 2 or 3 portions, and restriction enzyme digestions (AluI, HinfI, MboI, DdeI, TaqI, and RsaI) were performed according to the manufacturer's instructions with the buffer provided (Pharmacia, Uppsala, Sweden). Initially, the

Table 1. Populations of potato cyst nematode, *Globodera pallida*, used in this study.

Code	Pathotype	Virulence[a]	Origin
Pa1	Pa1	16	Scotland
P4A	P4A	6	South America
P5A	P5A	35	South America
D375	Pa2	2	The Netherlands
Coll 1077	Pa3	8	The Netherlands
Vp74-768-20	Pa3	32	The Netherlands
Germany (BBA1)	Pa2	5	Germany
Chavonery	Pa3	16	Switzerland
Lindley	Pa3	10	England
Halton	Pa2	2	England
Farcet	Pa3	2	England
Yapham	Pa3	4	England
Newton	Pa3	6	England
Bedale	Pa3	7	England
Derby	Pa3	6	England
Bryn Adda	Pa3	5	Wales
Luffness	Pa3	22	Scotland
Gourdie	Pa3	4	Scotland

[a] Virulence: the mean percentage of cyst production of each population on the differential host 62.33.3 (Kort et al., 1977) relative to cyst production on the susceptible cultivar Desiree.

digestion products were separated on 1% agarose TBE gels (Sambrook et al., 1989); however, the digestion patterns were difficult to visualize. Hence, discontinuous non-dissociating polyacrylamide gel electrophoresis with a 10% resolving gel was used. These gels were electrophoresed in 0.25M Tris/glycine buffer pH 8.3 for 6 hrs at 150V (Sambrook et al., 1989). DNA was visualized under UV light following staining with ethidium bromide (0.5 μg/ml) and then silver-stained (Merril, 1990) to confirm the presence or absence of minor bands. Results were repeated to confirm that the digestion patterns were reproducible. Marker VIII (Boehringer) was co-fractionated as a size standard. However, the sizes of the markers did not correspond to the sizes of the digestion products predicted from the sequence analysis. This was probably due to differences in the buffer of the marker and the restriction digests.

Cloning and sequencing of PCR products: PCR products were excised from 1% TBE buffered agarose gels using the Wizard PCR Prep kit (Promega) and cloned into the vector pGEM-T (Promega, Madison, WI). Two clones of PCR products from each of the *G. pallida* populations (Halton, Luffness, Pa1, P4A and P5A) were isolated, and 12 were isolated from D375 (two were sequenced over the whole length of the PCR product while the remaining 10 were sequenced over the first 500-600 bp). DNA was prepared using the Wizard Midipreps DNA Purification System (Promega) and sequenced by cycle sequencing (Applied Biosystems, Foster City, CA) on both DNA strands. Sequence was determined from all clones using forward (5′-GTTTTCCCAGTCACGAC-3′) and reverse (5′-AACAGCTATGACCATG-3′) primers. The internal primers used were ribo f2 (5′-CGATTGCTGTTGTCGTCG 3′), ribo f4 (5′ TATCGGTGGATCACTCGG 3′), ribo r2 (5′ GATGTCACTCCAATGGCG 3′), and 2043 (5′ GTCGAGTCACCCATTGGG 3′).

Sequence information was assembled using the Staden (1982) package of programs, and further analysis was performed with STADEN, UWGCG (Devereux et al., 1984) and FASTA (Devereux et al., 1984) programs provided by the Daresbury Laboratory, Warrington, United Kingdom. The genetic similarity between the sequences was calculated with the DNADIST program from PHYLIP 3.5 supplied by J. Felsenstein, University of Washington, Seattle, Washington.

Results

The sensitivity in detecting the products of restriction enzyme digestion and their resolution was superior following discontinuous non-denaturing polyacrylamide gel electrophoresis followed by ethidium bromide staining or silver staining when compared with agarose gel electrophoresis followed by ethidium bromide staining and UV light illumination (results not shown). Hence, the former method was used for the RFLP analyses.

Comparison of RFLP and sequence information: Four restriction enzymes that recognize four-base-pair target sequences (AluI, MboI, TaqI, and RsaI), and two that recognize a five-base-pair site (HinfI and DdeI) were used to test for RFLPs in the rDNA PCR product from the different populations of *G. pallida*. There were restriction sites in the PCR products for all of these enzymes. Sequence alignment of the amplification products from seven *G. pallida* populations are displayed in Fig. 1. A map indicating the positions of these restriction sites based on this sequence information is shown in Fig. 2. The six enzymes varied in the degree to which they distinguished populations. No RFLP differences were observed when the PCR product was digested with AluI, DdeI, or HinfI (results not shown), and no sequence differences between the populations at the sites recognized by these two enzymes were observed (Fig. 1). With MboI, the P5A population lacked the 125- and 431-bp digestion products present in all of the other populations but had a product of 556 bp (results not shown). This was confirmed in the sequence analysis, which identified GACC at position 288 instead of GATC, which is the recognition site for this enzyme. One of the Halton sequences also had the P5A type sequence at this point, but the 556-

