New Host Races of *Meloidogyne arenaria*, *M. incognita*, and *M. javanica* from Horticultural Regions of Spain

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ABSTRACT

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In total, 140 populations of the genus *Meloidogyne* collected from representative horticultural regions of Spain and maintained at the Department of Agroecology, Institute of Agrarian Sciences, Madrid were characterized using perineal morphology, sequenced characterized amplified region polymerase chain reaction with species-specific primers, and bioassay. Using a modified North Carolina differential host test as a basis, 13 *Meloidogyne* populations did not fit into the published race scheme. These populations had very limited host ranges, reproducing only on susceptible tomato cv. Marmande and sometimes also on tobacco cv. NC95 and the pepper cv. Sonar in the case of *Meloidogyne arenaria*. They did not reproduce on cotton or peanut. The species and new races were identified as *M. incognita* races 5 (6 populations) and 6 (2 populations) and *M. javanica* race 5 (2 populations), with new records of *M. arenaria* race 3 (2 populations) and *M. javanica* race 1 (23 populations) for Spain.

The root-knot nematodes *Meloidogyne arenaria*, *M. incognita*, and *M. javanica* are plant-parasitic nematode species of major importance because of the economical losses that they cause. As an alternative to chemical control, the use of resistant or nonhost crop cultivars is highly desirable. However, for this to be successful, the species, race, and virulence characteristics of the locally occurring nematode population need to be known. The North Carolina (NC) differential host test (9), which is based on the ability of a species or race to reproduce on a given host, was successfully used to separate the four main *Meloidogyne* spp., *M. arenaria*, *M. incognita*, *M. javanica*, and *M. hapla*, as well as host races of *M. incognita* (four races) and *M. arenaria* (two races). The NC differential host test continues to be used to characterize *Meloidogyne* spp. in conjunction with other species-specific morphological and biochemical markers (4,10,12). To date, morphological and biochemical markers are available to distinguish *Meloidogyne* spp. but there are currently no molecular

methods to separate the host races. Two additional host races of *M. javanica*—*M. javanica* race 2, which reproduces on pepper, and race 3, which reproduces on peanut but not pepper—were described using the NC differential host test (12). No morphological differences were found between the three races of *M. javanica*. Later, Carneiro et al. (2) described *M. javanica* race 4, which reproduced on both pepper and peanut. In this case, the species was confirmed by morphological and biochemical analysis.

The difficulties in characterizing *Meloidogyne* populations are highlighted by Stanton and O'Donnell (16) who, after studying 41 populations of *Meloidogyne* in Australia using the NC differential hosts, found several atypical populations: one *M. javanica* population that did not parasitize cotton and two populations of *M. hapla*, of which one did not parasitize pepper and the other tobacco.

Atypical populations were also described by Fargette (7). This author studied 57 populations of *Meloidogyne* spp. from West Africa and reported that some of them fit into the *M. incognita* race 2 group according to the NC differential host test but morphological and biochemical analysis revealed that the population was, in fact, a new *M. arenaria* race 3 that reproduced on pepper cv. California Wonder but not peanut cv. Florunner.

Genomic and morphological techniques to characterize *Meloidogyne* populations

have led to valuable information about the phylogenetic relationships and biogeography of *Meloidogyne* populations but, as yet, no differences have been found to characterize virulence (3,5,12). Developments in functional genomics may allow us to differentiate the virulence of *Meloidogyne* populations in the future. Currently, due to the lack of molecular markers, the only method to determine the virulence characteristics of *Meloidogyne* populations is to use a bioassay, which provides a characterization at a functional level, taking into account local requirements and crop cultivars. The objects of this work were to characterize atypical *Meloidogyne* populations which did not fit in to the current race scheme.

MATERIALS AND METHODS

In total, 140 populations currently maintained at the Department of Agroecology, Institute of Agrarian Sciences, Madrid were used in this study. All populations were collected from the root system of a wide range of horticultural and fruit crops, and single-egg-mass lines of each population were produced by inoculating susceptible *Solanum lycopersicum* cv. Marmande with an egg mass of the original collected material. The identification of *Meloidogyne* spp. was performed based on perineal pattern morphology according to Eisenback et al. (6) and sequenced characterized amplified region polymerase chain reaction (SCAR-PCR) based molecular techniques according to Zjilstra et al. (20).

