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Genetics / Génétique

AFLP analysis of the genetic diversity of *Meloidogyne chitwoodi* and *M. fallax*, major agricultural pests

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Abstract

M. chitwoodi and *M. fallax* populations are clustered and separated from the other species studied. The genetic diversity observed for *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*, and *M. mayaguensis* correlates well with the previously validated species. Two main groups can be identified within the *M. chitwoodi*/*M. fallax* cluster, the first group comprises only *M. chitwoodi* populations whereas the second group is made of *M. chitwoodi* and *M. fallax* populations. Moreover, *M. chitwoodi* displays a higher genetic diversity than *M. fallax* and is characterised by the presence of several clusters. **To cite this article: M. Fargette et al., C. R. Biologies 328 (2005).**

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Résumé

Analyse AFLP de la diversité génétique de *Meloidogyne chitwoodi* et *M. fallax*, ravageurs importants des cultures. Les populations de *M. chitwoodi* et *M. fallax* se regroupent séparément des autres espèces étudiées. La diversité génétique observée chez *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla* et *M. mayaguensis* est corrélée avec les espèces établies. Deux groupes principaux sont identifiés à l'intérieur de l'ensemble *M. chitwoodi*/*M. fallax*, le premier comprenant uniquement des populations de *M. chitwoodi*, et le second des populations de *M. chitwoodi* et *M. fallax*. De plus, *M. chitwoodi* montre une diversité génétique supérieure à celle de *M. fallax* et se structure en plusieurs sous-ensembles. **Pour citer cet article : M. Fargette et al., C. R. Biologies 328 (2005).**

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1. Introduction

The root-knot nematode *Meloidogyne chitwoodi* was first isolated and characterised on potato in northern USA [1]. Since then, this nematode has established itself as a major multi-million dollars pest on potato tubers and is also recorded in western USA, Mexico, Argentina and South Africa [2–6]. More recently, *M. chitwoodi* was found in the Netherlands, Belgium, Germany and Portugal, where it is considered an emerging pest threatening potato, but also ornamental productions. *M. chitwoodi* remained unnoticed for long, probably because of populations in relatively low numbers and possible confusion with the prevalent nematode in Northern Europe, *Meloidogyne hapla*. Economical impact in Europe, through direct loss and downgraded infested tuber stocks, is of concern. In addition, *M. chitwoodi* is able to develop on other major crops such as alfalfa, wheat, maize, beet, carrot, etc. [7–9]. The risk of spread throughout the European continent is increased by the ability of *M. chitwoodi* to develop over a large range of temperature. It threatens southern as well as northern Europe, where it can cohabit and be confused with other species of nematodes prevailing in milder climates *Meloidogyne arenaria*, *M. incognita*, and *M. javanica*. A critical issue will therefore be to ensure an early and reliable detection of *M. chitwoodi*.

Identification of species within the genus *Meloidogyne* mostly relied on esterase banding patterns in polyacrylamide gel electrophoretic analysis [10–12]. Using this technique, the species *M. chitwoodi* was characterised by an esterase pattern designated S1 [4]. A second population of nematodes attacking potatoes in Europe was characterised by the absence of an esterase band (null phenotype) and by a different MDH pattern [13]. This biochemical variant was first assigned to *M. chitwoodi* and was later described as *Meloidogyne fallax* [14]. Molecular diagnostics aiming at discriminating *M. chitwoodi* and *M. fallax* were developed, based on ITS [15,16], IGS [17,18] and SCARs [19].

However a broad view of the genetic diversity of *M. chitwoodi* and *M. fallax* is required and more discriminative markers are needed to investigate the genetic diversity at both inter- and intra-specific levels. Different strategies were developed using RFLP [20], RAPD [21] and AFLP markers [22–25] to analyse the

genetic diversity of various species of *Meloidogyne*. They further proved useful to distinguish between as well as within species.

AFLP markers were proved useful to analyse the inter- and intra-specific genetic diversity of various organisms such as plants [26,27], insects [28], fishes [29, 30], snakes [31], fungi [32] or bacteria [33]. Different levels of discrimination and analysis were achieved such as identification of interspecific hybrids [30], analysis of patterns of genetic differentiation within an insect species complex [28], phylogeny of rapidly evolving clades [29] or discrimination between closely related species [33]. At the intra-specific level, genetic diversity was assessed and structured into sub-specific taxa [27–29,31,32].

