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Identification of species specific Gnomic DNA fragments for Meloidgyne incognita, M.javanica and M. arenaria, in Ismailia and El Sharquia Governorates, Egypt

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Abstract

Sequence characterised amplified regions (SCARs) technique was used to identify the root knot nematodes, Meloidogyne species. Fourteen samples were collected from Papaya, Grape vine, Ullayq plant, Egg plant, Tomato plant and Banana roots infected by the root-knot nematodes and grown at three different localities, Faculty of Agriculture Suez Canal University and Abouswer region in Ismailia Governorate and El Salhia region, El Sharquia Governorate. The developed sets of SCAR primers were used for identification of the three species of Meloidogyne, Meloidgyne incognita, *M.javanica* and *M. arenaria* fast reliable and useful for identification at any developmental life stage. The results revealed that papaya grown in Fac. of Agric. farm was infested by either *M. incognita*, or *M. arenaria*, while *M. javanica* was found infecting Grape vine and Ullayq grown in Abousower and Egg plant planted in El-Salhia. On the other hand *M. incognita* was found infecting Grape vine and Banana root grown in Fac. of Agric farm. At El Salhia region *M. javanica* was found infecting Egg plant, while *M. incognita* was found infecting Banana and Tomato roots. Five samples out of the fourteen did not give any reaction with the three used primers.

Introduction

The root knot nematode, Meloidogyne species is a major yield-limiting pathogen in many crop production areas worldwide. Four species (*M. incognita*, *M. javanica* , *M. arenaria* and *M. hapla*) among more than 80 nominal species on record are considered of major economic importance (Eisenback,1997, Karssen and Van Hoenselaar, 1998). The identification of Meloidogyne species includes morpholgical characters such as larval measerments, perineal patterns of the females and the host range used to differentiate the species of this genus (Eisenback, 1985). Other identification techniques, which are more reliable such as isozyme electrophorasis and nucleic acids technology, allowed new approaches to get a reliable and precise nematode identification (Harris *et al* , 1990). The use of the polymeras chain reaction (PCR) and DNA marker in gentic diagnosis allows the identification of some Meloidogyne stages as single eggs or juvenile (Harris *et al*., 1990, Cenis, 1993 and Willamson *et al*., 1997). Zijlstra, (1997) cloned and sequenced the amplified rDNA-ITS fragment of *M. chitwoodi*, *M. fallax*, M.*hapla* and *M. incognita*. Haroon and Zijlstra (1998 a&b) used the ITS and restriction fragment length polymorphism RFLP of rDNA to distinguished between species of *Meloidgyne* which found infecting vegetable crops in Fayoum, Egypt. They found that ITS region of all isolates was digested with four restriction enzymes primers (Hind III, Eco RI and Dral). They used two sets to distinguished these species by the size of their fragment in a single PCR reaction, (SCAR primer and Multiplex) to differentiate between *M. javanica* and *M. incognita* which revealed the size of the cloned amplified ITS region of *M. incognita* at 760 bp. El-Amawey, (2000) used ITS-RFLPs multiplex PCR, SCARs and RAPD-PCR to identify the three species of Meloidagyne *M. javanica*, *M. incognita* and *M. hapla* by using DNA fragments.

Available expressed sequence tags (ESTs) data from plant parasite nematodes are derived from root knot nematodes (four species, 25,9000 ESTs) (Dautova *et al*., 2001)

The developed sets of the squence characterised amplified regions (SCARs) primers were successfully used in straightforward, fast and reliable PCR asays to identify *Meloidgyne incognita*, *M. javanica* and *M. arenaria*. The length variant SCAR markers can be amplified from DNA from egg masses, second stage juveniles and females. Identification technique of this species is therefore independent of the nematode life cycle stage. Morcover the SCAR-PCR assay was successfully applied using DNA extracts from infested plant material (Zijlstra *et al*., 2000).

In Egypt, the root knot nematode is recognized as major agriculture pest of a wide range of crops including field, vegetable, fruit and ornamental crops (Oteifa. 1964; Oteifa and Tarjan, 1965). The three species of *Meloidgyne*, *Meloidgyne incognita*, M.*javanica* and *M. arenaria* are highly distributed in northern Egypt (Ibrahim *et al*., 1986a,1994) and El-Saedy *et al* 1993. They added that *M. arenaria* was restricted in its distribution.

El Gindi *et al*., 1980; Mahrous 1991 and El Shawadfy, 1997, reported that the geographical distribution of the root knot nematode *M. javanica* is most abundant in sand soil and newly reclaimed land such as Nubaria, Tahrir province and Salhia

districts followed by *M. incognita.*

The objective of this study was to determine species specific gnomic markers for *Meloidgyne incognita*, *M. javanica* and *M. arenaria* by using species specific pairs of perimers (Table 1).These primers would be used to amplify the sequence chaecterised amplified region (SCARs), enabling a straight forward fast and rebiable identification of the three species.

