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Molecular and Morphometric Identification of *P. Thornei* and *P. Neglectus* in Southwest of Iran

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Abstract

Root lesion nematodes are considered as important agents of wheat yield reduction in most parts of the wheat growing areas. To elucidate disease situation in Khuzestan, a southwestern province of Iran, 40 soil & wheat root samples were collected. Morphological studies indicated that disease casual agents belong to *Pratylenchus thornei* and *P. neglectus* species. Morphometric studies showed that Differences exist in the body length compared with the studies done so far on these two species of nematode. The DNA of the two species, namely *Pratylenchus thornei* and *P. neglectus*, were extracted concidering Madani et al. [1], Silva et al. [2] and Waeyenberg et al. [3] plus some modifications. The quantity and quality of extracted DNA and its ability in DNA amplification and clearance of PCR bands were compared and the results showed that modified methods of Madani et al. [1] and Waeyenberg et al. [3] were the best methods for *P. thornei* and *P. neglectus* species. Polymerase chain reaction (PCR) and species-specific primers were used to identify *P. thornei* and *P. neglectus*.

Keywords: DNA; PCR; *P. neglectus; P. thorn*

Introduction

Wheat (*Triticum aestivum* L.) is an important grain in Iran. Iran has been ranked as the 12th producer of wheat, for producing more than 13.5 Mt during 2008-2009 [4]. Khuzestan, which is located in the southwest of Iran, is the second most important cereal producing province in the country due to the total production of 1.2 Mt during 2008-2009 [5].

Pratylenchus nematodes are common endoparasite of plant all around the world. The mentioned genus cause necrotic lesions due to being migratory and obligating parasites that invade the cortex of roots, tubers, and bulbs of plant. This reduces the yields [6,7]. Several cereal and legume crops are damaged by the cereal and legume root-lesion nematodes, namely *P. thornei* and *P. neglectus*, in many parts of the world such as the Mediterranean region, America, the Indian Subcontinent and Australia [8,9]. The two species *P. thornei* and *P. neglectus* are the most common species of Pratylenchus in Iran [10]. Ahmadi et al. [11] showed that the population density of *P. thornei* in wheat root samples ranged from 1-351 nematodes/g of root sample in Khuzestan.

Loof (1991) reported currently that the genus *Prtylenchus* that includes more than 60 species could be differentiated only by means of minor morphological and morphometric differences of adult females and males. Several characters can be used in the distinction of various species of this genus from each other. They are as follow: the number of annuli in the lip region, the presence or the absence of a spermatheca in females, the presence or the absence of males, the number of lines in the lateral field, and the shape of the tail [12,13]. Intraspecific morphological variability within the genus *Pratylenchus* is well documented for the most of the characters used in species identification; hence, causes difficulties in identifying species [13]. The use of molecular diagnostic tools is a practical solution to overcome such a problem. PCR-based methods are relatively rapid and very reliable; the possess high discriminating potentials, do not rely on the expressed products of the genome, and are independent of the environmental influence and the stage of the nematode life cycle. A PCR-based assay was used for the identification of six species of *Pratylenchus* through forward and reverse species-specific primers that were designed from the internal variable portion of the D3 expansion region of the 26S rDNA [14]. Al-Banna et al. [14] distinguished *P. neglectus* and *P. thornei* along with four other *Pratylenchus* spp. using PCR and species-specific primers derived from the internal variable portion of the D3 expansion region of the 28S rDNA. The detection and identification of *P. thornei* and *P. neglectus* from soil developed and two species were differentiated by PCR products of 144 bp for *P. neglectus* and 288 bp for *P. thornei* [15].

In the present study, using nucleotide sequences of the D3 expansion region of 28S rRNA, molecular methods were investigated for the aim of identifying *P. thornei* and *P. neglectus*.

