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A Root-Knot Nematode, *Meloidogyne arenaria* on Apple nurseries from Tunisia

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Abstract – Root-knot nematodes (RKN: *Meloidogyne* spp.) have a wide host range, what implies that multiple crops may be affected among them apple trees. Between 2013 and 2014, a nematological survey was carried out in apple trees from nurseries in Tunisia. Root galling caused by root-knot nematodes was observed. Infected tissues were examined and RKN species were identifiedThe morphological study based on perineal patterns of the females confirmed the presence of M. arenaria. In addition, the molecular identification was carried out and based on the internal transcribed spacer 1 (ITS1-5.8S-ITS2) gene. The sequences were compared with those of *Meloidogyne arenaria* in the GenBank database with high similarity (95%). This comparison confirmed the species identification and reconfirmed the morphological identification. Phylogenetic studies placed those populations with *M. arenaria*. To the best of our knowledge, this is the first report of this nematode associated with apple plant in Tunisia.

Keywords: ITS1, morphology, molecular identification, Phylogeny, Apple, *Meloidogyne arenaria*

1. Introduction

Apple is an important crop widely produced throughout the world including Tunisia (Goulão and Oliveira, 2001). Apple trees are affected by several diseases and pathogens among them plant parasitic nematodes, which are considered a serious problem of this crop (Utkhede et *al*., 1992; Karanastasi et *al*., 2006).

Root-knot nematodes (*Meloidogyne* spp.), belong to the genus *Meloidogyne Göldi* 1887 and are obligate plant parasites that feed on roots and are able to parasitize almost every species of vascular plants (Jones et al., 2013). Root-knot nematodes affect a wide range of host plants, and represent a worldwide concern for pome, stone and nut fruit growers (Askary et *al*., 2012). Root-knot nematodes are obligate plant parasites that feed on roots and are able to parasitize almost every species of hosts (Jones et *al*., 2013). Yield losses depend on the nematode species, population level, and crop species and may range from 10% (Collange et *al*., 2011) to 50%–80% (Stirling, 1991; Siddiqi, 2000).

Perineal pattern morphology of adult females is most frequently used for the identification of root-knot nematodes (Jepson, 1987; Hunt and Handoo, 2009). This method is often inconclusive for distinguishing closely related *Meloidogyne* species, because individuals within a population often vary considerably (Zijlstra et *al*., 2000).

PCR methods based on DNA have also been widely used for the identification of nematodes (Zijlstra et al., 2004; Powers et al., 2005; Adam et al., 2007; Devran and Söğüt, 2009). *M.incognita, M. arenaria* and *M. javanica* are regarded as the most common species reported in the Mediterranean region (La Massése et *al*., 1984).

The aim of this study was to characterize morphologically and compare with previous records the root knot nematode populations causing galls formation on apple roots in Tunisian nurseries and to molecularly identify these populations using ITS 1 gene sequences.

2. Materials and methods

2.1. Nematodes collection and extraction

Nematode surveys were conducted during 2013/2014 in four different nurseries located in central Tunisia (Kairouan (1 nursery), Zaghouan (3 nurseries)) Samples were collected with a shovel from the upper 50 cm of soil and roots from four orientations of the plant. Nematodes were extracted from 1g of infected roots (Figure 1) by a modified sugar centrifugal-flotation method (De Grisse, 1969). Population

of root nematodes was evaluated under an optical microscope. Nematode cultures were then established from single egg mass whose adult females had previously been identified by observation of the morphological characteristics of their perineal patterns (Taylor and Sasser, 1978) and reared on tomato plants cv. Riogrande in a glasshouse at 28±1ºC. All populations obtained from single egg masses were maintained continuously in plastic pots at sterilized mixture of peat and sand (2:1). After two months, nematodes extracted from roots and used for further morphological and molecular analysis.

2.2. Perineal pattern confection

Female nematodes obtained from the first individuals of single egg mass cultures were used for perineal pattern studies. Indeed, the females were recovered from infected tomato roots. Ten individual females from each population were selected randomly and perineal patterns were cut in 45% lactic acid and mounted in glycerin (Taylor and Netscher, 1974; Hartman and Sasser, 1985). Perineal pattern studies were conducted under a light microscope according to Eisenback et *al.* (1981) and Jepson (1987).

