Research Article

Study on Populations of Cabbage Cyst Nematode *Heterodera cruciferae* Franklin, 1945 from Iran

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Abstract | Cabbage cyst nematode, *Heterodera cruciferae* Franklin, 1945 causing serious damage on different cabbage cultivars and other host plants. High incidence of this nematode revealed by soil and root samples in the vegetable growing farmlands in Tabriz, Iran. Different populations of *H. cruciferae* were collected from the region and characterized based on morphological, morphometric and molecular features, four out of those populations isolated from the rhizosphere and roots of four different cabbage cultivars were subjected to further studies. All the populations showed same range of morphometric and morphological characters fitted thoroughly with the identity of *H. cruciferae*. Different primers for amplification of *ITS1-5.8S-ITS2*, *D2-D3* rDNA and *hsp* 90 genes, showed similarity between the nematode populations. ITS-RFLP profiles generated by 16 different restriction enzymes did not differentiate the populations, too. In phylogenetic analyses of partial sequences of 28S rDNA *D2-D3*, a clade clustering the four populations of cabbage cyst nematode was formed along with the sequences of *H. cruciferae* populations deposited in NCBI. These results showed morphometrical, morphological and molecular characters of under study populations are same and the host is the only differences between pathogenicity populations.

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Introduction

Cyst forming nematodes, heteroderids, are sedentary endoparasites of plants having ability to establish specific feeding sites in their host cells to achieve necessary nutrition for their development and growth. Some species such as *Heterodera glycines* Ichinohe, 1952 and *H. schachtii* (Schmidt, 1871) Örley, 1880 cause high damages and yield losses on different host plants worldwide (Subbotin *et al.*, 2010). Cabbage cyst nematode, *H. cruciferae* Franklin, 1945 has been reported from different continents (Subbotin *et al.*, 2010; Shurtleff and Averre, 2000) causing economically significant damages on cabbage and some other crops such as radish, cauliflower, *etc.* (Ravichandra, 2014; Mennan and Handoo, 2012). In Iran, occurrence of the nematode has been reported from rhizosphere and roots of two different varieties of cabbages (*Brassica oleracea* L. var. *capitata* and *Brassica oleracea* L. *var. gongylodes*) (Jabbari and Niknam, 2008),

and rhizosphere of sugar beet (Mahdikhani, 1998) and peanut (Mirgasemi *et al*., 2014). *Chenopodium album*, *Raphanus sativus*, *Coriandrum sativum*, *Sonchus oleraceus*, *Sisymbrium loeselii* and *Lepidium sativum* are also naturally infected by the nematode in the region (Khanzad-Bonab and Jabbari, 2019).

Study on occurrence and presence of *H. cruciferae* based on morphometric and morphological data in different regions of the world have been documented (Jones, 1950; Fenwick and Franklin, 1951; Franklin, 1951; Oostenbrink and den Ouden, 1953; Macdougal, 1960; Sturhan and Das, 1960; Grujicic and Krnjaic, 1966; Stone and Rowe, 1976; Riggs *et al*., 1982; Shahina and Maqbool, 1995; Brzeski, 1998; Bello *et al*., 1999; Jabbari and Niknam, 2008) but reports on molecular diagnosis of the nematode, are very scare (Chizhov *et al*., 2010; Sasanelli *et al*., 2013; Escobar *et al*., 2018; Mehalaine *et al*., 2020; Toktay *et al*., 2022). Although different cyst nematodes have been subjected of molecular identification studies in Iran (Tanha-Maafi *et al*., 2003, 2007), *H. cruciferae* was not included in the studies. Molecular studies based on ribosomal and mitochondrial genes are frequently used in different groups of nematode species identification (Tanha-Maafi *et al*., 2003, 2007; Chizhov *et al*., 2010; Sasanelli *et al*., 2013; Escobar *et al*., 2018; Mehalaine *et al*., 2020; Toktay *et al*., 2022). Ribosomal ITS genes having high variability in the region, so can be use for nematode identification even at subspecies level (Toktay *et al*., 2022). On the other hand, only using some sequences for nematode identification is not sufficient for identification at species level and morphological and morphometrical characters are needed for accurate and reliable identification. In current research, the main aims were (i) characterization of main populations of the nematode recovered from roots of four different cabbage cultivars in the region by using *ITS1-5.8S-ITS2* of rRNA, *D2-D3* rDNA and *hsp* 90 sequences, (ii) ITS-RFLP, (iii) morphologic and morphometric study on populations.

