Inter- and Intraspecific Variation Between Populations of *Globodera rostochiensis* and *G. pallida* Revealed by Random Amplified Polymorphic DNA

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ABSTRACT

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The genetic relationships between populations of the potato cyst nematode species *Globodera rostochiensis* and *G. pallida* were analyzed using random amplified polymorphic DNA (RAPD). Only nine of 250 amplified DNA fragments were common to both species. The intraspecific variation was small. The proportions of shared DNA fragments among *G. rostochiensis* populations ranged from 0.870 to 0.967 and those for *G. pallida* populations, from 0.829 to 1.000. Unweighted pair group method with arithmetic mean analysis of RAPD data showed that *G. rostochiensis* populations cluster in groups with similar pathotype designations. No similarity between RAPD data and pathotype classifications of the *G. pallida* populations was found. The origin of the observed inter- and intraspecific variation and the value of the RAPD technique to determine these variations are discussed.

Additional keywords: molecular evolution, morphological evolution, population genetics, sibling species.

The potato cyst nematodes *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone are successful colonizers in temperate zones of the world (16). Both sibling species originate from the Andean region of South America and were probably introduced into Europe after 1850 (15). From the sites of introduction, primary founders dispersed mainly by passive spread (15).

Control is based on crop rotation, soil disinfection, and the use of resistant potato cultivars. In Europe, a wide application of resistant cultivars is hampered by the occurrence of various virulent populations. Populations of both potato cyst nematode species are classified into pathotypes (24). These are defined by their multiplication rates on a standard set of differentials with resistance derived from *Solanum tuberosum* subsp. *andigena* Juz. & Buk. CPC 1673, *S. kurtzianum* Bitt. & Wittm. hybrid 60.21.19, *S. multidissectum* Hawkes hybrid P55/7, and several *S. vernei* Bitt. & Wittm. accessions.

Currently, eight pathotypes are recognized in Europe by this international pathotype scheme, five within *G. rostochiensis* (Ro_1–Ro_5) and three within *G. pallida* (Pa_1–Pa_3) (24). The international pathotype scheme is not capable of revealing the genetic diversity that has been introduced into Europe (4). Additional pathotypes were recognized by using extra differentials (9). Other drawbacks of the scheme are the laborious way virulence characteristics of populations are determined, the arbitrary way pathotypes are delineated, and the rather variable expression of the nematode and host genotypes, which is influenced by various environmental factors (4,12,28).

Differences in proteins between *G. rostochiensis* and *G. pallida* were analyzed with a variety of electrophoretic techniques (3,18). The discrimination between the sibling species is currently routine practice, with a serological assay using monoclonal antibodies raised against thermostable species-specific proteins (33). Also on the DNA level, molecular differences were demonstrated by a number of approaches (e.g., 6,7,13,30).

The intraspecific genetic variation of both species was assessed using isoelectric focusing (17) and two-dimensional gel electrophoresis (2,4) of proteins and isozyme analysis (39). These techniques, however, resulted in a limited number of discriminating characters. Also, restriction fragment length polymorphisms (RFLPs) were used to trace genetic differences between conspecific potato cyst nematode populations (29,32,35). These techniques, however, are laborious and require a substantial amount of DNA, which hampers genetic analysis of populations on a large scale.

This report evaluates the application of random amplified polymorphic DNA (RAPD) to assess inter- and intraspecific variation of potato cyst nematodes. The RAPD assay uses single random primers to amplify DNA fragments from minute amounts of template DNA with the polymerase chain reaction (PCR), resulting in DNA fingerprints of a species or population (36,37).

MATERIALS AND METHODS

Populations. Samples (>100 cysts) of nine populations of *G. rostochiensis* and 17 of *G. pallida* were collected from heavily infested spots in the field in different localities in the Netherlands (Table 1) and surveyed for variation in genomic DNA. Populations A5, B5, C5, E5, A7, B7, C7, D7, E7, A9, B9, C9, D9, E9, A11, and B11 were collected by the Plant Protection Service, Wageningen; populations A1, B1, C1, D1, E1, A3, B3, C3, and D3 by the DLO-Centre for Plant Breeding and Reproduction Research, Wageningen; and population C11 by the Hilbrands Laboratory, Assen. Populations were maintained in a greenhouse on potato cultivars susceptible to all pathotypes.

