Journal of Nematology 46(3):275–280. 2014.

Identification of *msp1* Gene Variants in Populations of *Meloidogyne incognita* Using PCR-DGGE

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Abstract: Effectors of root-knot nematodes are essential for parasitism and prone to recognition by adapted variants of the host plants. This selective pressure initiates hypervariability of effector genes. Diversity of the gene variants within nematode populations might correlate with host preferences. In this study we developed a method to compare the distribution of variants of the effector gene *msp1* among populations of *Meloidogyne incognita*. Primers were designed to amplify a 234-bp fragment of *msp1*. Sequencing of cloned PCR products revealed five *msp1* variants from seven populations that were distinguishable in their reproduction on five host plants. A protocol for denaturing gradient gel electrophoresis (DGGE) was developed to separate these *msp1* variants. DGGE for replicated pools of juveniles from the seven populations revealed ten variants of *msp1*. A correlation between the presence of a particular gene variant and the reproductive potential on particular hosts was not evident. Especially race 3 showed substantial variation within the population. DGGE fingerprints of *msp1* tended to cluster the populations according to their reproduction rate on pepper. The developed method could be useful for analyzing population heterogeneity and epidemiology of *M. incognita*.

Key words: genetics, host preference, *Meloidogyne incognita*, pathogenicity gene, PCR-DGGE, root-knot nematode, technique.

The root-knot nematode *Meloidogyne incognita* is one of the most economically damaging agricultural pests worldwide, with a wide host range of at least 1,700 plant species (Sasser et al., 1983). This sedentary endoparasite has evolved a highly specialized and complex relationship with its host plants by inducing the root tissue to form specific feeding sites, the so-called giant cells (Williamson and Hussey, 1996; Hussey and Grundler, 1998). Although *M. incognita* reproduces by obligate mitotic parthenogenesis, it exhibits high genome plasticity and adaptive capacity, which presumably is the basis for the extremely wide host range of the species (Castagnone-Sereno, 2006; Castagnone-Sereno and Danchin, 2014). However, some populations were reported to reproduce only on few host plants (Robertson et al., 2009), which is a hint for diversity among populations. Various populations of *M. incognita* have been differentiated into races based on their susceptibility to differential hosts (Robertson et al., 2009; Devran and Sogut, 2011), or into virulent (aggressive) and avirulent (nonaggressive) populations based on their reproduction on cultivars carrying a resistance gene (Anwar and McKenry, 2007; Olowe, 2010). Genome plasticity and exchange of individuals between local populations will lead to heterogeneous populations that will hardly be classifiable in a simple race scheme. The use of resistant or nonhost crops is an effective and environmentally friendly method to manage *M. incognita* on many crops and at the same time to reduce the use of chemical nematicides (Williamson and Kumar, 2006). For successful nematode management using resistant plant cultivars or appropriate crop rotations, the intraspecific diversity and heterogeneity of local populations of *M. incognita* need to be understood. Analysis on the level of individuals or populations of *M. incognita* based on distinct morphological and biochemical characters is difficult or impossible. Bioassays on differential host plants are time consuming and laborious, and within-population diversity remains unresolved.

A molecular assay that can determine the race or virulence of populations of the same species of *Meloidogyne* has not been obtained yet (Cortada et al., 2011). Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is a molecular method that can detect differences in DNA sequences or mutations of various genes based on differential denaturing characteristics of the DNA. Despite its speed and potential to discern changes in a single nucleotide base pair of a gene fragment, PCR-DGGE in nematology has so far been limited to analysis of soil or marine nematode communities based on 18S rRNA genes (Okada and Oba, 2008).

