RESEARCH NOTE – NOTA INVESITIGATIVA

IDENTIFICATION OF THE PEACH ROOT-KNOT NEMATODE, *MELOIDOGYNE FLORIDENSIS,* USING mtDNA PCR-RFLP

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ABSTRACT

Smith, T., J. A. Brito, H. Han, R. Kaur, R. Cetintas, and D. W. Dickson. 2015. Identification of the peach root-knot nematode, *Meloidogyne floridensis*, using mtDNA PCR-RFLP. Nematropica 45:138-143.

The peach root-knot nematode, *Meloidogyne floridensis*, is an emerging pathogen of peach (*Prunus persica*) and other crops currently known only to occur in Florida, USA. Molecular and morphological analyses are commonly used to differentiate *M. floridensis* from other root-knot nematodes species found in Florida, but it may be difficult to distinguish *M. floridensis* from *M. arenaria* without DNA sequencing because of the similarity of the DNA fragment size obtained with the commonly used DNA primer sets. An economical, reliable, and rapid method has been routinely used in our laboratory and allows these two nematode species to be differentiated without sequencing using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The mitochondrial (mtDNA) region between COII and 16S was amplified using the C2F3/1108 primer set and digested with *Hinf* I and *Ssp* I. Digestion of the PCR product with *Hinf* I yielded two unique fragments of approximately 770 bp and 370 bp for *M. floridensis*, which consistently allows for a fast and easy distinction between the two nematode species regardless of whether the sample is comprised of female or juvenile.

Key words: identification, *Meloidogyne arenaria*, *Meloidogyne floridensis*, mtDNA, PCR, mtDNA-RFLP.

RESUMEN

Smith, T., J. A. Brito , H. Han, R. Kaur, R. Cetintas, y D. W. Dickson. 2015. Identificación del nemátodo formador de agallas en las raíces del duraznero, *Meloidogyne floridensis*, mediante mtDNA PCR-RFLP. Nematropica 45:138-143.

El nemátodo formador de agallas en las raíces del duraznero, *Meloidogyne floridensis*, es un patógeno emergente del duraznero o melocotonero (*Prunus persica*) y otros cultivos, aunque actualmente solo se ha citado en Florida, USA. Análisis morfológicos y moleculares se usan comúnmente para diferenciar *M. floridensis* de otras especies de nemátodos formadores de agallas en las raíces encontradas en Florida, pero puede ser difícil distinguir *M. floridensis* de *M. arenaria* sin la secuenciación de ADN debido a la similitud en el tamaño de fragmento de ADN obtenido son los cebadores de ADN comúnmente usados. Un método fiable, rápido y económico se ha usado rutinariamente en nuestro laboratorio y permite distinguir estas dos especies de nemátodos sin tener que usar la secuenciación tras (PCR-RFLP). La región de ADN mitocondrial (mtDNA) entre COII y 16S se amplificó usando el cebador C2F3/1108 y fue digerida con *Hinf* I y *Ssp* I. La digestión del producto de la PCR con *Hinf* I produjo dos fragmentos únicos de aproximadamente 770 bp y 370 bp para *M. floridensis*, lo que consistentemente permitió una rápida y fácil diferenciación entre las dos especies de nemátodos, sin importar si la muestra estaba compuesta de hembras o juveniles.

Palabras clave: identificación, *Meloidogyne arenaria*, *Meloidogyne floridensis*, mtDNA, PCR, mtDNA-RFLP

Meloidogyne floridensis (Handoo *et al.*, 2004) is an important soilborne pathogen of peach (*Prunus persica* (L.) Stokes) in Florida. Although it was reported in the 1960s, this nematode was only described as a new species in 2004. Based on differential host tests, *M. floridensis* was initially misidentified as *M. incognita* race 3 (Sherman and Lyrene, 1983), but after more careful examinations that involved host range, morphology, and molecular analyses, this nematode was considered to be a new species, and named *Meloidogyne floridensis* (Handoo *et al.*, 2004) with the common name of peach root-knot nematode.

