Cytogenetic, Enzymatic, and Restriction Fragment Length Polymorphism Variation of *Meloidogyne* spp. from Spain

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ABSTRACT

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A survey of the cytogenetic, enzymatic, and DNA variation of root-knot nematodes, *Meloidogyne* spp., involved a total of 19 populations from various hosts in several regions of Spain. Of these, nine populations were identified as *M. arenaria*, six as *M. incognita*, three as *M. javanica*, and one as *M. hapla*. All *M. javanica* populations were hypotriploids with 43–46 chromosomes. The *M. arenaria* populations were hypotriploids with 40–48 chromosomes, except one diploid population with 34. The *M. hapla* population was a hypotriploid with 43–45 chromosomes. To assess isozyme variation, we assayed 11 enzyme systems. Only three systems showed some variation. Esterase patterns allowed separation of the four *Meloidogyne* species and two different forms in *M. incognita* and *M. arenaria*. Malate dehydrogenase patterns separated *M. hapla* from the rest of the species. Glutamate oxaloacetate transaminase separated *M. incognita* from the other species. Variation at the nucleic acid level was examined with a 1.8-kb mitochondrial DNA probe, IB2-37, which allowed the differentiation of *M. incognita*, *M. arenaria*, and *M. javanica*, after digestion of nucleic acids with either *Eco*RI or *Hin*fI. Oligonucleotide primers based on IB2-37 were used in polymerase chain reactions. DNA, amplified from 1–10 juveniles, was digested with *Hin*fI and produced patterns identical to those obtained in hybridization experiments. This procedure also allowed separation of *M. hispanica* and *M. microcephala* from the four major species. These results demonstrate that enzyme phenotyping and nucleic acid analysis provide consistent species identification in *Meloidogyne*.

Additional keyword: diagnostics.

Root-knot nematodes, *Meloidogyne* (Goeldi) spp. are recognized as important parasites of field, vegetable, and fruit crops in Spain. The first report of the root-knot nematode was made in 1922 (9), and, since then, it has been detected in a number of crops, including tomato, sweet melon, tobacco, carnation, and plantations of peach, grapes, and pomegranate (4,13). *Meloidogyne* spp. are widely distributed on the Iberian Peninsula, but the main losses are observed on tobacco in the Extremadura region and on tomato and peach along the Mediterranean coast (4,13). The root-knot nematode is also known to occur in the Canary Islands.

In spite of the economic importance of root-knot nematodes in Spain, little is known about the taxonomic status of these pathogens. In the most comprehensive work, Jiménez-Millán et al (13) described the morphology of several Spanish populations and reported the presence of *M. incognita acrita*, *M. incognita bauruensis*, *M. javanica*, *M. hapla*, *M. arenaria*, and *M. thamesi*. Hirschmann (12) described *M. hispanica* isolated from peach in the Andalucía region. No attempts have been made to characterize the cytogenetic or enzymatic variations among populations, now considered to be standard criteria for identification of *Meloidogyne* species (7,22). Moreover, little is known about the existence and geographic distribution of intraspecific variants. This lack of knowledge is a serious disadvantage for proper identification of species and races, and, consequently, for effective control of these nematodes through crop rotation and use of resistant cultivars.

The cytogenetic and enzymatic variations of *Meloidogyne* spp. have been studied on a considerable number of populations from around the world, thus providing a comprehensive picture of the variation in this genus (8,22). More recently, new techniques have been developed that allow the study of variation at the nucleic acid level. This can be done in several ways, but the most economical is the detection of restriction fragment length polymorphisms (RFLPs). RFLP analysis is used in taxonomy and permits the detection of infraspecific variation of plant-parasitic nematodes (1-3,5,14,18,23). With respect to *Meloidogyne* species, RFLPs have proved more effective in the detection of infraspecific variation than enzyme data. Separation of races 1, 2, 3, and 4 of *M. incognita*, races 1 and 2 of *M. arenaria*, and races A and B of *M. hapla* was achieved by identifying RFLPs of repetitive DNA sequences (6). However, only a single isolate of each population was examined. Intraspecific differences in *Meloidogyne* were detected in RFLPs of mitochondrial DNA (mtDNA) from several isolates of each of the four major species (16,17). However, as promising as these techniques are, they require large numbers of nematodes to work with, and, sometimes, results are not reproducible.