Materials and Methods

Nematode samples

Cysts were isolated from roots and rhizosphere of four different varieties of cabbage (Table 1) in vegetable farmlands in Tabriz, East Azarbaijan province, Iran (GPS coordinates 38° 06′ N, 46°15′E, by mean temperature of 6.6-20°C and sandy-loam soil type) during 2020-2022. Cysts were extracted by Fenwick (1940) method and were picked up from extracted material with a fine brush or needle under steriomicroscope.

Table 1: *List of cabbage cultivars grown in Tabriz vegetable farmlands.*

S.	Cultivars	Growing period	Cultivars code
1	*Brassica oleracea* L. var. *gongylodes*	Six months	K6
2	*Brassica oleracea* L. var. *gongylodes*	Three months	K3
3	*Brassica oleracea* L. var. *capitata*	Six months	C6
4	*Brassica oleracea* L. var. *capitata*	Three months	C3

Nematode identification

Morphological and morphometric characters: Species identification was carried out based on morphological and morphometrical characters of cysts, J_2 and male according to Subbotin *et al*. (2010).

Molecular diagnosis

DNA Extraction and PCR: The genomic DNA was extracted from J_2 by lysing method (Subbotin *et al*., 2010). Extracted DNA from the four populations were kept in -20°C until use.

The PCR reaction carried out in 25µl per reaction containing 1µl DNA, 16.35µl ddwater, 5µl PCR buffer (Promega, USA), 0.5 dNTPs (Promega, USA), 0.15 Taq DNA polymerase (5U µl/1- Promega, USA) and 2µl of each primer (10 pmol/µl). In all PCR reactions, positive and negative controls were included. PCR products were run on 1% agarose gel, stained with ethidium bromide and the results visualized using Bio Rad Molecular image®-Gel DocTMXR+ Gel documentation. The expected bands were extracted from gel using cutting tools and purified using illustrate GFX® PCR DNA and Gel Band Purification Kit, GE Healthcare-UK. Quality and quantity of purified DNA were evaluated by nanodrop (Nanodrop 2000c- USA) and the process followed by cloning. Out of nine different primer combinations applied for molecular diagnosis and phylogenetic studies of cyst forming nematodes (Subbotin *et al*., 2010), three primers (Table 2) were used for amplifying *ITS1-5.8S-ITS2* of rRNA, *D2-D3* 28S rRNA and *hsp* 90 genes. The PCR was programmed for all the reactions as shown in Table 3.

PCR-RFLP

For each enzyme, 2µl of each PCR product (0.2-1µg),

Table 2: *List of primers used for amplification of rRNA-ITS, D2-D3 and hsp 90 of Heterodera cruciferae populations in this study.*

Target region	Primers	Reference
ITS1-5.8S-ITS2	F TW81: GTTTCCGTAGGTGAACCTGC	Curran *et al.* 1994
	R AB28: ATATGCTTAAGTTCAGCGGGT	
D2-D3 28S rRNA	F D2A: ACAAGTACCGTGAGGGAAAGTTG	Nuun, 1992
	R D3B:TCGGAAGGA ACCAGCTACTA	
hsp 90	F U831: GAYACVGGVATYGGNATGACYAA	Skantar and Carta, 2005
	R L1110:TCRCARTTVTCCATGATRAAVAC	

Table 3: *PCR program for amplification of the ITS1-5.8S-ITS2 of rRNA, D2-D3 28S rRNA and hsp 90 genes (A: Calculated based on primer sequences; B: Depends on expected size of amplification in PCR, for each 1Kb equal to 1 min.).*

Initial denaturation	Denaturation	Annealing	Extension	Final extension
3 min - 94˚C	1 min - 94˚C	1.5 min - (A) ˚C	(B) min - 72˚C	10 min - 72˚C
		30- <40X		