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          0                                                                 60
          18S
Luffness  GTACACACCG  CCCGTCGCTG  CCCGGGACTG  AGCCATTTCG  AGAAACTCGG  GGACGATTAT
P4A       ..........  ..........  ..........  ..........  ..........  ..........
Halton    ..........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  ..........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ..........  ..........  ..........  ..........  ..........
Ferris    ----------  ----------  ----------  ----------  ----------  ----------

          61                                                               120
Luffness  GCGTGTCGGC  TTCGGTCGTC  GCGTTGATTG  GAACCGATTT  AATCGCAGTG  GCTTGAACCG
P4A       ..........  ..........  ..........  ..........  ..........  ..........
Halton    ..........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  ..........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ..........  ..........  ..........  ..........  ..........
Ferris    ----------  ----------  ----------  ----------  ----------  ----------

          121                                                              180
                                                                18S ITS1
Luffness  GGCAAAAGTC  GTAACAAGGT  AGCTGTAGGT  GAACCTGCTG  CTGGATCATT  ACCCAAGTGA
P4A       ..........  ..........  ..........  ..........  ..........  ..........
Halton    ..........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  ..........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ..........  ..........  ..........  ..........  ..........
Ferris    ---------.  ..........  ..........  ..........  ..........  ..........

          181                                                              240
Luffness  TACCAATTCA  CCACCTACCT  GCTGTCCAGT  TGAGTCAGTG  TGGGCAACAC  CACATGCCTC
P4A       ..........  ..........  ..........  ..........  ..........  ..........
Halton    ..........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  ..........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ....T.....  ..........  ..........  ..........  ..........  ..........
Ferris    ..........  ..........  .....T....  ..........  ..........  ..........

          241                                                              300
Luffness  CGTTTGTTGT  TGACGGACAC  ATGCCCGCTA  TGTTTGGGCT  GGCACATTGA  TCAACAATGT
P4A       ..........  ..........  ..........  ..........  ..........  ..........
Halton    ..........  ..........  ..........  ..........  ..........  C.........
Pa1       ..........  ..........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ..........  ..........  ..........  ..........  C.........
Ferris    ..........  ..........  ..........  ..........  ..........  ..........

          301                                                              360
          RsaI
Luffness  ATGGACAGCG  CCCTGTGGGC  ACATGAGTGT  TGGGGTGTAA  CCGATGTTGG  TGGCCCAATG
P4A       ..........  ..........  ..........  ..........  ..........  ..........
Halton    .C........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  T.........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       .C........  ..........  ..........  ..........  ..........  ..........
Ferris    ..........  ......--..  G.........  ..........  ..........  .........-
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            361                                                                 420
Luffness    GGTGACTCGA  CGATTGCTGT  TGTCGTCGGG  TCGCTGCACC  AACGGAGGTG  GCACGCCCAC
P4A         ..........  ..........  ..........  ..........  ..........  ..........
Halton      ..........  ..........  ......A...  ..........  ..........  ..........
Pa1         ..........  ..........  ..........  ..........  ..........  ..........
D375        ..........  ..........  ..........  ..........  ..........  ..........
P5A         ..........  ..........  ..........  .T........  ..........  ..........
Ferris      ..........  ..........  ..........  ..........  ..........  ..........

            421                                                                 480
Luffness    AGGGCACCCT  AACGGCTGTG  CTGGCGTCTG  TGCGTCGTTG  AGCGGTTGTT  GCGCCTTGCG
P4A         ..........  ..........  ..........  ..........  ..........  ..........
Halton      ..........  ..........  ..........  ..........  ..........  ..........
Pa1         ..........  ..........  ..........  ..........  ..........  ..........
D375        ..........  ..........  ..........  ..........  ..........  ..........
P5A         ..........  ..........  ..........  ..........  ..........  ..........
Ferris      ..........  ..........  ..........  ..........  ..........  --........

            481                                                                 540
                                                            RsaI        RsaI
Luffness    CGGATATGCT  GACATGGAGT  GTAGGCTACT  ATTCCATGTC  GTACGTGCCG  TACCCAGCGG
P4A         ..........  ........A.  .......G..  .........T  ..........  .....G....
Halton      ..........  .....T..A.  ........T.  .........T  ..........  .....G....
Pa1         ..........  ..........  ..........  ..........  ..........  ..........
D375        ..........  ..........  .......G..  ..........  ..........  ..........
P5A         .T........  ..........  .......G..  .........T  ..........  .....G....
Ferris      ..........  ........A.  .......G..  .........T  ..........  .....-....