A substantial improvement of the RFLP approach for practical nematode identification has been attained by the application of the polymerase chain reaction (PCR). A 1.8-kb mtDNA fragment of *M. incognita* race 2, which contains restriction sites diagnostic for the four major species, has been cloned and sequenced (11). Primers based on the termini of this fragment are capable of target DNA amplification from a single egg or juvenile.

The objectives of the present study were to clarify the taxonomic status of *Meloidogyne* spp. populations in Spain and to observe the consistency of the data obtained through the different taxonomic approaches.

MATERIALS AND METHODS

Nematode cultures. Nematode populations were obtained from infected plants collected from several locations from the Spanish peninsula. The nematodes were maintained on tomato (cv. Rutgers) at the Instituto Nacional de Investigaciones Agrarias, Madrid. Some populations, mainly from the Catalonia region,

were collected and maintained by S. Verdejo from Institut de Recerca i Tecnologia Agroalimentaries, Barcelona. Three additional populations were obtained from the collection of A. Navas from Consejo Superior de Investigaciones Cientificas, Madrid. All populations were sent to North Carolina State University (NCSU) where they were maintained on tomato (cv. Rutgers) under normal greenhouse conditions.

A preliminary characterization of populations and species assignment were made by observing female perineal patterns and male heads, as well as by measuring second-stage juvenile lengths.

For the molecular study, additional populations were used from the NCSU worldwide collection of root-knot nematodes. These populations had been well characterized earlier and served as a reference.

Cytogenetic study. Cytological preparations for the study of gametogenesis and for chromosome counting were made according to established procedure (21). Egg-laying females were smeared on slides, hydrolyzed with 10% HCl for 10 min, fixed in a 3:1 mixture of absolute ethanol/glacial acetic acid for 40 min, and stained with propionic orcein for 20 min. After being rinsed with 45% acetic acid, slides were sealed and observed in a compound microscope.

Enzymatic variation. Electrophoresis for detection of enzymes was performed in precast polyacrylamide gels, 4.3 × 5 cm in size and 0.5 mm thick, in a PhastSystem (Pharmacia Ltd., Uppsala, Sweden). Several samples of single female homogenates were electrophoresed to verify the purity of each population. Then, the extract of 15 females was pooled in 15 μl of extraction buffer and stored at −20 C until needed. For comparison of populations, 1 μl of extract was applied to each well. The system was programmed to run at 130 V for 10 min, 20 V for 2 min during sample application, and 200 V for 30 min for a total of 166 V h. Enzymatic activity was detected by incubation at 37 C (10). The 11 enzyme systems evaluated were: catalase (CAT, EC 1.11.1.6), esterase (EST, EC 3.1.1.1), glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1), glutamate dehydrogenase (GDH, EC 1.1.2.4), isocitrate dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), superoxide dismutase (SOD, EC 1.15.1.1), phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), phosphohexose isomerase (PHI, EC 1.4.6.8), and hexokinase (HK, EC 2.7.1.1).

Extraction of DNA and Southern analysis. A preparation of total DNA was obtained from each of the 13 NCSU populations. Multiplication of Spanish nematode populations proved difficult; from only two populations could sufficient total DNA be extracted for hybridization experiments. For the remaining Spanish populations, second-stage juveniles were collected and frozen at −80 C for use in PCR reactions.

