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Full Length Research Paper

Comparative d2/d3 LSU–rDNA sequence study of some Iranian *Pratylenchus loosi* populations

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The D_2/D_3 LSU rDNA expansion segment of 13 isolates attaching tea shrubs roots in tea gardens that verified by morphological and morphometrical studies as *Pratylenchus loosi* Loof, 1960 from Guilan province, North of Iran, were amplified and sequenced. Amplification of the D_2/D_3 LSU rDNA expansion segments yielded one fragment at over all sequenced isolates as 787 bp in size. The DNA sequences were aligned using Clustral X1.81 together and with three sequences of similar region of *P. loosi* isolates available in Genbank database (Isolate T from Serilanka and Isolates N1 and N2 from Florida, USA). Also the genetic distance between sequences data were calculated through four methods as following; Uncorrected distance (UC), Jukes-Cantor (JC) Kimura distance (K) and Jin-Neigamma distance (JNG). For generating phyllogenetic trees both Neighbor-joining (NJ) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) were used. The results indicated that very short genetic distance exist among the Iranian isolates and between the Iranian isolates and isolate T from Serilanka whereas the Iranian isolates and isolate T were genetically distinct from isolates N_1 and N_2. The phyllogenetic analyses revealed relationship not only among Iranian isolates but also between Iranian isolates and isolate T.

Key words: Tea, *Pratylenchus loosi*, D_2/D_3 LSU rDNA, sequencing, Iran

INTRODUCTION

The tea root lesion nematode, *Pratylenchus loosi* Loof, 1960 is considered one of the most important and destructive pathogen attacking tea shrubs roots in tea gardens of North of Iran (Hajieghrari et al., 2005) as well as Serilanka and Japan (Sivapalan et al., 1986). It causes a sever decline of tea shrubs where it infects all commercial tea orchards. In Iran, this species is one of the quarantine pests and were found in rooted tea slips imported from Japan (Maafi, 1993). Nowadays, it has been distributed in some tea growth areas of north Iran (Hajieghrari et al., 2005).

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Abbreviations: LSU, Large subunit; SSU, Small subunit; ITS, Internal transcribed spacer; UC, Uncorrected distance; JC, Jukes-Cantor; K Kimura distance; JNG, Jin-Neigamma distance; NJ, Neighbor-joining; UPGMA, Unweighted Pair Group Method with Arithmetic Mean.

Identification of *Pratylenchus* species is essential for facility diagnosis of potential pest problems as well as improving prediction about pathogenicity and host range. In the other hand, species identification in the genus *Pratylenchus* is particularly difficult because of a little morphological diversity exhibition between species. Intraspecific variability of certain morphological characters among genus *Pratylenchus* used for classical distinguishing species is well known and has been adequately documented (Roman and Hirschmann, 1969; Tarjan and Frederick, 1978).

Biochemical methods such as soluble protein analysis and isozyme markers useful for inter- and intraspecific differentiation of plant parasitic nematodes (Hussay, 1979; Fox and Atkinson, 1986) as well as useful for diagnosis of *Pratylenchus* species (Payan and Dickson, 1990; Jaumot et al., 1997; Ibrahim et al., 1995; Andres et al., 2000) but these methods are time consuming for culturing of nematode and gathering a sufficiently abundant sample because a large number of individuals are needed for biochemical analyses.

Table 1. Origin of the different *Pratylenchus loosi* isolates used in this study.

Species (Based on morphological and morphometricalstudies)	Location	Host	Code
Pratylenchus loosi	Phashalem	Tea	P1
Pratylenchus loosi	Lishavandan	Tea	P2
Pratylenchus loosi	Jirdeh	Tea	P3
Pratylenchus loosi	Lakan shahr	Tea	P4
Pratylenchus loosi	Lahijan	Tea	P5
Pratylenchus loosi	Zemidan	Tea	P6
Pratylenchus loosi	Koomleh	Tea	P7
Pratylenchus loosi	Otaghvar(South)	Tea	P8
Pratylenchus loosi	Otaghvar(Central)	Tea	P9
Pratylenchus loosi	Otaghvar(North)	Tea	P10
Pratylenchus loosi	Rood sar	Tea	P11
Pratylenchus loosi	Amlash	Tea	P12
Pratylenchus loosi	Alborz	Tea	P13

