Development of specific SCAR-markers for *Meloidogyne incognita* and *Meloidogyne javanica*

(Received: 30.05.2003, Accepted; 10.07.2003)

Akmal A. El-Ghore*, Sanaa Haroon, Magdy Abd El Rheem*** and Ehab Abdella*****
*Zoology Department, Faculty of Science, Cairo University, Cairo, Egypt
**Plant Protection Department, Faculty of Agriculture, El Fayoum Branch, Egypt
***Zoology Department, Faculty of Science, Bani-Sweef Branch, Egypt

ABSTRACT

Plant parasitic nematodes are major pests and cause diseases in many crops of economic importance. The annual losses world wide caused by plant parasitic nematodes are estimated to be approximately 77 billion US dollars. A substantial part of the losses is due to infestations by root-knot nematodes (Meloidogyne spp.) because they are parasites of a multitude of host plants. The most important and widely distributed root-knot nematode species are M. incognita, M. javanica, M. arenaria and M. hapla. For the development of specific sequence characterized amplified region (SCAR) markers from specific RAPD markers, samples of 20 populations of root knot nematodes were collected from heavily infested spots in the fields of nine different localities in four centres of Bani-Sweef governorate and 11 different localities in five centres of El Fayoum governorate. Such samples were inspected and the associated root knot nematodes species were isolated and identified depending upon the perennial patterns and two polymerase chain reaction (PCR) methods, which are ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA). The random amplified polymorphic DNA (RAPD) method was used to investigate the genetic diversity in Meloidogyne species isolates. Populations of each of M. incognita and M. javanica were distinguished by differences in fragment patterns with any of the ten RAPD primers used. From analysis of RAPD fingerprints of all tested populations, two RAPD markers were detected, one was specific for M. incognita populations with primer OPK-2 at fragment size of 1000 bp and the second was specific for M. javanica populations with primer OPB-3 at size of 1100 bp. These two RAPD markers were converted into SCAR (specific characterized amplified region) markers, which were sequenced and two PCR primer pairs were designed for each of M. incognita (MIE-for and MIE-rev) and M. javanica (MJE-for and MJE-rev). These primers can be used to detect these nematode species in the fields.

Key words: *SCAR-markers, specific PCR primers, plant parasitic nematodes.*

INTRODUCTION

Root-knot nematodes (RKN) constitute the most widely distributed group of plant-parasitic nematodes. These biotrophic endoparasites cause extensive damage to a wide variety of economically important plants and are responsible for world wide annual loss, estimated at about 5% (Sasser and Carter, 1985).

Species identification in Meloidogyne has been a major component of taxonomic research in Nematology. Although there are approximately 60 described species of RKNs, most taxonomic attention has been focused on

less than a dozen that are typically associated with diseases of agronomically important plant species. *M. incognita, M. javanica, M. arenaria* and *M. hapla* account for the majority of crop losses caused by RKNs. An extensive survey of about 1300 Meloidogyne populations from over 70 countries representing the primary food production regions of the world found at least one of these four species in 95% of the samples (Carter and Sasser; 1982; Cenis, 1993).

Species identification of plant parasitic nematodes was the most important step in monitoring their infections in order to develop strategies to control the nematodes in the field. Since the diagnostic tests like perennial patterns of adult females or the random amplified polymorphic DNA (RAPD) method, that was used to investigate the genetic diversity in different plant parasitic nematodes were not accurate. So, it is important to develop a rapid and specific test for the identification of species based on PCR techniques. To achieve this goal, the genetic diversity within certain species was assessed by RAPD, then RAPD-markers specific to these species were selected in order to convert them into specific characterized amplified region (SCAR) markers (Paran and Michelmore, 1993), which would be useful in the identification scheme of any plant parasitic nematodes.

Zijlstra *et al.* (2000) studied the identification of *Meloidogyne incognita, M. javanica* and *M. arenaria* using sequence characterized amplified region (SCAR) based-on PCR assay. Three randomly amplified polymorphic DNA (RAPD) markers, OPA-12, OPB-06 and OPA-01, species specific to the root-knot nematode species *Meloidogyne . arenaria, M. incognita* and *M. javanica*, respectively, were identified. After sequencing these RAPD-PCR products, longer primers of 18 to 23 nucleotides were designed to complement the terminal DNA sequences of the DNA fragments. This resulted in three pairs of species-specific primers (SCAR primers) that were successfully used in straightforward, fast and reliable PCR assays to identify *Meloidogyne incognita, M. javanica* and *M. arenaria.* The length of variant SCAR markers can be amplified from DNA egg masses, second stage juveniles and females. This species identification technique is therefore, independent of the nematode's life cycle stage. Moreover, the SCAR-PCR assay was successfully applied using DNA extracts from infested plant material. The method has a potential to be optimized for routine practical diagnostic tests facilitating the control of these economically important pest organisms.

