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First report of *Pratylenchus vulnus* associated with apple in Tunisia

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Abstract

The root-lesion nematode of the genus *Pratylenchus* Filipjev (1936) has a worldwide distribution and cause severe production constraints on numerous important crops. In 2013-14, during a survey of the apple nurseries and orchards in center of Tunisia (Kairouan, Zaghouan, Monastir and Kasserine), 70 different roots and soil samples were collected. The populations of root-lesion nematode were identified on the basis of their morphological and morphometric characters, and by molecular methods. Microscopic observation of females and males demonstrated the occurrence of *Pratylenchusd vulnus* on apple trees. The ribosomal DNA D2-D3 expansion segments of the 28S rRNA and of the *Pratylenchus* populations were PCR amplified and sequenced. The sequences were compared with those of *Pratylenchus* species in the GenBank database with high similarity (99%). This comparison reconfirmed the morphological identifications. Phylogenetic studies placed those populations with *P. vulnus*. This is the first report of *P. vulnus* infecting apple in Tunisia.

Key words

Pratylenchus, Morphometry, Molecular identification, Apple, Tunisia.

Along with dates and citrus, which represent the main fruit tree commodities produced and exported in Tunisia, apples have an important place in the fruit sector. Pome and stone fruit trees can be infected by plant parasitic nematodes which are a serious problem influencing the growth and production of trees in all major fruit producing area (Askary et al., 2012). The root-lesion nematodes, *Pratylenchus* genus, are common endoparasites of plants worldwide. These nematodes cause severe production constraints and have great economic impact on numerous important crops (Castillo and Vovlas, 2007). They are migratory parasites, cause local lesions, and affect root growth by penetrating and feeding on young roots of host plant (Pinochet et al., 1996).

Within the genus *Pratylenchus*, 12 species have been reported as potential pathogens on apple (Castillo and Vovlas, 2007). Moreover, root-lesion nematodes, particularly *Pratylenchus vulnus*, is a major pest attacking pome and stone fruit crops in warm Mediterranean environments (Mckenry, 1989; Pinochet et al., 1992; Nyczepir and Becker, 1998; Askary et al., 2012).

The purpose of this study is to identity morphologically and by molecular tools the population of plant parasitic nematode (*Pratylenchus* spp.) in apple nurseries and orchards in Tunisia.

Materials and methods

Nematodes collection and extraction

Nematode surveys were conducted during 2013-14 in three different sites (stoolbeds, nurseries, and orchards) located in central Tunisia (Kairouan, Zaghouan, Kasserine, Jendouba, and Monastir). Samples were collected with a shovel from the upper 50 cm of soil and roots of four to five holes. Nematodes were extracted from 500 g of soil and from 1 g of roots by a modified sugar centrifugal-flotation method (De Grisse, 1969). Single females of root-lesion nematodes were transferred to carrot discs at 25°C