DNA extraction. Adult white females were reared on the susceptible *S. tuberosum* subsp. *tuberosum* L. cultivar Eigenheimer in a growth chamber at 18 C and 16-h day length. They were harvested approximately 6 wk after inoculation, and aliquots of

250 mg of air-dried females were stored at −80 C. No deterioration was observed at this temperature after 3 yr. Genomic DNA was isolated according to De Jong et al (13), with some modifications. A single phenol extraction was performed after proteinase K digestion. The subsequent phenol and chloroform extractions were replaced by an extraction with a saturated 6 M NaCl solution. RNA was removed by incubation with 20 μg of RNase A at 37 C for 30 min. The DNA concentration was estimated with a fluorometer (TKO 100, Hoefer Scientific Instruments, San Francisco, CA) and use of a fluorescent dye (No. 33258, Hoechst-Roussel Pharmaceuticals, Somerville, NJ). At this stage, the DNA was sufficiently pure for amplification experiments.

RAPD PCR. The lyophilized oligonucleotide decamer primers (Operon Technologies, Alameda, CA), were resuspended in TE buffer, pH 7.2, (31) to a final concentration of 0.5 μg/μl. Before use, the primers were diluted to a concentration of 50 ng/μl. Table 2 lists the primers used.

The PCR amplification reactions were performed in 50 μl of 10 mM Tris-HCl (pH 8.8); 50 mM KCl; 1.5 mM $MgCl_2$; 0.1% Triton X-100; 2% gelatin; 200 μM of dATP, dTTP, dCTP, and dGTP each (Pharmacia LKB, Uppsala, Sweden); 50 ng of primer; 10 ng of genomic DNA; and 0.5 units of SuperTaq DNA polymerase (Sphaero Q, Leiden, Netherlands). Control reactions were included to avoid misinterpretations of the RAPD patterns due to PCR artifacts. These control reactions contained all the components except template DNA. The samples were overlaid with light mineral oil to prevent evaporation.

Amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 45 cycles of 1 min at 94 C, 2 min at 38 C, and 3 min at 72 C, with a temperature ramp of 1 C per 5 s for the 38–72 C transition. After amplification, DNA products were loaded on a 1% agarose gel in TAE buffer and the DNA fragments were separated (5 V/cm) (31). Lambda DNA digested with the restriction enzymes *Bgl*II and *Hpa*I (lambda 2) or *Hin*dIII, *Bam*HI, and *Eco*RI (lambda 3) was used as the molecular weight standard. The gels were stained with ethidium bromide (0.5 μg/ml) and photographed with Polaroid 665 film under UV light.

TABLE 1. The nine *Globodera rostochiensis* and 17 *G. pallida* populations used in this study, their pathotype classifications, and their site of collection in the Netherlands

Species Code	Pathotype	Location
G. rostochiensis		
D7 C295	Ro_3	Gramsbergen
E7 G1527	Ro_3	Anlo
A9 C286	Ro_3	Hoogeveen
B9 C293	Ro_3	Hoogeveen
C9 C294	Ro_3	Oosterhesselen
D9 A56	Ro_1	Bergh
E9 A50	Ro_1	Weert
A11 F539	Ro_4	Emmen
B11 Mierenbos	Ro_1	Wageningen
G. pallida		
A1 1095	Pa_2	?
B1 P2-22	Pa_2	Coevorden
C1 Rookmaker	Pa_3	Valthe
D1 A75-250-39	Pa_3	Gasselte
E1 1077	Pa_3	Anjum
A3 1112	Pa_3	Westerbork
B3 74-768-20	Pa_3	Sleen
C3 75-884-4	Pa_3	Vriezeveen
D3 1097	Pa_3	Hardenberg
A5 D383	Pa_2	Smilde
B5 D372	Pa_2	Anlo
C5 D370	Pa_2	Emmen
E5 D350	Pa_2	Avereest
A7 D353	Pa_2	Hardenberg
B7 D354	Pa_2	Oosterhesselen
C7 D371	Pa_2	Ommen
C11 HPL-1	Pa_2	Veendam