The importance of the *M. floridensis* became apparent when it was found infecting peach rootstocks 'Nemaguard' and 'Okinawa' (Sharpe *et al.*, 1969) and as well as 'Nemared' and 'Guardian' (Sherman *et al.*, 1991; Nyczepir *et al.*, 1998), all of which are resistant to both the southern (*Meloidogyne incognita*) (Kofoid & White, 1919) Chitwood, 1949 and Javanese (*Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 root-knot nematodes. Because of their susceptibility to *M. floridensis*, these popular rootstocks are no longer recommended as rootstocks for Florida stone fruits, including peaches, nectarines, and plums.

A new root-knot nematode resistant peach rootstock,'Flordaguard' was released in 1991 (Sherman *et al.*, 1991) and is currently the only commercial rootstock recommended by the University of Florida Extension service for planting in the state. The 'Flordaguard' rootstock is adapted to the warm environmental conditions of Florida and is reportedly resistant to *M. floridensis*. 'Flordaguard' is self-fertile, but pollen from other peach cultivars can fertilize flowers on this rootstock resulting in outcrossing. Outcrossing could result in a response on 'Flordaguard' that is not true-to-type (Olmstead *et al.*, 2007). This appears to be the case as a recent report (Brito and Stanley, 2011; Dickson, personal communication) indicates that *M. floridensis* was found infecting this rootstock. Further studies are needed to fully understand the factors leading to 'Flordaguard' infection by this nematode species.

Proper identification of *Meloidogyne* spp. is necessary for the implementation of effective nematode management and regulatory programs. It is also critical that plant breeders know the correct identification of target species in their breeding programs for the selection of resistant cultivars. The extensive morphological variations that are common among and within root-knot nematode species make their identification difficult (Hartman and Sasser, 1985; Jepson, 1987). Studying these characters requires specialized personnel and the use of high-quality microscopes, and the procedures that are involved are time consuming. Consequently, species identification by morphology alone may be inconclusive due to a high degree of overlap among required characters for species identification. Another approach used in species identification, esterase and malate dehydrogenase profiles via polyacrylamide gel electrophoresis, is limited because it is only useful if egg-laying females are available.

DNA sequencing of several different genes provides enough taxonomic and phylogenetic data to separate species of root-knot nematodes (Blok *et al.*, 1997; Wisehart *et al.*, 2002; Tigano *et al.*, 2005; Jeyaprakash *et al.*, 2006; Skantar *et al.*, 2008; McClure *et al.*, 2012). Sequencing can, however, be time consuming and expensive, therefore, a faster and more economical diagnostic procedure such as size polymorphism of a region instead of sequence polymorphism is more useful with a high volume of samples. The variable region of the mitochondrial (mtDNA) genome between cytochrome oxidase II and 16S when amplified with C2F3/1108 (Powers and Harris, 1993) provides enough length polymorphism to successfully distinguish several root-knot nematode species, including distinguishing *M. incognita* and *M. javanica* from *M. arenaria* (Neal, 1889) Chitwood, 1949 and *M. floridensis* in the first step (Powers and Harris, 1993; Stanton *et al.*, 1997; Blok *et al.*, 2002; Xu *et al.*, 2004; Powers *et al.*, 2005, Jeyaprakash *et al.*, 2006). Unfortunately, complications arise in distinguishing *M. floridensis* from *M. arenaria* using mtDNA length polymorphisms without sequencing because the fragment length of the amplicon produced (approximately 1.1 kb) is identical for both species (Jeyaprakash *et al.*, 2006). Restriction endonuclease digestion of the PCR product using different enzymes can result in unique restriction fragment length polymorphism (RFLP) patterns that can be used to successfully identify nematode species that PCR length polymorphism will not distinguish (Powers *et al.*, 1986; Powers and Harris, 1993; Powers *et al.*, 2005; McClure *et al.*, 2012; Jeyaprakash *et al.*, 2006; Zijlstra *et al.*, 1997). The focus of this work was to report a rapid and economical mtDNA-RFLP protocol able to distinguish *M. floridensis* from *M. arenaria* using either a single female or juvenile.

