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Gene Pool Similarities of Potato Cyst Nematode Populations Assessed by AFLP Analysis

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AFLP was used to characterize 24 potato cyst nematode populations. This novel DNA fingerprinting technique enabled the identification of 987 marker loci by screening only 12 primer combinations. Data on presence or absence polymorphisms and data on the intensities of corresponding DNA fragments were collected. Separate analysis of both data sets revealed similar dendrograms for the nine *G. rostochiensis* populations included in this study. Both dendrograms consisted of two groups containing three and five related populations, respectively. One population differed from either of these groups. Each group represented a different pathotype as defined by Kort et al. (J. Kort, H. Ross, H. J. Rumpenhorst, and A. R. Stone, Nematologica 23:333-339, 1977). Previously, a similar arrangement was found after analysis of the genetic variation using random amplified polymorphic DNA (RAPD) (R. T. Folkertsma, J. N. A. M. Rouppe van der Voort, M. P. E. van Gent-Pelzer, K. E. de Groot, W. J. van den Bos, A. Schots, J. Bakker, and F. J. Gommers, Phytopathology 84:807-811, 1994). For the 15 *G. pallida* populations analyzed, complex AFLP patterns were obtained and therefore only qualitative AFLP data were used. Incongruities were observed between clustering on the basis of AFLP data and classical pathotyping. This strongly confirms earlier findings obtained with RAPDs, because the AFLP markers used in this study outnumbered the population characteristics revealed by RAPDs by a factor of five. To arrive at a reliable pathotype designation of potato cyst nematode populations molecular data and virulence characteristics should be integrated. Possible causes for the difference in distribution of polymorphisms among *G. rostochiensis* and *G. pallida* populations are discussed.

Additional keywords: DNA fingerprinting, *Globodera*, neutral markers, pathotype, virulence genes.

The potato cyst nematodes *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone (Loof and Bakker 1992) both originate from the Andean region of South America (Evans and Stone 1977). They were introduced in Europe after 1850 (Evans et al. 1975). Potato cyst nematodes are apparently well adapted to survive in the temperate zones and cause substantial losses in potato crops.

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Potentially, plant resistance is an effective and durable means to control potato cyst nematodes (Jones et al. 1981). Selection towards alleles for virulence is slow since both nematode species produce only one generation in a growing season, their multiplication rate is low, ranging from 5 to 140 (Seinhorst 1992), the time between the generations is 2 to 4 years in a normal crop rotation, and their mobility is limited to about 20 cm per growing season. Because of their recent introduction and on the basis of the aforementioned arguments, it is unlikely that mutation and selection have changed the allele frequencies noticeably (Bakker et al. 1993).

Currently, potato cyst nematode populations are classified into pathotypes on the basis of their multiplication rates on 8 different potato lines. The international pathotype scheme recognizes 5 pathotypes within *G. rostochiensis* and 3 within *G. pallida* (Kort et al. 1977). Resistance breeding is severely hampered by shortcomings in the pathotype scheme. The scheme is not able to reflect the genetic diversity that was introduced into Europe (Bakker et al. 1993). Additional drawbacks are the arbitrary way pathotypes are delineated and the laborious and time-consuming way virulence is determined (Trudgill 1985; Phillips and Trudgill 1985; Bakker and Gommers 1989).

As a consequence of potato cyst nematodes being introduced into Europe, both variation in (a)virulence characteristics and selectively neutral markers are determined by the genotypes of the primary founders, random genetic drift, and gene flow. These processes influence the European gene pool. Under the condition that potato cyst nematode populations were not exposed to resistant host plants, molecular data from populations are informative for assessing interpopulation variation at (a)virulence loci, including those not yet revealed by the current pathotype scheme (Bakker et al. 1993). Obviously, under the presence of genes for resistance an increase in the frequency of virulence alleles will no longer be reflected by selectively neutral markers. In that case only markers closely linked to the (a)virulence alleles would be indicative for the virulence characteristics of a population.

Intraspecific genetic variation of a large number of potato cyst nematode populations has been assessed by two-dimensional gel electrophoresis (2-DGE) of proteins (Bakker and Bouwman-Smits 1988; Bakker et al. 1992), restriction

fragment length polymorphisms (RFLPs) (Schnick et al. 1990; Burgermeister et al. 1992) and randomly amplified polymorphic DNA (RAPD) (Folkertsma et al. 1994). Although these techniques are useful, several shortcomings should be mentioned. Both the 2-DGE technique and RFLP analysis are laborious and time consuming, and therefore unsuitable for large-scale population analyses. RAPD analysis, a polymerase chain reaction (PCR)-based technique, enables the amplification of numerous markers (Williams et al. 1990, Welsh and McClelland 1990; Folkertsma et al. 1994). However, a reliable estimate of relatedness between species or populations can seriously be hampered by preferential amplification of DNA fragments (Wilkerson et al. 1993).