Direct examination of the genetic material especially DNA sequence comparison are being used to examine relationship among taxa, even among diverse taxa that cannot readily be compared with morphological analysis (Chaswell-Chen et al., 1993) and a powerful tool to analyze genetic variation (Waeyenberge et al., 2000; Williamson and Westerdahl, 1993). In recent years sequence analysis of coding and non-coding region of nuclear ribosomal DNA (rDNA) have became a popular tools for species and subspecies identification of plant parasitic nematode from many genera (Ferris et al., 1993; Caswell-Chen et al., 1993; Cherry et al., 1997; De Ley et al., 2002) and has been evaluated as a means to clarify phyllogenetic relationships among population of species of nematode (Kaplan et al., 2000) because of highly stability and exhibition a mosaic of conserved and diverse regions (Powers et al., 1997). Each repeat consist of transcribed units (small subunit or SSU or 18S; large subunit or LSU or 28S; 5.8S; internal and external transcribed spacers) and an external non-transcribed or intergenic spacer (Power et al., 1997; De Ley et al., 1999). The D_2/D_3 expansion domains of the nuclear 28S rDNA subunit are sequence region that has been successfully used for diagnosing *Pratylenchus* species as well as other phytoparasitic nematodes (Mizuku et al., 1997; Handoo et al., 2001; Inserra et al., 2001).

The D_2/D_3 expansion segments of the 28S rDNA subunit (D_2/D_3 LSU-rDNA) are the longest expansion fragments in the LSU and are the most rapidly evolving coding region of the rDNA genes (De Ley et al., 2002; Kaplan et al., 2000; Al Banna et al., 2004; Subbotin et al., 2005). It is demonstrated that it is most useful for characterizing species of *Pratylenchus* and their phyllogenetic relationships (Al-Bana et al., 1997; Mizuku et al., 1997; Duncan et al., 1999; Carta et al., 2001; De Luca et al., 2004).The purpose of this study was to determine the nucleic acid sequence of D_2/D_3 fragment of some Iranian isolates and to compare D_2/D_3 LSU-rDNA homologues amplified for multiple *P. loosi* isolates available in the Genbank database.

MATERIAL AND METHODS

Original DNA sequence data were collected from 13 Iranian tea root lesion nematode isolates that verified by morphological and morphometrical studies as a *P. loosi* using three *Pratylenchus* genus diagnostic key (Café-filho and Huang, 1989; Frederick and Tarjan, 1989; Handoo and Goldon, 1989) and original description of *P. loosi* (Loof, 1960; Seinhorst, 1997). These *P. loosi* populations were isolated from different geographical location from tea shrubs infested roots of Guilan province, Iran (Table 1).

For DNA extraction, ten individuals from each isolates were hand-picked and placed in 10 µl double distilled water on slide glass and cut them into two or more pieces. Nematode pieces in 10 µl double distilled water were transfer into a sterile eppendorf tube containing 8 µl lysis buffer which consist of 500 mM KCl, 100 mM Tris-Cl pH 8.3, 15 mM $MgCl_2$ 10 mM DTT, 4.5 % Tween 20 and 0.1% gelatin (Waeyenberge et al., 2000), then 2 µl of proteinase K (600 µl/ml) were added into each samples and were stored at -80°C for 10 min for several days. After freezing, the tube were thawed and incubated for 1 h at 65°C in water bath followed by 10 min at 95°C for denaturing proteinase K before centrifugation for 5 min at 13000 rpm. The supernatant were transferred to PCR reagent mixture. Forward primer D_2A 5'- ACA AGT ACC GTG AGG GAA AGT TG -3' and reverse primer D_3B 5'- TCG GAA GGA ACC AGC TAC TA -3'(Kaplan et al., 2000; Courtright et al., 2000; Tenente et al., 2004) were used for amplification of the D_2/D_3 expansion region of the 28S RNA gene. All PCRs consisted of 50 µl reagent mixture containing; 37 µl dd H_2O, 5 µl 10X reaction buffer, 1 µl 15 mM $MgCl_2$, 1 µl dNTPs (10 mM), 0.3 µl D_2A primers 0.3 µl D_3B primers and 0.5 µl (2.5 unit) Taq-polymerase enzyme. The PCR reaction tubes were placed in a palm thermal cycler model GP001, Correbett research, Australia. Thermal cycling was done as follows: an initial denaturetion at 95°C for 10 min, 40 amplification cycles (denaturizing at 95°C for 30 s, annealing at 60°C for 45 s and extension at 72°C for 45 s) and a final step at 72°C for 10 min. Amplified products were separated on 1% TAE-buffered agarose gels, stained with ethidium bromide and visualized with UV illumination, and then excised from agarose gels using the Qiaquick Gel Extraction Kit (Qiagen Benelux B.V., the Netherlands), cloned into the pGEM-T vector and transformed into JM 109 High Efficiency Competent Cells (Promega, Leiden, the Netherlands). Ten colonies of each population were isolated using blue/white selection and submitted to PCR with vector primers (pGEM-T forward primer 5'GTTTTCCCAGTCACGAC-3' and pGEM-T reverse primer 5'-CAGGAAACAGCTATGAC-3'). Amplified products were purified using a Qiaquick PCR Purification Kit (Qiagen Benelux B.V., the Netherlands). DNA fragments were sequenced using the Big Dye Terminator V3.1 Cycle Sequencing Ready Reaction Kit and purified according to manufacturer's instructions (PE Applied Biosystems, Foster City, CA, USA). The resulting products were analyzed using an ABI Prism 310 Genetic analyzer.