            541                                                                 600
Luffness    CATGTCTGCG  CTTGTGTGCT  ACGTCCGTGG  CCGTGATGAG  ACGACGTGTT  AGGACCCGTG
P4A         ..........  .C........  .........A  ..........  ..........  ..........
Halton      ..........  ..........  ..........  ..........  ..........  ..........
Pa1         ..........  ..........  ..........  ..........  ..........  ..........
D375        ..........  ..........  ..........  ..........  ..........  ..........
P5A         ...A......  ..........  ..........  ..........  ..........  ..........
Ferris      ..........  ..........  .........A  ..........  ..........  ..........

            601                                                                 660
Luffness    CCTGGCATTG  GCACGCGGTT  TAAGACTTGA  TGAGTGCCCG  CAGGCACCGC  CAGCTTTTTC
P4A         ..........  ...T......  ..........  ..........  ..........  ..........
Halton      ..........  .....T....  ..........  ..........  ..........  ..........
Pa1         ..........  ..........  ..........  ........T.  ..........  ..........
D375        ..........  ..........  ..........  ........T.  ..........  ..........
P5A         A.........  .....T....  ..........  ..........  ..........  ..........
Ferris      ..........  .....T....  ..........  ...TGC..G-  ...-..-...  ..........

            661                                                                 720
                                                          ITS1   5.8S
Luffness    TCATTTTTAT  AAATTTTTTA  ATGCAATTTG  ATTGCTAAAA  TATTCTAGTC  TTATCGGTGG
P4A         .......C..  ..........  ..........  ..........  ..........  ..........
Halton      ..........  ..........  ..........  ..........  ..........  ..........
Pa1         ..........  ..........  ..........  ..........  ..........  ..........
D375        ..........  ..........  ..........  ..........  ..........  ..........
P5A         ..........  .........T  ........C.  ..........  ..........  ..........
Ferris      ..........  ..........  ..........  ..........  ..........  ..........
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          721                                                                     780
Luffness  ATCACTCGGC  TCGTGGATCG  ATGAAGAACG  CAGCCAACTG  CGATAATTAG  TGTGAACTGC
P4A       ..........  ..........  ..........  ..........  ..........  ..........
Halton    ..........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  ..........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ..........  ..........  ..........  ..........  ..........
Ferris    ..........  ..........  ..........  ..........  ..........  ..........

          781                                                                     840
Luffness  AGAAACCTTG  AACACAGAAC  TTTCGAATGC  ACATTGCGCC  ATTGGAGTGA  CATCCATTGG
P4A       ..........  ..........  ..........  ..........  ..........  ..........
Halton    ..........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  ..........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ..........  ..........  ..........  ..........  ..........
Ferris    ..........  ..........  ..........  ..........  ..........  ..........

          841                                                                     900
                                5.8S ITS2
Luffness  CACGCCTGGT  TCAGGGTCGT  AACCAAAAAA  TGCACTGCAT  GTGCGTGTTT  TATTTGCTAA
P4A       ..........  ..........  ..........  ..........  ..........  ..........
Halton    ..........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  ..........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ..........  ..........  ..........  ..........  ..........
Ferris    ..........  ..........  ..........  ..........  ..........  ..........

          901                                                                     960
Luffness  GATCACGCTT  CGGCGTGTTC  TTGCATACCA  TTGAATGCTA  CGCTGTGTAG  TGTTGGACGT
P4A       ..........  ..........  ..........  ..........  ..........  C.........
Halton    ..........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  ..........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ..T.......  ..........  ..........  ..........  C.........
Ferris    ...-......  ..........  ..........  ......CG..  ..........  ..........

          961                                                                     1020
Luffness  GCTGGCGCGA  AAATGTGTTG  TCTTTCGCGC  TTTACAGACC  GTAATTTAGG  CACGCCCTTC
P4A       ..........  ..........  ..........  ..........  ..........  ....A.....
Halton    ..........  ..........  ..........  ..........  ..........  ....T.....
Pa1       ..........  ..........  ..........  ..........  ..........  ....T.....
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ....A.....  ..........  ..........  ..........  ..........
Ferris    CG........  ..........  ..........  ..........  ..........  ..........

          1021                                                                    1080
                                                                ITS2  28S
Luffness  GTTCACATGC  GATAGCTGAA  TGCCTCGCCA  ATAGGCATTC  GCAATTGAAC  ATTTCGACCT
P4A       .C........  ..........  ..........  .........T  ..........  ..........
Halton    ..........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  ..........  ..........  .........T  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ..........  ..........  .........T  ..........  ..........
Ferris    ..........  ..........  ..........  .........T  ..........  ..........
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          1081                                                                     1140
Luffness  GAACTCAGAC  GTGAACACCC  GCTGAACTTA  AGCATATCAG  TAAGCGGAGG  AAAAGAAACT
P4A       ..........  ..........  ..........  ..........  ..........  ..........
Halton    ..........  ..........  ..........  ..........  ..........  ..........
Pa1       ..........  ..........  ..........  ..........  ..........  ..........
D375      ..........  ..........  ..........  ..........  ..........  ..........
P5A       ..........  ..........  ..........  ..........  ..........  ..........
Ferris    ..........  ..........  .........-  .........T  .T........  .---------