Data analysis. In assessing the inter- and intraspecific variability, we: 1) scanned negative films with a video densitometer (Model 620, Bio-Rad Laboratories, Richmond, CA), 2) selected DNA fragments showing an optical density of at least 1.0 on the film, and 3) evaluated the selected DNA fragments in all other populations and scored the presence or absence of these DNA fragments; fragments with an optical density less than 1.0 were also scored as present.

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 $F = 2N_{xy}/(N_x + N_y)$, in which N_x and N_y refer to the number of DNA fragments generated by the RAPD assay in populations X and Y, respectively, and N_{xy} is the number of DNA fragments shared by the two populations (27). Dendrograms were constructed from the F values using the unweighted pair group method with arithmetic mean (UPGMA) in the Clustan3.2 VAX-VMS program (34).

RESULTS

The 14 primers listed in Table 2 resolved 250 different DNA fragments. The number of DNA fragments produced per primer varied from three to 16 and ranged in size from 0.30 to 2.78 kb. For example, primer OPG-10 generated 15 DNA fragments (10 of which were polymorphic) in nine populations of *G. rostochiensis* and 16 DNA fragments (eight of which were polymorphic) in 17 populations of *G. pallida* (Fig. 1).

Only nine DNA fragments were common to all populations of both species. The F value, expressing similarity between *G. rostochiensis* and *G. pallida*, averaged 0.052. Of the 105 DNA fragments specific to *G. rostochiensis*, 85 were shared by all populations. Of the 127 specific fragments found in *G. pallida*, 69 were monomorphic. The intraspecific similarities (F values) of the *G. rostochiensis* populations ranged from 0.870 to 0.967 (av. 0.922) and those of the *G. pallida* populations, from 0.829 to 1.000 (av. 0.911).

The dendrogram of both the *G. rostochiensis* and *G. pallida* populations (Fig. 2) clearly expresses their wide divergence. The *G. rostochiensis* populations with similar pathotype classifications cluster together, illustrating similarity between both data sets. *G. rostochiensis* populations with identical pathotype classifications can be distinguished by a number of unique RAPD fragments. The Ro_1 populations are characterized by fragment OPG-19_{1391}, a 1,391-bp DNA fragment amplified with primer OPG-19. Populations classified as Ro_3 share the unique fragments OPG-06_{1817}, OPG-06_{1612}, OPG-12_{617}, OPG-12_{505}, and OPG-12_{452}.

Cluster analysis of the RAPD data of the *G. pallida* populations revealed limited resemblance with their pathotype classifications (Fig. 2). The genetic distance between populations with different pathotype classifications is often smaller than that between populations with the same pathotype classification. For example,

TABLE 2. The oligonucleotide decamer primers used to assess the inter- and intraspecific variation of *Globodera rostochiensis* and *G. pallida* and the number of DNA fragments produced per primer

Primer	Sequence	Number of DNA fragments	
		G. rostochiensis	*G. pallida*
OPG-02	GGCACTGAGG	9	9
OPG-03	GAGCCCTCCA	6	10
OPG-04	AGCGTGTCTG	3	9
OPG-05	CTGAGACGGA	4	10
OPG-06	GTGCCTAACC	8	5
OPG-08	TCACGTCCAC	4	15
OPG-10	AGGGCCGTCT	15	16
OPG-11	TGCCCGTCGT	9	7
OPG-12	CAGCTCACGA	10	10
OPG-13	CTCTCCGCCA	8	10
OPG-15	ACTGGGACTC	6	7
OPG-16	AGCGTCCTCC	14	12
OPG-17	ACGACCGACA	8	9
OPG-19	GTCAGGGCAA	10	13

