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Cluster analysis of 36 *Globodera pallida* field populations using two sets of molecular markers

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Abstract

Thirty-six populations of the potato cyst nematode *Globodera pallida*, all collected in the Netherlands, were analysed twice: by two-dimensional gel electrophoresis of proteins (2-DGE) and by random amplified polymorphic DNA fingerprinting (RAPD). Two-DGE revealed frequencies of 21 alleles at eight putative loci in each population. The same populations were subjected to RAPD analysis. This qualitative technique revealed 38 polymorphic DNA fragments. Both datasets were independently processed to determine the intraspecific variation. UPGMA analysis resulted in a 2-DGE- and a RAPD-based dendrogram with cophenetic correlation coefficients of 0.755 and 0.838 respectively. The correlation between the genetic similarity values for the populations was 0.572. Comparison between the 2-DGE- and the RAPD-based dendrogram revealed that only thirteen of the 36 populations analysed were clustered identically. It is concluded that the gene pool similarity concept is only in some instances applicable to Dutch populations of *G. pallida*. For populations that could not be differentiated unequivocally on the basis of molecular markers, markers closely linked to avirulence genes should be identified. Approaches that will lead to the identification of such markers are discussed.

Introduction

The potato cyst nematodes *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone originate from the Andean region in South America and were introduced into Europe after 1850 (Evans et al., 1975; Evans and Stone, 1977). Populations of both species are well adapted to the European climatological and environmental circumstances and cause substantial potato crop losses (Oerke et al., 1994).

The growth of potato cultivars with resistance to potato cyst nematodes is potentially an effective and durable means of control (Jones et al., 1981). Breeding for resistance against potato cyst nematodes and efficient application of host plant resistance genes require a reliable determination of virulence characteristics of potato cyst nematode populations. Currently, populations are classified by a pathotype scheme designed by Kort et al. (1977). The scheme recognizes five *G. rostochiensis* (Ro_1-Ro_5) and three *G. pallida* (Pa_1-Pa_3) pathotypes. These pathotypes are defined by their (in)ability to multiply on a range of differential potato clones (Kort et al., 1977). Unfortunately, the pathotype scheme reflects only a part of the variation at (a)virulence loci among potato cyst nematode populations in Europe (Bakker et al., 1993).

In the absence of selection pressure by host resistance genes, both (a)virulence alleles and molecular markers behave as neutral characters. Under this condition, the presence and frequency of (a)virulence alleles and molecular markers (linked or non-linked to (a)virulence alleles) within European potato cyst

nematode populations are determined by three processes: the genetic structures of the primary founder populations, random genetic drift and gene flow (Bakker, 1987; Bakker et al., 1993). Thus, degrees of divergence between populations revealed by molecular techniques should reflect their variation at (a)virulence loci, irrespective of the (non-)linkage between molecular markers and virulence genes. This concept has been formulated as the gene pool similarity concept (Bakker et al., 1993).

The intraspecific molecular variation among potato cyst nematode populations was investigated previously using 2-DGE (Bakker, 1987; Bakker et al., 1992; De Boer et al., 1992), multilocus enzyme electrophoresis (Phillips et al., 1992), RFLPs (Schnick et al., 1990; Burgermeister et al., 1992; Stratford et al., 1992), RAPDs (Folkertsma et al., 1994), PCR of satellite sequences Blok and Philips, 1995) and, recently, AFLPs (Folkertsma et al., 1996). In contrast to most PCR-based techniques, 2-DGE enables the estimation of allele frequencies at putative loci. These data allow an efficient and accurate assessment of gene pool similarities (Bakker, 1987; Bakker et al., 1992). However, the number of discriminating characters among conspecific populations is limited. Obviously, proteins represent expressed genes and constitute only a small fraction of the nematode genomic variation. Using random amplified polymorphic DNAs (RAPDs), a relatively large number of polymorphisms was demonstrated among the nematode populations analysed (Folkertsma et al., 1994). This PCR-based technique amplifies DNA fragments in both coding and non-coding regions of the genome (Williams et al., 1990).

