The Effects of Founding Events and Agricultural Practices on the Genetic Structure of Three Metapopulations of *Globodera pallida*

Rolf T. Folkertsma, Paul van Koert, Jeroen N. A. M. Rouppe van der Voort, Koen E. de Groot, Jan E. Kammenga, Johannes Helder, and Jaap Bakker

Laboratory of Nematology, Wageningen University, P.O. Box 8123, NL-6700 ES Wageningen, the Netherlands.

Current address of R. T. Folkertsma: Enviro-flor Ltd., P.O. Box 30260, Lusaka, Zambia.

Current address of P. van Koert and J. N. A. M. Rouppe van der Voort: Keygene N.V., Agrobusiness Park 90, P.O. Box 216, NL-6700 AE Wageningen, the Netherlands.

Accepted for publication 24 April 2001.

ABSTRACT

Folkertsma, R. T., van Koert, P., Rouppe van der Voort, J. N. A. M., de Groot, K. E., Kammenga, J. E., Helder, J., and Bakker, J. 2001. The effects of founding events and agricultural practices on the genetic structure of three metapopulations of *Globodera pallida*. *Phytopathology* 91: 753-758.

To assess the genetic constitution of the *Globodera pallida* populations in the Netherlands and the effects of agricultural practices, three geographically separated metapopulations, in total consisting of 226 local populations, were analyzed by two-dimensional gel electrophoresis (2-DGE) of total proteins. This technique allows the accurate assessment of allele frequencies in homogenates of mixtures of individuals. Based on the estimated average heterozygosity, the average proportion of polymorphic loci and the average number of alleles per locus, the genetic diversity among 226 local *G. pallida* populations was small. The small genetic basis of *G. pallida* in the Netherlands will facilitate the identification of *Solanum*

genotypes with a broad and durable resistance to *G. pallida*. Instead of clusters of local populations with unique alleles, a continuous range of allele frequencies was observed. Analysis of the three metapopulations by the Shannon-Weaver index and Nei's G_{ST} revealed that the metapopulation from a region with sandy-loam soils was clearly distinguishable from the remaining two; the local populations within this metapopulation were more similar and the genetic diversity within the individual local populations was significantly higher than the local populations from the two remaining regions. These regions are characterized by wider crop rotation schemes and a very limited use of nematicides. The less intensive cultivation of potatoes in these regions with sandy-clay soils resulted in relatively little variation within and more variation between local nematode populations. To our knowledge, the effects of agricultural practices on the genetic constitution of potato cyst nematode populations have not been pinpointed before.

A considerable number of plant-parasitic nematode species cause most serious losses outside their centers of origin, for example: the pinewood nematode, *Bursaphelenchus xylophilus*, which has been introduced from North America to Europe and Japan (12,34); the soybean cyst nematode, *Heterodera glycines*, which is thought to originate from China (26) and causes major problems in soybean production in the United States and Canada; and the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. Both *Globodera* spp. originated from the Andes region in South America and have been spread to potato production areas all over the world (14). These introductions, or primary founding events, often imply a substantial decrease in genetic variation compared with the source populations (25). Other temporal reductions in population size may occur when further colonization is realized by secondary founding, upon the application of nematicides or upon the growth of resistant host plants. Such bottlenecks will result in a further decrease of genetic variation through random genetic drift (25). A limited genetic variation of the nematode populations outside their centers of origin would increase the chances of successful control by resistant host plants.

Genetic drift is thought to be one of the major forces determining the genetic structure of nonindigenous plant-parasitic nematode populations. However, limited data are available on the effects of bottlenecks on the genetic variation within and between conspecific populations. Insight into the extent of random genetic

drift is important for the development of control strategies based on host plant resistance. Control by means of host plant resistance is ideally based on (i) unambiguous pathotype classification schemes; (ii) the availability of a representative set of pathotype populations to screen wild species or breeding lines for suitable resistance; and (iii) diagnostic assays to identify field population pathotypes, enabling growers to select appropriate resistant cultivars. In many situations, these requirements are not met; in the case of *G. pallida*, there is an apparently continuous range of variation in virulence relative to the available resistance genes (3).