The DNA sequences of all *P. loosi* populations were aligned using Clustal X1.81 (default options) together and with three sequences of *P. loosi* from Genbank (AF170439 isolate T from Serilanka, AF170438 isolate N_2 and AF170437 isolate N_1 from Florida, USA reported by Duncan et al., 1999). Also four types of genetic distance analyses were applied to analyze the alignment; uncorrected distance (UC), Jukes cantor (JC), Kimura distance (K) and Jin-Nei gamma distance (JNG). For generating phyllogenetic trees both Neighbor-joining (NJ) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) were used.

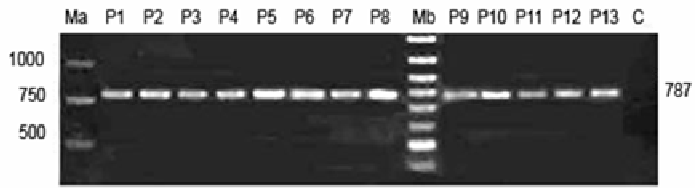

Figure 1. PCR products of D_2/D_3 LSU-rDNA region of *P. loosi* populations (P1-P13) from different geographical areas in Guilan province, Iran using specific D_2/D_3 LSU-rDNA primer pair (D_2A, D_3B). Ma, 1000 bp DNA ladder; Mb, 100 bp DNA ladder; C, control reaction without nematode DNA.

RESULTS

The 13 isolates showed that the qualitative characters of the populations such as number of lip annuli, spermatheca shape and tail shape agreed with original description of *P. loosi* Loof, 1960. The amplification of the D2/D3 LSU-rDNA expansion segments yielded one fragment at over all isolates as 787 bp in size (Figure 1). Control reaction without nematode DNA template never gives any PCR product.

Shown in Figure 2 are aligned sequences of the D_2/D_3 expansion segment of LSU-rDNA for Iranian isolates of *P. loosi* compared with three isolates from the Genbank database (AF170439 isolate T, AF170438 isolate N_2 and AF170437 isolate N_1) by using Clustral X 1.81. There is some sequence variability among studied *P. loosi* isolates within this cluster. The comparisons of the aligned sequences demonstrate that very high sequence similarity was detected with Iranian isolates and isolate T from Serilanka. On the other hand sequence variability was observed within Iranian and American isolates (N_1 and N_2) where differences was found not only between Iranian and American isolates but also with in American isolates and isolate T. It is interesting to note that all populations from Iran were replaced by G at position 320 instead of T which is present in *P. loosi*, isolate T. On the other hand, nucleotide T is missing at position 311 in all Iranian isolates. Within this cluster, sequence divergence within the Iranian isolates ranged from complete identity between P1, P3, P5, P8, P9, P12 and P13 therefore indicating that in these isolates the D_2/D_3 LSU rDNA expansion segment is completely homogeneous, until from 1 to 3 nucleotide differences between P2, P4, P6, P7, P10 and P11 were detected between some of the Iranian isolates of *P. loosi* (Table 2).