Differential host tests. Test plants with four true leaves—*Capsicum annuum* cv. Sonar (Clause Seeds, Almería, Spain), *S. lycopersicum* cv. Marmande, cotton (*Gossypium hirsutum* cv. DP 61), *Nicotiana tabacum* cv. NC 95, and *Arachis hypogaea* cv. Florunner—were used as differential host plants. Egg masses were collected and hatched in tap water in glass staining dishes. One thousand second-stage juveniles were added to each of five pots per differential containing 300 g of sterile sandy soil. All experiments were conducted in a growth chamber at 24 ± 1°C and a 16-h photoperiod with a daily watering with tap water for 45 days. The tests

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were repeated in batches with each line at least three times, using a known *M. incognita* race 1 and *M. arenaria* race 2 population as controls. Watermelon used in the NC differential host test was omitted because it is a differential only for *M. hapla*. The characterization of each line was performed based on the NC differential host test (9). The two pepper cvs. replaced cv. Carolina Wonder and the tomato cv. Marmande replaced cv. Rutgers because these cultivars in previous experiments have been shown to react in a similar fashion to those used in the original NC differential host test (10). Gallings were examined under a stereo microscope and a galling index on a scale of 0 to 10 was used (1) where 0 = no galls or fully developed egg masses containing juveniles; 1 to 4 = galling of secondary roots, with fully developed egg masses containing juveniles; and 5 to 10 = galling of primary lateral and tap roots, with an index of 5 indicating that 50% of the roots are galled. Populations which did not fit into the current host race scheme

and controls were processed for PCR and perineal pattern analysis.

Perineal patterns. All populations were maintained on *S. lycopersicum* cv. Marmande. Ten egg-laying females were dissected from infected roots and cleared in lactophenol (8) at 75°C for 30 min. The perineal region was cut from the cleared females and mounted in lactophenol DC and examined using a Dialux microscope equipped with Nomarsky interference optics.

DNA extraction. Three individual adult females of each ambiguous population of *M. incognita* (races 5 and 6) and *M. javanica* (races 1 and 5) and two control females from a population of *M. incognita* race 1 and *M. arenaria* (races 2 and 3) were dissected from infected tomato roots, washed in sterile water, and placed in Eppendorf tubes containing 20 µl of lysis buffer (1× PCR buffer, protease K at 60 mg/ml). The females then were frozen overnight at -20°C, heated to 60°C for 1 h, and boiled for 15 min to inactivate the

protease (19). The nematodes were centrifuged at 14,000 rpm for 1 min and 5 µl of supernatant from each species and race was used for each PCR amplification.

PCR amplification. All PCR reactions were performed in a final volume of 25 µl in a 0.2-ml PCR tube containing 2.5 µl of 10× PCR buffer (10 mM Tris-HCl, pH 8.3; 1.5 mM MgCl₂; and 50 mM KCl); 200 mM each dATP, dCTP, dGTP, and dTTP (Biotools S.L., Madrid); 200 nM each primer; and one unit of *Thermus thermophilus* DNA polymerase (Biotools S.L., Madrid, Spain). For *M. incognita*, *M. javanica*, and *M. arenaria*, the primers MIGF 5'-ACACAGGGGAAAGTTTGC CA-3' and MIGR 5'-GAGTAAGGC GAAGCATATCC-3' (11), which were developed from a random amplified polymorphic DNA fragment, were used under the following amplification conditions. Initial denaturing was carried out for a period of 3 min at 94°C; followed by 35 cycles of 94°C for 0.5 min, 58°C for 1 min, and 72°C for 1 min; followed by a

Table 1. Population and locality of *Meloidogyne arenaria* race 3, *M. incognita* races 5 and 6, and *M. javanica* races 1 and 5 from this study and their reaction in a modified North Carolina differential host test^a