Materials and Methods

Forty soil and wheat root samples were collected from Masjed Solleiman, Behbahan, Bagh Mallek and Shoushtar to elucidate disease situation in Khuzestan. Nematodes were extracted from soil samples using the Jenkins [16] method. We examined the standard morphological and morphometric characters of root-lesion nematodes [13]. Adult females were morphologically identified as either *P. thornei* or *P. neglectus*. Root-lesion nematodes were reared on carrot discs [17,18]. Then nematodes were extracted from carrot discs through Whitehead and Hemming methods [19].

Specimens used in this study were extracted from infested roots 24 to 48 hrs after the incubation [20]. They were then handpicked,

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characters	counties				Total
	Masjed Solleiman	Behbahan	Bagh Mallek	Shoushtar	Mean ± SD
n	10	10	10	10	40
L	715-552(547.2 ± 6.4)	595-690(576.2 ± 6.4)	425-620(616 ± 6.4)	512-730(616 ± 6.4)	606 ± 27.6(425-730)
a	33.9-40(30.9 ± 6.3)	27.6-35.6(25.7 ± 6.4)	30-39.8(35 ± 5.6)	27.5-40.2(31 ± 5.4)	33.9 ± 5.4 (27.5-35.6)
b	5.8-6.9(5 ± 0.9)	6.2-6.9(6.4 ± 0.7)	4.1-7.3(6.1 ± 0.6)	5.4-6.7(5.7 ± 0.8)	6.3 ± 0.7 (4.1-7.3)
b′	3.8-6.2(5 ± 0.9)	4-5.5(4.6 ± 0.7)	3.4-5.5(4.8 ± 1.1)	5.2-5.5(4.1 ± 1.1)	4.6 ± 1 (3.4-6.2)
c	18.9-22.1(20.1 ± 2.9)	12.8-22.3(20 ± 2)	19.9-23.8(20 ± 1.5)	18-20.9(21 ± 2.5)	20.5 ± 2.1 (12.8-23.8)
c′	1.3-2.6(1.9 ± 0.5)	1.7-2.5(1.9 ± 0.5)	1.6-2.1(2 ± 0.4)	1.5-2.6(2.2 ± 0.4)	2.1 ± 0.4 (1.3-2.6)
V	77-79(77.6 ± 1.4)	76-78(77 ± 1.2)	79-79.9(77.3 ± 1.05)	76-78(78 ± 1.3)	77.6 ± 1.2 (76-79.9)
Stylet	15-16(15.3 ± 0.5)	15-16(16 ± 1.1)	16-18(16.3 ± 0.7)	15-17(15.9 ± 1)	16.1 ± 1 (15-18)
m	44.4-44.6(44 ± 2.4)	45-50.3(45.5 ± 2.6)	41-48(44.4 ±3)	45.2-48.9(47 ± 2.6)	46 ± 2.6 (41-50.3)
Oeso[1]	99-103(100 ± 2.1)	97-99(98.3 ± 4.4)	83-96(95 ± 6.4)	93-110(103.5 ± 8.4)	99.6 ± 6.5 (83-110)
Over[2]	30-45(36 ± 7.7)	30-39(38 ± 2.1)	35-49(47 ± 8.4)	25-55(41.8 ± 11.54)	41.7 ± 8.9 (25-55)
Exc.pore	65-90(86.5 ± 8.7)	87-95(88.2 ± 1.5)	78-90(75.8 ± 1.1)	90(90 ± 0)	85.1 ± 9.3 (65-95)
Bw	17-20(18 ± 1.5)	19-26(19.4 ± 1)	17-21(8 ± 1.9)	15-19(17 ± 1.7)	18.9 ± 1.9 (15-26)
G%	23(22.4 ± 2.9)	25.3(24.4 ± 1.2)	18.9-19(19 ± 5.4)	21-24.2(22.12 ± 2.7)	22.1 ± 4.6 (18.9-24.2)
Pus	15-18(16 ± 4.7)	20-27(19.4 ± 2)	14-20(19.4 ± 2.9)	19-27(18.7 ± 4.9)	19.6 ± 4.3 (14-27)
Tail	25-45(34.2 ± 7.2)	35-40(35 ± 10.5)	30(30 ± 0)	26-30(30.6 ± 4.7)	34.4 ± 6.8 (25-45)
ABW	11(11 ± 0)	11-13(12.7 ± 2.2)	11-13(13 ± 0.5)	11-14(11.9 ± 1.5)	14.8 ± 1.3 (11-14)
DGO	2	2	2	2	2± 0 (2)
mB%	52.4-53.6(53 ± 0.4)	51-62.4(59 ± 5.1)	51(51 ± 0)	51.9-57(55.3 ± 3)	55.3 ± 4.3 (51-62.4)
GI	90-125(107 ± 12.1)	95-99.1(99 ± 6.3)	125(125 ± 0)	95-110(109 ± 14.5)	107.6 ± 12.8 (90-125)
V-an	79-125(108 ± 17)	90-120(100 ± 12.9)	78-110(199.3 ± 14.8)	98-119(116.9 ± 6.8)	104.1 ± 15.6 (78-125)