The secreted protein MSP1 of *M. incognita* belongs to the SCP/TAPS proteins that have been proposed to play key roles in host–pathogen interactions and defense mechanisms (Castillo et al., 2010). The *msp1* gene is highly expressed in preparasitic and parasitic second-stage juveniles (J2) of *M. incognita* but not in adults (Ding et al., 2000). The *msp1* cDNA contained an open reading frame encoding 231 amino acids with the first 21 amino acids being a putative secretion signal. The secreted protein was shown to be essential for the initial infection of the host plant (Ding et al., 2000). Thus, it might be prone to diversifying selection for evasion of the plant immune system and host range extension of the population. The objective of this study was to explore sequence diversity of *msp1* in *M. incognita* populations, which showed variability in their reproduction rate on different hosts. For rapid analysis of the distribution of *msp1* variants within and among populations, a PCR-DGGE system was developed to fingerprint *msp1* gene variation.

Received for publication February 17, 2014.

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The authors warmly thank Prof. Stephen Thomas (New Mexico State University) for providing populations representing three races of *Meloidogyne incognita*.

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This paper was edited by Andrea Skantar.

Materials and Methods

Nematode sources: The seven nematode populations and races used in this study originated from three different countries (Table 1). Four populations (E1, E2, G1, G2) were identified as *M. incognita* by the molecular diagnostic key of Adam et al. (2007), except that primer 195 was modified to 195M (ATTGTAATGAGCCGTT CGC). The populations representing three races were identified and supplied by Prof. Stephen Thomas, New Mexico State University in the United States. All populations were isolated from single egg masses and propagated on tomato (*Solanum lycopersicum*) cv. Moneymaker under greenhouse conditions.

Greenhouse test: Different crops/cultivars were used to differentiate between populations and/or races, including pepper (*Capsicum annuum* cv. California wonder), cotton (*Gossypium hirsutum* cv. DP 61) and three cultivars of tomato (*Solanum lycopersicum*) namely cv. Moneymaker (susceptible), cv. Tomasa (tolerant), and cv. Sparta (resistant). Two week-old seedlings were transplanted into 11-cm-diam. plastic pots containing about 400 g of pasteurized field-soil:sand mix (1:1, v:v). Two weeks later, each seedling was inoculated with 200 freshly hatched J2 in 2-ml water by pipetting into four 3-cm-deep holes around the plant base. The inoculum was prepared by extracting nematode eggs from tomato roots using 1.5% NaOCl as described by Hussey and Barker (1973). The suspension of eggs was placed on a modified Baermann dish and incubated at 25 ± 2°C for 7 to 10 d to separate hatched J2 from eggs (Hooper et al., 2005). Only freshly hatched J2 collected within 48 h were used for the experiments. Eight replicate pots for each host plant and nematode population were arranged in a randomized block design. The plants were watered as needed and fertilized weekly with 10 ml of commercial fertilizer (WUXAL® Super NPK fertilizer, 8-8-6 with micronutrients, 2.5 g liter^{-1}). Pots were kept in the greenhouse at 22 ± 2°C and 16-h photoperiod.

The experiment was terminated 50 d after inoculation when 30% to 50% of the eggs from one egg mass produced on Moneymaker showed folded juveniles inside the eggs. Plants were removed from their pots, and root systems were washed to remove adhering soil. Egg masses were stained for 15 min with 4% cochenille red (Brauns-Heitmann, Warburg, Germany). The number of galls and egg masses on each root system was counted. Roots were transferred into 2% chlorine solution and vigorously shaken for 3 min to free the eggs from the gelatinous matrices. The suspension was washed with tap water through a 250-μm sieve to remove root debris. Embryonic eggs (black inside), juvenile eggs (folded juveniles recognizable within eggs), and hatched juveniles were collected on a 20-μm sieve and counted.

Table 1. *Meloidogyne incognita* populations used in this study and their origin.

Code	Geographic origin	Original host
E1	Sekem organic farm, El-Sharkia, Egypt	Pepper
E2	Sekem organic farm, El-Sharkia, Egypt	Tomato
G1	Reichenau, Baden-Württemberg, Germany	Bur cucumber
G2	Reichenau, Baden-Württemberg, Germany	Cucumber
R1	Ken Barker, USA[a]	Tomato
R2	Ken Barker, USA[a]	Tobacco
R3	New Mexico, USA[a]	Chili pepper

[a] Kindly provided by Prof. Stephen Thomas (New Mexico State University).

