Genetic diversity of the potato cyst nematode in the Netherlands

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Stellingen

1. In tegenstelling tot wat algemeen wordt aangenomen is de resistentieveredeling tegen het aardappelcystenaaltje niet een op voorhand verloren wapenwedloop.

   dit proefschrift

2. De aardappelcystenaaltjes *Globodera rostochiensis* en *G. pallida* hebben Nederland onafhankelijk van elkaar gekoloniseerd.

   dit proefschrift

3. De aardappelzetmeelindustrie heeft een significante bijdrage geleverd aan de variatie binnen en tussen populaties van het aardappelcystenaaltje in Noord-Oost Nederland.

   dit proefschrift

4. Nu het verzadigen van genetische kaarten met DNA markers onder handbereik ligt, wordt de bottleneck in het veredelingsonderzoek het ontwerpen van betrouwbare 'groene' experimenten.

5. Uitgaande van Haeckel's definitie van fylogenie is het onjuist een fylogenetische boom van DNA sequenties te presenteren zonder deze vanuit evolutionair oogpunt te interpreteren. Zhou en medewerkers hadden kunnen volstaan met een fenetische benadering van het geheel.

   Haeckel E (1866). Generelle Morphologie der Organismen. Reimer Berlin.
   Zhou *et al.* (1997). EMBO Journal 16:3207-3218.

6. De veelgehoorde klacht dat medisch onderzoek zich voornamelijk richt op het mannelijk deel van de samenleving geldt zeker niet voor moleculair onderzoek aan populaties van het aardappelcystenaaltje.

7. Slechts fietsend op de Mont Ventoux, na het bos, openbaart zich de ware aard van de wetenschapper.

   Tim Krabbé (1978). De Renner.

8. De sympatrische divergentie tussen 'hervormden' en 'gereformeerden' heeft tot een dermate groot aantal autapomorfe kenmerken geleid dat pogingen tot lumping van beide in een 'Samen op Weg' gemeente alleen al om taxonomisch redenen onverantwoord is.

9. Het is eenvoudiger een BAC te isoleren dan een BAC te formeren.

10. Het zou fietsers verplicht moeten worden bij naderend onheil van achteren eerst naar rechts uit te wijken alvorens een blik over de linker schouder te werpen.

11. De groei fan de 'wite skimmel' yn in protte Fryske doarpen hat mear fan dwaan mei in waaksende honkfêstheit fan de jongerein yn Fryslân as mei een waaksende wurkgelegenheit.

Stellingen behorend bij het proefschrift getiteld "Genetic diversity of the potato cyst nematode in the Netherlands', door Rolf Folkertsma.

Wageningen, 10 oktober 1997

Voorwoord

Uiteindelijk is het schrijven van dit boekje toch de beklimming van een berg van de buiten categorie gebleken, en ik spreek nu uit een dubbele ervaring. Na de passage van een haarspeldbocht volgde steeds een nieuwe en bleef de top op enorme afstand liggen. Soms leek geen eind aan de beklimming te komen. De voldoening de top te bereiken is dan ook erg groot. Dat de beklimming van zo'n berg, evenals de uitvoering van het onderzoek beschreven in dit boekje, een eenmansaktie is, is een illusie. Zonder een initiator van de fietstocht, zonder mechanicien en zonder gezelschap tijdens het fietsen, zou die top nooit gehaald zijn. In dit voorwoord wil ik kort stilstaan bij diegenen die bewust of onbewust een bijdrage hebben geleverd tot het tot stand komen van dit boekje en in het bijzonder bij diegene die mijn onderzoeksperiode op de vakgroep Nematologie als een zeer 'fietsvriendelijke' hebben gemaakt.

Jaap Bakker is op twee terreinen belangrijk geweest voor de uitvoering van dit onderzoek. Allereerst als initiator, het onderzoek beschreven in dit boekje is ten slotte te beschouwen als een voortzetting van het onderzoek waar hijzelf in 1987 op gepromoveerd is. Jaap is echter vooral belangrijk geweest als de aandrager van materiaal voor de aanpak van het onderzoek. Zijn ideeën en optimisme gaven mij iedere keer de energie om de draad van het onderzoek weer op te pakken. Van hem heb ik geleerd dat onderzoek doen meer is dan het uitvoeren van proefjes. De vele gesprekken met hem van grote betekenis geweest voor het verloop van mijn onderzoek.

Fred Gommers is erg belangrijk geweest voor de initiatie van het onderzoek. Voor het vertrouwen wat hij in mij stelde bij de aanstelling in 1991 en bij de verlenging op een EC-project in 1995 ben ik hem dankbaar. Zijn manier van werken heeft mij wel eens bijna tot wanhoop gedreven. Tegelijkertijd was hij wel degene die ervoor zorgde dat de soms moeizaam lopende besprekingen met de overige partners binnen het EC-project in goede banen werden geleid. Bovendien heb ik altijd erg genoten van onze uitstapjes, of die nu in Spanje, Frankrijk, Portugal, of dichter bij huis in het park de Hoge Veluwe waren.

Hans Helder's nuchtere kijk op mijn onderzoek, in de periode van Jaap's afwezigheid, heeft ervoor gezorgd dat ik wat objectiever naar het gene pool similarity concept ging kijken. Zijn kitische inbreng is van grote invloed geweest op de vorm en inhoud van met name het derde en vierde hoofdstuk.

Jeroen Rouppe van der Voort is gedurende de hele periode belangrijk geweest voor het uitwisselen van ideeën, voor het maken van onderzoeksopzetten, voor het analyseren van onderzoeksgegevens en voor het afreageren van frustraties. Gezamenlijk hebben we het derde hoofdstuk voor onze rekening genomen. Ik had mij geen betere compagnon kunnen wensen.

Voorts wil ik Marga van Gent-Pelzer, Koen de Groot, Paul van Koert en Peter van Zandvoort bedanken voor hun belangrijke bijdrage aan dit proefschrift. Zij hebben met zijn vieren gezorgd voor een enorme hoeveelheid data (2-DGE, RAPD, AFLP) waarop het overgrote deel van het in dit boekje gepresenteerde werk is gebaseerd. Jan Verhaaf wil ik hier ook graag noemen. Hij heeft zich vooral de laatste jaren bezig gehouden met de doorvermeerdering, opschoning, en bewaring van onnoemelijk veel aardappelcystenaaltjes populaties. Ik heb de plezierige sfeer waarin de samenwerking plaats heeft gevonden altijd erg op prijs gesteld.

Naast bovengenoemde medewerkers, wil ik ook de overige medewerkers van de vakgroep Nematologie en het Laboratorium van Monoklonale Antilichamen niet onvermeld laten. Zij hebben er met zijn allen voor gezorgd dat ik met veel plezier terug kijk op de periode die ik op de vakgroep door mocht brengen.
Twee mensen wil ik in het bijzonder noemen: Ineke de Jong en Arjen Schots. Ineke heeft een hele reeks sequenties opgehelderd van kloons van, zeer waarschijnlijk, mitochondriaal DNA van het aardappelcystenaaltje. Arjen was samen met Jaap de begeleider van het MJPG project, waarbij hij het monoklonalen werk onder zijn hoede nam.

Ook ik heb een aantal studenten van de LUW begeleid tijdens mijn verblijf op de vakgroep Nematologie: Rieneke van den Bosch, Bart Thoma, Bastiaan Hoogendoorn, Kees Swaans en Gerard Koorevaar. Ik heb het werken met jullie als zeer waardevol beschouwd.

Verder wil ik Rob Goldbach noemen. Ik dank hem voor het feit dat hij volledig belangeloos heeft willen fungeren als 'hulp-sinterklaas' (zijn eigen citaat). Daarnaast stel ik het erg op prijs dat hij mij in mijn huidige funktie binnen zijn tospogroep in staat heeft gesteld mijn werkzaamheden bij de vakgroep Nematologie af te ronden.

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Leendert Molendijk, Thea van Galen-van Beers en medewerkers van het PAGV te Lelystad voor de samenwerking en uitvoering van de virulentieproef die in het laatste hoofdstuk beschreven wordt. De leden van de WACO voor het kritisch volgen van mijn onderzoek.

Furthermore, I would like to thank the participants in the EC-project 'Genotyping potato cyst nematode populations: a search after the initial introductions' for the valuable co-operation and the pleasent atmosphere in which the annual meetings took place. I am especially grateful to Mark Phillips. The discussions we had on the variation of potato cyst nematodes and potato cyst nematode research in general have been very useful and stimulating.

Van niet te onderschatten belang voor het uiteindelijk voltooien van dit proefschrift zijn mijn vrienden (m/v) geweest. Bedankt voor de interresse in de voortgang van het onderzoek, maar vooral bedankt voor de aktiviteiten buiten het onderzoek om. Zo'n berg beklim je niet alleen.

Papa, mama, Baukje, Ria en Nynke. Ik hoef jimme net te bitankjen foar jimme ynhaldelike bydrage oan dit wurk. Jim wieden foaral bilangrik yn de wat dreechere riten. Dan stieden jimme der en koe ik op jimme bouwe. Dat had folle wichtiger west foar it ôfmeitsjen fan dit wurk dan my oan it ferstân te bringen dat 'random genetic drift' in grutte ynfloed hat op de populaesje struktuer fan it ierappelcysteieltsje.

Karin, jouw steun was onmisbaar,
van dichtbij en heel ver af.

Contents

chapter 1

Introduction

The potato cyst nematodes *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone are obligate parasites and are able to reproduce on various Solanaceous species. In The Netherlands potato cyst nematodes affect potato cultivation both directly by causing yield losses, and indirectly because of their quarantine status. Control of potato cyst nematodes is based on combinations of i) crop rotation, ii) soil disinfestation with nematicides and iii) the growth of resistant cultivars. From an economical point of view, crop rotation is for most farmers not feasible. Potatoes are an important crop in the Netherlands and determine on average 50% of the annual income (Anonymous, 1994a). The application of nematicides, both fumigants and granulates, is since 1989 officially restricted. In the period 1984/1988, the annual amount of active compounds used was 68.5x103 kg. According to the 'Meerjarenplan Gewasbescherming', the application of nematicides should decrease with 31.0% in 1995, 45.0% in 2000 and 55.0% in 2010 (Anonymous, 1989). To cope with these reductions, the growth of resistant cultivars becomes increasingly important. Consequently, there is an urgent need to obtain insight in the spatial distribution patterns of virulent potato cyst nematode populations.

a historical account: spread of potato cyst nematodes in Western Europe

Potato cyst nematodes originate from the Andes region in South America where they coevolved with their Solanaceous hosts (Stone, 1979; Stone, 1985). The nematode has very likely been introduced in Western Europe from 1850 onwards (Jones & Jones, 1974). At that time farmers needed potato cultivars resistant to *Phytophthora infestans* (Mont.) de Bary, the causal agent of 'the Great Famine' in Ireland. Before 1850 most potato cultivars were highly susceptible to *Phytophthora infestans* (Addens, 1952; Drenth *et al.*, 1993). Potato seeds and seed potatoes were imported from South America by breeders from England and Germany, the leading potato breeding countries in the 19th century (Addens, 1952). Selection of the imported material resulted in a number of resistant cultivars (Salaman, 1910) which were distributed all over Europe (Addens, 1952). It is likely that with the introduction of these new cultivars, the nematode was spread throughout Europe.

The first reports on infestations of potato cyst nematodes in various European countries are summarized by Oostenbrink (1950): Germany (Rostock, 1913), Scotland (1913), England (Yorkshire, 1917), Ireland (1922), Sweden (1922), Denmark (1928), The Netherlands (1938), Finland (1946), France (1948) and Belgium (1949). These observations coincide with the import of new breeding material from South America. Most of these infestations were reported from fields or gardens with severe damage of the potato crops (Oostenbrink, 1950). Because of the relatively slow multiplication rate of potato cyst nematodes, it is likely that the initial infection of these fields or gardens took place before 1900 (Oostenbrink, 1950).

resistant cultivars

Shortly after the first reports of potato cyst nematodes in The Netherlands, potato cultivation was restricted on infested soils (Oostenbrink, 1950). At the same time efforts were made to breed for resistant cultivars. Numerous *Solanum* accessions were tested for resistance against potato cyst nematode populations. Monogenic resistance was found in *Solanum tuberosum* ssp. *andigena* (*H1* gene) against, in retrospective, *G. rostochiensis* pathotype Ro1 (Ellenby, 1952) and in *Solanum multidissectum* (*H2* gene) against, in retrospective, *G. pallida* pathotype Pa1 (Dunnett, 1961). Oligogenic resistance was found in a range of *S. vernei* accessions (Dellaert & Vinke, 1987; Dellaert *et al.*, 1988).

However, the increasing application of soil fumigants in the fifties and sixties and their success in the control of potato cyst nematodes, curbed the efforts to develop resistant potato cultivars in this period. It was not until the early seventies, when an increasing number of infestations was observed (Mulder & Veninga, 1988), that a renewed interest for resistant cultivars arose.

durability of resistant cultivars

The growth of resistant cultivars is in theory an effective means to control potato cyst nematodes. The low multiplication rate (5-140 per generation), the limited active migration and the low frequency of potato cultivation (on average one crop every 2-4 years) contribute to the potential success of resistant cultivars. The durability of resistance genes in area is determined by two features: 1) the spatial variations in virulence among potato cyst nematode populations before the introduction of the resistant cultivars and 2) the selection rate of virulent genotypes upon exposure to resistance genes.

Spatial variation in virulence is a common feature for potato cyst nematode populations and is often observed before the introduction of a new resistant cultivar. Although *G. rostochiensis* and *G. pallida* have been spread all over Europe (Evans & Stone, 1977), their pathotypes have not been distributed uniformly. Regional differences became apparent after the growth of resistant cultivars. For example, only *G. rostochiensis* populations avirulent for the *H1* gene were introduced into the United Kingdom (UK), whereas both virulent and avirulent populations are found on the continent (Bakker, 1987). *G. pallida* populations avirulent for the *H2* gene were found only in northern Scotland and Northern Ireland, and not elsewhere in Europe (Stone *et al.*, 1986; Zaheer *et al.*, 1993).

The selection rate for virulence upon exposure to resistance genes has been studied in field trials and in pot experiments (Turner *et al.*, 1983; Turner, 1990; Whitehead, 1991; Beniers *et al.*, 1993). Also computer simulations (Jones, 1985; Spitters & Ward, 1988; Schouten, 1993, 1994) have been used to analyse the increase of virulent genotypes by assuming a gene-for-gene relationship. These studies have shown that the selection rate varies between populations. Populations with comparable virulence levels do not necessarily show similar selection rates (Turner *et al.*, 1983). The selection rate also depends on the genetic structure of the resistance (Spitters & Ward, 1988).

pathotypes

With the discovery of virulent populations, classification schemes were independently developed in the United Kingdom, Germany and The Netherlands. These schemes discriminated populations from each other on their ability to reproduce on a number of resistant clones (Kort *et al.*, 1977). Shortly after the development of these pathotype schemes, it became apparent that some of the pathotypes did not interbreed freely and that two sibling species of potato cyst nematodes were present in Europe: *G. rostochiensis* and *G. pallida* (Stone, 1972). From 1977 onwards an international pathotype scheme has been used for the intraspecific classification of potato cyst nematodes, integrating the national pathotype schemes (Kort *et al.*, 1977). This scheme recognizes eight different pathotypes, five within *G. rostochiensis* and three within *G. pallida*.

Soon after its introduction, the international pathotype scheme has been seriously criticized (Trudgill, 1985; Nijboer & Parlevliet, 1990; Bakker *et al.*, 1993). According to this scheme, a population is considered virulent for a differential if the Pf/Pi is > 1 and avirulent if the Pf/Pi <1. This approach is analogous to the way plant pathogenic fungi are classified and where pathotypes are defined as 'population[s] of a pathogen in which all individuals have a particular character of pathogenicity in common [..].' (Robinson, 1969). This definition is commonly used in phytopathology and i) requires that genes for resistance and genes for (a)virulence are known and ii) assumes that virulence characteristics are fixed in field populations.

However, apart from the gene-for-gene relationship between *S. tuberosum* ssp. *andigena* and *G. rostochiensis* (Janssen *et al.*, 1990), little is known about the genetics of the interaction between potato cyst nematodes and their Solanaceous hosts. Furthermore, partial resistance against potato cyst nematode populations has often been reported. Mixtures of avirulent and virulent genotypes are assumed to occur in such populations (Trudgill, 1985), seriously hampering the applicability of the scheme.

The second point of concern is that measuring virulence characteristics of populations is labourious and strongly influenced by environmental variation. Under the auspices of the European Plant Protection Organisation (EPPO) several groups have independently determined the virulence characteristics of a number of test populations. It was found that the absolute multiplication rate varies often between experiments carried out at different institutes. Expression of the multiplication rates on resistant cultivars as percentages of those on susceptible controls resulted in a reduction of the variation, but the values were still too variable for statutory use (Mugniéry *et al.*, 1989).

Third, the resistance spectra of commercial cultivars often show no resemblance to those of the differentials used in the pathotype scheme, because the resistance in some differentials is oligogenic or polygenic, which implies that cultivars derived from these differentials do not necessarily have the same resistance spectra. Another drawback is that breeders also use additional resistance genes distinct from those in the differentials of the international pathotype scheme.

Despite the aforementioned points of concern, current breeding programs and the diagnosis of potato cyst nematode populations are still based on the international pathotype scheme.

outline of this thesis

The aim of this thesis was to study the intra- and interspecific variation of *G. rostochiensis* and *G. pallida* in The Netherlands and to develop a framework for the identification of potato cyst nematode populations to optimize the control by means of host plant resistance. The second chapter describes the application of Random Amplified Polymorphic DNA (RAPD) for the amplification of species specific sequences from pools of individuals and single juveniles. The genetic variation between and within the two nematode species, as revealed by RAPDs and AFLPs, is studied in chapter 3 and 4, respectively. In chapter 5 the variation among *G. pallida* populations as revealed by RAPDs and two-dimensional gel electrophoresis of proteins (2-DGE) is compared. Chapter 6 describes the influence of random genetic drift on the secondary founders of *G. pallida* in The Netherlands. In chapter 7 the quantitative variation in protein polymorphism, as revealed by 2-DGE, and virulence towards two resistant potato cultivars are compared by studying 102 *G. pallida* populations from The Netherlands.

chapter 2

Single juveniles of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* differentiated by randomly amplified polymorphic DNA

abstract

Random amplified polymorphic DNA (RAPD) offers a potential basis for the development of a diagnostic assay to differentiate the potato cyst nematode species *Globodera rostochiensis* and *G. pallida*. Nine decamer primers have been tested for their ability to amplify species specific DNA sequences. Primer OPG-05 produced two discrete DNA fragments which were consistently present in five *G. rostochiensis* populations and absent in five *G. pallida* populations. These fragments were detectable in single females as well as in single second stage juveniles. Their amplification is extremely efficient and reproducible over a wide range of template concentrations. One fifth of a single juvenile is sufficient to generate reproducible RAPD markers. The amplification from single juveniles requires no DNA isolation. The use of a crude homogenate does not impair the polymerase chain reaction.

introduction

The potato cyst nematodes *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone are morphologically nearly indistinguishable and were until 1973 considered as races of *Heterodera rostochiensis* (Stone, 1973). Both species can be divided into pathotypes, which are defined by their ability to multiply on a standard set of resistant cultivars (Kort *et al.*, 1978). Currently eight pathotypes are recognized, five within *G. rostochiensis* (Ro1-Ro5) and three within *G. pallida* (Pa1-Pa3).

Because both species have their own virulence spectra, a proper identification is important in the control of potato cyst nematodes by means of resistance. Morphological characterisation is laborious and requires specialist knowledge for accurate identification. Therefore, a variety of biochemical techniques has been used to distinguish the sibling species (Fox & Atkinson, 1986). Protein analysis by isoelectric focusing (Fox & Atkinson, 1984), sodium dodecyl sulphate (SDS) electrophoresis (Bakker *et al.*, 1988), and high resolution two dimensional gel electrophoresis (Bakker & Bouwman-Smits, 1988a) revealed that *G. rostochiensis* and *G. pallida* are quite distinct at the protein level. A number of species specific proteins appeared to be homologous, abundant and thermostable and these were used to develop an enzyme linked immunosorbent assay (ELISA) with monoclonal antibodies for routine species identification (Schots *et al.*, 1989).

The extensive genetic differentiation of the sibling species on the basis of proteins was con-

firmed by analysing restriction fragment length polymorphisms (Burrows & Boffey, 1986; De Jong *et al.*, 1989; Burrows, 1990; Schnick *et al.*, 1990). Species specific DNA probes allowed the differentiation of single cysts of *G. rostochiensis* and *G. pallida* (Stratford *et al.*, 1992).

Another approach to develop sensitive detection systems is based on the amplification of DNA sequences by the polymerase chain reaction (PCR) with specific primers (Harris *et al.*, 1990) or arbitrary primers (Welsh & McClelland, 1990; Williams *et al.*, 1990). The latter procedure permits the detection of genetic markers for genome mapping and diagnostic purposes using random amplified polymorphic DNA (RAPD). The RAPD technique seems a promising approach to distinguish plant parasitic nematode species and has already been applied to discriminate two *Heterodera* species by using single cysts containing approximately 100 juveniles (Caswell-Chen *et al.*, 1992).