Table 4: *The restriction enzymes and related cutting sites used in PCR-RFLP.*

S.	Enzyme	Cutting site	S.	Enzyme	Cutting site	S.	Enzyme	Enzyme
1	*Hinf* I	5' -G ANTC- 3' 3' -CTNA G- 5'	7	*Alu* I	5' -AG CT- 3' 3' -TC GA- 5'	12	*Bsp143I*	5' - GATC- 3' 3' -CTAG - 5'
2	*Mva* I	5' -CC WGG- 3' 3' -GGW CC- 5'	8	*Msp* I	5' -C CGG- 3' 3' -GGC C- 5'	13	*Hind* III	5' -A AGCTT- 3' 3' -TTCGA A- 5'
3	*Xba* I	5' -T CTAGA- 3' 3' -AGATC T-5'	9	*Sa* II	5' -G TCGAC- 3' 3' -CAGCT G- 5'	14	*Pvu* I	5' -CGAT CG- 3' 3' -GC TAGC- 5'
4	*Hin*6 I	5' -G CGC- 3' 3' -CGC G-5'	10	*Kpn* I	5' -GGTAC C- 3' 3' -C CATGG- 5'	15	*BamH* I	5' -G GATCC- 3' 3' -CCTAG G- 5'
5	*EcoR* I	5' -G AATTC- 3' 3' -CTTAA G- 5'	11	*Rsa* I	5' -GT AC- 3' 3' -CA TG- 5'	16	*Pst* I	5' -CTGCA G- 3' 3' -G ACGTC-5'
6	*BusR* I	5' -GG CC- 3' 3' -CC GG- 5'						

1µl restriction enzyme (2-10 unit), 1µl buffer 10X and 6µl ddwater were added to a sterilized PCR tube and all tubes incubated at least eight hours in 37˚C for. None of the used enzymes (Table 4) had star activity. Digested DNA fragments present in PCR tubes (10 µl) were loaded on a 1.5% agarose gel and the results were visualized under Bio Rad Molecular image®-Gel Doc™XR+ Gel documentation.

Cloning and sequencing

PCR products were cloned by pGEM-T® easy vector system (pGEM®-Tand pGEM®-TEasy vector System, Promega-USA). Ligation process was carried out based on the kit instruction. Transformation was achieved using heat shock by 3µl of ligation mix and 50µl of *Escherichia coli* competent cell (Homemade, DH5α). The transformed bacteria were cultured in liquid LB media contained in special falcon tubes and were shaken at the speed of 120 rpm for 90 minutes. The bacteria were cultured on plates containing solid LB with agar. White-blue screening was implemented after keeping the plates in 37ºC at least for 12 hours. Colony PCR for blue colonies as DNA template along with specific primers of inserted sequences (Tables 2 and 3) was done. The insertion of the sequence into the bacteria was visualized on PCR products. The residue of the colonies cultured in liquid LB media under the same situation that cited above were kept overnight (at least 8 hours). The plasmid extraction of the cultured bacteria carried out using Nucleospin®, Plasmid, DNA, RNA and protein purification kit, Mchery-Nagel, Germany according to the kit manual. In order to more confirmation of sequences insertion into plasmids, one more PCR reaction was conducted on the extracted plasmid as DNA template in the PCR and its specific primers (Tables 2 and 3). All transformed plasmids were sent to GATC Biotech-Germany for sequencing by Sanger method

Table 5: *Morphological and morphometric features of Heterodera cruciferae populations collected from Tabriz vegetable growing farmlands (ll measurements are in µm).*