DNA extraction: Genomic DNA was extracted from J2 using the ZR Tissue and Insect DNA MicroPrep™ kit (ZYMO RESEARCH, Irvine, CA). Ten J2 for each of the populations were transferred into a ZR BashingBead™ lysis tube and then lysed in a FastPrep instrument (MP Biomedicals, Heidelberg, Germany) for 40 sec at high speed. The tubes were centrifuged for 1 min at 10,000 *g*, the supernatant transferred to a Zymo-spin™ IV Spin Filter and processed according to the manufacturer's instructions.

PCR–DGGE to differentiate msp1 gene variants: Based on an alignment of published and generated *msp1* sequences of *Meloidogyne hapla* and *M. incognita*, the primers msp410f (5′-TTGATGATTGATGCCTGTAATGC-3') and MImsp596r (5'-ATAACGACAATCAATCAAAT-3') were designed targeting conserved sites. To amplify products for DGGE analysis a modified forward primer msp410fGC with a 5' GC-clamp (CGCCCGGGGCGCG CCCCGGGCGGGGCGGGGGCACGGGGGG) was used. The *msp1* gene fragments were amplified in a 25-μl volume of 1 μl of template DNA, 1× TrueStart buffer, 0.2 mM deoxynucleoside triphosphates, 3.75 mM $MgCl_2$, 4% (vol/vol) acetamid, 0.2 μM of each primer, and 1.25 U TrueStart Taq polymerase (Fermentas, St. Leon-Rot, Germany), with thermal cycles: 95°C for 5 min, then 40 cycles at 94°C for 45 sec, 46°C for 30 sec, and 72°C for 30 sec, and a final extension step of 72°C for 5 min. PCR products were examined by running 5-μl aliquots of the reaction mixtures in a 1% agarose gel. DGGE was performed with a gradient of 29% to 56% of denaturants (where 100% denaturant was defined as 7 M urea and 40% formamide) and 6.2% to 9% acrylamide—N,N9-methylenebisacrylamide (37,5:1) as previously described (Weinert et al., 2009). Similar amounts of PCR product were loaded on a DGGE gel with four replicates per population, each derived from ten J2. DGGE was performed in a $PhorU_2$ apparatus (Ingeny, Goes, The Netherlands), in 1× Tris-acetate-EDTA buffer at 60°C with a constant voltage of 100 V for 16 hr. DNA in the gel was detected by acid silver staining as described by Heuer et al. (2001). The dried and scanned gels were analyzed using the software GelCompar II version 6.6 (Applied Maths, Ghent, Belgium). Lanes were normalized with common bands as internal standard. Pairwise similarities of the DGGE profiles by Pearson correlation were determined, and cluster analysis was done by the

unweighted pair group method with arithmetic averages (UPGMA).

Cloning and sequencing: For the sequencing of the different bands of *msp1* gene fragments observed at different positions in the DGGE gel, PCR products obtained with the primers msp410f and MImsp596r were cloned using the vector pGEM-T and *Escherichia coli* JM109 high-efficiency competent cells according to the instructions of the manufacturer (Promega, Madison, WI). Based on PCR-DGGE, cloned amplicons corresponding in electrophoretic mobility to different bands were sequenced (Macrogen, Amsterdam, The Netherlands). Sequences were aligned using Mega version 6 (Tamura et al., 2013).

Statistical analysis: The numbers of egg masses, embryonic eggs, juvenile eggs, and hatched J2 from each of the five plants were compared among the seven nematode populations. To account for correlations in this multivariate dataset and to reduce dimensionality, principal component analysis using SPSS Statistics 19 was performed. The first two principal components, which explained 89% of the variance, were used for univariate analyses of variance with Tukey adjustment to test for significant differences between the nematode populations.