Independent classifications of *G. rostochiensis* populations on the basis of selectively neutral markers (2-DGE, RAPDs) and virulence characteristics resulted in similar groupings (Bakker, 1987; Folkertsma et al., 1994). This example clearly illustrates the usefulness of the gene pool similarity concept. When *G. pallida* is concerned, a sibling species of *G. rostochiensis*, inconsistencies were reported between classifications based on 2-DGE or RAPDs and virulence characteristics (Bakker et al., 1992; Folkertsma et al., 1994). The limited number of differential potato clones was held responsible for the observed dissimilarity. In this report 36 Dutch field populations of *G. pallida* are characterized twice using virtually independent molecular marker techniques, 2-DGE and RAPD-PCR. We will compare both classifications and discuss the implications of the outcome.

Materials and methods

Populations

Samples of 36 populations of *G. pallida* from different localities in the Netherlands, listed in Table 1, were obtained from: the Plant Protection Service, Wageningen (population no. A5, B5, A7, B7, C7 and all populations labelled Ve**), the DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Wageningen (population no. A1, B1, C1, D1, E1, A3, B3), and the Hilbrands Laboratory, Assen (population no. C11). The original samples (> 100 cysts/population) were obtained from heavily infested spots in the field. Populations were maintained in a greenhouse on potato cultivars susceptible to all pathotypes.

Adult white females of each population, required for DNA and protein extraction, were reared on the susceptible cultivar *S. tuberosum* ssp. *tuberosum* L. cv. 'Eigenheimer' in a growth chamber at 18 °C and 16 h daylength. The females were harvested approximately 6 weeks after inoculation. Aliquots of air-dried white females were stored at –80 °C.

Protein sample preparation and mini two-dimensional gel electrophoresis of proteins

Protein samples from a mixture of 50 females (approximately 200 μg fresh weight) per population were prepared as described by Bakker and Bouwman-Smits (1988). The protein samples were either stored at –80 °C or immediately used for electrophoresis. Mini two-dimensional gel electrophoresis of proteins (2-DGE) was essentially performed as described by De Boer et al. (1992). The capillary tubes, used for isoelectric focusing, were cleaned by immersion in ethanol, and washed in deionized, distilled water. Protein samples were applied on top of the focusing gel with a Hamilton syringe (type #705, Hamilton Company, Nevada USA). Focusing was performed without a pre-run and was accomplished with the following voltage schedule: 16.5 h 10 V, 90 min 180 V, 30 min 270 V and 80 min 603 V using a D.C. Buchler Instruments power supply (Chicago, California USA). The focusing of proteins was performed in series of eight populations. In each series, C11 was included as a standard population. After focusing, the gels were extruded in an equilibration buffer containing 62.7 mM Tris HCI (pH 6.8), 2.3% (w/v) SDS and 7.8% (v/v) glycerol. Separation of proteins on the basis of differences in molecular weights was done using two

Table 1. The 36 *G.pallida* populations analysed in this study with their site of collection in the Netherlands

Population-code		Site of collection
A1	1095	?
B1	P2-22	Coevorden
C1	Rookmaker	Valthe
D1	A-75-250-39	Gasselte
A3	1112	Westerbork
B3	74-768-20	Sleen
D3	1097	Hardenberg
A5	D383	Smilde
B5	D372	Anlo
A7	D353	Hardenberg
B7	D354	Oosterhesselen
C7	D371	Ommen
C11	HPL-1	Veendam
VeD1	90-607-12	Klijndijk
VeA4	90-607-10	Valthermond
VeB4	90-607-8	2 Exloërmond
VeC4	90-607-4	1 Exloërmond
VeD4	90-607-6	Exloo
VeE5	90-607-2	Odoorn
VeA8	90-607-14	Odoorn
VeB8	90-227-14	Emmen
VeA11	90-628-2	Zwenderen
VeB11	90-621-2	Beerze
VeD13	90-51-2	Balkbrug
VeB15	90-80-2	Wedde
VeD15	90-394-2	Elim
VeE15	90-167-2	Dalen
VeA16	90-126-2	Eesergroen
VeC17	90-37-18	Gasteren
VeB18	90-78-2	Beilen
VeC19	90-250-2	Gasselternijveen
VeE19	90-768-4	Noord Sleen
VeA20	90-266-2	Gieterveen
VeD22	90-707-2	Rolde
VeA23	90-787-2	Onstwedde
VeE23	90-1013-18	Meppen