2.3. Molecular identification

DNA was extracted from the juvenile J2. Nematodes specimens were each transferred to an Eppendorf tube containing 30 µL 10×PCR buffer (100 mMTris-HCl, pH 9.0 at 25 °C, 500 mMKCl, 15 mM MgCl2), 10 µL Proteinase K (1 mg/mL), 50 µL distilled water. Specimens were crushed during 3 min with an ultrasonic homogenizer. The tubes were incubated at 68°C for 2 h, then at 100 °C for 15 min and stored at -20 °C.

The internal transcribed spacer 1 (ITS1) -5.8S gene was amplified using the forward primer TW81 (5′ GTTTCCGTAGGTGAACCTGC-3′) and reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) as described in Subbotin et *al*. (2001) are used for purified, quantified and used for direct sequencing. The amplification condition was: 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 45 s at 60 °C and 2 min at 72 °C, with final extension of 10 min at 72 °C. All PCR reactions were performed in 25 µl volumes including 3µl DNA, 2.5 µl 10×PCR buffer, 1.25 µl of 2.5 mM dNTPs, 0.4µl from each primers and 0.25 µl Titanium Taq.

The PCR products were separated by electrophoresis (110V, 45min) in 2.0% agarose gels in TAE buffer with 2.5 µl DNA Ladder gels are stained with Ethidium bromide, visualized and photographed under UV-light (Bio-rad DX, USA). All reactions were repeated twice for clear and stable banding patterns. The presence or absence of DNA fragments was scored as one or zero, respectively, in the binary matrix. Simple matching coefficients (SM) (Digby & Kempton, 1987).

2.4. Sequence alignment and phylogenetic analysis

The 18S fragments are sequenced with an ABI Prism 377 sequencer (Perkin Elmer) in both directions and unambiguous consensus sequences obtained. The sequences are deposited into the GenBank database. DNA sequences were aligned by Clustal W (http://workbench.sdsc.edu, Bioinformatics and Computational Biology group, Dept. Bioengineering, UC San Diego, CA). The sequences were compared with those of the other *Meloidogyne* species available at the GenBank sequence database using the BLAST homology search program. The *Meloidogyne* sequences are aligned using CLUSTALW implemented in the MEGA package (Kumar *et al*., 2008). Clade reliability is examined to through a nonparametric bootstrap with 1000 replicated samples. The phylogenetic tree was constructed by neighbor joining method with MEGA package v.7 (Kumar et *al*., 2016).

3. Results and Discussion

3.1. Perineal patterns and molecular study

Roots of apple plants showed galls and egg masses of *Meloidogyne* (Figure 1). Perineal pattern variability is observed within and between populations isolated from the four apple nurseries showing galled roots (Figure 2).

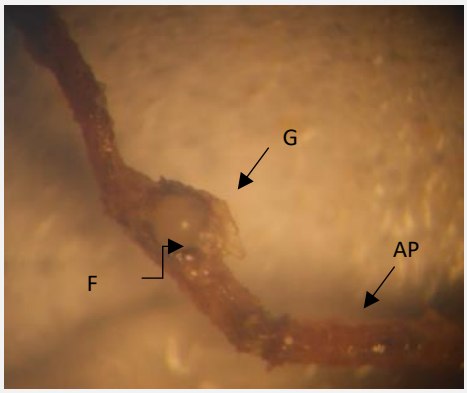

Figure1. Root of apple plant disfigured with galls containing female of *Meloidogyne* (F: female, G: galls, AP: apple root)

females perineal patterns from each *Meloidogyne* population were grouped into one root-knot species *M. arenaria* according to similar morphology by a main diagnostic feature for species identification. These patterns showed a generally low, round to indented dorsal arch near the lateral field with irregular forks in the lateral field, fine smooth striae (Figure 2). root knot nematode associated with apple tree had been reported in numerous studies but Itoh et *al*. (1969) were the first researchers who reported the presence of two species of *Meloidogyne* (*M. mali* and *M. ulmi*) on apple roots in Japan.