The AFLP assay is a new PCR-based approach to DNA fingerprinting (Zabeau and Vos 1993). In contrast to the RFLP procedure, the AFLP technique generates virtually unlimited numbers of DNA fragments from nanogram quantities of genomic DNA. In comparison to the RAPD approach, the AFLP technique uses stringent reaction conditions which guarantees a better reproducibility. Furthermore, this technique is quantitative and AFLPs can therefore be used as codominant markers (Van Eck et al. 1995). This report evaluates the usefulness of the AFLP technique in pathotyping potato cyst nematode populations.

RESULTS

AFLP fingerprinting of 9 *Globodera rostochiensis* and 15 *G. pallida* populations revealed a total number of 513 and 551 amplified DNA fragments, respectively. Twelve primer combinations were used to generate these fragments. Figure 1A shows fingerprints of the populations after PCR with primer combination E+GA/M+AC (for definitions, see Materials and Methods). Figure 1B shows fingerprints of the *G. rostochiensis* populations after PCR with primer E+GA in combination with M+AG. It can be seen that one nucleotide change in the primer from set M caused a complete change of the AFLP fragment pattern. The number of amplified fragments ranged from 22 to 105 and depended on length of the extension at the 3′-end of the primer. An increase of the extension length reduced the number of amplified fragments for populations of both species. Under the reaction conditions described the size of the DNA fragments ranged from 50 to 500 bp.

Among the polymorphic DNA fragments two subsets can be distinguished: presence/absence and band intensity polymorphisms (see Materials and Methods). In Figure 1A both types of polymorphisms are illustrated. It is noted that the variation in the band intensities between duplicates was small.

Variation among *G. rostochiensis* populations.

Comparison of the DNA fingerprints among *G. rostochiensis* populations revealed that 15.8% of the fragments were polymorphic. The estimated similarity among *G. rostochiensis* populations based on the shared presence of 81 polymorphic DNA fragments averaged 0.587 and ranged from 0.167 (population C9 and population B11) to 0.938 (population A9 and population C9). The band intensity polymorphisms found were taken as a measure to estimate the frequencies of corresponding DNA fragments in a population. In this way 25 DNA fragments were identified, corresponding to 12 putative loci. The similarity among *G. rostochiensis* populations based on these intensity polymorphisms was computed according to Rogers (1984), and averaged 0.600 ranging from 0.335 (population D7 and B11) to 0.860 (population D7 and B9).

Variation among *G. pallida* populations.

The proportion of polymorphic DNA fragments in *G. pallida* was 23.0%. Only qualitative polymorphisms were recorded. Band intensity polymorphisms could not be identified for the *G. pallida* populations with the primer combinations used. The estimated similarity based on 127 polymorphic DNA fragments averaged 0.739 and ranged from 0.524 between population A3 and A5 to 0.976 between population A5 and B5.

Clustering of *G. rostochiensis* populations.

Figure 2A shows a dendrogram of the investigated *G. rostochiensis* populations based on variants expressed by the presence or absence of DNA fragments. The populations were clustered into three groups which coincide with their pathotype classification as defined by Kort et al. (1977). The three groups were distinguished by 3, 7, and 12 unique DNA fragments, respectively. As an example, Figure 1B shows fragment GA/AG-130, a 130-bp sized DNA fragment that was amplified using primers E+GA and M+AG. This fragment was one of those specific for the Ro_1 populations tested. Figure 1A shows also two fragments, GA/AC-140 and GA/AC-130, specifically present in the Ro_3 populations tested. No Ro_4 population specific fragments were amplified with the primer combinations E+GA/M+AG and E+GA/M+AC.

Cluster analysis of the similarities between nine *G. rostochiensis* populations based on quantitative polymorphisms, resulted in a dendrogram as depicted in Figure 2B. The overall topology of the dendrogram based on band intensity polymorphisms is similar to the one based on the presence or absence of DNA fragments.

Clustering of *G. pallida* populations.

