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Investigation on Iranian *Pratylenchus vulnus* populations by morphological and molecular marker (RAPD- PCR)

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Morphological characteristics and genetic variability of two isolates of root-lesion nematode *Pratylenchus vulnus*, collected from the rhizospher of apple trees in Moghan and from maple trees in Behshar area's, were analyzed. Morphological studies of cultured population on carrot disks showed that there are minor differences between two isolates. In both isolates, spermatheca are oval, relatively narrow and oval to round, head with three or four annules and in some specimens with three annules on one side and four annulus on the other side of the head. Tail shape was variable. Scanning electron microscopy (SEM) showed no considerable morphological difference of head in the two isolates. The investigation of genetic structure with RAPD-PCR marker indicated that two isolates have relatively high genetic variability (45%). The results of this research demonstrated morphometric similarity and genetic variability in the Iranian isolates of *P. vulnus*. This variability was not enough to allow us to divide them into two different species.

Key words: Morphological, *Pratylenchus vulnus,* Iranian Populations, Molecular marker

Introduction

Root-lesion nematodes (*Pratylenchus* spp.) are economically the most importance in world after root-knot nematodes (*Meloidogyne* spp.) and cyst-nematodes (*Heterodera* spp.) (Davis and MacGuidwin, 2000). Identification of root-lesion nematodes is difficult due to overlapping of morphometric characters and little morphological diversity (Roman and Hirschmann, 1969; Ryss, 2002). *P. vulnus* (Allen and Jensen, 1951) is a species with a wide host range and cause of severe losses in the crops, fruit trees and ornamental plants. The two populations of *P. vulnus* reported from Iran (Pourjam *et al*., 1997;

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Borhani *et al*., 1999) showed some variations in morphological features as well as host plant, geographical and environmental conditions. The variation is very important in *Pratylenches* species and for regulatory purposes need to be specifically determined.

Species identification in nematodes are initially on morphologically In addition, nematode relationship to other microorganism may effect its morphological characters, as well (Baldwin and Mundo-ocampo, 1991). Biotechnology and molecular markers used widely in different taxonomical studies. Information from differences in DNA molecules provides a useful tool for differentiating recently diverged taxa at species/subspecies or even pathotype/race level. Knowledge of genetic variability within and between phytophagous nematode populations and current detection of species for the selection of suitable control strategies (Mai, 1985; Hyman, 1996). However, most studies of population genetics of these nematodes have focused on the identification of molecular markers that correlate with species and pathotypes (Anderson *et al.,* 1998).

Approaches such as the use of restriction fragment length polymorphism (RFLP) analysis (Caswell-Chen *et al.,* 1992; Fallas *et al.,* 1996; Kaplan *et al.,* 1996; Allen *et al.,* 1997; Ibrahim *et al.,* 1997; subbotin *et al.,*1997; 1999), random amplified polymorphic DNA (RAPD) analysis (Hahn *et al.,* 1995; Pinochet *et al.,* 1994; Fallas *et al.,* 1996; Ibrahim *et al.,*1997), and sequencing of ITS regions have been applied successfully in nematode taxonomy and detecting genetic variability among nematodes. This method was used in the identification of the intraspecific variation within population (Caswell –Chen *et al.,* 1992; Cenis, 1993), studying the genetic structure of population (Williams *et al.,* 1990) and estimating differentiation among nematode population (Nadler *et al.,* 1995). Thus, the purpose of this study was to evaluate morphological variability of the two Iranian *P. vulnus* populations and to compare their genetic relationships using RAPD-PCR markers.

Material and methods

Two *P. vulnus* populations one collected from apple trees in Moghan and the other from maple trees (*Acer velutinum*) in Behshar region. Nematodes were extracted from soil and roots by a modified Baerman funnel techniques (White head and Hemming, 1965). The specimens were killed and fixed by hot solution of FGA 4:1:1 (formaldehyde, glycerin and acetic acid), processed to anhydrous glycerin by the modified Seinhorst method (De Grisse, 1969) and studied by light microscopy. Some studied and measured specimens were selected for SEM studies.

