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Comparison of molecular patterns and virulence behaviour of potato cyst nematodes

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Summary - Seven populations of potato cyst nematode belonging to *Globodera rostochiensis* and *G. pallida* were analyzed by means of RAPD markers, microsatellite primed PCR and 2-D gel electrophoresis of total proteins. A high degree of polymorphism was detected in all types of markers. Furthermore, virulence reactions of the nematode populations on a set of differential potato genotypes were recorded. Significant population and host effects as well as a highly significant interaction between these two variables were observed. Genetic distances between nematode populations based on molecular data and virulence reactions were computed. Cluster analyses were performed and the resulting distance matrices compared. A good consistency between the different dendrograms and highly significant correlation coefficients were observed when comparing the distance matrices derived from molecular data and virulence reactions. Practical applications of such molecular analyses to the study of nematode populations could provide indirect virulence testing and therefore recommendations of the appropriate potato cultivars for cultivation in infested soils.

Résumé - ***Comparaison entre profils moléculaires et comportement de virulence des nématodes à kyste de la pomme de terre*** - Sept populations de nématodes à kyste de la pomme de terre (*Globodera rostochiensis* et *G. pallida*) ont été analysées au moyen de marqueurs provenant de fragments d'ADN amplifiés au hasard (RAPD), d'amorces de microsatellites amplifiés en chaîne par réaction de polymérase (PCR) et d'électrophorèse de protéines totales en deux dimensions sur gel. Un degré élevé de polymorphisme a été détecté chez tous les types de marqueurs. De plus, des réactions de virulence des populations de nématodes ont été estimées sur une série de génotypes différentiels de pomme de terre. Des effets significatifs de la population et de l'hôte, de même qu'une interaction hautement significative entre ces deux variables, ont été observés. Les distances génétiques entre populations de nématodes, fondées sur les données moléculaires et les réaction de virulence ont été estimées. Des analyses en grappes ont été réalisées et les matrices de distance en résultant comparées. Une bonne consistance entre les différents dendrogrammes, de même que des coefficients de corrélation hautement significatifs ont été observés lorsque l'on a comparé les matrices de distance dérivées des données moléculaires, et les réactions de virulence. L'application pratique de telles analyses moléculaires à l'étude des populations de nématodes permettrait d'obtenir des tests indirects de virulence et donc de pouvoir recommander les cultivars appropriés de pomme de terre dans le cas de sols infestés.

Key-words: 2-DGE, genetic distance, *Globodera*, microsatellite primed PCR, nematodes, potato, RAPD, virulence.

Potato cyst nematodes (PCN) are considered to form a genetic complex with two sibling species, *Globodera rostochiensis* and *G. pallida* (Sturhan, 1985). Both species contain subspecific categories which are related to virulence (Dropkin, 1988). Such categories are distinguished on their ability to reproduce on a set of differential potato clones or cultivars, which implies that virulence genes in the parasite match to resistance genes in the host. So far, H1, K1, Fa-Fb, H2 and H3 genes in potato have been reported to be involved in the resistance mechanisms (Phillips, 1994). H1 and H2 are single dominant genes and related to recessive virulence counterparts in a gene-for-gene relationship in *G. rostochiensis* and *G. pallida*, respectively (Parrot, 1981; Janssen *et al.*, 1991). These two genes confer resistance against Ro1/Ro4 (H1) and Pa1 (H2) pathotypes following the Kort's international scheme (Kort *et al.*, 1977). The other pathotypes (Ro2, Ro3, Ro5, Pa2, Pa3) are called "virulence groups" (Trudgill, 1985) or "virulence phenotypes" (Sturhan, 1985). Both, the European and Andean pathotyping schemes of Kort *et al.* (1977) and Canto Saenz and Mayer de Scurrah (1977) apparently do not reflect precisely the genetic variability in the PCN population complex (Bakker *et al.*, 1992).

Molecular markers for resistance genes against *G. rostochiensis* have been described in potato (Gebhardt *et al.*, 1993; Pineda *et al.*, 1993; Niewohner *et al.*, 1995). Isozymes, 2-DGE of proteins, RFLP and RAPDs have been reported as efficient tools for population grouping (Bakker *et al.*, 1992; Burgermeister *et al.*, 1992; Phillips *et al.*, 1992; Folkertsma *et al.*, 1994).