The origin, host plants, and designations of four isolates of *M. floridensis* used in this study were: isolate 1 (N05-227-17B) from tomato (*Solanum lycopersicum* L.), Seminole County, FL, isolate 2 (N04-627-5B) from cucumber (*Cucumis sativus* L.), Hendry County, FL, isolate 3 (N03-1582-3B) from tomato, Indian River County, FL, and isolate 4 (N03-1894) from peach, Alachua County, FL. Three isolates of *M. arenaria* were used; isolate 1 (N06-543-13B) from tomato, Brevard County, FL, isolate 2 (N13-1014-3B) from peanut (*Arachis hypogaea*

L.), Marian County, FL, and isolate 3 (EN0101) from peanut, Alachua County, FL. All isolates used in this study were collected as part of a cooperative *Meloidogyne* spp. survey carried out in the state of Florida by the Department of Agriculture Division of Plant Industry (FDACS- DPI) and the University of Florida, Department of Entomology and Nematology (Brito *et al.*, 2008). Isolates of both nematode species were well characterized and identified previously using morphological and isozyme analyses (esterase and malate hydrogenase) (Brito *et al.*, 2008, Stanley *et al.*, 2009). The *M. floridensis* isolate 4 is the topotype of this nematode species with published sequences for the mtDNA region between COII and 16S (GenBank Accession #DQ228697.1) as well as the whole genome (Lunt *et al.*, 2014). Nematode isolates of each species were reared on tomato cv. Agriset 334 in two separate greenhouses.

Nematode DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen®, Valencia, CA) with modifications to the preparation of the sample and the addition of an overnight proteinase K digestion to ensure that the tissue was completely lysed. Individual females were dissected by hand from infected roots and placed in a 1.5 mL tube containing 180 µL of Buffer ATL. Juveniles were prepared for DNA extraction using a method adapted from McClure *et al.*, 2012 as follows; a single juvenile was placed in a 10-µL drop of buffer ATL on a clean cover slip and sliced into multiple pieces using a scalpel sterilized with DNase Displace (Fisher Scientific, Pittsburgh, PA). The solution containing the sliced nematode was pipetted into a 1.5-mL tube containing 170 uL of Buffer ATL. This DNA extraction method results in 50 uL of DNA. Forward primer C2F3 (5'-GGTCAATGTTCAGAAATTTGTGG-3')and reverse primer 1108 (5'-TACCTTTGACCAATCACGCT-3') (Integrated DNA Technologies, Coralville, IA) was used to amplify the mtDNA region between COII and 16S (Powers and Harris, 1993). Each 50 µL PCR reaction included 10 µL of DNA template, 1X Phusion High Fidelity buffer, 20 µM dNTP, 3 mM MgCl2, 0.5 µM of each primer, and 0.5 U of Phusion High Fidelity polymerase (Thermo Fisher Scientific, Inc., Waltham, MA). DNA amplification was performed in a Applied Biosystems® GeneAmp® PCR System 9700 thermocycler (Thermo Fisher Scientific, Inc., Waltham, MA) as follows: initial denaturation for 1 min at 98°C, followed by 40 cycles of denaturation for 10 sec at 98°C, annealing for 30 sec at 57°C, extension for 2 min at 68°C, and finally an extension at 68°C for 2 more min. PCR products were digested with Thermo Scientific FastDigest enzymes *Hinf* I and *Ssp* I (Thermo Fisher Scientific, Inc., Waltham, MA) by combining 17µL of nuclease free water (HyClone Laboratories, Logan, UT), 2 µL 10X Fast Digest Green Buffer, 10 µL PCR product, and 1 µL Fast Digest Enzyme overnight at 37°C. All products were separated in 1.5% agarose gel for 75 min at 80 volts in 1X TBE buffer and stained with GelGreen (Biotium, Inc., Hayward, CA).