Cluster analysis of the AFLP data of 15 *G. pallida* populations showed limited resemblance to their pathotype classification (Fig. 2C). The distance between populations with different pathotype classifications is often smaller than between populations with the same classification. This is illustrated by the populations A7 D353 and D3 1097, classified as Pa_2 and Pa_3, respectively, that were clustered together in the dendrogram. While all the populations could be differentiated on the basis of AFLP fragments, only a few were differentiated by specific fragments: cluster A1, B1 was specified by 1, cluster A5 and B5 by 4, population C1 by 4, population A3 by 1 and population B3 by 2 specific AFLP fragments.

The intraspecific similarities of both potato cyst nematode species based on AFLPs were compared with the results obtained in a previous study using RAPDs (Folkertsma et al. 1994). The Spearman rank correlation coefficients between the intraspecific similarity based on AFLPs and RAPDs were 0.80 for *G. rostochiensis* and 0.86 for *G. pallida*.

Divergence between *G. rostochiensis* and *G. pallida* populations.

The wide divergence of *G. rostochiensis* from *G. pallida* at

the molecular level using AFLP's is clearly illustrated by the observation that the primer combinations used in this study resolved only 64 (6.4%) DNA fragments common to all populations of both sibling species. DNA fragments observed in populations of both species were monomorphic.

DISCUSSION

This study has shown that the AFLP technique is a powerful method for the characterization of intraspecific variation among populations of *G. rostochiensis* and *G. pallida*. Fifty nanograms of genomic DNA sufficed to generate template for an unlimited amplification of restriction fragments. Twelve different primer combinations enabled the amplification of 987 marker loci in 24 potato cyst nematode populations (marker loci consisting of multiple bands are included).

In case of the *G. rostochiensis* populations analyzed, close agreement was found between the dendrogram based on the presence or absence of AFLP markers and the one based on band intensity polymorphisms. Both data sets resulted in the discrimination of three similar groups containing three, five and one population, respectively. These were also identified