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In the present work, we attempt to assess the possible relationships between molecular patterns and virulence characteristics of PCN populations.

Material and methods

PCN POPULATIONS

Seven different PCN isolates were used for molecular analyses and virulence assays. They include five *G. rostochiensis* populations (Lagran, Angostina, Xinzo, Valencia of the virulence group Ro1, and a Ro5 reference population) and two populations of *G. pallida* (Mataró and the reference population German). Reference populations were made available by SCRI (Dundee, UK) while the other populations were obtained from the CIT-INIA collection (Madrid, Spain) and from our own field work. PCN populations were multiplied in a growth chamber (constant temperature of 18°C, 16 h light per day) in 12 cm clay pots on the potato cv. Desirée and harvested after 3 months.

DNA EXTRACTION

For PCR analysis total genomic DNA was extracted from approximately 200 cysts of each nematode population. The cysts were crushed in 500 µl extraction buffer (10 mM tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 50 mM NaCl and 10 mM ß-mercaptoethanol). The extract was transferred to 1.5 ml microtubes and SDS was added to a final concentration of 0.6%. The mixture was incubated at 65°C for 15 min. After adding 50 µl 3 M potassium acetate and refrigeration at 0°C for 10 min, the homogenate was centrifuged at 13 000 rpm for 20 min at 4°C. After addition of 285 µl of isopropanol the tubes were stored 30 min at -20°C for DNA precipitation. The pellet was dissolved in TE and extracted once with phenol:chloroform:isoamylalcohol (25:24:1), precipitated with ethanol and dissolved in double distilled water. Finally the DNA was quantified in a UV spectrophotometer.

DNA AMPLIFICATION AND GEL ELECTROPHORESIS

All PCR reaction mixtures had a total volume of 50 µl. The mixtures contained 0.6 units of Taq DNA