Population and locality ^b	Cotton cv. DP61	Tobacco cv. NC95	Pepper cv. Sonar	Peanut cv. Florunner	Tomato cv. Marmande
<i>M. arenaria</i> race 3					
B30 Losar de la Vera (CA)	0	6.7 ± 2.9	6.1 ± 2.3	0	6.7 ± 2.3
B93R. Caríñena (Z)	0	4.6 ± 2.1	1.8 ± 0.9	0	7.9 ± 2.1
<i>M. incognita</i> race 5					
BM8. Perelló El (V)	0	0	0	0	7.6 ± 1.5
BM9. Mareny de Barraquetes (V)	0	0	0	0	7.6 ± 2.5
BM9M2. Mareny de Barraquetes (V)	0	0	0	0	7.4 ± 1.9
BM9M5. Mareny de Barraquetes (V)	0	0	0	0	7.0 ± 2.7
BM11. Mareny de Barraquetes (V)	0	0	0	0	6.5 ± 2.7
BM13. Mareny de Barraquetes (V)	0	0	0	0	7.4 ± 2.4
<i>M. incognita</i> race 6					
B2R. Talavera de la Reina (TO)	0	3.5 ± 1.7	0	0	5.7 ± 2.1
B94b. Villa del Prado (M)	0	6.0 ± 3.6	0	0	7.3 ± 3.0
<i>M. javanica</i> race 1					
B126. Cañada La (AL)	0	8.2 ± 0.9	0	0	8.5 ± 1.0
B96. Uleila del Campo (AL)	0	7.0 ± 0.0	0	0	5.0 ± 2.8
MJ1. Pineda de Mar (B)	0	5.8 ± 2.8	0	0	6.9 ± 2.5
B106R. Chipiona (CA)	0	4.9 ± 2.6	0	0	4.8 ± 2.2
B183. San Lúcar de Barrameda (CA)	0	1.0 ± 0.0	0	0	2.4 ± 1.5
B61A. Santa Eulalia (Ibiza)	0	6.5 ± 0.7	0	0	4.7 ± 1.5
B88. Puebla La (Mallorca)	0	5.7 ± 2.1	0	0	7.4 ± 2.8
B67. Águilas (MU)	0	4.0 ± 2.8	0	0	5.1 ± 2.6
B71. Águilas (MU)	0	5.0 ± 0.0	0	0	5.8 ± 3.5
B81. Águilas (MU)	0	4.0 ± 1.8	0	0	6.5 ± 4.0
B33A. Mazarrón (MU)	0	2.5 ± 1.7	0	0	8.8 ± 1.5
B4R. Árico (Tenerife)	0	8.0 ± 0.0	0	0	7.3 ± 2.3
B4R1. Árico (Tenerife)	0	6.3 ± 3.1	0	0	7.8 ± 1.6
B4R2. Árico (Tenerife)	0	8.0 ± 2.8	0	0	3.0 ± 2.0
B4R3. Árico (Tenerife)	0	6.4 ± 2.5	0	0	7.0 ± 2.5
B4R4. Árico (Tenerife)	0	5.7 ± 1.8	0	0	6.5 ± 2.7
B9. Árico (Tenerife)	0	6.5 ± 1.3	0	0	6.2 ± 2.8
B9M2. Árico (Tenerife)	0	6.8 ± 0.5	0	0	5.3 ± 2.2
B41. Árico (Tenerife)	0	2.3 ± 1.5	0	0	6.8 ± 2.6
B21. Árico (Tenerife)	0	5.0 ± 3.6	0	0	7.6 ± 3.0
B195. Arona (Tenerife)	0	4.4 ± 1.9	0	0	6.8 ± 2.5
B92. Fasnia (Tenerife)	0	6.5 ± 0.6	0	0	5.4 ± 2.2
B9M4. Guía de Isora (Tenerife)	0	2.3 ± 1.5	0	0	6.0 ± 3.5
<i>M. javanica</i> race 5					
B64. Lepe (H)	0	0	0	0	9.0 ± 1.4
BM6 Arico (Tenerife)	0	0	0	0	5.0 ± 2.0

^a Data are means of galling indices on a scale from 0 to 10 (1) of three replicates.