The effect of bottlenecks on genetic variation within and between populations has been studied extensively with a range of molecular techniques (4,13,20,21). Hubby and Lewontin (18) were among the first to study the genetic variation among *Drosophila* populations by allozyme electrophoresis (18). They detected differences in allele frequencies between natural populations of *D. pseudoobscura* by analyzing numerous individual specimens per population (23). The differences in allele frequencies could be attributed to recent founder events (22,27).

Although numerous articles have been published on the genetic variation between plant-parasitic nematode populations, and in particular potato cyst nematode populations (5,6,9,15,17,31,36), none of these studies considered the use of allele frequencies to investigate genetic variation. Estimation of allele frequencies from analyses of single nematodes is a tedious task and has only been accomplished in a few studies (7,11). An alternative is to analyze pools of individuals of a population with two-dimensional gel electrophoresis (2-DGE) of total proteins. The ratios of the protein quantities of codominant alleles at a locus were an appropriate measure for estimating allele frequencies at that locus (1). Further-

Corresponding author: J. Bakker; E-mail address: jaap.bakker@nema.dpw.wau.nl

Publication no. P-2001-0621-01R

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more, comparison of the allele frequencies estimates determined by 2-DGE on pools of individuals of a population and of single individuals of the same population revealed no significant variation in allele frequency estimates between the methods (11).

We investigated the genetic structure of three metapopulations of *G. pallida* in the Netherlands. These metapopulations originate from three geographically separated regions. From these regions, 226 local populations (i.e., subpopulations) were analyzed by 2-DGE, and the allele frequencies at eight protein loci were estimated. For decades, the potato growing practices in these three regions have been different, and here we relate the differences to the genetic constitutions of *G. pallida* metapopulations.

MATERIALS AND METHODS

Nematode metapopulations. In 1990 to 1992, samples were taken from 226 *G. pallida*-infested sites in three geographically distinct areas in the Netherlands: north Friesland (29 local populations); the IJsselmeerpolders (48 local populations); and north-eastern Netherlands (149 local populations) (Fig. 1). Most samples consisted of at least 100 cysts (with a minimum of 25). A sample was considered to represent a local population if cysts were taken from a site bordered by an uninfested area. Local populations from north Friesland were provided by the General Netherlands Inspection Service for Agricultural Seeds and Seed Potatoes (NAK, district Noord, Buitenpost) and the Institute of Plant Protection Research (IPO-DLO, Wageningen). Local populations from the IJsselmeerpolders were provided by NAK (district Midden, Emmeloord), the Research Station for Arable Farming and Field Production of Vegetables (PAV, Lelystad), and de Groene Vlieg BV (Dronten). Samples from the northeastern Netherlands were obtained from heavily infested spots in the field. These local populations were collected by members of the Landbouwwer vereniging Borgercompagnie, IPO-DLO (Wageningen), and the potato breeding institute, Karna (Valthermond). HPL-1, used as a standard or reference population in this study, originated from a site in the northeastern Netherlands (from the Hilbrands Laboratory collection, Assen, the Netherlands).

Fig. 1. Map of the Netherlands indicating the sampling sites of the three *Globodera pallida* metapopulations: north Friesland, IJsselmeerpolders, and the northeastern Netherlands.

Preparation of protein samples and mini 2-DGE of proteins.

Local populations of *G. pallida* were maintained on susceptible cv. *Solanum tuberosum* spp. *tuberosum* L. Eigenheimer in a growth chamber at 18°C with 16 h day length. For protein extraction, 50 adult white females per local population were collected approximately 6 weeks after inoculation. At that age, the white females were supposed to be virgin, although in some instances males were observed. The air-dried females were stored at -80°C until used.

Protein samples from 50 pooled females of each local population (~200 mg fresh weight) were prepared as described by Bakker et al. (2). The protein samples were either stored at -80°C or used immediately for electrophoresis. Mini 2-DGE of proteins was performed as described by de Boer et al. (11) with some minor modifications (16). Protein samples were electrophoresed in series of eight local populations (16), and the standard population HPL-1 was included as the control. After being stained, the gels were dried on a vacuum dryer (Model 543; Bio-Rad, Hercules, CA).

Data collection. Protein profiles were evaluated from dried gels with a bench viewer. The molecular weight and the pH of isoelectric point (IP) variants were estimated as described by de Boer et al. (11). In a previous study (1), it was demonstrated that IP variants are encoded by corresponding alleles from a single locus. We examined the allele frequencies at loci *C*, *F*, *H*, *I*, *J*, *N*, and *M*, which were previously described (11,16). Two alleles (X_1 and X_2) of previously undescribed locus *X* were observed. Both alleles at locus *E* could not unambiguously be scored in all populations. A protein spot unrelated to locus *E* was consistently present at the position of E_1 , thus hampering the determination of the frequency of this allele.