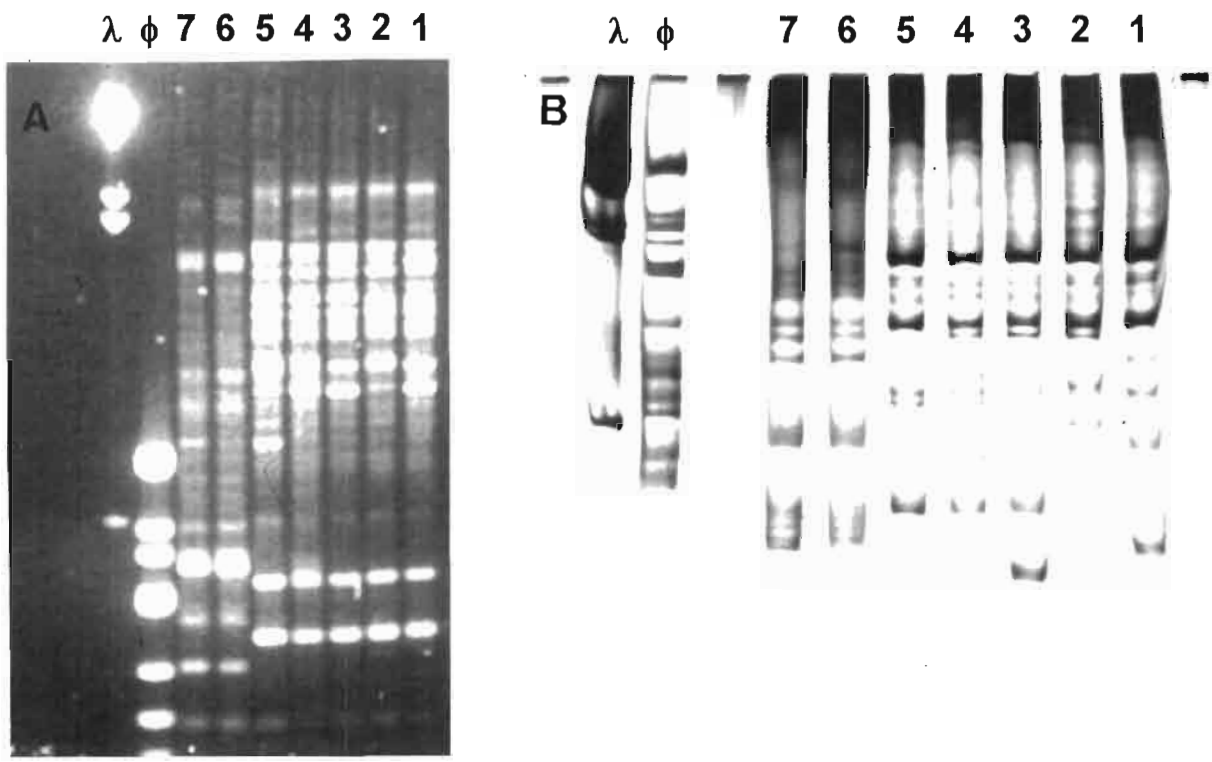

Fig. 1. *Molecular patterns of seven PCN populations. A: RAPD patterns obtained with primer OP-G10; B: Amplified fragments obtained with microsatellite-complementary primer* $(AGG)_5$. *DNA size markers: lane λ, (HindIII-digested λ DNA), lane ϕ (X174 digested with HinfI). PCN populations: 1=Lagran, 2=Angostina, 3=Xinzo, 4=Valencia, 5=Ro5, 6=Mataró, 7=German.*

Polymerase (Sphaero Q, Leiden, The Netherlands), 50 ng primer, 200 µM dNTPs (Pharmacia LKB), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and approx. 25 ng of template DNA. Reaction mixtures were overlayed with 50 µl mineral oil, before being placed in a Linus Autocycler plus FTS-1.

A total of eleven 10-mer primers of arbitrary sequence (Operon Technologies, Alameda, CA, USA) were used for PCR amplification to produce RAPDs (random amplified polymorphic DNAs; Williams *et al.*, 1990). The PCR programme had an initial cycle of 4 min at 94°C, followed by 45 cycles of 1 min at 94°C, 2 min at 38°C, and 3 min at 72°C. A final elongation step of 10 min at 72°C followed. Amplification products (15 µl) were loaded on a 1.5% agarose gel and stained with ethidium bromide after electrophoresis, using standard methodology (Sambrook *et al.*, 1989).

In addition, the following seven microsatellite-complementary primers (MS-primers) $(AGC)_5$, $(GTG)_5$, $(CAG)_5$, $(CCA)_8$ $(AGG)_5$, $(GTG)_5$, $(CA)_5$ (Weising *et al.*, 1995) were used to amplify repetitive sequences. The amplification conditions consisted of one initial cycle of 4 min at 94°C, followed by 45 cycles –1 min at 94°C, 1 min annealing at temperatures calculated according to the Wallace rule of Weising *et al.* (1995), 2 min at 72°C –, and a final elongation step of 3 min at 72°C. Finally, 10 µl of the amplification products were loaded on a acrylamide/bisacrylamide (29:1) gel (8%, TBE 1X), separated by electrophoresis and detected by silver staining.

Protein analysis

For protein analysis on two-dimensional polyacrylamide gels (2-DGE), total proteins were extracted according to Bossis and Mugniéry (1993) but using cysts instead of white females. 2-DGE was performed following the protocol of Bossis and Mugniéry (1993) with slight modifications and three replications per population. Bisacrylamide instead of PDS was used at the same concentration in IEF and SDS gels. The voltage schedule in the first dimension run was: 17 h - 10 V; 1 h 30 min - 180 V; 0 h 30 min - 270 V; 1 h 20 min - 600 V. Proteins were stained with ammoniacal silver solution (Oakley *et al.*, 1980), using the protocol of De Boer *et al.* (1992), as modified by Bossis and Mugniéry (1993).

Table 1. *Observed polymorphisms among seven PCN populations obtained with different types of molecular markers.*

Number	Primer code	Primer sequence	Total number of markers	Number of polymorphic markers	Number of patterns
RAPDs					
1	OP-G3	GAGCCCTCCA	26	25	6
2	OP-G4	AGCGTGTCTG	18	18	7
3	OP-G5	CTGAGACGGA	17	14	7
4	OP-G8	TCACGTCCAC	17	14	7
5	OP-G9	CTGACGTCAC	22	22	7
6	OP-G10	AGGGCCGTCT	28	26	6
7	OP-G11	TGCCCGTCGT	25	23	7
8	OP-G13	CTCTCCGCCA	19	19	5
9	OP-G15	ACTGGGACTC	22	20	7
10	OP-G16	AGCGTCCTCC	36	31	7
11	OP-G17	ACGACCGACA	36	33	7
Mean			24.2	22.3	6.6
Sum			266	245	
MS-primers					
1		$(GTG)_5$	17	16	5
2		$(AGG)_5$	23	23	7
3		$(CCA)_6$	25	23	7
4		$(CAG)_5$	20	16	3
Mean			21.3	19.5	5.5
Sum			85	78	
Proteins			>250	34	7