Luffness  AACGAGGATT  C
P4A       ..........  .
Halton    ..........  .
Pa1       ..........  .
D375      ..........  .
P5A       ..........  .
Ferris    ----------  -
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FIG. 1. Sequences of ITS1, 5.8S, and ITS2 regions and partial sequence of 18S and 28S regions from five *Globodera pallida* populations, and sequence of *G. pallida* from Ferris et al. (1995). The 18S, 5.8S, and 28S sequences are in bold. Actual and potential RsaI sites are underlined. Sequence differences between clones are shown in Table 2.

bp product was not obvious in the digestion patterns. The P5A population was distinguished from all the other populations with the enzyme TaqI by the absence of a product of 371 bp. The sequence of the P5A clones had an additional TaqI site at 688 bp, which accounts for the RFLP difference with this population.

Of the six enzymes used, the enzyme that discriminated among most of the populations was RsaI. RFLP patterns were produced that distinguished Pa1, P5A, and D375 from the remaining populations (Fig. 3). The differences in the RFLP patterns were due to the presence or absence of RsaI sites within the 520-bp digestion product that spans part of the 18S gene and part of the ITS1 region. No restriction sites for RsaI

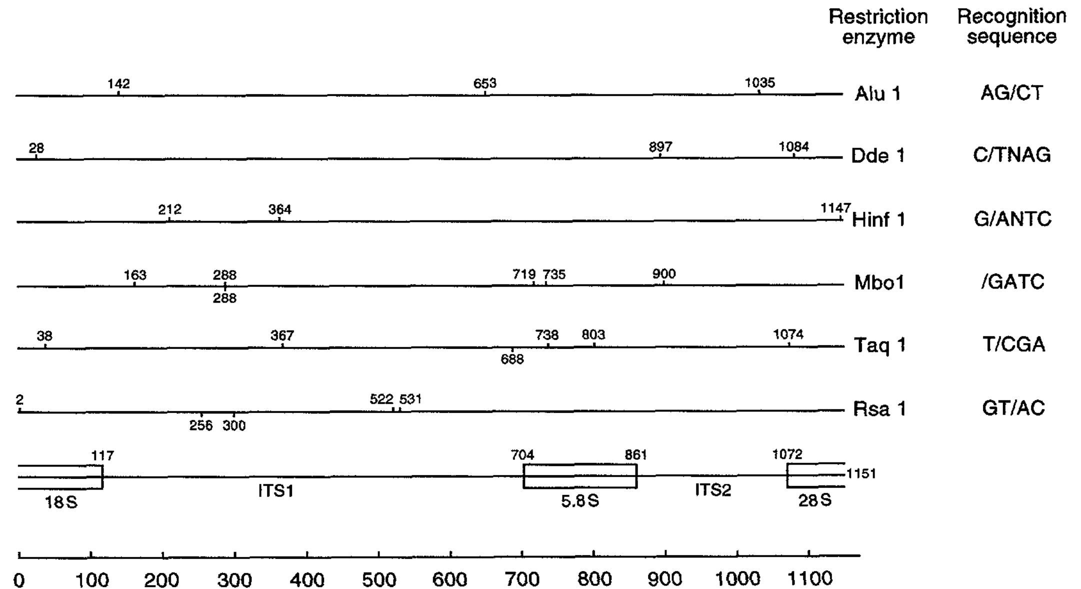

FIG. 2. Schematic diagram showing restriction sites in *Globodera pallida* for Alu1, Dde1, Hinf1, Mbo1, Taq1, and Rsa1 in the PCR products amplified with the ribosomal primers. The primers are not included at the extremities of the map. Positions of sites common to all populations are shown above the lines, and known variable sites are shown below.