1: Length of esophagus
2: The esophageal glands overlap the intestine

Table 1: Morphometric data of females of *Pratylenchus thornei* from 4 counties were examined. Measurements are in µm and expressed as means ± standard deviation (range).

killed and fixed with a solution of 10% formaline + 1% acetic acid+ 1% glycerine+ 88% distilled water, heated to 80°C, then processed to pure glycerine for microscope observations according to De Grisse [21]. Measurements were done with the aid of a camera Lucida and an ocular micrometer. Abbreviations used are defined by Loof [13].

In this study, three different modified DNA extraction methods were used. The first method was a modificated protocol adapted from Madani et al. [1]. Ten microliter dH_2O, 3µl Proteinase K (0.6 mg/ml) and 3µl 1X Mg-free thermophilic DNA polymerase reaction buffer were added to each sterile 1.5ml microtube containing ten adult nematodes. Then dH_2O was added to the sample to the final volume of 41µl and then sample was incubated at 65°C for 1hr. After the first incubation accomplished, the sample was incubated further at 95°C for 15 minutes before storing it at -20°C. In the second method, DNA was extracted from nematodes regarding the protocol described by Waeyenberg et al. [3] with some modification. Ten adult nematodes were put into 20 µl of sterilized water on a concave glass slide. Then 10 µl of suspended nematode pieces were pipetted into a 0.2 ml sterile microtube containing 8 µl of lyses buffer (500 mM KCl; 100mM Tris-Cl, pH 8.3; 15mM $MgCl_2$; 10mM dithiothreitol [DTT]; 4.5% Tween 20; and 0.1% gelatin). The tube content was frozen at -20°C for at least 20 min, thawed, and then 2 µl of proteinase K (600 µg/ml) were added. The tubes were incubated at 65°C in a water bath for 1 h, and consecutively at 95°C for 10 mins to inactive proteinase K. The nematode lyses mix was centrifuged at 16,000 × g for 5 min and the supernatant was transferred to a new 0.2 ml tube and stored in -20°C until it used as the DNA template.

In the third method, modifying Silva et al. [2] method, DNA was extracted from nematodes. Ten sterilized adult nematodes with 10 µl of sterilized water were put into 0.2 ml microtube with 20 µl of lyses buffer (100mM Tris-Cl, pH 8.3; 1.4mM NaCl; 20mM EDTA; and 0.1% B-Mercaptioethanol). Afterwards, samples were frozen for 20 mins and then were immediately placed at 65°C for 1 h. The volumes of 30 µl, 24 µl and 6 µl phenol, chloroform and isoamilalcohol were added to samples respectively. After applying severe shock and complete mixture, the samples centrifuged for 15 mins at 13000 rpm and the supernatant phase was transferred to the new microtube. Then, 30 µl of cold isopropanol were added and frozen for 10 min.