Bio-Rad power supplies (Model 1000/500, Bio-Rad Laboratories, Richmond, California USA). Two Mini-Protein II Cells (Bio-Rad) were attached to each power supply. This allowed the simultaneous electrophoresis of eight populations. Silver nitrate, used for gel staining, was purchased from Merck (Darmstadt, FRG).

After staining, the gels were dried on a vacuum dryer (model 543, Bio-Rad) in order to store the gels for a prolonged period of time.

DNA extraction and RAPD PCR

DNA samples from a mixture of approximately 250 mg females, were prepared as described by Roosien et al. (1993). DNA samples were stored at 4 °C. The conditions for the amplification of arbitrary primed DNA fragments were as described by Folkertsma et al. (1994). The decamer oligonucleotides were from the commercially available RAPD primer kit G. Primer numbers OPG2, OPG3, OPG4, OPG5, OPG6, OPG9, OPG10, OPG11, OPG12, OPG13, OPG15, OPG16, OPG17 and OPG19 (Operon Technologies, Alameda, California USA) were used. After amplification the reaction products were separated by electrophoresis in 1.0% agarose gels. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV light with Polaroid 665 film.

Data processing

Protein profiles were evaluated visually by superimposing dried gels on a bench viewer. The molecular weights and iso-electric points of the different iso-electric point variants (IP-variants) were estimated as described by De Boer et al. (1992). The frequencies of the IP-variants (encoding alleles at putative loci) were estimated by the ratios between the protein quantities of the corresponding variants. The sums of the relative protein quantities of the corresponding IP-variants in a population was defined to be unity. For details see elsewhere (Bakker et al., 1992). The 2-DGE data set was analysed using various similarity indices (Nei identity (Nei, 1972), Nei unbiased identity (Nei, 1978), Rogers similarity (Rogers, 1984)) and several cluster algorithms (Unweighted Pair-Group Method with Arithmetic mean (UPGMA), Single linkage and Complete linkage (Sneath and Sokal, 1973)). The clusters of the nine possible combinations were expressed in separate dendrograms using the SAHN procedure in NTSYS-pc Version 1.80 (Rohlf, 1994). The cophenetic correlation coefficient (CCC) was computed to evaluate the quality of the cluster analyses. This product-moment correlation coefficient is a measure for the agreement between the original similarity matrix and the similarity values implied by the dendrogram (Sneath and Sokal, 1973). Rohlf (1994) presents an interpretation of the CCC values for the degree of agreement between both matrices. The dendrogram with the highest CCC value was used for further evaluation.

Using the RAPD data, the intraspecific variability was essentially assessed as described by Folkertsma et al. (1994). The RAPD fragments identified in a set of 17 *G. pallida* populations (Folkertsma et al. 1994) were

also evaluated for the 36 populations in this study. Only polymorphic DNA fragments unambiguously identified in both sets of populations were considered in the determination of the similarity between populations. The RAPD data set was analysed using two similarity indices (the Jaccard coefficient (Jaccard, 1908) and the Dice coefficient (Dice, 1945)) and three cluster algorithms (UPGMA, Single linkage, Complete linkage). The results were visualized in a dendrogram using the SAHN procedure in NTSYS (Rohlf, 1994). The cophenetic correlation coefficient was computed as described above. The dendrogram with the highest CCC value was further evaluated.

To compare the resulting 2-DGE and RAPD similarity matrices the normalized Mantel statistic 'r', which is equivalent to the Pearson product-moment correlation, was computed using the MXCOMP procedure in NTSYS (Rohlf, 1994).

