Identification of PCN species by PCR Identification of Potato Cyst Nematode species by Polymerase Chain Reaction

P. J. R. Njau

Department of Crop Science and Production, Faculty of Agriculture, Sokoine University of Agriculture, P.O. Box 3005, Chuo Kikuu, Morogoro, Tanzania.

Abstract

In a study conducted on cysts collected from the eastern and western Flanders regions of Belgium in 1998 and 1999. Globodera pallida and G. rostochiensis were the only two Globodera species collected and identified based on PCR (Polymerase Chain Reaction) criteria. G. rostochiensis was the predominant species forming 98% (n = 2500) of the total cysts collected. G. pallida was encountered in 2% (n=2500) of the total cysts tested. The results suggest that G. rostochiensis is the dominant member of the potato cyst nematodes in the eastern and western Flanders regions of Belgium. Unlike G. pallida, G. rostochiesis is highly prolific and this behavior may be useful in targeting control measures for this specie such as use of resistant cultivars and crop rotation. This is the first longitudinal study to be carried out in Belgium using multiplex PCR..

Keywords: Belgium, Potato cyst nematodes, Polymerase chain reaction.

Introduction

Potato cyst nematode (PCN) species, *Globodera pallida* (Wollenweber, 1923) and *G. rostochiensis* (Stone, 1972) originated from the Andean region in South America from where they have spread to many countries around the world (Brodie *et al.*, 1993). They are major agricultural pests and the distinction of the two species is essential for proper application of control and quarantine measures (Curtis *et al.*, 1996). However, the correct identification of these species is extremely difficult because the morphological differences between the two species are very small and within species variations that occur make identification using morphological characters specialized and time consuming exercise. Potato cyst nematodes, therefore, remain the major cause of yield and quality reduction of potato crop in all countries where potato is grown (Schots *et al.*, 1988; Haydock and Evans, 1998).

For many years, the two species of PCN were considered to be the potato strain of the sugar beet cyst nematode, *Heterodera Schachtii* (Schmidt). However, in 1923 Wollenweber classified this potato strain as *Heterodera rostochiensis* (Woll). In the seventies, it became apparent that in Europe two species of PCN were present (Jones *et al.*, 1970) as, increasingly workers described subtle differences between the PCN populations. Guile (1970) noted that females of certain PCN became golden-yellow after rupturing the root cortex while females of other PCN population became white or cream after emergency from the root. Variations in the size and morphology of juvenile stylet (Evans and Webley, 1970; Webley, 1970) as did structural differences in the vulval and lip region of the nematodes (Green, 1971, Stone, 1972) also indicated a degree of heterogeneity within *H. rostochiensis.* A genetic basis for these differences was indicated by the work of Trudgill and Carpenter (1971), who found consistent differences in the electrophoretic profiles of soluble proteins from populations producing golden and cream or white females and by Parrot (1972), who

*Corresponding author

demonstrated that mating between the two types were unsuccessful. The accumulated evidence of heterogeneity within *Heterodera rostochiensis* eventually led to the description of two sibling species *Heterodera rostochiensis*, with golden colored females, and *Heterodera pallida* with white or creamy colored females. Subsequently *H. rostochiensis* and *H. pallida* were assigned to the genus *Globodera* (Behrens, 1972; Mulvey and Stone 1976; Brodie *et al.*, 1993) along with other round cyst nematodes that lacked a terminal cone on the mature cyst. This separated PCN and their related species from the genus *Heterodera* containing the lemon-shaped cyst nematodes.

Due to variations in biology and crop culture and physiological conditions, the composition of the sibling species may differ within a given area therefore precise species identification to determine the composition of the nematodes is central to successful PCN control. Failure to recognize the species composition in any given potato production area may lead to control effort being directed at a minor pest at the expense of the main pest. Several procedures have been proposed to distinguish between potato cyst nematode species and pathotypes using protein polymorphism obtained by electrophoresis technique based on characteristic bands or spots of total protein extracts (Bakker & Gommers, 1982; Fleming & Marks, 1983) or isozyme (Fox and Atkinson, 1984). Serological procedures such as ELISA using monoclonal antibodies have been used for species identification (Schots *et al.*, 1989; Brodie *et al.*, 1993). Other techniques allow the identification of PCN species at the DNA level. Polymerase chain reaction (PCR), a DNA based technique has been found to be an important tool for the identification of the sibling species of potato cyst nematodes (Mulholland *et al.*, 1996; Bulman & Marshall, 1997).