Results

Phenotypic differentiation among M. incognita populations: The patterns of embryonic eggs, juvenile eggs, and hatched J2 generated on the five host plants varied among populations (Fig. 1A). As expected, all populations reproduced well on the susceptible tomato cultivar 'Moneymaker' showing the highest number of eggs. Populations G1, G2, R2, and E1 produced fewer eggs and J2 on tomato cv. Moneymaker than R3 ($P \leq 0.05$). Eggs produced by population E2 on Moneymaker developed faster to juvenile eggs than those from the other populations and races, with 23% of the total eggs

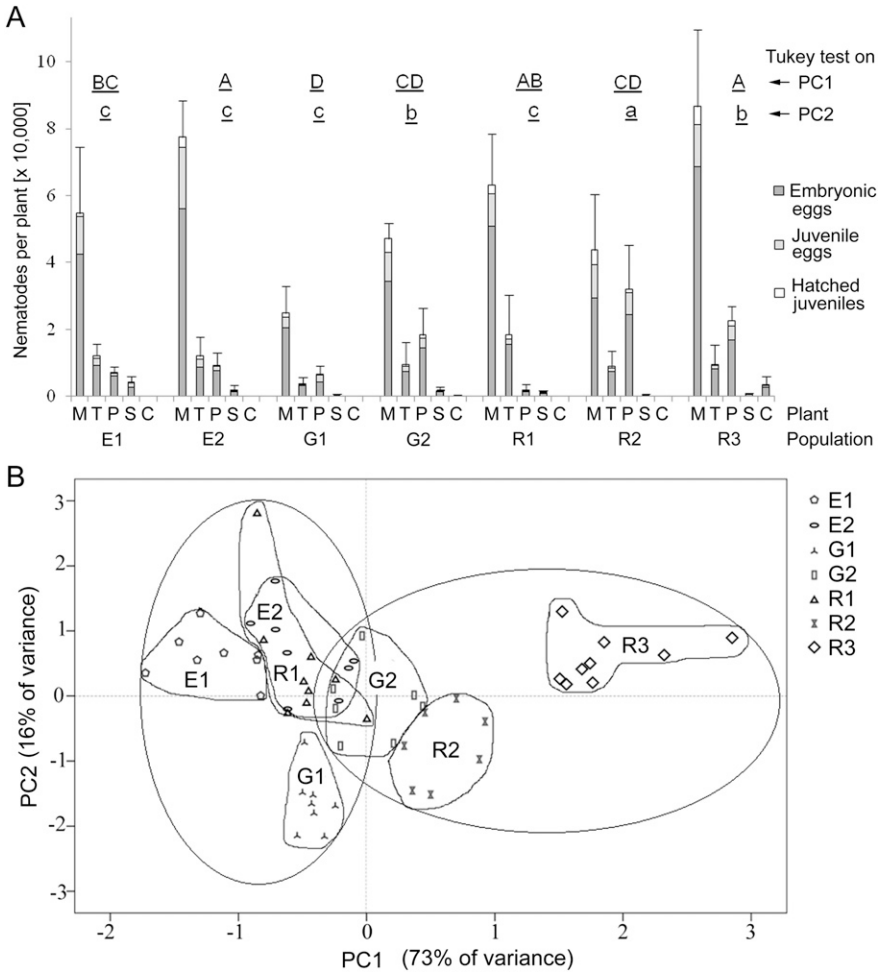

Fig. 1. Phenotypic characterization of *Meloidogyne incognita* populations by their reproduction on tomato cv. Moneymaker (M), tomato cv. Tomasa (T), tomato cv. Sparta (S), pepper cv. California wonder (P), and cotton cv. DP61 (C). A. Progeny and developmental stages of eggs were determined for each host plant 50 d after inoculation of 200 J2 of Egyptian populations (E1, E2), German populations (G1, G2), or American populations representing the races R1, R2, or R3. The reproduction pattern of the populations on the different host plants was compared by principal component analysis of the multivariate dataset and pairwise analysis of variance of principal components 1 (PC1) and 2 (PC2) using Tukey's adjustment ($n = 10$, $P < 0.05$). Different upper or lower case letters in a row indicate significant differences between populations with respect to PC1 or PC2, respectively. Error bars represent SD of total numbers of eggs. B. Biplot of PC1 and PC2.