The allele frequencies for each local population were estimated with the aid of an image analysis system. This system included a macroscope (Wild M420; Leica Heerburg AG, Heerburg, Switzerland), a CCD camera (Sony, Tokyo, Japan), a camera control unit (DXC-750 P; Sony), and a Quantimet 570 (Cambridge Instruments Ltd., Cambridge, UK). The area and staining intensity of an IP variant were measured, and the integrated optical density (IOD) of each IP variant was calculated. The frequency of an allele was determined by dividing the IOD of the corresponding IP variant by the total IOD of the different IP variants representing the corresponding putative locus.

Data analysis and validation. To validate the estimation of allele frequencies by 2-DGE, 30 separate protein samples of standard population HPL-1 were electrophoresed in 30 independent runs and the allele frequencies were estimated. Observed allele frequencies were converted by an arcsine $\sqrt{p_{ij}}$ transformation (32), where p_{ij} represents the allele frequency for allele *i* at locus *j* ($0 \leq p_{ij} \leq 1$). To determine the variation in genetic distance between different 2-DGE gels from a single population, Rogers genetic distance (29) between pairs of gels of standard population HPL-1 from the 30 aforementioned runs was determined by the DISTANCE procedure in NTSYS-pc (version 1.80, Applied Bio-statistics, Exeter Software, Setauket, NY). In the following sections, all procedures written in capitals refer to routines included in this software package. Rogers genetic distances among the 435 pairs of gels from the subpopulation HPL-1 was assumed to follow a normal distribution, and the average genetic distance, standard deviation, and 95% confidence intervals were determined with SAS (version 6.09, SAS Institute, Cary, NC).

Genetic structure of *G. pallida* metapopulations. To analyze the genetic structure of the three metapopulations and the differences in genetic structure between them, a number of parameters were evaluated. First, the Shannon-Weaver index corrected for sample size and fitted with standard errors (19) was evaluated. Presence (1) or absence (0) of alleles on each locus was determined for each local population. This resulted in a local population haplotype. Upon grouping the local populations from each

region according to their haplotype, the Shannon-Weaver index was calculated. A *t* test was performed for a pair-wise comparison of the genetic variation among the three metapopulations as described previously (19).

Second, the coefficient of gene differentiation (G_{ST}) was evaluated for each metapopulation (24). To estimate the G_{ST} of each metapopulation over 354 monomorphic and 8 polymorphic loci, three parameters were calculated: (i) the average gene diversity within the local populations from that particular metapopulation (H_S); (ii) the average gene diversity between local populations from the metapopulation (D_{ST}); and (iii) the gene diversity (H_T) in the total population, where $H_T = H_S + D_{ST}$. The extent of absolute gene differentiation of each metapopulation was calculated as $D_m = D_{ST} \times s/(s - 1)$, where s is the number of local populations within the metapopulation (24). For each metapopulation, $R_{ST} (=D_m/H_S)$ was used to quantify the gene diversity within local populations relative to the gene diversity between local populations (24).

Genetic variation within two regions. A detailed analysis of the connection between sampling site and genetic diversity was carried out for two areas, the IJsselmeerpolder and the northeastern Netherlands. Because the exact sample location of the individual local populations was known, the product moment correlation r between Rogers distance matrix and the location distance matrix (in kilometers) among the local populations from these two areas could be determined with the matrix comparison MXCOMP option in NTSYS-pc.

RESULTS

Validation. 2-DGE enabled the resolution of 21 IP variants, encoded by 21 alleles at 8 putative loci, and 354 monomorphic protein spots in 226 *G. pallida* local populations. These 354 monomorphic protein spots were assumed to represent 354 monomorphic loci.

Slight differences in allele frequency estimates will arise when different samples of 50 females are taken from a local population. In addition, minor variation in electrophoresis and staining conditions can be expected between runs. To quantify both sources of variation, 30 separate protein samples of standard population HPL-1 were electrophoresed in 30 runs, and the allele frequencies at 21 alleles encoded by 8 putative loci were estimated. The range in allele frequency estimates for each polymorphic locus was used as a measure for the expected variation in allele frequency for each local population (Table 1). The average genetic distance ($\pm$ standard deviation) among the 30 gels of reference population HPL-1 was 0.039 ± 0.012 , resulting in a 95% confidence interval with an upper limit of 0.064.