The species-specific forward primer PTHO and the common reverse primer D3B [14] were used to identify *P. thornei*. The species-specific forward primer PNEG-F and the common reverse primer D3B5 were used to identify *P. neglectus* [15]. The species-specific forward primers PTHO and PNEG-F1 were designed based on the variable region in the alignment of the 28S rRNA D3 expansion domains. The species-specific backward primers D3B5 and D3B were selected from the conserved region of the same D3 expansion domain in order to produce a PCR fragment with different sizes [15].

Four isolates of *P. thornei* and *P. neglectus* from Masjed Solleiman (Pt1, Pn1), Behbahan (Pt2, Pn2), Bagh Mallek (Pt3, Pn3) and Shoushtar (Pt4, Pn4) were used to examine the specificity of the *Pratylenchus* primers. PCR reactions of 25 µl contained the DNA template (5 µl), 0.3 µl units of *Taq* polymerase, 0.5 µl dNTPs, 1 µl each primer, 2.5 µl 1X PCR buffer with 7.5µl $MgCl_2$. PCR amplification was performed in a thermocycler (Bio-Rad,) as follows: 95°C for 3 mins followed by 35 cycles of 95°C for 30 s, 60 0C (PNEG/D3B5) or 62°C (PTHO/D3B) for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min [15]. PCR products were separated in 1% standard agarose gels mixed with safe stain. Molecular size was estimated by a 100-bp DNA ladder (Fermentase). Besides, band pattern was photographed under UV light. In this study 140 nematodes were studied.

Results

All the qualitative characters including the number of lip,

spermatheca (presence and shape), and the body length of the 2 species of *Pratylenchus* conformed to the original descriptions. Most of the quantitative characters of the populations also agreed with the original descriptions. However, a few characters fell outside the range of the original descriptions. For example, individuals of the both populations of *P. thornai* and *P.neglectus* had greater body lengths (425 to730 µm and 450 to720 µm) than those reported in the original description. Morphometrics of the females *P. thornei* and *P. neglectus* are reported in Tables 1 and Table 2.

All three DNA extraction methods which were used in this experiment were successful and the nematodes were tracked. Each species-specific primer was constructed to amplify DNA from the target species but to preclude the amplification of non-target *Pratylenchus* species

As a result, in comparison with Silva et al. [2] method for *P. thornei* and *P. neglectus*, modified methods of Madani et al. [1] and Waeyenberg et al. [3] were the best DNA extraction methods according to the band resolution which were created by gel electrophoresis and quantitative/qualitative analysis of extracted DNA (Table 3). A common need in molecular diagnostics of lesion nematodes is to distinguish *P. thornei* from *P. neglectus* in field samples. Using PNEG and D3B5 species-specific primers produced a 144 bp band for *P. neglectus* (Figure 1). However, these primers produced no band with extracted DNA from the species *P. thornei*. As it was expected, PTHO/D3B species-specific primers produced a 288 bp specific band with *P. thornei* (Figure 2) but no band was amplified with extracted DNA of *P. neglectus* (Figure 3). Each of the optimized primers amplified a unique PCR product from its respective target and did not produce an applicant from other *Pratylenchus* species. Furthermore, the presence and the size of the amplification product obtained from individual female nematodes were similar to those obtained from the D3 amplification products. This assay showed that conserved D3 primers amplified DNA from all individuals of the three species, thus confirming the overall reliability of the PCR for a specimen tested.

Discussion

Morphological identification of *Pratylenchus* species relies on the observation of a specific stage of its life cycle. In addition, there is strong intra-specific variation along with few species-specific diagnostic characteristics [22,23]. The Morphometric differences have been reported to be the result of geographical distribution, ecophenotypic effects and different-hosts [24].