developed to J1 ($P \leq 0.05$). By contrast, the resistant tomato cultivar Sparta suppressed reproduction of all *M. incognita* populations and races, except for population E1. For the tolerant tomato cultivar Tomasa, no significant differences in the quantity or development of eggs were observed among all populations, except that the total number of eggs produced by G1 was significantly lower than that produced by R1 ($P = 0.002$). Pepper cultivar California wonder differentiated between the three races but did not distinguish between German and Egyptian populations. On cotton cv. DP61 only R3 and G2 were able to reproduce, with R3 resulting in a significantly higher number of eggs than G2. Principal component analysis on the selected nematode parameters for the different host plants and analysis of variance of the first and second principal component (PC1 and PC2) showed significant differences between all populations, except that E1 and E2 were not different from R1 (Fig. 1A). The biplot of PC1 and PC2 showed a good discrimination between the two populations E1 and G1 and the two races R2 and R3, but was overlapping for the populations E2 and G2 and race R1 (Fig. 1B). The PC1 that explained 73% of the total variance was mainly based on the number of embryonic eggs on tomato cv. Moneymaker, whereas PC2 explained an additional 16% of the total variance and was mainly based on the number of embryonic eggs on pepper. On the right side of the biplot are the populations G2, R2, and R3 that had in common a relatively high reproduction on pepper in contrast to the other populations.

Differentiation of M. incognita populations based on msp1 gene variation: Five cloned variants of the *msp1* gene with different sequences, which were derived from four populations of *M. incognita*, were used to establish a DGGE gradient that electrophoretically separated all variants (Fig. 2A, lane M). DGGE profiles from four replicate mixes of ten J2 from each population revealed ten bands in total, with different electrophoretic mobility. Among them, the variant R1-A was most dominant in all populations. R2-B was also abundant in all samples from all populations. Other gene variants seemed to be more abundant in some populations than in others. For example, gene variants G2-A and G2-B were abundant in the population G2, R2, and R3 (but only in two of the replicates from ten J2), but much less abundant in the populations E1, E2, G1, and R1. Band E1-B was weak and only appeared for population E1 and R2 (Fig. 2A). Especially populations E1, G2, and R3 showed substantial variation among the replicate pools of ten J2.

UPGMA analysis of DGGE patterns of the different populations revealed a separation in two main clusters (Fig. 2B). One large cluster was formed by G2, R2, R3 (two replicates) and E1 (one replicate) and the other one by G1, E2, R1, E1 (except for one replicate) and R3 (two replicates). Thus, DGGE fingerprints of *msp1* tended to cluster the populations according to their reproduction rate on pepper. With the exception of R3, replicates of each population (at least three replicates) were forming a cluster with > 95% similarity.

Sequencing of cloned amplicons, which corresponded to different bands, revealed point mutations at several positions (Fig. 2C). All sequences were 97% to 99% similar to sequences of *msp1* genes of *M. incognita* in GenBank (AF013289, ASM18041v1). Two C to A conversions close to the end that is not stabilized by the GC-clamp could explain the shorter migration distance in the denaturing gradient of variant G2-A compared with the other sequenced variants. Similarly, R2-B shows five G/C to A conversions compared with G2-B, R1-A, and E1-B, and thus partially melted earlier in the denaturing gradient to form a band. The fuzzy band between G2-A and R2-B could be identified as single-stranded DNA by its reddish color and thus was not an additional gene variant.