Table 2. *Multiplication of different PCN populations on a set of differential potato genotypes in the virulence assays.*

Differential potato genotypes	PCN Populations							Mean Value
	Angostina	Lagran	Xinzo	Valencia	Ro 5	German	Mataro	
Average number of cysts per pot								
CLON12380	0.3	0.0	0.0	0.0	0.8	16.3	13.0	4.33
CORSAIR	2.3	4.6	13.2	11.8	43.0	99.0	117.8	41.66
CROMWELL	1.0	0.0	2.6	0.8	241.4	295.4	312.3	121.91
MORAG	1.5	5.8	49.2	35.5	97.0	171.8	365.0	103.69
VANTAGE	3.0	4.8	55.3	13.3	81.3	64.7	420.5	91.82
DESIREE	491.0	826.0	1073.4	590.4	942.0	821.6	1425.2	881.37
Mean value	83.17	140.20	198.94	108.61	234.23	244.80	442.29	207.46
Relative Multiplication Rates (Desirée =100%)								
CLON12380	0.05	0.00	0.00	0.00	0.08	1.98	0.91	0.43
CORSAIR	0.45	0.55	1.22	1.99	4.56	12.05	8.26	4.16
CROMWELL	0.20	0.00	0.24	0.12	25.64	35.97	22.11	12.04
MORAG	0.28	0.70	4.40	6.01	10.41	20.92	25.62	9.76
VANTAGE	0.61	0.58	5.19	2.24	8.62	7.87	29.51	7.80
Mean value	0.32	0.37	2.21	2.07	9.86	15.76	17.28	6.84
Relative Multiplication Indices								
CLON12380	11.6	0.0	0.0	0.0	18.0	459.6	210.9	100
CORSAIR	10.8	13.3	29.5	47.8	109.7	290.1	198.8	100
CROMWELL	1.7	0.0	2.0	1.0	213.0	298.8	183.6	100
MORAG	2.8	7.2	45.0	61.6	106.7	214.3	262.5	100
VANTAGE	7.8	7.4	66.5	28.7	110.5	100.9	378.2	100
Mean value	6.93	5.57	28.59	27.82	111.56	272.74	246.80	100

Virulence essay

The seven PCN populations mentioned above were multiplied on a set of five differential potato cultivars with varying general susceptibility to PCN (Corsair, Cromwell, Morag, Vantage, Desirée) and on the differential potato clone (Clone 12380 provided by M.S. Phillips). The virulence assays were performed in a growth chamber under the above-mentioned conditions with five replications per each combination of population and differential potato genotype. The initial inoculum consisted of twenty viable juveniles per gram of soil. The resulting cysts were harvested after 3 months and counted to calculate the final infestation (Pf values).

Data Analysis

The presence and absence of each amplified PCR fragment on the gels was visually scored for each population. For 2-DGE analysis, the software programm "2-D Analyzer" (Version 6.03, BioImage) was used on a Sun Workstation to compare presence and absence of the numerous protein spots present on each of the gels corresponding to the different PCN populations. Only the best of the three repetitions was processed for each population.

Data were collated and analysed on an IBM compatible PC with in-house developed software. The NTsys-PC program (Rohlf, 1993) was used to calculate genetic distances, for cluster analysis, and for comparison of distance matrices. SAS software was employed to perform analyses of variances.