The work presented in this article was aimed at investigating the genetic diversity of *M. chitwoodi* and *M. fallax* using AFLP markers. We report here that the group comprising *M. chitwoodi* and *M. fallax* separates clearly from the other *Meloidogyne* species. We also report that *M. chitwoodi* is characterised by a high genetic diversity and is structured into subgroups that may be undergoing speciation.

2. Material and methods

2.1. Populations of nematodes

Nematode samples originated from farm fields. Codes, specific diagnosis and geographical origins are provided in Table 1. Lines of *M. incognita*, *M. arenaria*, *M. javanica*, *M. mayaguensis* and *M. hapla* originate from nematode cultures raised from single egg mass lines, whereas *M. chitwoodi* and *M. fallax* correspond to sampled populations. Nematodes were multiplied on susceptible tomato in greenhouses.

2.2. Molecular marker analyses

Genomic DNA from lines of *M. arenaria*, *M. javanica*, *M. incognita*, *M. mayaguensis* and *M. hapla* was extracted and prepared as described by Fargette et al. [20], and genomic DNA from *M. chitwoodi* and *M. fallax* populations was extracted and prepared as described by Zijlstra et al. [16]. DNA samples extracted from *M. chitwoodi* and *M. fallax* isolates were checked for contamination as previously described

Table 1
Populations of *Meloidogyne* nematodes used for genetic diversity analysis

Nematode species	Sample code	Geographic origin
<i>M. javanica</i>	22	Burkina Faso
	23	Burkina Faso
<i>M. arenaria</i>	10	Ivory Coast
	28	French West Indies
	31	French West Indies
	32	French West Indies
<i>M. incognita</i>	9	Ivory Coast
	15	Thailand
<i>M. mayaguensis</i>	3	Ivory Coast
	13	Puerto Rico
<i>M. hapla</i>	33	The Netherlands
<i>M. chitwoodi</i>	cce	The Netherlands
	ck	The Netherlands
	cu	The Netherlands
	cl	The Netherlands
	cp	The Netherlands
	ca	The Netherlands
	cx	The Netherlands
	cz	The Netherlands
	cch	Portugal
	cch	Argentina
	cbh	California
<i>M. fallax</i>	fa	The Netherlands
	fb	The Netherlands
	fh	France

[16,19,34]. The AFLP procedure was conducted under standard conditions as recommended by the manufacturer (AFLP Analysis System I kit, BRL). 150 ng of primary templates were digested by *EcoRI* and *MseI* in the reaction buffer provided with the kit for 2 h at 37 °C in a final volume of 25 µl. The mixture was incubated at 70 °C for 15 min to inactivate the restriction endonucleases, put on ice and ligated to *EcoRI* and *MseI* adapters with T4 DNA ligase for 2 h at 20 °C in a final volume of 50 µl. A 1:10 dilution of the ligation mixture was performed in TE buffer [35] and 5 µl of diluted mixture was used as template for the non-selective preamplification reaction. Preamplification was performed in the presence of the pre-amp primer mix (containing the preamplification primers) provided by the supplier in a final volume of 51 µl and 1 unit of Taq DNA polymerase for a total of 20 cycles under the following conditions: 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s. After completion of the 20 cycles, samples were maintained at 4 °C and preamplification was checked by electrophoresis in 2% agarose

gel at 11 v cm⁻¹ and staining with ethidium bromide along with DNA from *Arabidopsis thaliana* as a control sample and a DNA ladder ranging from 75 bp to 4 kb. Preamplification was considered successful when bands from 100 to 1000 bp were clearly visible. Primers were end-labelled with (γ -³³P) ATP as recommended by the supplier (Amersham) and selective amplifications were conducted using a total of seven combinations. The primers contained two selective bases on the *EcoRI* side and three selective bases on the *MseI* side. The selective nucleotides of the seven primers were TG/CAA, TC/CTG, AC/CAT, TC/CAA, TC/CAT, AC/CTG, TA/CAA. Selective amplification was conducted as specified by the supplier in a final volume of 20 µl first for 1 cycle at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s, then for 12 cycles, where the annealing temperature is lowered by 0.7 °C at each cycle and finally for a series of 23 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. After PCR amplification, samples were analysed by electrophoresis in a 0.4-mm-thick, 5% polyacrylamide gel under denaturing conditions (7.5 M urea). Migration was performed in 0.5 × TBE buffer [35] at 1.4 W cm⁻¹ until bromophenol blue is removed from the gel. The gel was then transferred onto Whatman paper, dried and exposed to autoradiography film (Fuji) for one week.