In this report a RAPD-PCR method is presented, which enables the differentiation of single juveniles and females of *G. rostochiensis* and *G. pallida*.

materials and methods

nematodes

Five populations of *G. rostochiensis* and five populations of *G. pallida* were obtained from different localities. The *G. rostochiensis* populations (no.1 - no.5) were kindly supplied by J. Bakker, Plant Protection Service, Wageningen, The Netherlands and the *G. rostochiensis* population Harmerz (no.11) was obtained from dr. H.J. Rumpenhorst, Department of Nematology, Münster, Germany. The *G. pallida* populations (no.6 - no.10) were supplied by H.J. Vinke, Centre for Plant Breeding and Reproduction Research (CPRO), Wageningen, The Netherlands. These populations are designated as follows in the original collection : Ro1 A50 (no.1), Ro1 Mierenbos (no.2), Ro3 C286 (no.3), Ro3 C294 (no.4), Ro4 F539 (no.5), Ro5 Harmerz (no.11), Pa2 D350 (no.6), Pa2 D383 (no.7), Pa2 1095 (no.8), Pa3 1077 (no.9) and Pa3 Rookmaker (no.10).

The populations were maintained on *Solanum tuberosum* ssp. *tuberosum* L. Eigenheimer, susceptible to all pathotypes. Clay pots of 1 L volume were filled with a sandy loam soil and inoculated with approximately 200 cysts. The pots were placed in a growth chamber at 18°C and 16h daylength. Young white females were harvested 35 days after inoculation by gently washing the roots. They were separated from root debris by sieving (0.75 mm pore diameter) and centrifugation in a 35 % sucrose solution at 1000 g for 5 minutes. The floating females were collected, rinsed with tap water and remaining root debris was removed manually under a dissecting microscope. The females were stored in 0.25 g aliquots at -80°C until use.

Second stage juveniles were obtained by crushing eggs from wet young cysts in distilled water (Janssen *et al.*, 1987) or by hatching from 1-year-old dried cysts in potato root diffusate.

DNA isolation

Genomic DNA was isolated from approximately 6000 young females (250 mg) according to De Jong *et al.* (1989) with the following modifications. After proteinase K digestion, one phenol extraction was performed to remove fatty components. The subsequent phenol and chloroform extractions were replaced by a salt extraction procedure by adding 1/3 volume 6M NaCl and centrifugation at 10,000 g for 5 minutes.

DNA was precipitated by addition of one volume isopropanol, washed twice with 70% ethanol, dried under vacuum, and resuspended in approximately 50 µl 10 mM Tris-HCl, 1 mM EDTA pH 7.5. Finally, RNase treatment (20 mg RNase A) was performed at 37°C for 30 minutes. Aliquots of the DNA solutions were mixed with a fluorescent dye (Hoechst 33258) and DNA concentrations were determined with a mini-Fluorometer (TKO-100, Hoefer Scientific Instruments U.S.A.).

DNA from single females was isolated by using a slight modification of the sperm lysis procedure described by Li *et al.* (1988). One female was transferred to a miniature glass mortar (Karl-Heinz Müller KG, Hannover Münden, Germany) containing 5 µl of lysis buffer and homogenized with a small glass pestle. The lysis buffer consisted of PCR reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.1% Triton X-100) and 0.1 mg/ml proteinase K, 20 mM dithiothreitol (DTT) and 1.7 mM sodium dodecyl sulphate (SDS). The 5 µl homogenate was transferred to a fresh microcentrifuge tube and the pestle and mortar were rinsed with another 20 µl of lysis buffer. Subsequently the homogenate with a total volume of 25 µl was incubated for one hour at 37°C and for five minutes at 85°C. Remaining cell debris was removed by centrifuging 5 minutes at 10,000 g. Twenty µl of the supernatant were used for amplification reactions.

Single juveniles were transferred to a series of miniature glass mortars and homogenized in 2 µl distilled water. The crude DNA extract was suspended in 100 µl distilled water and 20 µl was directly used in the RAPD assay.

primers

The oligonucleotide decamer primers were purchased from Operon Technologies Inc. (Alamada, U.S.A.). The lyophilized primers were dissolved in 10 mM Tris-HCl, 1 mM EDTA pH 7.2 at a (stock)concentration of 0.5 mg/ml. The primers are listed in Table 2.1.

Table 2.1
Primers tested for PCR amplification of genomic DNA of *G. rostochiensis* and *G. pallida*.

primer	*sequence*	%GC
OPE-04	5'-GTGACATGCC-3'	60
OPG-02	5'-GGCACTGAGG-3'	70
OPG-03	5'-GAGCCCTCCA-3'	70
OPG-04	5'-AGCGTGTCTG-3'	60
OPG-05	5'-CTGAGACGGA-3'	60
OPG-06	5'-GTGCCTAACC-3'	60
OPG-07	5'-GAACCTGCGG-3'	70
OPG-08	5'-TCACGTCCAC-3'	60
OPG-09	5'-CTGACGTCAC-3'	60

PCR technique

Amplification reactions were performed in 50 µl reactions containing final concentrations of 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia LKB), 50 ng primer, 1.25 U Taq DNA Polymerase (Sphaero Q, Leiden, The Netherlands) and 3 ng genomic DNA. Control reactions were essential 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 carried out in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 45 cycles after initial denaturation for 4 minutes at 94°C. Each cycle consisted of 1 min

at 94°C, 2 min at 38°C and 3 min at 72°C. The fastest available transition between each temperature was used, except for the transition between 38°C and 72°C which had an increase of 1°C per 5 sec. Twenty µl aliquots of the amplification products were loaded on a 1% agarose gel, separated by electrophoresis (5 V cm^{-1} for 2.5 hours) and detected by ethidium bromide staining.

results

Initial experiments showed that the reaction conditions described by Williams *et al.* (1990) were not suitable for the amplification of DNA fragments from potato cyst nematodes. The PCR technique was optimized using total genomic DNA extracts from females. Nine primers (Table 2.1) were used to amplify DNA fragments from females of five *G. rostochiensis* populations and five *G. pallida* populations. Template DNA was isolated from a batch of approximately 6000 females per population. The nine primers revealed a total of 148 fragments of which only 5 fragments were common to the *G. rostochiensis* and *G. pallida* populations. Although the sibling species were discriminated by the majority of the RAPD fragments, most primers (*e.g.* OPG-04, OPG-06, OPG-07, OPG-09) were not suitable for diagnostic purposes because of the extensive intraspecific variation of their amplification products (data not shown).

An additional complication was the influence of the template concentration on the polymerase chain reaction. For example, varying the template concentrations from 0.1 ng to 50 ng per 50 µl reaction volume for primer OPG-02 resulted in different amplification products (Fig. 2.1). Some of these fragments were hardly visible when low concentrations were used and increased rapidly in intensity when template concentrations were raised. The amplification of other fragments was negatively correlated with the template concentration.

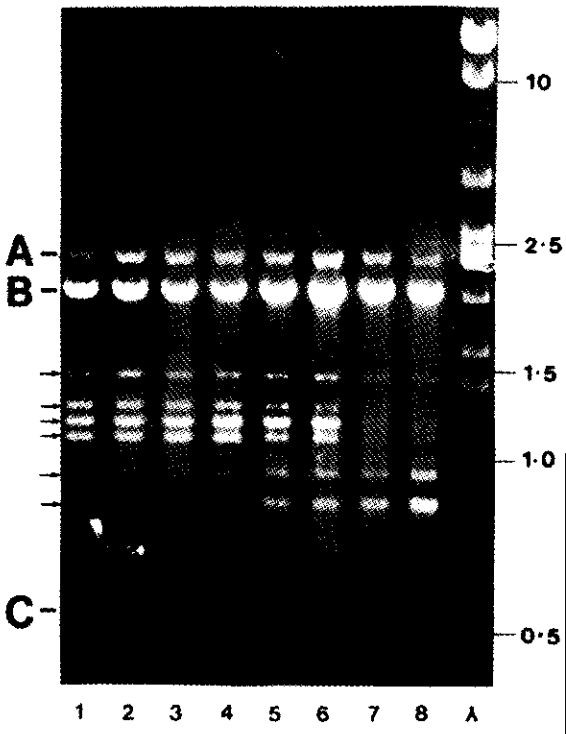

Figure 2.1

Effect of template concentration on the amplification of fragments from genomic DNA from batches of females of G. rostochiensis *(population no. 11) with primer OPG-02. DNA concentrations in lane 1 - 8 are respectively 0.1, 0.5, 1.0, 2.5, 5.0, 10, 25 and 50 ng per 50 µl reaction volume. Arrows indicate PCR fragments of which the appearance is positively or negatively correlated with the concentration of genomic DNA. Lane λ represents Eco RI\Bam HI\Hind III digested lambda DNA as a standard for molecular weights which are given in kilobase pairs.*

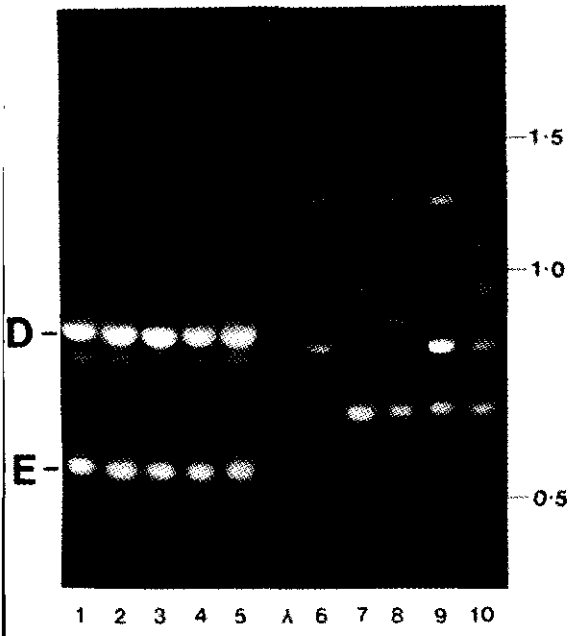

Figure 2.2
Amplification of genomic DNA from batches of females of five G. rostochiensis *populations (lane 1 - 5) and five* G. pallida *populations (lane 6 - 10) with primer OPG-05. For each reaction 3 ng genomic DNA was used. Fragment D and E are specific for* G. rostochiensis. *Lane λ represents EcoRI\BamHI\HindIII digested lamba DNA as a standard for molecular weights which are given in kilobase pairs.*

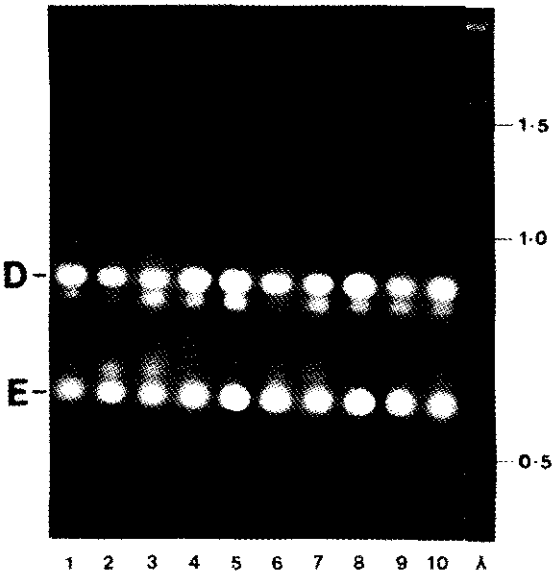

Figure 2.3
Amplification of fragment D and E from two juveniles of G. rostochiensis *with primer OPG-05. Five replicates of one juvenile are shown on the left (lane 1 - 5) and of another juvenile on the right (lane 6 - 10). The two juveniles were obtained from population no. 11. For each amplification one-fifth of a crude homogenate of a juvenile was used. Lane λ represents EcoRI\BamHI\HindIII digested lamba DNA as a standard for molecular weights which are given in kilobase pairs.*

With primer OPG-05 two distinct DNA fragments (D and E, Fig. 2.2) were resolved which were consistently present in the five *G. rostochiensis* populations and absent in the five *G. pallida* populations. Their amplification was not affected by variations in template concentrations. High (Fig. 2.2) and low (Fig. 2.3) template concentrations revealed identical patterns. Even one-fifth of a homogenate of a single juvenile was sufficient to obtain a reproducible pattern (Fig. 2.3). Similar results were obtained with single females (data not shown).

The suitability of primer OPG-05 to identify individuals was evaluated by analysing 120 juveniles. Fragments D and E were observed in all 80 juveniles tested from *G. rostochiensis*. None of the RAPD patterns of the 40 juveniles sampled from *G. pallida* contained fragments similar to D and E. Examples of the amplification of fragments D and E from 10 different individuals of G. rostochiensis are shown in Fig. 2.4.

No DNA isolation is required for the identification of single juveniles. The PCR mixture could be added directly to the crude homogenate. This simple procedure is not applicable to single females, probably due to undesirable substances in the homogenate which interfere with the amplification reaction.

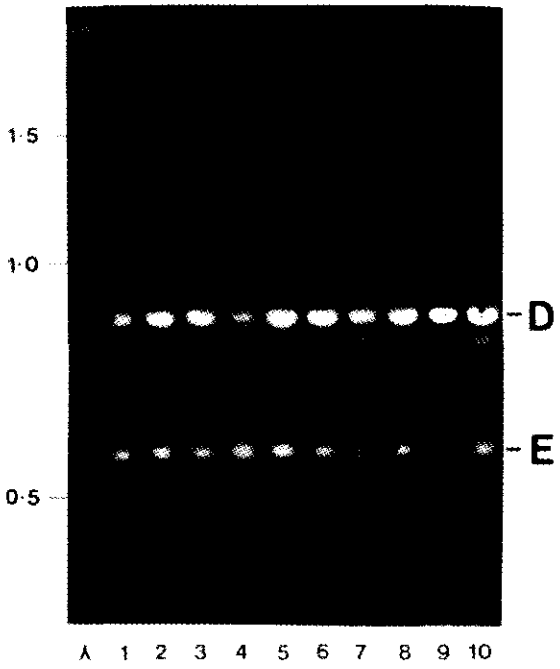

Figure 2.4
Amplification of fragment D and E using primer OPG-05 from ten different juveniles (lane 1 - 10) of G. rostochiensis. *For each amplification one-fifth of a crude homogenate of a juvenile from population no. 2 was used. Lane λ represents EcoRI\BamHI\HindIII digested lamba DNA as a standard for molecular weights which are given in kilobase pairs.*

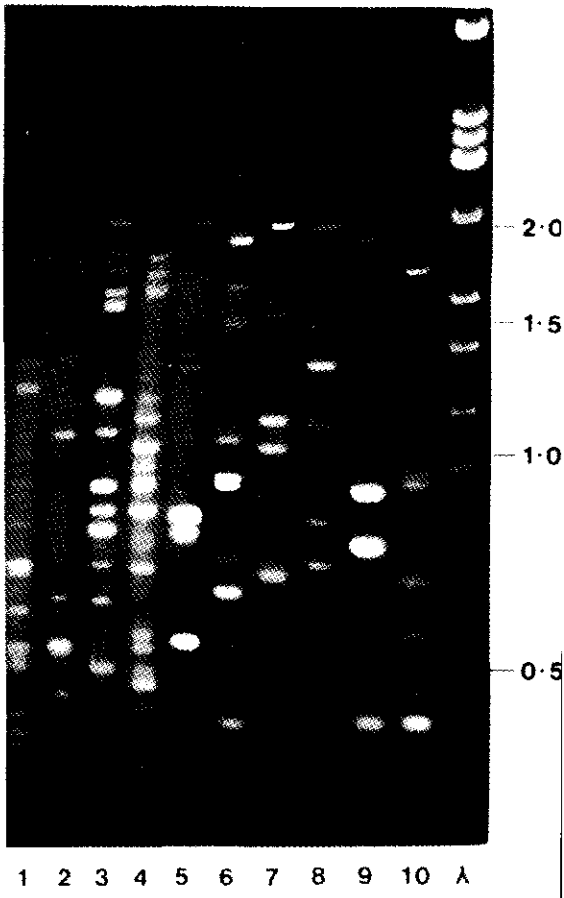

Figure 2.5
Five RAPD patterns from a single juvenile of G. rostochiensis *(lanes 1, 3, 5, 7 and 9) and five RAPD patterns from a single juvenile of* G. pallida *(lanes 2, 4, 6, 8 and 10). The patterns were obtained with OPG-02 (lane 1,2), OPG-03 (lane 3,4), OPG-05 (lane 5,6), OPG-08 (lane 7,8) and OPE-04 (lane 9,10). For each amplification one-fifth of a crude homogenate of one juvenile was used. The* G. rostochiensis *and* G. pallida *juvenile were obtained from population no. 1 and no. 7, respectively. Lane l represents EcoRI\BamHI\HindIII digested lamba DNA as a standard for molecular weights which are given in kilobase pairs.*

The potential of the RAPD technique to discriminate between individuals of *G. rostochiensis* and *G. pallida* is illustrated in Fig. 2.5. Comparison of one *G. rostochiensis* individual of population no. 1 and one *G. pallida* individual of population no. 7 with five primers revealed 94 bands. Only seven out of the 94 RAPD fragments were common to both juveniles.
Thirty three fragments were also amplified when higher genomic template concentrations (up to 10 ng) from batches of females were used.

discussion

The polymerase chain reaction in combination with arbitrary primers is suitable for the discrimination of *G. rostochiensis* and *G. pallida*. Fragments D and E, revealed by RAPD-PCR with primer OPG-05, are excellent diagnostic characters for females and juveniles of *G. rostochiensis*. No intraspecific variation in these fragments is observed between and within the *G. rostochiensis* populations.

Various RAPD markers are vulnerable to changing experimental conditions. Differences in the template concentration, the source and purity of the DNA, may result in distinct fingerprints. For example, a crude homogenate of one *G. rostochiensis* and one *G. pallida* juvenile (Fig. 2.5) revealed a total of 94 DNA fragments, of which only 33 were resolved when higher template concentrations from batches of females were used. To obtain versatile and reproducible markers, we selected RAPD fragments which are hardly affected by template concentrations and the DNA extraction procedure. Fragments D and E (Fig. 2.2, Fig. 2.3, Fig. 2.4 & Fig. 2.5) can be amplified from crude homogenates of single juveniles and DNA extracted from single females. Also high template concentrations (up to 10 ng) from batches of females allowed a proper amplification.

The observation that even 1/5 of a juvenile is sufficient to reveal a clear RAPD pattern (Fig. 2.3, Fig. 2.4 & Fig. 2.5) indicates that the amplification with arbitrary primers requires only a small number of templates. Juveniles of *Caenorhabditis elegans* contain less than 1000 nuclei (Wood, 1988) and therefore it may be assumed that the number of nuclei in potato cyst nematode juveniles is also of that order. Since potato cyst nematodes are diploid organisms, this implies that 1/5 of a homogenate of a juvenile probably contain less than 400 copies of each chromosome. In addition, the vast majority of the RAPD fragments originate from low-copy sequences. Hybridization of total genomic DNA of *G. rostochiensis* to 58 RAPD fragments by reverse Southern blotting (Chang *et al.*, 1988) revealed only 12 repetitive signals (Roosien, unpublished data). This indicates that the complexity of the genome of potato cyst nematodes seems similar to other species within the Heteroderidae (Pableo & Triantaphyllou, 1989).

The sibling species *G. rostochiensis* and *G. pallida* may have diverged millions of years ago and accumulated numerous molecular differences without major morphological changes (Bakker & Bouwman-Smits, 1988a). This explains the extensive genetic differentiation measured with the RAPD technique in spite of their morphological similarity. Testing nine RAPD primers on genomic DNA from batches of females of five *G. rostochiensis* and five *G. pallida* populations revealed a total of 148 fragments. Five fragments were common to both species and 143 occurred in one or more populations of either *G. rostochiensis* or *G. pallida*. This high level of genetic variation between the siblings is in agreement with other molecular data. For example, high resolution two dimensional gel electrophoresis resolved approximately 250 gene products and showed that the sibling species shared only 30 % of their polypeptides (Bakker & Bouwman-Smits, 1988a).

Previous studies have already shown that DNA techniques are suitable to differentiate nematode species by using small amounts of nematological material (Burrows, 1990; Harris *et al.*, 1990; Stratford *et al.*, 1992; Caswell-Chen *et al.*, 1992). In this report we developed a RAPD method which allows the identification of species by using single individuals. A major additional advantage of the RAPD technique is that it provides opportunities to analyse intraspecific variation. Based on crosses between

homozygous virulent and avirulent potato cyst nematodes (Janssen *et al.*, 1990), linkage studies between RAPD markers and virulence genes may result in diagnostic assays to pathotype field populations and may lead to the characterisation of virulence genes. The ability to assess genetic variation between single individuals will probably also find applications in the epidemiology and ecology of plant parasitic nematodes.

chapter 3

Inter and intraspecific variation between populations of *Globodera rostochiensis* and *G. pallida* revealed by random amplified polymorphic DNA

abstract

The genetic relationships between populations of the potato cyst nematode species *Globodera rostochiensis* and *G. pallida* were analysed using random amplified polymorphic DNA (RAPD). Only nine out 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 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.

introduction

The potato cyst nematodes, *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone are successful colonizers in temperate zones of the world (Evans *et al.*, 1977). Both sibling species originate from the Andes region of South America and were probably introduced into Europe after 1850 (Evans *et al.*, 1975). From the sites of introduction, primary founders dispersed mainly by passive spread (Evans *et al.*, 1975).

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 (Kort *et al.*, 1977). These are defined by their multiplication rates on a standard set of differentials with resistance derived from *Solanum tuberosum* ssp *andigena* Juz. and Buk., *S. kurtzianum* Bitt. and Wittm. hybr. 60.21.19, *S. multidissectum* Hawkes hybr. P55/7, and several *S. vernei* Bitt. and Wittm. accessions.