Genetic variation of *G. pallida* in the Netherlands. Among the 362 putative protein loci resolved by the 2-DGE technique, only 8 loci were polymorphic. The average heterozygosity (24) of the population was 1.0%, assuming that each local population was in Hardy-Weinberg equilibrium, with an average of 1.024 alleles per locus and 1.7% polymorphic loci. It is noted that populations are not in equilibrium immediately after a severe reduction of the population (a bottleneck). However, within a very few generations (≤ 3) this equilibrium is restored. The size of the local populations strongly suggests the restoration of the Hardy-Weinberg equilibrium.

Rogers genetic distance among the local populations ranged from 0.03 to 0.374. Among the 226 *G. pallida* subpopulations, 149 local populations were genetically identical to at least one other subpopulation (66%), i.e., had a Rogers distance smaller than 0.064. These identical subpopulations were distributed over 53 groups. The number of identical subpopulations within a group ranged from 2 to 8 and averaged 2.8. Approximately 25% of the pairs of identical populations were sampled in different regions.

Genetic structure of three *G. pallida* metapopulations. To unravel the genetic structure of the three metapopulations, the Shannon-Weaver index and the coefficient of gene differentiation (G_{ST}) were evaluated. The Shannon-Weaver index enabled the differentiation of groups of populations based on their haplotypes, as defined by the presence or absence of alleles. Using the Shannon-Weaver index, it was concluded that there was a significant difference in population structure between the different metapopulations ($P < 0.01$) (Table 2).

Secondly, assuming that the 226 *G. pallida* subpopulations are in Hardy-Weinberg equilibrium, the coefficient of gene differentiation (G_{ST}) was evaluated for each metapopulation (24) (Table 2). A significant difference in genetic differentiation between the metapopulation from northeastern Netherlands and the metapopulations from north Friesland and the IJsselmeerpolders was observed. Although the gene diversity in the three regions (H_T) was similar, the average gene diversity within subpopulations (H_S) was higher and the absolute gene differentiation (D_m) lower in the northeastern Netherlands compared with north Friesland and the IJsselmeerpolders (Table 2).

Genetic variation within two regions. To study the spatial variation between local populations in more detail, two separate areas, Oostelijk Flevoland located within the IJsselmeerpolders (≈ 20 km²) and the Veendam area located in the northeastern Netherlands (≈ 20 km²), were chosen.

TABLE 2. The genetic diversity of three geographically separated *Globodera pallida* metapopulations^a

	Metapopulation		
	North Friesland	IJsselmeerpolders	Northeastern Netherlands
Haplotypes	25	44	111
Shannon-Weaver	3.176 (0.165)	3.756 (0.119)	4.578 (0.094)
H_S	0.0073	0.0068	0.0085
D_{ST}	0.0030	0.0032	0.0021
H_T	0.0103	0.0100	0.0107
D_M	0.0031	0.0033	0.0022
G_{ST}	0.71	0.68	0.80
R_{ST}	0.43	0.48	0.25

^a Genetic parameters: the Shannon-Weaver index corrected for sample size and fitted with standard errors in parentheses, average gene diversity within the local populations within a metapopulation (H_S), and average gene diversity between local populations within a metapopulation (D_{ST}). The gene diversity in the total population (H_T) is calculated by adding H_S and D_{ST} . The coefficient of gene differentiation; $G_{ST} = 1 - (D_{ST}/H_T)$ and $R_{ST} = D_M/H_S$, whereas $D_M = D_{ST} \times s/(s - 1)$, in which s is the number of local populations within the metapopulation.