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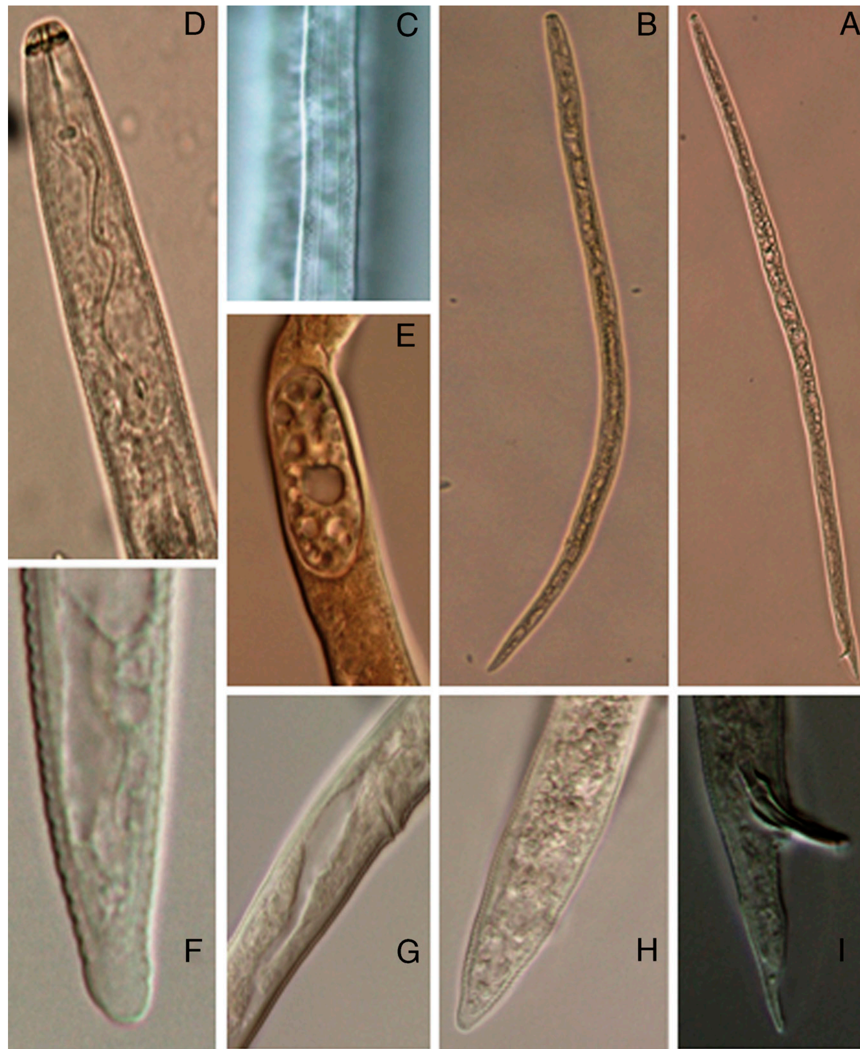

Figure 1: Photo micrographs of *Pratylenchus vulnus* specimens: females (B) and males (A), (C) lateral field at mid – body; (D) spermatheca; (E) pharyngeal region; (F and H) female tails; (G) vulval region; (I) male tail (Scale bars: A, B = 100 mm; C–I = 20 mm).

(Castillo et al., 1995). After incubation of carrot cultures for up 8 wk, the carrot discs were cut into pieces in petri dishes containing sterile water. Then, the petri plates were incubated for 24 hr and collected by sterile Pasteur pipettes. Finally, purified nematodes extracted from carrot discs were used for further morphological and molecular analysis.

Morphological identification

Nematodes were preliminarily identified by morphological features. For that, five females and five males of each population of *Pratylenchus* were killed by gentle heat, fixed in buffered formalin (Seinhorst, 1959), and processed to pure glycerin. Specimens were examined using a Nikon Eclipse 80i compound microscope with Normarski differential interference contrast at powers up to 1,000× magnification. Measurements were made on glycerin infiltrated specimens with Nikon Digital Sight DS-FI2 K10410 camera and expressed in µm. The used measurement's abbreviations were defined as Siddiqi (2000). Camera observations were performed as defined by De Man (1880). Nematodes were identified to the species level using Castillo and Vovlas (2007) diagnostic keys.

The scanning electron microscopy (SEM) of *Pratylenchus* males and females were assessed as described by Abolafia (2015).