Nematode populations were surface sterilized with 6000 ppm streptomycin sulfate during 2 hours and rinsed three times by sterile distilled water. Twenty individuals of each population reared monoxenically on carrot disk culture (Moody *et al.,* 1673) and incubated at 23±1°C for several generations. DNA was extracted according to the modified Sulston and Hodgkin (1988) method. For DNA extraction approximately several thousand of each population of nematodes (extracted from carrot disks) were used. Six random primers were used for RAPD analysis (Pinochet *et al.,* 1994). Primers were synthesized by Cinagen in Iran (Table 1).

Table 1. Random primers using in genetic diversity assessment of two Iranian isolates *Pratylenchus vulnus* by RAPD-PCR

Primer Sequence	Code
5′-GTTTCGCTCC-3′	OPB-1
5′-GGACTGGAGT-3′	OPB-4
5′-GGTGACGCAG-3′	OPB-7
5′-CCTTGACGCA-3′	OPB-12
5′-GGAGGGTGTT-3′	OPB-15
5′-AGGGAACGAG-3′	OPB-17

Amplification reactions were performed using 25 µlit of PCR mix containing 2.5 µlit Buffer PCR (10x), 0.6 µlit $MgCl_2$ (50 mM), 0.5 µlit dNTP mix (10 mM), 5 µlit primer, 0.5 µlit DNA taq polymerase, 1 µlit template (200 ng) and 14.9 µlit double distilled water. Control samples containing all reagents except DNA were included in all assay to test for contamination. Amplifications were performed in a Master cycler epgradient Eppendorf thermocycler programmed for: an initial denaturation at 94°C for 5min, followed by 35 cycles of one min at 94°C with an annealing temperature of 34°C for one min and extension at 72°C. All reactions were repeated 2 times. 15 µlit from the PCR products were loaded with loading buffer on 1.5% agarose gel in 1X TBE buffer and separated through electrophoresis at 100 V/cm. 1 kb DNA Ladder was used as a size marker. Gels were stained with ethidium bromide and were visualized under ultraviolet light and photographed.

RAPD marker was described as a dominant marker. If the band exists as 1 consider and if does not exist as 0 consider. Initially for matrix of 1 and 0 were used from photocapt software. Using this software, the generated bands weight were determinated on the gels on the basis of applied molecular marker weight (1 kb DNA Ladder). After determinating bands weight, similarity matrix of 1 and 0 were generated on the basis of presence or absence of the similarity bands and the results introduced in Exel software.

For similarity determination between the two studied population were used from different methods. For cluster analysis was used from similarity matrix of 1 and 0 on the basis of the similarity matrix results of 1 and 0, Cluster analysis with UPGMA method in Mvsp-32 software were performed using three Jacard, Simple-Matching and Nei and Li similarity coefficient and similarity amount of the isolates were investigated with given six primer individually and with the sum of them.

Result and discussion

Morphological and morphometrical variations were also seen in Iranian populations of *P. vulnus*. Some Moghan specimens (morphologically) were closely related to original description (Allen and Jensen, 1951); very slender body, tail tapering gradually to smooth conical tip (Fig. 1: H), oval spermatheca and typically rounded stylet knobs (Fig. 1: I). However, in other population from Behshahr, the tail shape (hemispherical with more or less round tip *vs* tapering with conical tip), and shape of spermatheca (oval to rounded) show variation and were resemble to those populations described by Gao *et al.* (1999). However the comparative morphological and morphmetrical characters of Iranian populations of *P. vulnus* confirmed their relationship but, were not considered as identical characters (Tables 2 and Fig. 1 and 2). Therefore the above characters were not sufficient to delineate the whole Iranian populations of *P. vulnus*. Tentative light microscopy was supported by scanning electron microscopy. Head on face view of all studied populations of the species was the same (Figs. 3 and 4) and SEM photography also confirmed the closely relationship of the two populations. The areolation of Mid bond (Corbett, 1974) or outer bonds of lateral fields (Gao *et al.,* 1999), was not seen in Iranian population of *P. vulnus*. So far some reports refer to morphological variation of *P. vulnus* populations from different geographical areas (Corbett, 1974; Loff, 1978; Café-filho and Huang, 1989; Frederick and Tarjan, 1989; Handoo and Golden, 1989; Loof, 1991; Gao *et al.,* 1999).

Other than morphological and morphometrical variations molecular studies using RAPD-PCR markers also, demonstrated relatively high genetic variability (45%) among these two population (Table 1). But, this was not enough to separate them into two different epithet.