TABLE 1. Univariate statistics of the arcsine $\sqrt{(p_{ij})}$ -transformed allele frequencies at polymorphic loci estimated from gels of a standard population, HPL-1

Statistics	C		F			H			X		I		J			N		M			
	1	2	1	2	3	1	2	3	1	2	1	2	1	2	3	1	2	1	2	3	4
Average	0.25	1.32	0.54	0.45	0.83	0.74	0.8	0.14	0.71	0.86	1.57	0	0.98	0.59	0	0.8	0.77	0	0.82	0.75	0
Minimum	0.1	1.15	0.39	0.31	0.69	0.58	0.63	0	0.56	0.79	1.57	0	0.82	0.36	0	0.7	0.67	0	0.73	0.45	0
Maximum	0.42	1.47	0.69	0.57	1.01	0.94	0.96	0.33	0.79	1.01	1.57	0	1.21	0.75	0	0.9	0.87	0	1.12	0.84	0
Standard deviation	0.07	0.07	0.08	0.07	0.08	0.08	0.07	0.11	0.05	0.05	0	0	0.1	0.1	0	0.04	0.04	0	0.09	0.09	0
Variance	0.01	0.01	0.01	0	0.01	0.01	0.01	0.01	0	0	0	0	0.01	0.01	0	0	0	0	0.01	0.01	0

Figure 2A shows an unweighted pair-group method with arithmetic average (UPGMA) dendrogram, illustrating the genetic variation among 26 subpopulations from Oostelijk Flevoland. The UPGMA dendrogram reveals three groups of identical local populations, representing 31% of the subpopulations analyzed from this area. No correlation was observed between the genetic and geographic distances of the local populations ($r = 0.049$, $Z = 561$ with p [random $Z \geq$ observed Z] = 0.229 over 1,000 permutations).

From the Veendam area in northeastern Netherlands, 23 local populations were analyzed. Among the subpopulations from this area, four groups of identical local populations were found, representing 52% of the populations. Figure 2B shows the UPGMA dendrogram illustrating the genetic differentiation among the 23 subpopulations analyzed. A small but significant correlation was observed between the genetic and geographic distances of the 23 populations from the area in the northeastern Netherlands ($r = 0.544$, $Z = 141$, and p [random $Z \geq$ observed Z] = 0.002 over 1,000 permutations).

DISCUSSION

The comparison of allele frequency estimates among the 226 *G. pallida* subpopulations, as revealed by 2-DGE, suggests that the number of distinct introductions of *G. pallida* in the Netherlands

has been limited. The genetic variation among the local populations of *G. pallida* was predominantly expressed by quantitative differences in allele frequencies rather than qualitative differences. No clusters of populations with unique alleles were observed. These data strongly suggest that the Dutch *G. pallida* subpopulations originate from one source or, in case of multiple introductions, from a number of sources with a similar genetic structure.

The overall genetic constitution of local *G. pallida* populations in the Netherlands. As with many other colonizers, colonization of a country or region by potato cyst nematodes starts with a limited number of primary settlements followed by population extensions and secondary founder events. Random genetic drift will change the allele frequencies of each secondary founded population compared with the source population, and the extent of drift depends on the size of the bottleneck and the expansion rate of the population (24,25). The 2-DGE data suggest that the colonization of the Netherlands has been accompanied by extensive random genetic drift. In the absence of drift or a limited effect of drift, a small number of clusters of identical populations would be expected. However, the similarity dendrogram constructed from the genetic distances of 226 local *G. pallida* populations revealed a continuous range of variation (dendrogram available upon request). From our data, it was concluded that local populations with a genetic distance smaller than 0.064 are likely to be genetically identical. Using this threshold value, 66% of the local populations appeared to be identical to one or more subpopulations. However, these identical subpopulations were distributed over 53 small clusters. Clusters were discriminated from each other by differences in allele frequencies at various loci. Thus, the genetic diversity of the *G. pallida* population in the Netherlands, as revealed by 2-DGE, is predominantly determined by the genetic constitution of the primary founders and the effect of random genetic drift on the secondary founders.

The estimated heterozygosity, as revealed with 2-DGE for the *G. pallida* population in the Netherlands (1.0%), is small in comparison with the estimated heterozygosity of other organisms based on 2-DGE of proteins. The average heterozygosity of natural *D. melanogaster* populations ranged from 4% for an American population (22) to 2.5 and 1.2% for populations from Benin and France, respectively (10). The average heterozygosity of two natural *D. simulans* populations from France and Congo was estimated to be 2.9 and 2.7%, respectively (10). Racine and Langley (28) estimated the heterozygosity of a natural *Mus musculus* population at 2%. The low heterozygosity of *G. pallida* compared with these outbreeding metazoans, which did not encounter recent founder effects, suggests that the *G. pallida* subpopulations in the Netherlands have encountered substantial genetic bottlenecks since their introduction. European and South American populations have been compared with random amplified polymorphic DNA and amplified fragment length polymorphism analyses (6; R. Folkertsma, J. Rouppe van der Voort, and M. Phillips, unpublished data). These molecular data revealed that the genetic variation of *G. pallida* in South America is large compared to Europe.