Results

Two-dimensional gel electrophoresis of proteins

Comparison of 36 populations of *G. pallida* with 2-DGE of total protein extracts revealed 21 IP-variants. These were assumed to be encoded by 21 alleles at 8 putative loci (Bakker and Bouwman-Smits, 1988, De Boer et al., 1992). The IP-variant loci indicated with the capitals C, E, F, H, I, J, and M were previously described by De Boer et al. (1992). We were able to identify two additional alleles (M_1 and M_4) at the M-locus and two alleles (N_1 and N_2) at a previously undescribed locus N. In comparison with De Boer et al. (1992) we were unable to resolve allele 4 at locus J and both alleles at locus L unambiguously. In Figure 1, A and B, 2-DGE patterns representing the protein compositions of two different populations, population C11 and D1 respectively, are shown. Comparison between the gels of these two populations revealed 354 invariant protein spots.

The nine different combinations between similarity indices and clustering algorithms resulted in dendrograms with nearly identical topologies (data not shown). The UPGMA dendrogram constructed from the similarity matrix based on Rogers distance had the highest CCC value and was therefore used for further evaluation. The intraspecific similarity based on estimated allele frequency differences between the *G. pallida* populations averaged 0.723 and ranged from 0.495 (between populations D1 and VeC17) to 0.969 (between populations A5 and B5). Clustering of the 36 *G. pallida* populations resulted in a dendrogram with a low CCC: 0.755, indicating poor agreement between the similarity values implied by the dendrogram and those of the original similarity matrix. At a similarity of 0.90, six different clusters representing 13 populations could be identified (Figure 2A).

RAPD fingerprinting

Fourteen RAPD primers resulted in the amplification of 96 different DNA fragments in the 36 *G. pallida* populations. Among the identified RAPD fragments, 38 were found to be polymorphic. Of the 58 polymorphic DNA fragments previously identified among 17 *G. pallida* populations (Folkertsma et al., 1994), 20 could not be reproduced in this study using the same primer set. The number of DNA fragments produced per primer varied from 4 to 8 and ranged in size from 0.30 to 2.78 kb. The six different combinations between similarity coefficient and clustering algorithm resulted in dendrograms with similar topologies (data not shown). The UPGMA dendrogram constructed from a similarity matrix based on the Dice coefficient had the highest CCC value and was therefore used for further evaluation. The estimated similarity among 36 *G. pallida* populations, based on 38 polymorphic DNA fragments, ranged from 0.545, between populations B5 and VeE15, to 1.000 between A5 and B5 (average 0.802). Clustering of the similarity values resulted in a dendrogram with a CCC value of 0.838, slightly higher than the CCC value of the dendrogram based on protein data. At a similarity of 0.90, eight clusters of populations were identified representing 26 populations (Figure 2B).

Comparison between both techniques

The relationship between the estimated similarities based on 2-DGE allele frequency data and on polymorphic RAPD fragments, was evaluated by the determination of the normalized Mantel statistic 'r'. The magnitude of this coefficient was 0.572, assuming a normal distribution of the similarities in both similarity matrices. Despite the low correlation between both datasets, thirteen of the 36 populations analysed are arranged in a similar manner on the dendrogram using both techniques. All populations clustered at a similarity >0.90 in the dendrogram based on 2-DGE are also clustered in the dendrogram based on RAPD data. The identical clusters are shaded in Figure 2A and B.