^b Population codes and localities: AL, Almería; B, Barcelona; CA, Cáceres; HU, Huelva; LO, Logroño; M, Madrid; MU, Murcia; TO, Toledo; V, Valencia; and Z, Zaragoza.

Fig. 1. Perineal patterns of **A**, *Meloidogyne incognita* race 5 (Perelló El); **B**, *M. incognita* race 6 (Talavera de la Reina); **C**, *M. javanica* race 1 (Mazarrón); **D**, *M. javanica* race 5 (Lepe); and **E**, *M. arenaria* race 3 (Losar de la Vera).

final 5-min extension period at 72°C. For *M. javanica*, the SCAR primers Fjav 5'-GGTGGCGGATTGAAGTGAAGC-3' and Rjav 5'-CAGGCCCTTCAGTGGAACTATAC-3' (20) were also used with the same cycling program as for *M. incognita*, but with an annealing temperature of 62°C. For *M. arenaria*, the SCAR primers Far 5'-TCGGCGATAGAGGTAAATGAC-3' and Rar 5'-TCGGCGATAGACACTACAAC-3' (20) were used with the same cycling program as for *M. incognita* but with an annealing temperature of 60°C. *M. hapla* and water were used as negative controls and *M. incognita* race 1 and *M. arenaria* race 2 were used as positive controls. No possible control was available for *M. javanica* because race 1 has not been previously cited in Spain. Amplified fragments were separated by electrophoresis in TRIS-borate-EDTA (TBE)-buffered 1.2% agarose gel, stained with ethidium bromide, and visualized with UV illumination (14).

RESULTS

Differential host tests. Six populations of *M. incognita* (4.3%) reproduced on tomato but not on pepper, cotton, tobacco, or peanut, indicating that these populations were a new race (Table 1). Two populations of *M. incognita* (1.4%) reproduced on tomato and tobacco but not on pepper, cotton, or peanut, indicating that these populations were another new race (Table 1). Two populations (1.4%) of *M. javanica* reproduced on tomato but not on pepper, cotton, tobacco, or peanut, indicating that these populations were a new race of *M. javanica* (Table 1). Three *M. arenaria* populations (2.1%) reproduced on tomato, pepper, and tobacco but not on cotton or peanut, indicating that these populations were a new race of *M. arenaria* (Table 1). The geographical origin of the populations and the average galling index on the differential hosts are also shown in Table 1. No association was found between geographical location and race.

Perineal patterns. Under the light microscope, the perineal patterns of *M. incognita* showed the characteristic high dorsal arch with wavy striae which bend toward the lateral lines and the absence of distinct lateral line incisures which are characteristic of this species (Fig. 1A and B). Perineal patterns of *M. javanica* showed a typical low to flattened dorsal arch and conspicuous lateral line incisures separating the pattern into dorsal and ventral regions (Fig. 1C and D). The *M. arenaria* populations had the characteristic rounded to flattened, low dorsal arch, with some of the striae forming a small shoulder just above the lateral lines. This species tends to be confused with *M. incognita* because perineal patterns show large morphological variability (Fig. 1E).

SCAR amplification. PCR amplification products using the primer set

MIGF/MIGR (Fig. 2) yielded a single product of 550 bp for *M. incognita*, *M. javanica*, and *M. arenaria*, which was consistent with the results of previous researchers (11). The Fjav/Rjav primer set yielded a single product of 670 bp (Fig. 3), the size expected for *M. javanica*. These primers did not amplify products from *M. incognita* or *M. arenaria*, consistent with the results reported by Zjilstra et al. (20), and the primer set Far/Rar yielded a single product of 420 bp for *M. arenaria* only, which was consistent with the results reported by Zjilstra et al. (20) (Fig. 4). Non-specific amplification products from *M. hapla* were not observed with any of the primer sets used.