PCR amplification with the mtDNA primer set C2F3/1108 resulted in products with lengths of approximately 1.1 kb for both *M. floridensis* and

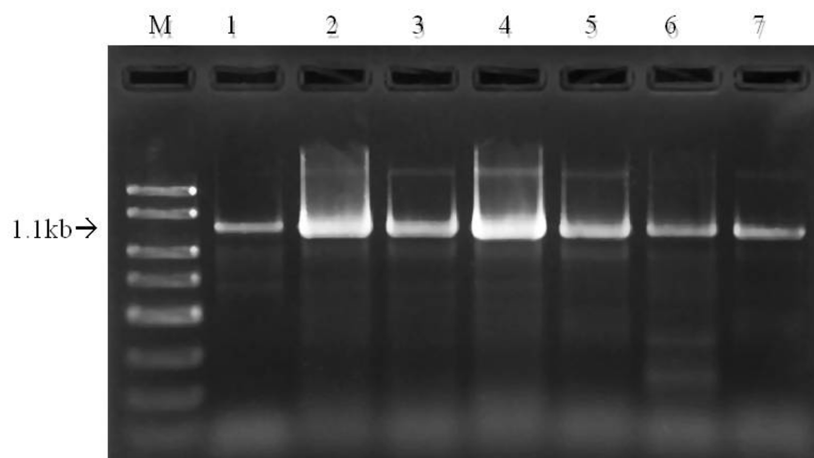

Fig. 1. Mitochondrial DNA product generated with primer set C2F3/1108 using a single juvenile (1) *Meloidogyne floridensis* isolate 1; (2) *M. floridensis* isolate 2; (3) *M. floridensis* isolate 3; (4) *M. floridensis* isolate 4; (5) *M. arenaria* isolate 1; (6) *M. arenaria* isolate 2; (7) *M. arenaria* isolate 3; (M) exACTGene low-range DNA ladder 2,000 bp.

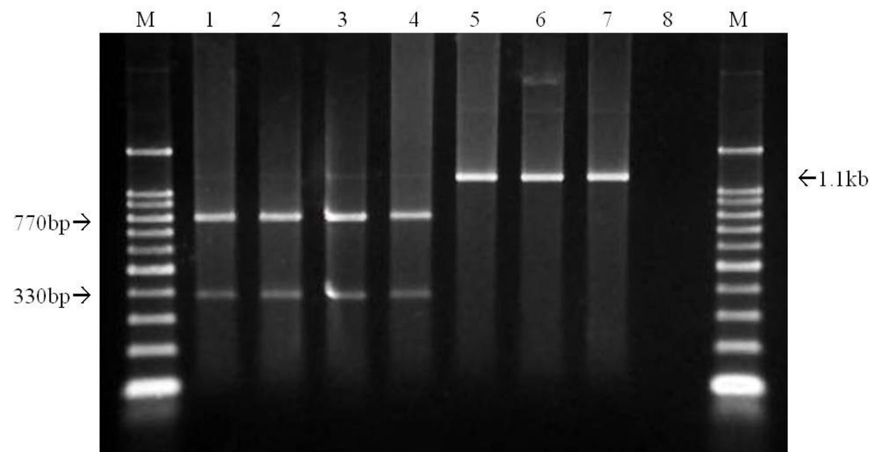

Fig. 2. Restriction enzyme profiles of the mitochondrial DNA products generated from single juveniles of each nematode species digested with *Hinf* I. (1) *Meloidogyne floridensis* isolate 1; (2) *M. floridensis* isolate 2; (3) *M. floridensis* isolate 3; (4) *M. floridensis* isolate 4; (5) *M. arenaria* isolate 1; (6) *M. arenaria* isolate 2; (7) *M. arenaria* isolate 3; (8) Negative control; and (M) GelPilot 100 bp plus ladder 1500 bp.