2.3. Data analysis

Scoring for presence/absence of AFLP bands and data analysis was carried out as described in previous studies [20,21]. These data were analysed using the statistical package Genstat 5 [36] to produce similarity matrices [37]. Dendrograms were constructed according to UPGMA. The topology of the dendrogram shown in Fig. 1 was assessed by a bootstrap test (1000 bootstraps).

3. Results

3.1. Genetic relatedness of *M. chitwoodi* and *M. fallax* to other *Meloidogyne* species

The genetic relatedness of *M. chitwoodi* and *M. fallax* to other *Meloidogyne* species was investigated by

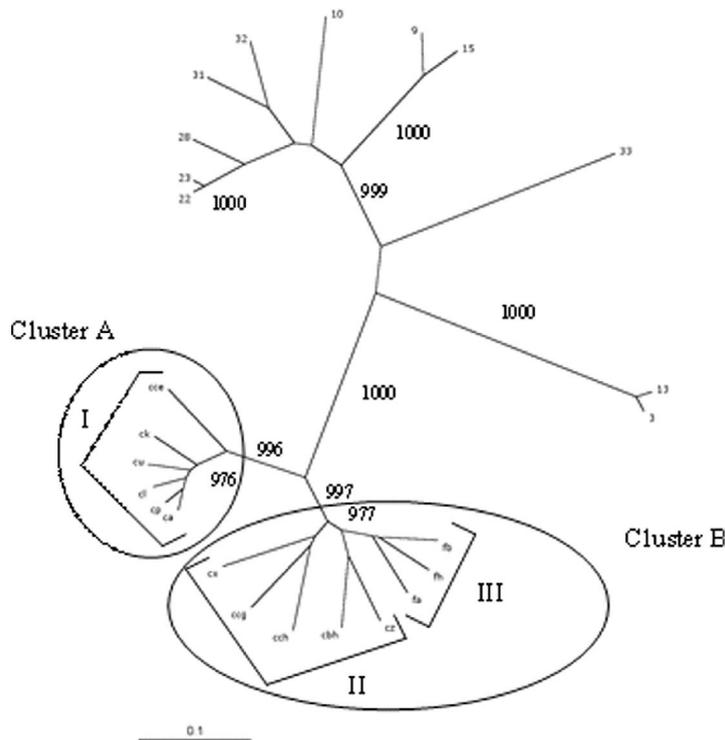


Fig. 1. Dendrogram of the relationships between *Meloidogyne* species. Only bootstrap values higher than 950 are shown.

comparing a total of 14 populations to 11 lines belonging to five species (two lines of *M. incognita*, four lines of *M. arenaria*, two lines of *M. javanica*, one line of *M. hapla* and two lines of *M. mayaguensis*). The distribution of the various samples as shown by AFLP analysis is presented in Fig. 1. The distribution shows that the selected representatives of *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*, and *M. mayaguensis* fall into distinct groups. The *M. mayaguensis* lines (samples 3 and 13) are clearly separated from the others with a bootstrap value of 1000. Similarly, the *M. incognita* lines make a separate group characterised by a similar bootstrap value. Although *M. arenaria* and *M. javanica* appear clustered, the very high bootstrap value (1000) that supports the *M. javanica* branch indicates that they are clearly separate groups. *M. chitwoodi* and *M. fallax* populations are clustered and clearly separated from the other species of *Meloidogyne* with a bootstrap value of 1000.

Data from the similarity matrix (Table 2) indicate that the percentage of similarity between the *M. chit-*

woodi/*M. fallax* group and *M. hapla*, the *M. javanica*/*M. arenaria* group, *M. mayaguensis* and *M. incognita* is 49.92%, 37.55%, 46.11% and 42.68%, respectively. Samples 22 and 23 which are *M. javanica* lines are shown in Fig. 1 to be more closely related to sample 28 (*M. arenaria*) than the latter is to other *M. arenaria* representatives (10, 31, and 32). Furthermore, the intraspecific similarity within *M. arenaria* is lower (79.32%, Table 3) and diversity not as stringently structured (bootstrap values of 849, 637 and 847) than between *M. arenaria* and *M. javanica* (82.53% and bootstrap value of 1000).