DISCUSSION

One of the original aims of the NC differential host test in conjunction with the use of morphological characteristics such

as perineal pattern was to detect host races and other pathogenic variations within the four most common *Meloidogyne* spp. based on unexpected host responses. One of the most striking results from this study was the finding that the two new host races of *M. incognita* did not parasitize susceptible pepper cultivars and, therefore, are more like the majority of *M. javanica* populations which do not reproduce on pepper. In a previous study (13), none of the *M. javanica* populations reproduced on susceptible pepper, which led us to the assumption that the new races of *M. incognita* populations were *M. javanica*. During the International *Meloidogyne* Project, only 7% of the *M. javanica* populations tested parasitized pepper (9). In the original NC differential host test published by Taylor and Sasser (17), *M. javanica* was shown not to parasitize cotton; however, Eisenback et al. (6) suggested that some

Fig. 2. Amplification products obtained with the MIGF/MIGR primer set. Lane M is a 100-bp molecular marker; Mi1, *Meloidogyne incognita* race 1 (Perelló El); Mi5, *M. incognita* race 5 (Perelló El); Mi6, *M. incognita* race 6 (Talavera de la Reina); Mj1, *M. javanica* race 1 (Mazarrón); Mj5, *M. javanica* race 5 (Lepe); Ma2, *M. arenaria* race 2; and Ma 3, *M. arenaria* race 3 (Losar de la Vera). Amplification product, 550 bp.

Fig. 3. Amplification products obtained with the Fjav/Rjav primer set. Lane M is a 100-bp molecular marker; Mi1, *Meloidogyne incognita* race 1 (Perelló El); Mi5, *M. incognita* race 5 (Perelló El); Mi6, *M. incognita* race 6 (Talavera de la Reina); Mj1, *M. javanica* race 1 (Mazarrón); Mj5, *M. javanica* race 5 (Lepe); Ma2, *M. arenaria* race 2; and Ma 3, *M. arenaria* race 3 (Losar de la Vera). Amplification product, 670 bp.

Fig. 4. Amplification products obtained with the Far/Rar primer set. Lane M is a 100-bp molecular marker; Mi1, *Meloidogyne incognita* race 1 (Perelló El); Mi5, *M. incognita* race 5 (Perelló El); Mi6, *M. incognita* race 6 (Talavera de la Reina); Mj1, *M. javanica* race 1 (Mazarrón); Mj5, *M. javanica* race 1 (Lepe); Ma2, *M. arenaria* race 2; and Ma 3, *M. arenaria* race 3 (Losar de la Vera). Amplification product, 420 bp.

populations of *M. javanica* do reproduce on cotton. Confusion over the published data and the race nomenclature should be revised, as was recently done with the soybean cyst nematode *Heterodera glycines*. Race 1 of *M. javanica* reacts in the same way in the NC differential host test as the Australian *M. javanica* populations (16) which, without the use of other diagnostic methods, would be impossible to separate from *M. arenaria* race 2. The new races were only found in the Mediterranean regions of Spain, with 3 isolates of *M. arenaria* race 3, 6 isolates of *M. incognita* race 5, 2 isolates of *M. incognita* race 6, 23 isolates of *M. javanica* race 1, and 2 isolates of *M. incognita* race 5.

Another surprising result was that the new race 6 of *M. incognita* and race 1 of *M. javanica* reproduced only on tomato and tobacco, which is the same reaction in the NC differential host test as *M. arenaria* race 2. Therefore, these races of three different *Meloidogyne* spp. are functionally similar. Race 5 of *M. incognita* and *M. javanica* reproduce only on tomato. These populations may be of use in the future in selection studies to investigate at a molecular level the appearance of virulence in populations.

Based on the NC differential host test and perineal pattern morphology, an *M. incognita* race 5 has been previously described parasitizing coffee (18). However, no biochemical or molecular studies were performed to confirm the morphological data. Race 3 of *M. arenaria* would appear to be more common than first expected because other reports can be found in the literature of an atypical *M. arenaria*. To date, race 3 populations have been identified only in Africa, Portugal, and Spain (7,13,15). *M. arenaria* race 1 has not been reported in Spain.

Although dated, the NC differential host test still has an important role in the functional characterization of the four major *Meloidogyne* spp., *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*. To date, no molecular markers are available to determine race or virulence within these species. The differential host test should take into account new local cultivars because many of the differentials are now becoming obsolete.

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