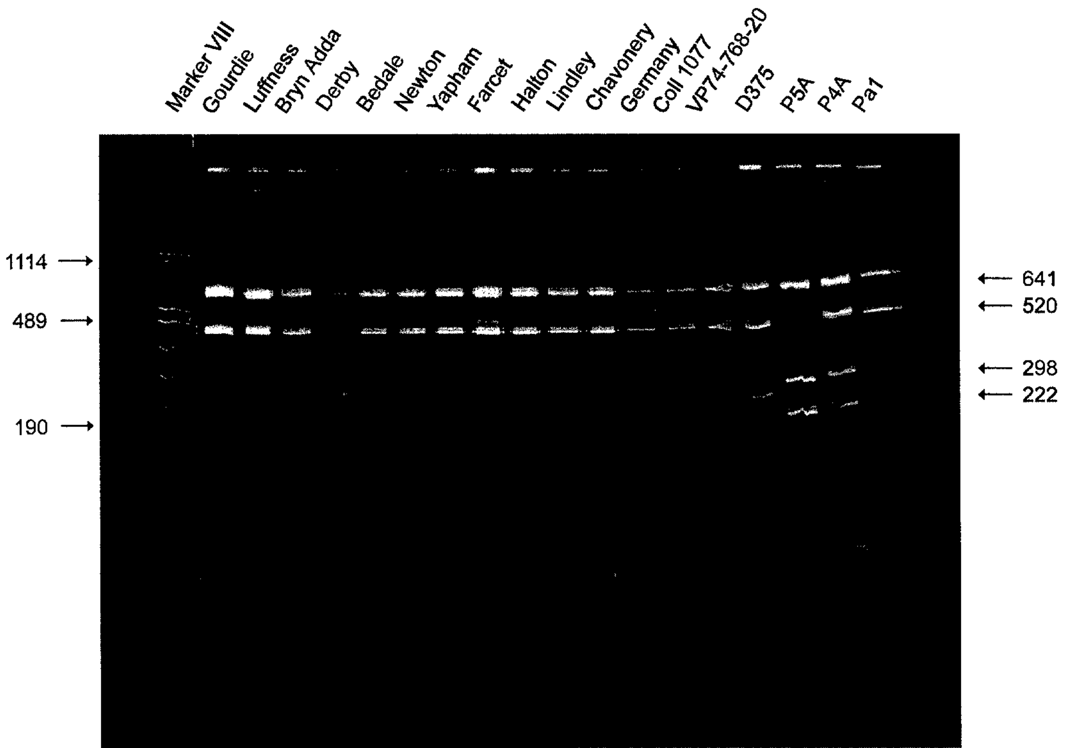

FIG. 3. RsaI digestion products of ribosomal PCR products from 17 *Globodera pallida* populations. Sizes of digestion products based on sequence information are indicated. (Size marker: Marker VIII, Boehringer).

were observed in the sequence of the other region (620-bp fragment) of the PCR product for any of the isolates. With the Pa1 population the 520-bp band was not digested, and the sequence confirms the absence of RsaI sites in this region. With the P5A population, an internal RsaI site (position 300) gives rise to products of 222 and 298 bp in size, which is confirmed in the P5A sequence. With P4A, a mixture of presumably digested and undigested 520-bp bands was seen, but neither of the two sequences obtained from this population showed a RsaI site in this region. In the D375 population both digested and undigested products of the 520-bp band were seen, with the digested product of 260 bp presumably a doublet. It is possible that some copies of the 520-bp fragment have a RsaI site at position 255. With the remaining populations, digestion patterns in which there are apparently mixtures of products, some of which do not have RsaI sites within the 520-bp fragment and a small proportion with one or the other RsaI site, were observed in the RFLP patterns. Twelve clones of D375 were sequenced in the region of the 520-bp fragment, but no RsaI sites were found.

rDNA of individuals: To determine if the complex patterns observed with the RsaI digests were the result of populations being mixtures of individuals with different types of rDNA or whether the rDNA of an individual had different types of repeat units, PCR was performed on indidividual nematodes. Digestion of the PCR products produced from individual nematodes from the population Halton with RsaI again produced the 520-bp band and the smaller bands presumably resulting from internal digestion of some copies of this fragment (Fig. 4).

Comparison of sequences: Sequence comparisons from two clones of PCR products from the populations P5A, P4A, Pa1, D375, Luffness, and Halton were aligned with the sequence of Ferris et al. (1995) and are