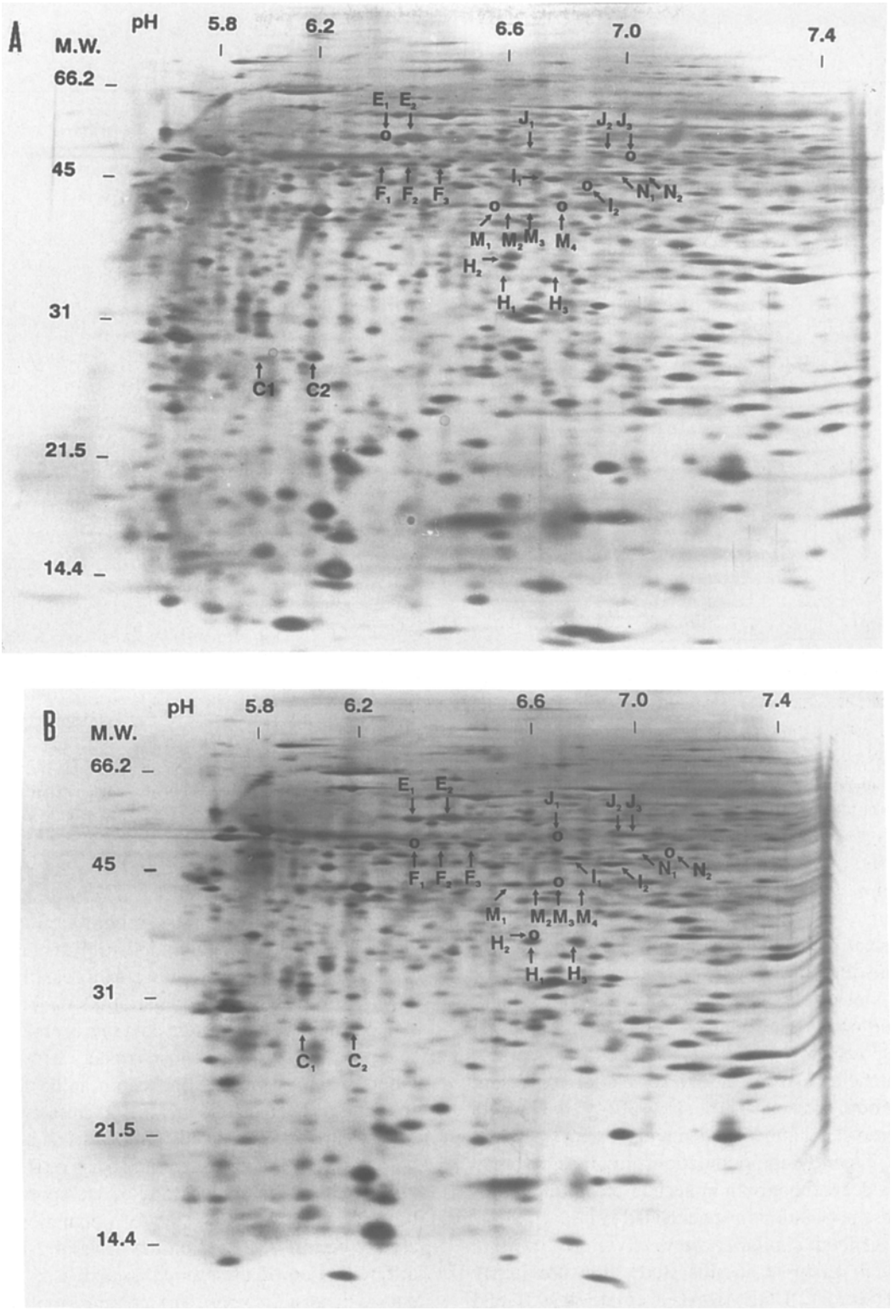

Figure 1. Two-dimensional protein patterns of a mixture of 50 females from population C11 HPL-1 (A) and D1 A75-250-39 (B). IP-variants are indicated as described in the text. Spots marked with arrows indicate IP-variants present in these populations. The open circles indicate the absence of an IP-variant.