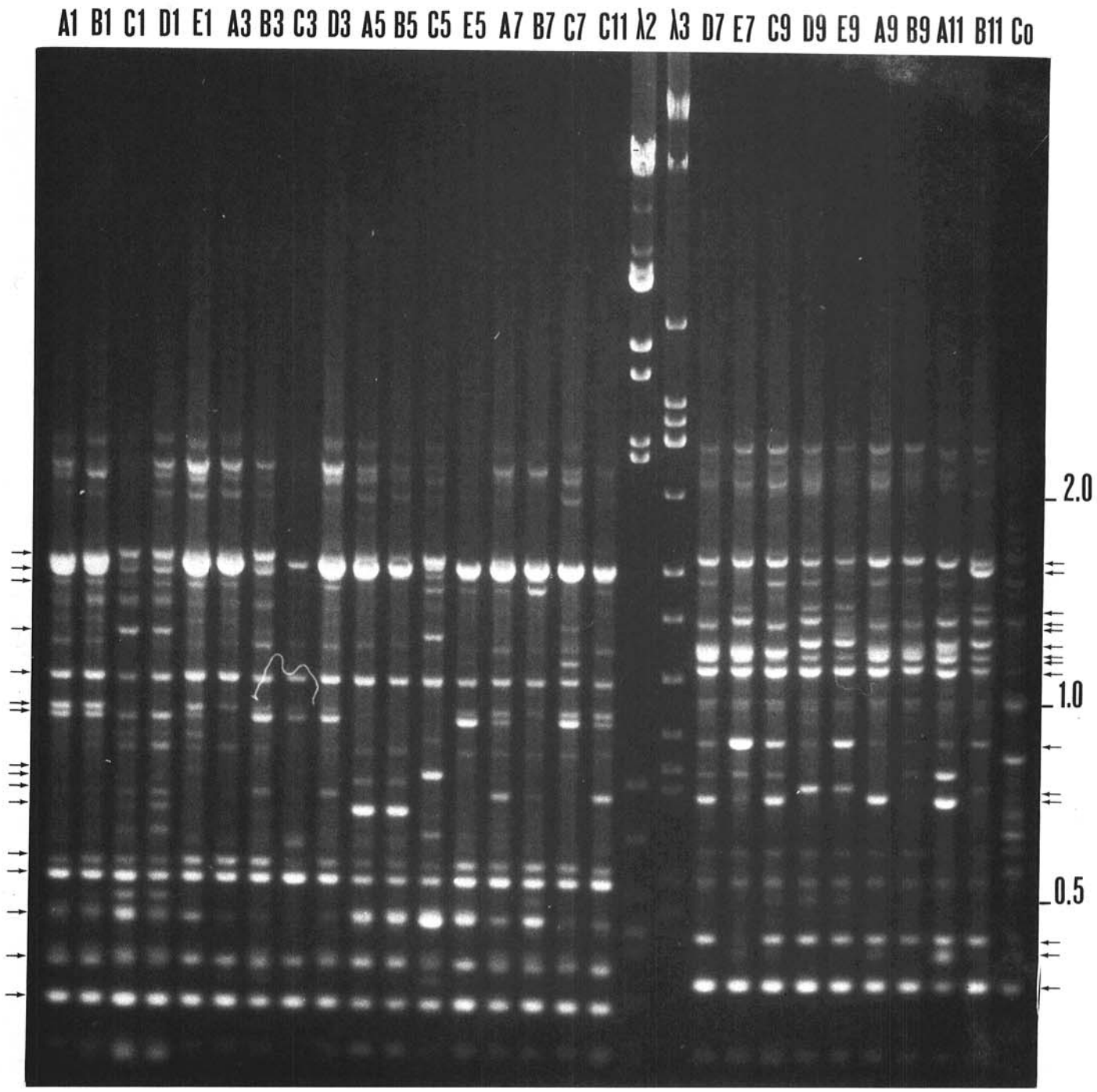

Fig. 1. DNA fingerprints of nine *Globodera rostochiensis* and 17 *G. pallida* populations after PCR with primer OPG-10. Arrows indicate 15 (right) and 16 (left) DNA fragments scored to be present in at least one population of *G. rostochiensis* and *G. pallida*, respectively. Lanes A1–C11, *G. pallida* populations coded according to Table 1; λ2, lambda DNA digested with *Bgl*II and *Hpa*I; λ3, lambda DNA digested with *Hin*dIII, *Bam*HI, and *Eco*RI; lanes D7–B11, *G. rostochiensis* populations coded according to Table 1; and Co, control sample without template DNA. Molecular weights are indicated in kilobases.

populations E5 D350 and E1 1077, classified as Pa_2 and Pa_3, respectively, are clustered together in the dendrogram (Fig. 2). A number of DNA fragments were found to be unique for a *G. pallida* population or a cluster of populations: $OPG\text{-}03_{1248}$ for B7 D354; $OPG\text{-}08_{752}$, $OPG\text{-}10_{807}$, $OPG\text{-}12_{1874}$, and $OPG\text{-}17_{564}$ for C5 D370; $OPG\text{-}02_{745}$ for cluster C1 Rookmaker and D1 A75-250-39; and $OPG\text{-}08_{1101}$ for cluster A5 D383 and B5 D372.

DISCUSSION

The two potato cyst nematode species exhibit exceptionally similar morphologies and were, until 1970, considered as pathotypes of the species *Heterodera rostochiensis* Woll. (20). The observation that both sibling species share only nine RAPD fragments on a total of 250 confirms their wide divergence at the molecular level as established before on the basis of proteins (e.g., 3,18) and RFLPs (7,13,35).

Contrasts between morphological and molecular evolution are described for a wide variety of taxonomic groups, e.g., fish, frogs, reptiles, and snails (38). Extensive molecular variation is also reported for a number of morphologically nearly indistinguishable nematode species, e.g., *Caenorhabditis elegans* (Maupas) Dougherty and *C. briggsae* Dougherty & Nigon (8) and *Brugia malayi* Brug and *B. pahangi* Buckley & Edison (25). These findings indicate that genetic differentiation measured with molecular techniques is not necessarily correlated with the evolution of morphological characters (22). Morphological evolution primarily depends on regulatory mutations that alter patterns of gene expression (23). Both potato cyst nematode species have apparently accumulated DNA sequence differences during millions of years without major effects on the regulatory genes. The slow evolution of the regulatory system in both nematode species is confirmed by their potential to hybridize and to produce viable second-stage juveniles (26).