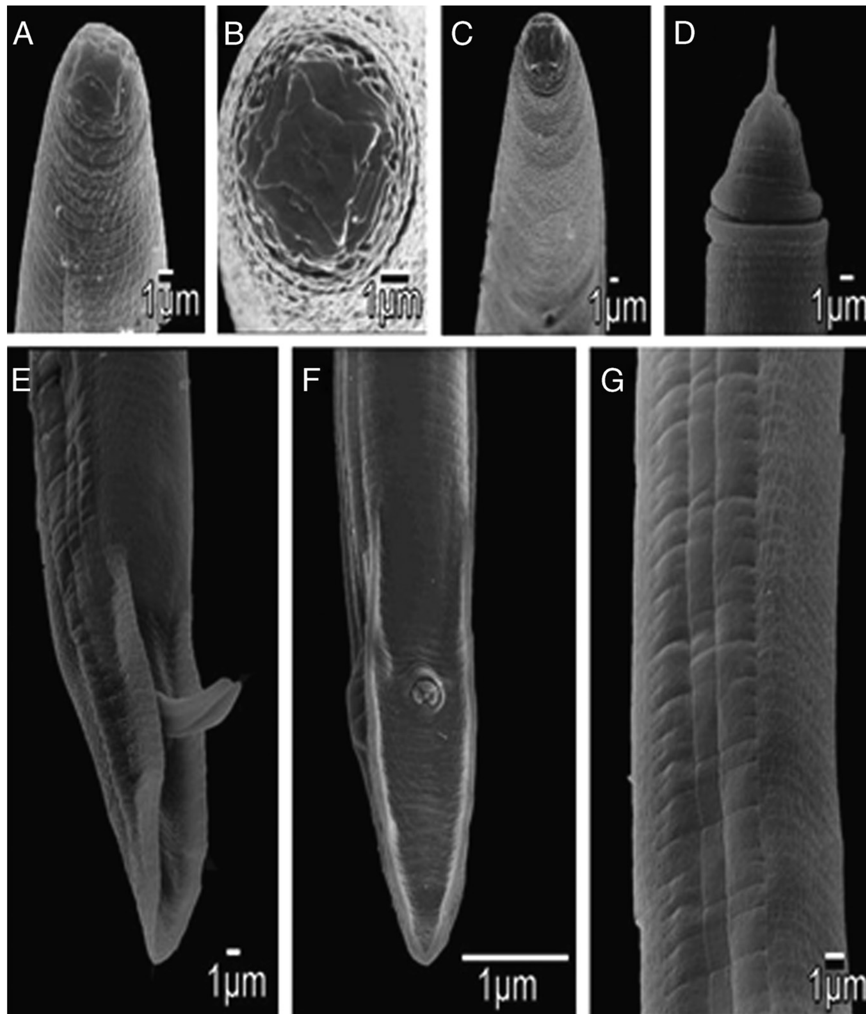

Figure 2: SEM micrograph of P. vulnus male morphology from Tunisia (A,B,C,D) anterior region, (E,F) tail, (G) lateral fields; sp: spicules, lig: 4 lateral lines; anx: 3 labial annuli).

Molecular identification

DNA extraction

DNA was extracted from several specimens from each sample. Each nematodes specimen was transferred to an Eppendorf tube containing 30 µL 10× PCR buffer (100 mMTris-HCl, pH 9.0 at 25°C, 500 mMKCl, 15 mM $MgCl_2$), 10 µL Proteinase K (1 mg/mL), 50 µL distilled water. Specimens were crushed for 3 min with an ultrasonic homogenizer. The tubes were incubated at 68°C for 2 hr, then at 100°C for 15 min and stored at –20°C.

DNA amplification and cloning

The forward 18S-F (5′-TTGGATAACTGTGGTTTA ACTAG-3′) and the reverse 18S-R (5′-ATTTCACCTCT CACGCAACA-3′) primers and the forward D2a (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and the reverse D3b (5′-TCGGAAGGAACCAGCTACTA-3′) were used for amplification and sequencing of the partial 18S r RNA genes and the D2-D3 expansion segments of the 28S r RNA, respectively. The amplification condition were: 95°C for 3 min, followed by 40 cycles of 30 sec at 95°C, 45 sec 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 3 µl DNA, 2.5 µl 10× PCR buffer, 1.25 µl of 2.5 mM dNTPs, 0.4 µl from each primers and 0.25 µl Titanium Taq.

The PCR products were separated by electrophoresis (110 V, 45 min) in 2.0% agarose gels in TAE buffer with 2.5 µl DNA Ladder. The gels were stained with Ethidium bromide, visualized, and photographed

