Nematol. medit. (2001), 29: 91-97

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MELOIDOGYNE CHITWOODI AND *M. FALLAX* IN BELGIUM

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Summary. During a *Meloidogyne* survey in Belgium a total of 2877 soil and root samples were taken. Root-knot nematodes were found in 9.2% of the samples. The species were identified according to morphological, morphometrical, biochemical, and molecular characters. Ten percent of the positive samples contained *M. chitwoodi* and/or *M. fallax*. Among the enzymes used, only *Tru* 9I clearly separated *M. chitwoodi, M. fallax* and *M. hapla* from each other. RAPD was used to examine the molecular diversity between the populations. The tree constructed on the basis of the RAPD patterns grouped the populations according to the species. It revealed considerable intraspecific variation supporting the idea that *M. chitwoodi* and *M. fallax* have been present in Belgium for a long time.

Root-knot nematodes are widely distributed and cause substantial reduction of crop yield and quality. In 1974 a root-knot species was found on potato tubers in North-America and grouped in the *Meloidogyne hapla* complex. Later, the nematode population was described as a new species, *M. chitwoodi* (Golden *et al.*, 1980). Since its description, *M. chitwoodi* has been reported in several European countries: the Netherlands (Brinkman and Van Riel, 1990), Germany (Heinicke, 1993; Müller *et al.*, 1996) and France (Marzin, pers. com.). In 1992 it was detected for the first time in Belgium (M. Moens, unpublished).

During the period 1992-1995 a root-knot nematode population closely related to *M. chitwoodi* was detected in the Netherlands. Because of differences in morphology and isozyme patterns, this population was later described as *M. fallax* (Karssen, 1996).

In the present paper the distribution and the molecular diversity of both *M. chitwoodi* and *M. fallax* populations in Belgium are reported.

Material and methods

Soil and root samples, in total 2877, were collected throughout Belgium during 1996-1997. Potato and vegetable producing areas were sampled more intensively and the number of samples was increased in areas where *M. chitwoodi* had previously been detected. Soil samples were put in pots and planted with tomato (cvs Marmande or Moneymaker). Two months later, roots were washed and examined for the presence of root-knots.

To increase the possibility for detecting mixed populations 30 young females were collected from each population and kept for bio-

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chemical identification. Corresponding egg masses were used to build up monospecific populations. This was done on potato tubers (c, Bintje) in closed plastic containers filled with sterilised sand (method originally developed by J. J. Van De Haar, The Netherlands).

M. chitwoodi Golden, O' Bannon, Santo *et* Finley and *M. fallax* Karssen, were morphologically and morphometrically identified according to Jepson (1987) and Karssen (1999). The technique described by Karssen *et al.* (1995) was used for biochemical characterisation.

DNA was extracted from five juveniles of the same egg mass according to Joyce *et al.* (1994) and used in PCR. Two 21 nucleotide sequences complementary to conserved sequences in the 18S and 26S rDNA genes of *Xiphinema bricolensis* (Vrain *et al.*, 1992) served as primers. Upon completion, 5 ml of the PCR mixture was used for electrophoresis in a 1% agarose gel containing ethidium bromide. Following electrophoresis, DNA fragments were visualised under UV light and photographed. The concentration of the DNA bands was estimated with a DNA mass ladder on the gels. Equal concentrations of each PCR product were digested with different restriction enzymes (*Alu*I, *Bsh*1236I, *BsiZI*, *Cfo*I, *Hin*fI, *Hpa*II, *Mva*I and *Tru9*I) in the appropriate buffer according to manufacturer's instructions. RFLP patterns were visualised under UV light after electrophoresis on a 2% agarose gel with ethidium bromide and photographed. To check repeatedly the identity and purity of the monospecific cultures, PCR with species-specific primers for *M. chitwoodi* and *M. fallax* (Petersen *et al.*, 1997) were used.