In this study the specimens conform closely to the earlier descriptions of *P. thornei,* but there exist some variations. Body length and breadth were measured 425-730 µm and 4.1-7.3; while Sher and Allen [25] and Pourjam et al. [26] reported them to be 450-770 µm, 5.5-8 and 420-680 µm, 5.2-8 respectively. Also compared with reports Yu [27] and Castillo & Vovlas (2007) stylet length was samed (16 µm) but c and body length were smaller and greater respectively. Also ć were measured 2.1 µm that it was similar to Castillo & Vovlas (2007) reports and V (77.6 µm) was larger than Yu [27] reports with 76 µm. Specimens also conform closely to the earlier descriptions of *P. neglectus* but with some variations. The body length, the tail length and bw were measured 450-720 µm, 20-30 µm and 22-14; while in Pourjam et al. [10] studies 340-590 µm, 13-33 and 12-22 µm were reported respectively, and Filipjev and Schuurmans Stekhoven [28] reported the body length 0.31-0.5 mm and the tail length 20-18 have been reported. Also compared with reports Doucet and Cagnolo [29] Mizukubo and Minagawa [30] and Zedan and Geraert [31] body length and a were greater. Stylet length (16.7 µm) in this study was greater than Mizukubo and Minagawa [30] with 16 µm and Doucet and Cagnolo [29] with 16.6 µm reports also it was in the same range (16.5-17.5 µm) of Zedan and

characters	counties				Total
	Masjed Solleiman	Behbahan	Bagh Mallek	Shoushtar	Mean ± SD
n	5	5	5	5	20
L	504-624(569.3 ± 642.9)	490-637(568 ± 51.8)	528-720(595 ± 80.3)	450-637(614 ± 99)	583.8 ± 68.9 (450-720)
a	30.3-51.4(38 ± 8.4)	25.2-32.8(28.9 ± 3.3)	24.5-33.5(29.2 ± 3.5)	21.6-31.5(26.7 ± 3.7)	30.7 ± 6.5 (21.6-51.4)
b	4.9-6.1(5.5 ± 0.5)	3.8-5.2(4.6 ± 0.5)	5.2-6.1(5.7 ± 0.4)	5-6.1(5.1 ± 0.6)	5.2 ± 0.7 (3.8-6.1)
b′	3.9-11.9(6.2 ± 3.3)	3.9-4.8(4.3 ± 0.3)	3.2-6.4(5.1 ± 1.3)	3-10(8.7 ± 3.4)	6.1 ± 2.8 (3-11.9)
c	18.9-26.2(22.2 ± 2.7)	18.1-27.2(22.4 ± 3.9)	18.1-27.2(21.4 ± 4.4)	18.3-25(22.2 ± 3.5)	22.1 ± 3.3 (18.1-27.2)
c′	1.1-1.8(1.4 ± 0.3)	1.2-1.5(1.4 ± 0.1)	1.3-2.6(2.3 ± 0.6)	2.5(2.5 ± 0)	1.8 ± 0.6 (1.1-2.6)
V	81.7-81.9(81 ± 0.9)	81.2-83.6(82 ± 1)	81.3-83(82 ± 0.8)	82-83.6(82.6 ± 0.4)	82.5 ± 0.9 (81.2-83.6)
Stylet	13-20(16.8 ± 3.1)	15-18(16.6 ± 1.3)	15-18(16.4 ± 1.5)	19(17 ± 2.3)	16.7 ± 2 (13-20)
m	43.2-46(44.9 ± 2.9)	40.8-48.2(46.7 ± 1.2)	46-48.5(44.2 ± 1.8)	45.7-47(45 ± 0.9)	45.2 ± 1.9 (40.8-48.5)
Oeso[1]	90-122(111 ± 13.6)	95-120(108 ± 11.5)	75-110(99.6 ± 15.3)	78-105(96.8 ± 12.2)	103.6 ± 13.6 (75-122)
Over[2]	25-30(28 ± 2.7)	20-45(30 ± 9.3)	20-40(31.8 ± 9.9)	22-43(39.4 ± 3.9)	32.3 ± 8 (20-45)
Exc.pore	70-79(75.4 ± 4.5)	71-89(81.6 ± 8.4)	67-75(72.8 ± 8.7)	69-82(77.4 ± 5.1)	76.6 ±7.1 (67-89)
Bw	14-19(16.8 ± 2.3)	19-22(20 ± 1.2)	18-21(19.8 ± 1.1)	15-22(20.6 ± 2.5)	19.3 ± 2.3 (14-22)
G%	10.6-21.7(15 ± 4.1)	13.8-16(13.5 ± 2)	11-14.5(14 ± 2.5)	12-14.5(13.2 ± 3.4)	15.41 ± 3.9 (10.6-21.7)
Pus	20-25(21.8 ± 2.6)	19-25(20.7 ± 2.2)	19-23(22.4 ± 2.4)	20(23 ± 2.1)	22.1 ± 2.3 (19-25)
Tail	22-30(25.4 ± 4.2)	25-30(27 ± 2.7)	20-27(24.4 ± 3)	23-27(25 ± 1.6)	25.5 ± 2.9 (20-30)
ABW	13-15(14 ± 1)	12-16(15.2 ± 0.8)	12-17(13.6 ± 2.1)	13-17(15 ± 1.9)	14.6 ± 1.6 (12-17)
DGO	2	2	2	2	2 ± 0 (2)
mB%	42.3-66.7(53.3 ± 9.9)	51.3-63.9(59.2 ± 5.6)	54.5-62(51.3 ± 4.3)	44-63.9(52.8 ± 8.9)	54.2 ± 7.5 (42.3-66.7)
GI	60-95(86 ± 14.7)	65-85(78.2 ± 13.8)	60-90(85 ± 11.2)	90(90 ± 0)	84.8 ± 11.4 (60-95)
V-an	50-95(76 ± 16.4)	77-95(88.8 ± 8.6)	55-85(78.6 ± 13.9)	53-91(89.8 ± 7)	83.3 ± 12.7 (50-95)