Currently eight pathotypes are recognized in Europe by this international pathotype scheme, five within *G. rostochiensis* (Ro1-Ro5) and three within *G. pallida* (Pa1-Pa3) (Kort *et al.*, 1977). The international pathotype scheme is not capable of revealing the genetic diversity that has been introduced into Europe (Bakker *et al.*, 1992). Additional pathotypes were recognized by using extra differentials (Canto Saenz & De Scurrah, 1977). 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 (Bakker *et al.*, 1992; Dale & Phillips, 1985; Phillips, 1985).

Differences in proteins between *G. rostochiensis* and *G. pallida* were analysed with a variety of electrophoretic techniques (Bakker & Bouwman-Smits, 1988b; Fox & Atkinson, 1986). The discrimination between the sibling species is currently routine practise, with a serological assay using monoclonal antibodies raised against thermostable species-specific proteins (Schots *et al.*, 1990). Also on the DNA level, molecular differences were demonstrated with a number of approaches (*e.g.* Burrows, 1990; Burrows & Perry, 1988; De Jong *et al.*, 1989; Roosien *et al.*, 1993).

The intraspecific genetic variation of both species was assessed using isoelectric focussing (Fox & Atkinson, 1985) and two-dimensional gel electrophoresis of proteins (Bakker & Bouwman-Smits, 1988a; Bakker *et al.*, 1992) and isozyme analysis (Zaheer *et al.*, 1992). 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 (Phillips *et al.*, 1992; Schnick *et al.*, 1990; Stratford *et al.*, 1992). 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 (Welsh & McClelland, 1990; Williams *et al.*, 1990).

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 from different localities in the Netherlands (Table 3.1) and surveyed for variation in genomic DNA. Population A5, B5, C5, E5, A7, B7, C7, D7, E7, A9, B9, C9, D9, E9, A11, B11 were collected by the Plant Protection Service, Wageningen; A1, B1, C1, D1, E1, A3, B3, C3, D3 by the DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO), 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 cultivar *S. tuberosum* ssp. *tuberosum* L. Eigenheimer in a growth chamber at 18°C and 16 h daylength. They were harvested approximately 6 weeks after inoculation and aliquots of 250 mg air-dried females were stored at -80°C. No deterioration was observed at this temperature after 3 years. Genomic DNA was isolated according to De Jong *et al.* (1989) 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 mg RNase A at 37°C for 30 min. The DNA concentration was estimated

with a fluorometer (TKO 100, Hoefer Scientific Instruments), using a fluorescent dye (Hoechst 33258). At this stage the DNA was sufficiently pure for amplification experiments.

Table 3.1
The nine *G. rostochiensis* and 17 *G. pallida* populations used in this study, their pathotype classification and their site of collection in the Netherlands.

Species Code	*Pathotype*	*Location*
G. rostochiensis		
D7 C295	Ro3	Gramsbergen
E7 G1527	Ro3	Anlo
A9 C286	Ro3	Hoogeveen
B9 C293	Ro3	Hoogeveen
C9 C294	Ro3	Oosterhesselen
D9 A56	Ro1	Bergh
E9 A50	Ro1	Weert
A11 F539	Ro4	Emmen
B11 Mierenbos	Ro1	Wageningen
G. pallida		
A1 1095	Pa2	?
B1 P2-22	Pa2	Coevorden
C1 Rookmaker	Pa3	Valthe
D1 A75-250-39	Pa3	Gasselte
E1 1077	Pa3	Anjum
A3 1112	Pa3	Westerbork
B3 74-768-20	Pa3	Sleen
C3 75-884-4	Pa3	Vriezeveen
D3 1097	Pa3	Hardenberg
A5 D383	Pa2	Smilde
B5 D372	Pa2	Anlo
C5 D370	Pa2	Emmen
E5 D350	Pa2	Avereest
A7 D353	Pa2	Hardenberg
B7 D354	Pa2	Oosterhesselen
C7 D371	Pa2	Ommen
C11 HPL-1	Pa2	Veendam

RAPD PCR

The lyophilized oligonucleotide decamer primers (Operon Technologies, Alameda, USA), were resuspended in TE-buffer, pH 7.2 (Sambrook *et al.*, 1989) to a final concentration of 0.5 mg/ml. Before use, the primers were diluted to a concentration of 50 ng/ml. Table 3.2 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% gelatine; 200 mM of each dATP, dTTP, dCTP and dGTP (Pharmacia LKB, Sweden); 50 ng primer; 10 ng genomic DNA; and 0.5 U SuperTaq DNA polymerase (Sphaero Q, Leiden, The Netherlands). Control reactions were included to avoid misinterpretations of the RAPD patterns due to PCR artefacts. 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, 3 min at 72°C with a temperature ramp of 1°C per 5 sec for the 38°C to 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) (Sambrook *et al.*, 1989). Lambda DNA digested with the restriction enzymes BglII and HpaI (λ 2) or HindIII, BamHI and EcoRI (λ 3), was used as the molecular weight standard. The gels were stained with ethidium bromide (0.5 mg/ml) and photographed with Polaroid 665 film under UV light.

data analysis

In assessing the inter- and intraspecific variability, we: 1) scanned negative films with a video densitometer (Model 620 Bio-rad, Richmond, USA), 2) selected DNA fragments showing an Optical Density (O.D.) 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 O.D. smaller 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=2Nxy/(Nx+Ny) in which Nx and Ny refer to the number of DNA fragments generated by the RAPD assay in populations X and Y, respectively, and Nxy is the number of DNA fragments shared by the two populations (Nei & Li, 1979). Dendrograms were constructed from the F-values using the unweighted pair group method with arithmetic mean (UPGMA) in the Clustan3.2 VAX-VMS program (Sneath & Sokal, 1973).

Table 3.2
The oligonucleotide decamer primers used to assess the inter and intraspecific variation of *G. rostochiensis* and *G. pallida*. Indicated is the number of DNA fragments produced per primer.

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

results

The 14 primers listed in Table 3.2 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 (of which 10 were polymorphic) in nine *G. rostochiensis* populations and 16 DNA fragments (eight being polymorphic) in 17 *G. pallida* populations (Fig. 3.1).

Only nine DNA fragments were common to

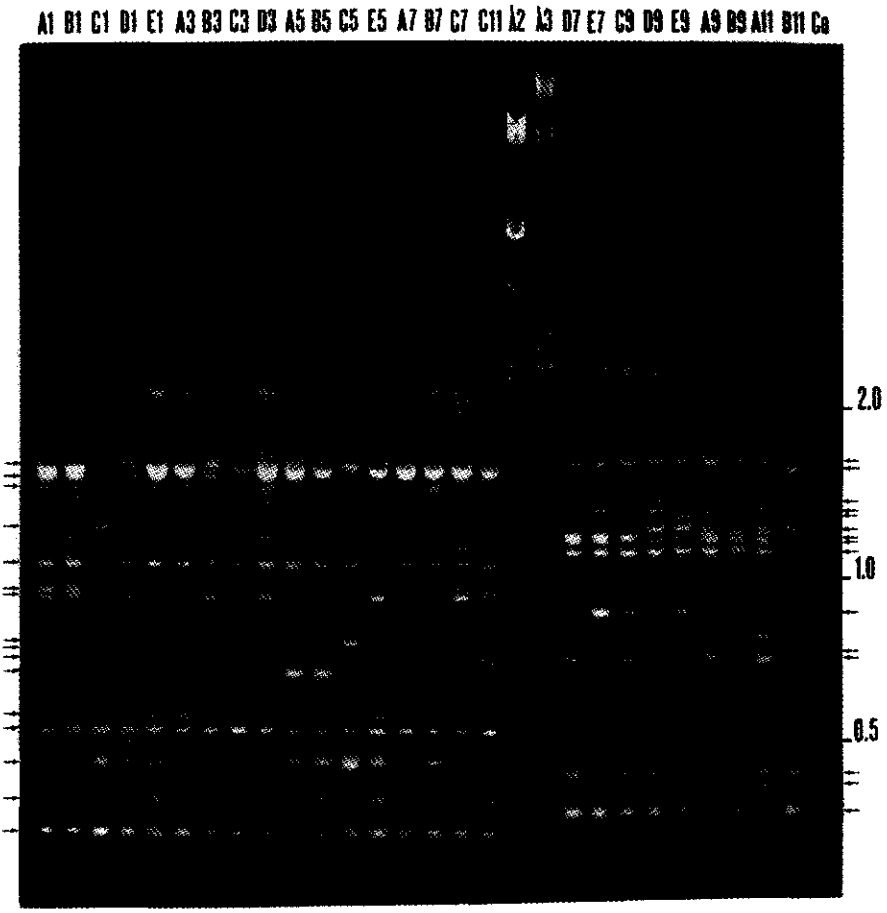

Figure 3.1

DNA fingerprints of 17 G. pallida *and nine* G. rostochiensis *populations after PCR with primer OPG-10. Indicated with arrows are 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 3.1; λ 2, phague λ DNA digested with BglII and HpaI; λ 3, phague λ DNA digested with HindIII, BamHI and EcoRI; lanes D7 - B11,* G. rostochiensis *populations coded according to Table 3.1; Co, control sample without template DNA. Molecular weights are indicated in kilobases (kb).*

all populations of both species. The F-value, expressing similarity between *G. rostochiensis* and *G. pallida*, averaged 0.052. Of the 105 DNA 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 of the *G. pallida* populations from 0.829 to 1.000 (av. 0.911).

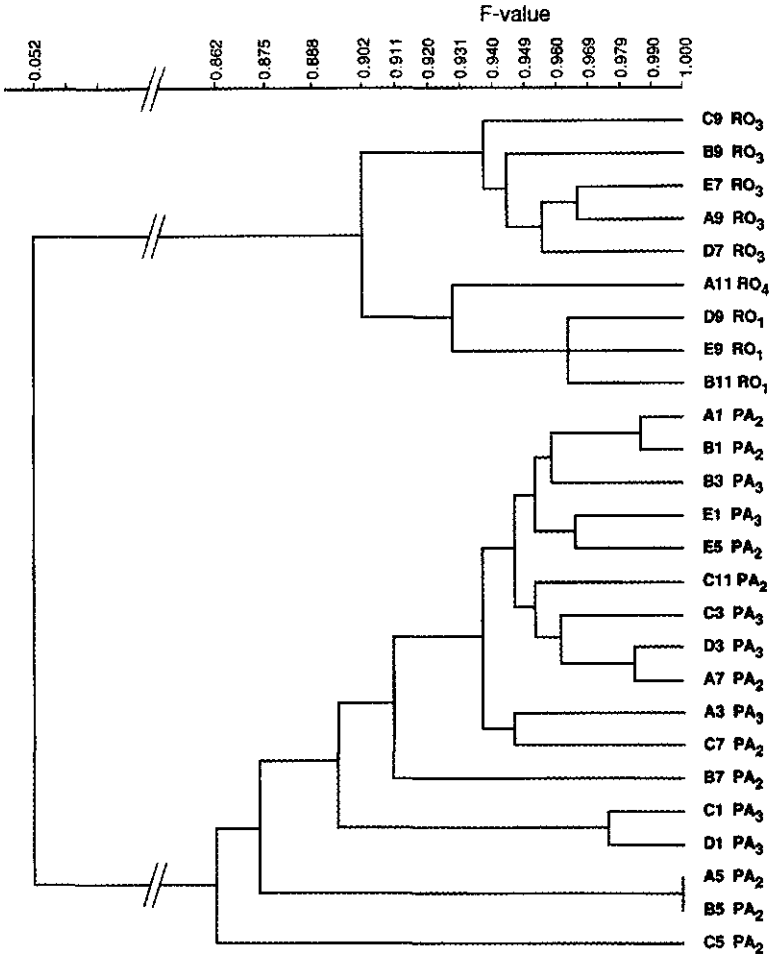

Figure 3.2
Similarity dendrogram of nine G. rostochiensis *and 17* G. pallida *populations constructed from the genetic similarity based on 241 RAPD DNA fragments, using UPGMA.*

The dendrogram of both the *G. rostochiensis* and *G. pallida* populations (Fig. 3.2) clearly express 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 Ro1 populations are characterized by fragment OPG-19$_{1391}$, a 1,391 bp sized DNA fragment amplified with primer OPG-19. Populations classified as Ro3 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 classification (Fig. 3.2). The genetic distance between populations with different pathotype classifications is often smaller than that between populations with the same pathotype classification. For example, populations E5 D350 and E1 1077, classified as Pa2 and Pa3, respectively, are clustered together in the dendrogram (Fig. 3.2). A number of DNA fragments were found to be unique for a *G. pallida* population or a cluster of populations (OPG-03$_{1248}$ for B7 D354; OPG-08$_{752}$, OPG-10$_{807}$, OPG-12$_{1874}$ and OPG-17$_{564}$ for C5 D370; OPG-02$_{745}$ for cluster C1 Rookmaker and D1 A75-250-39; OPG-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.) (Jones *et al.*, 1970). 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.* Bakker & Bouwman-Smits, 1988b; Fox & Atkinson, 1986) and RFLPs (Burrows & Perry, 1988; De Jong *et al.*, 1989; Stratford *et al.*, 1992).

Contrasts between morphological and molecular evolution are described for a wide variety of taxonomic groups, *e.g.* fish, frogs, reptiles, and snails (Wilson, 1976). Extensive molecular variation is also reported for a number of morphologically nearly indistinguishable nematode species, *e.g. Caenorhabditis elegans* Maupas and *C. briggsae* Dougherty and Nigon (Butler *et al.*, 1981) and *Brugia malayi* Brug and *B. pahangi* Buckley and Edison (McReynolds *et al.*, 1986). These findings indicate that genetic differentiation measured with molecular techniques is not necessarily correlated with the evolution of morphological characters (Kimura, 1983). Morphological evolution primarily depends on regulatory mutations that alter patterns of gene expression (King & Wilson, 1975). 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 (Mugniéry, 1979). This occurs only when both parental species show a similar gene regulation (Wilson, 1976).

Several groups (Demeke *et al.*, 1992; Goodwin & Annis, 1991) 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 (Caswell-Chen *et al.*, 1992; Demeke *et al.*, 1992; Goodwin & Annis, 1991; Kambhampati *et al.*, 1992). We agree with Chapco *et al.* (1992), that until a theoretical framework is developed for the variation generated by the RAPD assay, the statistic F=2Nxy/(Nx+Ny) (Nei & Li, 1979) 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 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) (Caswell -Chen *et al.*, 1992) and of populations of grasshopper species (*Melanoplus* spp.) (Chapco *et al.*, 1992). 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 (Bakker *et al.*, 1993). 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. Within both species distinct clusters were observed. The clustering of the populations 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 (on average 25x), exclude mutation to explain the observed intraspecific variation. Mutation rates vary from 10^{-4} - 10^{-6} per gene per gamete for most eukaryotic organisms (Ayala, 1976). 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 with 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* (Kort *et al.*, 1977). This number is too small to properly reflect the genetic variation of *G. pallida* populations in Europe (Bakker *et al.*, 1992). Also, the genetic variation in *G. pallida* seems to be larger than in *G. rostochiensis*. Our data are corroborated by Schnick *et al.* (1990) who reported smaller proportions of polymorphic DNA fragments from *G. rostochiensis* populations than from *G. pallida*.

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.

chapter 4

Gene pool similarities of potato cyst nematode populations assessed by AFLP analysis

abstract

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

introduction

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

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

the allele frequencies noticeably (Bakker *et al.*, 1993).

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

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

Intraspecific genetic variation of a large number of potato cyst nematode populations has been assessed by two-dimensional gel electrophoresis (2-DGE) of proteins (Bakker & Bouwman-Smits, 1988b; Bakker *et al.*, 1992), restriction fragment length polymorphisms (RFLPs) (Schnick *et al.*, 1990; Burgermeister *et al.*, 1992) and randomly amplified polymorphic DNA (RAPD) (Folkertsma *et al.*, 1994). Although these techniques are useful, several shortcomings should be mentioned. Both the 2-DGE technique and RFLP analysis are laborious and time consuming, and therefore unsuitable for large scale population analyses. RAPD analysis, a polymerase chain reaction (PCR)-based technique, enables the amplification of numerous markers (Williams *et al.*, 1990; Welsh & McClelland, 1990; Folkertsma *et al.*, 1994). However, a reliable estimate of relatedness between species or populations can seriously be hampered by preferential amplification of DNA fragments (Wilkerson *et al.*, 1993).

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

materials and methods

populations

Samples (> 100 cysts) of nine *G. rostochiensis* populations and fifteen *G. pallida* populations from different localities in the Netherlands (Table 4.1) were surveyed for variation in genomic DNA. The populations were obtained from the Plant Protection Service, Wageningen (population A5, B5, E5, A7, B7, C7, D7, E7, A9, B9, C9, D9, E9, A11, B11), the DLO Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Wageningen (population A1, B1, C1, D1, E1, A3, B3, C3, D3), and the Hilbrands Laboratory, Assen (population C11). The populations collected from heavily infested spots in the field were sampled before the growth of resistant cultivars. Populations were maintained in a greenhouse on potato cultivars susceptible to all pathotypes.

Table 4.1
The nine *Globodera rostochiensis* and 15 *G. pallida* populations used in this study, their pathotype classification and their site of collection in the Netherlands.

Code	*Species*	*Pathotype*	*Location*
D7 C295	*G. rostochiensis*	Ro3	Gramsbergen
E7 G1527	*G. rostochiensis*	Ro3	Anlo
A9 C286	*G. rostochiensis*	Ro3	Hoogeveen
B9 C293	*G. rostochiensis*	Ro3	Hoogeveen
C9 C294	*G. rostochiensis*	Ro3	Oosterhesselen
D9 A56	*G. rostochiensis*	Ro1	Bergh
E9 A50	*G. rostochiensis*	Ro1	Weert
A11 F539	*G. rostochiensis*	Ro4	Emmen
B11 Mierenbos	*G. rostochiensis*	Ro1	Wageningen
A1 1095	*G. pallida*	Pa2	?
B1 P2-22	*G. pallida*	Pa2	Coevorden
C1 Rookmaker	*G. pallida*	Pa3	Valthe
E1 1077	*G. pallida*	Pa3	Anjum
A3 1112	*G. pallida*	Pa3	Westerbork
B3 74-768-20	*G. pallida*	Pa3	Sleen
C3 75-884-4	*G. pallida*	Pa3	Vriezeveen
D3 1097	*G. pallida*	Pa3	Hardenberg
A5 D383	*G. pallida*	Pa2	Smilde
B5 D372	*G. pallida*	Pa2	Anlo
E5 D350	*G. pallida*	Pa2	Avereest
A7 D353	*G. pallida*	Pa2	Hardenberg
B7 D354	*G. pallida*	Pa2	Oosterhesselen
C7 D371	*G. pallida*	Pa2	Ommen
C11 HPL-1	*G. pallida*	Pa2	Veendam

isolation of genomic DNA

Prior to DNA extraction, adult white females were reared on the susceptible cultivar *Solanum tuberosum* ssp. *tuberosum* L. Eigenheimer in a growth chamber at 18°C and 16 h daylength. DNA samples from approximately 250 mg (fresh weight) adult females were essentially prepared as described by Roosien *et al.* (1993). The females were homogenized in proteinase K buffer (Sambrook *et al.*, 1989). A single phenol extraction was performed to remove fatty components. After an extraction with a 6 M NaCl solution (Roosien *et al.*, 1993), the DNA was precipitated by addition of one volume of isopropanol. The pellet was washed twice with 70% ethanol, dried under vacuum and resuspended in 50 µl TE buffer (pH 7.5) (Sambrook *et al.*, 1989). DNA samples were stored at 4°C.

AFLP procedure

1 generation and selection of fragments

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

5'-biotin-CTCGTAGACTGCGTACC
CTGACGCATGGTTAA

The sequence of the MseI-adapter was:

5'-GACGATGAGTCCTGAG
TACTCAGGACTCAT

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

2 non-selective amplification of template DNA

Non-selective amplification of secondary template was performed with primers complementary to the core of the adapter sequences. Bead suspension (5 µl) was mixed with 50 ng primer E+0 (5'-GACTGCGTACCAATTC), 50 ng primer M+0 (5'-GATGAGTCCTGAGTAA), 0.08 U Taq polymerase (Perkin Elmer, USA), 2 µl 10x PCR-buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin) and 0.8 µl 5 mM dNTPs in a final volume of 20 µl. The PCR reaction was performed in a PE-9600 thermal cycler (Perkin Elmer, Norwalk, USA) using the following temperature profile: 30 s at 94°C; 30 s at 56°C; 60 s at 72°C for 20 cycles.

To verify the production of secondary template, 12 µl of the PCR mixture was electrophorized on a 1% agarose gel in TAE buffer stained with 0.5 mg/ml ethidium bromide (Sambrook *et al.*, 1989). The lengths of the most abundant DNA fragments of the secondary template varied between 50 and 200 bp and was observed as a smear.

3 selective amplification of restriction fragments

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

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

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

data analysis

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

Among the polymorphic DNA fragments two subsets can be distinguished: presence/absence and band intensity polymorphisms.
Presence/absence polymorphisms result from:
1) the disappearance of a restriction site,
2) insertions or deletions between restriction sites, larger than approximately 50 nucleotides, or 3) non-complementarity between the selective nucleotides of the primer and the internal sequences of the restriction fragment. The latter can be illustrated by primer M+AC; in case of a mismatch at the annealing site due to a change from TG to, *e.g.*, TC, amplification of this restriction fragment is prevented. The intensities of the amplified bands will vary according to the proportion of non-mutated individuals within the population analyzed. Nevertheless, only the presence or absence of fragments of similar lengths was scored and, therefore, these polymorphisms are called qualitative polymorphisms.