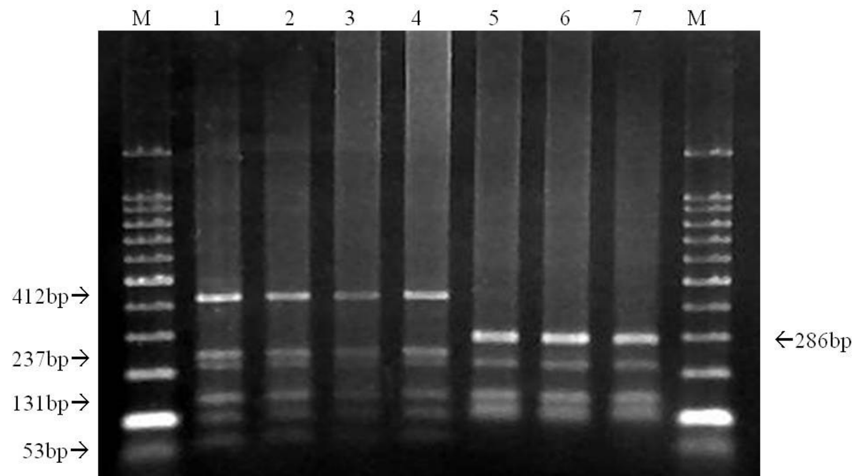

Fig. 3. Restriction enzyme profiles of the mitochondrial DNA products generated from single females of each nematode species digested with *Ssp* I. (1) *Meloidogyne floridensis* isolate 1; (2) *M. floridensis* isolate 2; (3) *M. floridensis* isolate 3; (4) *M. floridensis* isolate 4; (5) *M. arenaria* isolate 1; (6) *M. arenaria* isolate 2; (7) M. arenaria isolate 3; and (M) GelPilot 100 bp plus ladder 1500 bp.

M. arenaria juveniles (Fig. 1) and females (data not shown). Restriction maps were generated for *Hinf* I and *Ssp* I with a mtDNA sequence previously published from an isolate from our laboratory currently available in GenBank for *M. floridensis* (GenBank Accession #DQ228697.1) and a published *M. arenaria* sequence (GenBank Accession #AY635610). The published sequences were approximately 1.1 kb in length and predicted *Hinf* I to produce fragments with lengths 771 bp and

370 bp for *M. floridensis* and no restriction site for *M. arenaria*. Digestion of our PCR product with *Hinf* I produced fragments of approximately 770 bp and 370 bp for both *M. floridensis* juveniles (Fig. 2) and females (data not shown). *Hinf* I digestion of *M. arenaria* resulted in a fragment with length of 1.1 kb, indicative of the absence of a restriction site on the sequence for the enzyme. The enzyme map produced for *Ssp* I predicted fragments of 412, 237, 211, 131, 97, and 53 bp for *M. floridensis* and 286, 276, 212, 131, 110, and 97 bp for *M. arenaria*. Similar fragments were obtained in all of the digests of both females (Fig. 3) and juveniles (data not shown) with *Ssp* I, which confirmed this prediction.

The restriction enzyme *Hinf* I has high diagnostic value for distinguishing *M. floridensis* from *M. arenaria* because it lacks a restriction site on *M. arenaria* and results consistently in two fragments for *M. floridensis*. Digestion with *Ssp* I produces a unique fragment for *M. floridensis* of 412 bp; nevertheless, the other fragments are very similar in size and are not visually distinct enough to be useful for diagnostic purposes. Our results for digestion with the *Ssp* I enzyme were congruent with the result previously published by Jeyaprakash *et al.* (2006). RFLP with *Hinf* I is useful for both females and juveniles, whereas *Ssp* I digest performed on juveniles lacks resolution. Attempts were made to improve this protocol, but none were successful.

For the past three years, mtDNA-RFLP with the PCR primer set C2F3/1108 and *Hinf* I digestion enzyme has been routinely employed in our Nematode Diagnostic Laboratory to distinguish different populations of these two root-knot nematode species from several crops in different counties throughout Florida. It has proved to be an inexpensive, repeatable, and reliable diagnostic test that can distinguish these two nematode species using either a single egg-laying female or a single second-stage juvenile (J2).

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