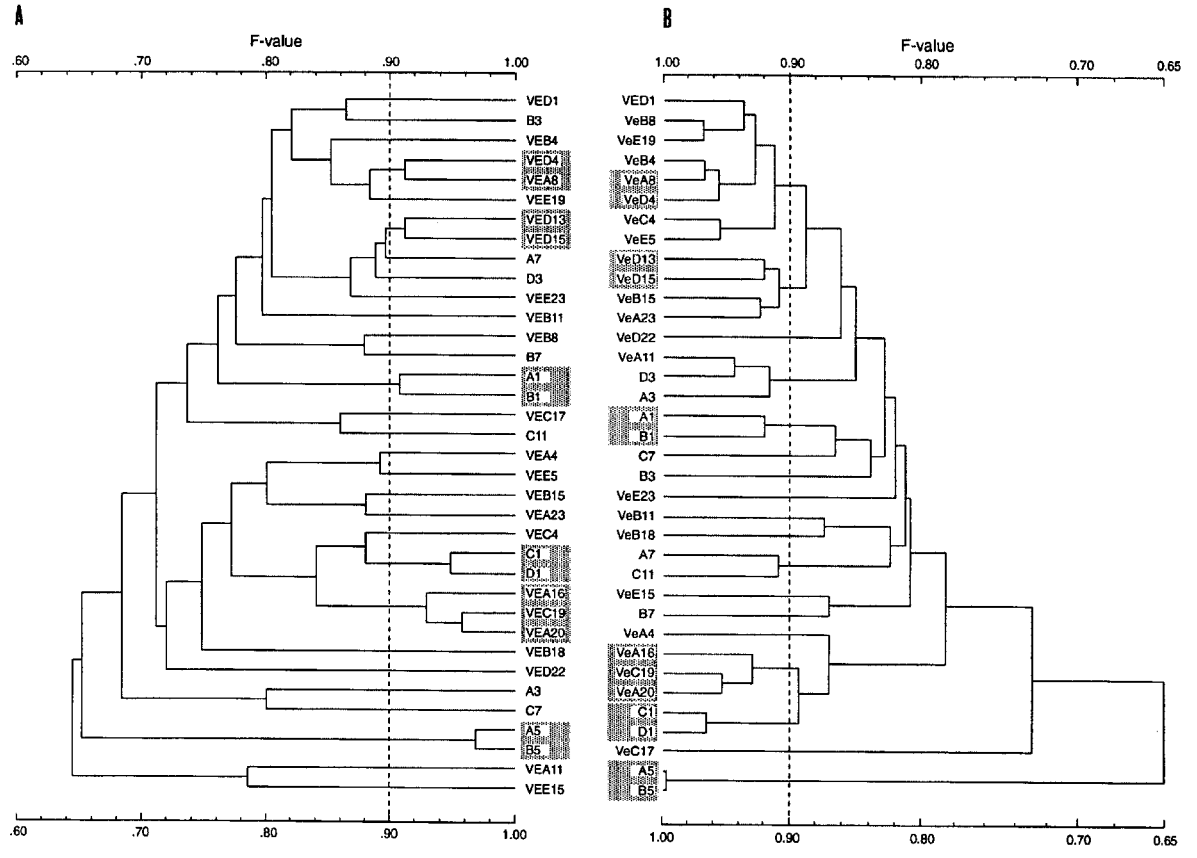

Figure 2. Similarity dendrograms of the 36 *G. pallida* populations based on two-dimensional gel electrophoresis of proteins (A) and random amplified polymorphic DNA (B). Clusters of populations are discriminated at an F-value of 0.90. Shaded boxes indicate populations similar clustered using both data sets.

Discussion

Two virtually independent data sets were used to determine the intraspecific variation among 36 *G. pallida* populations. The two resulting dendrograms, one based on 2-DGE allele frequencies, the other on polymorphic DNA fragments, showed considerable inconsistencies. Despite the low correlation coefficient between both datasets and the low CCC value of both dendrograms, a number of identical clusters was identified. Apparently, the populations involved diverged to such a degree that both molecular techniques identified these populations as phenetically similar.