Alternatively the lengths of the amplified sequences can change due to small insertions and/or deletions. AFLP patterns are complex and, therefore, only changes less than approximately 50 nucleotides could be detected. The central criterion for the identification of these length polymorphisms was the uniformity of the sums of the intensities of the corresponding bands, irrespective of whether the population under investigation was mono- or polymorphic at that particular locus. These polymorphisms were quantitatively scored by estimating the ratio between the intensities of the corresponding DNA fragments. The sums of the staining intensities of corresponding DNA fragments in a population was defined to be unity. Quantitative polymorphisms might represent putative alleles at one locus.

The presence or absence of a DNA fragment in a population was treated as a binary character. The data were converted to similarity values using the formula F=2Nxy/(Nx+Ny). Nx and Ny refer to the number of DNA fragments generated by the AFLP assay in populations X and Y, respectively, whereas Nxy is the number of DNA fragments shared by the two populations (Nei & Li, 1979). F-values were used to construct dendrograms with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in the Clustan32 VAX-VMS program (Sneath & Sokal, 1973). The presence or absence of DNA fragments was scored for all *G. rostochiensis* and *G. pallida* populations.

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

results

AFLP fingerprinting of nine *Globodera rostochiensis* and 15 *G. pallida* populations revealed a total number of 513 and 551 amplified DNA fragments, respectively. Twelve primer combinations were used to generate these fragments. Figure 4.1A shows fingerprints of the populations after PCR with primer combination E+GA/M+AC (For definitions, see Materials and Methods). Figure 4.1B shows fingerprints of the *G. rostochiensis* populations after PCR with primer E+GA in combination with M+AC or M+AG. It can be seen that one nucleotide change in the primer from set M caused a complete change of the AFLP fragment pattern. The number of amplified fragments ranged from 22 to 105 and depended on length of the extension at the 3'-end of the primer. An increase of the extension length reduced the number of amplified fragments for populations of both species. Under the reaction conditions described the size of the DNA fragments ranged from 50 to 500 bp.

Among the polymorphic DNA fragments two subsets can be distinguished: presence/absence and band intensity polymorphisms (see Materials and Methods).
In Fig. 4.1A both types of polymorphisms are illustrated. It is noted that the variation in the band intensities between duplicates was small.

variation among *G. rostochiensis* populations

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

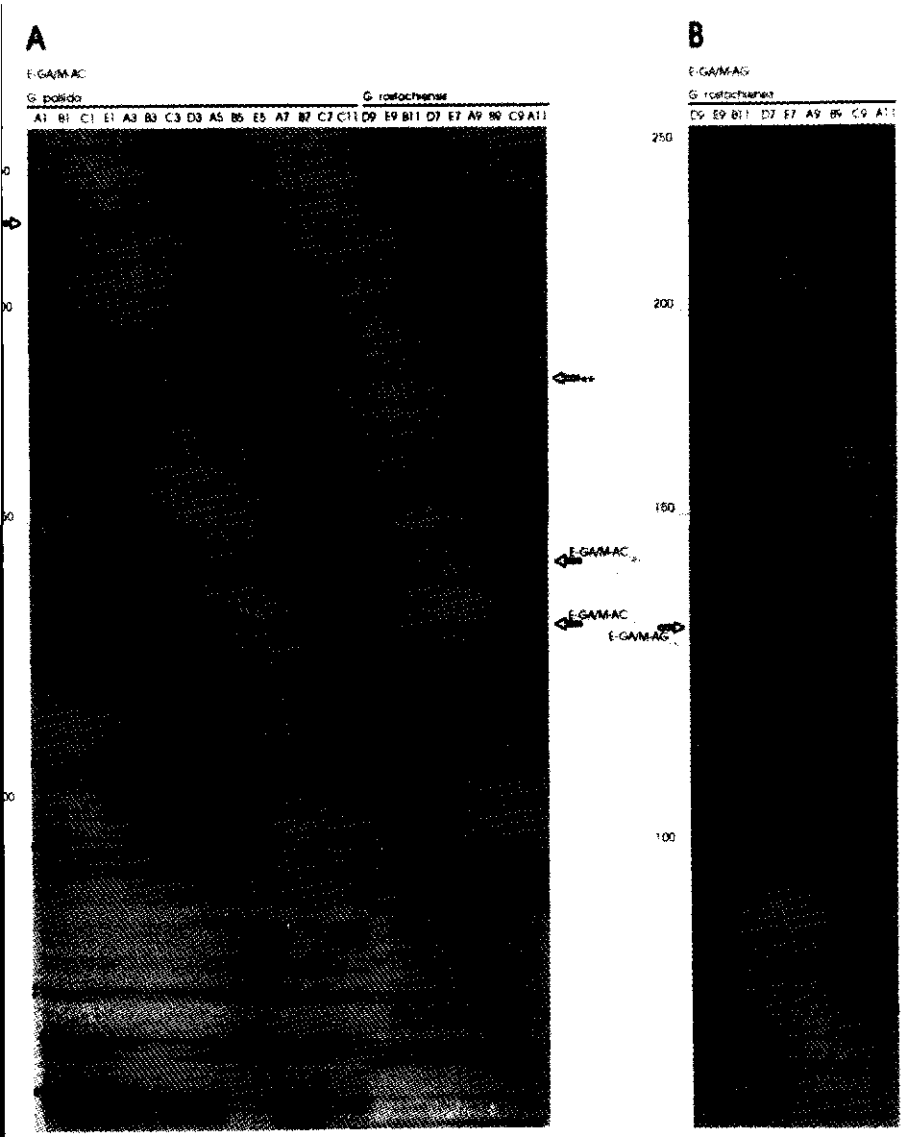

Figure 4.1

A: *AFLP fingerprints of nine* G. rostochiensis *and 15* G. pallida *populations after selective amplification with primer-combination E+GA/M+AC. Arrows with one and two asterisk(s) indicate a presence/absence polymorphism and two corresponding DNA fragments, respectively. Two Ro3 specific fragments, GA/AC-140 and GA/AC-130, are indicated with an arrow. Lanes A1-C11 represent the* G. pallida *populations encoded in Table 4.1; lanes D9-A11 represent the* G. rostochiensis *populations encoded in Table 4.1. Molecular weights are indicated in basepairs.*

B: *AFLP fingerprints of nine* G. rostochiensis *populations after selective amplification with primercombination E+GA/M+AG. One Ro1 specific fragment, GA/AG-130, is indicated with an arrow. Lanes D9-A11 represent the* G. rostochiensis *populations encoded in Table 1. Molecular weights are indicated in basepairs.*

variation among *G.pallida* populations

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

clustering of *G. rostochiensis* populations

Figure 4.2A shows a dendrogram of the investigated *G. rostochiensis* populations based on variants expressed by the presence or absence of DNA fragments. The populations were clustered into three groups which coincide with their pathotype classification as defined by Kort *et al.* (1977). The three groups were distinguished by three, seven and twelve unique DNA fragments, respectively. As an example, Figure 4.1B shows fragment GA/AG-130, a 130 bp sized DNA fragment that was amplified using primers E+GA and M+AG. This fragment was one of those specific for the Ro1 populations tested. Figure 4.1A shows also two fragments, GA/AC-140 and GA/AC-130, specifically present in the Ro3 populations tested. No Ro4 population specific fragments were amplified with the primer combinations E+GA/M+AG and E+GA/M+AC.

Cluster analysis of the similarities between nine *G. rostochiensis* populations based on quantitative polymorphisms, resulted in a dendrogram as depicted in Figure 4.2B. The overall topology of the dendrogram based on band intensity polymorphisms is similar to the one based on the presence or absence of DNA fragments.

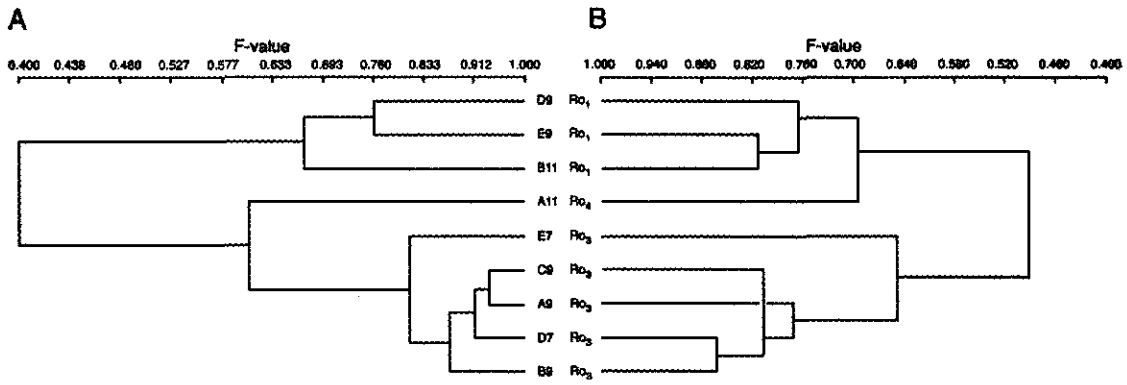

Figure 2

A: *The similarity dendrogram of nine* G. rostochiensis *populations based on presence/absence polymorphisms.*

B: *The similarity dendrogram of nine* G. rostochiensis *populations based on corresponding DNA fragments.*

clustering of *G. pallida* populations

Cluster analysis of the AFLP data of 15 *G. pallida* populations showed limited resemblance to their pathotype classification (Figure 4.2C). The distance between populations with different pathotype classifications is often smaller than between populations with the same classification. This is illustrated by the populations A7 D353 and D3 1097, classified as Pa2 and Pa3 respectively, that were clustered together in the dendrogram. Whilst all the populations could be differentiated on the basis of AFLP fragments, only a few were differentiated by specific fragments: cluster A1, B1 was specified by one, cluster A5 and B5 by four, population C1 by four, population A3 by one and population B3 by two specific AFLP fragments.

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

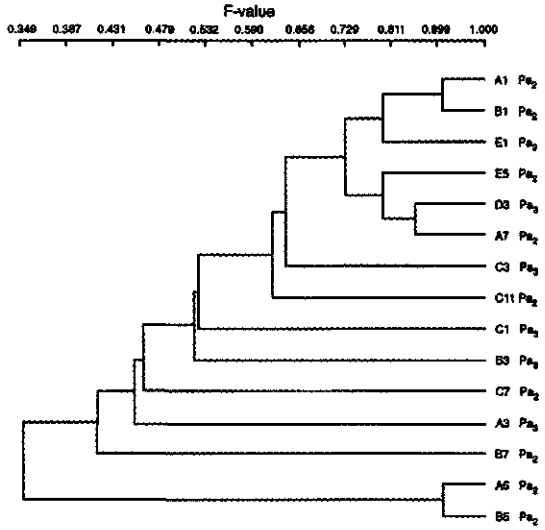

Figure 4.2
C: *The similarity dendrogram of 15* G. pallida *populations based on presence/absence polymorphisms.*

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

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

discussion

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

In case of the *G. rostochiensis* populations analyzed, close agreement was found between the dendrogram based on the presence or absence of AFLP markers and the one based on band intensity polymorphisms. Both data sets resulted in the discrimination of three similar groups containing three, five and one population, respectively. These were also identified in a previously described dendrogram based on RAPD data (Folkertsma *et al.*, 1994) and correspond to the pathotype classification of these populations. The Spearman rank correlation coefficient between the similarities based on AFLPs and RAPDs was 0.80. Hence, both types of DNA marker techniques result in similar classifications.

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

Agreement between the dendrograms based on AFLP and RAPD data (taken from Folkertsma *et al.* (1994)) was also found for the *G. pallida* populations analysed. This is illustrated by a Spearman rank correlation coefficient of 0.86. In contrast to *G. rostochiensis*, the clustering of the *G. pallida* populations revealed limited resemblance with their pathotype classification. Similar observations were previously made with 2-DGE (Bakker *et al.*, 1992), multilocus enzyme electrophoresis (Phillips *et al.*, 1992), RFLPs (Schnick *et al.*, 1990; Phillips *et al.*, 1992) and RAPD analyses (Folkertsma *et al.*, 1994). An explanation for this lack of concordance is the inadequacy of the pathotype scheme for *G. pallida*. A recently described major resistance locus from *S. tuberosum* ssp. *andigena* CPC 1673 (Arntzen *et al.*, 1993) discriminates the cluster A5-B5. This illustrates that degrees of similarities revealed by AFLP markers can also be indicative for virulence characteristics of *G. pallida* populations. An additional explanation for the observed incongruity between clusters of *G. pallida* populations based on molecular and virulence characteristics may be the structure of the genetic variation between *G. pallida* populations. The proportion of polymorphic DNA fragments among *G. rostochiensis* populations (15.8%) was low as compared to *G. pallida* (23.0%). This difference in the level of intraspecific variation of both species was also observed for RAPD loci (Folkertsma *et al.*, 1994). Although the proportion of polymorphic DNA fragments for *G. rostochiensis* populations was lower than for *G. pallida*, this smaller proportion allowed a clear clustering of the *G. rostochiensis* populations into three defined groups. A considerable portion of the polymorphic DNA fragments among *G. pallida* populations appeared to be scattered. This hampers an unambiguous classification of the *G. pallida* populations analysed.

The inability to cluster *G. pallida* populations may indicate a higher level of gene flow among the characterized *G. pallida* populations as

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

To differentiate conspecific populations with a high degree of overall similarity Avise (1975) and Ayala (1983) argued that frequency distributions of alleles at polymorphic loci are most effective. This has encouraged us to develop methods for *G. pallida* to identify AFLP loci at which frequencies of corresponding DNA fragments in populations could be determined. Currently, additional primer combinations are being tested to identify such fragments in individuals and populations.

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

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

The divergence between both sibling species determined by the AFLP analysis is in agreement with previous investigations (Bakker & Bouwman-Smits, 1988b; De Jong *et al.*, 1989; Folkertsma *et al.*, 1994). Both species share only 64 comigrating AFLP fragments on a total of 1000 which confirms their extensive differentiation at the molecular level. All comigrating AFLP fragments were monomorphic. It should be noted that both species are morphologically nearly identical and were, until 1970, considered as pathotypes of the species *Heterodera rostochiensis* Woll. (Jones *et al.*, 1970; Stone, 1973).

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

chapter 5

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

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. 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 Andes region in South America and were introduced into Europe after 1850 (Evans *et al.*, 1975; Evans & 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.* (1978). The scheme recognizes five *G. rostochiensis* (Ro1-Ro5) and three *G. pallida* (Pa1-Pa3) 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 & Phillips, 1995) and, recently, AFLPs (Folkertsma *et al.*, 1996b). 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.

material and methods

populations

Samples of 36 populations of *G. pallida* from different localities in The Netherlands, listed in Table 5.1, 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 *Solanum tuberosum* ssp. *tuberosum* L. 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 mg fresh weight) per population were prepared as described by Bakker & Bouwman-Smits (1988a). 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 (Chicago, California USA) power supply. 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 HCl (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 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.

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

Code		*Site of collection*
A1	1095	?
B1	P2-22	Coevorden
C1	Rookmaker	Valthe
D1	A75-250-39	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

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 OPG-02, OPG-03, OPG-04, OPG-05, OPG-06, OPG-09, OPG-10, OPG-11, OPG-12, OPG-13, OPG-15, OPG-16, OPG-17 and OPG-19 (Operon Technologies, Alameda, CA) were used. After amplification the reaction products were separated by electrophoresis in 1.0% agarose gels. Gels were stained with ethidium bromide (0.5 mg/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 isoelectric 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 & 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 & 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-pc version 1.80 (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 & Bouwman-Smits, 1988a, 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 (M1 and M4) at the M-locus and two alleles (N1 and N2) 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 Fig 5.1A 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.

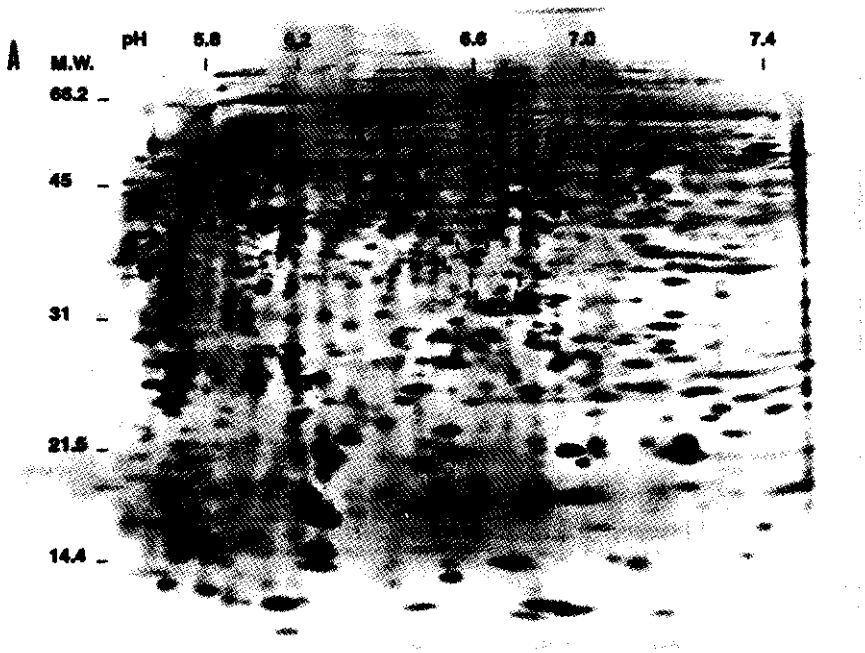

Figure 5.1

A: *Two-dimensional protein patterns of a mixture of 50 females from population C11 HPL-1 . 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 5.1
B: *Two-dimensional protein patterns of a mixture of 50 females from population 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.*

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 5.2A).

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 combina-

tions 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 5.2B).

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 5.2A and B.

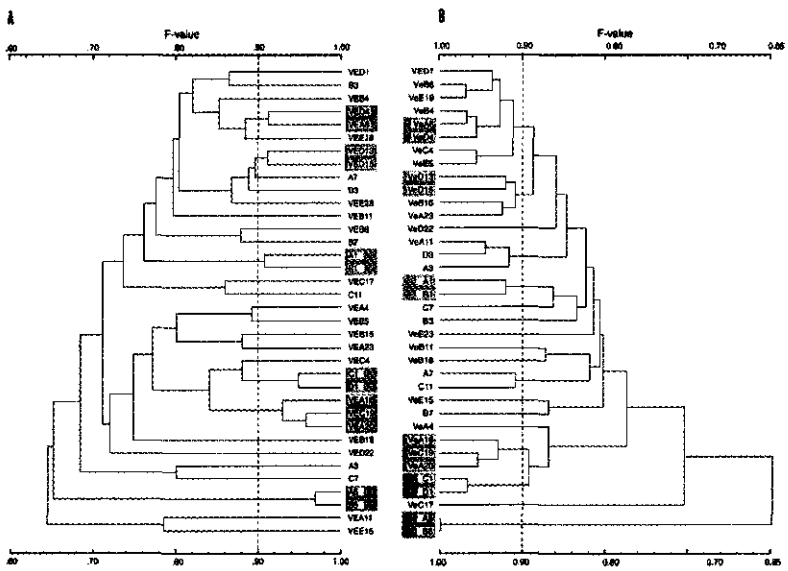

Figure 5.2
Similarity dendrograms of the 36 G. pallida *populations based on two-dimensional gel electrophoresis of proteins (A) and random amplified polymorphic DNA (B) data. Clusters of populations are discriminated at an F-value of 0.90. Shaded boxes indicate populations similar clustered using both 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 & Langley, 1979; Bakker & Bouwman-Smits, 1988a). Furthermore, Mickevich & 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 qualitatively 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.*, 1996b). 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 (*avrH1*) 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 *avrH1*. 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.

CHAPTER 6

Bottleneck effects on secondary founders of *Globodera pallida*

abstract

The genetic variation among 226 *Globodera pallida* populations from the Netherlands was studied by analysing the allele frequencies at 8 polymorphic loci revealed by two dimensional gel electrophoresis (2-DGE) of total proteins. The 2-DGE data indicate that the genetic diversity, directly or indirectly introduced from South-America has been limited and that the genetic variation among the Dutch *G. pallida* populations is predominantly the result of random genetic drift. The population bottlenecks have generated a continuous range of quantitative variation in allele frequencies. No clusters of populations with unique alleles are observed. Crop rotation schemes seem to have a significant influence on population structures. The heterozygosity level, the number of alleles per locus and the number of polymorphic loci were significantly lower in areas where the recovery of populations after a bottleneck is retarded, among others by wider crop schemes.

introduction

A considerable part of the economically important plant parasitic nematode species causes serious losses outside its centre of origin. For example, *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease in Japan has been introduced from North-America (De Guiran & Bruguier, 1989; Tarès *et al.*, 1994). *Heterodera glycines*, a major problem in soybean cultures in the US and Canada was presumably introduced from Japan (Riggs, 1977). *Globodera rostochiensis* and *G. pallida*, both originating from the Andes region in South-America, have been spread to potato growing areas all over the world (Evans & Stone, 1977). The numerous secondary founding events by which areas are colonized are presumbly accompanied by various bottlenecks. Control measures, such as the application of nematicides or the growth of resistant cultivars, are probably also responsible for significant reductions in population sizes. All these processes may result in changes in allele frequencies caused by random genetic drift. Although random genetic drift is probably one of the major forces determining the genetic makeup of plant parasitic nematode populations, hardly no 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, among others, of importance in developing control strategies based on host plant resistance. Control by means of 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 on suitable resistance, iii) diagnostic assays to pathotype field populations enabling growers to select the appropriate resistant cultivar. In many situations these requirements are difficult to achieve because of an apparently continuous range of variation in virulent genotypes for various resistance genes. The present view is

that drift plays a dominant role in these spatial variation in virulence. Nematode populations are often not fixed for their avirulence and virulence genes, which offers ample opportunity for random genetic drift to operate.