For all molecular marker types, similarity coefficients (SC) between the seven PCN populations were calculated according to Jaccard (1908) and transformed into dissimilarities using its complement (1-SC). In the virulence assays, multiplication rates relative to the propagation of the PCN populations on the cv. Desirée (=100%) were computed (Table 2) in order to minimize distortions due to highly dispersed absolute multiplication values. From these data, relative multiplication indices were calculated, expressed as the multiplication of a PCN population relative to the average propagation of all populations on each differential potato genotype (=100%). Analyses of variances were performed after appropriate transforma-

Table 3. *Characteristics of distance values among PCN populations calculated from molecular patterns and from virulence behaviour and correlation between the obtained distance matrices.*

Derived from	Characteristics of distance values *	C.V.	Correlation coefficients between distance matrices				
			Proteins	RAPDs	Microsat. primed PCR	Rel. Multiplic. Rates	Rel. Multiplic. Indices
Proteins	0.270(0.111-0.419)	34.83		0.894	0.868	0.700	0.800
RAPDs	0.569(0.234-0.829)	34.84		----	0.973	0.779	0.860
Microsattelite primed PCR	0.582(0.176-0.917)	50.74				0.868	0.907
Rel. Multiplication Rates	26.050(0.480-44.940)	59.38				----	0.922
Rel. Multiplication Indices	349.600(12.72-654.800)	63.90					
Average Correlation	------	------	0.816	0.877	0.904	0.817	0.872

* Distances based on dissimilarity coefficients expressed as Mean values, Range (in brackets) and Coefficients of variation (C.V.%). Distances were derived from presence and absence of protein spots from 2-DGE, RAPDs and amplification products from microsatellite primed PCR, or based on Euclidean distances derived from multiplication values in the virulence assays (see text for details).

tions with these different types of multiplication values, and genetic distances using Euclidean distances were computed between the seven PCN populations.

Cluster analysis was performed based on the dissimilarity coefficients from molecular data or Euclidean distances from multiplication values and using UPGMA as clustering method. In each case, a cophenetic matrix was computed from the tree matrix and compared to the original similarity matrix in order to measure the goodness of fit (Rohlf, 1972).

Finally, distance matrices obtained from molecular marker analyses and from the virulence assays were compared by performing Mantel (1967) correlation tests between each pair of matrices.

Results

The eleven different 10-mer primers used in the analysis revealed a total of 266 different RAPD fragments, of which 245 were polymorphic. Only reproducible and well marked bands were considered further, while faint bands were ignored. An example of polymorphic RAPD patterns obtained with primer OP-G10 is shown in Fig. 1A. In our set of populations, each of the tested primers revealed from 17 to 36 different RAPD fragments. Eight of the eleven primers generated RAPD patterns which clearly distinguished between all nematode populations used in this assay.

In microsatellite primed PCR analysis, only the four MS primers listed in Table 1 provided repetitive banding patterns and revealed 78 polymorphic bands from a total of 85 amplified bands (Fig. 1B). The number of polymorphic DNA fragments ranged from 17 to 25 with an average of 21.3 fragments per primer.

Over 250 reproducible protein spots, including minor and faint spots, were detected on the two-dimensional gels. Only 34 clearly marked spots were scored for the different PCN populations. An example of a two dimensional protein gel is shown in Fig. 2. Table 1 summarizes the polymorphisms observed between the seven PCN populations.

The range of the different distance values between PCN populations, the corresponding mean distance, and the coefficient of variation are indicated in Table 3 for each marker type. Cophenetic matrices always showed a significant correlation ranging from 0.943 to 0.994. The results of the cluster analyses are presented in Fig. 3. In all dendrograms, the *G. pallida* populations are clearly separated from the *G. rostochiensis* populations.

The results of the virulence assays are presented in Table 2. The overall average final infestation (*Pf*) was 207 cysts per pot; in the cv. Desirée, which was by far the most susceptible, *Pf* ranged from 491 to 1425 cysts per pot. Average *Pf* values were the lowest for the nematode population Angostina and were the highest in both *G. pallida* populations. The transformation of *Pf* values into relative multiplication values led to some differences in the ranking of the individual and in the mean values (particularly for relative multiplica-

tion indices), as seen in Table 2. Analyses of variance showed in all cases a highly significant effect on the PCN populations (except for relative multiplication indices) and on the differential potato genotypes. A highly significant interaction between these two variables, *Pf* and relative multiplication value, was observed in each case. In the dendrograms derived from Euclidean distances (based on relative multiplication values), a clustering similar to the dendrograms derived from molecular markers (based on genetic distances), particularly PCR markers, was observed (Fig. 3).

A higly significant Mantel correlation coefficient was obtained for comparisons between each pair of distance matrices (Table 3). The highest correlation coefficient is that between the two types of PCR markers. Values between protein and DNA markers were marginally lower. The lowest values were obtained when comparing distances derived from relative multiplication rates with those derived from molecular markers. However, they increased substantially when considering relative multiplication indices.