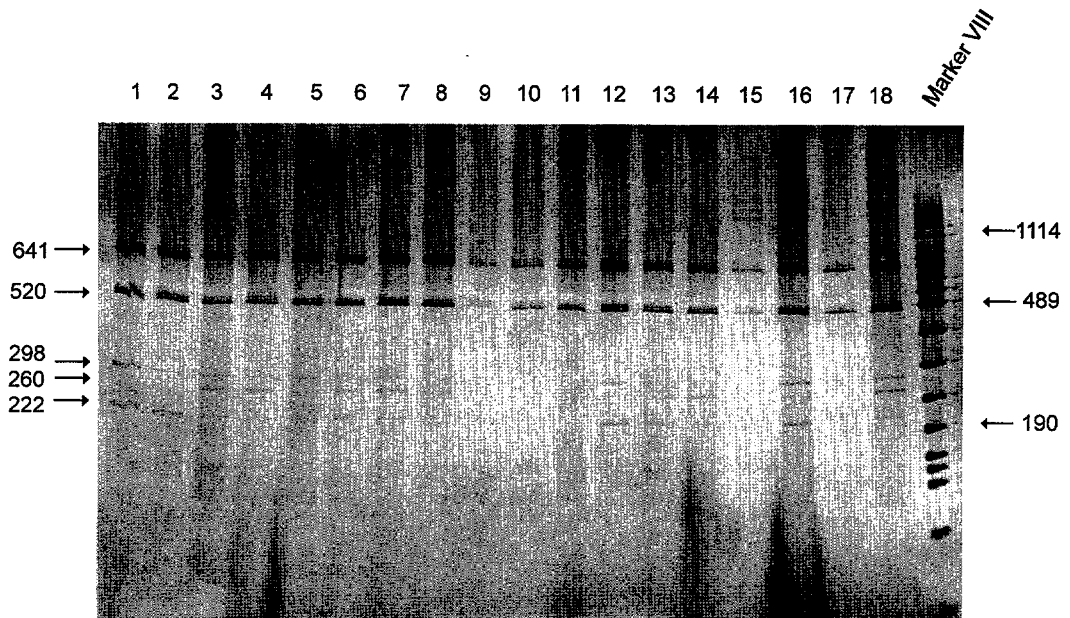

FIG. 4. RsaI digestion products of ribosomal PCR products from 18 individual *Globodera pallida* juveniles. Each track (1-18) represents products from an individual nematode from population Halton. The gel was silver-stained. (Size marker: Marker VIII, Boehringer).

shown in Fig. 1. There were no differences between any of the sequences in the 171 bp of 18S, 79 bp of 28S, or 157 bp of 5.8S sequence. There were more sequence differences in the ITS1 region than in ITS2. The two P5A sequences were identical as were the D375 sequences; there was one difference between the two Luffness sequences, four between the two Pa1 clones, and eight between the two P4A clones. The Halton clones were the most variable with 13 differences (Table 2). Overall, the South American P5A sequence is most distinct from the others. In the ITS1 region there were 10 positions that differentiated P5A from all the other sequences, one position that differentiated the Pa1 clones from all of the rest, six sites that differentiated the P5A clones from one or both of the P4A and Halton clones; in the ITS2 region there were two positions at which the P5A sequences were different from all of the rest and two where one of each of the Halton, P4A, and Pa1 were different from the rest. With the P4A sequences, neither showed RsaI sites in the 520-bp region. With the RsaI digest for P4A there appeared to be two types of PCR products in similar proportions—those without a RsaI site and those with the 300-bp site. There were 11 differences between the two clones of P4A (eight in ITS1 and three in ITS2) (Table 2). The dissimilarities between the clones and populations are shown in Table 3. The sequence from Ferris et al. (1995) was most different, being 2.4% different from the sequences examined in this study.

DISCUSSION

This study on *G. pallida* in Europe aims to analyze genetic diversity and relate this to the likely introductions from South America and to their virulence characteristics. Our knowledge of South American *G. pallida* is limited, and the two populations included here may be unrepresentative. The P5A population from South America is clearly most distinct from the others, both with RFLP and sequence analysis. This population was also found to be most distinct in previous work assessing relationships among populations of *G. pallida* with simple sequence repeat (SSR) primers (Blok and

TABLE 2. Sequence differences between pairs of clones from six populations of *Globodera pallida*.

Population	Positions of sequence differences between clones																		
	291	302	387	496	499	508	509	519	536	552	569	614	616	638	668	951	1015	1022	1060
P5A																			
P4A										T/C	G/A	T/C			T/C	T/C	C/A	T/C	C/T
Pa1						A/G								C/T			C/T		C/T
D375																			
Luffness						A/G													
Halton	T/C	T/C	C/A	G/T	G/A	A/G	C/T	C/T	A/G				C/T			T/C	C/T		C/T

Phillips, 1995) and RAPDs (Blok et al., 1997). P5A is also distinct biologically, being highly virulent on clones bred from *S. vernei* and *S. tuberosum* spp. *andigena* (Phillips and Trudgill, in press). The P4A population, also from South America, was found to be distinct from the other populations in an RAPD study (Blok et al., 1997), but here the separation from the European populations is less clear, particularly with regard to the sequence analysis. The Pa1 population possesses only two distinguishing nucleotides from the sequences of the other populations in this study. However, biologically it is distinguished from the other populations by lacking virulence to the H2 gene derived from *Solanum multidissectum*. The D375 population from the Netherlands, which has very low virulence (F. Arntzen, pers. comm.), was distinguished from the others in the digestion patterns of the rDNA PCR product obtained with both RsaI and HinfI. However, previous studies with SSR primers and RAPDs did not separate this population from the other Dutch populations included in this study. With the remaining populations from Europe, the RsaI digests indicated that the proportion of different types of repeat units within an array varied, with some populations displaying little or no evidence of the minor repeat types. Thiéry and Mugniéry (1996) reported no intraspecific variation in their rDNA analysis, which included six *G. pallida* populations (Pa1, Pa2, and Pa2/3 pathotypes), although with RsaI digests they found additional weak polymorphic bands, suggesting differences in some of the repeat units within their populations. There were no restriction patterns with fragment sizes consistent with both potential internal RsaI sites within the 520-bp fragment.