The effect of bottlenecks on the genetic variation within and between populations can be studied with the aid of molecular techniques. Lewontin and coworkers were among the first to study genetic variation by allozyme electrophoresis on numerous single individuals (Hubby & Lewontin, 1966). By doing so, differences in allele frequencies between natural populations of *Drosophila pseudoobscuris* could be attributed to recent founder events (Lewontin & Hubby, 1966; Prakash *et al.*, 1969). Although a substantial number of studies report on the molecular variation between plant parasitic nematode populations, none of these studies considered the use of allele frequencies to study genetic variation. Both at the protein and DNA level, research was focussed on the identification of qualitative diagnostic characters (*e.g.* Schnick *et al.*, 1990; Phillips *et al.*, 1992; Shields *et al.*, 1996).

Estimating allele frequencies by analysing single nematodes is a tedious task and has only been accomplished in a few studies (Bossis & Rivoal, 1989, De Boer *et al.*, 1992). An alternative to monitor changes in allele frequencies between a large number of populations is to analyse pools of individuals with two dimensional gel electrophoresis of proteins (2-DGE). The ratios between the protein quantities produced by codominant alleles proved to be an appropriate measure for the allele frequencies. The accuracy of this method was confirmed by 2-DGE of single individuals (De Boer *et al.*, 1992).

In this report we studied the effects of bottlenecks on *G. pallida* in the Netherlands with 2-DGE by analyzing the allele frequencies at 8 putative loci. As with many other recently introduced colonizers, the genetic variation among these secondary founders is predominantly determined by two processes: the genetic constitution of the primary founders, directly or indirectly introduced from South-America, random genetic drift and gene flow. Mutation and selection are of minor importance in explaining the observed variations in proteins (Bakker *et al.*, 1993).

For various other species it has been shown that the level of heterozygosity and the degree of similarity decreases in the presence of bottlenecks. A commonly applied procedure to obtain insight in bottleneck effects on colonizing species is to compare the genetic variation of the primary and secondary founders with the variation present in their centre of origin (*e.g.* Demelo & Hebert, 1994; Balanya *et al.*, 1994). Such comparisons are difficult to establish for *G. pallida*, because of the patchy distribution patterns of plant parasitic nematodes, which hinders the selection of a representative set of source populations. In addition, the original locations in South America from where the potato cyst nematodes have been introduced are unknown.

To study the extent of random genetic drift, we analyzed the genetic differentiation of 226 Dutch *G. pallida* populations with 2-DGE. The rationale behind this approach is that, inherent in the way of spread, only a small number of initial populations have been introduced in the Netherlands and that large proportions of the 226 Dutch populations are related by descent. As a consequence, the role of random genetic drift can be inferred from the number of identical populations found.

In this report it is shown that the descendants of the primary founders have been subjected to extensive random genetic drift. In addition, it is demonstrated that the levels of heterozygosity, numbers of polymorphic loci and average genetic distances differ significantly between regions and can be related to bottleneck effects caused by crop rotation schemes and the length of the growing season.

materials and methods

populations

In the period 1990-1992, samples were taken from 226 *G. pallida*-infected sites in three regions in the Netherlands; north Friesland (29 populations), the IJsselmeerpolders (48 populations) and the northeast Netherlands (149 populations) (Figure 6.1). Most samples consisted of at least 100 cysts (with a minimum of 25).

In north Friesland and the IJsselmeerpolders AM-I was used to take samples. AM-I is a soil-sampling strategy designed to detect relatively small infestations. This method is in use for years and was published recently (Been & Schomaker, 1996). 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). Populations from the IJsselmeerpolders were placed at our disposal by the NAK (district 'Midden', Emmeloord), the Research Station for Arable Farming and Field Production of Vegetables (PAGV, Lelystad) and 'de Groene Vlieg BV' (Dronten).

Samples from the northeast Netherlands were obtained from the centres of heavily infested spots in the field. These populations were collected by members of the 'Landbouwvereniging Borgercompagnie', IPO-DLO (Wageningen), and the potato breeding institute 'Karna' (Valthermond). The standard population HPL-1, originating from the northeast Netherlands, was obtained from the Hilbrands Laboratory, Assen.

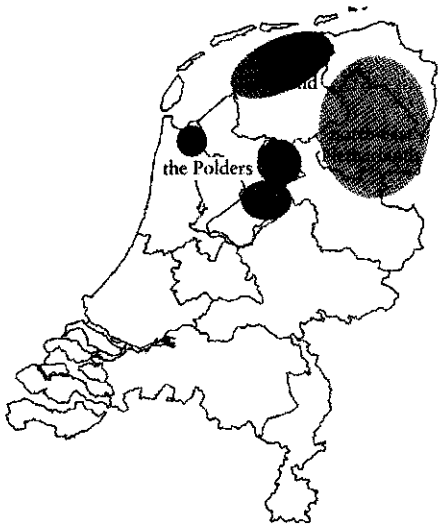

Figure 6.1
Map of the Netherlands.
Indicated are the three regions (north Friesland, the IJsselmeerpolders and northeast Netherlands) from where the G. pallida *populations were collected.*

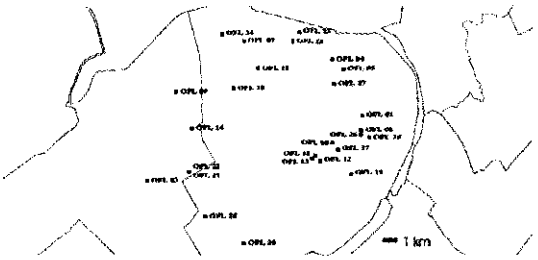

Figure 6.2
Map of the area in Oostelijk Flevoland, region the IJsselmeerpolders. Indicated are the approximate locations from where the 26 G. pallida *populations (OFL01-26) were collected.*

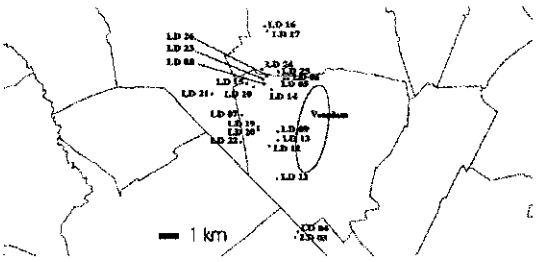

Figure 6.3
Map of the area near Veendam, region northeast Netherlands. Indicated are the approximate locations from where the 23 G. pallida *populations (LD03-26) were collected.*

origin of the populations

This paper compares the genetic diversity among *G. pallida* populations from three geographically separated potato growing regions in the Netherlands (Figure 6.1): north Friesland, the IJsselmeerpolders and the northeast Netherlands. *G. pallida* populations are exposed to different conditions in these regions.

In north Friesland and the IJsselmeerpolders mainly certified seed and ware potatoes are cultivated, whereas in the northeast Netherlands starch potatoes are prevalent (Anonymous, 1995). In starch potatoes, new cultivars harbouring different kinds of resistance towards potato cyst nematodes were commercialized earlier and grown on a larger scale (Anonymous, 1996). Furthermore, the cultivation frequency of potatoes in the northeast Netherlands is in general higher (every 2-3 years) than in north Friesland or the IJsselmeerpolders (every 3-4 years). The wide-scale application of nematicides in the northeast Netherlands, where sandy soils with substantial amounts of organic matter prevail, contributed to this more intensive cultivation. North Friesland and the IJsselmeerpolders have soils consisting of heavy marine clay and light marine clay, respectively. Efficient application of nematicides is hampered on such soils (Munnecke & Van Gundy, 1979).

The first reports on potato cyst nematodes in north Friesland date back to the fifties (Oostenbrink, 1950). Before the growth of cultivars with the *H1* resistance gene, most of the potato cyst nematode populations (70%) were pathotyped Ro1 in the Netherlands (Hietbrink, pers. comm.). Nevertheless, the introduction of *H1*-harbouring cultivars in the fifties did not contribute to a reduction of potato cyst nematode populations in north Friesland (Hietbrink, pers. comm.). Nowadays, most potato cyst nematode populations in north Friesland are diagnosed *G. pallida*. This suggests that *G. pallida* has been the predominant species in this region since the first reports of potato cyst nematodes. It is noted that infection sites are generally small.

The IJsselmeerpolders, including the Wieringermeer, the Noordoost-polder and Oostelijk Flevoland, represent virgin soils that were reclaimed in 1934, 1942 and 1958, respectively. The first reports on potato cyst nematodes date from 1967, 1964 and 1977 (J. Bakker, Plant Protection Service, pers. comm.). *G. rostochiensis* is the dominating potato cyst nematode species in this region, although *G. pallida* becomes increasingly abundant (Anonymous, 1994). The infection sites of potato cyst nematodes are still small.

The first observations of potato cyst nematodes in northeast Netherlands were reported in the forties (Oostenbrink, 1950). The introduction of cultivars with the *H1* resistance gene severely reduced most of the populations, indicating that a considerable part of these populations consisted of the pathotype Ro1 and Ro4 of *G. rostochiensis* (Kort, pers. comm.; Hietbrink, pers.comm.). However, from their introduction onwards, populations multiplying on cultivars with the *H1* resistance gene were observed as well. In the seventies *G. pallida* became the predominant cyst nematode species in this region (Van der Burgt *et al.*, 1995). In contrast to north Friesland and the IJsselmeerpolders, infection sites are often large in the northeast Netherlands, causing significant growth reduction of potato plants in the focal centres.

sample preparation and mini two-dimensional gel electrophoresis of proteins

Nematode populations were maintained on the susceptible cultivar *Solanum tuberosum* ssp. *tuberosum* L. Eigenheimer in a growth chamber at 18°C and 16 h day length. For protein extraction a total number of 50 adult white females per population were collected approximately 6 weeks after inoculation. At that stage, the white

females are supposed to be virgin, although in some instances males were observed. The air-dried females were stored at -80°C until use.

Protein samples from pooled females (~200 mg fresh weight) were prepared as described by Bakker & Bouwman-Smits (1988a). The protein samples were either stored at -80°C or immediately used for electrophoresis. Mini two-dimensional gel electrophoresis of proteins (2-DGE) was performed as described by De Boer *et al.* (1992) with some minor modifications (Folkertsma *et al.*, 1996a). Populations were electrophorized in series of eight (Folkertsma *et al.*, 1996a) always including population HPL-1. After staining, the gels were dried on a vacuum dryer (model 543, Bio-Rad, USA).

data collection

First, 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). This study examined the allele frequencies at the IP-variant loci C, F, H, I, J, N and M which were previously described by De Boer *et al.* (1992) and Folkertsma *et al.* (1996a). In this study we were able to identify two alleles (X1 and X2) at a previously undescribed locus X. Unlike De Boer *et al.* (1992) and Folkertsma *et al.* (1996a) we could not resolve both alleles at locus E unambiguously in all populations. A protein spot unrelated to locus E was constantly present at the position of E1, thus hampering the determination of the frequency of this allele.

Subsequently, the frequencies of the IP-variants for each population were estimated with the aid of an image analysis system. This system included a macroscope (Wild M420, Leica Heerburgg AG, Switzerland), a CCD camera (Sony, Japan), a camera control unit (DXC-750 P, Sony, Japan) and a Quantimet 570 (Cambridge Instruments Ltd, United Kingdom). 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 each IP-variant was determined by dividing the IOD of that variant by the total IOD of the corresponding IP-variants.

data analysis

1 quantification of the bias introduced by sampling errors and electrophoresis conditions

In the case of polymorphic loci, slight differences in allele frequency estimates will occur when different samples of 50 females are taken from a population. In addition to this, minor variation in electrophoresis conditions and staining can be expected between runs. To quantify both sources of variation, population HPL-1 was co-electrophorized 30 times and the allele frequencies at 21 alleles encoded by 8 putative loci were estimated. Observed allele frequencies were converted by an arcsin $\sqrt{(pij)}$ transformation (Sokal & Rohlf, 1995), where pij represents the allele frequency for allele i at locus j ($0<pij<1$). If a binomial distribution of the allele frequencies in a population is assumed, the hypothesis that the observed variance is due to sampling variation only, can be tested. For this purpose a Chi-square test was performed ($a=0.05$).

To determine the variation in genetic distance between different gels from a single population, Rogers' genetic distance (Rogers, 1984) between pairs of HPL-1 gels from the 30 aforementioned runs was determined using the DISTANCE procedure in NTSYS-pc Version 1.80 (Applied Biostatistics Inc., USA). In the following sections all procedures written in capitals refer to routines included in this software package. The Univariate procedure in SAS 6.09 was applied to evaluate the distribution of the genetic distances among the 435 pairs of gels. Average genetic distances, standard deviations and 95% confidence intervals were determined.

2 variation between samples from a single field

To estimate the variation in allele frequencies between different samples from a single field, cysts were collected from two lots, one in Friesland (lot 1) and one in the Noordoost-Polder (lot 2, NOP 01). Of lot 1 samples were taken every 10 m along an imaginary line crossing two infections sites (A and B). FG24, FG25 and FG26 are samples from site A. Sample FG27 originated from site B. The samples E3, F3, G3, H3, A5 and C5 of lot 2 were collected as described for lot 1, with the imaginary line crossing one infection site. For every sample the allele frequencies were determined.

3 genetic variation within and between populations from three different regions

To evaluate the genetic diversity within populations from each region, the estimated heterozygosity, the average number of alleles per locus and the proportion of polymorphic loci were estimated per population (Nei, 1987) over eight polymorphic loci. Heterozygosity levels were calculated from the allele frequency data.
The genetic differentiation of the populations was evaluated by determining Rogers' genetic distance.

The significance of variations between the regions was analysed using the SAS 6.09 statistical package. The variation in estimated heterozygosity, average number of alleles per locus and the proportion of polymorphic loci among populations from the three regions was tested using a Wilcoxon's two-sample test with a continuity correction of 0.5 (Sokal & Rohlf, 1995). The variation in average genetic distance between the regions was tested using the *t*-test (Sokal & Rohlf, 1995).

4 genetic variation among populations from two relatively small areas: Oostelijk Flevoland (the IJsselmeerpolders) and Veendam (the northeast Netherlands)

To enable a detailed analysis of the genetic variation within relatively small areas 26 populations from Oostelijk Flevoland (region 'the IJsselmeerpolders', Figure 6.2), and 23 populations from the vicinity of Veendam (region 'northeast Netherlands', Figure 6.3) were taken. For each population the allele frequencies were estimated and Rogers' genetic distance was determined. Moreover, the geographic distances (in km) between pairs of populations within each area were determined.

To evaluate the correlation between the resulting genetic and geographic distance matrices, the normalized Mantel statistic (Z), which is equivalent to the Pearson product-moment correlation (r) (Rohlf, 1994), was determined.
To test the significance of this correlation, 1000 permutations with a fixed geographic distances matrix and a permutated genetic distances matrix were performed (Dietz, 1983; Livshits *et al.*, 1991).

Dendrograms and nonmetric multidimensional scaling (MDS) plots were used to evaluate the genetic diversity among populations from both areas. The dendrograms were based on cluster analyses of the genetic distances using the unweighted pair group method with arithmetic mean (UPGMA; Sneath & Sokal, 1973) in the SAHN procedure. The resulting matrices were converted into dendrograms using TREE. Genetic distance matrices and the corresponding eigenvalues of these matrices (obtained with procedures DCENTRE and EIGEN) were used as input for a MDS analysis (Sneath & Sokal, 1973). Stress 2 (Rohlf, 1994) was used to evaluate the goodness of fit between the distances in the configuration space and the monotone function of the original distances.

results

Comparison of the *G. pallida* populations with 2-DGE revealed 21 IP-variants. These were encoded by 21 alleles at 8 putative loci.

To quantify the bias introduced by sampling errors and variation in electrophoresis conditions, allele frequencies between 30 samples of population HPL-1 were evaluated. All samples were electrophoresed separately (Table 6.1). The Chi-square test shows that the observed variance in allele frequencies for the majority of the alleles analyzed is larger than the expected variance (P = 0.05). The only exception are the loci X and N where no significant differences between the observed and expected variance in allele frequencies were found. As to be expected, technical variation influences the allele frequency estimate. However, the observed increase in variance was small for most evaluated alleles.

Table 6.1

The average, minimum, maximum, standard deviation and variance of the arcsin$\sqrt{(p_{ij})}$-transformed allele frequencies at polymorphic loci in gels of population HPL-1. The statistic X^2 ($[n-1]\cdot var_{observed}/var_{expected}$), with n = number of gels analysed and $var_{expected}$ = 1/8n, is also given. *X^2 values printed in italics are significantly different from* X^2 (n-1, a=0.05).

Locus	C		F			H			X	
Allele	C1	C2	F1	F2	F3	H1	H2	H3	X1	X2
Average	0.25	1.32	0.54	0.45	0.83	0.74	0.80	0.14	0.71	0.86
Minimum	0.10	1.15	0.39	0.31	0.69	0.58	0.63	0.00	0.56	0.79
Maximum	0.42	1.47	0.69	0.57	1.01	0.94	0.96	0.33	0.79	1.01
Stand. dev.	0.07	0.07	0.08	0.07	0.08	0.08	0.07	0.11	0.05	0.05
Variance	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.00	0.00
X^2	*59.15*	*59.14*	*79.71*	*52.23*	*78.76*	*68.68*	*60.74*	*136.50*	29.12	29.12

Locus	I		J			N		M			
Allele	I1	I2	J1	J2	J3	N1	N2	M1	M2	M3	M4
Average	1.57	0	0.98	0.59	0	0.80	0.77	0	0.82	0.75	0
Minimum	1.57	0	0.82	0.36	0	0.70	0.67	0	0.73	0.45	0
Maximum	1.57	0	1.21	0.75	0	0.90	0.87	0	1.12	0.84	0
Stand. dev.	0.00	0	0.10	0.10	0	0.04	0.04	0	0.09	0.09	0
Variance	0.00	0	0.01	0.01	0	0.00	0.00	0	0.01	0.01	0
X^2	0.00	0	*106.33*	*106.33*	0	22.31	22.31	0	*104.38*	*104.38*	0

Since the values for skewness and kurtosis are respectively 0.605 and 0.214, the Rogers' genetic distances among the 435 pairs of gels of population HPL-1 are assumed to follow a nearly normal distribution. The average genetic distance among the 30 gels of population HPL-1 is 0.039 ± 0.012, resulting in a 95% confidence interval with an upper limit of 0.064. Based on these results it is concluded that populations with a larger genetic distance than 0.064 are genetically distinct.

To study the variation between samples taken from a single field, two lots were selected. Lot 1 is located in Friesland and lot 2 in the Noordoost-Polder. Samples were taken over a length of 100 metres. Only samples with 25 or more cysts were analysed. In lot 1 three samples and in lot 2 six samples with 25 or more cysts were found. The distance between these samples ranged from 10 to 20 metres. The observed variation in allele frequencies between the samples was small. The average Rogers' distance between these samples of lot 1 is 0.029, with a maximum distance of 0.036 and for lot 2 these values are 0.039 and 0.055, respectively. Since the maximum distances are smaller than 0.064, the samples from lot 1 and lot 2, respectively, are considered as genetically identical. These data show that the applied methodology enables the recognition of identical populations which are derived from the same source.

To study the genetic diversity within and between populations from three different regions, 226 *G. pallida* populations from three geographically isolated regions were studied; 29 populations from north Friesland, 48 from the IJsselmeerpolders and 149 from the northeast Netherlands. The genetic distances among the populations ranged from 0.03 to 0.374. Joined analysis of the allele frequencies of the 226 *G. pallida* populations revealed 149 genetically identical populations (66%), i.e. with a mutual Rogers' distance < 0.064. These identical populations were distributed over 53 groups. The number of populations within a group ranged from 2 to 8 and averaged 2.8. (data available upon request). The genetically identical populations were not always derived from the same region. Sixty-three pairs of genetically identical populations (25%) were collected in different regions. The average heterozygosity of the populations analysed was 0.8%, with 1.024 alleles per locus and 1.7% polymorphic loci.

The variation within populations from north Friesland and the IJsselmeerpolders appeared to be larger than the variation within populations from the northeast Netherlands. The average heterozygosity, the average number of alleles per locus and the average number of polymorphic loci are lower in north Friesland and the IJsselmeerpolders than in the northeast Netherlands (Table 6.2, 6.3 and 6.4, respectively). Analysis of the genetic variation between populations from the three regions, in terms of the average genetic distance (Rogers') per region, indicated also a significant (a = 0.01) difference (Table 5). The average genetic distance between populations from north Friesland and the IJsselmeerpolders is larger than the average genetic distance between populations from the northeast Netherlands.

Table 6.2
Average estimated heterozygosity per population per region

Region	*N pop*	*Av. ± st.dev.*)*
north Friesland	29	0.0073 ± 0.0026 a
IJssel.polders	48	0.0068 ± 0.0021 a
northeast Netherlands	149	0.0085 ± 0.0018 b

*) Entries with the same letter are not significantly different from each other (a = 0.01).

Table 6.3
Average number of alleles per locus per region.

