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Author(s): Devran, Z., Söğüt, M. A., Gözel, U., Tör, M., and Elekçioğlu, I. H.

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Analysis of genetic variation between populations of *Meloidogyne* spp. from Turkey

Zübeyir Devran,* Mehmet Ali Söğüt,** Uğur Gözel,*** Mahmut Tör,****
and İ. Halil Elekçioğlu*****

*West Mediterranean Agricultural Research Institute, 07100 Antalya, Turkey, e-mail: zubeyird@yahoo.com,

**Plant Protection Department, Faculty of Agriculture, Süleyman Demirel University, 32260 Isparta, Turkey,

***Plant Protection Department, Faculty of Agriculture, Çanakkale Onsekiz Mart University, 17020 3anakkale, Turkey,

****The University of Warwick, Warwick HRI Wellesbourne Warwick CV35 9EF, UK,

*****Plant Protection Department, Faculty of Agriculture, Çukurova University 01330 Adana, Turkey

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Summary. Genetic variation among different 29 root-knot nematode populations was characterized by using amplified fragment length polymorphisms (AFLP). Twenty-two nematode samples collected from infected vegetables (tomato, cucumber, eggplant, pepper and melon) at various locations of the Mediterranean Region in Turkey were screened together with seven reference populations including *Meloidogyne arenaria*, *M. javanica* and *M. incognita*. Genetic polymorphism differentiation of *Meloidogyne* species was determined using 11 AFLP primer combinations. Depending on the primer combination and the genotypes, single lanes with 15-45 amplified DNA fragments were produced. Furthermore, genetic intra-specific similarity of *M. arenaria*, *M. incognita*, and *M. javanica* ranged from 40 to 67%, 45 to 56% and from 41 to 73%, respectively. These findings showed an absence of general correlation between genetic similarity and geographical origin of the populations within species, which was demonstrated using the AFLP patterns.

Key words: AFLP-genetic variations, *Meloidogyne arenaria*, *M. javanica*, *M. incognita*, root-knot nematodes.

Root-knot nematodes (*Meloidogyne* spp.) are the most economically destructive genus of plant-parasitic nematodes in the world (Sasser, 1989). They cause yield losses on many different crops and four species in particular out of the 80+ described species are responsible for at least 90% of the estimated crop losses caused by root-knot nematodes. Important species include *Meloidogyne arenaria*, *M. javanica* and *M. incognita* in temperate and tropical regions, and *M. hapla*, *M. chitwoodi* and *M. fallax* encountered mainly in cooler climates (Netscher & Sikora, 1990; Siddiqi, 2000).

In Turkey, the Mediterranean region is the most important region for open field and enclosed production of vegetables. *Meloidogyne arenaria*, *M. javanica* and *M. incognita* cause economic problems on crop production in this region (Elekçioğlu & Uygun, 1994; Elekçioğlu *et al.*, 1994; Söğüt & Elekçioğlu, 2000). An effective soil fumigant, methyl bromide (MeBr), gave good control of *Meloidogyne* spp. but its use was officially restricted, except for exceptional situations, such

as for quarantine purposes at the end of 2007 because of its adverse environmental and human impact. It has gradually been either removed from the market or drastically restricted. Therefore, nematicides application of soil solarisation combined with MeBr alternatives and resistant varieties are recommended in order to control nematodes in this region. However, soil solarisation can only be applied effectively in summer, which is insufficient time to provide protection during the growing season. Nematode resistant varieties are used in this region but are limited to only a few crops such as tomato. Plant resistance may only be to specific populations or races of a particular nematode species and may be compromised by resistance-breaking virulent populations. In fact, field populations that completely overcome the *Mi* resistance have been reported for *Meloidogyne* spp. from different regions of the world (Eddaoudi *et al.*, 1997; Tzortzakakis *et al.*, 1999). Investigations on intraspecific genetic variability in order to identify subspecific groups of *Meloidogyne* are essential for

the design of successful management practices (Castagnone-Sereno *et al.*, 1995; Semblat *et al.*, 1998).

No previous studies have been done to investigate intraspecific genetic variation of *Meloidogyne* spp. in Turkey. The aim of this study was an estimation of the genomic relationships among 22 populations of *Meloidogyne* spp. (*M. arenaria*, *M. javanica*, *M. incognita*) collected from the Mediterranean Region in Turkey by using AFLPs; isolates of *Meloidogyne* spp from seven other countries were included for comparison.