1: Length of esophagus
2: The esophageal glands overlap the intestine

Table 2: Morphometric data of females of *Pratylenchus neglectus* from 4 counties were examined. Measurements are in µm and expressed as means ± standard deviation (range).

DNA extraction method	Quantity (ng/µl)	Quality ($OD_{260/280}$)
Madani et al. [1]	22.2-24.2	0.57-0/76
Waeyenberge et al. [3]	310.6-346.9	0.8-1.04
Silva et al. [2]	476-477.3	1.5-1.67

Table 3: Quantity and quality of DNA extraction methods.

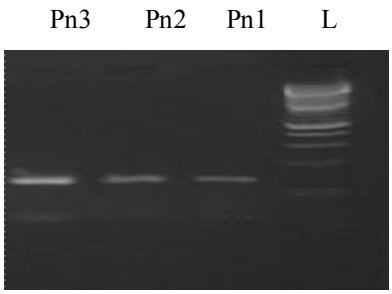

Figure 1: Specific polymerase chain reaction amplification for *P. neglectus* (144 bp) from pure cultures using species-specific primers PNEG/D3B5. DNA templates from the isolates Pn1, Pn2, Pn3; L: 100-bp DNA molecular weight ladder.

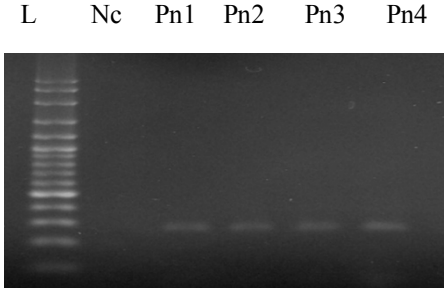

Figure 2: Specific polymerase chain reaction amplification for *P. thornei* (288 bp) from pure cultures using species-specific primers PTHO/D3B. DNA templates from the isolates Pn1, Pn2, Pn3, Pn4; Nc: negative control without DNA template; L: 100-bp DNA molecular weight ladder.