A modified extraction procedure was used to isolate total nematode DNA from frozen eggs (20). A purification step was added by ultracentrifugation in isopycnic gradient. After the addition of 0.9 g/ml of CsCl and 0.5 μg/ml of ethidium bromide (EtBr) to the DNA solution, centrifugation was carried out in a VTi-90 rotor at 55,000 rpm for 18 h. Then, the EtBr was extracted with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and (TE)-CsCl saturated isoamyl alcohol; the DNA was dialyzed extensively in TE and stored at 4 C.

DNA from each nematode population was digested with *Eco*RI and *Hin*fI (Promega Corp., Madison, WI). Five units of enzyme was added to 50 μl of reaction mixture that contained 1 μg of nematode DNA, and the mixture was incubated for 10 h at 37 C. DNA fragments were separated by electrophoresis in 0.8% agarose gels in 1× TBE (0.09 M Tris-borate, 0.09 M boric acid, 0.002 M EDTA) running buffer at 15 mA during 12 h. Digested DNA was transferred to Nytran filters (Schleicher and Schuell Inc., Keene, NH) by standard procedures (19), baked under vacuum at 80 C for 2 h, and stored.

The DNA used as a probe was a 1.8-kb mtDNA fragment from *M. incognita* race 2, cloned in the *Hin*dIII site of pUC18 (11). After digestion and electrophoretic separation of the plasmid, the insert was recovered from the gel with Geneclean II (Bio-101, La Jolla, CA). The probe was radioactively labeled with [^{32}P]dCTP by the random primer protocol, according to the manufacturer's instructions (U.S. Biochemicals, Cleveland, OH).

After the Nytran filter was washed in 3× SSC (0.45 M sodium chloride, 0.045 M sodium citrate, pH 7.0) and 0.1% sodium dodecyl sulfate (SDS) for 2 h and was prehybridized in a solution composed of 6× SSC, 5× Denhart's solution (0.5 g/L of Ficoll 400, 0.5 g/L of polyvinylpyrrolidine, 0.5 g/L of bovine serum albumin), 1% SDS, 0.05% sodium pyrophosphate, and 100 μg/ml of denatured salmon sperm DNA at 60 C for 1 h, hybridization was carried out at 60 C for 12 h in the same solution. Radioactive probe was added to an activity of 5,000 cpm/cm^2 of filter. The hybridized filters were washed four times for 45 min each in 2× SSC at 60 C. Then, filters were exposed wet to X-ray film for 24 h at −70 C.

PCR reactions. The primers for the PCR reactions were the two 20 mers described by Harris et al (11): 5′-TAAATCAATC-TGTTAGTGAA-3′ and 5′-ATAAACCAGTATTTCAAACT-3′. Primers were synthesized and purified by the DNA Synthesis Facility of the Department of Genetics, NCSU.

Template DNA was obtained from 1–10 juveniles of each nematode population, disrupted with a micropipette tip in 10 μl of water, and transferred to a cold 0.5-ml microfuge tube. Other extraction methods assayed included the disruption of juveniles fixed in 50% ethanol or formalin and of single females. The reaction mixture was composed of two units of Taq DNA polymerase (Promega Corp.), 0.2 mM dNTPs, 0.1% Triton X-100, 0.4 μM of each primer, and 1× reaction buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 20 μg/ml of gelatin). The volume of the reaction mixture was completed with sterile distilled water to 50 μl, and 50 μl of mineral oil was added on top. The amplification reaction was run for 35 cycles under the following conditions: denaturation at 94 C for 1 min; annealing at 50 C for 2 min; extension at 68 C for 3 min. A final incubation at 72 C for 5 min was allowed after the 35 cycles for completion of partially extended amplification products.

After the amplified product was precipitated in ethanol, 5 μl of it was digested with *Hin*fI for 1 h at 37 C. DNA fragments were separated on a 1.5% agarose gel and stained with ethidium bromide.