The present study was undertaken to determine the PCN species composition in the eastern and western Flanders potato growing regions of Belgium using multiplex PCR.

Materials and methods

Origin of potato cyst nematode populations

Five of 14 populations of potato cyst nematodes, which were collected in 1998 by Dr. N. Viaene, Agriculture Research Station (ARS), Merelbeke, Belgium from potato growing fields in the eastern and western Flanders regions of Belgium were used in this study. The five populations namely, Aarsele, AX1, Bevere, Pop 104 Gr and Wannegem were selected based on their considerably higher mean and relatively lower coefficient of variations of eggs and juvenile contents (Table 1). The dry cysts of the populations used in the study were extracted from soil using the high-pressure water method as described by Hendrickx and Moens (1996). For comparison of reactions, authentic cysts of *G. pallida* and *G. rostochiensis* were used. The cysts of those species were kindly provided by Dr. Kenneth Evans, Rothamsted Experimental station, Harpenden, Hertfordshire, England.

Table 1. Mean, Standard deviation and Coefficient of variations of eggs and juveniles contents of 14 populations of potato cyst nemetodes[1]

Population	Full egg (E)	Empty egg	Full juvenile (j2)	Empty juvenile (j2)	Full E+j2	Empty E+j2	
Aarcele	119,7	90,0	120,9	6,3	240,6	96,3	mean
	102,3	77,0	82,2	4,5	136,1	76,1	stdev
	85%	86%	68%	72%	57%	82%	cv
Wannegem	102,4	353,8	409,2	38,8	511,6	392,6	mean
	55,7	100,5	58,8	25,3	95,1	95,6	stdev
	54%	28%	14%	65%	19%	24%	cv
Ichtegem	11,8	374,4	125,1	3,2	136,9	377,6	mean
	18,1	140,5	181,0	6,3	190,2	138,1	stdev
	154%	38%	145%	197%	139%	37%	cv
Kanegem	10,6	401,2	41,3	3,8	51,9	405,1	mean
	14,6	186,5	64,9	4,5	77,4	187,8	stdev
	137%	46%	157%	117%	149%	46%	cv
Wevelgem	110,6	248,72	484,24	19,4	594,8	268,1	mean
prei	102,3	97,2	780,8	16,2	833,7	95,2	stdev
	92%	39%	161%	83%	140%	35%	cv
AB2	96,4	59,9	384,2	55,2	480,6	115,0	mean
Lo-Reninge	73,5	31,6	198,6	80,9	246,1	93,2	stdev
	76%	53%	52%	147%	51%	81%	cv
Bevere	132,0	136,0	327,2	98,1	459,2	234,1	mean
extra stalen	70,4	60,2	111,6	57,7	129,9	102,3	stdev
	53%	44%	34%	59%	28%	44%	cv
POP104	108,3	164,1	257,9	78,1	366,2	242,2	mean
Gr.	71,4	77,5	86,9	37,8	110,8	102,4	stdev
	66%	47%	34%	48%	30%	42%	cv
BA 16	50,3	108.5	155,1	22,7	194,5	126,2	mean
STL 44	48,9	124,0	197,2	30,0	230,1	131,0	stdev
	97%	114%	127%	132%	118%	104%	cv
AZ 6	43,4	106,8	154,9	23,5	188,6	124,5	mean
Sint-Laurent	45,1	123,2	195,1	29,3	228,2	130,3	stdev
	104%	115%	126%	125%	121%	105%	cv
AY3	40,6	97,8	150,2	23,1	181,1	115,1	mean
Sint-Laurent	43,3	112,0	187,7	29,1	217,6	117,7	stdev
	106%	115%	125%	126%	120%	102%	cv
POP 104	42,8	85.6	151.5	24,8	184,0	103,8	mean
Veld	42,5	95,6	187,0	28,6	217,5	104,0	stdev
	99%	112%	123%	115	118%	100%	cv
AX1	130,1	270,9	335,6	60,7	465,7	331,6	mean
Assenede	108,6	67.2	80,6	27,2	160,7	85,7	stdev
	106%	37%	15%	26%	35%	26%	cv
Lange Reep	0.04	257,24	2,68	0,48	2,7	257,7	mean
Assened	0,2	83,5714	11,3347	0,71414	11,5	83,7	stdev
		7	5	3			
	500%	32%	423%	149%	424%	32%	cv