To study the molecular diversity of *M. chitwoodi* and *M. fallax*, total DNA was extracted from egg masses with the Puregene DNA isolation Kit (Animal Tissue Protocol, Biozym, Landgraaf, The Netherlands). In addition to the collected Belgian populations, DNA was also extracted from *M. chitwoodi* race 1, 2 and 3 (USA), *M. chitwoodi* (Rips, The Netherlands) and *M. fallax* (Baexem, The Netherlands). One microlitre DNA extract was used for the PCR. The PCR mixture contained 3 µl 10X PCR buffer, 4 mM $MgCl_2$, 250 µM of each dNTP, 0.2 µM of a ten-mer primer of random sequence (OPA2, OPA3, OPA4, OPA7, OPA9, OPA10, OPA11, OPA12, OPA13, OPE9, OPE19, OPK2, OPM12, Operon Technologies, Alameda, USA), 2 units *Taq* DNA-polymerase (Qiagen, Crawley, West Sussex, UK) and double distilled water up to a total volume of 30 µl. The PCR programme was carried out in a DNA thermal cycler (PTC 200, Biozym, Landgraaf, The Netherlands) with an initial denaturation at 93 °C for 5 min, followed by 45 cycles of 93 °C for 30 s, 36 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 8 min. Ten microlitres of PCR mixture were used for electrophoresis in a 2% agarose gel containing ethidium bromide. Following electrophoresis, DNA fragments were visualised under UV light and photographed. The patterns were recorded manually as a binary matrix. The RAPDistance Programs, version 1.04 (Armstrong J., Gibbs A., Peakall Rod and Weiller G. of the Australian National University, Canberra, Australia) was used for the analysis of the RAPD fragments. Genetic distances among the 22 populations were calculated by means of pairwise comparison using the Dice coefficient. Cluster analysis was performed using TREEVIEW (Page, 1996).

Results and discussion

Identification of root-knot nematodes using morphological and morphometrical characteristics requires skill, is time-consuming and often inconclusive. Biochemical techniques using esterase and malate dehydrogenase patterns have proven to be suitable for the characterisation of *Meloidogyne* species (Esbenshade and Triantaphyllou, 1985; Pais and Abrantes, 1989; Esbenshade and Triantaphyllou, 1990; Ibrahim and Perry, 1993). Although Karssen *et al.* (1995) adapted this technique to an automated elec-

trophoretic apparatus it still has its limits as it requires young females. The technique was adopted because it allowed the use of the corresponding egg masses of identified females to initiate pure cultures.

Molecular tecniques are potentially more powerful for nematode diagnosis. Comparitive analysis of coding and non-coding regions of ribosomal DNA (rDNA) became a popular tool for species and subspecies identification of plant-parasitic nematodes from many genera (Vrain *et al.*, 1992; Wendt *et al.*, 1993; Zijlstra *et al.*, 1995; Subbotin *et al.*, 2000; Waeyenberge *et al.*, 2000).

Studies on the genus *Meloidogyne* by Zijlstra *et al.* (1995) and Schmitz *et al.* (1998) revealed that only one (*Rsa*I) out of 33 restriction enzymes differentiated *M. chitwoodi* from *M. fallax*. However, the *Rsa*I pattern of *M. fallax* was not clearly distinguishable from that of *M. hapla*. *Tru*9I undoubtedly separated the three species (Fig. 1). PCR with the species specific primers designed by Petersen *et al.* (1997) repeatedly confirmed the purity of the cultures and proved that these primers are valid for the identification of Belgian populations of *M. chitwoodi* and *M. fallax*.

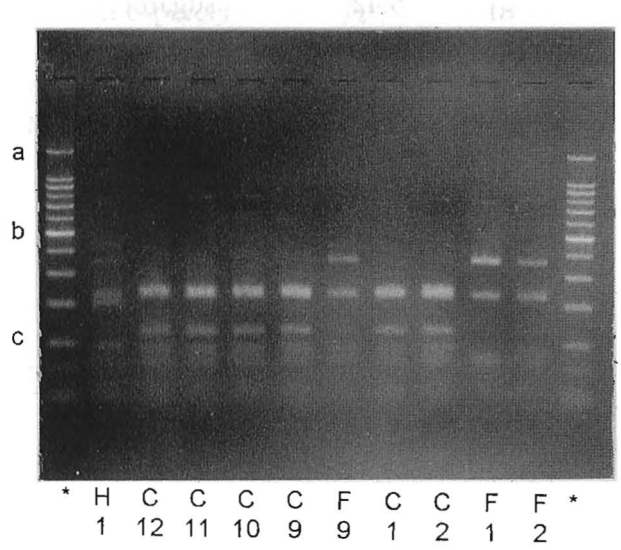

Fig. 1 - *Tru*9I RFLP pattern of *Meloidogyne* species - see Table III for codes; *: 100bp DNA-ladder (a = 1.5Kb, b = 500bp, c = 100bp).

Fig. 2 - Locations of *M. chitwoodi* (•) and *M. fallax* (▼) populations detected in Belgium.

Root-knot nematodes were present in 9.2% of the samples and *M. hapla* was the predominant species. *M. chitwoodi* and/or *M. fallax* were found in 10% of the positive samples. In certain areas *M. chitwoodi* and *M. fallax* were widespread and caused more problems than *M. hapla*. *M. chitwoodi* and *M. fallax* were detected mainly in areas of intensive vegetable growing for the canning industry in the provinces of Antwerp and Limburg (Fig. 2, Table I and II). In these parts of Belgium, non-infected fields are becoming rare. *M. chitwoodi* was also found in the heart of a potato producing area of the province of West-Flanders.