The genetic distance between sequences data were calculated through four methods as following; uncorrected distance (UC), Jukes-Cantor (JC) Kimura distance (K) and Jin-Neigamma distance (JNG). The results showed very short genetic distance among Iranian isolates and within Iranian isolates and isolate T from Serilanka (less than 0.53% distance). Also the longest distance is between N_1 and N_2 isolates with Iranian isolate and isolate T. Phyllogenetic analyses with Neighbor–Joinig (NJ) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) yielded very similar topologies for the phyllogenetic relationship of *P. loosi* isolates by using calculated genetic distances (available on request). Therefore only one phyllogenetic tree are presented (Figure 3).

Table 2. Sequence differences between D_2/D_3 LSU-rDNA expansion segments of Iranian *P. loosi* isolates (P1-P13).

Isolate	Position	Substituted nucleotide	Substituting nucleotide
P2	677	T	C
P2	492	A	G
P4	414	C	T
P6	450	A	T
P7	267	T	C
P10	701	A	G
P11	672	G	A
P11	152	C	T
P11	142	--	T

Two mainly clades are particularly strongly supported, one of them includes the N_1 and N_2 isolates (supported with 1.82, 1.79, 1.92 and 1.90% distance calculated with JNG, K, JC and UC, respectively) and any one include Iranian isolates and isolate T (supported with 0% to 0.53% distance analyzed with each four methods) In this clade the most genetic distance based on D_2/D_3 LSU rDNA were obtained between P2 and P11 isolates (0.53%). The genetic distance between these clades were calculated as 8.87, 8.35, 8.33 and 7.88% distances with JNG, K, JC and UC methods, respectively.

DISCUSSION

The *P. loosi* was first described from tea hosts in Serilanka. Nowadays this species is reported from Japan, India, Korea, and Iran, and recently from native plants on Florida, USA. Useful diagnostic characters for identification plant parasitic nematodes such as *Pratylenchus* sp. are remarkably few because of the small size and simple anatomy of phytoparasitic nematodes (Chitwood, 2003). Intraspecific variability of certain morphological characters presently used for describing *Pratylenchus* species present difficulties in identification of species. After analyzing intraspecific morphological and morphometrical variation, Pourjam et al. (1999) demonstrated that some morphological and morphometrical similarity were observed between Iranian isolates of *P. loosi* and American populations from the native plants in Florida described as *P. loosi* by Inserra et al. (1996). Morphological studies confirmed their closely relationships, therefore despite some morphological and morphometrical variations between them, Pourjam et al. (1999) proposed that the American isolates as a subspecific rank of *P. loosi*. It seems that there are difficulties in identify *P. loosi*-like population

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P11;  ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
P13;  ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
P4;   ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
P10;  ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
P2;   ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
P6;   ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
P5;   ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
P12;  ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
P9;   ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
P8;   ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
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P1;   ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
P7;   ACAAGTACCGTGAGGGAAAGTTGAAAAGCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
T;    ----------GCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
N1;   ----------GCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA
N2;   ----------GCACTTTGAAGAGAGAGTTAAAGAGGACGTGAA

P11;  ACCGATGAGATGGAAACGGACAGAGCTAGCGTATCTGGCTTGCATTCAGCTTGCGCAGTC
P13;  ACCGATGAGATGGAAACGGACAGAGCTAGCGTATCTGGCTTGCATTCAGCTTGCGCAGTC
P4;   ACCGATGAGATGGAAACGGACAGAGCTAGCGTATCTGGCTTGCATTCAGCTTGCGCAGTC
P10;  ACCGATGAGATGGAAACGGACAGAGCTAGCGTATCTGGCTTGCATTCAGCTTGCGCAGTC
P2;   ACCGATGAGATGGAAACGGACAGAGCTAGCGTATCTGGCTTGCATTCAGCTTGCGCAGTC
P6;   ACCGATGAGATGGAAACGGACAGAGCTAGCGTATCTGGCTTGCATTCAGCTTGCGCAGTC
P5;   ACCGATGAGATGGAAACGGACAGAGCTAGCGTATCTGGCTTGCATTCAGCTTGCGCAGTC
P12;  ACCGATGAGATGGAAACGGACAGAGCTAGCGTATCTGGCTTGCATTCAGCTTGCGCAGTC
P9;   ACCGATGAGATGGAAACGGACAGAGCTAGCGTATCTGGCTTGCATTCAGCTTGCGCAGTC
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N2;   ACCGATGAGATGGAAACGGACAGAGCTAGCGTATCTGGCTTGCATTCAGCTTGCACAGTC