Table 1. *Meloidogyne* species and populations used in this study.

Code	Species	Host plants	Geographic Origin
*A53	<i>M. arenaria</i>	Unknown	Ivory Coast
*A54	<i>M. arenaria</i>	Unknown	28 French West Indies
*A55	<i>M. arenaria</i>	Unknown	32 French West Indies
A25	<i>M. arenaria</i>	Melon	Kumluca, Antalya
A36	<i>M. arenaria</i>	Melon	Aksu, Antalya
A30	<i>M. arenaria</i>	Tomato	Kumluca, Antalya
A1	<i>M. arenaria</i>	Cucumber	Aksu, Antalya
J2	<i>M. javanica</i>	Tomato	Kazanlı, İçel
J3	<i>M. javanica</i>	Cucumber	Adanalıoğlu, İçel
J5	<i>M. javanica</i>	Cucumber	Kazanlı, İçel
J12	<i>M. javanica</i>	Eggplant	Kumluca, Antalya
J13	<i>M. javanica</i>	Cucumber	Kumluca, Antalya
J14	<i>M. javanica</i>	Tomato	Kocahasanlı, İçel
J16	<i>M. javanica</i>	Tomato	Kocahasanlı, İçel
J18	<i>M. javanica</i>	Cucumber	Hamurlu, İçel
J20	<i>M. javanica</i>	Cucumber	Erdemli, İçel
J21	<i>M. javanica</i>	Tomato	Erdemli, İçel
J23	<i>M. javanica</i>	Eggplant	Kumluca, Antalya
J31	<i>M. javanica</i>	Melon	Aksu, Antalya
*J60	<i>M. javanica</i>	Unknown	L 22
*J57	<i>M. javanica</i>	Unknown	107-2 Trinidad
*151	<i>M. incognita</i>	Unknown	9 Ivory Coast
*152	<i>M. incognita</i>	Unknown	111 USA (Race 3)
I24	<i>M. incognita</i>	Pepper	Hacıaliler, Antalya
I26	<i>M. incognita</i>	Pepper	Mavikent, Antalya
I28	<i>M. incognita</i>	Pepper	Kazanlı, İçel
I29	<i>M. incognita</i>	Eggplant	Kumluca, Antalya
I33	<i>M. incognita</i>	Cucumber	Aksu, Antalya
I35	<i>M. incognita</i>	Tomato	Mavikent, Antalya

* References populations from other countries.

MATERIAL AND METHODS

Nematode populations: The name and geographical origin of nematode populations are shown in Table 1. Each nematode population used in this study was collected directly from the field. Identification of species was confirmed by PCR

using egg masses obtained from each plant root. All collected samples were checked to ensure that pure populations were obtained and to eliminate individuals belonging to other species. Identification of the root-knot nematode isolates collected from Turkey was confirmed by analysis of ITS region of rDNA (Zijlstra *et al.*, 1995) and by mtDNA (Stanton *et al.*, 1997). Furthermore, seven populations were provided by Dr. Vivian C. Blok (Scottish Crop Research Institute) as reference isolates that were used in the identification of species and AFLP studies.

Table 2. Sequences of EcoRI and MseI adaptors and primers used for AFLP.

Primers/Adapters	Sequences (5'-3')
EcoRI adapter	CTCGTAGACTGCGTACC CTGACGCATGGTTAA
MseI adapters	GACGATGAGTCTGAG TACTCAGGACTCAT
EcoRI+T	GACTGCGTACCAATTCT
EcoRI+A	GACTGCGTACCAATTCA
EcoRI+TCT	GACTGCGTACCAATTCTCT
EcoRI+TAA	GACTGCGTACCAATTCTAA
EcoRI+TGC	GACTGCGTACCAATTCTGC
EcoRI+TCT	GACTGCGTACCAATTCTCT
EcoRI+AGA	GACTGCGTACCAATTCAGA
EcoRI+ATC	GACTGCGTACCAATTCATC
EcoRI+ACC	GACTGCGTACCAATTCACC
EcoRI+ATT	GACTGCGTACCAATTCATT
MseI+C	GATGAGTCCTGAGTAAC
MseI+G	GATGAGTCCTGAGTAAG
MseI+CTT	GATGAGTCCTGAGTAAGTT
MseI+CAA	GATGAGTCCTGAGTAACAA
MseI+CTA	GATGAGTCCTGAGTAAGTA
MseI+GGC	GATGAGTCCTGAGTAAGGC
MseI+GCG	GATGAGTCCTGAGTAAGCG