The nematodes were extracted from sandy to sandy-loam soils which were slightly acidic. Both species were isolated from different hosts (Table I and II). A note in the FAO Plant Protection Bulletin (Anonymus, 1991) suggests that *M. chitwoodi* was already present in The Netherlands in 1930. The nematode would have been able to remain undetected because of its resemblance to *M. hapla*. The same situation might also have occurred in Belgium as *M. chitwoodi* juveniles were isolated from oak forest soil (code C1).

For all populations listed in Table III, reproducible RAPD patterns were obtained with all

TABLE I - *Origin of* Meloidogyne chitwoodi *populations detected in Belgium.*

Province	Location	Soil type	pH	Host
Antwerp	Balen	Sandy	5.92	maize
	Kasterlee	Sandy	5.83	black salsify
	Poederlee	Sandy	5.02	fallow
	Postel	Sandy	5.55	black salsify
	Postel (*)	Sandy	6.21	black salsify
	Postel	Sandy	5.72	black salsify
	Postel	Sandy	5.83	black salsify
	Postel	Sandy	5.56	black salsify
	Postel	Sandy	5.80	bean
	Postel (*)	Sandy	5.78	carrot
	Postel	Sandy	6.82	fallow
	Putte	Sandy-loam	5.49	bean
Limburg	Hasselt	Sandy	6.81	sugar beet
	Houthalen	Sandy	5.92	oak forest
	Lommel	Sandy	6.43	maize
West-Flanders	Waregem	Sandy-loam	6.04	fallow

* Population mixed with *M. fallax*.

TABLE II - *Origin of* Meloidogyne fallax *populations detected in Belgium.*

Province	Location	Soil type	pH	Host
Antwerp	Arendonk	Sandy	5.42	sugarbeet
	Kasterlee	Sandy	6.59	maize
	Postel (*)	Sandy	5.78	carrot
	Postel	Sandy	5.39	grassland
	Postel	Sandy	5.67	grassland
	Postel	Sandy	5.30	carrot
	Postel (*)	Sandy	6.21	black salsify
Limburg	Hasselt	Sandy	6.81	sugarbeet
	Lommel	Sandy	5.79	grassland
	Stokkem	Sandy-loam	6.82	maize

(*) Population mixed with *M. chitwoodi*.

of the primers tested. The RAPD patterns revealed considerable intraspecific variation among the populations (Fig. 3). The tree is composed of three main branches grouping the populations of *M. hapla, M. chitwoodi* or *M. fallax* (Fig. 4). Sub-clusters are not related to any geographic origin. Studies by Schmitz *et al.* (1998) grouped the three American *M. chitwoodi* populations in one cluster. In our results *M. chitwoodi* races 1 and 2 are separated from

TABLE III - *List of* Meloidogyne *populations used for RAPD.*

Species	Origin	Code
M. hapla	Belgium, Lommel	H1
M. chitwoodi	Belgium, Houthalen	C1
M. chitwoodi	Belgium, Postel	C2
M. chitwoodi	Belgium, Waregem	C3
M. chitwoodi	Belgium, Hasselt	C4
M. chitwoodi	Belgium, Putte	C5
M. chitwoodi	Belgium, Kasterlee	C6
M. chitwoodi	Belgium, Lommel	C7
M. chitwoodi	Belgium, Balen	C8
M. chitwoodi	The Netherlands, Rips	C9
M. chitwoodi race 1	USA, Oregon	C10
M. chitwoodi race 2	USA, Oregon	C11
M. chitwoodi race 3	USA, California	C12
M. fallax	Belgium, Hasselt	F1
M. fallax	Belgium, Stokkem	F2
M. fallax	Belgium, Postel	F3
M. fallax	Belgium, Postel	F4
M. fallax	Belgium, Postel	F5
M. fallax	Belgium, Lommel	F6
M. fallax	Belgium, Arendonk	F7
M. fallax	Belgium, Kasterlee	F8
M. fallax	The Netherlands, Baexem	F9