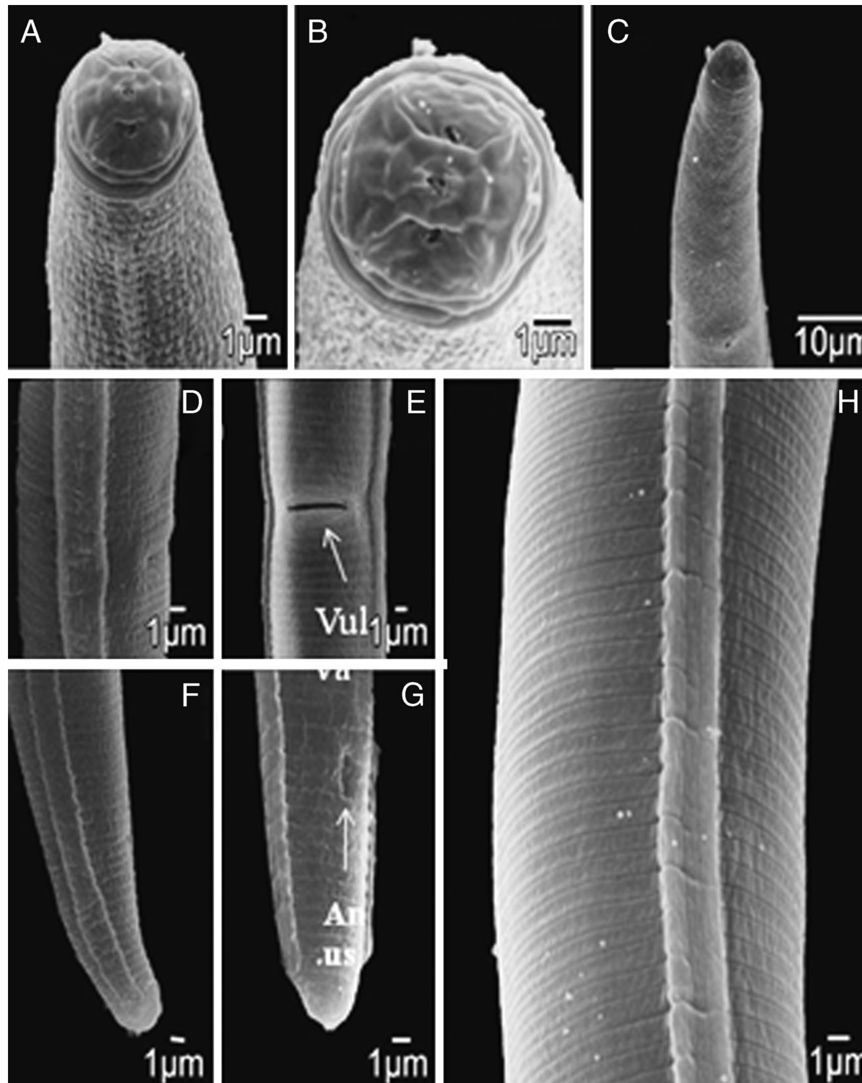

Figure 3: SEM micrograph of *P. vulnus* female morphology from Tunisia (A,B,C) anterior region, (D) lateral lines at vulva region, (E) vulva, (F,G) tail, (H) lateral fields).

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 1 or 0, respectively, in the binary matrix. Simple matching coefficients (SM) was performed with NTSYS2.1 (Exeter Software, Setauket, NY). (Digby and Kempton, 1987).

Sequence alignment and phylogenetic analysis

The 18S and 28S fragments were sequenced in-house with an ABI Prism 377 sequencer (Perkin Elmer) in both directions and unambiguous consensus sequences obtained. The sequences were deposited into the GenBank database. DNA sequences were aligned by Clustal W (http://workbench.sdsc.edu, Bioinformatics and Computational Biology group, Department of Bioengineering, UC San Diego, CA). The sequences were compared with those of the other *Pratylenchus* species available at the GenBank sequence database using the BLAST homology search program. The model of base substitution was evaluated using MODELTEST (Posada and Crandall, 1998). The *Pratylenchus* sequences were aligned using CLUSTALW implemented in the MEGA package (Kumar et al., 2008). Clade reliability was examined though a nonparametric bootstrap with 1,000 replicated samples. The phylogenetic tree was constructed by neighbor-joining method with MEGA package v.7 (Kumar et al., 2016).

Table 1. Morphometrics of *Pratylenchus vulnus* from apples stoolbeds, nurseries (males and females), and orchards from four Tunisian regions (Kasserin, Kairouan, Monastir, and Zaghouan, Tunisia).