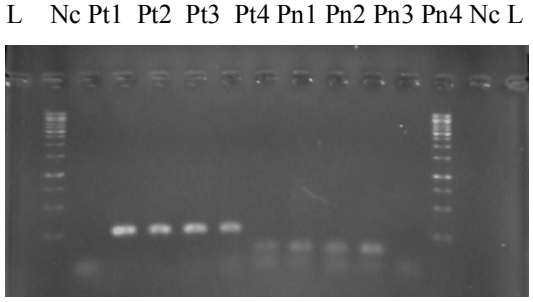

Figure 3: Specific polymerase chain reaction amplification for *P. thornei* (288 bp) and *P. neglectus* (144bp) from pure cultures using species-specific primers PTHO/PENG. DNA templates from the isolates Pt1, Pt2, Pt3, Pt4, Pn1, Pn2, Pn3, Pn4; Nc: negative control without DNA template; L: 1000-bp DNA molecular weight ladder.

Geraert [31] reports. In this study b were measured 5.2 µm that was greater than Zedan and Geraert [31] reports with 3.6-3.8 µm but was smaller than Mizukubo and Minagawa [30] with 5.4 µm and Doucet and Cagnolo [29] with 5.8 µm reports. These variations could be related to the high variability, diversity and wide distribution of *Pratylenchus spp.* Under different climatic conditions, certain morphological characters, such as the number of lip annuls, the shape of the spermatheca, and the structure of the lateral field, are generally reliable for the identification of *Pratylenchus* species [22]. However, the recognition of these characters requires substantial and specialized training and, even then, multiple adult female specimens are necessary for reliable species diagnosis. The qualitative morphological characteristics of the *Pratylenchus* populations used in this study followed the original species descriptions, but some morphometric discrepancies were found. Such morphometric variations in *Pratylenchus* species have been previously reported [32]. Townshend [33] reported that morphometric variations existed among populations of *P. penetrans* associated with strawberry and those associated with celery in Ontario, Canada. Furthermore, variations in size were also found among populations recovered from the same host (strawberry) but collected from different geographical areas Townshend [33]. Similarly, Doucet et al. [34] reported that temperature significantly influences the morphometrics of individuals derived from a single isolate of *P. vulnus*. All these findings indicate that morphometric characters are not always reliable as primary characters for the *Pratylenchus* species identification.

One of the best choices for diagnostic purposes, i.e. the specific identification of several nematode species, is the application of molecular genetics techniques, especially those which are PCR-based [35].

Manual methods for nematodes DNA extraction were used in the present study. Madani et al. [1] and Waeyenberg et al. [3] along with some modification were the best DNA extraction methods for *P. thornei* and *P. neglectus*. Therefore, these methods are recommended for the both mentioned species, but the other species of genus *Pratylenchus* should be reviewed for DNA extraction methods. Latter methods had the highest sensitivity and validity for identifying and tracking nematodes. These manual methods are recommended due to their low cost in comparison with expensive commercial kits. In addition, the quality and quantity of the samples were also competitive with commercial kits.

In this study, two pairs of species-specific primers were used for the PCR amplification. PTHO/D3B specific primers described by Al-Banna et al. [14] were used for P. thornei and produced a 288 bp band. However, no band was amplified with the extracted DNA of *P. neglectus*. PNEG/D3B5 primers also produced a 144 bp band with *P. neglectus* while no band was amplified with the extracted DNA of *P. thornei*. These results are consistent with results of Yan et al. [15] study. These species were separate due the differences in the size of the pieces after electrophoresis of PCR. Moreover, the forward primers (PTHO, PNEG) used in the study are designed from domain variable parts D3 in large subunit nuclear ribosomal RNA, because this variable portion can make the identification of different species possible. Backward primers are designed into a fixed part of the domain D3. Because the use of two different conserved areas in the domain D3, we can use the factor 'fragment length difference' as a factor in identifying various species. D3 expansion region for *Pratylenchus* is specific to the level of the species and does not vary among populations of conspecifics [36].

These species-specific primers should be applicable to diagnostics of both pure and mixed *Pratylenchus* populations, since single females can be tested. However, until the PCR identification has been validated on a wide collection of field isolates, parallel identification with qualitative morphological techniques is recommended for key samples [14].

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