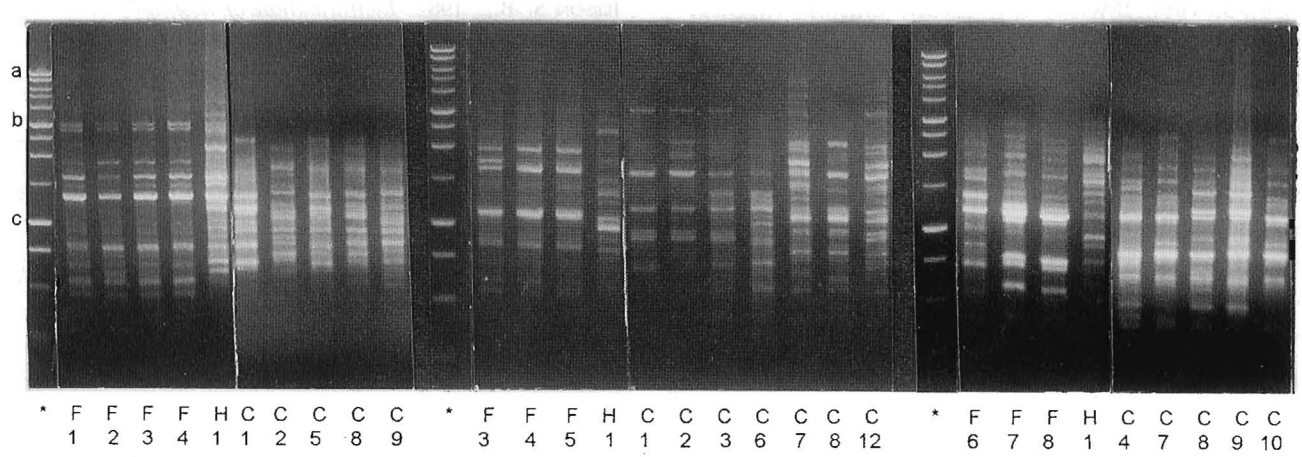

Fig. 3 - RAPD patterns for *Meloidogyne* species. (A) OPA2, (B) OPA3, (C) OPA4; see Table III for codes; *: 1Kb DNA-ladder (a = 10Kb, b = 3Kb, c = 1Kb).

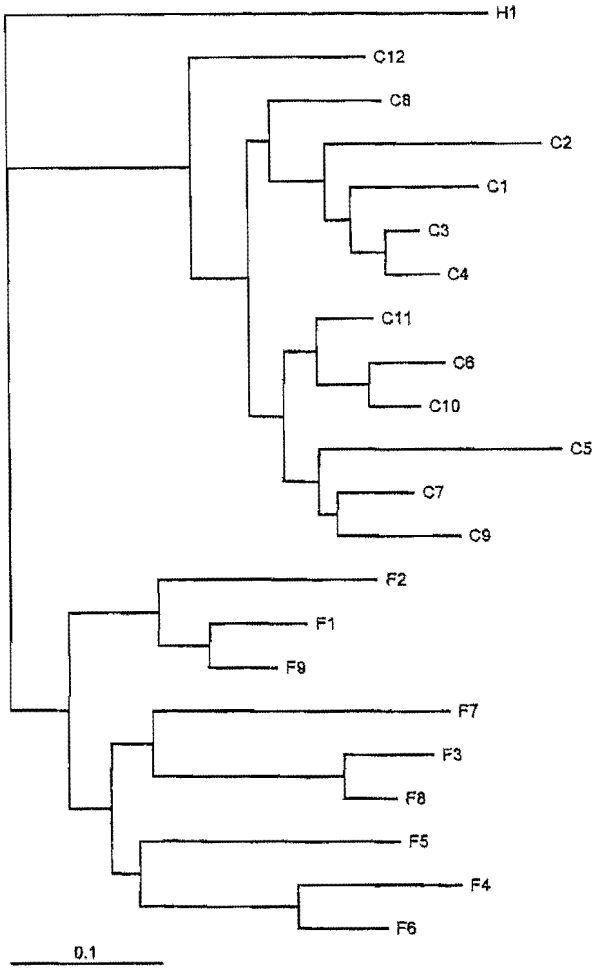

Fig. 4 - Tree of genetic distances between 22 Belgian *Meloidogyne* populations calculated according to Dice coefficient with TREEVIEW.

race 3. This difference can be due to differences in primers. The relative high genetic distances observed between the populations suggest a long presence of *M. chitwoodi* and *M. fallax* in Belgium allowing differentiation of populations. These results support the idea of Schmitz *et al.* (1998) that if *M. chitwoodi* has an American origin, the species was introduced and established in Europe at least twice with a long interval between the two introductions. More populations from Europe and USA have to be examined before definite conclusions can be drawn.

Acknowledgements. This research was financed by "Begrotingsfonds voor de productie en bescherming van planten en plantaardige producten", "Ministerie van Middenstand en Landbouw - Bestuur voor Onderzoek en Ontwikkeling" and "N. V. La Corbeille". We are grateful to Drs H. Mojtahedi, G. S. Santo and G. Karssen for providing *Meloidogyne* populations from the USA and the Netherlands.

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Accepted for publication on 12 February 2001.