Legend

- 1=Twenty five cysts from each population were randomly selected and of which their eggs and juvenile
- Contents were counted
- Populations in bold were used for PCR analysis
- CV = Coefficient of variation
- Stdev = Standard deviation
- E = Eggs
- J2 = Juvenile stage 2

Extraction of Nematode DNA

Nematode cysts of each of the five populations were inspected for exclusion of empty cysts, and from each population, 500 cysts were selected at random and used for PCR test. The cysts were soaked in distilled water overnight at room temperature or placed in a refrigerator at 6°C for 12 hours. DNA was extracted from single cysts by the procedure as described by Bulman and Marshall (1997) with slight modification as

follows: Cysts were ground using an electric grinder instead of plastic micro pestles and in 20 µl of solution containing 10µl double distilled water, 8µl worm lysis buffer (100mMKcl + 100mMTris. HCl pH 8.3 + 15mM Mgcl2 + 10 mMDTT + 4.5% Tween 20 + 0.1% Gelatin) and 60ug/ml Protenaise K (instead of 50ul of guanidine isothiocynate Tris-Hcl buffer). The crude DNA extract was stored at -7°C overnight prior to incubation at 65°C for one hour and subsequently at 94°C for 10 minutes. Extracts were centrifuged at 14 x 10^3 rpm for one minute and used for PCR mediated amplification of DNA and species speciation without further treatment.

Polymerase Chain Reaction

The PCR reactions to distinguish between *G. pallida* and *G. rostochiensis* were carried out as described by Bulman and Marshall (1997) with slight modification using Primers PITSr3 (5'-AGCGCAGACATGCCGCAA-3') and PITSp4 (5'-CAACAGCAATCGTCGAG-3') in combination with primer ITS5. Each reaction component was prepared on ice and consisted of 5µl 1x PCR Taq buffer, 10µl Q solution, 200mM dNTPS, 0.3µg each primer, 1 unit of Taq DNA polymerase, 8 µl of nematode template and sterile double distilled water to a final volume of 42µl. Controls with and without nematodes were always included with each test. Denaturation of double strand DNA took place at 94°C (2min.), primer annealing at 60°C (30s) followed by polymerization at 72°C each for 30s. The reactions were performed in a programmable thermocyler (Biozym, The Netherlands).

Agarose gel electrophoresis

To analyze the PCR amplification products, 5µl of each were mixed with 1µl of loading buffer (Promega, Madison, WI, USA) and carefully loaded into individual slots of 1% agarose gels containing ethidium bromide at a final concentration of 0.05µg/ml. As positive controls, DNA samples of authentic *G. pallida* and *G. rostochiensis* were used. A standard DNA marker 100bp was also run on each gel. Electrophoresis was performed in 1x TAE buffer at 100V, 100W for 0.8 hour. Reactions showing amplification in the negative control were discarded. DNA was visualized on ultra-violet transluminator and photographed using Polaroid film. Electrophoretic migration of DNA in agrose gels varies with the size of DNA fragments hence it is easy to distinguish between *G. pallida* and *G. rostochiensis* specific PCR products. All samples giving a PCR product of 265 bp were considered *G. pallida* whereas samples giving a PCR product of 434 bp were considered *G. rostochiensis* (Bulman & Marshall, 1997). All reactions giving no PCR product or giving PCR product beyond these ranges were considered to belong to species other than *G. pallida* and *G. rostochiensis*.

Results

A total of 4167 cysts belonging to 14 populations were isolated from soil samples collected from the potato growing fields in the eastern and western Flanders regions of Belgium. About 2500 cysts were morphologically identified to belong to potato cyst nematodes and were further analyzed by PCR to characterize them into respective PCN species. This represented 60% of the total number of cysts collected in the eastern and western Flanders region of Belgium.

Out of the total 2500 cysts characterized in PCR, 2000 were identified as *G. rostochiensis* whereas the remaining 500 cysts were *G. pallida*. The DNA bands in ethidium stained agrose gel for these species are shown in Fig. I. The length of sequence in nucleotides amplified between UN primers and each of the species specific primers is 264 for *G. pallida* and 434 for *G. rostochiensis*.

The results obtained in this study indicate that *G. rostochiensis* is the most prevalent sibling species of PCN in the potato growing areas in Belgium although pockets of *G. pallida* can be encountered in a few fields. These species are responsible for the huge loss in yield of potato crop in Belgium.