Discussion

In this study, a PCR-DGGE technique was developed to characterize the distribution of *msp1* gene variants within and between populations of *M. incognita*. With one exception, all *M. incognita* populations analyzed could be distinguished by their reproduction potential on five hosts. This was expected from the populations that represented different races (Hartman and Sasser, 1985). Substantial variations among populations in qualitative host range and quantitative reproduction potential on different hosts have been reported in previous studies (Ehwaeti et al., 1999; Anwar and McKenry, 2007; Robertson et al., 2009; Olowe, 2010; Verdejo-Lucas et al., 2012). Here we used a combination of parameters that were related to egg production and embryonic development to discriminate populations, which might result in amore sensitive discrimination than with numbers of eggs or galls alone. None of the selected host plants on its own was able to differentiate among all populations. Reproduction on tomato cv. Moneymaker discriminated well among some populations, whereas mostly pepper allowed to distinguish among the races. Tomato cv. Sparta differentiated population E1 from others, whereas cotton cv. DP61 differentiated race 3 from other populations. Phenotypic variation among replicates within populations was substantial, as typically experienced in such bioassays (Verdejo-Lucas et al., 2012), so that most populations were not clearly separated in PC analysis (Fig. 1B). Natural field populations should be even more diverse than the single-egg-mass lines used in bioassays because genetic heterogeneity is not reduced through the artificial population size bottleneck. Even though bioassays with differential hosts have been useful to classify populations into a race scheme and to identify *M. incognita* populations (Anwar and McKenry, 2007; Olowe, 2010; Thies, 2011), they are