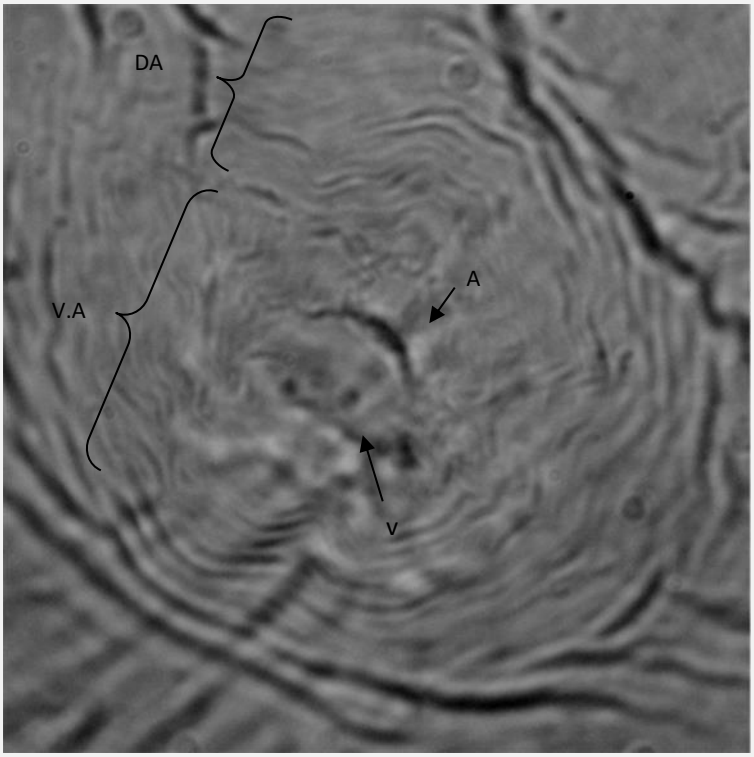

Figure 2. Female perineal pattern (bar: 100 x) (A: anus, V: vulve, D.A: dorsal arch, V.A: ventral arch)

In Pakistan, Khan et *al*. (2010) have proven the presence of *M. incognita* associated with apple roots (*Malus pumila* Mill.). Similarly, Ranjan (2005) reported the presence of *M. incognita* in apple rootstocks in India. Mokbel et *al*. (2006) suggested that this specie (*M. incognita*) are found at high frequency associated with apple trees. Juveniles of the *M. hapla* root-knot nematode have been found in the soil of apple orchards in Slovakia (Liskova, 2007).

The morphological and molecular identification have confirmed the presence of *M. arenaria* associated with apple roots. The nuclear ribosomal DNA genes (internal transcribed spacer 1 or ITS1) of *M.*

arenaria from Tunisia (KJ572384.1) were 95% similarity with those of *Meloidogyne arenaria* in the GenBank database. The phylogenetic tree based on ITS1 and obtained with the partial 18S confirmed a similar phylogenetic relationship with *M. arenaria* (Figure 3). In addition, the phylogenetic analysis showed the strong relationship of our *M. arenaria* with *M. incognita* (Figure3).