3.2. Genetic diversity of *Meloidogyne chitwoodi* and *Meloidogyne fallax*

Two main and distinct clusters can be identified in Fig. 1. Cluster A comprises the cce, ck, cu, cl, ca, and cp populations of *M. chitwoodi* (group I). Cluster B is more diverse and comprises populations of *M. chitwoodi* (group II: cx, ccg, cch, cbh and cz) and *M. fallax* (group III: fa, fb and fh). Similarity values in

Table 2
Genetic relatedness of the *M. chitwoodi*/*M. fallax* group to other *Meloidogyne* species

Mc/Mf	77.07				
Mh	49.92	100.00			
Mj/Ma	37.55	55.55	82.27		
Mm	46.11	52.18	45.30	97.11	
Mi	42.68	53.70	71.09	48.96	92.61
	Mc/Mf	Mh	Mj/Ma	Mm	Mi

Data represent the percentage of similarity between species of *Meloidogyne* when considering that *M. chitwoodi* and *M. fallax* represent a single species and *M. javanica* and *M. arenaria* represent another single species. Mc/Mf: *M. chitwoodi*/*M. fallax* group (cce, ck, cu, cl, cp, ca, cx, ccg, cch, cbh, cz, fa, fh, fb). Mh: *M. hapla* [33]. Mj/Ma: *M. Javanica*/*M. arenaria* group [10,22,23,26,28,31,32]. Mm: *M. mayaguensis* [3,13]. Mi: *M. incognita* [9,15].

Table 3
Percentage of similarity between groups of *Meloidogyne* species

I	90.11							
II	69.34	81.40						
III	72.05	80.34	88.43					
Mh	40.57	54.80	60.50	100.00				
Mj	35.97	47.92	54.87	53.89	97.84			
Ma	25.42	38.76	44.04	56.38	82.53	79.32		
Mm	37.93	50.42	55.31	52.19	54.09	40.91	97.11	
Mi	35.14	46.46	51.48	53.70	75.92	68.68	48.96	92.61
	I	II	III	Mh	Mj	Ma	Mm	Mi

Groups I, II and III are identified in Fig. 1. *M. arenaria* and *M. javanica* are considered as separate groups. Group I: *M. chitwoodi* populations (cce, ck, cu, cl, cp, ca). Group II: *M. chitwoodi* populations (cx, ccg, cch, cbh, cz). Group III: *M. fallax* populations (fa, fh, fb). Mh: *M. hapla* [33]. Mj: *M. javanica* [22,23]. Ma: *M. arenaria* [10,26,28,31,32]. Mm: *M. mayaguensis* [3,13]. Mi: *M. incognita* [9,15].

Table 4
Percentage of similarity between clusters of *M. chitwoodi* and *M. fallax*

A	90.11	
B	70.36	81.59
	A	B

Clusters A and B are identified in Fig. 1. Cluster A comprises *M. chitwoodi* populations only (cce, ck, cu, cl, cp, ca), whereas cluster B contains populations of both *M. chitwoodi* (cx, ccg, cch, cbh, cz) and *M. fallax* (fa, fh, fb).

Table 4 reflect the presence of these two clusters, characterised by high bootstrap values (998 and 977). With a percentage of similarity of 77% (Table 2), the internal variation within the overall *M. chitwoodi*/*M. fallax* group is higher than those of clusters A and B (90.11 and 81.59%, respectively).

Within group I, the isolate cce branches separately from isolates ck, cu, cl, cp and ca with a bootstrap value of 976 (Fig. 1). Group III is more homogenous (88.43% similarity) than group II (81.4% similarity). Groups I and II (*M. chitwoodi*) are more distant to each

other than group III (*M. fallax*) is from group II (Table 3).

4. Discussion

The first evidence arising from the data presented here is that outside from the *M. chitwoodi*/*M. fallax* group, the observed genetic diversity correlates well with the previously validated species, i.e. *M. incognita*, *M. javanica*, *M. arenaria*, and *M. mayaguensis*. These species are clearly clustered within groups separated by high bootstrap values (999 or 1000). The only exception to this stringent clustering is the *M. hapla* line (line 33) with a relatively low value of bootstrap (i.e., 677). This lack of stringency might be a consequence of having only one population of *M. hapla* in the study, making impossible to assess the intraspecific variability.