In summary, these data support already existing studies that suggest that the majority of European populations of *G. pallida* derive from one source. There are a few exceptions: Pa1 has been found to be distinct in other studies but not D375. The Luffness population also has shown dissimilarity to most European populations, but this was not confirmed here. The distinctness of P5A is apparent, but that of P4A is not.

TABLE 3. Between and within population dissimilarity matrix for seven populations of *Globodera pallida.*

	Luffness	P4A	Halton	Pal	D375	P5A
Luffness	0.0009[a]					
P4A	0.0057	0.0105[a]				
Halton	0.0057	0.0081	0.0114[a]			
Pal	0.0026	0.0072	0.0070	0.0035[a]		
D375	0.0013	0.0061	0.0066	0.0026	0.0000[a]	
P5A	0.0145	0.0149	0.0136	0.0158	0.0149	0.0000[a]
Ferris[b]	0.0180	0.0180	0.0195	0.0190	0.0174	0.0269

[a] A number for a population vs. itself shows the dissimilarity between the two cloned PCR products from each of six populations used in this study. The other numbers show the average dissimilarity between populations.
[b] Population of Ferris et al. (1995) included for comparison.

It is tempting to speculate that the populations displaying several repeat types arose through the hybridization of ancestors with different repeat types. One of each of the Halton and P4A clones showed some sequence similarities with those of the P5A clones, but these differences were scattered throughout the sequence.

Based on the sequence analysis and the restriction enzyme digestions in this work, the P5A population shows little variation between repeat units. Arnheim et al. (1980) reported low variation among rDNA arrays within individuals; the multiple copies of rDNA are homogenized in a process called concerted evolution. The multiple copies of rDNA do not evolve independently, and a number of molecular mechanisms have been postulated to account for this process (Hillis and Dixon, 1991). Given that there is a tendency for homogenization of rDNA repeats, then the results of this current work would suggest that in many of the British *G. pallida* populations, the process of homogenization to a uniform repeat type has not been completed, and represented in their arrays are small numbers of repeat types possibly arising from hybridization in their ancestry between individuals with distinct ribosomal genotypes. Work by Grisi (1995) indicates that the ribosomal repeats in *G. pallida* occur only in one chromosomal location; hence, the processes of ribosomal gene evolution in this species are not likely to be further complicated by recombination between different chromosomes (Arnheim et al., 1980).

Digestion of the rDNA PCR product from individual nematodes from the Halton population indicated that there are different repeat types in a single individual. Multiple digestion patterns generated from the PCR product from rDNA from a single nematode also have been observed by Zijlstra et al. (1995) with *M. hapla.* They proposed that in one individual there could be different ITS types with digestion patterns yielding composite patterns of *M. chitwoodi* and *M. incognita* or *M. javanica* types. The arrangement of these different types is unknown. A considerable amount of sequencing would be required to gain an accurate picture of the amount of variation between different repeats within an individual. Ideally, multiple clones of genomic DNA would be used to minimize the interference of sequencing artifacts arising during amplification and subsequent cloning of the PCR products.

In this study the ITS1 region showed the most sequence variation and the 5.8S gene showed the least, confirming reports with other organisms where ribosomal gene sequences have been found to be highly conserved but the spacer regions are more variable (Hillis and Dixon, 1991). Ferris et al. (1993, 1994), with *H. glycines* and *H. avenae,* also reported more variation in the ITS1 region than in ITS2 and more conservation in the 5.8S gene. In a comparison of the ITS regions and 5.8S genes of *G. pallida, G. rostochiensis,* and *G. virginia,* Ferris et al. (1995) also found the ITS1 region to be more variable than the ITS2. They found no differences between *G. pallida* and *G. rostochiensis* in the 5.8S sequence but did report a 5% dissimilarity in the ITS1 region. Their sequences for a British isolate of *G. pallida* did

not extend into the 18S and 28S regions quite as far as the sequences in the present study. However, the sequence of Ferris et al. (1995) contained eight nucleotide deletions not observed in this study. There were also four sequence differences: two changes shared with two or more of our sequences, and two pairs of nucleotide inversions relative to our sequences. The explanation for these sequence differences is unclear.

The study of rDNA using PCR to examine phylogenetic relationships between and within species is now widely used. This approach is particularly attractive with small organisms like nematodes, where the quantities of DNA available are a major technical limitation. However, there are some limitations with this approach that should be considered, particularly when examining closely related isolates. Artifacts during amplification, cloning, and propagation in *E. coli* may contribute to sequence variation. When there are small amounts of sequence polymorphism between isolates, differences arising from artifacts may account for some of the polymorphism. The study of rDNA also can be complicated by the presence of sequence and length variants in different units. Analysis can be straightforward when there is total uniformity in the repeat units, both within an individual and among individuals in a species. However, as was found with *G. pallida,* there can be more complicated rDNA arrangements that confound interpretations. Given the complexity of the RFLP patterns that can arise with multiple repeat units differing in their restriction digestion patterns, interpreting relationships must be approached with caution. Analysis of ribosomal gene arrangements in different populations gives insight into the relationships of these populations; however, extrapolation of evolutionary relationships based on ribosomal genes to genes involved in phenotypes such as virulence must be done with care as the pressures driving their evolution are different.