Stoolbed			
Locality	MM106 Chiha		
Characters	Females	Males	
n	5	5	
L	444.4 ± 29.36 (415.71–485.86)	419.83 ± 32.43 (395.01–475.2)	
Stylet length (µm)	14.29± 0.16 (14.17–14.55)	14.85 ± 0.21 (14.56–15.1)	
Spicules (µm)		16.88± 0.26 (16.58–17.2)	
Gubernaculum (µm)		4.8± 0.39 (4.2–5.25)	
V or T	81.22± 0.73 (80.55–82.39)	34.02± 1.17 (32.11–35.2)	
a	26.73 ± 1.32 (24.59–28.1)	28.99 ± 2.76 (25.2–31.4)	
b	7.62 ± 0.55 (6.87– 8.16)	6.72 ± 0.66 (5.88–7.5)	
b′	4.92 ± 0.9 (3.76–5.66)	4.85 ± 0.27 (4.55–5.22)	
c	19.11 ± 0.44 (18.57–19.5)	20.75 ± 0.55 (19.88–21.3)	
c′	2.29 ± 0.22 (2.01–2.58)	2.14 ± 0.1 (2.01–2.28)	
Nursery females			
Locality	Lorca Chiha	Meski SMCCSPS	
Characters	Females	Females	
n	5	5	
L	556.71 ± 33.21 (521.32–607.49)	512.48 ± 51.46 (450.11–578.12)	
Stylet length (µm)	15. 45± 0.31 (15.17–15.93)	14. 44± 0.6 (13.69–15.19)	
Spicules (µm)			
Gubernaculum (µm)			
V or T	78.77± 1.31 (76.95–80.55)	79.14 ± 1.74 (76.58–81.3)	
a	28.8 ± 4.12 (25.93–35.73)	28.88 ± 3.72 (25.72–35.33)	
b	7.82 ± 1.33 (6.1–9.56)	7.97 ± 0.84 (7.12–9.18)	
b′	5.26 ± 0.11 (5.16–5.41)	4.27 ± 0.27 (3.88–4.55)	
c	20.22 ± 1.91 (18.17–22.7)	21.79 ± 2.81 (18.68–25.5)	
c′	2.44 ± 0.27 (2.08–2.78)	2.33 ± 0.33 (2.04–2.91)	
Nursery males			
Locality	Lorca Chiha	Anna Manzel Nour	Meski SMCCSPS