The present work was carried out on two species of root-knot nematodes (RKNs) *(Meloidogyne incognita and Meloidogyne javanica)* and the RAPD data analysis with ten oligonucleotid primers was done on 20 RKNs populations that obtained by El Ghor *et al.* (2003) (Unpublished data). We also report the development of specific pairs of primers to amplify and detect variation at genetically defined loci that have advantages over RAPD markers. These markers are sequence characterized amplified regions (SCARs). SCARs that are well optimized tend to be less sensitive than RAPDs to varying reaction conditions. SCARs can be selected for co-dominance, amplifying more than one allele, which help greatly in population and mapping studies.

MATERIALS AND METHODS

Development of specific SCAR-markers from characteristic RAPD markers

According to RAPD amplification products, a fragment size 1100 bp, with primer OPB-3 was specific for *M. javanica*

populations, while a fragment at size 1000 bp with primer OPK-2 was specific for *M. incognita* populations.

Purification of DNA from agarose gel

The two previously mentioned RAPD amplification products were cutout from the agarose gel and DNA of each was purified from gel slices using GFX – PCR DNA and gel band purification kit (Amersham Pharmacia Biotech, Ltd., England).

Cloning and transformation of RAPD fragments

The purified DNA products were cloned into pMOSBlue (2887 bp)cloning vector and transformed into MOSBlue competent cells, using pMOSBlue blunt ended cloning kit (Amersham Pharmacia biotech, Ltd., England). Then, 100 µl from each transformed MOSBlue cells were spreaded onto LB media plates contining ampicillin (50 (g/(l), tetracyline (15 · (g/(l), IPTG (23.8 mg/ml) and X-gal (50 mg/ml) and incubated overnight at 37°C.

Isolation of plasmid DNA from transformed cells

From LB plates, plasmid DNA was purified from white colonies using plasmid mini-prep purification protocol system (Promega, Madison, and U.S.A). This protocol allows the rapid isolation of small amounts of plasmid DNA without the need for column purification or banding in CsCl gradients.

Screening of positive colonies

The cloned inserts were screened by amplification of mini-prep plasmid DNA with the original RAPD primers (OPB-3 and OPK-2 primers). The amplification processes were carried out tusing 50 µl Ready-To-Go RAPD analysis Beads Kit (Amersham Pharmacia biotech, Ltd., England). The amplification reaction was carried out using a Perkin-Elmer 480 thermalcycler (Norwalk, CT). Initially the PCR amplification conditions were one cycle at 95°C for 5 min, followed by 10 cycles of 95°C for 1 min, 36°C for 30 sec. and 72°C for 30 sec, then 30 cycles of 95°C for 10 sec, 35°C for 30 sec, and 72°C for 30 sec.

After DNA amplification, 10 µl of the PCR products were analyzed on 1.5 % agarose gels using electrophoresis. The resulted bands were analyzed using Gel Documentation system (Gel Doc., 2000).

Sequence analysis

About 800 ng miniprep DNA products were sequenced using cycle sequencing kit (Amersham Pharmacia biotech, Ltd., England) on the Automated Laser Fluorescent DNA Sequencer (ALF, Amersham). Before sequencing, miniprep products were amplified using the following PCR program, one cycle at 95°C for 3 min, followed by 19 cycles of 94°C for 15 sec, 45°C for 15 sec. and 72°C for 40 sec, then one cycle at 72°C for 5 min.

Fluoreceine labelled T7 promoter and U-19 primers (2 pmol/(l) were used for amplification of the insert DNA.

T7 promoter primer:

5'-FICTAATACGACTCTATAGGGA-3'

U-19 mer primer: 5'-FIACGTCGTGACTGGGAAAAC-3'

DNA sequences were analyzed using laser gene software package (DNA STAR, Madison, USA).

Specific primers

From the sequence results of each sample, two specific primer sequences of 19-21 bases were chosen to minimize primer-pair annealing, beginning on the RAPD primer sequence or within 100 bp of the primer sequence. The pairs of specific primers were MIE-for and MIE-rev primers for *M. incognita* and MJE-for and MJE-rev primers for *M.*

javanica.