The main branch comprising *M. chitwoodi* and *M. fallax* is clearly separated from the other *Meloidogyne* species (large distance and high bootstrap values).

The clear separation observed between *M. chitwoodi* and *M. hapla* is in agreement with the theory of an early separation of these two sympatric species considered to have occurred between 35 and 43 million years ago [38]. The high level of heterogeneity observed within *M. chitwoodi* (groups I and II) also confirms the concept of *M. chitwoodi* being an ancient organism as shown by a high diversity of the mitochondrial DNA [39] and of the *AluI* satellite DNA [40].

The genetic diversity revealed by AFLP markers within the *M. chitwoodi*/*M. fallax* branch indicates the presence of well-structured populations. The main *M. chitwoodi*/*M. fallax* group cannot be considered a single species since *M. chitwoodi* and *M. fallax* have been shown to be reproductively isolated [41–43]. The minimal clustering must then be considered at the separation of *M. fallax* samples (group III) from the *M. chitwoodi* isolates, which is characterised by a bootstrap value of 977. In contrast to the homogeneity of the *M. fallax* group, *M. chitwoodi* displays a large heterogeneity with isolates ranging into two different clusters separated by high bootstrap values, i.e. groups I and II. Unlike interspecific markers, AFLP are specifically useful for investigating intraspecific variations and relatedness between closely related entities. Therefore, similarity matrices allow an insight on the relatedness between the different groups through a comparative way. In the present study, we observed that *M. fallax* is more similar to one subset of *M. chitwoodi* (group II) than this same subset is to the group I of *M. chitwoodi*. This observation is in agreement with the analysis of *M. fallax* satellite DNA, which was found to be more closely related to the two members of the pMcCo2 subfamily of *M. chitwoodi* satellite DNA than pMcCo2 was to the pMcCo1 subfamily of *M. chitwoodi* satellite DNA [44]. Although, unlike satellite DNA, AFLP data are not meant for phylogenetic analysis, this organisation of *M. chitwoodi* into distinct clusters may represent speciation events under process. However, in contrast to *M. fallax*, it is not possible to define their degree of achievement. Further crossing experiments will be needed to investigate the existence of reproductive isolation between the *M. chitwoodi* clusters if any. Such a distribution may reflect the facultative parthenogenetic reproductive behaviour of *M. chitwoodi* as well as the poor ability of these organisms to move and mix and would be compatible with the concept of complex of

species. Owing to this low mobility, low encounter probability and trends to parthenogenesis, these populations could be considered as being ‘pseudoclonal’. Furthermore, beside amphimixy, facultative parthenogenesis most likely has a non negligible share in the reproductive process, as shown by the low number of males in *M. chitwoodi* populations [41].

The observed variation in the width of the genetic basis between groups I and II could also reflect the coexistence of populations characterised by different modes of reproduction. Indeed, biodiversity within amphimictic populations would be displayed differently than in parthenogenetic ones. Under such a hypothesis, these two groups may correlate with different modes of reproduction, hence displaying different degrees of heterogeneity. On the other hand, one cannot exclude either a structuring effect of the host plants on which populations were sampled. However, appropriately designed experiments will be needed to investigate these various hypotheses.

M. fallax is considered to have achieved speciation from at least one population of *M. chitwoodi* [44], displaying thus a narrower genetic basis as shown in the similarity matrices presented in Table 3. According to the analysis of the *AluI* satellite DNA from *M. fallax*, which was found to be less variable than that of *M. chitwoodi*, this speciation was described as a recent event [44]. The structure of the *M. chitwoodi* populations could be compatible with a paraphyletic organisation. However, further studies using phylogenetic markers, i.e. DNA sequences, must be conducted to unravel this evolutionary aspect. Associating these phylogenetic analyses to interfertility tests would definitely demonstrate whether the high genetic diversity observed within *M. chitwoodi* is indeed an image of populations currently undergoing speciation or recently separated as species, a status which could ultimately lead to a revision of the nomenclature, as done with *M. fallax*.

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