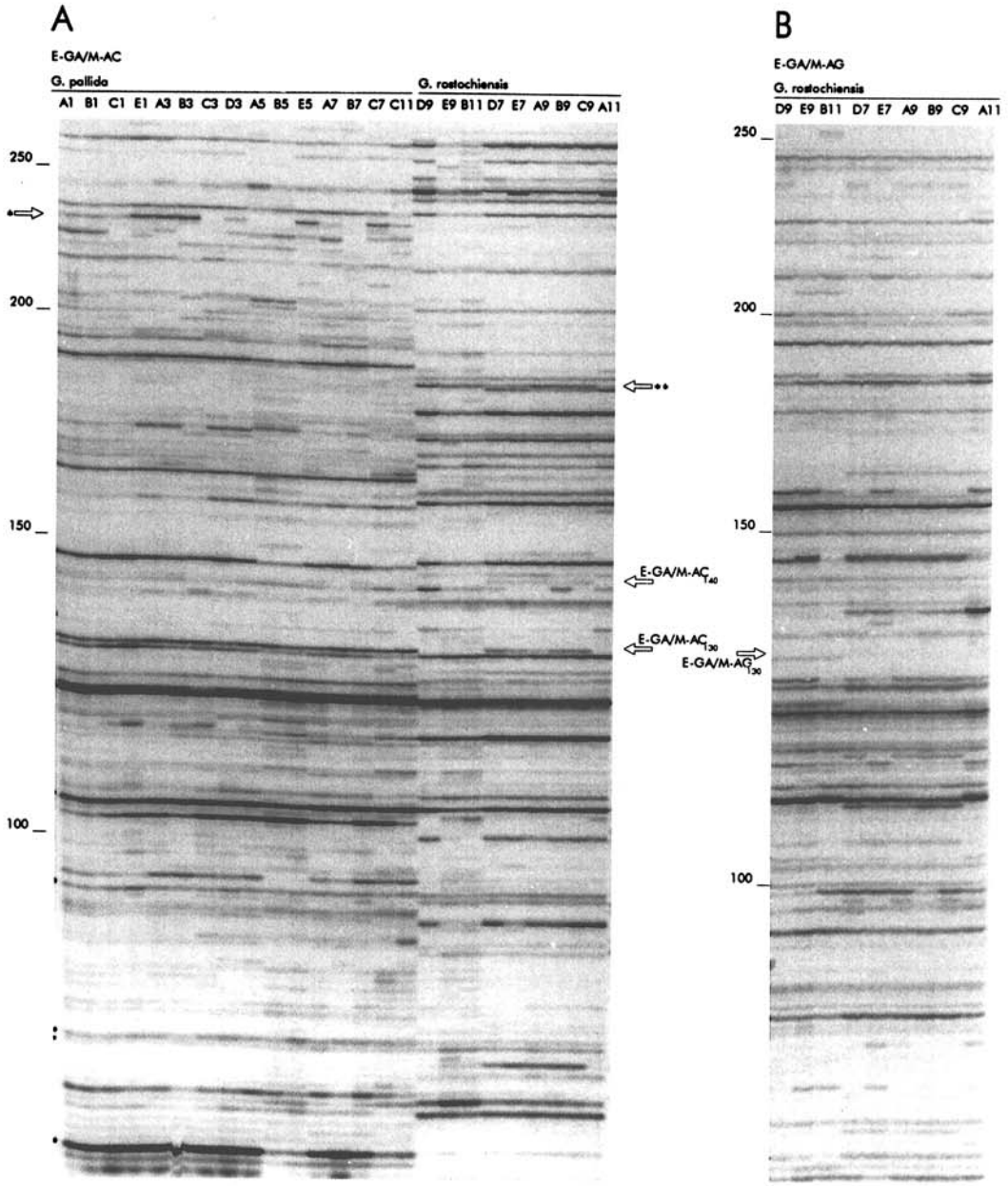

Fig. 1. A, AFLP fingerprints of 9 *Globodera rostochiensis* and 15 *G. pallida* populations after selective amplification with primer combination E+GA/M+AC. Arrows with one and two asterisk(s) indicate a presence/absence polymorphism and two corresponding DNA fragments, respectively. Two Ro_3 specific fragments, GA/AC-140 and GA/AC-130, are indicated with an arrow. Lanes A1-C11 represent the *G. pallida* populations encoded in Table 1; lanes D9-A11 represent the *G. rostochiensis* populations encoded in Table 1. Molecular weights are indicated in base pairs. **B,** AFLP fingerprints of 9 *Globodera rostochiensis* populations after selective amplification with primer combination E+GA/M+AG. One Ro_1 specific fragment, GA/AG-130, is indicated with an arrow. Lanes D9-A11 represent the *G. rostochiensis* populations encoded in Table 1. Molecular weights are indicated in base pairs.

in a previously described dendrogram based on RAPD data (Folkertsma et al. 1994) and correspond to the pathotype classification of these populations. The Spearman rank correlation coefficient between the similarities based on AFLPs and RAPDs was 0.80. Hence, both types of DNA marker techniques result in similar classifications.

A number of AFLP markers specific to the three *G. rostochiensis* groups was identified. Since the majority of the molecular markers are considered to be selectively neutral (Kimura 1983) these markers may be indicative for the virulence of these populations (Bakker et al. 1993). More *G. rostochiensis* populations need to be examined, however, to establish whether these markers are likely to have universal application.

Agreement between the dendrograms based on AFLP and RAPD data (taken from Folkertsma et al. 1994) was also found for the *G. pallida* populations analyzed. This is illustrated by a Spearman rank correlation coefficient of 0.86. In contrast to *G. rostochiensis*, the clustering of the *G. pallida* populations revealed limited resemblance with their pathotype classification. Similar observations were previously made with 2-DGE (Bakker et al. 1992), multilocus enzyme electrophoresis (Phillips et al. 1992), RFLPs (Schnick et al. 1990; Phillips et al. 1992), and RAPD analyses (Folkertsma et al., 1994). An explanation for this lack of concordance is the inadequacy of the pathotype scheme for *G. pallida*. A recently described major resistance locus from *S. tuberosum* ssp. *andigena* CPC 1673 (Arntzen et al. 1994) discriminates the cluster A5-B5. This illustrates that degrees of similarities revealed by AFLP markers can also be indicative for virulence characteristics of *G. pallida* populations. An additional explanation for the observed incongruity between clusters of *G. pallida* populations based on molecular and virulence characteristics may be the structure of the genetic variation between *G. pallida* populations. The proportion of polymorphic DNA fragments among *G. rostochiensis* populations