The SCAR amplification

The above mentioned pair of primers specific for each species were examined with the DNA samples of the 20 nematode populations using ready°-To-Go PCR beads kit (Amersham Pharmacia biotech, Ltd, England). The amplification was carried out in a Perkin-Elmer 480 thermal cycler (Norwalk, CT). Initially the PCR amplification conditions (as were as follows: one cycle of 94°C for 40 sec, 62°C for 20 sec and 72°C for 2 min, followed by 5 cycles of 94°C for 20 sec, 60°C for 20 sec and 72°C for 2 min. With a decrease of 2°C per cycle for the annealing temperature and afterward 25 cycles of 94°C for 20 sec, 52°C for 20 sec and 72°C for 2 min, Followed by extension step at 72°C for 5 min.

The reaction products were resolved by electrophoresis on 1.5% agarose gels in 1X TBE, stained with ethidium bromide and visualized with UV light of Gel Documentation system (Gel Doc., 2000) image analysis software (BioRad Laboratories, Inc., California, USA).

RESULTS AND DISCUSSION

Development of specific SCAR-markers from characteristic RAPD markers

RAPD markers were useful to characterize the genetic diversity and relationships of root knot nematodes (*Meloidogyne* spp.) (Randing *et al.,* 2002). However, RAPD primers were nonspecific and could amplify DNA fragments from any genome (Fegan *et al.*, 1998; Samac *et al.*, 1998; Verdier et al., 1998) and concerns exist about the reproducibility of RAPD/ PCR reaction. This is a reason why we had to convert RAPD markers characteristic *of M. incognita* and *M. javanica* into sequence characterized amplified region (SCAR) markers. The use of specific SCAR primer pairs in standard PCR reactions has been suggested for reliable amplification of characterized polymorphic RAPD sequences.

In the present work, the RAPD results (that obtained by El Ghor *et al.*, 2003, unpublished data) were used to investigate the genetic diversity in *Meloidogyne incognita* and *Meloidogyne javanica* populations. This RAPD analysis was also intended to identify molecular markers characteristic of these species, in order to develop PCR-based markers, which can be used to detect these Meloidogyne species in the field. Such tests are reported to be sensitive, reliable and fast, which explains why PCR-based techniques have become more and more widespread for detecting plant parasites (Henson and French, 1993; Trebaol *et al.,* 2001). In order to develop PCR-markers characteristic to each of *M. incognita* and *M. javanica* a RAPD marker shared by all *M. incognita* populations and not present in all *M. javanica* populations and a second RAPD marker shared in all *M. javanica* populations and not present in all *M. incognita* populations were identified.

From the analysis of RAPD amplification products (El Ghor *et al.,* 2003 unpublished), a fragment size of 1100 bp obtained with primer OPB-3 was found to be specific for *M. javanica* populations, while a fragment at size of 1000 bp with primer OPK-2 was specific for *M. incognita* populations (Fig.1).

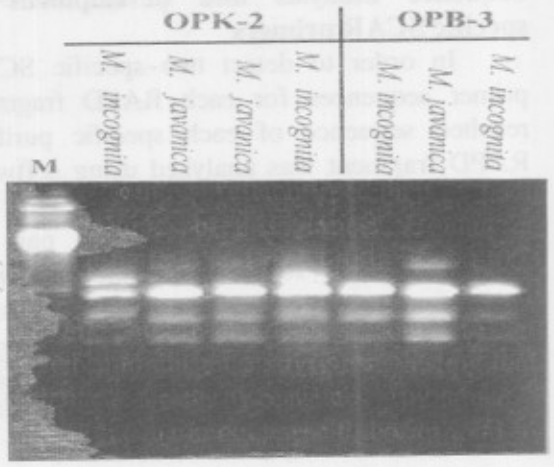

Fig. (1): Agarose gel of random amplified polymorphic DNA fragments generated from DNA of different populations of M. incognita and M. javanica originating from different geographic locations with random decamer primers OPK-2 and OPB-3. M is 100-bp marker.

Screening of positive colonies

On LB plates, many growing white and blue colonies were observed. The white colonies were positive and may have the right insert. So, 4 colonies of white bacterial colonies from each LB plates were screened using the original RAPD primers for detecting the white positive colonies that had the right insert. The screening products were checked on 1.5% agarose gel and the resulted band size in each reaction was compared with the related band size in RAPD reactions. As shown from PCR amplification products (Fig. 2), seven screening reactions give single clear band at size 1100 bp in lanes number 2, 3, 4 and 5 with primer OPB-3 and single clear band at size 1000 bp in lanes 6 and 7 with primer OPK-2. From these results, all picked white colonies had the right insert.