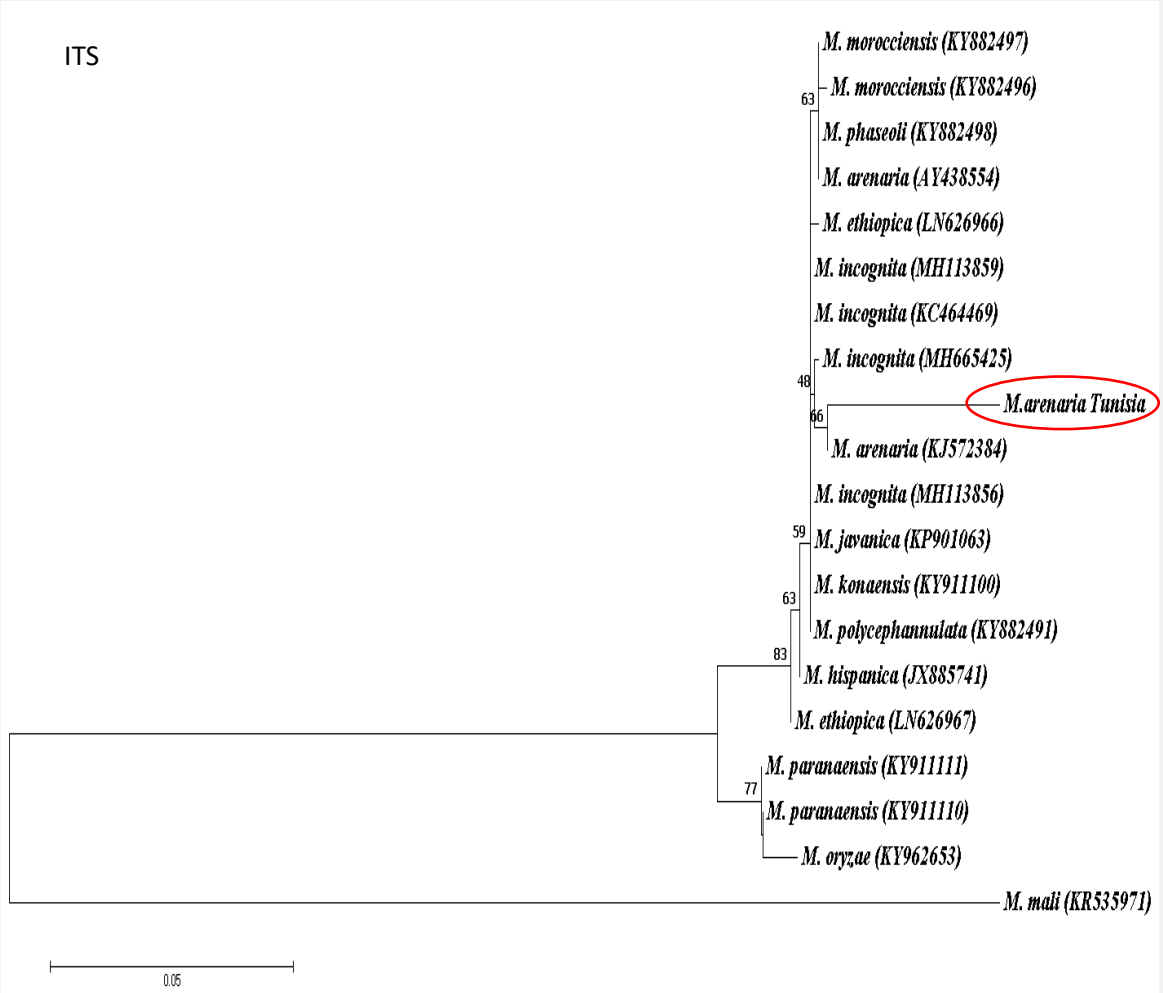

Figure 3. Phylogenetic relationships between *Meloidogyne arenaria* identified in Tunisia and other *Meloidogyne* species already deposited in GenBank. The *Meloidogyne* sequences aligned in MEGA with CLUSTALW and the plylogenetic tree made by MEGA version 7.0 based on the Neighbor-Joining method as inferred from partial 18S rRNA gene.

3.2. Populations Distribution

The perineal patterns and DNA analyses were evaluated mutually to confirm the identification of *Meloidogyne arenaria* present in four surveyed nurseries of apple trees.

Results indicated that only *M. arenaria* was found in 2 surveyed localities with different roots abundance: 3.25 % in Zaghouan region and 2.64% in Kairouan region. These results could be explained by the soil texture which is sandy- loamy in the Kairouan region whereas the region of zaghouan is characterized by clay-loam soil. According to Karssen et *al*. (2013), *Meloidogyne* spp. occurred on a wide range of soil types, but their association with crop damage is more readily observed in sandy and sandy-loamy soils.

4. Conclusion

The root knot nematode *M. arenaria* is commonly found on fruit tree roots especially peach and almond, whereas, this is the first report of this nematode associated with apple roots in Tunisia. This knowledge is important to understand the role of *M. arenaria* as stress factor on apple tree and for of the assessment of future control strategies.

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