RESULTS

Cytogenetic study. Cytological observations to determine the chromosome number of each population were made primarily on oocytes located in the anterior part of the uterus, adjacent to the spermatotheca (Tables 1,2). Such oocytes usually were at late prophase or metaphase of the first maturation division. All populations showed a similar configuration of metaphase chromosomes that consisted of two chromatids each (dyads) and therefore represented univalents. The presence of univalent chromosomes at metaphase I suggests that no pairing of homologous chromosomes had occurred earlier and that all populations reproduce obligatorily by mitotic parthenogenesis (22).

Observed populations of *M. incognita* showed the typical distribution of chromosomes for this species, which is characterized by a prolonged prophase and an unusual clustering of the chromosomes (21). These features are peculiar to this species and make counting difficult; no accurate chromosome counts were made. The three populations of *M. javanica* had chromosome numbers ranging from 43 to 46. These data are within the range of the only cytogenetic form of this species considered to be a hypotriploid (22). Of the nine populations of *M. arenaria*, all except one had chromosome numbers ranging between 40 and 48 and are considered to be hypotriploids. One population showed 34 chromosomes. Apparently, it represents the diploid form that is quite rare worldwide. The *M. hapla* population had 43–45 chromosomes and can reproduce also by mitotic parthenogenesis; it represents the hypotriploid form of cytogenetic race B of this species.

Enzymatic variation. Of the 11 enzyme systems assayed, only three, esterase, malate dehydrogenase, and glutamate dehydrogenase, showed variation among populations. All populations

TABLE 1. Origin, host, chromosome number, and esterase pattern of the Spanish *Meloidogyne* populations

Species	Location[a]	Host	Chromosome number[b]	Esterase pattern[c]
1. *M. arenaria*	Catalonia	Tomato	48	A.2
2. *M. arenaria*	Catalonia	Tomato	40–41	A.2
3. *M. arenaria*	Catalonia	Peach	48	A.3
4. *M. arenaria*	Catalonia	Muskmelon	43–46	A.2
5. *M. arenaria*	Murcia	Peach	46–48	A.3
6. *M. arenaria*	Ebro	Peach	NA	A.3
7. *M. arenaria*	Extremadura	Tomato	NA	A.2
8. *M. arenaria*	Castilla	Sugarbeet	34	A.3
9. *M. arenaria*	Castilla	Grape	NA	A.3
10. *M. incognita*	North	Beet	Inc. type	I.1
11. *M. incognita*	Catalonia	Kiwifruit	Inc. type	I.1
12. *M. incognita*	Ebro	Peach	30	Atyp.
13. *M. incognita*	Castilla	Tomato	Inc. type	I.1
14. *M. incognita*	Extremadura	Tomato	Inc. type	I.1
15. *M. incognita*	Murcia	Lettuce	Inc. type	I.1
16. *M. javanica*	Catalonia	Fig tree	43	J.3
17. *M. javanica*	Catalonia	Almond	43	J.3
18. *M. javanica*	Andalucía	Carnation	43–46	J.3
19. *M. hapla*	Galicia	Kiwifruit	43–45	H.1

[a] Locations in Spain: Catalonia: NE; Galicia: NW; Castilla and Ebro: Central; Murcia: SE; Andalucia: S; Extremadura: W.
[b] Inc. type: typical configuration of chromosomes in *M. incognita*; NA: not analyzed.
[c] I1: specific pattern from *M. incognita* with one band. A2 and A3: specific patterns from *M. arenaria* with two and three bands, respectively. J3: specific pattern from *M. javanica* with three bands. H1: pattern from *H. hapla* with one band (7). Atyp.: atypical pattern.