According to the screening results, the plasmids DNA of the right colonies were isolated using plasmid miniprep purification protocol system and the miniprep products were sequenced.

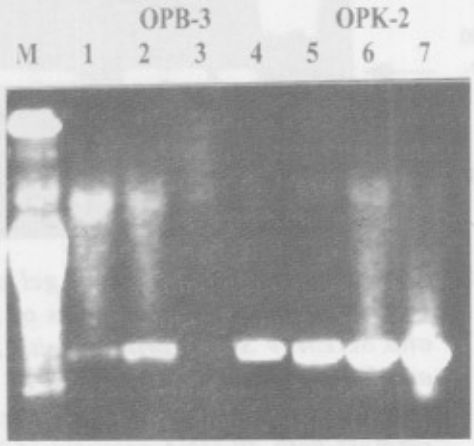

Fig. (2): Agarose gel of amplified screening products run on 1.5 % agarose gel after 30 min at 90 volt. Lanes number 1, 2 ,3 and 4 are screening products of plasmid DNA that amplified with OPB-3 primer and lanes number 5, 6 and 7 are screening products of plasmid DNA that amplified with OPK-2 primer. M is 100-bp marker.

Sequence analysis and development of specific SCAR primers

In order to detect two specific SCAR primer sequences for each RAPD fragment resulted sequence of each specific purified RAPD fragment was analyzed using software DNA-Star program.

The sequences of the developed pair of primers specific for *Meloidogyne incognita* populations *were:*

MIE-for:
5`-ATCCGTGCTGTAGCTTGCCC-3`
MIE-rev:
5`-CACCATCCGTTATAAGCTCTG-3`

The sequences of the developed pairs of primers specific for *Meloidogyne javanica* populations were:

MJE-for: 5`-GTCCGTTATCTGAGCTTAT-3`
MJE-rev:5`-AGTCACTCCATCACCTTCA-3`

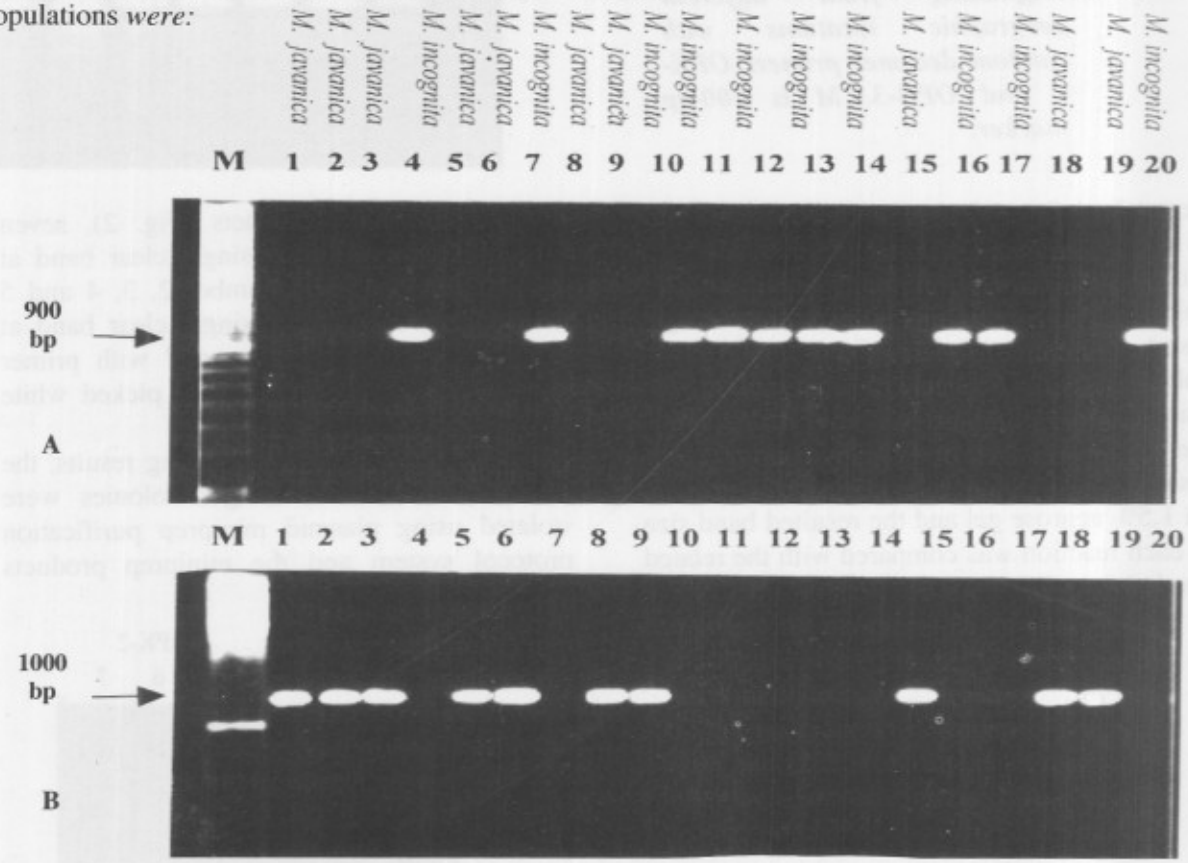

Fig. (3): Separation on 1.0% agarose gel of products from PCR amplification of certain DNA fragments with two pairs of SCAR primers (A) indicates results of MIE-for and MIE-rev SCAR primers with 20 DNA samples of 20 populations, which they amplifiede single band at size of 900 bp with DNAs of Meloidogyne incognita populations only, while (B) indicates results of MJE-for and MJE-rev SCAR primers with 20 DNA samples of 20 populations in which they amplified a single band at size of 1000 bp with DNAs of Meloidogyne javanica populations only. M is 100 bp marker.

PCR analysis of the 20 samples of genomic DNA, was carried out using each pair of the developed SCAR primers, MIE-for and MIE-rev yielded 900 bp products with all M. incognita populations, only (Fig. 3A), while SCAR primers MJE-for and MJE-rev yielded 1000 bp products with all *M. javanica* populations, only (Fig. 3 B). From a practical point of view, the ability to accurately detect polymorphisms between root-knot nematode populations is of outstanding importance for the design of effective integrated control of these parasites. Plant resistance is currently the most efficient and environmentally sound method of controlling root- knot nematodes, but the implementation of crop rotations including resistant cultivars requires specific and sub-specific identification of these pests.

REFERENCES

Carter, C.C. and Sasser, J.N. 1982. Research on integrated crop protection systems with emphasis on the root-knot nematodes (Meloidogyne species) affecting economic food crops: Developing nations. Raleigh: North Carolina State University Graphics.

Cenis, J.L. 1993. Identification of four major Meloidogyne spp. by random amplified polymorphic DNA (RAPD-PCR). Phytopathology 83: 76.

Fegan, M., Croft, B.J., Teakle, D.S., Hayward, A.C. and Smith, G.R. 1998. Sensitive and specific detection of *Clayibacter xyli* subsp. *xyli,* causal agent of ratoon stunting disease of sugarcane, with a polymerase chain reaction-based assay. Plant Pathol., 47: 495-504.

Henson, J.M. and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. Annu. Rev. Phytopathol., 31: 82-109.

Paran, I. and Michelmore, R.W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet., 85: 985-993.

Randig, O., Bongiovanni, M., Carneiro, R.M. and Castagnone-Sereno, P. 2002. Genetic diversity of root-knot nematodes from Brazil and development of SCAR markers specific for the Coffee-damaging species. Genome ,45 (5): 862-870.

Sasser, J.N. and Carter, C.C. 1985. Overview of the international Meloidogyne project 1975-84. In: An advanced treatise on Meloidogyne, Vol. I (eds. Sasser, J.N. and Carter, C.C.), PP. 19-24. NC State University Graphics, Raleigh.

Samac, D.A., Nix, R.J. and Oleson, A.E. 1998. Transmission frequency of *Clayibacter michiganensis* subsp. insidiosus to alfalfa seed and identification of the bacterium by PCR. Plant Dis., 82: 1362-1367.

Trebaol, G., Manceau, C., Tirilly, Y. and Boury, S. 2001. Assessment of the genetic diversity among strains of *Xanthomonas cynarae* by random amplified polymorphic DNA analysis and development of specific characterized amplified regions for the rapid identification of *X. cynarae*. Appl. and Environ. Micro., 67(8): 3379-3384.