Region	*N pop*	*Av. ± st.dev.*)*
north Friesland	29	1.0210 ± 0.0077 a
IJssel.polders	48	1.0196 ± 0.0058 a
northeast Netherlands	149	1.0253 ± 0.0051 b

*) Entries with the same letter are not significantly different from each other (a = 0.01).

Table 6.4
Average proportion of polymorphic loci per region.

Region	*N pop*	*Av. ± st.dev.*)*
north Friesland	29	0.0156 ± 0.0047 a
IJssel.polders	48	0.0152 ± 0.0039 a
northeast Netherlands	149	0.0185 ± 0.0027 b

*) Entries with the same letter are not significantly different from each other (a = 0.01).

Table 6.5
Average genetic distance (Rogers) between pairs of populations per region.

Region	*N pop*	*Av. ± st. dev.*)*
north Friesland	406	0.175 ± 0.051 a
IJssel.polders	1128	0.179 ± 0.056 a
northeast Netherlands	11026	0.148 ± 0.044 b

*) Entries with the same letter are not significantly different from each other (a = 0.01).

To study the spatial variations in more detail, populations from two small areas (approximately 20 km2) in the IJsselmeerpolders (Figure 6.2) and the northeast Netherlands (Figure 6.3) were selected. Figure 6.4 shows an UPGMA dendrogram, illustrating the genetic variation among 26 *G. pallida* populations from Oostelijk Flevoland. The Cophenetic Correlation Coefficient (CCC) of this dendrogram is 0.79, indicating a nearly good fit (Rohlf, 1994). The UPGMA dendrogram reveals three groups of identical populations, representing 31% of the populations analysed from this area. No correlation was observed between the genetic and geographic distances of the populations analysed (r = 0.049, Z = 561 with p[random Z ≥ observed Z] = 0.229 over 1000 permutations).

From the region in the northeast Netherlands 23 populations were selected. Among the populations from this area four groups of identical populations were found, representing 52% of the populations analysed. Figure 6.5 shows the UPGMA dendrogram illustrating the genetic differentiation among the 23 populations analysed. The CCC of this dendrogram is 0.80, indicating a good fit (Rohlf, 1994). A small, but significant

correlation was observed between the genetic and geographic distances of the 23 populations from the vicinity of Veendam (r = 0.544, Z = 141 and p[random Z ≥ observed Z] = 0.002 over 1000 permutations).

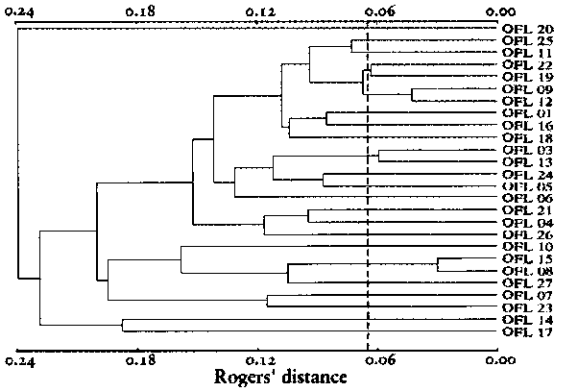

Figure 6.4

Similarity dendrogram based on 2-DGE data of 26 G. pallida *populations sampled in Oostelijk Flevoland, region the IJsselmeerpolders. Populations with a mutual distance < 0.064, roughly indicated by the line, are con-*

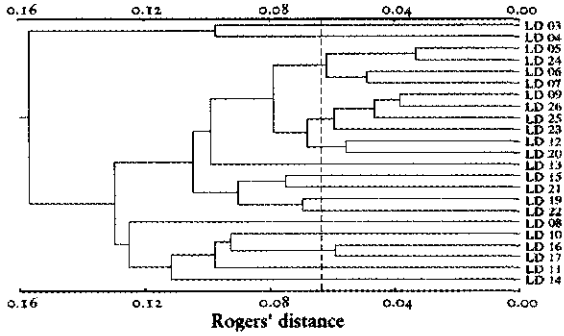

Figure 6.5

Similarity dendrogram based on 2-DGE data of 23 G. pallida *populations sampled nearby Veendam, region northeast Netherlands. Populations with a mutual distance < 0.064, roughly indicated by the line, are considered to be genetically identical.*

discussion

The intraspecific variation among the *G. pallida* populations in Europe 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 distribution of the polymorphisms revealed by 2-DGE among the 226 populations suggests that the number of distinct introductions of *G. pallida* in the Netherlands has been limited. The genetic variation among the *G. pallida* populations 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* populations originate from one source or, in case of multiple introductions, from a number of sources with a similar genetic makeup. This conclusion is corroborated by previous investigations with RAPDs and AFLPs (Folkertsma *et al.*, 1994, 1996b).

As with many other colonizers, colonization of a country or region by potato cyst nematodes starts with a limited number of primary foundations followed by population extensions and secondary foundations. Random genetic drift will change the allele frequencies of each secondary founded population as compared to the source population, and the extent of drift depends on the size of the bottleneck and the expansion rate of the population (Nei *et al.*, 1975). 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 small effect of drift, a small number of clusters with large numbers of identical populations would be expected. However, the similarity dendrogram constructed from the genetic distances of 226 *G. pallida* populations revealed a continuous range of variation. Although 66% of the populations appeared to be identical to one or more populations, these identical populations were distributed over 52 small clusters. The majority of the 226 populations were discriminated from each other by differences in allele frequencies at various loci.

These data indicate that the high infestation levels of potato cyst nematodes in The Netherlands are often derived from secondary foundations with extremely low initial population densities. For example, bottleneck sizes as small as 2000 individuals have no measurable effect on allele frequencies, even when extended over 150 generations (Bodmer & Cavalli-Sforza, 1976). Potato cyst nematodes have been introduced into Europe after 1850 and since they have only one generation in a growing season and potatoes are not grown every year, the maximum number of generations in the Netherlands will be less than 150. Preliminary estimates based on computer simulations using the allele frequency data of the 226 populations suggest that population sizes during bottlenecks have often been smaller than 100 individuals (unpublished results).

Significant variation in population structure was observed between the three investigated regions with regard to heterozygosity level, number of alleles per locus and proportion of polymorphic loci. *G. pallida* populations from north Friesland and the IJsselmeerpolders show in general less variation within populations as compared to their counterparts in the northeast Netherlands. Also, the average genetic distance between populations in north Friesland and the IJsselmeerpolders is larger than the average genetic distance observed in the northeast Netherlands. These data demonstrate that the impact of random genetic drift on the secondary founders has been larger in north Friesland and the IJsselmeerpolders than in the northeast Netherlands. The regional differences are most

likely caused by wider crop rotations and shorter growing seasons in north Friesland and the IJsselmeerpolders, resulting in lower multiplication rates. In these regions potatoes are in general cultivated every 3-4 years and harvested in the beginning of July, hampering completion of the life cycles. In the northeast Netherlands potatoes are grown once every 2 to 3 years and harvested during 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. It is well known, that a slow recovery after a bottleneck enhances the effect of drift (Nei *et al.*, 1975).

Studying two small areas (approximately 20 km^2) in detail, revealed no large positive correlations between geographic and genetic distances. These findings are corroborated by the analysis of the 226 populations from north Friesland, the IJsselmeerpolders and the northeast Netherlands. The clusters of identical populations demonstrated that secondary foundations take place in nearby fields as well as in fields at large distances. Seventy-five percent of the identical populations are derived from the same region. These findings illustrate the mode of colonization of potato cyst nematodes. The passive way of spread *e.g.* by soil adhering to seed tubers or machineries is a stochastic process and results in mosaic distribution patterns.

The estimated heterozygosity as revealed with 2-DGE for the *G. pallida* populations analyzed (0.8%) is small in comparison with other organisms. The average heterozygosity of natural *Drosophila melanogaster* populations, as revealed by 2-DGE, ranged from 4% (Leigh Brown & Langley, 1979) for an American population to 2.5% and 1.2% for a population from France and Benin, respectively (Coulthart & Singh, 1988). The average heterozygosity of two natural *D. similans* populations from France and Congo was found to be 2.9% and 2.7%, respectively (Coulthart & Singh, 1988). Racine & Langley (1980) estimated the heterozygosity of a natural *Mus musculus* population to be 2%. The low heterozygosity of *G. pallida* corroborates the impression that the founding and subsequent spread of *G. pallida* in the Netherlands has been accompanied with loss of variation due to random genetic drift. Additional research in the centre of origin, South America, is required to confirm this conclusion.

Recently, Hyman & Whipple (1996) and Nadler (1996), made a plea for the study of genetic structures of plant-pathogenic nematode populations. They urged for the use of allele frequencies to describe the variation within and among populations. This study is the first attempt to obtain insight in the impact of random 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. Since avirulence alleles are often not fixed, the extensive drift observed at loci revealed by 2-DGE will also be reflected at avirulence loci, which hampers a proper delineation of pathotypes.

chapter 7

Integrated analysis of quantitative variation in virulence, fitness and protein polymorphism among 102 *Globodera pallida* populations

abstract

In the absense of selection pressure by host plant resistance, the genetic variation in virulence among European *Globodera pallida* populations and variation revealed by molecular techniques are predominantly determined by: i) the genetic structures of the primary founders introduced South America, ii) random genetic drift and iii) gene flow. Since these processes affect the whole gene-pool of a population, it has been assumed that similarities revealed by molecular techniques are also reflected at virulence loci. To test this hypothesis the variation in allele frequencies at 8 polymorphic loci resolved by 2-DGE of 102 Dutch *G. pallida* populations was compared with the variation in virulence towards the resistant cultivars Santé, Elles, Seresta and Darwina. Linear regression analysis showed that the genetic similarities revealed by 2-DGE are positively correlated with similarities at (a)virulence loci.

introduction

Although resistance genes against nematode species have been identified in various host plant species (Fassuliotis, 1987; Williamson & Hussey, 1996) control by means of host resistance remains a continual challenge. The various sources of nematode resistance presently known confer only pathotype specific resistance. In addition, the variability at the (a)virulence level for most nematode species is insufficiently known. As with many other soilborne pathogens, nematodes have patchy distribution patterns. Small areas or even single fields can be infested with populations having distinct genetic structures with regard to virulence loci. Breeders, growers and researchers are facing the question how to handle this spatial variation in virulence.

In order to assess and classify this variation in virulence, pathotype schemes have been developed for various species, *e.g. Heterodera avenea*, *H. glycines*, *Globodera rostochiensis* and *G. pallida* (Triantaphyllou, 1987). In these schemes pathotypes are delineated by their (in)ability to reproduce on a standard set of differentials. However, a classification of plant parasitic nematode populations into pathotypes has been criticised (Trudgill, 1985; Nijboer & Parlevliet, 1990; Bakker *et al.*, 1993) because of the arbitrary way pathotypes are classified and the laborious and time-consuming way virulence is determined.

As an alternative strategy, molecular techniques have been applied to assess the intraspecific classification of plant parasitic nematodes

(*e.g.*, Abad, 1994; Baum *et al.*, 1994; Blok & Phillips, 1995; Li *et al.*, 1996). The majority of these molecular studies focussed on the analysis of qualitative rather than quantitative variation. Also quantitative variation in virulence has hardly been addressed in these molecular studies. A qualitative approach is recommended for species with a high degree of intraspecific variation, such as *G. rostochiensis* (Folkertsma *et al.*, 1994; 1996b). However, a quantitative approach is recommended to classify populations of species, such as *G. pallida* (Folkertsma *et al.*, 1994; 1996a), with a high degree of similarity (Avise, 1975; Ayala, 1983). Recently, Hyman & Whipple (1996) noticed an urgent need for quantitative molecular studies of plant parasitic nematodes.

This report is the first attempt of an integrated analysis of quantitative variation in protein polymorphism and virulence. The European populations of the potato cyst nematodes *G. rostochiensis* and *G. pallida* are a suitable model to study such relationships. In previous reports it has been hypothesized, that in the absence of the selection pressure by host resistance genes, intraspecific variation in virulence and variation revealed by molecular techniques are predominantly determined by three processes: i) the genetic structures of the primary founders introduced from South America, ii) random genetic drift and iii) gene flow (Bakker *et al.*, 1993). Since these processes affect the whole gene pool of a population, similarities revealed by molecular techniques are also reflected at virulence loci. This concept has been formulated as the gene pool similarity concept (Bakker *et al.*, 1993). This concept does not exclude other mechanisms, like mutation, balanced polymorhisms or other selection mechanisms, but states that the major part of the observed variation between the *G. pallida* populations can be explained by aforementioned processes.

To test the gene pool similarity concept, allele frequencies at 8 loci of 102 *G. pallida* populations from The Netherlands, which had not or hardly been exposed to the relevant resistance genes, were assessed with 2-DGE and compared with variation in virulence. In addition we tested whether the gene pool similarity concept can also be applied to loci determining the fitness of a population.

materials and methods

origin *G. pallida* populations

Samples (>25 cysts) of 87 field populations (labelled Ve**) were sampled in the autumn of 1990 in the northeast Netherlands. The samples were obtained from the Plant Protection Service Wageningen. Samples of three field populations from the same region (labelled Feunekes, Nijboer and Begeman) were obtained from the potato breeding institute 'Karna' (Valthermond). Three additional samples (labelled Campmans, Wentink, Janssen) were sampled in the IJsselmeerpolders and obtained from the Research Station for Arable Farming and Field Production of Vegetables (PAGV, Lelystad). The remaining nine populations (labelled D**, E** and IPO-Pa3) were originally sampled from heavily infested spots in fields in the northeast Netherlands. These samples were obtained from the DLO-Institute of Plant Protection Research (IPO-DLO, Wageningen), the DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO, Wageningen) and the Plant Protection Service (PD, Wageningen). Most populations analysed were sampled before the growth of resistant cultivars. However, the three populations obtained from 'Karna' were sampled in a field where cultivar Seresta was grown. The populations were maintained on the non-resistant culti-

var *S. tuberosum* ssp. *tuberosum* L. Eigenheimer in a growth chamber at 18°C and 16 h daylength.

two-dimensional gel electrophoresis of proteins (2-DGE)

The genetic relationships between the 102 *G. pallida* populations as revealed by 2-DGE were analysed as described previously (Folkertsma *et al.*, 1997).

the virulence tests

The virulence of 102 *G. pallida* populations was assessed on four different potato cultivars with different genetic backgrounds. Cultivar Désirée was used as a non-resistant control. Cultivar Santé was used as a Pa2 resistant cultivar. Resistance in Santé is derived from *S. vernei* clone 62.33.3. Cultivar Elles was used as the second Pa2 resistant cultivar, with resistance derived from from *S. vernei* clone 62.33.3 and *S. vernei* clone 24/20. Seresta, the fourth cultivar, is a Pa3 resistant cultivar with resistance derived from *S. vernei* clone 24/20, *S. vernei* ssp. *ballsii* clone 2/1, *S. vernei* clone LGU 8, *S. oplocence* clone EBS 1786, *S. spegazzinii* clone 440 and *S. tuberosum* ssp. *andigena* clone CPC 1673.

The *G. pallida* populations were reared on cultivar Désirée the year preceding the realization of the virulence test. The virulence test was performed in pots containing 5 kg of soil composed of silversand, hydroculture granules (2-4 mm in size) and claypowder (4:1:0.7). Steiner nutrient solution (0.02 v/w) and additional fertilizer (0.001 v/w; N:P:K 12:10:18) were added before inoculation. The moisture level in the pot was kept at 18% during the whole experiment.

One year old eggs+juveniles (J2) of each population were used as an inoculum. Eggs+J2 suspensions were made and the number of viable eggs+J2 per ml suspension was determined visually. Pots were inoculated with 5 eggs+J2 per g soil. Small tubers (approximately 28 mm) of each cultivar with one sprout were used as planting material. The tubers were disinfected with a 0.5% Solacol treatment (Hoechst Schering AgrEvo, The Netherlands) for 20 sec.

The experiment was performed in two different glass houses, both considered as a block. In each block, the experiment was laid out in a complete randomized block design, with one observation per cultivar-population combination. Five replicates for population Janssen and E408 on each cultivar were laid out to obtain an impression of the experimental variation. Three replicates (1, 3, and 5) were placed in block one and two replicates (2 and 4) in block two.

After 14-16 weeks the plants died and the soil in the pots was dried. The cysts were extracted by elutriation, using a Seinhorst elutriator, and the final number of newly formed cysts and newly formed eggs+J2 per g soil were determined. Because of the expected large numbers of cysts on the non-resistant cultivar Désirée, three subsamples of 400 grams of soil were taken per pot and analysed seperately. The average number of newly formed cysts per g soil and the average number of newly formed eggs+J2 per g soil were determined over the three subsamples.

A second virulence test was performed on the resistant cultivars Santé and Darwina, with cultivar Eigenheimer as the non-resistant control. Darwina is a Pa2 resistant cultivar derived from *S. vernei* and *S. tuberosum* ssp. *andigena*. From the 87 populations labelled Ve**, 20 were randomly chosen. Population D350 (Pa2), obtained from the Plant Protection Service, was used as a reference population. The virulence test was performed in pots containing 1 kg sandy clay. Pots were inoculated with 5 eggs per g soil and one tuber of the resistant or non-resistant cultivar was planted. The experiment was performed in a growth chamber at 18°C and

16 h daylenght. After three months the plants died and the soil was dried. The newly formed cysts were collected using a Kort Elutriator and the final number of newly formed cysts and eggs+J2 per g soil was determined.

data analysis

NTSYS-pc Version 1.80 (Exeter software, Setauket, NY) was used to assess the genetic variation between the populations analysed based on 2-DGE data, applying Rogers' distance (Rogers, 1984; Folkertsma *et al.*, 1997).

The analysis of the virulence data necessitates the definition of a number of parameters. The relative reproduction for each population on the resistant cultivars was expressed as the quotient between the final population density on the resistant cultivar and the final population density on the non-resistant cultivar Désirée. The final population density was expressed as the total number of newly formed cysts per g soil or the total number of newly formed eggs+J2 per g soil, respectively. The fecundity of each population on each cultivar was expressed as the average number of eggs+J2 per cyst. Finally, the fitness of each population was expressed as the quotient between the final and initial population density (in eggs+J2 per g soil) on the non-resistant cultivar Désirée (Pf/Pi) (Beniers *et al.*, 1995).

The statistical analysis of the virulence data was performed with the aid of Genstat (version 3.1, Payne *et al.*, 1987). The analysis of variation was performed with two observations per population/cultivar combination. Missing values were omitted from the data set when non-parametric tests were performed. Both Genstat and NTSYS-pc (Rohlf, 1994) were used to analyse the integrated virulence/2-DGE dataset.

results

2-DGE of 102 *G. pallida* populations revealed 8 polymorphic loci with a total of 21 alleles. Large variation in allele frequencies was observed among the 102 *G. pallida* populations. The genetic distances based on these frequencies ranged from 0.03 to 0.34. Construction of a similarity dendrogram revealed a continuous range of variation (available upon request). No clear clusters were observed. The vast majority of this variation is due to genetic differences and not to experimental variation (Folkertsma *et al.*, 1997). In a previous report, the bias introduced by sampling errors and electrophoresis conditions was quantified and it was shown that populations with a mutual genetic distance of 0.064 or smaller were genetically identical. Twenty-one populations, distributed over 10 groups, were found to be identical.

The variation in virulence among the 102 *G. pallida* populations towards the resistant cultivars Santé, Elles and Seresta is shown in Figure 7.1A-C. The virulence characteristics of the populations were evaluated by determining the relative reproduction (RV), which was calculated by expressing the Pf/Pi values on the resistant cultivars as a percentage of the Pf/Pi values on the susceptible cultivar Désirée. The relative reproduction based on eggs and J2 gram ranged from 0.3% to 68.8% for Santé and from 0.3% to 38.3% for Elles (see Fig. 7.1A and B). For Seresta these figures varied from 0.1% to 6.7% (Fig. 7.1C).

The fitness of the populations was evaluated by analysing the absolute Pf/Pi values on the susceptible cultivar Désirée, which ranged from 0.97 to 105.51 and averaged 43.1.