DNA extraction: DNA extraction from egg masses was performed as described by Ceniz (1993). Frozen egg masses were crushed in liquid nitrogen with a mortar and pestle and incubated for 1 h at 55°C in 1000 µl of extraction buffer (100 mM Tris-HCl, pH 8.5; 50 mM EDTA, pH 7.4; 100 mM NaCl; 1% SDS; and 100 µg ml⁻¹ of Proteinase K). The lysate was extracted once with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1). Nucleic acids were precipitated from the aqueous layer with 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volume of absolute ethanol at -20°C. After centrifugation at 8000 g for 15 min, the pellet was washed two times with 70 % ethanol and dissolved in 100 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0).

AFLP protocol: Template DNA preparation: The AFLP procedure was performed essentially as

described by Vos *et al.* (1995). Genomic DNA (200 ng) was digested with 15 units *EcoRI* and 10 unit *MseI* for 4 h at 37°C in NE Buffer 3 (New England Biolab, UK) in a final volume of 50 µl.

Ligation of Adapters: 50 µl of a ligation mixture contained 5xT4 ligase buffer (GibcoBRL, Gaithersburg USA), 5 pmol *EcoRI* adapter, 50 pmol *MseI* adapter (Table 2), 1 unit of T4 DNA ligase (GibcoBRL, Gaithersburg USA) and 50 µl digested DNA and the samples were incubated for 15 h at 20°C. After ligation, the reaction mixture was diluted 10-fold in TE buffer (10 mM Tris pH 8.0, 0.1 mM EDTA).

Preamplification: The first step included preamplification of the adapter ligated DNA in a PCR reaction with *EcoRI* and *MseI* primers which each has one selective nucleotide (Table 2). Each 25 µl PCR reaction contained 2.5 µl of DNA, 10x Reaction buffer, 50 mM MgCl₂, 5 mM of dNTP (Bioline), 10 µM of each+1 primer (*EcoRI*+N, *MseI*+N; Table 2) and 1 unit of Taq DNA polymerase (Bioline, UK). Samples were run on a PCR machine (PTC-0220 DNA Engine Dyad, Massachusetts, USA) for 20 cycles of 94°C (30 s), 56°C (1 min), 72°C (1 min). A 5 µl DNA aliquot of the preamplified DNA was checked on a 1.5% agarose gel, where a slight DNA smear was visible. The remaining PCR reaction was diluted 50-fold in TE buffer.

Selective amplification: Selective primers included eight *EcoRI*+3 primers and five *MseI*+3 primers, with a total of 11 *EcoRI*/*MseI*+3 primer combinations (Table 2). Selective amplification was performed with a (P³³) ATP labelled *EcoRI*+3 primers and unlabelled *MseI*+3 primers. The 10 µl PCR mix consisted of 2.5 µl DNA, 10xPCR buffer, 50 mM MgCl₂, 5 mM each dNTP, 10 µM labelled *EcoRI*+3 primer, 10 µM *MseI*+3 primers and 1 unit Taq DNA polymerase (Bioline, UK). DNA was amplified using the following temperature cycle: one cycle, 94°C (30 s), 65°C (30 s), 72°C (60 s) followed by nine cycles of 0.7°C lower annealing temperature each cycle and 25 cycles of 94 (30 s), 56°C (30 s), 72°C (60 s).

Gel analysis: 10 µl PCR products were mixed with 10 µl formamide dye (98% formamide, 10 mM EDTA, 0.025 % each of xylene cyanol and bromophenol blue) denatured for 5 min at 94°C. A subsample (4 µl) of the mix was loaded into a 6% denaturing polyacrylamide gel (200 ml 30% acrylamide, 400 g urea) in 1x TBE electrophoresis buffer, using a vertical gel apparatus (Model S2, Life Tech.). The gels were dried with a gel dryer (Model 583, Bio Rad), and X ray films (Kodak, Japan) were exposed at room temperature.

Data analysis: The presence or absence of a DNA fragment in a population was treated as binary characters. The data were converted to similarity values using the formula $F = 2N_{xy} / N_x + N_y$, N_x and N_y refer to number of DNA fragments generated by the AFLP assay in populations X and Y, respectively, whereas N_{xy} is the number of DNA fragments shared by the two populations (Nei & Li, 1979). F values were used to construct a dendrogram with the unweighted pair group method with arithmetic mean (UPGMA) in the NTSYS (Numerical Taxonomy and Multivariate System)-pc version 1.80 (Rohlf, 1994).