TABLE 2. Additional populations used in the restriction fragment length polymorphism study

Code (NCSU)[a]	Species	Location	Chromosome number[b]	Esterase pattern[c]	Race[d]
I-68	*M. incognita*	N. Carolina	41–43	I.1	Race 1
I-2152	*M. incognita*	Nigeria	45	I.1	Race 2
I-2337	*M. incognita*	Argentina	36	I.1	Race 3
I-527	*M. incognita*	Texas	42	I.1	Race 4
A-523	*M. arenaria*	Texas	53	A.2	Race 1
A-413	*M. arenaria*	Nigeria	53–54	A.2	Race 1
J-76	*M. javanica*	Georgia	42	J.3	
J-93	*M. javanica*	Brazil	42	J.3	
H-86	*M. hapla*	Virginia	17	H.1	Race A
H-230	*M. hapla*	Chile	48	H.1	Race B
2526	*M. chitwoodi*	Argentina	NA	S.1	
468	*M. hispanica*	Spain	NA	S.3	
2116	*M. microcephala*	Thailand	NA	A.1	

[a] North Carolina State University.
[b] NA: not analyzed.
[c] I1: specific pattern from *M. incognita* with one band. A1 and A2: specific patterns from *M. arenaria* with one and two bands, respectively. J3: specific pattern from *M. javanica* with three bands. H1: pattern from *M. hapla* with one band. S.1 and S.3: nonspecific bands (7).
[d] Race classification according to North Carolina differential test for *Meloidogyne* races and species, except races A and B of *M. hapla*, which are cytogenetic races.

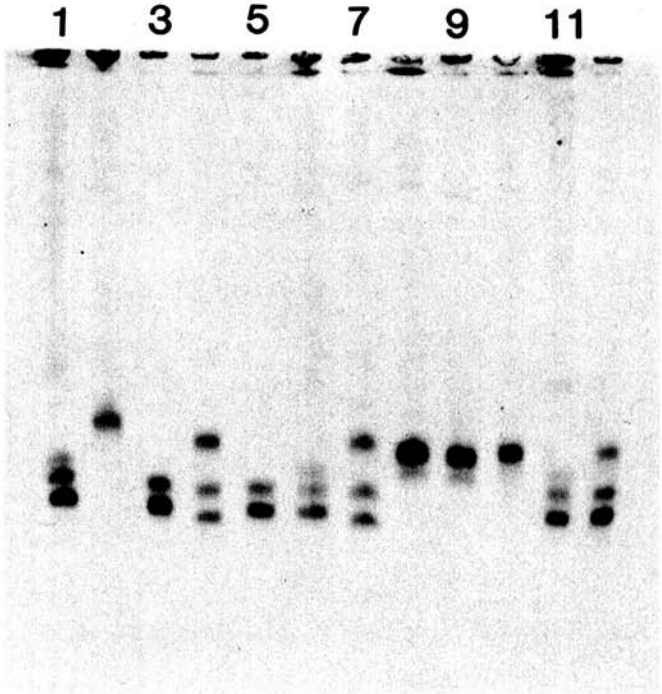

Fig. 1. Esterase phenotypes of 12 Spanish *Meloidogyne* populations. Phenotype A3: lanes 1, 6, and 11 (*M. arenaria* populations 5, 9, and 8, respectively). Phenotype A2: lanes 3 and 5 (*M. arenaria* populations 2 and 1, respectively). Phenotype J3: lanes 4, 7, and 12 (*M. javanica* populations 17, 16, and 18, respectively). Phenotype I1: lanes 2, 8–10 (*M. incognita* populations 12, 10, 11, and 15, respectively). The phenotype in lane 2 (*M. incognita* population 12) has a single band that is considerably slower than the normal type I1 band of all other *M. incognita* populations. Esterase phenotypes are as in Esbenshade and Triantaphyllou (7).

except one showed esterase phenotypes typical of the major species (Fig. 1). Population 12 (Fig. 1, lane 2), identified as *M. incognita* according to morphological and cytogenetic features, showed a single esterase band that was unusually slow for most of the *M. incognita* populations studied. Populations of *M. arenaria* showed two of the three different forms of the enzyme found in this species (7). The two forms, A2 (Fig. 1, lanes 3 and 5) and A3 (Fig. 1, lanes 1, 6, and 11), are characterized by the presence of two and three bands, respectively.