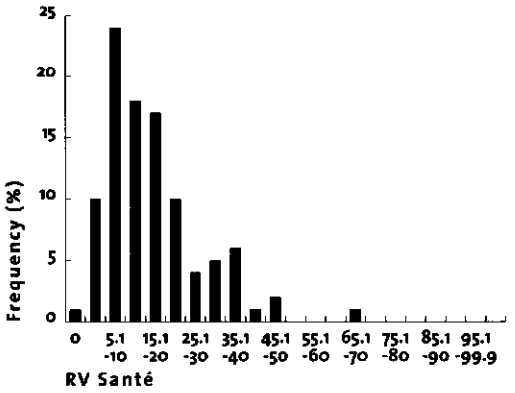

Figure 7.1A
Frequency distribution of the relative reproduction (RV) of 94 G. pallida *populations on cultivar Santé.*

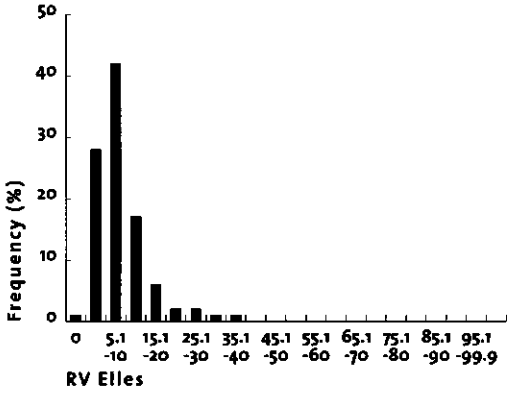

Figure 7.1B
Frequency distribution of the relative reproduction (RV) of 100 G. pallida *populations on cultivar Elles.*

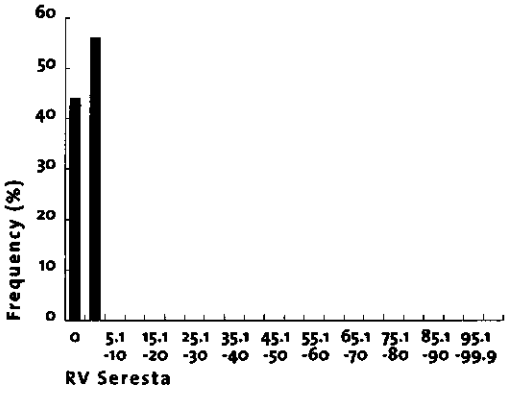

Figure 7.1C
Frequency distribution of the relative reproduction (RV) of 99 G. pallida *populations on cultivar Seresta.*

The gene-pool similarity concept was tested for variation at virulence loci by arranging 102 *G. pallida* populations in groups on the basis of genetic distances. Groups of populations were delineated at genetic distances of 0.064, 0.1, 0.15, 0.2 and 0.25 and the coefficient of variation (CV) of the relative reproduction was calculated for each group. Figure 7.2A-C show that genetic similarities at the 8 polymorphic loci revealed by 2-DGE are also reflected at avirulence loci. Linear regression analysis (Fig. 7.2A-C) showed that an increase in genetic distance is associated with an increase of the average variation coefficients. This pattern was observed for all resistant cultivars. The percentage variance explained by linear regression analysis was 15.0% for Santé, 14.2% for Elles and 5.9% for Seresta. These percentages were increased by diminishing the experimental variation in a second virulence tests. Therefore a set of 20

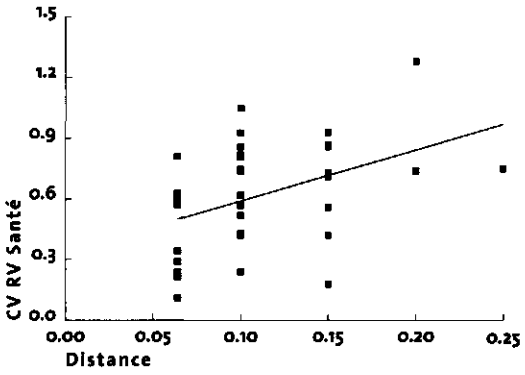

Figure 7.2A

*Linear regression (E [CV] = 0.346 + 2.480 * Distance) fitted to the relation between the coefficient of variation (CV) of the relative reproduction (RV) on Santé of 94* G. pallida *populations grouped at a genetic distance of 0.064, 0.1, 0.15, 0.2 and 0.25.*

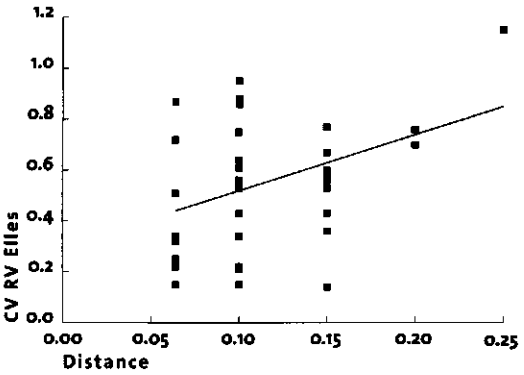

Figure 7.2B

*Linear regression (E [CV] = 0.269 + 2.330 * Distance) fitted to the relation between the coefficient of variation (CV) of the relative reproduction (RV) on Elles of 100* G. pallida *populations grouped at a genetic distance of 0.064, 0.1, 0.15, 0.2 and 0.25.*

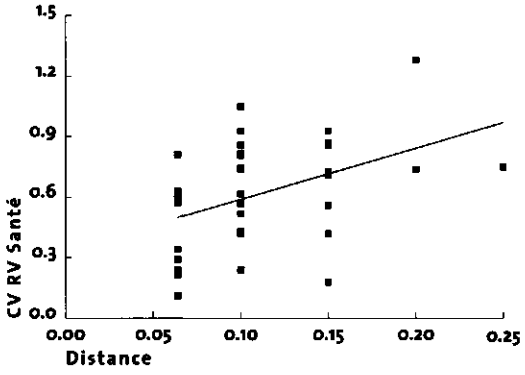

Figure 7.2C

*Linear regression (E [CV] = 0.608 + 1.821 * Distance) fitted to the relation between the coefficient of variation (CV) of the relative reproduction (RV) on Seresta of 99* G. pallida *populations grouped at a genetic distance of 0.064, 0.1, 0.15, 0.2 and 0.25.*

randomly chosen G. *pallida* populations and a reference population were tested. The percentage variance explained by linear regression analysis between the genetic distance among groups of populations and the coefficient of variation was 25.9% for the resistant cultivar Santé and 49.4% for the resistant cultivar Darwina (Fig. 7.3A and 7.3B, respectively). In this case the populations were grouped at each branch point of the dendrogram constructed for these populations, ranging from a distance of 0.078 to 0.209.

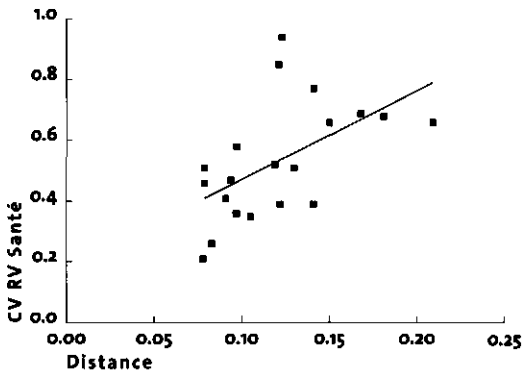

Figure 7.3A
*Linear regression (E [CV] = 0.183 + 2.91 * Distance) fitted to the relation between the coefficient of variation (CV) of the relative reproduction (RV) on Santé of 21* G. pallida *populations grouped at the branching points in the dendrogram.*

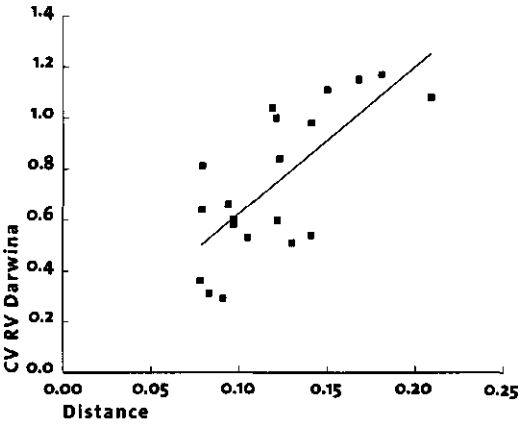

Figure 7.3B
*Linear regression (E [CV] = 0.050 + 5.73 * Distance) fitted to the relation between the coefficient of variation (CV) of the relative reproduction (RV) on Darwina of 21* G. pallida *populations grouped at the branching points in het dendrogram.*

The gene-pool similarity concept was tested for the variation in fitness in a similar way as was done for the variation in virulence data. For each group the variation coefficient of the absolute Pf/Pi values on the susceptible cultivar Désirée was calculated. Figure 7.4 shows that the genetic distance is positively correlated with the average variation coefficient. The percentage of variance explained by linear regression analysis is 6.9%. The fitness of the populations could not be associated with the heterogeneity of the populations. The heterozygosity levels of the populations, which ranged from 0.2% to 1.2%, were not correlated with the absolute Pf/Pi values on the susceptible cultivar Désirée. Analyses of the average number of alleles per locus and the proportion of polymorphic loci revealed the same result.

No evidence was obtained for linkage of one of the 21 alleles resolved by 2-DGE with (a)virulence loci. Analyses of the allele frequencies revealed no correlation with the relative reproduction on Santé, Elles or Seresta. Similar results were obtained for the fitness data.

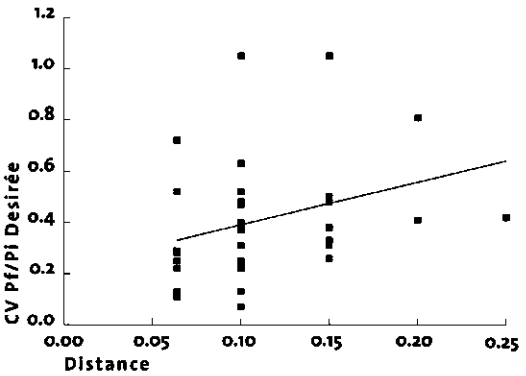

Figure 7.4
*Linear regression (E [CV] = 0.106 + 1.681 * Distance) fitted to the relation between the coefficient of variation (CV) of the Pf/Pi on Désirée of 102* G. pallida *populations grouped at a genetic distance of 0.064, 0.1, 0.15, 0.2 and 0.25.*

discussion

Control of plant parasitic nematodes by means of host plant resistance is often hampered by the mosaic distribution patterns of pathotypes (Folkertsma *et al.*, 1997). To address this problem, various molecular studies have analysed the genetic variation of pathotypes (*e.g.* Bolla *et al.*, 1988; Burrows, 1990; Carpenter *et al.*, 1992; Wendt *et al.*, 1993). To our opinion (Bakker *et al.*, 1993; Rouppe van der Voort *et al.*, 1994; Folkertsma *et al.*, 1996a), molecular techniques can only be used to pathotype field populations by: i) measuring gene-pool similarities, or ii) by tracing markers linked to avirulence genes or iii) by identifying the avirulence genes themselfes. In molecular studies on pathotypes, these possibilities are seldomly mentioned or considered to design the appropriate experiments. Elucidating the molecular nature of avirulence genes or obtaining informative markers is a daunting task for plant parasitic nematodes and requires a detailed knowledge of the genetics of virulence.

The strength of the gene-pool similarity concept is that it requires no knowledge about the genetic nature of the virulence loci. The concept states that in the absence of selection pressure by resistance genes, intraspecific variation at selectively neutral molecular loci should also be reflected at virulence loci, including those not resolved yet (Bakker *et al.*, 1993). To verify the gene-pool similarity concept, similarities in allele frequencies at 8 polymorphic protein loci were compared with the similarities at virulence loci. Our data confirm the concept and show that, despite the experimental variation, the genetic similarities revealed by 2-DGE are positively correlated with the similarities at (a)virulence loci.

The gene pool similarity concept is apparently also applicable to the variation in fitness between populations. The interpopulational variation with regard to the absolute Pf/Pi values on the susceptible cultivar Désirée increases as the genetic distance increases. These data indicate that fluctuations in Pf/Pi values are not entire-

ly due to environmental factors, but also have a genetic component. This result is rather remarkable, because, in contrast to polymorphisms revealed by 2-DGE, fitness is not a selectively neutral trait and directly affects the reproduction of a population. Apparently, the reproduction rates of the genotypes with the lowest fitness values were sufficient to survive and to cause a notable infestation. On the other hand it is also noted that fitness is determined here by measuring Pf/Pi values on the susceptible cultivar Désirée in the greenhouse. The outcome with regard to variation in fitness among the 102 *G. pallida* populations may be different if the experiment is performed with alternative susceptible cultivars or under field conditions. In contrast to other studies on heterozygosity and fitness (reviewed by Nei, 1987), no evidence was obtained that fitness can be related to the genetic heterogeneity of the populations. The heterozygosity, the number of alleles per locus and the proportion of polymorphic loci showed no correlation with the absolute Pf/Pi values on the susceptible cultivar Désirée.

A major problem in studying quantitative variation in virulence among plant parasitic nematode populations is the experimental variation. Pf/Pi values are strongly influenced by external factors, such as the age and storage conditions of the cysts, previous host and environmental influences (Mugniéry *et al.*, 1989). To minimize the influence of these factors, all populations were reared and harvested synchronously before performing the final experiment. Despite these precautions and the use of relative reproduction values as a measure for the virulence characteristics, considerable variation among replicates was observed. Nevertheless, it is feasible to assume that the percentage variance explained by regression analysis can be increased by developing more accurate bioassays.

The 2-DGE data of the 102 *G. pallida* populations suggest that the number of distinct introductions from South America has been limited. The similarity dendrogram based on the 2-DGE data reveals a continuous range of variation. No clusters of populations with unique alleles are observed. It seems that the Dutch *G. pallida* populations are the descendants of a single introduction or, in case of multiple introductions, from a number of sources with a similar genetic structure. The 2-DGE data of the 102 *G. pallida* populations are corroborated by the virulence data, which show also a continuous range of variation. This implies that the genetic variation among these populations is predominantly the result of random genetic drift. This conclusion has important consequences for the control by means of resistance and suggests that breeding for resistance is less complicated than previously thought.

The relationships based on 2-DGE data can be used as a guide in testing the effectiveness of new sources of resistance. Most important is that representatives of all distinct introductions are included. The diversity generated by random genetic drift during colonization is of secondary importance, because this process affects only the gene pool of single populations and does not change the total gene pool introduced in an area. Since the Dutch *G. pallida* populations seem to be derived from a single source, or rather similar sources, a representative set of test populations can be inferred from the 2-DGE data. Computer studies simulating the effect of drift are in progress to estimate the minimum number of test populations for a reliable survey.

The gene pool similarity concept is in principle also applicable to predict the effect of the growth of resistant cultivars by pathotyping soil samples. Unknown field populations can be identified by comparing their molecular characteristics with those of a set of reference populations with known virulence properties.
As shown in this report the interpopulational variation in virulence is positively correlated

with the mutual genetic distance. Thus, if 2-DGE analysis shows that an unkown field population is similar to one of the reference populations, the virulence characteristics of this field population should also be similar to the virulence characteristics of the reference population. In case the field population has already been exposed to the relevant resistance genes, a correction factor for the increase in virulence has to be taken into account. Such correction factors can be derived from simple selection experiments in pots, or when the genetics of the interaction is known, this factor can be estimated from computer simulations, as has been accomplished for the interaction of *G. rostochiensis* with the *H1* gene (Spitters & Ward, 1988).

Summary and concluding remarks

The potato cyst nematodes *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* (Stone) originate from the Andes region in South America and have been introduced into Western Europe since 1850. Both species are successful colonizers. Once primary founders have established vital populations, an area is rapidly colonized by secondary founding events. The mode of spread results in patchy distribution patterns. Analyses of the processes that influence the spatial variations in virulence are of major importance for the control by means of host plant resistance. The ability to unravel the mosaic distribution patterns of the two species and their pathotypes enables breeders and growers to anticipate on the dynamics of virulent populations. The aim of this thesis was to analyse the intra- and interspecific variation of *G. rostochiensis* and *G. pallida* in the Netherlands and to obtain insight in the processes that determine the spatial variations in protein, DNA and (a)virulence polymorphisms.

interspecific variation

The molecular variation between the sibling species *G. rostochiensis* and *G. pallida* is remarkably large. The RAPD technique revealed a total of 250 DNA fragments, of which only nine DNA fragments were common to both species (Chapter 3). Similar results were obtained with the AFLP assay. A total of 1000 AFLP fragments was amplified, of which only 64 fragments could be identified in both species (Chapter 4). These results agreed with previous investigations using 2-DGE and show that morphologically nearly indistinguishable organisms can be quite distinct the molecular level.

The extensive genetic differentiation of *G. rostochiensis* and *G. pallida* offers perspectives for the development of a diagnostic assay. In addition the PCR technique enables the amplification of species specific fragments from small numbers of cysts extracted from soil samples. For example, *G. rostochiensis* specific DNA fragments can be amplified from single juveniles (Chapter 1).

intraspecific variation

The intraspecific variation of *G. rostochiensis* as revealed by RAPDs and AFLPs is relatively low (Chapter 3 and 4). The proportion of polymorphic DNA fragments among nine *G. rostochiensis* populations was 19% and 15.8%, respectively. Three clusters of populations were identified and each cluster could be described by one or more specific DNA fragments.

The intraspecific variation of *G. pallida*, as revealed with RAPDs or AFLPs, is larger in comparison with *G. rostochiensis* (Chapter 3 and 4). The proportion of polymorphic RAPD fragments among 17 populations was 46%. For the AFLP assay this figure was 23% polymorphic AFLP fragments among 15 populations. The majority of the populations displayed a continuous range of variations. Only a few clearly diverged clusters could be discriminated on the basis of specific DNA fragments.

Various groups have applied the RAPD technique to study the genetic divergence among conspecific populations of plant parasitic nematodes. The validity of this technique was evaluated in chapter 4 by comparison of the clustering of 36 *G. pallida* populations based on RAPD and 2-DGE data. Both data sets demonstrated that the majority of the *G. pallida* populations were not clearly differentiated from each other. The overall correlation between the distance matrices derived from both data sets was low. Careful examination of the separate dendrograms showed similarity in clustering only for clearly diverged populations or groups of populations.

Although the AFLP and RAPD technique are simple, fast and require only minute amounts of biological material, they are not suitable to resolve the subtle differences among potato cyst nematode populations. Quantitative variation in allele frequencies is often not resolved with those techniques, which is, among others, due to the virtual inability to recognize co-dominant alleles and the non-linear amplification of DNA fragments. In chapter 6 and 7 the genetic variation was studied by analysing pools of individuals with 2-DGE. The ratios between the protein quantities produced by the codominant alleles are appropriate measures for the allele frequencies. The correctness of this method has been confirmed by 2-DGE of single individuals (De Boer *et al.*, 1992)

bottleneck effects on the secondary founders

The intraspecific variation among potato cyst nematode populations in Europe is predominantly determined by the genetic constitution of the primary founders, directly or indirectly introduced from South America, and the effect of random genetic drift on the secondary founders. To obtain insight in these processes, 226 *G. pallida* populations from the Netherlands were analyzed with 2-DGE. The results strongly suggest that these populations originate from one source, or in case of multiple introductions, from a number of sources with a similar genetic makeup.

The genetic differentiation of the 226 *G. pallida* populations indicate that the colonization of the Netherlands has been accompanied by extensive random genetic drift. Only a limited proportion of the populations appeared to be identical. It is also shown, that the bottleneck effects differ between regions. Significant variation in population structure was observed between the three investigated areas. The genetic variation within *G. pallida* populations from north Friesland and the IJsselmeerpolders is in general smaller than within populations from the northeast Netherlands. A plausible explanation for this phenomena is the low multiplication rate of potato cyst nematodes due to wider crop rotation schemes in the IJsselmeerpolders and north Friesland. These factors result in a slow expansion of newly founded populations, which enhances the effect of drift.

gene-pool similarity concept

The gene-pool similarity concept rests on the hypothesis that in the absence of selection pressure by host plant resistance, degrees of similarity between populations revealed by molecular techniques are also reflected at virulence loci, including those not yet resolved. To test this concept, the genetic variation revealed by 2-DGE among 102 *G. pallida* populations was compared with the variation in virulence towards two resistant cultivars. This analysis showed that a decrease in genetic distance among populations is accompanied with a decrease in variation in virulence. In addition it is demonstrated that the gene pool similarity concept is also applicable to loci determining the variation in fitness among populations. The variation in Pf/Pi values among the 102 populations on Désirée is in general smaller between closely related populations than between distantly related populations.

breeding for resistance

Breeding for resistance has been dominated by trial-and-error approaches, which has stimulated the view that control by means of host plant resistance is unavoidably a short-term approach due to the 'appearance' of virulent populations. The pervasive myth that breeding for resistance against potato cyst nematodes is a lost arms race is challenged by the results of this thesis. Until recently it was assumed that the genetic variation of *G. pallida* in the Netherlands was too large to produce potato cultivars with broad-spectrum resistance. In this thesis it is shown that the genetic diversity introduced from the Andes region has been limited and that the variation among the Dutch *G. pallida* populations is mainly the result of random genetic drift. The elaborate analysis of 226 Dutch *G. pallida* populations offers perspectives to obtain potato cultivars with broad and durable resistance. The gene pool similarities revealed by 2-DGE can be used as guidance in testing the effectiveness of new sources of resistance.

Samenvatting en conclusies

In deze samenvatting is getracht een indruk te geven van de achtergrond (paragraaf 1-5), de doelstellingen (paragraaf 6) en de resultaten (de paragraaf 7-11) van het onderzoek beschreven in dit proefschrift. In paragraaf 12 wordt stilgestaan bij de implicaties van dit onderzoek op de resistentie veredeling tegen aardappelmoeheid. De Nederlandstalige tekst is speciaal bedoeld voor niet-ingewijden. Voor meer technische details wordt verwezen naar de Engelstalige samenvatting en de artikelen.

aardappelmoeheid in Nederland

Aardappelmoeheid (AM) wordt veroorzaakt door de aardappelcystenaaltjes *Globodera rostochiensis* (Woll.) Skarbilovich en *G. pallida* Stone en vormt reeds decennia lang een groot probleem in de aardappelteelt in Nederland. Beide soorten stammen uit het Andes gebergte in Zuid-Amerika. Daar co-evolueerden zij met verwanten van de cultuuraardappel (*Solanum tuberosum* ssp. *tuberosum*). Waarschijnlijk werden beide soorten rond 1850 geïntroduceerd in West-Europa. Toen werd reeds een begin gemaakt met de resistentieveredeling tegen de aardappelziekte die veroorzaakt wordt door de schimmel *Phytophthora infestans* en werden wilde verwanten van de aardappel naar Europa gehaald. De eerste waarneming van een mogelijke aantasting van de aardappel door aardappelcystenaaltjes in Europa stamt uit Duitsland (Kühn, 1881; Oostenbrink, 1950). De eerste betrouwbare waarneming uit 1913, komt wederom uit Duitsland. Daarna werden deze cystenaaltjes gevonden in veel landen. In Nederland pas in 1938. Nu zijn beide soorten wijd verbreid in die gebieden van Nederland waar aardappelen worden geteeld. Noordoost-Nederland, het gebied van de fabrieksaardappelteelt, is voor een groot deel matig tot zwaar besmet. Ook daarbuiten, met name in Friesland en de IJsselmeerpolders, neemt de laatste jaren de besmettingsgraad toe.

schade door aardappelcystenaaltjes

De aaltjes veroorzaken directe schade aan aardappelplanten door het wortelstelsel te parasiteren. De wortels worden beschadigd bij binnendringing van de aaltjes en door onttrekking van voedingsstoffen aan de plant. Daarnaast wordt indirect schade veroorzaakt omdat AM in West-Europa als een quarantaineziekte wordt beschouwd. Dit houdt in dat van overheidswege wettelijke maatregelen uitgevaardigd zijn ter voorkoming van verspreiding. De teelt van pootaardappelen en ander vermeerderingsmateriaal is bijvoorbeeld niet toegestaan op besmet verklaarde percelen.