RESULTS AND DISCUSSION

Eleven AFLP primer combinations were used with genomic DNA isolated from seven *M. arenaria*, eight *M. incognita* and fourteen *M. javanica* populations; 15-45 amplified DNA fragments were resolved on a single lane, depending on the primer patterns used and the nematode genotype analysed. A similar matrix based on simple matching values was also constructed to estimate the level of DNA polymorphism among 29 root-knot nematode populations. Calculation of simple matching values was based on the presence or absence of discrete characters (AFLP fragments) from paired samples. Thus, the matrix values estimated the number of AFLP fragments shared (or not shared) between two individuals.

Table 3. Genetic similarity matrix for seven *M. arenaria* based on the formula of Nei and Li (1979).

	A53	A54	A55	A25	A36	A30	A1
A53	100						
A54	67	100					
A55	55	58	100				
A25	44	45	49	100			
A36	45	44	45	47	100		
A30	46	50	48	43	47	100	
A1	45	44	43	46	45	40	100

The highest genetic similarity was 67% between A53 (Ivory Coast) and A54 (28 French West Indies) of *M. arenaria* populations, 56% between I51 (9 Ivory Coast) and I52 (111 USA) of *M. incognita* populations and 73% between J60 (L22) and J57 (107-2 Trinidad) of *M. javanica* populations (Tables 3, 4, 5). The lowest genetic similarity was 40% between A30 (tomato, Kumluca) and A1 (cucumber, Aksu) of *M. arenaria* populations; 45% between I24 (pepper, Hacialiler) and I52 (unknown, 11 USA), between I33 (cucumber, Aksu) and I52 (unknown, 111 USA), and also between I28 (pepper, Kazanlı) and I33 (cucumber, Aksu) of *M. incognita* populations;

41% between J23 (eggplant, Kumluca) and J18 (cucumber, Hamurlu), also between J23 (eggplant, Kumluca) and J31 (melon, Aksu) of *M. javanica* populations (Tables 3, 4, 5). Genetic similarity of other populations ranged between 41 and 67% that showed high genetic differentiation levels related to characteristic properties. Some populations that were originally collected from close geographic areas displayed very low similarity values (*M. incognita* populations I29 (eggplant, Kumluca) and I35 (tomato, Mavikent) or *M. javanica* populations J20 (cucumber, Erdemli) and J21 (tomato, Erdemli)) (Tables 4, 5). By contrast, very high similarity values were observed on *M. javanica* population J3 (cucumber-Adanalioğlu) and J2 (tomato-Kazanlı) collected from different regions; these results may be associated with selection by intensive chemical application of frequent exposure to the same chemical compounds. Furthermore, reproduction of *Meloidogyne* spp (*M. incognita*, *M. javanica* and *M. arenaria*) is commonly parthenogenetic and may be sexual in adverse conditions. It may be assumed that adverse conditions may lead to an increase in genetic variation that may also be associated with intensive chemical applications, soil structure and climatic conditions.

Table 4. Genetic similarity matrix for eight *M. incognita* based on the formula of Nei and Li (1979).

	I51	I52	I24	I26	I28	I29	I33	I35
I51	100							
I52	56	100						
I24	50	45	100					
I26	53	49	51	100				
I28	54	47	47	52	100			
I29	50	49	54	54	49	100		
I33	46	45	53	47	45	47	100	
I35	46	50	52	54	47	49	50	100

The UPGMA analysis grouped the 29 root-knot nematode populations into clusters corresponding to their respective species. Cluster analysis (Fig. 1) on all seven *M. arenaria* population revealed two distinct groups. Population A53, A54 and A55 clustered on one side of the tree; however, isolates A25, A36 and A1 clustered on the other side. The eight *M. incognita* populations used in this study have mainly been classified into three different groups (I51, I52 and I28; I26 and I35; I24, I29 and I33). The *M. javanica* populations clustered into four groups. AFLP markers provided a high level of efficiency for detecting DNA polymorphism over a