Discussion

PCR analysis identified 2000 of 2500 cysts tested to be *G. rostochiensis* showing this species to be the dominant member of the PCN species represented in the potato growing areas in Belgium. *Globodera pallida* was, however, identified from cyst isolated from soil samples collected from only one area, Aarsele, in the eastern Flanders region.

Although the present study was not comprehensive in terms of space and time the results obtained, however, are in agreement with the findings of Pyone (1993) and Ramos (1995)

who reported G. *rostochiensis* as the dominant PCN species in the potato growing areas of Belgium. The sibling species were identified by the morphological parameters of second stage juveniles (J_2) and vulval fenestralia. As in my study, these workers detected *G. pallida* in cysts from soil samples collected from the Aarsele, potato growing area in the Flanders region.

The increase in relative frequencies of one PCN species over another in some parts of Europe has been observed to be associated with species genetic background (Hominick, 1982) and species biology (Haydock & Evans, 1998). However, the exact reason for the great preponderance of *G. rostochiensis* over *G. pallida* in Belgium is difficult to ascertain but it is believed that the use of control tactics for PCN might be working (overtime) selectively to the advantage of *G rostochiensis*. However, further studies in this area are justified because the information generated would have future significance especially in the designing of tactics for the management of potato cyst nematodes in this country.

The PCR analysis used in characterization of members of the potato cyst nematode species complex in the present study has been shown to have very high efficiency for precise identification of the sibling species (Mulholand *et al.*, 1996; Bulman & Mashall, 1997). Unlike morphometric characters which utilize J2 Juveniles and mature females, PCR has the advantage of using all developmental stages and may use very small portions of such specimens, giving a high opportunity for experimental replications. However, the high prices of reagents and expensive equipment may limit its large scale application a feature that offer morphological and morphometric taxonomy greater advantages.

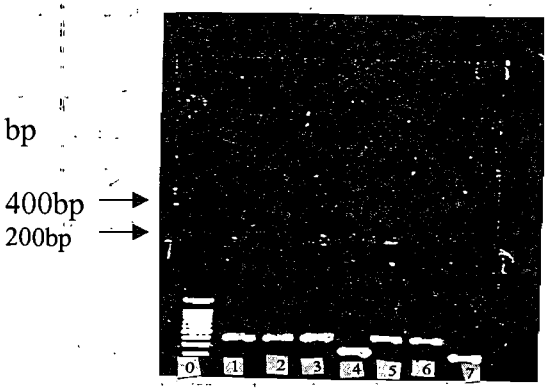

Fig.1. DNA bands of populations Ax1 Assenede, Bevere, Pop 104Gr, Aarsele, and Wannegem stained with ethidium bromide. Lane 0; DNA standard marker; Lane1, AX1 Assenede; Lane 2, Bevere; Lane 3, Pop 104Gr.; Lane 4, Aarsele; Lane 5,Wannegem; Lane 6, *G. rostochiensis* control; and Lane 7, *G. Pallida* control.

Conclusion

The results of the present study suggest that *G. rostochiensis* is the most prevalent PCN species present in the potato growing areas of Belgium. *G. pallida* is only of limited occurrence. Based on its higher prevalences relative to *G. pallida*, the species contributes significantly to loss in yield of potato crop in the potato growing areas surveyed.

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References

Bakker, J. and Gommers, F. J. 1982. Differentiation of the potato cyst nematodes.*Globodera rostochiensis* and *G. pallida* and two *Globodera rostochiensis* pathotypes by means of two dimensional electrophoresis. Proceedings Koninklije Nederlandse Akademie van Weternschappen, 85: 300-305.

Behrens, E. 1972. Morphologische Unersuchungen von Heterodera spp.an *Solanum tuberosum*. Probleme der Phytometologie, 2 Juni 1972; 76-85.

Brodie, B. B., Evans, K., and Franco, J. (1993). Nematode parasities of potatoes In: Evans, K.Trudgill, D.L. and Webster, J.M.(ed). Plant parasitic nemotodes in temperate agriculture. Wallingford; CABI pp 87-132.

Bulman, S. R. and Marshal, J. W. 1997. Differentiation of Australia potato cyst nematodes populations using polymerase chain reactions (PCR). New Zealand Journal of Crop and Horticultural Science 25: 123-129.