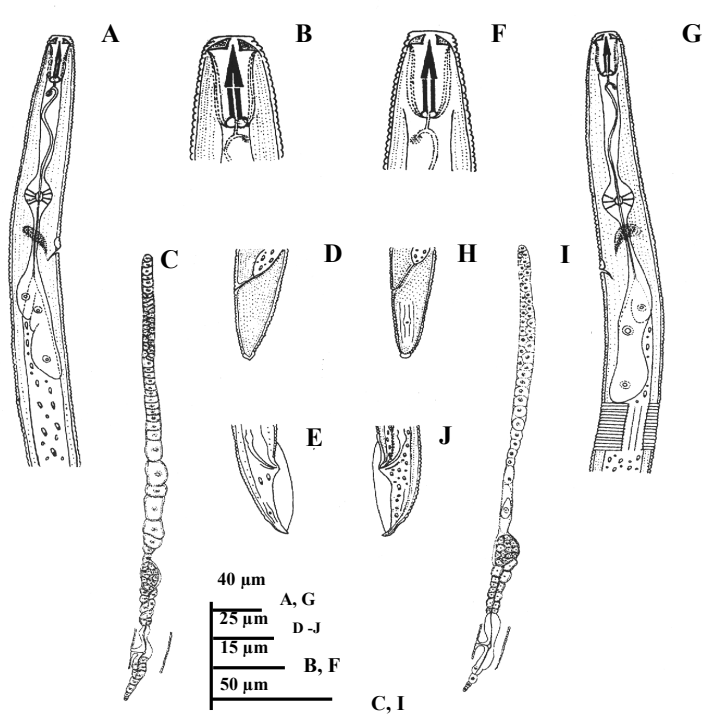

Fig. 1. *Pratylenchus vulnus*, Behshahr (A-E) and Moghan (F-J) isolates. A & G: oesophagus of female; B & C: anterior region of female; E & H: reproductive system; F & G: tail of female and I & J tail of male.