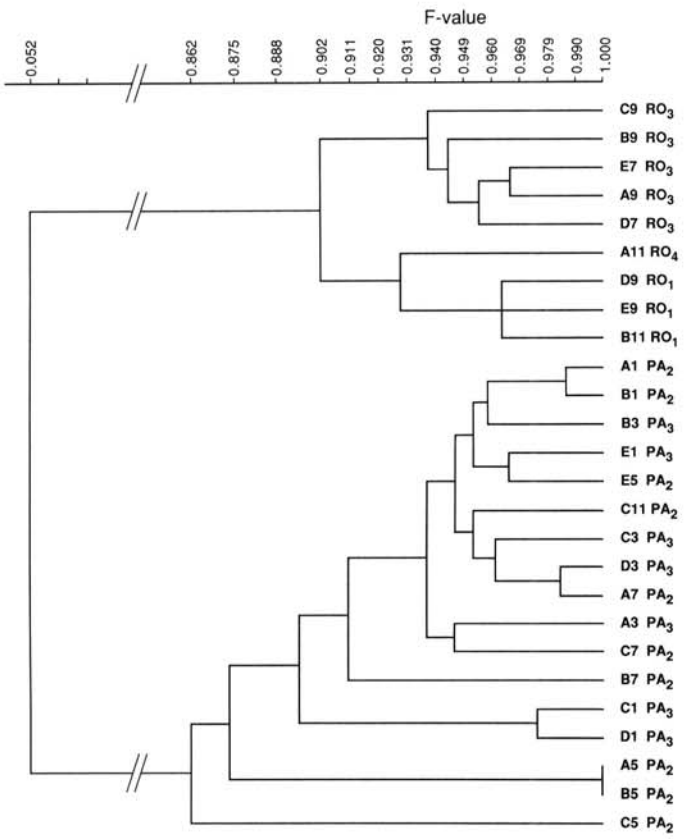

Fig. 2. Similarity dendrogram of nine *Globodera rostochiensis* and 17 *G. pallida* populations constructed from the genetic distance based on 241 random amplified polymorphic DNA fragments, using the unweighted pair group method with arithmetic mean (UPGMA) in the Clustan3.2 VAX-VMS program.

This occurs only when both parental species show a similar gene regulation (38).

Several groups (14,19) used the intensity of stained DNA fragments on an agarose gel as a measure for the allele frequencies. The RAPD assay, however, allows no inference of allele frequencies. The intensity of a DNA fragment on an agarose gel is not indicative of the copy number of the DNA fragment in the initial template DNA sample. Only presence or absence of DNA fragments can be recorded.

Various similarity measures have been used to determine the genetic divergence between organisms on the basis of RAPD data (10,14,19,21). We agree with Chapco et al (11) that until a theoretical framework is developed for the variation generated by the RAPD assay, the statistic $F = 2N_{xy}/(N_x + N_y)$ (27) is at present the most unambiguous formula to express inter- or intraspecific similarity.

The intraspecific similarity among *G. rostochiensis* and *G. pallida* populations ranged from 0.870 to 0.967 and from 0.829 to 1.000, respectively. These intraspecific similarities are high in comparison to RAPD data of populations of the beet cyst nematode (*Heterodera schachtii* Schmidt) (10) and of populations of grasshoppers (*Melanoplus* spp.) (11). It is assumed that the intraspecific variation between European populations predominantly results from the genetic structures of the primary founders, random genetic drift, and gene flow (5). Our data, though, suggest only restricted gene flow between conspecific populations. Due to their poor dispersal abilities, potato cyst nematode populations are able to maintain their genetic integrity for prolonged periods of time. Distinct clusters were observed within both species, and the clustering showed no correlation with geographic origin. Also, populations sampled at nearby sites, e.g., the *G. pallida* populations D3 1097 and C7 D371, are discriminated by various unique DNA fragments. These observations indicate a lack or restricted rate of exchange.

The brief space of time after the introduction of both species in Europe, approximately in 1850, and their low multiplication rate per potato crop (approximately 25×) exclude mutation to explain the observed intraspecific variation. Mutation rates vary from 10^{-4} to 10^{-6} per gene per gamete for most eukaryotic organisms (1). Mutations therefore can be neglected as an important running force for the observed genetic variation.

The RAPD data of the *G. rostochiensis* populations are in concordance with the pathotype classifications. Each cluster of populations with identical pathotype classification can be recognized by one or more unique RAPD markers. In contrast, clustering of the *G. pallida* populations on the basis of RAPD data reveals only limited resemblance to their pathotype classification. Explanations for this discrepancy may be found in the limited number of differential potato clones used to classify pathotypes of *G. pallida* (24). This number is too small to properly reflect the genetic variation of *G. pallida* populations in Europe (4). Also, the genetic variation in *G. pallida* seems to be larger than that in *G. rostochiensis*. Our data are corroborated by Schnick et al (32), who reported smaller proportions of polymorphic DNA fragments from *G. rostochiensis* populations than from *G. pallida* populations.

At present, we are integrating data from RAPDs, two-dimensional gel electrophoresis of proteins, and virulence tests to delineate groups of related potato cyst nematode populations. One of our goals is to estimate the number of initial introductions in Europe, knowledge that will contribute to efficient breeding programs aimed at broad and durable resistance.

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