K6						K3					
J2		Cyst		Male		J2		Cyst		Male	
L	351.15 ± 15 (333.3-420)	L	505.4 ± 58.5 (400-609.5)	L	961 ± 83 (828.5-1080.0)	L	360.15 ± 15 (340.0-410)	L	515.4 ± 52.2 (442.5-600)	L	971 ± 72.3 (828 -1180)
a	20 ± 1.5 (17.5-24)	W	398 ± 63.2 (305-571.5)	a	38.2 ± 2.5 (33-41)	a	19 ± 1.5 (17.5-22)	W	380.1 ± 57.3 (325-561.2)	a	36.2 ± 2.1 (30-39)
b	4.01 ± 0.1 (2.6-4.3)	neck length	66.5 ± 15 (50-84)	b	8.3 ± 0.9 (8-10)	b	3.8 ± 0.1 (2.4-4.1)	neck length	60.7 ± 11.2 (47.3-76.2)	b	7.9 ± 0.3 (7.6-9.8)
b'	2.4 ± 0.4 (2.06-2.7)	L/W	1.3 ± 0.1 (1.1-1.5)	b'	5.3 ± 0.6 (5-6)	b'	2.5 ± 0.5 (2.1-2.6)	L/W	1.1 ± 0.1 (0.8-1.3)	b'	6.1 ± 0.2 (4.7-5.3)
c	8.4 ± 0.7 (7.5-11)	Semienestral width	36.6 ± 5 (31-44)	Stylet	23 ± 1.6 (20-26)	c	8.0 ± 0.3 (6.2-10.7)	Semienestral width	33.2 ± 3.5 (28.6-40.2)	Stylet	24.7 ± 1.8 (19.2-28.3)
c'	4 ± 0.3 (3.0-4.4)	Semifenestral lenght	16.6 ± 4.6 (12.5-22)	Gubnaculum	7.07 ± 1.1 (6-8.4)	c'	3.3 ± 0.3 (31-4.2)	Semifenestral lenght	19.0 ± 5.7 (15.5-23.5)	Gubnaculum	6.07 ± 1.1 (5.4-7.7)
Stylet	21 ± 0.8 (20-24.0)	Vulval Slit	42.3 ± 5 (30-50)	Spicules	21 ± 4.4 (16-30)	Stylet	23 ± 0.4 (21-25.0)	Vulval Slit	37.3 ± 3.7 (32.5-47.6)	Spicules	25.1 ± 5.7 (20-32)
Tail	41 ± 5 (26.2-47)			T	48 ± 14.1 (35-68)	Tail	41 ± 5 (26.2-47)			T	42.7 ± 12.6 (37.3-64.5)
H	21.3 ± 1.9 (16-23.4)					H	23.1 ± 1.9 (17-22.4)				
H/Stylet	1.02 ± 0.1 (0.8-1.2)					H/Stylet	1.2 ± 0.1 (0.7-1.3)				
Head annules	3-4	Underbridge	Weak	Head annules	4-6	Head annules	3-4	Underbridge	Weak	Head annules	6
Lateral lines	4	egg sac	Present	Lateral lines	4	Lateral lines	4	egg sac	Present	Lateral lines	4
Phasmid position	33% of tail length	Cuticle surface pattern	Zig-Zag			Phasmid position	31% of tail length	Cuticle surface pattern	Zig-Zag		
		C6						**C3**			
L	355.2 ± 12 (337-420)	L	500.4 ± 54.5 (420-569.5)	L	922 ± 53 (768.3-975.2)	L	348. 5 ± 15 (313.5-400)	L	497.3 ± 48.1 (423.5-609)	L	947.9 ± 77.9(892.3-1180.0)
a	19.4± 0.9 (16.7-22.5)	W	381 ± 52.2 (305-491.5)	a	32.4 ± 1.9 (30-42)	a	18.3 ± 1.1 (15.7-23.1)	W	372.1 ± 57.2 (325-517.5)	a	40.2 ± 2.0 (31.3-43.1)
b	3.7 ± 0.3 (2.4-4.2)	neck length	60.5 ± 12 (52-78)	b	8.5 ± 0.7 (7.6-11)	b	3.3 ± 0.4 (2.2-3.7)	neck length	60.2 ± 11.6 (53.2-77.2)	b	9.1 ± 0.6 (7.9-12.4)
b'	2.1± 0.5 (1.8-2.4)	L/W	1.1 ± 0.1 (1.0-1.3)	b'	5.0 ± 0.4 (4.7-6.1)	b'	2.0 ± 0.7 (1.8-2.6)	L/W	1.5 ± 0.4 (1.2-1.6)	b'	5.7 ± 0.2 (5-7)
c	7.7 ± 0.7 (7.1-10.1)	Semienestral width	32.4 ± 5 (35-47)	Stylet	25 ± 1.2 (23.1-25)	c	8.6 ± 0.5 (7.0-12.2)	Semienestral width	40.6 ± 3.1 (33-43.7)	Stylet	21.7 ± 1.1 (18.5-23.6)
c'	4.5 ± 0.2 (4.0-4.8)	Semifenestral lenght	14.2 ± 4.1 (10.2-18)	Gubnaculum	5.9 ± 0.8(5.9-7.5)	c'	2.9 ± 0.4 (2.6-4.0)	Semifenestral lenght	18.2 ± 2.8 (16.5-20.5)	Gubnaculum	5.7 ± 0.8 (4.9-7.2)
Stylet	21 ± 0.5 (19-24.0)	Vulval Slit	37.3 ± 3.5 (24.8-46.5)	Spicules	23.2 ± 2.6 (19-26.3)	Stylet	24.2 ± 0.6 (22.2-25.7)	Vulval Slit	40.6 ± 2.8 (7.2-43.1)	Spicules	23.5 ± 2.9 (18.2-31.2)
Tail	38.4 ± 5 (30.8-44.6)			T	45.2± 12.9 (37-57.2)	Tail	43.5 ± 3.7 (30.2-47.6)			T	51.3 ± 12.1 (47.2-63.5)
H	19.8 ± 1 (16-21.2)					H	23.1 ± 1.7 (13.7-20.7)				
H/Stylet	1 ± 0.2 (0.7-1.1)					H/Stylet	1.6 ± 0.5 (1.1-1.9)				
Head annules	3-4	Underbridge	Weak	Head annules	5	Head annules	4	Underbridge	Weak	Head annules	5-6
Lateral lines	4	Underbridge	Present			Lateral lines	4	egg sac	Present	Lateral lines	4
Phasmid position	32% of tail length	egg sac	Zig-Zag			Phasmid position	33% of tail length	Cuticle surface pattern	Zig-Zag		