Characters	Males	Males	Males
n	5	5	5
L	483.75 ± 10.58 (468.63–492.7)	478.62 ± 30.09 (431.65–503.93)	485.52 ± 34.38(451.12–540.76)
Stylet length (µm)	14.81 ± 0.49 (14.41–15.66)	14.46 ± 0.75 (13.49–15.26)	14.31 ± 0.28 (14.01–14.66)
Spicules (µm)	17.07± 1.35 (15.47–19.14)	19.16± 0.61 (18.25–19.65)	18.15 ± 0.58 (17.37–19)
Gubernaculum (µm)	4.92± 0.34 (4.46–5.31)	5.26± 0.27 (4.95–5.66)	5.07 ± 0.43 (4.56–5.72)
V or T	35.38± 2.75 (32–38.17)	37.56± 2.55 (33.72–40.6)	36.42 ± 2.76 (34.05–40.54)
a	28.85 ± 1.45 (26.5–30.3)	29.37 ± 2.33 (25.87–32.15)	27.01 ± 1.37 (25.36–28.91)
b	8.24 ± 1.21 (7.23–10.22)	9.11 ± 0.38 (8.66–9.67)	9.2 ± 1.01 (8.14–10.5)
b′	5.31 ± 0.1 (5.2–5.43)	4.29 ± 0.45 (3.77–4.95)	4.85 ± 0.53 (4.47–5.75)
c	19.89 ± 1.48 (17.86–21.62)	20.95 ± 1.46 (18.7–22.47)	20.16 ± 1.83 (18.85–23.37)
c′	2.29 ± 0.18 (2.09–2.51)	2.06 ± 0.08 (2–2.21)	2.26 ± 0.21 (1.92–2.45)
Orchads			
Locality	Lorca Chiha	Anna Manzel Nour	Meski SMCCSPS
Characters	Males	Males	Males
n	5	5	5
L	483.75 ± 10.58 (468.63–492.7)	478.62 ± 30.09 (431.65–503.93)	485.52 ± 34.38(451.12–540.76)
Stylet length (µm)	14.81 ± 0.49 (14.41–15.66)	14.46 ± 0.75 (13.49–15.26)	14.31 ± 0.28 (14.01–14.66)
Spicules (µm)	17.07± 1.35 (15.47–19.14)	19.16± 0.61 (18.25–19.65)	18.15 ± 0.58 (17.37–19)
Gubernaculum (µm)	4.92± 0.34 (4.46–5.31)	5.26± 0.27 (4.95–5.66)	5.07 ± 0.43 (4.56–5.72)
V or T	35.38± 2.75 (32–38.17)	37.56± 2.55 (33.72–40.6)	36.42 ± 2.76 (34.05–40.54)
a	28.85 ± 1.45 (26.5–30.3)	29.37 ± 2.33 (25.87–32.15)	27.01 ± 1.37 (25.36–28.91)
b	8.24 ± 1.21 (7.23–10.22)	9.11 ± 0.38 (8.66–9.67)	9.2 ± 1.01 (8.14–10.5)
b′	5.31 ± 0.1 (5.2–5.43)	4.29 ± 0.45 (3.77–4.95)	4.85 ± 0.53 (4.47–5.75)
c	19.89 ± 1.48 (17.86–21.62)	20.95 ± 1.46 (18.7–22.47)	20.16 ± 1.83 (18.85–23.37)
c′	2.29 ± 0.18 (2.09–2.51)	2.06 ± 0.08 (2–2.21)	2.26 ± 0.21 (1.92–2.45)

All measurements in µm and in the format: mean±SD (range).
Abbreviations are defined in Siddiqi (2000).

Results and discussion

Morphological identification showed the presence of *P. vulnus* in root and soil samples of apple trees located in the four regions visited and their abundance compared to other plant parasitic nematode varied between 58.24% in orchard in the region of Kasserine and 91.45% in nursery located in the region of Kairouan. All the qualitative and quantitative characters including number of lip annuli, spermatheca (presence

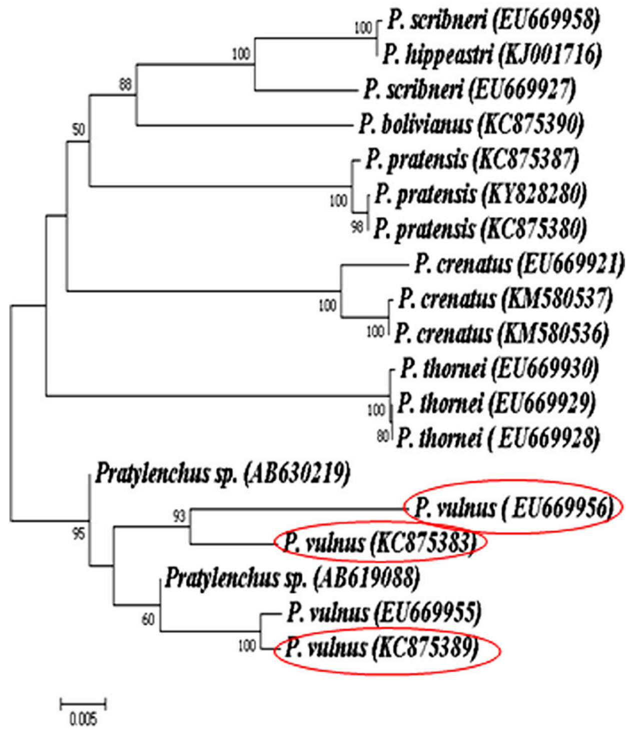

Figure 4: Phylogenetic relationships between *Pratylenchus vulnus* identified in Tunisia and other *Pratylenchus* already deposited in GenBank. The *Pratylenchus* sequences aligned in MEGA with CLUSTALW and the phylogenetic tree made by MEGA version 7.0 based on the Neighbor-Joining method as inferred from partial 18S rRNA gene.

and shape), post-vulval uterine sac, and tail shape of Tunisian populations of *P. vulnus* agreed with the original descriptions (Figs 1–3; Table 1). However, a few characters fell outside the range of the original descriptions. The measurements reported in the original description of Gao et al. (1999): 0.50 to 0.81 mm for female and 0.43 to 0.61 mm for male were similar to ours *Pratylenchus* specimens.