P11;  GCTACCGATGAATCGCTGATCTCCAGATTGGGACTGTTGACTAGTCGGT-GGTGGCTGCG
P13;  GCTACCGATGAATCGCTGATCTCCAGATTGGGACTGTTGACTAGTCGGTTGGTGGCTGTG
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P7;   GCTACCGATGAATCGCTGATCTCCAGATTGGGACTGTTGACTAGTCGGTTGGTGGCTGTG
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N1;   GCTGCCCATGAATCGCTGACCTCCAGATTGGGGCTGTTGACTAGTGGGCCGGTGGCGGTG
N2;   GCTGCCCATGAATCGCTGACCTCCAGATTGGGGCTGTTGACTAGTGGGCCGGTGGCGGTG

P11;  TTGTGCATTTGCAAGTGGAGTGCGTCGAGGCATCCGGTGCGGCGGAATGAACTTGGTTTT
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P7;   TTGTGCATTTGCAAGTGGAGTGCGTCGAGGCATCCGGTGCGGCGGAATGAACTTGGTTTT
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N1;   TAGTGCATTTGCAAGTGGAGTGCGTCGAGGCGCCGGGATGGCGGAATGAACTGGGCTTT
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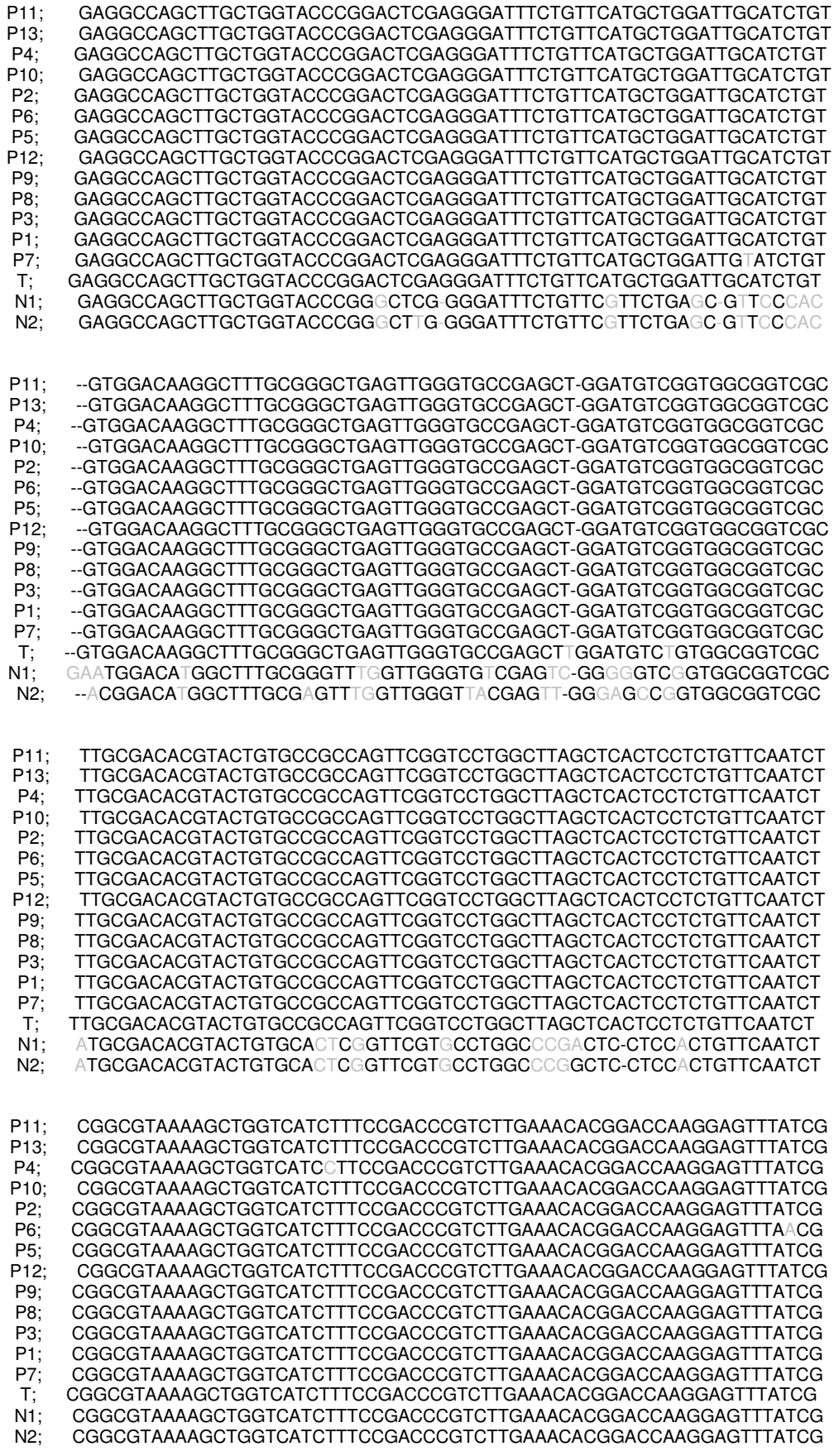
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N1; ATGCGACACGTACTGTGCACTCGGTTCGTGCCTGGCCCGACTC-CTCCACTGTTCAATCT
N2; ATGCGACACGTACTGTGCACTCGGTTCGTGCCTGGCCCGGCTC-CTCCACTGTTCAATCT
P11; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
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P4; CGGCGTAAAAGCTGGTCATCCTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
P10; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
P2; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
P6; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAACG
P5; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
P12; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
P9; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
P8; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
P3; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
P1; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
P7; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
T; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
N1; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG
N2; CGGCGTAAAAGCTGGTCATCTTTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTATCG

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P11;  TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P13;  TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P4;   TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P10;  TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P2;   TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATAAAAGTGAACGTATCCGC
P6;   TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P5;   TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P12;  TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P9;   TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P8;   TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P3;   TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P1;   TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
P7;   TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
T;    TGTGCGCGAGTCATT-GGGCGTTGAAAACCCAAAGGCGCAATGAAAGTGAACGTATCCGC
N1;   TGTGCGCGAGTCATT-GGGCGTTCAAAACCCAAAGGCGCAATGAAAGTGAACGTTTCCAT
N2;   TGTGCGCGAGTCATTTGGG-GTTCAAAACCCAAAGGCGCAATGAAAGTGAACGTTTCCAT

P11;  TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P13;  TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P4;   TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P10;  TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P2;   TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P6;   TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P5;   TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P12;  TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P9;   TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P8;   TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P3;   TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P1;   TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
P7;   TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
T;    TA-GGAGCCAACGTGCGATCCTGGTCATTGCGGTGGCCAGGCGCAGCATGGCCCCATCCT
N1;   TTCGGAGCCGACGTGCGATCCTGGTCACTGCGGTGGCCAGGCGCAGCATGGCCCCATCCC
N2;   TTCGGAGCCGACGTGCGATCCTGGTCACCGCGGTGGCCAGGCGCAGCATGGCCCCATCCC

P11;  GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P13;  GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P4;   GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P10;  GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P2;   GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P6;   GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P5;   GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P12;  GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P9;   GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P8;   GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P3;   GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P1;   GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
P7;   GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
T;    GACTGCTTGCAGTAGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
N1;   GACTGCTTGCAGTGGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT
N2;   GACTGCTTGCAGTGGGGTGGAGGAAGAGCGTACGCGATGAGACCCGAAAGATGGTGAACT

P11;  ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGGAGTCCGAAGCGATTCTGACGT
P13;  ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
P4;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
P10;  ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
P2;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCTGAAGCGATTCTGACGT
P6;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
P5;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
P12;  ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
P9;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
P8;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
P3;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
P1;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
P7;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
T;    ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
N1;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
N2;   ATTCCTGAGCAGGATAAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGT
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P11;    GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P13;    GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P4;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P10;    GCAAATCAATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P2;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P6;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P5;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P12;    GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P9;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P8;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P3;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P1;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
P7;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCT
T;      GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAAC------------
N1;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATC--------
N2;     GCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACC-TC--------