Two phenotypes of malate dehydrogenase were observed. One, designated H1 (7) and consisting of a single band, is specific of *M. hapla* and was found in the only population of this species detected in the survey. The other phenotype, N1, consisting of a single band much slower than the H1 band, is nonspecific and appeared in all populations of the other species.

Glutamate dehydrogenase migration phenotypes allowed separation of *M. incognita* populations, which are characterized by a single band, from populations of all other species, which showed two bands. The remaining enzyme systems assayed produced nonspecific phenotypes common to all the populations. Glutamate oxaloacetate transaminase showed a single band, which was a phenotype designated N1 by Esbenshade and Triantaphyllou (7) and observed in 81% of the 291 populations of their study. In contrast, the superoxide dismutase phenotype N2, observed in all populations of the present study, occurred in only 2% of the populations of that study.

DNA polymorphisms. Hybridization of total DNA digested with *Eco*RI or *Hin*fI with the mitochondrial probe IB2-37 produced distinctive patterns for *M. incognita*, *M. arenaria*, and *M. javanica* (Fig. 2). The results for all NCSU populations examined were consistent with the patterns depicted in Figure 2. *M. hapla* DNA hybridized poorly in all experiments, although an increased exposure period for autoradiograms did reveal bands of appropriate size. No differences were found in the restriction patterns of the four host races of *M. incognita* and the two cytological races of *M. hapla* (data not shown).

In the PCR experiments, DNA extracts from fresh juveniles of *M. incognita*, *M. javanica*, *M. hapla*, *M. chitwoodi*, *M. hispanica*, and *M. microcephala* were consistently amplified. It was also possible to amplify DNA from juveniles fixed in 50% ethanol but not from those fixed in formalin. Amplification from disruption of single females produced less product DNA in comparison with juveniles. No amplified DNA product could be obtained from *M. arenaria* populations despite repeated attempts. The amplified DNA was digested with *Hin*fI, producing the same restriction patterns found in the hybridization experiments (Fig. 3). To further evaluate the utility of PCR in *Meloidogyne* species diagnosis, we examined three additional populations of other species. The patterns of *Hin*fI digestion of *M. hispanica* and *M. microcephala*, which consisted of two fragments of approximately 900 and 800 bp, were identical and distinct from those of the other species. Digestion of *M. chitwoodi* DNA with *Hin*fI produced patterns identical to *M. javanica*.

DISCUSSION

The results of these experiments confirm the consistency of enzyme phenotyping and mtDNA RFLPs in *Meloidogyne* species identification. Furthermore, identification based on these protocols agrees with traditional methods such as perineal patterns, juvenile lengths, chromosome numbers, and host range tests. The rapidity with which enzyme and DNA assays may be applied suggests that these techniques may have a substantial impact on routine *Meloidogyne* species identification in diagnostic labs.

The present study revealed that all four major species of *Meloidogyne* occur in peninsular Spain, with a predominance of *M. arenaria* populations and only one population of *M. hapla*. Other species previously reported from Spain, such as *M. hispanica* and *M. thamesi*, were not detected. This result is not surprising, given the rarity of such species and the relatively low number of populations surveyed.

All populations studied reproduced obligatorily by mitotic parthenogenesis. No meiotic, facultatively amphimictic populations were found. There was a small amount of variation in chromosome number among populations of each species. Two cytogenetic forms of *M. arenaria* were observed: the hypotriploid, which is the most common, and the diploid, which is represented by only one out of nine populations studied. The triploid form, which is the most representative cytogenetic form of this species worldwide, was not found in the present survey. Similarly, the meiotic race A of *M. hapla*, which is the predominant and most typical cytogenetic form of this species worldwide, was not detected in this survey.