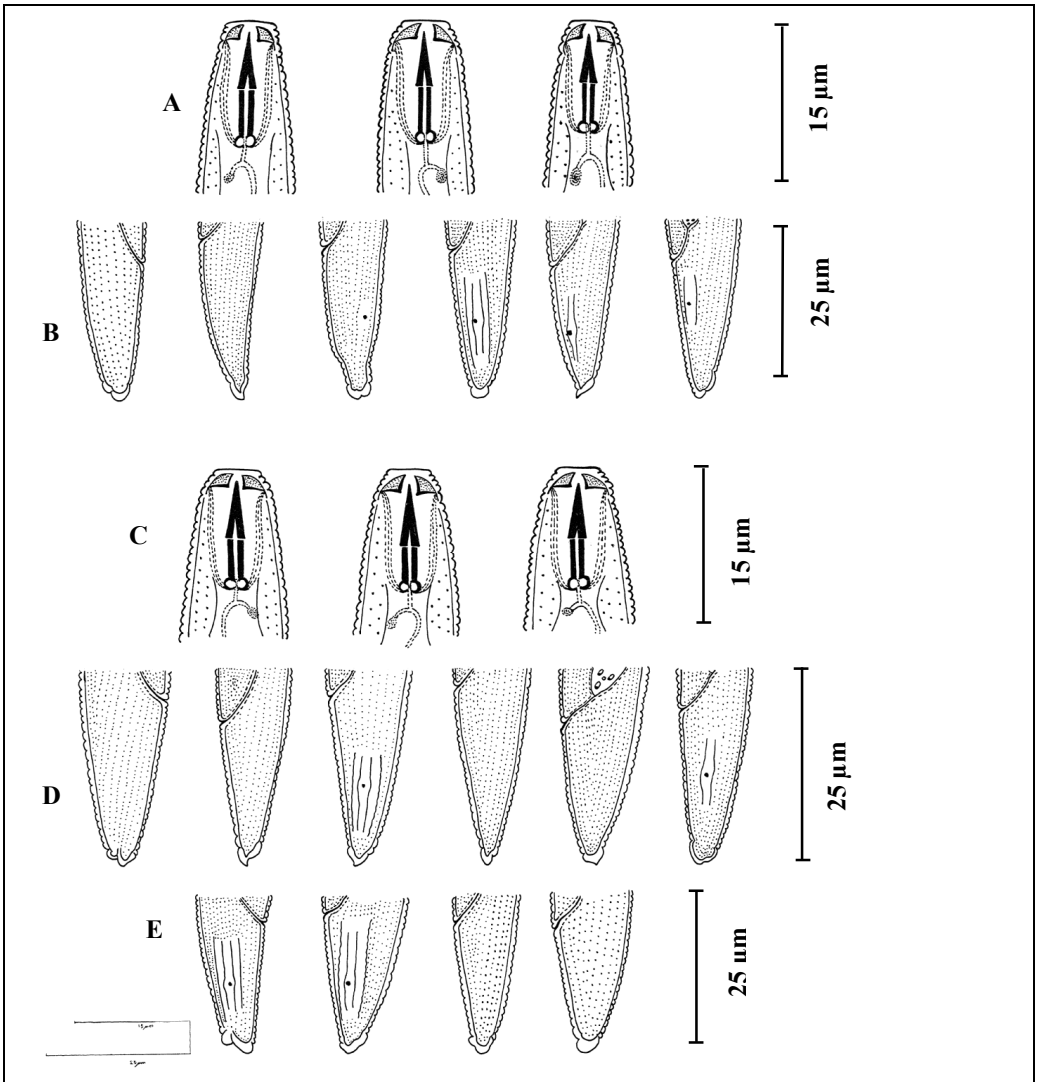

Fig. 2. *Pratlenchus vulnus*. Variation of head and tail shape of Moghan (A and B) and Behshahr (C-E) isolates.

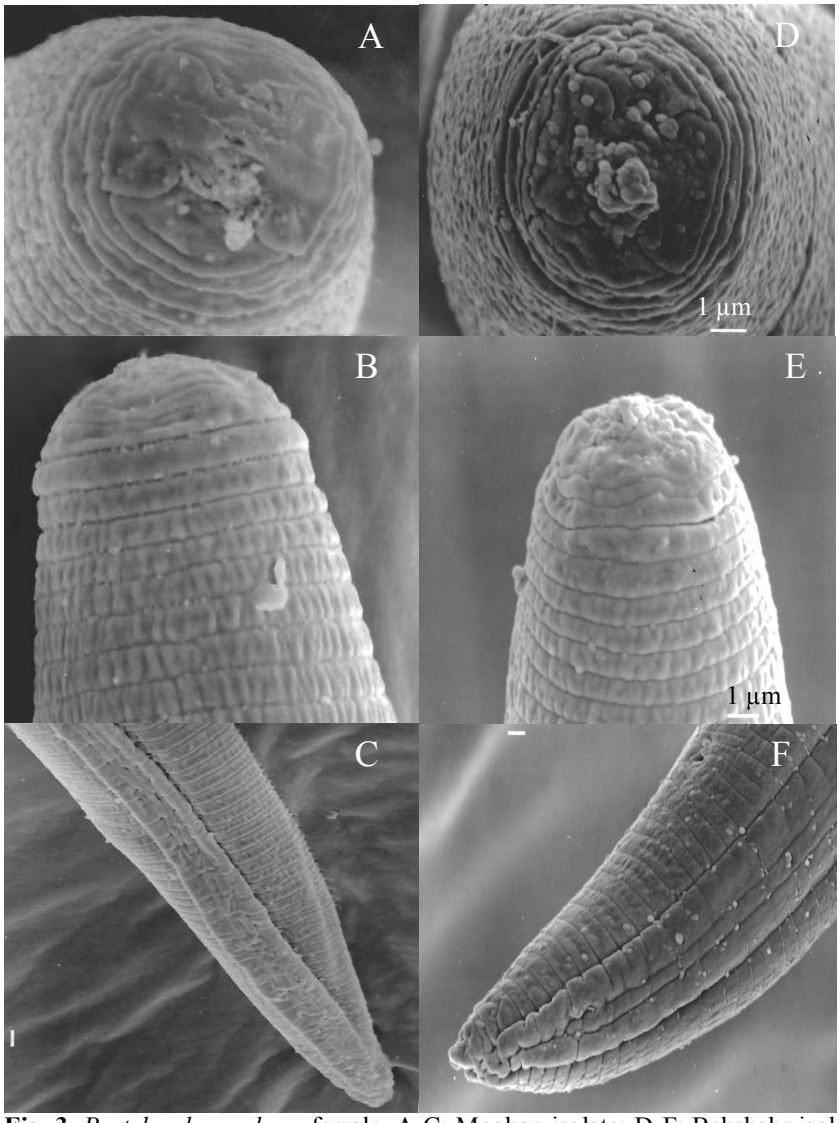

Fig. 3. *Pratylenchus vulnus*, female. A-C: Moghan isolate; D-F: Behshahr isolate. A & D: En face view. B & E: Lateral view of Head; C & F: Tails. (Bars equal to 1 µm).