Het gemiddelde jaarinkomen in de akkerbouw in Nederland wordt voor ongeveer 50% verkregen uit aardappelen. Door de wettelijke maatregelen in verband met AM worden de mogelijkheden voor een goede bedrijfsplanning beperkt. Dit vanwege de voorgeschreven vruchtwisseling, die de teelt van aardappelen beperkt en daarmee de hoogte van het inkomen.

bestrijding van aardappelmoeheid

AM kan bestreden worden met behulp van drie verschillende methoden:

1 Vruchtwisseling. Aardappelcystenaaltjes planten zich voort op een beperkt aantal vertegenwoordigers van de familie der Solanaceae, onder andere op aardappel en tomaat maar bijvoorbeeld niet op de zwarte nachtschade. Onder een ander gewas vindt een langzame natuurlijke afsterving plaats. In Nederland is de aardappel de belangrijkste waardplant. Aardappelcystenaaltjes zouden dan ook geen probleem vormen in de akkerbouw indien aardappelen bijvoorbeeld eens in de 10 jaar op hetzelfde perceel verbouwd werden.

2 Grondontsmetting. Twee typen nematiciden worden onderscheiden: fumigantia en granulaten. Fumigantia, zoals onder meer DD (dichloorpropeen), zijn aspecifieke biociden die al het bodemleven doden. Met behulp van dergelijke middelen is het mogelijk de grond van een perceel te 'steriliseren' en daarmee ook de aanwezige aaltjes te doden. Granulaten, zoals onder meer Aldicarb, zijn wat specifieker voor aaltjes dan fumigantia. Nematoden worden niet gedood maar tijdelijk verlamd waardoor hun ontwikkeling vertraagd op gang komt en de planten zich in de beginfase kunnen ontwikkelen zonder last te hebben van parasiterende aaltjes. Beide typen nematiciden zijn erg belastend voor het milieu en worden nu restrictief toegepast.

3 De teelt van resistente rassen. In het begin van de jaren zestig kwam een aantal rassen op de markt, zoals bij voorbeeld 'Saturna', met het *H1* resistentiegen afkomstig uit *S. tuberosum* ssp. *andigena*. Al snel werden populaties aangetroffen waartegen dit resistentiegen niet werkzaam was. Nieuwe vormen van resistentie werden geïntroduceerd (onder andere uit *S. vernei*) en telkens werden vrij snel veldpopulaties gevonden waartegen deze vormen van resistentie niet werkzaam waren. Later werd dan ook geconcludeerd dat deze agressieve populaties reeds aanwezig waren voordat resistente rassen werden verbouwd.

In de jaren zeventig werd gepoogd in deze complexe materie ordening aan te brengen middels het pathotypenschema. Populaties van beide soorten werden onderverdeeld in een aantal groepen (pathotypen) op basis van hun vermogen (virulentie) of onvermogen (avirulentie) zich te vermeerderen op een set aardappelplanten met verschillende vormen van resistentie ('differentials'). In dit pathotypenschema, een eerste poging tot kwantificering van inter- en intraspecifieke variatie van aardappelcystenaaltjes, worden vijf pathotypen binnen *G. rostochiensis* (Ro1, Ro2, Ro3, Ro4 en Ro5) en drie binnen *G. pallida* (Pa1, Pa2 en Pa3) onderscheiden. Criterium voor dit vermogen of onvermogen van een populatie is de vermeerderingsfactor (Pf/Pi) op een 'differential'. Is deze groter dan 1, dan wordt de eigenschap van deze populatie met een plus (+) gewaardeerd, is deze kleiner of gelijk aan 1 dan met een min (-). Combinaties van plussen en minnen bepalen uiteindelijk de pathotypenkwalificatie.

Vrij snel na de introductie van het pathotypenschema in 1977 (Kort *et al.*, 1978) werd kritiek geuit op dit schema. Eén van de voornaamste kritiekpunten is dat ten onrechte wordt uitgegaan van een fytopathologisch concept. Hierin hebben praktisch alle individuen binnen een populatie dezelfde (a)virulente eigenschappen. Voor een zich ongeslachtelijk voortplantende schimmelpopulatie is dit correct, voor aardappelcystenaaltjes, een bisexuele soort, absoluut niet. Een pathotype bestaat uit mengsels van virulente en avirulente individuen. Daarnaast is kritiek geuit op de bewerkelijkheid van het pathotypen schema. De pathotypentoets bestaat uit bepalingen van Pf/Pi op een serie 'differentials'. De Pf/Pi wordt daarnaast sterk beïnvloed door omgevingsfactoren. Ook worden nu aardappelgenotypen in de resistentieveredeling gebruikt die niet vertegenwoordigd zijn in de set 'differentials'

waardoor de praktische relevantie van het schema aanzienlijk beperkt wordt.

Meestal werden bovengenoemde bestrijdingsmethoden in combinatie toegepast. Wettelijke maatregelen, vervat in de wet op de aardappelmoeheid (Besluit 1952, sindsdien een aantal malen gewijzigd), bepaalden onder meer dat aardappelen in principe één maal in de vier jaren verbouwd mochten worden. Een ontheffing voor de één op drie teelt werd verleend in de fabrieksaardappelteelt mits grondontsmetting werd toegepast of resistente rassen werden verbouwd. In geval van combinatie van beide werd een één op twee teelt van aardappelen toegestaan.

Meerjarenplan Gewasbescherming en herziening wet op aardappelmoeheid

In 1989 werd het Meerjarenplan Gewasbescherming (MJPG) geformuleerd met als voornaamste doelstelling de toepassing van bestrijdingsmiddelen terug te dringen. Jaarlijks werd in de periode 1984-1988 10.000.000 kg actieve stof gebruikt. De doelstelling van het MJPG was het gebruik van bestrijdingsmiddelen te reduceren met 31,0 % in 1995 en 55,0 % in 2010. De consequentie van deze reductie was dat er een herziening van de regelgeving ten aanzien van aardappelmoeheid plaats vond. Sinds 1994 is het, op een aantal ontheffingen na, slechts toegestaan aardappelen één keer in de drie jaren te verbouwen. Dit voorschrift is onafhankelijk van de teelt van resistente rassen of het toepassen van grondontsmettingsmiddelen. Ten aanzien van de teelt van pootaardappelen en ander voortkwekingsmateriaal is sinds 1994 niets gewijzigd. Hierdoor vallen akkerbouwers voor de bestrijding van AM in feite terug op intensiever gebruik van resistente rassen. Uitbreiding van vruchtwisselingsschema's is in de gangbare landbouw meestal geen economisch haalbare optie.

'gene-pool similarity concept'

Op basis van onderzoek naar de variatie tussen populaties van beide aardappelcystenaaltjes op eiwit niveau werd begin jaren negentig het 'gene-pool similarity concept' geformuleerd (Bakker *et al.*, 1993). Dit concept biedt een alternatief voor het pathotypenschema en integreert moleculaire en virulentie eigenschappen van populaties. Het concept gaat uit van de veronderstelling dat rond 1850 in Europa een beperkt aantal introducties van aardappelcystenaaltjes populaties vanuit de Andes heeft plaats gevonden, slechts een klein deel van de totale 'gene-pool' in Zuid-Amerika. In afwezigheid van selectiedruk door resistentiegenen, langer dan een eeuw werden alleen vatbare rassen verbouwd, wordt dan de genetische samenstelling van de huidige populaties in Europa in hoofdzaak bepaald door de genetische constitutie van de oorspronkelijke introducties, 'random genetic drift' en 'gene flow'. Selectie en mutatie spelen een zeer waarschijnlijk een ondergeschikte rol. In dit geval gedragen (a)virulentie loci zich als neutrale kenmerken net zo als eiwit- en DNA-polymorfieen en komen moleculaire verwantschappen tussen populaties ook tot uiting op (a)virulentie loci en vice versa. Wanneer groepen van populaties gevormd kunnen worden op basis van moleculaire verwantschappen dan is de verwachting dat deze ook overeenkomstige eigenschappen vertonen voor wat betreft virulentie eigenschappen ten aanzien van alle resistentiegenen. Ook die welke momenteel nog niet in veredelingsprogramma's gebruikt worden. Begin jaren zestig werden de eerste resistente rassen

(aanvankelijk alleen *H1* resistentie) op grotere schaal verbouwd. Op die percelen heeft natuurlijk wel selectiedruk door resistentiegenen plaats gevonden.

doelstelling van het onderzoek

Het doel van dit project was i) bestudering van de mogelijkheden van diagnostisering van populaties binnen beide soorten aardappelcystenaaltjes (intraspecifieke variatie) (Hoofdstuk 2-5). Met behulp van een dergelijke toets, gebaseerd op eiwit- of DNA-polymorfismen, kan dan in principe van te voren vastgesteld worden welke typen (virulente) populaties aanwezig zijn en kan een perceelsgericht advies gegeven worden ten aanzien van de te telen (resistente) aardappelrassen. ii) Samenhangend met i) een schatting maken van de genetische diversiteit van *G. pallida* in Nederland, en van de frequenties van deze diversiteiten (Hoofdstuk 6). Zijn deze bekend dan kan tevens advies gegeven worden aan veredelaars welke populaties als toetspopulaties te gebruiken hun veredelingsprogramma's. iii) Toetsen van het 'gene-pool similarity concept' aan de hand van een aantal *G. pallida* veldpopulaties (Hoofdstuk 7).

variatie tussen soorten

Uit een vergelijking van DNA fingerprints, genetische vingerafdrukken, tussen populaties van beide soorten bleek dat slechts 3,6% (RAPDs, Hoofdstuk 3) en 6,4% (AFLP, Hoofdstuk 4) van de DNA fragmenten in populaties van beide soorten voorkomt. Hoewel de soorten morfologisch nauwelijks van elkaar zijn te onderscheiden, verschillen ze aanzienlijk van elkaar op moleculair niveau.

De ontwikkeling van een gevoelige diagnostische toets om de soorten van elkaar te onderscheiden is in principe mogelijk met behulp van de RAPD merkers. *G. rostochiensis* specifieke DNA fragmenten werden zelfs aangetoond in slechts 1/5 deel van het homogenaat van één larve (Hoofdstuk 2). Op dit moment wordt op grote schaal een ELISA toets, gebaseerd op specifieke monoklonale antilichamen, gebruikt om in een grondmonster de soorten aardappelcystenaaltjes te determineren.

variatie binnen *G. rostochiensis*

Zowel RAPD als AFLP analyses laten zien dat de intraspecifieke variatie in *G. rostochiensis* in Nederland laag is (Hoofdstuk 3 en 4). Het aandeel polymorfe DNA fragmenten in 9 onderzochte *G. rostochiensis* populaties bedroeg respectievelijk 19% en 15,8%. In beide gevallen werden twee clusters van populaties onderscheiden. Elke cluster wordt met een of meerdere specifieke DNA fragmenten gekarakteriseerd. Populaties binnen een cluster hebben bovendien vergelijkbare virulentie eigenschappen (pathotypen Ro1 en Ro3). Deze resultaten tonen aan dat een diagnostische toets voor deze clusters ontwikkeld kan worden.

variatie binnen *G. pallida*

De intraspecifieke variatie in *G. pallida* is eveneens laag maar groter dan die in *G. rostochiensis* (Hoofdstuk 3 en 4). Het aandeel polymorfe RAPD kenmerken in 17 populaties bedroeg 46%. In 15 van deze populaties bedroeg het aandeel polymorfe AFLP kenmerken 23%. In beide gevallen kunnen met behulp van specifieke DNA fragmenten slechts enkele duidelijk gedivergeerde clusters herkend worden. Evenwel bevat een aantal clusters populaties met een verschillende pathotypen classificatie.

Dit beeld wordt in Hoofdstuk 5 nog eens bevestigd in een analyse van de genetische variatie van 36 *G. pallida* populaties met behulp van twee verschillende moleculaire technieken: RAPDs en allel frequenties bepaald aan de hand van eiwitconcentraties van isoelectrisch punt (IP) varianten in 2-DGE. Analyse van beide datasets leerde dat de meerderheid van de populaties niet duidelijk van elkaar verschilt. Geconcludeerd moet dan ook worden dat voor een betrouwbare diagnostische toets voor *G. pallida* niet ontwikkeld kan worden met de hier gebruikte systemen maar dat gezocht moet worden naar DNA merkers die gekoppeld zijn met (a)virulentie.

de genetische diversiteit van *G. pallida* in Nederland

Omdat *G. pallida* momenteel het grootste probleem vormt in de aardappelteelt is de genetische diversiteit met behulp van 2-DGE nader onderzocht bij 226 populaties uit Noord-Friesland, de IJsselmeerpolders en Noordoost-Nederland (Hoofdstuk 6).

De relatief lage genetische diversiteit van *G. pallida* in Nederland impliceert dat de genetische verschillen tussen de oorspronkelijke introducties ('primary founders') eveneens gering geweest moet zijn.

De genetische structuren van de populaties verschillen per regio. In Noord-Friesland en de IJsselmeerpolders is de variatie binnen populaties lager dan in populaties uit Noordoost-Nederland. De variatie tussen populaties in Noord-Friesland en de IJsselmeerpolders evenwel is hoger dan in Noordoost-Nederland. Dit beeld wordt bevestigd indien meer gedetailleerd naar twee subregio's in de IJsselmeerpolders en Noordoost-Nederland gekeken wordt. Met name de effecten van 'random genetic drift' zijn groot geweest. Dit wordt toegeschreven aan verschillen in bedrijfsvoering in beide subregio's.

validatie van het 'gene-pool similarity concept'

De variaties op moleculair niveau, en de variaties in virulentie op drie resistente rassen (Santé, Elles en Seresta) tussen 102 *G. pallida* populaties zijn met elkaar vergeleken. Aangetoond werd dat als het verschil op moleculair niveau tussen populaties afneemt, ook de variatie tussen populaties in relatieve reproductie op de resistente rassen kleiner wordt. Het gene-pool similarity concept is dus bruikbaar voor het schatten van de variatie in virulentie. Tevens werd een vergelijkbare relatie gevonden tussen moleculaire kenmerken en de variatie in fitness (de vermeerdering van populaties op het vatbare ras Désirée). Hieruit blijkt dat de verschillen in reproductie van populaties op vatbare rassen gedeeltelijk genetisch bepaald zijn.

resistentieveredeling

De gen-om-gen relatie, zoals die geformuleerd is door Flor (1943, 1971), wordt in natuurlijke systemen en in de landbouw veelvuldig gebruikt als model om de 'wapenwedloop' tussen planten en hun belagers te verklaren. De gangbare modellen richten zich voornamelijk op de selectie van resistentie en/of virulentie genen in locale populaties. De invloed van de geografische structuren van de plant- en pathogeenpopulaties wordt hierdoor onderschat. Thompson & Burdon (1994) onderkenden deze lacune in het coevolutionaire denken. Zij benadrukken dat de metapopulatie structuur, met factoren als random genetic drift en gene flow, minstens zo belangrijk is voor het uiteindelijke resultaat als locale natuurlijke selectie.

Voor het resistentiemanagement van aardappelcystenaaltjes is het cruciaal dat de geografische component in de interaktie tussen aardappel en aardappelcystenaaltjes meegenomen wordt. Nog te vaak wordt het virulent zijn van een populatie op een resistente cultivar verklaard door selectie. Uit onderzoek naar de verspreidingspatronen van aardappelcystenaaltjes bleek dat virulente populaties vaak aanwezig waren in een gebied voordat cultivars met de corresponderende resistentiegenen verbouwd werden. Voor een succesvolle bestrijding van aardappelcystenaaltjes met resistente cultivars is het dus noodzakelijk rekening te houden met de geografische verspreidingspatronen van populaties met verschillende virulentie eigenschappen.

De veredeling op resistentie tegen aardappelmoeheid was de afgelopen decennia voornamelijk gebaseerd op een 'trial-and-error' benadering. Telkens werd het effect van resistente rassen teniet-gedaan door het 'opkomen van resistentie doorbrekende aaltjespopulaties'. Dit heeft veelal geleid tot de zienswijze dat de bestrijding van aardappelmoeheid met behulp van resistente rassen een korte termijn benadering is. Uit de resultaten van dit proefschrift blijkt echter dat de veredeling op resistentie tegen aardappelcysteaaltjes geen verloren 'wapenwedloop' behoeft te zijn. Tot voor kort werd aangenomen dat de genetische diversiteit van de aardappelcystenaaltjes, met name van *G. pallida*, te groot was om rassen te kweken met een brede en duurzame resistentie. In dit proefschrift is aangetoond dat het aantal verschillende introducties van *G. pallida* vanuit Zuid-Amerika, vrij gering is geweest en dat de genetische variatie van tussen de *G. pallida* populaties in Nederland voornamelijk veroorzaakt is door 'random genetic drift'. De uitgebreide analyse van 226 *G. pallida* populaties uit de IJsselmeerpolders, Noord-Friesland en Noordoost-Nederland biedt goede perspectieven voor het veredelen op brede en duurzame resistentie tegen aardappelmoeheid. De 'gene-pool similarities', zoals vastgesteld aan de hand van eiwitelectroforese, kunnen als leidraad gebruikt worden voor het testen van de effectiviteit van resistentiegenen alvorens deze op te nemen in een verdelingsprogramma, hetgeen de 'sleutel' is voor het veredelen op duurzame waardplantresistentie tegen aardappelmoeheid.

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Curriculum vitae

Roelof Tjallo Folkertsma werd geboren op 12 november 1963 te Workum. In 1982 werd het V.W.O. diploma behaald aan het Chr. Lyceum 'Oostergo' te Dokkum, waarna hij in 1986 de Bijzondere Hogere Landbouwschool te Leeuwarden succesvol afrondde. De goede resultaten in het laatste jaar werden beloond met de Boelstra-Olivier prijs. Hierna zette hij zijn studie voort aan de Landbouw Universiteit in Wageningen (LUW). In 1990 studeerde hij af als plantenziektekundige, gespecialiseerd in fysiologische en moleculair-biologische aspekten van de gewasbescherming. Tijdens zijn doctoraal heeft hij onderzoek verricht naar de variatie op eiwit en mtDNA niveau van isolaten van *Phytophthora infestans*. Dit onderzoek is uitgevoerd in Wales (UK) bij de groep van dr. R. Shattock (Department of Plant Pathology, UCNW, Bangor).
Vanuit Nederland is dit onderzoek begeleid door dr. ir. L. Davidse (vakgroep Fytopathologie, LUW). Ook heeft hij zich, onder begeleiding van dr. ir. J. Bakker (vakgroep Nematologie, LUW), beziggehouden met de ontwikkeling van een methode om het mitochondriaal DNA (mtDNA) van aardappelcystenaaltjes te isoleren. Bij de groep van dr. T. Bisseling (vakgroep Moleculaire Biologie, LUW) heeft hij onderzoek verricht naar de aanwezigheid van wortelhaar specifieke eiwitten in de erwt (*Pisum sativum*).
In 1990 is de auteur in dienst getreden bij de vakgroep Nematologie om onderzoek te doen naar de genetische diversiteit van het aardappelcystenaaltje in Nederland. Het onderzoek werd uitgevoerd in het kader van het Meerjaren Plan Gewasbescherming project 'detectie en identificatie van aardappelcystenaaltjes (*G. pallida* en *G. rostochienis*) populaties (AOB 1)'. Twee jaar na aanvang van het onderzoek werd het onderzoek geïntegreerd in het EC-projekt 'Genotyping potato cyst nematodes in Europe: a search after the initial introductions (AIR3 CT 92.006)', een samenwerkingsverband met onderzoeksgroepen afkomstig uit Frankrijk (dr. D. Mugniery, Rennes), Portugal (prof. dr. M.S.M. De Santos, Coimbra), Schotland (dr. D.L. Trudgill, Dundee) en Spanje (dr. E. Ritter, Vitoria). Tevens werd een deel van het onderzoek uitgevoerd in het kader van het Herstruktureringsproject 'Een goede en betrouwbare resistentie tegen aardappelmoeheid voor Nederland'. Dit project werd uitgevoerd in nauwe samenwerking met het CPRO-DLO (dr. ir. C. Hoogendoorn, dr. ir. P.J.C.C. Wolters, dr. ir. R. Janssen).
Sinds oktober 1996 is de auteur werkzaam als postdoc bij de vakgroep Virologie (LUW). Hier houdt hij zich bezig met het isoleren en karakteriseren van het *Sw-5* resistentiegen in de tomaat tegen het tomatenbronsvlekkenvirus.