P11;    GGTTCCTTCCGA
P13;    GGTTCCTTCCGA
P4;     GGTTCCTTCCGA
P10;    GGTTCCTTCCGA
P2;     GGTTCCTTCCGA
P6;     GGTTCCTTCCGA
P5;     GGTTCCTTCCGA
P12;    GGTTCCTTCCGA
P9;     GGTTCCTTCCGA
P8;     GGTTCCTTCCGA
P3;     GGTTCCTTCCGA
P1;     GGTTCCTTCCGA
P7;     GGTTCCTTCCGA
T;      ------------
N1;     ------------
N2;     ------------
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Figure 2. Sequence alignment of D_2/D_3 LSU r DNA with Clustral X 1.81 for 13 isolate of *P. loosi* (P1-P13) in compared with same position of three isolates AF170439 isolate T, AF170438 isolate N_2 and AF170437 isolate N_1 from Genbank database reported by Duncan et al.(1999).

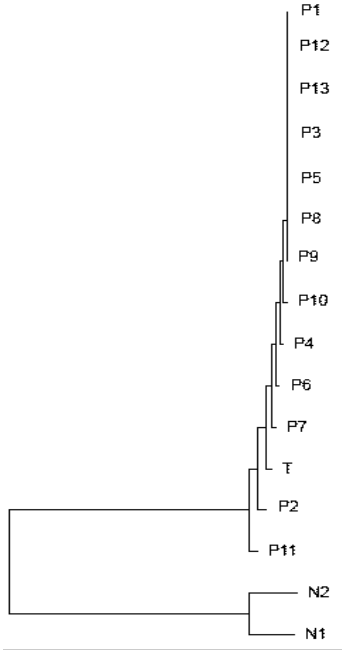

Figure 3. Phyllogenetic tree describing the relationships of *Pratylenchus loosi* isolates of this study in compared with three isolates AF170439 isolate T, AF170438 isolate N_2 and AF170437 isolate N_1 from Genbank database reported by Duncan et al. (1999) based on D_2/D_3 LSU rDNA sequences Jin-Neigamma distance (JNG) and generated by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analyses.

based on morphological and morphometrical methods without the aid of molecular markers. In the recent years, comparative analysis of the D_2/D_3 28S rDNA expansion segment sequence has became a popular tool to differentiate cryptic species which are morphological identical (or with some overlapped morphological variation) but genetically distinct (Subbotin et al., 2005).

Duncan et al. (1999) analyzed some species of *Pratylenchus* as well as tree isolates of *P. loosi*, isolate T from Serilanka (original description by Loof, 1960) and isolates N_1 and N_2 from central Florida, USA describing *P. loosi* (Inssera et al., 1996) by using D_2/D_3 LSU rDNA expansion segment sequence and found that there is substantial D_2/D_3 28S rDNA sequence difference between them. These datasets appear to indicate that N_1 and N_2 isolates from Florida do not consist of sibling species and proposed that the American isolates as an undescribed species of *Pratylenchus*.

In order to clarify the taxonomic status of tea infesting nematode from Guilan province, we characterize the D_2/D_3 expansion segment of large submit of nuclear DNA. Sequence dataset demonstrated a very low level of sequence diversity in Iranian isolates of *P. loosi* and isolate T from Serilanka strongly suggesting extensive genetic homogenization. These result provide evidence to support the proposal that Iranian isolate belong to *P. loosi* and phyllogenetically relationship exist between Iranian and

isolate T from Serilanka; and despite the morphologically similarity of *P. loosi* populations described from Iran and American isolate, there are substantial D_2/D_3 sequence difference between them, confirming Duncan et al. (1999) proposal that the American isolates as a undescribed species of *Pratylenchus*.

The presence of Iranian isolates and T isolate D_2/D_3 LSU-rDNA nucleotide sequences can be considered as the molecular signature of *P. loosi* and can be used as an additional tool for close identification of this species from other geographical regions and among other *P. loosi*-like species.

Al-Banna et al. (1997, 2004) considered that the D3 expansion segment does not show intra specific variation in *Pratylenchus* sp. Our also study showed that the D_2/D_3 LSU rDNA expansion segment is not a suitable region to use for intraspecific variation of *P. loosi* as well as some other plant parasitic nematodes (Subbotin et al., 2005) because the D_2/D_3 28S rDNA expansion segment is the most rapidly evolving coding region of the rDNA and is flanked by highly conserved sequences and can distinguish taxa at species level.

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