large number of randomly sampled loci, and proved to be very useful for detecting levels of genetic variation among natural populations of *Meloidogyne* spp. Many studies have been made in order to investigate genetic variation of *Meloidogyne* spp. Carpenter *et al.* (1992) reported the relatively high levels of variation in the *M. arenaria* populations with geographically closely related isolates by using RFLP. *Meloidogyne arenaria* and *M. javanica* were found to be closer to each other than to *M. incognita* (Baum *et al.*, 1994; Xue *et al.*, 1993). Some authors have suggested that these species can possibly be considered as one group. Both results are consistent with these RAPD and RFLP studies (Fargette *et al.*, 1996), which indicate that *M. arenaria* is a heterogeneous mixture of clonal lines and *M. javanica* a homogenous group of related lines. Studies of mitochondrial DNA, however, have not shown that *M. arenaria* is more closely related to *M. javanica* than *M. incognita* but they have shown that lines of *M. arenaria* can show as much or more intraspecific variation than in any interspecific comparisons (Hugall *et al.*, 1994; Hyman and Powers, 1991; Powers, and Sandall, 1988) and thus indicate that these two group have diverged. In this study, intraspecific variation (41-73%) was greatest within *M. javanica*. In contrast, the levels of variability were lower within *M. incognita* and *M. arenaria* 45-56% and 40-67% respectively.

Examination of the genetic variation of *Meloidogyne* spp. has been conducted in several studies. Blok *et al.* (1995) have suggested the presence of dissimilarity by 8% among four populations from different countries by using RAPD analysis and in another study the difference was 20% between two populations (Guirao & Cenis, 1995). Also, Garneiro *et al.* (1998) reported similarity which is >40% for some *M. javanica* populations collected from Brazil. In this study, intraspecific genetic variability of *M. arenaria*, *M. incognita* and *M. javanica* ranged from 40 to 67%, 45 to 56% and 41 to 73%, respectively (Tables 3, 4, 5). In contrast, intraspecific genetic variation showed much lower values of 9.7%, 4.4% and 3.3% in *M. arenaria*, *M. incognita* and *M. javanica*, respectively, by using AFLP (Semblat *et al.*, 1998). In the same way, Tzortzakakis *et al.* (1999) observed very high mean genetic similarity (99.4%) and a very homogeneous group of *M. javanica*. In our present study, we used not only different single egg masses but also the populations came from different cropping systems and different geographic origins. These may be important factors that can play

Table 5. Genetic similarity matrix for 14 *M. javanica* based on the formula of Nei and Li (1979).

	J2	J3	J5	J12	J13	J14	J16	J18	J20	J21	J23	J31	J60	J57
J2	100													
J3	68	100												
J5	67	54	100											
J12	64	50	70	100										
J13	60	57	56	69	100									
J14	64	56	55	58	50	100								
J16	57	52	57	61	54	60	100							
J18	61	52	56	50	53	57	60	100						
J20	55	54	55	56	52	53	64	52	100					
J21	57	50	55	58	52	59	61	47	51	100				
J23	51	55	54	68	50	49	45	41	44	43	100			
J31	60	52	53	55	56	52	54	49	50	48	41	100		
J60	52	53	52	57	55	53	52	49	56	49	42	53	100	
J57	54	55	53	57	59	54	58	48	51	51	45	58	73	100