Materials and Methods

Root-knot nematode collection: Sample of root-knot nematode were collected from root plants in El Sharquia and Ismailia Governorates 2002. These sample were collected from different hosts at the chosen localities; tomato and Egg plants grown in El-Salhia, El Sharquia Governorate . Papaya, Grape vine, Banana and Ullayq grown in the Farm of Fac. of Agric. Suez Canal Univ. and Grape vine, Banana and Ullayg grown in Abouswer, Ismailia Governorate. Samples were kept in normal saline or freezing until extraction of the DNA.

DNA extraction: DNA extraction was carried out according to (Zouhar *et al.*, 2000). In a mortars included 2 gram root-knot added 200-600 ul of CTAB (50mM Tris HCl ph 8.0, 0.7 M Nacl, 10 mM EDTA, 1% CTAB and 20 mM B-mercaptoethanol). The homogenate was put in water bath to 60°C for 2h. Mixed 1:1 with chloroform- isoamyl-alcohol (24:1), vortexes for 15 min and centrifuged 10 min at 3500g (7000 rpm). An equal volume of isoamylalcohol was added to the supernatant and DNA was precipitated at -20°C for 9-12 hrs. Then it was centrifuged (10 min at 7000 rpm) and the pellet was washed by 40 ul of 80% ethanol with 10 mM LiCl and 1 mM Tris. After 15 min the microtubes were centrifuged 10 min at 7000 rpm. The pellets were dried under vacuum and resuspended in 20 ul ddH_2O.

SCAr amplification and analysis: amplification reaction for SCAR were performed in 25 ul reaction volumes containing 10 mM Tris pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCL uM each of dATP, dCTP, dGTP and dTTP, 0.5 unit of Taq DNA polymerase (Pharmacia) and 1 to 10 ng of total DNA. For PCR reaction using the primers Far/Rar (Table1), they were used at a final concentration of 0.3u*M*. For PCR reaction using Finc/Rinc or Fjav/Rjav (Table1) a primer concentration of 0.24 uM was used. For SCAR amplification, the thermocycler was programmed for 2 min at 94°C followed by 35 cycles of 30s at 94°C at the annealing temperature and 1min at 72°C. Annealing temperatures were 54°C using the primers Fince/Rinc, 61°C using primers Far/Rar and 64°C using primers Fjav/Rjav. 5 ul aliquots were removed from the reaction and subjected to electrophoresis on a 1% agarose gel. Products were visualized by staining with ethidium bromide. A 100 bp ladder (Promega) was used as molecular size stander.

Table (1): Nucleotide sequence of primer used for each SCAR (After Zizlstra *et al.* 2000)

Name of SCAR primer*	Sequence of SCAR primer	Size of SCAR
Far	TEGGCGATAGAGGTAAATGAC	420
Rar	TEGGEGATAGACATACAACT	
Fjav	GGTGCGATTGAACTGAGC	670
Rjav	CAGGCCCTTCAGTGGAACTATAC	
Finc	ETETGCCCAATGAGCTGTCC	1200
Rinc	CTCTGCCCTCACATTAAG	

Table (2): Molecular weights of the amplification products from different Nematoda species.

Sample No.	Nematoda sample	Host	Localities	Nematoda species	Molcular weight bp.
1	Mixed culture	Papaya root	Fac. of Agriculture	*M. arenaria*	420
2	Mixed culture	Tomato root	El Salhia	-ve	-
3	Pure culture	Tomato root	El Salhia	*M. incognita*	1200
4	Culture	Grape vine	Fac. of Agriculture	*M. incognita*	1200
5	Culture	Grape vine	Abousower	*M. javanic*	670
6	Culture	Banana plant	Fac. of Agriculture	-ve	-
7	Culture	Ullayq plant	Abousower	*M. javanic*	670
8	Mixed egg	Papaya root	Fac. of Agriculture	*M. incognita*	1200
9	Culture egg	Egg plant	El Salhia	*M. javanic*	670
10	Pure culture	Ullayq root	Fac. of Agriculture	-ve	-
11	Pure culture	Babana root	Fac. of Agriculture	*M. incognita*	1200
12	Pure culture	Grape vine root	Abousower	-ve	-
13	Pure culture	Grape vine root	Fac. of Agriculture	-ve	-
14	Pure culture	Banana root	Abousower	*M. incognita*	1200

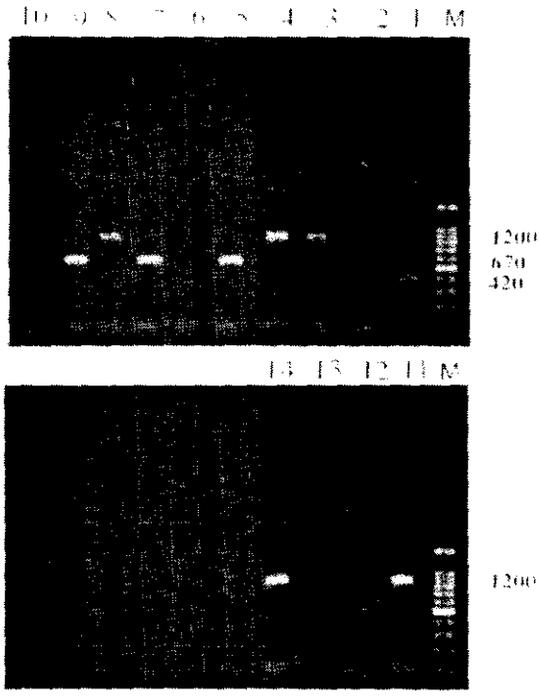

Fig (1): Typical amplification products of PCR reaction of 14 sample from Ismailia and El Sharquia Governorate