The genetic similarity between ten *G. pallida* populations analysed in this study was previously assessed using 2-DGE (Bakker et al., 1992) and RAPDs (Folkertsma et al., 1994). Despite differences in the number of loci or polymorphic DNA fragments resolved, respectively, the clustering of extensively diverged populations ((A1 and B1), (C1 and D1) and (A5 and B5)) was identical in both pairs of dendrograms. Differences in the composition of clusters arose when clusters of populations were less clearly differentiated from each other (populations A3, A7, D3, C11). As soon as slightly different data sets are used for cluster analysis this will result in clusters with an altered composition. A similar conclusion can be drawn from the datasets compared in this study. Clusters of populations clearly differentiated on the basis of 2-DGE data will also be differentiated on the basis of RAPD. This will result in similar clustering of such populations in both datasets. When clusters of populations can not clearly be differentiated on the basis of 2-DGE data, differentiation of these populations based on RAPD data will also be poor. The composition of clusters composed of such populations will be dissimilar after analysis of both data sets.

Which of the molecular techniques evaluated in this report is most valuable for the analysis of the intraspecific variation among *G. pallida*? The data presented here do not allow a clear answer. Both molecular techniques identify identical clusters composed of clearly diverged populations. Both techniques, however, fail to show similarities in clustering of populations when these populations have less well diverged. The virtual absence of cluster specific marker loci in *G. pallida*, both at the protein and DNA level, makes the determination of allele frequencies for these populations desirable. Avise (1975) and Ayala (1983) argued that to differentiate conspecific populations with a high degree of overall biochemical similarity, frequency distributions of alleles at polymorphic loci are most significant. Two-DGE enables quantitative evaluation of the genetic variation among populations on the basis of homologous characters (Leigh Brown and Langley, 1979; Bakker and Bouwman-Smits, 1988). Furthermore, Mickevich and Johnson (1976) argued that alleles at a genetic locus are in principle independent characters. However, the number of discriminative alleles resolved by 2-DGE is limited. RAPD-PCR, a qualitative technique (Williams et al., 1993), enables the analysis of the genetic variation among populations by a potentially unlimited number of loci. However, the status of these loci and their reliability for similarity estimates is to be questioned. Comigration of RAPD fragments amplified in different *G. pallida* populations is a weak basis to suppose homology between these fragments (Black, 1993). Without Southern analyses or sequence determinations of the DNA fragments under study, the assumption of homology may be false. The independence between characters of a population obtained with RAPD-PCR may be violated because of primer competition. Wilkerson et al. (1993) showed that the amplification of certain DNA fragments interferes with or precludes the amplification of other bands. This might occur through preferential annealing of the primer to repetitive sequences present on template DNA.

It is noted that the genetic variation between German or Dutch *G. pallida* populations, in terms of the proportion of polymorphic RFLP (Schnick et al., 1990) or RAPD fragments (Folkertsma et al., 1994) is high in comparison to the genetic variation between *G. rostochiensis* populations sampled in these countries (Folkertsma et al., 1996). In contrast to the *G. rostochiensis* populations studied, the substantial variation found among *G. pallida* populations did in general not result in clusters of populations having RAPD markers defining these clusters (Folkertsma et al., 1994). The high number of polymorphic DNA fragments in *G. pallida* appears to be scattered among the populations indicating a high level of gene flow among *G. pallida* populations.

For those *G. pallida* populations that cannot be identified unambiguously using the approach presented in this paper, we suggest the identification of markers closely linked to (a)virulence loci in *G. pallida* populations. In principal two different strategies can be followed to identify markers physically linked to (a)virulence. Analogous to the identification of markers linked to the H1 (a)virulence gene ($avrH_1$) in *G. rostochiensis* (Janssen, 1990; Rouppe van der Voort et al., 1994), 100% avirulent and virulent lines could be developed for *G. pallida*. The progeny of crosses between both lines should be screened for segregating markers. Using this approach, Rouppe van der Voort et al. (1994) have identified numerous RAPD markers linked to $avrH_1$. Alternatively, *G. pallida* field populations could be selected on resistant cultivars (Pastrik et al., 1995). Screening for differences between the selected and unselected populations, may lead to the identification of markers linked to (a)virulence. Pastrik et al. (1995) identified two RAPD markers that were preferentially amplified in the selected population. Crucial for both approaches is the choice of the population to be selected and the potato genotype to select on.

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