The qualitative morphological characteristics of the *Pratylenchus* populations used in this study followed the original species descriptions, but some morphometric discrepancies were found (Figs 2,3). Such morphometric variations in *Pratylenchus* confirmed the presence of different morphotypes used in this study. This intraspecific variability could be due different reproductive strategy of a population, isolates variation, environment conditions, and geographical localization (Pourjam et al., 1997; Troccoli et al., 2016).

Phylogenetic relationships within *Pratylenchus* species based on the 18S and 28S sequences were generated by MEGA and minimum evolution (Figs 4,5). The 18S and D2/D3 expansion fragments of the 28S rDNA idendity ranged from 98 to 99% identity with *P. vulnus* worldwide. The biogeography history of *P. vulnus* could be clarified by understanding the phylogenetic relationships among several species of nematodes. The 18S phylogenetic constructed tree showed that our *P. vulnus* is closely similar to two clades: *P. thornei*, clade included a group of *P. crenatus*, *P. pratensis* species. The phyologenetic tree based on 28S gene revealed the strong relationship *of P. vulnus* with *P. neglectus*, *P. parazae*, *P. thornei*, and *P. mediterraneus*.

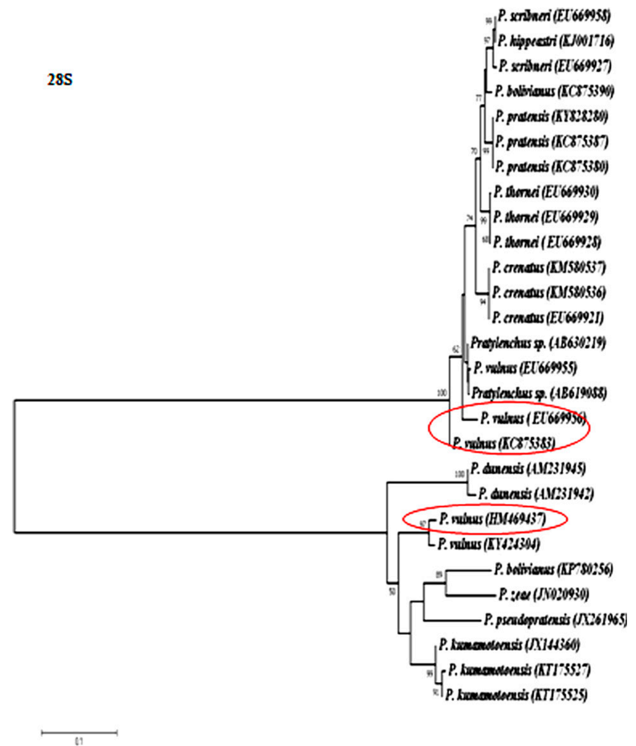

Figure 5: Phylogenetic relationships between Pratylenchus vulnus identified in Tunisia and other Pratylenchus already deposited in GenBank. The Pratylenchus sequences aligned in MEGA with CLUSTALW and the phylogenetic tree made by MEGA version 7.0 based on the Neighbor-Joining method as inferred from D2-D3 expansion segments of the 28S rRNA.

Morphometric informations and phylogenetic analysis clarified the unambiguous species identification and complemented to reveal the first report of *P. vulnus* infecting apple in Tunisia. The morphometry was reported not robust and must be complemented by molecular tools in *P. penetrans* group (Handoo et al., 2008; Janssen et al., 2017).

Acknowlegment

Compliance with ethical standards: This research does not contain any conflicts of interest, nor research involving humans or animals. Lobna Hajji-Hedfi and Noura Chihani-Hammas contributed equally to this work.

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