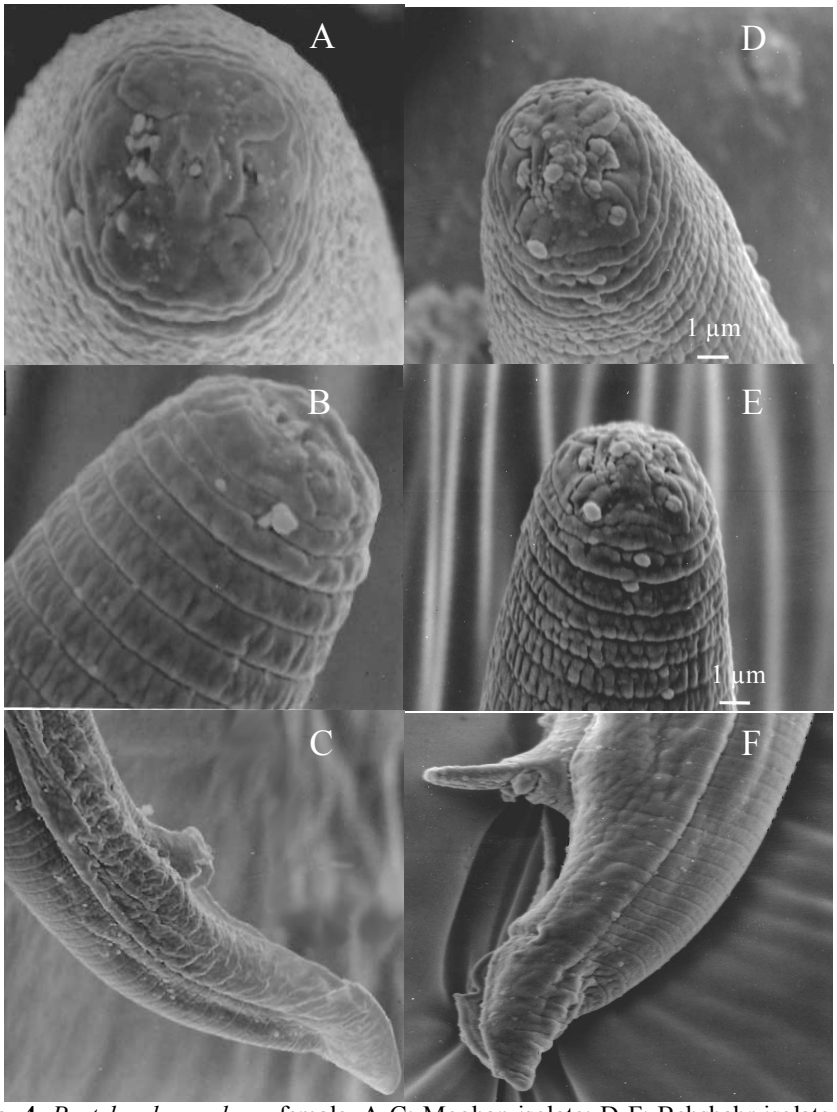

Fig. 4. *Pratylenchus vulnus*, female. A-C: Moghan isolate; D-F: Behshahr isolate. A & D: En face view. B & E: Lateral view of Head; C & F: Tails. (Bars equal to 1 µm).

Genetic variation of Iranian population of Pratylenchus vulnus

All the six random primers (Table 1) used in this research, were clearly showed polymorphism among two populations. In the all PCR reactions, was used negative control without nematode DNA that never gave any PCR product. The reactions with the six primers in two population, produced 57 bands, that their sizes were changeable between 250 to 4625 base pairs. Only

the bands were presented in two replicated reaction were scored (Figures 5: A , B) and non reproducible bands were considered. The highest bands and the most percent of polymorphism were produced with B-12 primer. The lowest polymorphism were produced with B-17 primer. The data of similarity among the population are presented in a similarity matrix in Table 3. These six primers were well estimated that these two populations are one same species, but have relatively high genetic (45%) variability.