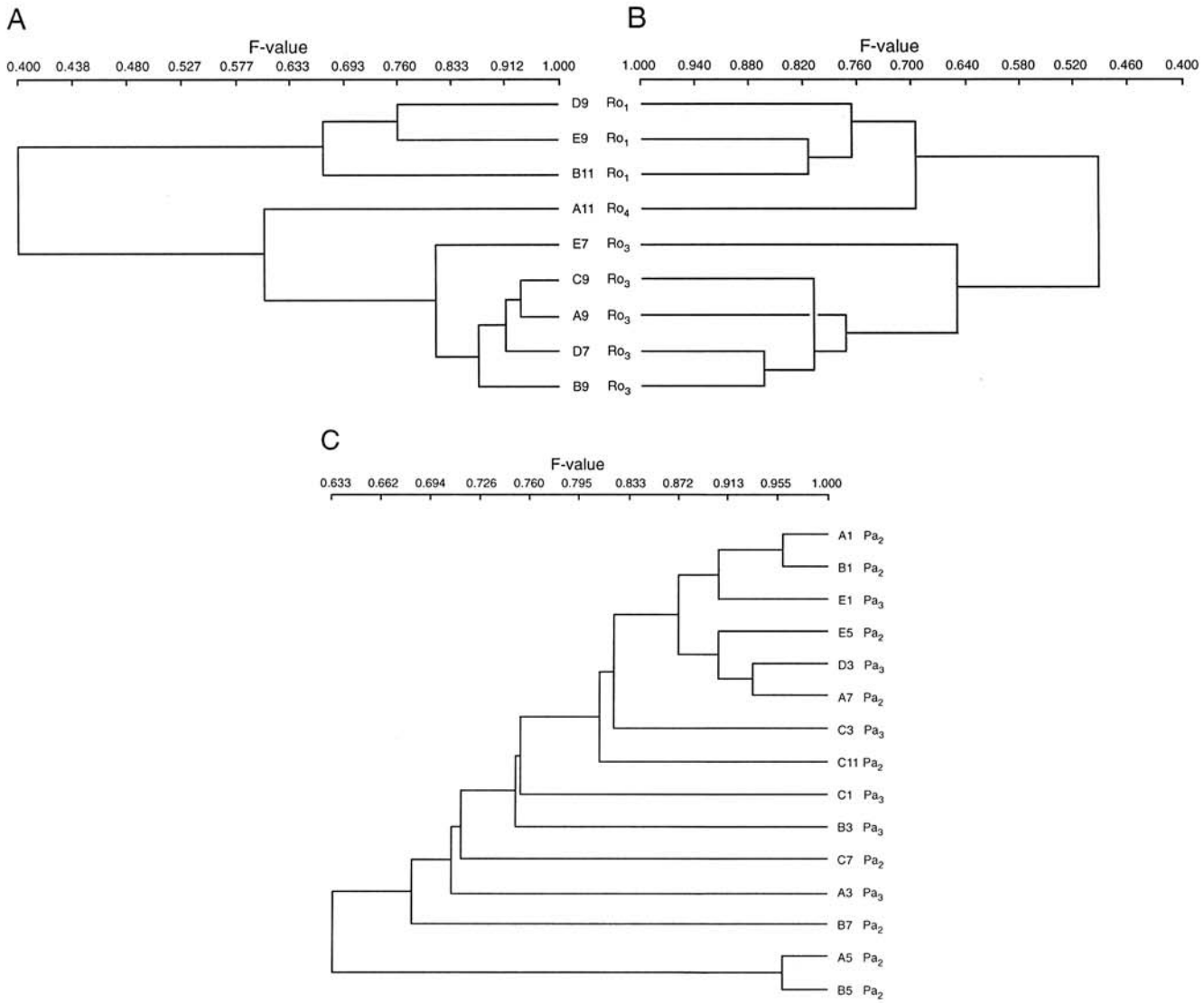

Fig. 2. A, The similarity dendrogram of 9 *Globodera rostochiensis* populations based on presence/absence polymorphisms. **B,** The similarity dendrogram of 9 *Globodera rostochiensis* populations based on intensities of corresponding DNA fragments. **C,** The similarity dendrogram of fifteen *Globodera pallida* populations based on presence/absence polymorphisms.

(15.8%) was low as compared to *G. pallida* (23.0%). This difference in the level of intraspecific variation of both species was also observed for RAPD loci (Folkerstma et al. 1994). Although the proportion of polymorphic DNA fragments for *G. rostochiensis* populations was lower than for *G. pallida*, this smaller proportion allowed a clear clustering of the *G. rostochiensis* populations into three defined groups. A considerable portion of the polymorphic DNA fragments among *G. pallida* populations appeared to be scattered. This hampers an unambiguous classification of the *G. pallida* populations analyzed.