Results and Discussion

Six SCAR primers, Far/Rar; Fjav/Rjav and Finc/Rinc were examined with 14 root-knot nematode Meloidogyne spp. (Table 1). The results of extracted DNA from the fourteen samples collected from 6 different hosts grown at 3 different localities are presented in Table (2).

The resulted banding pattern amplification products using primer in reaction with tested root-knot species were illustrated in Fig (1). The bands were ranged at size from 420 and 1200 bp. One strong species-specific band is 420 bp, which appeared with *M. arenaria*. The band of *M. javanica* was 670 bp while it was 1200 bp with *M. incognita*. These results are in agreement with the earliest studies involving the use of protein analysis (Dalmasso and Berge 1979 and Dicksen *et al.*, 1971) since they reported that *M. arenaria* and *M. javanica* are more closely related to each other than to *M. incognita*. Also the results of Zijlstra (1997) confirmed this results since she reported that the size of SCAR of *M.* arenaeia and *M. javanica* are 420 and 670 respectively, while *M. incognita* is 1200 bp.

The results indicate the ability of these primers to detect the polymorphic between the three species of Meloidogyne.

Methods of SCAR primers were successful by used in straightforward, fast and reliable PCR assays to identify nematode species. Moreover, the SCAR-PCR extracts from infested plant material. The method has potential to be optimized for routine practical diagnostic testes facilitating the control of these economically important pest organisms,(Zijlstra *et al.*, 2000).

Results shown in Table (2) indicated that *M. arenaria* was found infecting Papaya root grown in the farm. of Fac. of Agric. only. There was no any other sample positive to M.*arenaria*. On the other hand *M. incognita* was found infecting the roots of Grape vine, Banana and Ullayq in the same farm. While there were three samples, which were taken from the infected roots of Banana, Ullayq and Grape vine in the same Farm. gave negative reaction with the three used primers, which indicate that these three are different species other than the three mentioned infesting the soil of the Fac. of Agric. Farm.

As for the three samples which were taken from El-Salhia, one gave negative reaction which the two others were *M. incognita* and M.javanic which were collected from the infected roots of tomato and Egg plant respectively. Similar results were found in the third region Abouswer, since *M. javanica* was found infecting the root of Grape vine and Ullayq and *M. incognita* was found infecting Banana roots. While the fourth sample gave negative reaction with the three used primers.

In other words, *M. javanica* was found infecting the roots of Grapevine and Ullak grown in Abouswer, and Egg plant grown in El Salhia, while M.*incognita* was found infecting the root of tomatoes grown in El Salhia and Grape vine, Babaz and Banana grown in the Farm of Fac. of Agric.

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تصنيف لبعض أنواع المواقع الجينية لكروموسومات الملوجينا انيكوجينتا وملوجينا جافنيكا وملوجينا ارنيبربا لمحافظة الاسماعيلية والشرقية - مصر

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أستخدم ترتيب المواقع الجينية للكروموسومات كطريقة لتصنيف نيماتودا تعقد الجذور لأنواع الملوجين .ومن خلال أربعة عشرة عينة جمعت من جذور نباتات الباباز _ العنب - العلائق _ الباذنجان _ الطماطم والموز وجميعها مصابة نيماتودا تعقد الجذور وقد تم جمع العينات من مزرعة كلية الزراعة جامعة قناة السويس ومنطقة أبو صوير محافظة الإسماعيلية كما تم جمع عينات من منطقة الصالحية محافظة الشرقية .

وقد أستخدمه ثلاث أزواج من البادئات المتخصصة كأحد الطرق لتصنيف ثلاث أنواع من الملوجينا هى ملوجينا انيكوجينتا وملوجينا جافنيكا وملوجينا ارنيبربا وهى طريقة سريعة وناجحة لتحديد أي طور من أطوار حياة النيماتودا .

وقد أشارة النتائج أن الباباز النامي في مزرعة كلية الزراعة مصاب بالنوع ملوجينا انيكوجينتا وملوجينا ارنيبربا بينما ملوجينا جافنيكا كانت تصيب العنب والعلائق في منطقة أبو صوير ونبات الباذنجان في منطقة الصالحية .

ومن ناحية أخرى فإن ملوجينا أنيكوجيتا كانت تصيب العنب والموز بمزرعة كلية الزراعة. وفي منطقة الصالحية وجدة الملوجينا أنيكوجيتا كانت تصيب الموز والطماطم. كما وجد أن هناك خمس عينات من الأربعة عشرة لم تعطى آي تفاعل مع الثلاث بادئات المستخدمة .