Molecular and Morphometric Identification of *P. thornei* and *P. neglectus* in Southwest of Iran

Fatemeh Fayazi*1, Reza Farokhi-Nejd1, Ali Reza Ahmadi2, Hamid Rajabi Memari2 and Zeynab Bahmani1

1Department of Plant Protection, College of Agriculture, Shahid Chamran University, Ahvaz, Iran
2Department of Plant Protection, Agriculture and Natural Resources Research Centre of Khuzestan, Ahvaz, Iran
3Department of Agronomy and Plant Breeding, College of Agriculture, Shahid Chamran University, Ahvaz, Iran

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 causal 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 considering 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 *Pratylenchus* 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 Soleiman, 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.
and then were immediately placed at 65°C for 1 h. The volumes of 30 µl, 24 µl and 6 µl phenol, chloroform and isomyl alcohol were added to samples respectively. After applying severe shock and complete mixture, the samples centrifuged for 15 mins at 13000 rpm and the supernatant 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 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 Solleimam (Pt1, Pt1), 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 7.5µM MgCl2, 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°C (PNEG/D3BS) 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 40 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 both the populations of *P. thornei* and *P. neglectus* had greater body lengths (425 to 730 µm and 450 to 720 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 species-specific primer was constructed to amplify DNA from the target species but to preclude the amplification of non-target 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 named (16 µm) but c and body length were smaller and greater respectively. Also c 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

<table>
<thead>
<tr>
<th>characters</th>
<th>counties</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Masjed Solleiman</td>
<td>Behbahan</td>
</tr>
<tr>
<td>L</td>
<td>504-624(569 ± 642.9)</td>
<td>490-637(568 ± 51.8)</td>
</tr>
<tr>
<td>a</td>
<td>30.3-51(38 ± 8.4)</td>
<td>25.2-32.8(28.9 ± 3.3)</td>
</tr>
<tr>
<td>b</td>
<td>4.9-6(5 ± 0.5)</td>
<td>3.8-5.2(4.6 ± 0.5)</td>
</tr>
<tr>
<td>b’</td>
<td>3.9-11(6.2 ± 3.3)</td>
<td>3.9-4.8(4.3 ± 0.3)</td>
</tr>
<tr>
<td>c</td>
<td>18.9-26.2(22 ± 2.7)</td>
<td>18.1-27.2(22 ± 3.4)</td>
</tr>
<tr>
<td>c’</td>
<td>1.1-1.8(1 ± 0.3)</td>
<td>1.2-1.5(1 ± 0.4)</td>
</tr>
<tr>
<td>V</td>
<td>81.7-81.9(81 ± 1.9)</td>
<td>81.2-83.6(82 ± 1)</td>
</tr>
<tr>
<td>Stylet</td>
<td>13-20(16.8 ± 3.1)</td>
<td>15-18(16.6 ± 1.3)</td>
</tr>
<tr>
<td>m</td>
<td>43.2-46(44.9 ± 2.9)</td>
<td>40.8-48.2(46.7 ± 1.2)</td>
</tr>
<tr>
<td>Oeso</td>
<td>90-122(111 ± 13.6)</td>
<td>95-120(108 ± 11.5)</td>
</tr>
<tr>
<td>Over</td>
<td>25-30(28 ± 2.7)</td>
<td>20-45(30 ± 9.3)</td>
</tr>
<tr>
<td>Exc:por</td>
<td>70-79(75.4 ± 4.5)</td>
<td>71-89(81.6 ± 8.4)</td>
</tr>
<tr>
<td>Bw</td>
<td>14-19(16.8 ± 2.3)</td>
<td>19-22(20 ± 1.2)</td>
</tr>
<tr>
<td>G%</td>
<td>10.6-21.7(15 ± 4.1)</td>
<td>13-18(13.5 ± 2)</td>
</tr>
<tr>
<td>Pus</td>
<td>20-25(21.8 ± 2.6)</td>
<td>19-25(20 ± 2.2)</td>
</tr>
<tr>
<td>Tail</td>
<td>22-30(25 ± 4.4)</td>
<td>25-30(27 ± 2.7)</td>
</tr>
<tr>
<td>ABW</td>
<td>13-15(14 ± 1)</td>
<td>12-16(15.2 ± 0.8)</td>
</tr>
<tr>
<td>DGO</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>mB%</td>
<td>42.3-66.7(53.3 ± 9.9)</td>
<td>51.3-63.9(59.2 ± 5.6)</td>
</tr>
<tr>
<td>GI</td>
<td>60-95(86 ± 14.7)</td>
<td>65-65(78.2 ± 13.8)</td>
</tr>
<tr>
<td>V-an</td>
<td>50-95(76 ± 16.4)</td>
<td>77-95(88.8 ± 8.6)</td>
</tr>
</tbody>
</table>

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).
Under different climatic conditions, certain morphological characters, the high variability, diversity and wide distribution of smaller than Mizukubo and Minagawa [30] with 5.4 µm and Doucet and greater than Zedan and Geraert [31] reports with 3.6-3.8 µm but was Geraert [31] reports. In this study b were measured 5.2 µm that was Geraert [31] reports.

A wide collection of field isolates, parallel identification with qualitative and quantitative morphometric techniques is recommended for key samples [14]. 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 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 species 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. thornei and produced a 288 bp band. However, no band was amplified with the extracted DNA of P. neglectus. 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 after electrophoresis of PCR. Moreover, the forward primers (PTHO, PNEG) used in the study are designed from domain variable parts 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].

Acknowledgments
This research was supported by the Department of Plant Protection and
the Department of Agronomy and Plant Breeding, College of Agriculture, Shahid Chamran University, Ahvaz, Iran. We thank Yan, G., Smiley, W. R. and Safara, S. for their help.

References