The inability to cluster *G. pallida* populations may indicate a higher level of gene flow among the characterized *G. pallida* populations as compared to the *G. rostochiensis* populations. Potato cyst nematodes have poor dispersal mechanisms and only small proportions of field populations sampled contain cysts of both species (Kort and Bakker 1980; R. T. Folkertsma, unpublished). This indicates that genetic exchange between conspecific populations in the Netherlands is rare. Hence, it seems plausible that most gene flow occurred before introduction of *G. pallida* into the Netherlands.

To differentiate conspecific populations with a high degree of overall similarity Avise (1973) and Ayala (1983) argued that frequency distributions of alleles at polymorphic loci are most effective. This has encouraged us to develop methods for *G. pallida* to identify AFLP loci at which frequencies of corresponding DNA fragments in populations could be determined. Currently, additional primer combinations are being tested to identify such fragments in individuals and populations.

Selection on (a)virulence alleles can not be considered as a driving force for the genetic divergence among the conspecific potato cyst nematode populations analyzed because they were sampled before the growth of resistant cultivars. Currently, potato cultivars resistant against various potato cyst nematode genotypes are grown on a large scale in the Netherlands. Hence, selection will be important for the genetic make-up of potato cyst nematode populations in the near future. As an alternative, these populations can be pathotyped using markers linked to (a)virulence alleles. Pastrik et al. (1995) identified two RAPD markers in a selected *G. pallida* population. It is tempting to speculate that these markers are linked to virulence. However, the abundance of both markers in the selected population may also be caused by random genetic drift. It is therefore also possible that these markers are selectively neutral.

The way to identify markers linked to (a)virulence is to analyze populations derived from controlled matings between virulent and avirulent nematode lines (Bakker et al. 1993). These markers are currently not available for large-scale analyses. Under these circumstances, the highly informative AFLP fingerprinting technique may offer solutions.

The divergence between both sibling species determined by the AFLP analysis is in agreement with previous investigations (Bakker and Bouwman-Smits 1988; De Jong et al. 1989; Folkertsma et al. 1994). Both species share only 64 comigrating AFLP fragments on a total of 1,000, which confirms their extensive differentiation at the molecular level. All comigrating AFLP fragments were monomorphic. It should be noted that both species are morphologically nearly identical and were, until 1970, considered as pathotypes of the species *Heterodera rostochiensis* Woll. (Jones et al. 1970; Stone 1973).

The AFLP technique represents a conceptual and practical advance in DNA fingerprinting. It does not require prior knowledge of DNA sequences and produces an unlimited source of template DNA. From this source, DNA fingerprints are generated with greater resolution and information content than is possible by conventional RAPD and RFLP techniques (Zabeau and Vos 1993).

MATERIALS AND METHODS

Populations.

Samples (> 100 cysts) of nine *G. rostochiensis* populations and 15 *G. pallida* populations from different localities in the Netherlands (Table 1) were surveyed for variation in genomic DNA. The populations were obtained from the Plant Protection Service, Wageningen (population A5, B5, E5, A7, B7, C7, D7, E7, A9, B9, C9, D9, E9, A11, B11), the DLO Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Wageningen (population A1, B1, C1, D1, E1, A3, B3, C3, D3), and the Hilbrands Laboratory, Assen (population C11). The populations collected from heavily infested spots in the field were sampled before the growth of resistant cultivars. Populations were maintained in a greenhouse on potato cultivars susceptible to all pathotypes.

Isolation of genomic DNA.

Prior to DNA extraction, adult white females were reared on the susceptible cultivar *Solanum tuberosum* ssp. *tuberosum* L. 'Eigenheimer' in a growth chamber at 18°C and 16 h day length. DNA samples from approximately 250 mg (fresh weight) adult females were essentially prepared as described by Roosien et al. (1993). The females were homogenized in proteinase K buffer (Sambrook et al. 1989). A single phenol

Table 1. The 9 *Globodera rostochiensis* and 15 *G. pallida* populations used in this study, their pathotype classification and their site of collection in the Netherlands