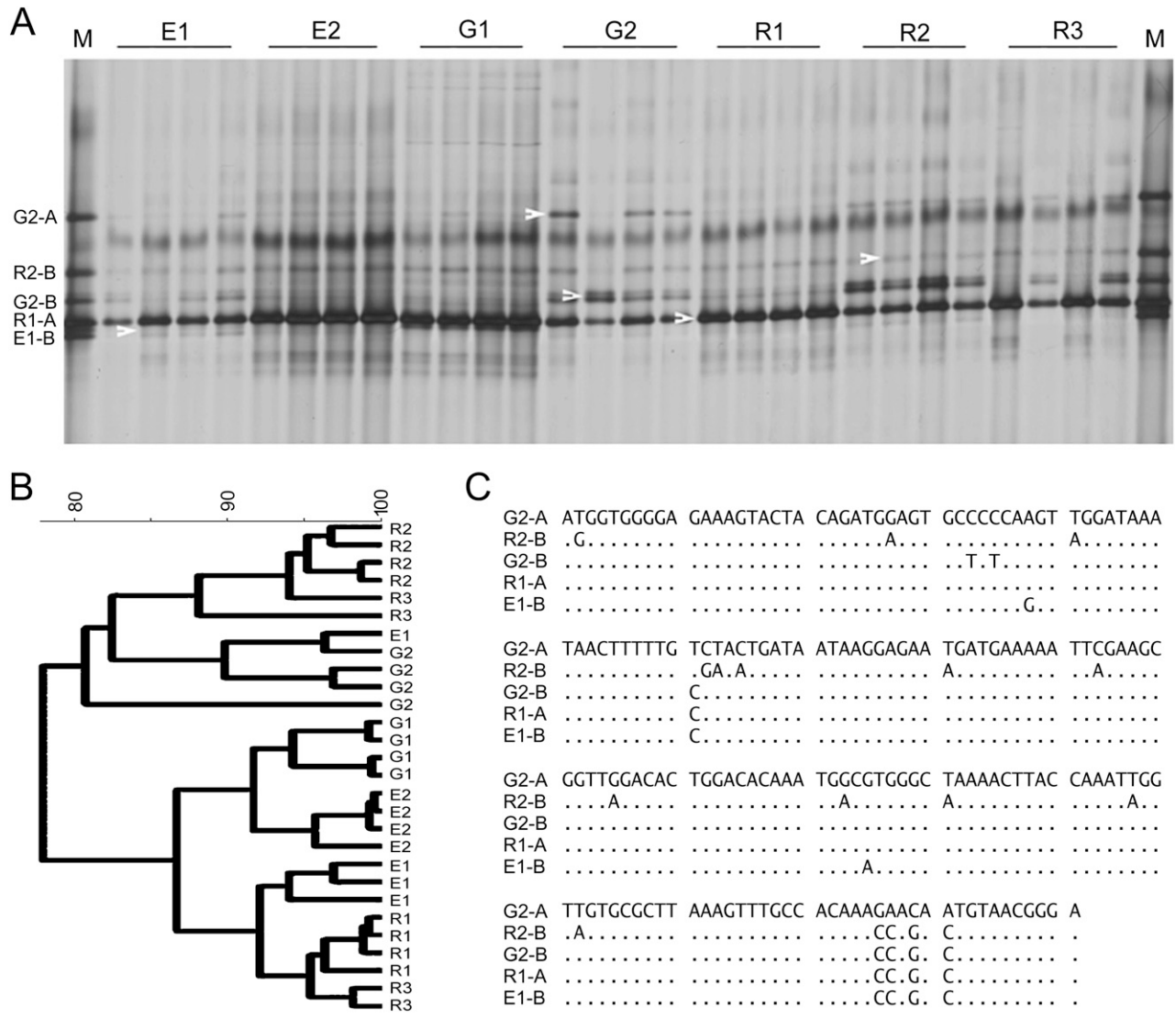

FIG. 2. Genetic differentiation of *Meloidogyne incognita* populations based on variants of their pathogenicity gene *msp1*. A. Denaturing gradient gel electrophoresis (DGGE) of the *msp1* genes amplified by PCR from two Egyptian populations (E1, E2), two German populations (G1, G2), and American populations representing the races R1, R2, and R3. Each of four replicates per population was derived from DNA of ten J2. Cloned and sequenced amplicons representing the different gene variants were combined in a marker (M) as indicated on the left side. B. UPGMA cluster analysis of the DGGE fingerprints. C. Alignment of DNA sequences of the *msp1* gene variants representing major bands in DGGE. Dots indicate the same base as in the sequence of the G2-A variant. Primer sequences were not included.

time-consuming and tend to hide variations within and between populations.

Despite its high rapidity and efficiency as a diagnostic tool, DGGE in nematology so far has been applied only to compare soil or marine nematode communities based on the 18S rRNA gene that is much too conserved to differentiate populations and often even does not distinguish between closely related species (Cook et al., 2005; Okada and Oba, 2008; Sato et al., 2009). The effector MSP1 of *M. incognita* belongs to the secreted pathogenicity factors that are prone to be sensed by pattern recognition receptors or resistance gene products of the plant (Bellafiore and Briggs, 2010). Thus, *msp1* presumably is under selective pressure to change. The high genome plasticity of *M. incognita* promotes extreme adaptive capacity, e.g., by divergence of pairs of homologous genome segments (Abad et al., 2008; Castagnone-Sereno and Danchin, 2014). The PCR-DGGE system is an ideal tool to explore *msp1* gene variation in many samples from populations or individuals. DGGE for replicated pools of juveniles from the seven populations revealed ten *msp1* variants. Specific patterns were detected for most populations, but especially for the population representing race 3 a substantial variation within the population was revealed. It is unknown whether variation of *msp1* plays a role in modulation of the host preference of *M. incognita*. We observed a tendency for clustering of the *msp1* patterns from populations reproducing well on pepper that may indicate such a role. However, this might also be explained by linkage of *msp1* variants with other host range modulating effector genes. Comparative genomic

studies will give further hints on which effector genes are most promising to be indicative for host specificity (Castagnone-Sereno et al., 2013; Danchin et al., 2013). The PCR-DGGE approach could then be adapted to that gene for efficient studies on population-level epidemiology and population-specific infectivity. Next generation sequencing may become a valuable alternative for DGGE but it is not yet a good choice to compare many samples each needing a unique barcode, because the cost per sample (although low per Mbp) is still too high.

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