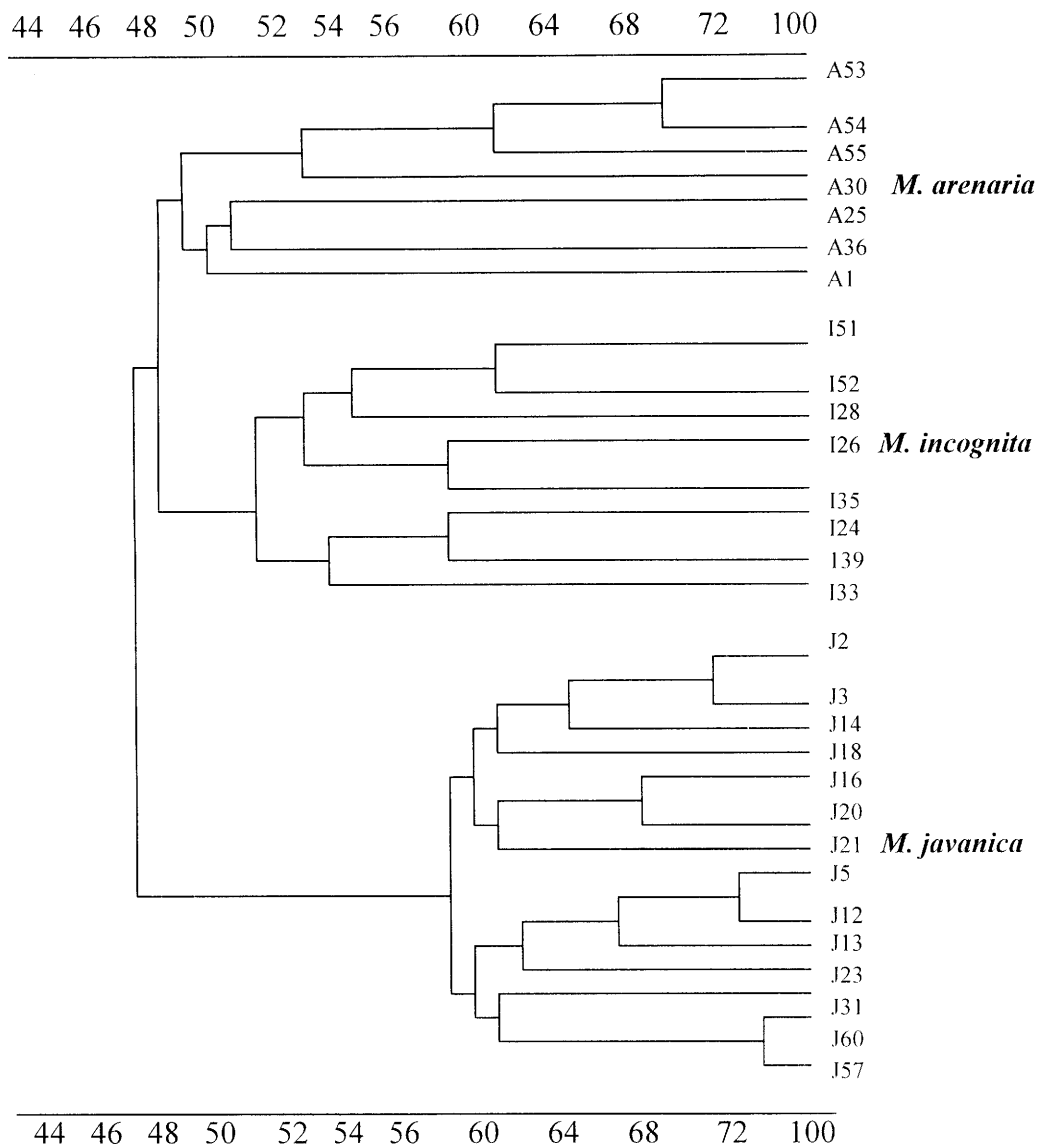


Fig. 1. Dendrogram of 29 *Meloidogyne* populations.

a role in the genetic variation in these species. Within each of the three root-knot nematode species, the cluster analysis showed an absence of correlation between genomic similarity and geographical origin of the populations. Populations which are very dissimilar were found in widely separated areas (Tables 3, 4, 5).

In conclusion, genetic variation within intra and inter species was determined as high level according to AFLP analysis. Considering the prominent situation faced with in greenhouse fields of Turkey, the most destructive pathogen on greenhouse cultivation is root-knot nematodes in south coast regions. Therefore, other studies are necessary in order to understand whether this case may result from intensively pesticide application or other factors.

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Devran Z., Söğüt, M.A., Gözel, U., Tör, M., Elekçioğlu İ. H. Анализ генетического разнообразия популяций *Meloidogyne* spp. в Турции.

Резюме. Генетическое разнообразие 29 популяций галлообразующих нематод было исследовано методом полиморфизма длины амплифицируемых фрагментов (amplified fragment length polymorphisms - AFLP). Двадцать два образца, полученные от зараженных растений (помидоры, огурцы, баклажаны, перец и дыня) в различных местах средиземноморского региона Турции, были изучены вместе с 7 уже определенными популяциями видов *Meloidogyne arenaria*, *M. javanica* и *M. incognita*, которые служили стандартами. Генетический полиморфизм видов *Meloidogyne* был определен на основе 11-и комбинаций праймеров для AFLP. В зависимости от примененных праймеров получали дорожки с 15-45 амплифицированными фрагментами ДНК. Генетическое внутривидовое сходство в пределах видов составляло у *M. arenaria*, *M. incognita* и *M. javanica* 40-67%, 45-56% и 41-73%, соответственно. Полученные AFLP-данные продемонстрировали отсутствие четкой корреляции между степенью генетического сходства и местом сбора популяций.