Code	Species	Pathotype	Location
D7 C295	*G. rostochiensis*	Ro_3	Gramsbergen
E7 G1527	*G. rostochiensis*	Ro_3	Anlo
A9 C286	*G. rostochiensis*	Ro_3	Hoogeveen
B9 C293	*G. rostochiensis*	Ro_3	Hoogeveen
C9 C294	*G. rostochiensis*	Ro_3	Oosterhesselen
D9 A56	*G. rostochiensis*	Ro_1	Bergh
E9 A50	*G. rostochiensis*	Ro_1	Weert
A11 F539	*G. rostochiensis*	Ro_4	Emmen
B11 Mierenbos	*G. rostochiensis*	Ro_1	Wageningen
A1 1095	*G. pallida*	Pa_2	?
B1 P2-22	*G. pallida*	Pa_2	Coevorden
C1 Rookmaker	*G. pallida*	Pa_3	Valthe
E1 1077	*G. pallida*	Pa_3	Anjum
A3 1112	*G. pallida*	Pa_3	Westerbork
B3 74-768-20	*G. pallida*	Pa_3	Sleen
C3 75-884-4	*G. pallida*	Pa_3	Vriezeveen
D3 1097	*G. pallida*	Pa_3	Hardenberg
A5 D383	*G. pallida*	Pa_2	Smilde
B5 D372	*G. pallida*	Pa_2	Anlo
E5 D350	*G. pallida*	Pa_2	Avereest
A7 D353	*G. pallida*	Pa_2	Hardenberg
B7 D354	*G. pallida*	Pa_2	Oosterhesselen
C7 D371	*G. pallida*	Pa_2	Ommen
C11 HPL-1	*G. pallida*	Pa_2	Veendam

extraction was performed to remove fatty components. After an extraction with a 6 M NaCl solution (Roosien et al. 1993), the DNA was precipitated by addition of one volume of isopropanol. The pellet was washed twice with 70% ethanol, dried under vacuum and resuspended in 50 µl TE buffer (pH 7.5) (Sambrook et al. 1989). DNA samples were stored at 4°C.

AFLP procedure.

1. Generation and selection of fragments.

The AFLP procedure was performed following the protocol of Keygene N.V. (Zabeau and Vos 1993). Primary template DNA was prepared in a one-step restriction-ligation reaction. Approximately 50 ng of genomic DNA was digested at 37°C for 1 h, using 10 U of *Eco*RI, 5 U of *Mse*I, and 8 µl of 5× restriction-ligation buffer (50 mM Tris-acetate, 50 mM magnesium acetate, 250 mM potassium acetate, 25 mM DTT, and 0.25 µg/µl BSA) in a final volume of 40 µl. After adding 5 pmol of *Eco*RI-adapter, 50 pmol of *Mse*I-adapter, 0.1 µl of 100 mM ATP, 0.2 U of T4-DNA ligase and 2 µl of 5× restriction-ligation buffer the ligation reaction was performed for 3 h at 37°C in a final volume of 50 µl. The sequence of the *Eco*RI-adapter was:

5′-biotin-CTCGTAGACTGCGTACC
CTGACGCATGGTTAA

The sequence of the *Mse*I-adapter was:

5′-GACGATGAGTCCTGAG
TACTCAGGACTCAT

Dynabeads M-280 streptavidine (Dynal, Oslo, Norway) were used to select biotinylated DNA fragments. Before use, the beads were washed once in 10 volumes STEX (1 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 0.1% (v/v) Triton X-100) and resuspended in 5 volumes of 2× STEX. Per DNA sample 50 µl resuspended beads were used in a final volume of 100 µl. The suspension was incubated for 30 min at room temperature. Gentle agitation was applied to ensure proper binding of biotinylated DNA. The beads were collected with a magnet (Dynal MPC). The supernatant was discarded and the beads were washed in 100 µl of STEX. Resuspended beads were transferred to new reaction vials. The beads were washed twice with 100 µl of STEX and transferred to new tubes again, to prevent adhesion of the beads to the tube wall. After the final wash step, the beads were resuspended in 200 µl of TE buffer (10 mM Tris pH 8.0, 0.1 mM EDTA).

2. Nonselective amplification of template DNA.

Nonselective amplification of secondary template was performed with primers complementary to the core of the adapter sequences. Bead suspension (5 µl) was mixed with 50 ng primer E+0 (5′-GACTGCGTACCAATTC), 50 ng of primer M+0 (5′-GATGAGTCCTGAGTAA), 0.08 U of *Taq* polymerase (Perkin Elmer, USA), 2 µl of 10× PCR-buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin) and 0.8 µl 5 mM dNTPs in a final volume of 20 µl. The PCR reaction was performed in a PE-9600 thermal cycler (Perkin Elmer, Norwalk, USA) using the following temperature profile: 30 s at 94°C; 30 s at 56°C; 60 s at 72°C for 20 cycles. To verify the production of secondary template, 12 µl of the PCR mixture was electrophorized on a 1% agarose gel in TAE buffer stained with 0.5 µg/ml ethidium bromide (Sambrook et al. 1989). The lengths of the most abundant DNA fragments of the secondary template varied between 50 and 200 bp and was observed as a smear.