Table 3. Percent of similarity between two Iranian isolates *Pratylenchus vulnus* on the RAPD-PCR reaction

Primer / similarity coefficient	OPB-1	OPB-4	OPB-7	OPB-12	OPB-15	OPB-17	B_1-B_{17}
Jaccard	50%	33%	16%	14%	28%	75%	38%
Simple Matching	50%	33%	16%	14%	28%	75%	38%
Nei & Li	66%	50%	28%	25%	44%	85%	55%

However Pinochet *et al.* (1994) 63 to 96% similarities using showed in the seven different population of *P. vulnus* from different geographic locations and different hosts. Among the seven populations the only one dissimilar population was which had similarity values from 0/51 to 0/57 as compared with the rest of the other population. Where as the greatest similarity was 85% among Iranian population that obtained with primer B-17 and the mean similarity was 55% with the sum of primers. These results were indicated that two populations had relatively high genetic (45%) variability. The choice of the class of genetic markers to be used for a particular project depends on many features, including the type of populations that are available, the amount of biological material of the investigators disposal the genetics of the trail-and the organism to be studied and cost. The number of primers able to produce polymorphic markers in RAPD reactions is virtually unlimited and in comparison with the biochemical techniques is a good alternative for detection of genetic variation. RAPD techniques is sensitive to different variables, including the quality and quantitative of genomic DNA, and used enzyme. In these studies, attempt for DNA extraction from small quantities of initial samples were not successive. The observation of the band resulted from genomic DNA on the agarose gel major step for ensure to DNA extraction, and its efficiency for PCR reaction.

The use of template DNA different amount (15, 60, 200 ng) in the PCR reactions were showed that using small quantities, the number and polymorphism of the bands were not suitable for genetical variation studies and

the best results were found in the high quantities of templates (200 ng), repeatedly, the number and polymorphism. In the PCR reaction with template DNA 60 ng, in the spite of, in some primers amplified bands numbers were large, but reproducibility and polymorphism were low. In the template DNA quantities 200 ng, the best results were obtained, reproducibility and polymorphism (Figures 5).