Curtis, R. H. Seger, C., I. and Evans, K. 1996. Observations on the specificity of *Globodera rostochiensis* and *Globodera pallida* diagnostic proteins to amphids of *Globodera* species. Nematologica, 42: 466-480.

Evans, E. B. and Webley, D. P.1970. A guide to morphological differences between pathotypes of *Heterodera rostochiensis* larvae. Plant Pathology 19: 171-173.

Evans, K. Trudgill, D. L. and Webster, J.M. (ed). Plant parasitic nemotodes in temperate agriculture. Wallingford; CABI pp 87-132.

Fleming, C. C. and Marks, P. J. 1983. The identification of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* by isoelectric focusing of proteins on polyacrylamide gels. Annals of Applied Biology 103: 277-287.

Fox, P. C. and Atkinson, H. J. 1984. Glucose phosphate isomerase polymorphism in field populations of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. Parasitology 91: 499-506.

Green, C. D. 1971. The morphology of the terminal area of the round cyst nematodes *Heterodera rostochiensis* and allied species. Nematologica 17: 34-46.

Guille, C. T. 1970. Further observations on cyst colour changes in potato cyst eelworm. pathotypes. *Pl. Path.* 20: 43-46.

Hendrickx, G. and Moens, M. 1996. A high pressure injector simplifies the extraction of lobodera cysts from soil. Nematologica 20: 43-57.

Hominick, W. H. 1982. Selection of a rapidly maturing population of *Globodera rostochiensis* by continuous cultivation of early potatoes in Ayshire, Scotland. Annals of Applied Biology 100: 345-351.

Hydock, P. P. and Evans, K. 1998. Management of potato cyst nematodes in the UK. An integrated approach? Outlook on Agriculture. 20: 253-260.

Jones, F. G. W., Carpenter, J. M., Parrot D. M., Stone, A. R. and Trudgill, D. L. 1970. Potato cyst nematodes: one species or two? *Nature* (Lond.) 227: 83-84.

Mulholand, V.; Carde, L.; O'Donnel, K. J.; Fleming, C. L. and Powers, T. O. 1996.Use of polymerase chain reaction to discriminate potato cyst nematodes at the species level In: Proceedings of Diagnostics in Crop Production Ed. Marshal G. British Crop Production Council, Furham, UK, pp. 247-252.

Mulvey, R.H. and Stone,A.R. 1976. Description of *Punctodera matadorensis* n.gen.n.sp (Nematoda:Heteroderidae) from Saskatchewan, with lists of species and generic diagnoses of Globodera, Heterodera and Sarisodera. Can, Zool. 43: 772 -785.

Parrot, D. M. 1972. Mating of *Heterodera rostochiensis* pathotypes. Ann. Appl. Biol., 71: 271-273.

Pyone, P. K. 1993. On the identity of some potato cyst nematode populations from Belgium. MSc. Thesis, Postgraduate International Nematology Course (PINC), State University of Gent, Belgium pp. 1-30.

Ramos, G.A.S. 1995. *Globodera rostochiensis* in early potatoes. MSc. Thesis. International Nematology Course, State University of Ghent, Ghent, Belgium. Pp 1-30. Schots, H.; Hermsen, T; Schuten, S. Gommers, F. J. and Egberts, E. 1989. Serological differentiation of the potato cyst nematodes *Globodera pallida and G. rostochiensis* II. Preparation and characterization of species specific monoclonal antibodies. Hybridoma, 8: 401-413.

Schots, H.; Hermsem, T; Schuten, S.Gommers, F.J. and Egberts, E.1989. Serological differentiation of the potato cyst nemotodes *Globodera pallida* and *G.rostochiensis* II. Preparation and characterization of species specific monoclonal antibodies. Hybridoma 8:401-413.

Stone, A. R. 1972. *Heterodera pallida* n.sp. (Nematoda: Heteroderidae), a second

species of potato cyst nematodes. *Nematologica* 18: 591-606.

Trudgil, D. L. and Carpenter, J .M. 1971. Disc electrophorsis of proteins of Heterodera species and pathotypes of *Heterodera rostochiensis*. Annals of Applied Biology 129: 315 – 322.

Webley, D.1970. A morphometric study of three pathotypes of the potato cyst eelworm. *Heterodera rostochiensis* recognised in Great Britain. Nematologica 16, 107-112.

Wollenweber, H. W. 1923. Krankheiten und Beschädigugen der kartoffel. Arb. Forsch. Ist. Kartofffl., Berlin, 7: 1 – 56.