Two of the three known forms of esterase phenotypes were detected in *M. arenaria* populations studied. Two esterase phenotypes were observed also in *M. incognita*. One of them, I1, represents the only described phenotype of the species (7), and the other was observed for the first time in population 12. This nematode also had only 30 chromosomes. Additional study may be required, however, to verify the species identification of this population. Two forms of glutamate dehydrogenase, not observed previously, were found in other Spanish populations. The remaining enzyme systems assayed showed no variation at all, although many forms of them are known to exist as shown in previous studies (8). There did not appear to be a relationship between geographic distribution of species and esterase variants.

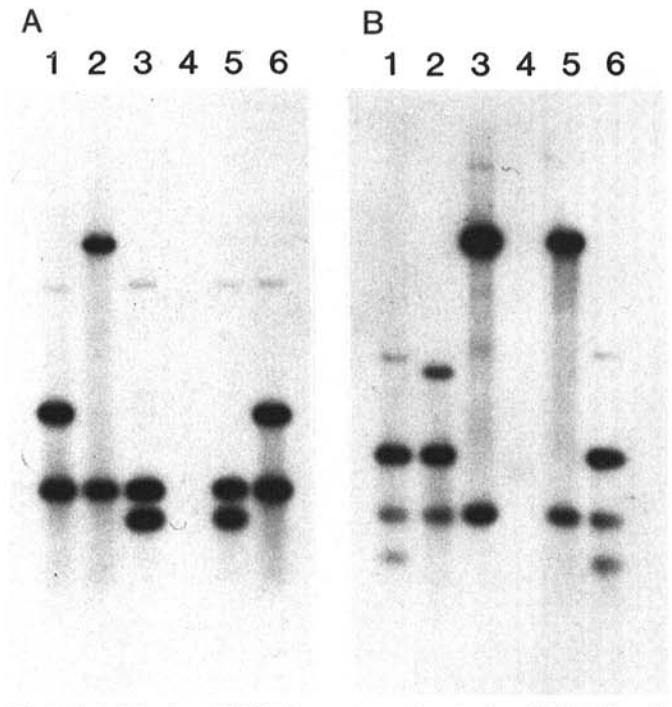

Fig. 2. Hybridization of *Meloidogyne incognita* mitochondrial DNA probe IB2-37 to DNA of the four major *Meloidogyne species*. **A,** Restriction fragment length polymorphisms generated with *Eco*RI. **B,** Restriction fragment length polymorphisms generated with *Hin*fI. Lane 1: *M. incognita* (North Carolina State University [NCSU] I-68); lane 2: *M. arenaria* (NCSU A-413); lane 3: *M. javanica* (NCSU J-93); lane 4: *M. hapla* (NCSU H-86); lane 5: *M. javanica* (Spanish population 16); lane 6: *M. incognita* (Spanish population 11).