Literature Cited

Arnheim, N., M. Krystal, R. Schmickel, G. Wilson, O. Ryder, and E. Zimmer. 1980. Molecular evidence for genetic exchanges among ribosomal genes on nonhomologous chromosomes in man and ape. Proceedings of the National Academy of Science USA 77:7323–7327.

Blok, V. C., and M. S. Phillips. 1995. The use of repeat sequence primers for investigating genetic diversity between populations of potato cyst nematodes with differing virulence. Fundamental and Applied Nematology 18:575–582.

Blok, V. C., M. S. Phillips, and B. E. Harrower. 1997. Comparison of British populations of *Globodera pallida* with populations from continental Europe and South America using RAPDs. Genome 40:286–293.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acid Research 12:387–395.

Evans, K., J. Franco, and M. M. De Scurrah. 1975. Distribution of species of potato cyst-nematodes in South America. Nematologica 21:365–369.

Ferris, V. R., J. M. Ferris, and J. Faghihi. 1993. Variation in spacer ribosomal DNA in some cyst-forming species of plant-parasitic nematodes. Fundamental and Applied Nematology 16:177–184.

Ferris, V. R., J. M. Ferris, J. Faghihi, and A. Ireholm. 1994. Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. Journal of Nematology 26:144–151.

Ferris, V. R., L. I. Miller, J. Faghihi, and J. M. Ferris. 1995. Ribosomal DNA comparisons of *Globodera* from two continents. Journal of Nematology 27:273–283.

Grisi, E. S. 1995. Nematology News 57:41.

Hillis, D. M., and M. T. Dixon. 1991. Ribosomal DNA: Molecular evolution and phylogenetic inference. Quaterly Review of Biology 66:411–453.

Ibrahim, S. K., R. N. Perry, P. R. Burrows, and D. J. Hooper. 1994. Differentiation of species and populations of *Aphelenchoides* and of *Ditylenchus angustus* using a fragment of ribosomal DNA. Journal of Nematology 26:412–421.

Kort, J., H. Ross, H. J. Rumpenhorst, and A. R. Stone. 1977. An international pathotype scheme for identifying and classifying pathotypes of potato cyst nematodes *Globodera rostochiensis* and *G. pallida.* Nematologica 23: 333–339.

Merril, C. R. 1990. Silver staining of proteins and DNA. Nature 343:779–780.

Phillips, M. S., B. E. Harrower, D. L. Trudgill, R. Waugh, and M. A. Catley. 1992. Genetic variation in British populations of *Globodera pallida* as revealed by isozyme and DNA analyses. Nematologica 38:304–319.

Phillips, M. S., and D. L. Trudgill. 1998. Variation of virulence, in terms of quantitative reproduction of *Globodera* populations from Europe and North America, in relation to resistance from *Solanum vernei* and *S. tuberosum* ssp. *andigena* CPC 2802. Nematologica 44, in press.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. 2nd edition. New York: Cold Spring Harbor Laboratory Press.

Staden, R. 1982. Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. Nucleic Acid Research 10: 4731–4751.

Thiéry, M., and D. Mugniéry. 1996. Interspecific

rDNA restriction fragment length polymorphism in *Globodera* species, parasites of solanaceous plants. Fundamental and Applied Nematology 19:471–479.

Vrain, T. C., D. A. Wakarchuk, A. C. Lévesque, and R. L. Hamilton. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. Fundamental and Applied Nematology 15:563–573.

Wendt, K. R., T. C. Vrain, and J. M. Webster. 1993. Separation of three species of *Ditylenchus* and some host races of *D. dipsaci* by restriction fragment length polymorphism. Journal of Nematology 25:555–563.

Xue, B., D. L. Baillie, and J. M. Webster. 1993. Amplified fragment length polymorphisms of *Meloidogyne* spp. using oligonucleotide primers. Fundamental and Applied Nematology 16:481–487.

Zijlstra, C., A. E. M. Lever, B. J. Uenk, and C. H. Van Silfhout. 1995. Differences between ITS regions of isolates of root-knot nematodes. *Meloidogyne hapla* and *M. chitwoodi.* Phytopathology 85:1231–1237.

Zijlstra, C., B. J. Uenk, and C. H. Van Silfout. 1997. A reliable, precise method to differentiate species of rootknot nematodes in mixtures on the basis of ITS-RFLPs. Fundamental and Applied Nematology 20:59–63.