Discussion

Virulence behaviour of the studied PCN populations showed a large variability and highly significant population × host interactions in good agreement with previous reports (Phillips & McNicol, 1986; Schouten, 1994; Seinhorst, 1995). Standardization and performance of the assays under controlled conditions, as in the present work, is necessary since they might also be influenced by enviromental factors such as site, season, etc. (Phillips, 1985; Phillips *et al.*, 1989 *a*, *b*, *c*; Salazar, 1991). The observed host-parasite interactions suggest that the present classification scheme of PCN species and pathotypes needs further and more accurate differentiation. Molecular markers could offer the basis for such an approach since in our assay as well as in previous studies (Burgermeister *et al.*, 1992; Phillips *et al.*, 1992; Folkerstma *et al.*, 1994) a high degree of polymorphism among PCN populations has been detected at the DNA and protein level. Such polymorphism was useful even for genotyping individual populations. The observed variability and the fact that molecular differences can be found between populations previously identified as belonging to the same pathotype (Bakker *et al.*, 1993), suggest the use of PCN populations instead of artificial entities (namely the pure pathotypes from collections) for PCN identification, resistance screening and potato breeding, as proposed previously by Dropkin (1988).

Genetic distances have been estimated between individual populations derived from Jaccard's similarity coefficient based on amplified DNA fragments and proteins. According to Link *et al.* (1995) this coefficient is appropriate for the dominant marker types used in our molecular assays.