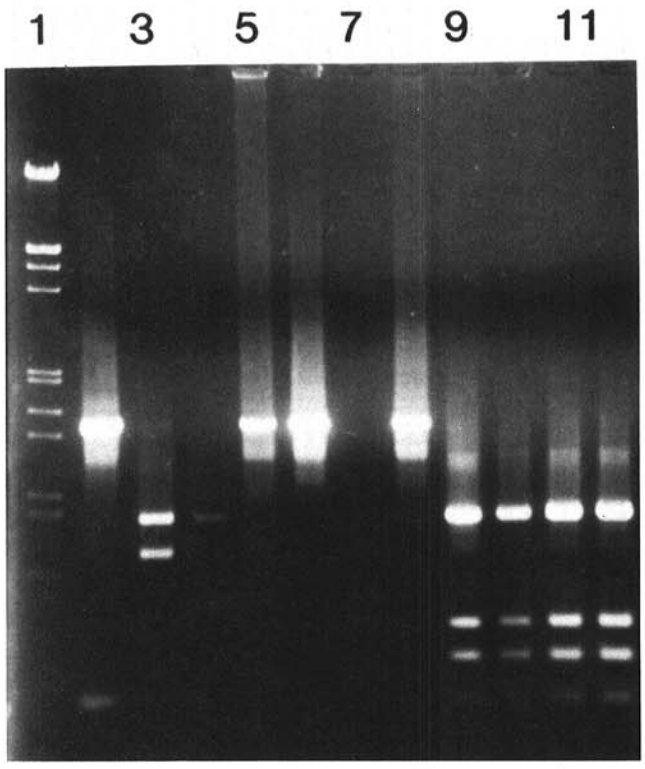

Fig. 3. Restriction fragment length polymorphisms obtained from polymerase chain reaction amplified mitochodrial DNA of several *Meloidogyne* populations digested with *Hin*fI. Lane 1: lambda *Hin*dIII/*Eco*RI; lane 2: *M. chitwoodi* (NCSU 2526); lane 3: *M. hispanica* (NCSU 468); lane 4: *M. microcephala* (NCSU 2116); lanes 5, 6, and 8: *M. javanica* (Spanish populations 16, 18, and 17, respectively); lanes 9–12: *M. incognita* (Spanish populations 10, 12, 13, and 15, respectively). Lane 7: blank.

The east part of the country, where many of the populations were collected, is quite constant with respect to the agroclimatic conditions, and there is frequent movement of propagation material. The nematode populations found there include the three species common to warm climates, *M. arenaria*, *M. incognita*, and *M. javanica*. The Galicia (NW) region, where the only *M. hapla* population was found, is very different and isolated from the east coast as it has more acidic soil and a colder climate. The same is true for the Castilla region, where the only diploid form of *M. arenaria* was found. Clearly, though, many more populations would need to be examined to get a definitive picture of *Meloidogyne* species distribution in Spain.

The mtDNA probe was not adequate to detect subspecific variation. However, it proved useful in the separation of the major species. The same sequence, when amplified by PCR, was also useful in differentiating *M. hispanica* and *M. microcephala* from the major species. This feature is useful because no specific enzyme pattern is able to separate these two species. The pattern obtained for *M. chitwoodi*, identical to that of *M. javanica*, is unexpected. According to the enzymatic phylogeny of the genus, the two species are quite different, with a coefficient of similarity of 0.16 (8). Moreover, the species differ in mode of reproduction, *M. chitwoodi* being a facultative meiotic parthenogenetic species with a chromosome number of $n = 14$–18 (22). Other parts of the genome and additional populations must be studied before reaching phylogenetic conclusions by this approach.

The PCR results were consistent with the hybridizations, although the failure in the amplification of DNA of *M. arenaria* is a serious drawback. The entire 1.8-kb fragment has been sequenced, and some parts of the sequence are known to be inadequate as primers for race 1 of this species and also some populations of *M. hapla*. This problem can be circumvented by using an internal downstream primer, but at the cost of losing accuracy in the separation of *M. arenaria* from *M. incognita* (T. O. Powers, *personal communication*). Other parts of the sequence should be investigated in the design of primers. Notwithstanding, the technique confirmed its usefulness in situations in which nematode material is limited. Moreover, it makes possible the study of competition of different species in the soil, using RFLPs as markers. Another important feature of PCR is the possibility of amplifying DNA from fixed specimens. This property makes the conservation of material easier and facilitates the comparison of populations worldwide.

The use of a combination of morphological, cytogenetic, enzymatic, and DNA data presents a clearer picture of the taxonomic status of *Meloidogyne* spp. in Spain. It also shows the consistency of the three different approaches in the identification of *Meloidogyne* spp. At present, the most economical approach is the use of esterase electrophoretic patterns, which allows the determination of the major species in a short time and with experimental simplicity. Nevertheless, the PCR technique has enormous potential. As new probes are developed and improved, new levels of infraspecific variation can be detected. It may also be possible to develop universal primers to regions highly conserved in different taxa. This approach would allow amplification of regions with abundant RFLPs. The use of universal primers currently has many applications in taxonomic and phylogenetic studies in plants and animals (15).

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