Verdier, V., Mosquera, G. and Assigbetse, K. 1998. Detection of the cassava bacterial blight pathogen, *Xanthomonan axonopodis* pv. Manilotis, by polymerase chain reaction. Plant Dis., 82: 79-83.

Zijlstra, C., Donkers-Venne, D. and Fargette, M. 2000. Identification of *Meloidogyne incognita, M. javanica and M. arenaria* using sequence characterized amplified region (SCAR) based PCR assay. Nematology, 2(8): 847-853.

الملخص العربي

تقييم لدور SCAR كأحد التقنيات الحديثة التي تستخدم PCR للمقارنة بين نوعين من نيماتودا تعقد الجذور

*أكمل الغر، ** سناء هارون، ***مجدى عبدالرحيم ،***ايهاب عبداللا
*قسم علم الحيوان-كلية العلوم- جامعة القاهرة-مصر
**قسم وقاية النبات-كلية الزراعة- جامعة القاهرة-فرع الفيوم-مصر
***قسم علم الحيوان-كلية علوم-جامعة القاهرة-فرع بنىسويف-مصر

تعتبر نيماتودا تعقد الجذور من الآفات الهامة التي تصيب العديد من المحاصيل الهامة وخاصة محاصيل الخضر (الطماطم والبامية والباذنجان.....وغيرها). لذا فإن أعراض الإصابة بنيماتودا تعقد الجذور تظهر على هيئة تورمات على جذور النباتات المصابة وكذلك إصفرار الأوراق وضعف المجموع الخضري مما يؤدى إلى نقص فى إنتاجية المحصول وهذا يؤدى بدوره إلى أضرار مادية كبيرة. ومن ضمن أنواع النيماتودا شائعة الانتشار بمصر والتي تتبع جنس *M eloidogyne* ثلاث أنواع هي *M. javanica M. arenaria M. incognita*,. تتميز العزلات الجغرافية لنيماتودا تعقد الجذور فى هذه الأنواع لجنس Meloidogyne فى مصر بتنوعها الوراثي ويعتبر اكتشاف التنوع الوراثي الموجود داخل وفيما بين المجتمعات الجغرافية للنيماتودا من الأهمية فى فهم عملية تطور النيماتودا وكذلك فى إدارة عملية المكافحة الغير كيماوية.

بدراسة نتائج البصمات الوراثية RAPD-fingerprint المتحصل عليها من خلال نتائج مجموعة العمل القائمة بالبحث (وهى نتائج لم تنشر بعد) مع كل المجتمعات التي تم اختبارها تمكنا من تحديد زوج من قطع الدنا المميزة RAPD-markers إحدى هذه القطع مميزة للمجتمعات من نوع *M.incognita* وقد ظهرت عند تفاعل الدنا المستخلص من تلك المجتمعات مع OPK-2 primer عند المسافة ١٠٠٠ قاعدة نيتروجينية والقطعة الأخرى مميزة للمجتمعات من نوع *M. javanica* و ظهرت عند تفاعل الدنا مع OPB-3 primer عند المسافة ١١٠٠ قاعدة نيتروجينية . باستخدام تلك القطع المميزة و دراسة تركيبها عن طريق الحصول على ترتيب قواعدها النيتروحينية تمكنا من الحصول على نوع جديد من التقنيات التي تمكنا من تحديد التباين بين جزيئات الدنا لأفراد النوع الواحد .

تلك التقنية تسمى SCAR-marker وهذا النوع من تقنيات الPCR-markers هو الأدق لتحديد النوع عن APD markers وذلك لأننا بدراسة تتابع القواعد النيتروجينية لقطع الدنا السابق ذكرها سوف تمكنا من الحصول على زوج من البادئات لكل قطعة على حده و بتفاعل كل زوج من هذه البادئات مع الدنا بإستخدام جهاز التفاعل المتسلسل ثم تفريد الناتج على جهاز التفريد الكهربي حصلنا على نتائج جيدة بحيث زوج البادئات المميز للمجتمعات من نوع *M incognita* أعطت قطعة دنا واحده عند المسافة ٩٠٠ قاعدة نيتروجينية وفى حالة *M. javanica* البادئات المميزة لها أعطت قطعة دنا واحده عند المسافة ١٠٠٠ قاعدة نيتروجينية . ومن خلال تلك النتائج يتضح لنا أن تقنية الSCAR-markers لها أفضلية في تحديد النوع داخل الجنس الواحد و لكنها لا تستطيع إظهار التباين بين الأفراد داخل النوع الواحد .