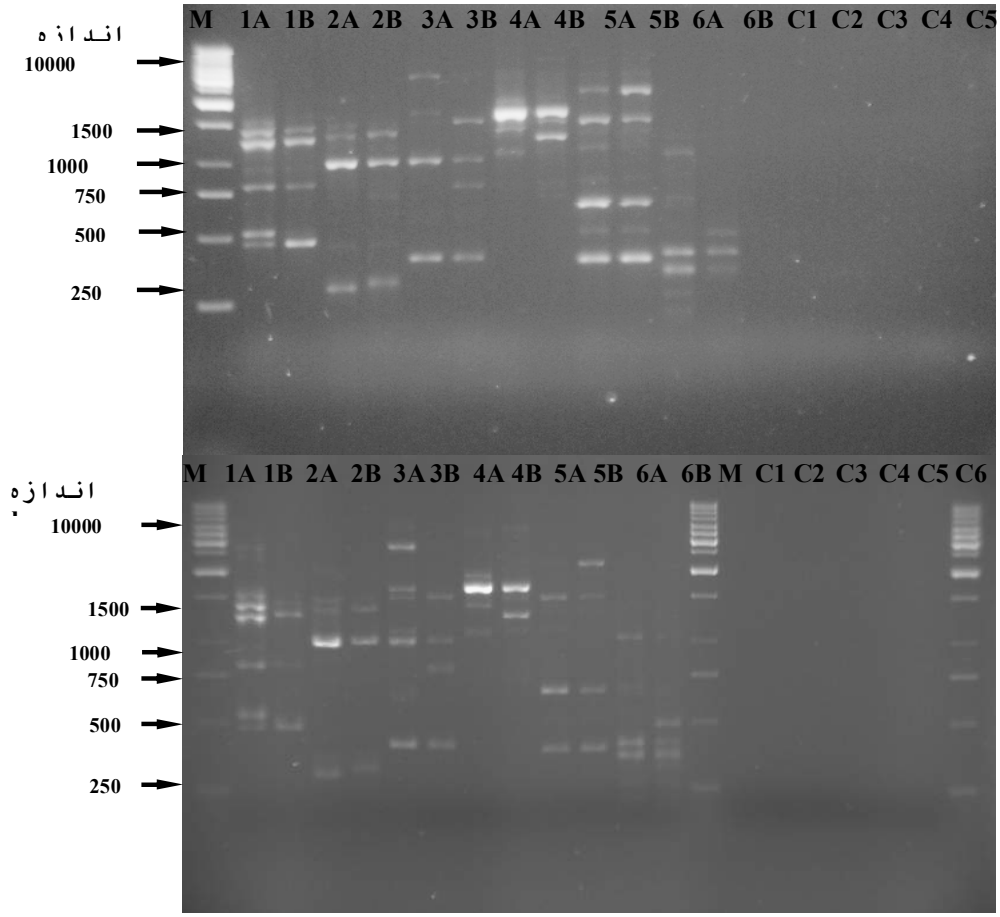

Fig. 5. Results of amplified DNA pattern of RAPD-PCR reaction with six random primers in the two Iranian isolates *Pratylenchus vulnus*. M lane: Marker (1000bp). A and B letters Behshahr and Moghan isolates. 1= OPB-1 primer, 2= OPB-12 primer, 3= OPB-4 primer, 4= OPB-7 primer, 5= OPB-17 primer and 6= OPB-15 primer. C1-C6 control (A: one repeat, B: two repeat).

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Table 2. Morphometric characters of Iranian populations of *Pratylenchus vulnus* cultured in the carrot disk media in comparing to some other populations (all measurements in micrometer)

Origin	**Moghan Population**				**Behshahr Population**			
Characters	Female	CV	Male	CV	Female	CV	Male	CV
n	50	-	20	-	50	-	20	-
L	604 (461-702)	8.5	509 (449-577)	6.3	642 (554.5 781)	7.0	535 (482-648)	7.6
a	31.4 (25.6-38.5)	9.3	30.9 (26.4-34.4)	6.7	27.9 (23-35.6)	8.7	31.6 (26-46.4)	14.0
b	7 (5.2-8.3)	9.1	6.3 (5.9-6.6)	3.5	7.4 (6.1-8.2)	6.5	6.7 (6-7.5)	5.9
b′	4.5 (3.7-5.4)	8.3	4.3 (3.7-5)	8.3	4.8 (4.1-5.7)	8.0	4.4 (3.9-4.9)	5.3
c	23.2 (16.8-26.6)	9.6	21.7 (18.6-29.9)	11.2	24.3 (20.5-29.3)	8.2	22 (18.9-28.9)	10.3
c′	2.5 (1.7-4.1)	17.8	2.1 (1.5-2.4)	11.0	2.1 (1.8-2.7)	9.6	2 (1.5-2.6)	14.1
V	81.1 (74.8-84.2)	2.0	-	-	81.3 (74.8-91.4)	2.8	-	-
V′	84.8 (78.8-88)	2.2	-	-	84.8 (78.1-95)	3.0	-	-
G or T	43.6 (25.3-56.1)	15.4	48 (34.2-94.7)	28.2	52.4 (32.8-82.1)	22.7	55.9 (40.2-75.5)	15.5
Stylet	14.1 (12-17)	5.8	13.3 (12-14)	5.4	14.5 (13.1-16.5)	5.6	13.6 (13-15)	4.5
Oesophagus	86.9 (75-100)	6.4	81.7 (71-89)	6.8	87.6 (75-104)	7.4	79.6 (75-87)	4.4
Overerlapping	46.7 (27-69)	17.3	-	19.8	46.7 (33-61)	13.2	-	64.0
Excretory Pore	85.5 (71-102)	7.7	75.6 (65-84)	7.5	85.1 (66-96)	7.7	74.8 (56-85)	9.8
PUS	36.2 (16-56)	22.0	-	-	45 (35-59)	13.3	-	-
PUS/Body Width	1.9 (0.9-2.7)	22.3	-	-	2 (1.5-2.9)	13.6	-	-
Vulva-anus	86.5 (53-123.5)	15.5	-	-	96.7 (73-134)	13.8	-	-
Tail	25.9 (20-37)	12.7	23.8 (15-27)	11.0	26.7 (21-32)	9.9	24.2 (20-28)	9.0
Spicule	-	-	16.7 (14-22)	11.1	-	-	17.1 (15-19)	7.0
Gubernaculum	-	-	4.7 (4-6)	14.4	-	-	4.6 (4-5)	11.2