Metapopulation differences among three geographically separated regions. A significant variation in population structure between the three metapopulations was found with the Shannon-Weaver index. Numerous haplotypes could be discriminated in either of the three regions, indicating large differentiation within and between the three metapopulations. The use of the Shannon-Weaver index, however, does not allow for the analyses of the subtle changes in allele frequencies. For instance, allele C1 is scored "present" in both a population with allele frequency C1 0.05 and in a population with allele frequency C1 0.95, resulting in an identical haplotype for locus C.

The analysis of the coefficient of genetic differentiation (G_{ST}) for the three metapopulations revealed that the populations from north Friesland and the IJsselmeerpolders show, in general, less

Fig. 2. A, Unweighted pair-group method with arithmetic average (UPGMA) dendrogram showing the genetic variation among 26 *Globodera pallida* populations sampled in Oostelijk Flevoland. B, UPGMA dendrogram showing the genetic variation among 23 *G. pallida* populations sampled in the northeastern Netherlands in the vicinity of Veendam.

variation within subpopulations and more variation between subpopulations than their counterparts in the northeastern Netherlands. Correction of G_{ST} for the number of local populations sampled, with R_{ST} , strengthened the observation. With D_{ST} as a parameter to quantify the genetic differentiation between local populations from a metapopulation, the variation among subpopulations in north Friesland and the IJsselmeerpolders is significantly larger than in northeastern Netherlands metapopulation. The data demonstrate that the impact of random genetic drift on the secondary founders has been larger in north Friesland and the IJsselmeerpolders than in northeastern Netherlands. The regional differences are most likely due to wider crop rotations and shorter growing seasons in north Friesland and the IJsselmeerpolders, resulting in lower multiplication rates. In these regions, potatoes are generally cultivated every 3 to 4 years and harvested in the beginning of July, hampering the completion of the life cycles. In northeastern Netherlands, potatoes are grown once every 2 to 3 years and harvested in the autumn. These factors are a plausible explanation for the observed regional differences because they have a significant influence on the expansion rates of populations after a founding event. A slow recovery after a bottleneck enhances the effect of drift (25).

An alternative explanation for the observed difference in metapopulation structure is a larger impact of gene flow on the genetic constitution of the local populations from northeastern Netherlands than with north Friesland and the IJsselmeerpolders. The sandy-loam soils prevalent in the northeastern Netherlands are more vulnerable to wind dispersion than the clay and sandy-clay soils in north Friesland and the IJsselmeerpolders. Cysts are readily dispersed by the wind (33,35), which occurs on a wider scale in the northeastern Netherlands than in north Friesland and the IJsselmeerpolders, thus facilitating spatial genetic exchange between adjacent local populations. In addition, farmers in the northeastern Netherlands are often self-supporting in that individual farmers produce their own seed potatoes, whereas farmers in the other regions are often obliged to use certified seed potatoes. The lack of control on the presence of potato cyst nematodes in batches of seed potatoes grown for personal use may facilitate temporal gene flow (8). These differences in agricultural practices are reflected in the correlations between the geographical and Rogers distance among the local populations. No correlation was observed for the subpopulations from Oostelijk Flevoland (an area within the IJsselmeerpolders). However, a significant correlation between the geographical distribution and genetic makeup was detected among local populations from Veendam (an area within the northeastern Netherlands).

Implications for R genes based control strategies. The small genetic basis of *G. pallida* in the Netherlands will facilitate the identification of *Solanum* genotypes with a broad and durable resistance to *G. pallida*. Indeed, recent studies revealed that a limited number of resistance loci in potato suffice to control the range of *G. pallida* populations present in the Netherlands (30).

Our study provides insight into the impact of genetic drift on plant-parasitic nematode populations. If the results for *G. pallida* populations are representative for other plant-parasitic nematode species, there will be a need for a major revision of current strategies to classify pathotypes. Because avirulence alleles are often not fixed (3), the extensive drift observed at loci revealed by 2-DGE will be reflected at avirulence loci, which hampers a proper delineation of pathotypes.

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