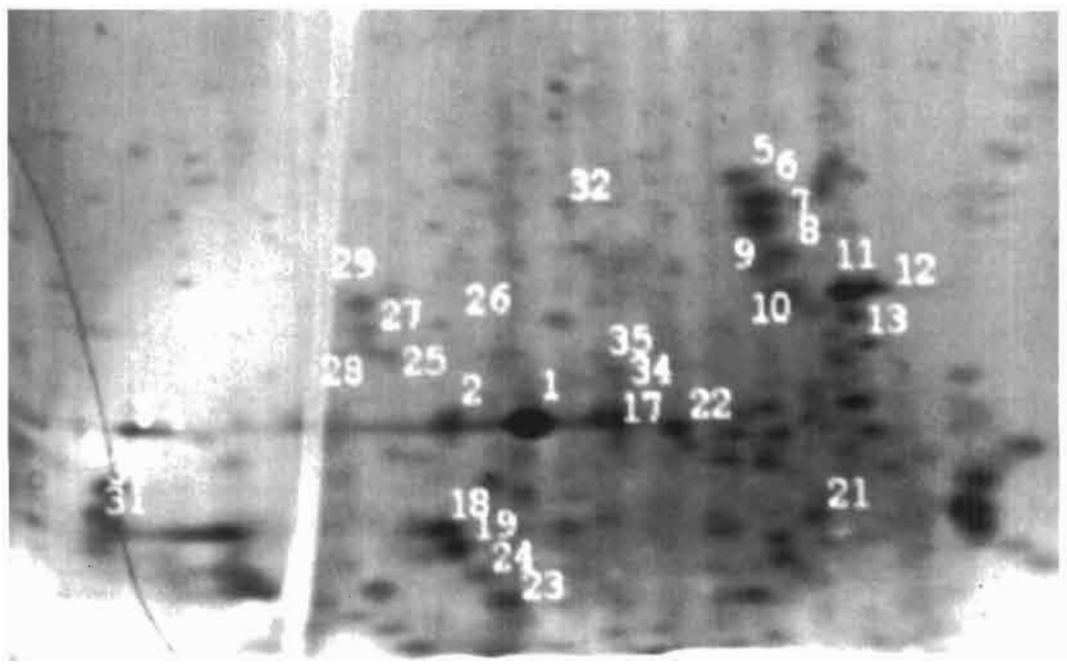

Fig. 2. *Two-dimensional protein gel of population Angostina.*

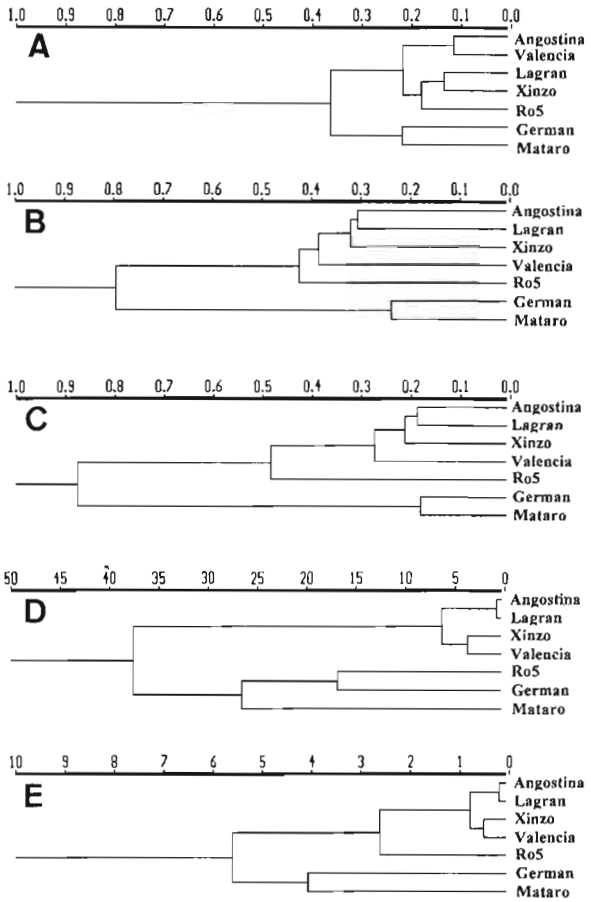

Fig. 3. *Dendrograms of seven PCN populations derived from molecular markers. (A: Protein markers; B: RAPD markers; C: Microsatellite primed PCR markers) based on dissimilarity coefficients and the UPGMA clustering method and derived from Euclidean distances based on multiplication values in a virulence assay (D: Relative multiplication rates; E: Relative multiplication indices; see text for details on calculating these values).*

Certain relationships observed in the dendrogram can be explained by the origin of the PCN populations. The populations Angostina and Lagran, both from northern Spain and belonging to *G. rostochiensis*, also cluster together in most dendrograms, whereas the Ro5 population from a British collection clusters mostly apart.

Problems associated with the use of RAPD markers, such low reproducibility of the results or lack of sequence homology between equal-sized markers, are well known and have been summarized by Black (1993). Reproducibility and specifity can be increased by the use of larger primers as in the case of MS primers. Comparison of the dendrograms based on genetic distances revealed some smaller differences in the cluster formation, but it is documented that clustering also depends on the particular method used (Hubalek, 1982). Nevertheless, when comparing the correlation between the distance matrices from the different marker types, highly significant correlation coefficients were obtained. Therefore RAPDs as well as microsatellite primed PCR seem to represent valuable tools for this kind of analysis. The somewhat lower correlation values between the DNA markers and the protein data could result from the limited number of scored protein spots

From the multiplication values of the virulence assay and the different molecular marker types, a good coincidence of genetic distances could be observed when looking at the corresponding dendrograms (Fig. 3). This is particularly true when considering the correlation coefficients (Table 3), the correlation between genetic distances based on molecular patterns and virulence behaviour (which is even higher when considering relative multiplication indices). This seemingly surprising finding might result from linkage between markers and minor virulence genes in the PCN populations under study. Correlation coefficients may be influenced by the heterogeneous structure of the choosen PCN populations which include three main groups: four populations of *G. rostochiensis* Ro1 (representing quite different regions of Spain), one Ro5 population and two populations of *G. pallida*. However, a separate analysis based only on the five *G. rostochiensis* populations gave similar correlation coefficients ranging from 0.72 to 0.95, except for the correlations involving protein data where only a reduced number of data values were left after removal the *G. pallida* populations from the analysis.

A potential application of this finding is that the traditional testing for determining virulence of PCN populations could be replaced by molecular analysis, since nematode populations with similar molecular-patterns are expected to show a similar virulence behaviour against determined cultivars. Additionally, by knowing the molecular characteristics of a particular nematode population, it should be possible to make a recommendation on which cultivar should be cropped to reduce efficiently the multiplication rates of nematodes in infested soils.

Nevertheless, only a limited number of PCN populations and differential potato genotypes have been assayed in this study and it will be necessary to verify these findings within a larger set of objects.

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