3. Selective amplification of restriction fragments.

For selective amplification of restriction fragments two sets of primers were used. Set E and set M contain primers which are derived from primer E+0 and M+0, respectively, with additional selective nucleotides at the 3′-end. The code following the E or M refers to the selective nucleotides at the 3′-end (e.g., primer E+GGA refers to a primer from set E with the selective nucleotides GGA at its 3′-end).

One primer was labeled according to the manufacturers' recommendations using 0.2 U of T4-kinase (Pharmacia LKB, Uppsala, Sweden) and 1 µCi ^{33}P-ATP (Isobio, Charleroi, Belgium). Amplification was performed using 5 ng of labeled and 30 ng of unlabeled selective primer in the reaction mixture as described above. The following PCR profile was used: 14 cycles 30 s at 94°C, 30 s at 65°C and 60 s at 72°C. Per cycle, the annealing temperature was decreased by 0.7°C per cycle. The initial cycles were followed by 24 cycles of 30 s at 94°C, 30 s at 56°C and 60 s 72°C.

Reaction products were loaded on a 5% polyacrylamide gel (Sequagel-5, BioZym, Georgia, USA) in 1× TBE electrophoresis buffer (Sambrook et al. 1989), using a Bio-Rad (Richmond, CA) sequence gel system. The gels were dried (Model 583, Bio-Rad), and X-Ray films (Konica, Tokyo, Japan) were exposed at room temperature.

Data analysis.

DNA fingerprints were evaluated visually by inspection of autoradiographs on a bench viewer. Every experiment was at least repeated once, and only DNA fragments consistently present or absent were evaluated.

Among the polymorphic DNA fragments two subsets can be distinguished: presence/absence and band intensity polymorphisms. Presence/absence polymorphisms result from: (i) the disappearance of a restriction site, (ii) insertions or deletions between restriction sites, larger than approximately 50 nucleotides, or (iii) noncomplementarity between the selective nucleotides of the primer and the internal sequences of the restriction fragment. The latter can be illustrated by primer M+AC; in case of a mismatch at the annealing site due to a change from TG to, e.g., TC, amplification of this restriction fragment is prevented. The intensities of the amplified bands will vary according to the proportion of nonmutated individuals within the population analyzed. Nevertheless, only the presence or absence of fragments of similar lengths was scored and, therefore, these polymorphisms are called qualitative polymorphisms.

Alternatively the lengths of the amplified sequences can change due to small insertions and/or deletions. AFLP patterns are complex and, therefore, only changes less than approximately 50 nucleotides could be detected. The central criterion for the identification of these length polymorphisms was the uniformity of the sums of the intensities of the corresponding bands, irrespective of whether the population under

investigation was mono- or polymorphic at that particular locus. These polymorphisms were quantitatively scored by estimating the ratio between the intensities of the corresponding DNA fragments. The sums of the staining intensities of corresponding DNA fragments in a population was defined to be unity. Quantitative polymorphisms might represent putative alleles at one locus.

The presence or absence of a DNA fragment in a population was treated as a binary character. The data were converted to similarity values using the formula $F = 2N_{xy}/(N_x + N_y)$. N_x and N_y refer to the number of DNA fragments generated by the AFLP assay in populations X and Y, respectively, whereas N_{xy} is the number of DNA fragments shared by the two populations (Nei and Li 1979). F-values were used to construct dendrograms with the unweighted pair group method with arithmetic mean (UPGMA) in the Clustan32 VAX-VMS program (Sneath and Sokal 1973). The presence or absence of DNA fragments was scored for all *G. rostochiensis* and *G. pallida* populations.

The intensity of corresponding DNA fragments was scored only for *G. rostochiensis* populations. The similarity among *G. rostochiensis* populations based on the intensity of corresponding DNA fragments was determined using Rogers' similarity index (1984). From the resulting similarity matrix a dendrogram was constructed using UPGMA and the Biosys-I software package (Swofford and Selander 1981).

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