(Sanger and Coulson, 1975). The sequences were aligned and blasted by selected sequences from NCBI and phylogenetic trees created using Mega-6® (Tamura *et al*., 2011) and CLC®Genomic Workbench, v7 (CLC Inc, Aaraus, Denmark) softwares considering the default parameters.

Results and Discussion

Nematode identification

Morphology and morphometric characters: The morphometric and morphological features of the populations collected from the region were shown in Table 5. In comparison with previous records and descriptions (Chizhov *et al*., 2010; Stone and Rowe, 1976; Subbotin *et al*., 2010) it was found that all the populations collected from the region was *H. cruciferae* and their morphometrical and morphological data were fully corresponded with those of the other reports documented for this species. No significant variation was found among the populations as the morphological and morphometrical characteristics.

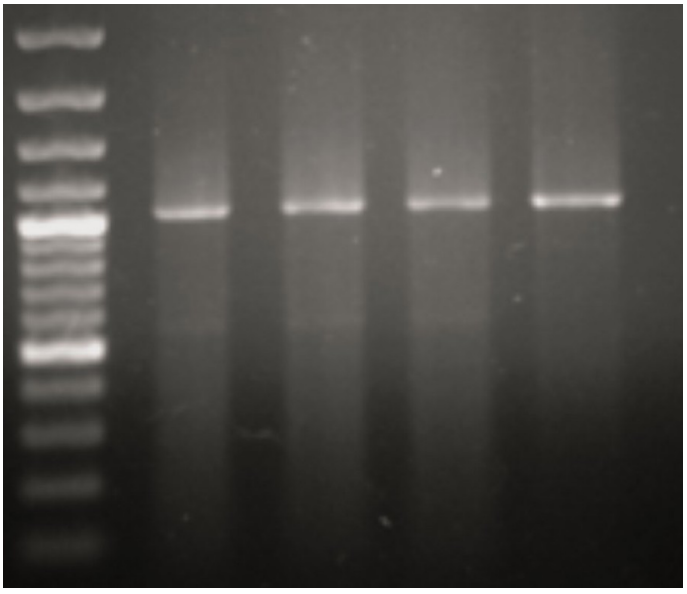

Figure 1: *The amplified ITS1-5.8S-ITS2 of rRNA region of four different populations of H. cruciferae using universal primers (TW81- AB28). (1: Ladder, 2: K6, 3: K3, 4: C6, 5: C3).*

Molecular diagnosis

ITS1-5.8S –ITS2 region: Amplification of the *ITS1-5.8S-ITS2* regions of rRNA gene yielded a single fragment (1250 bp) for all studied populations (Fig. 1). Sequencing, blasting and alignment results approved it. Alignment of the sequence with similar sequences, 1041 bp (Subbotin *et al*., 2010), 1040 bp (Chizhov *et al*., 2009), ~ 1000 bp (Sasanelli *et al*., 2013) and 816-825 bp (Toktay *et al*., 2022) showed more than 95 percent similarity between the sequences. The results of sequencing and blasting of *ITS1-5.8S-ITS2* of rRNA obtained from the four populations of the cabbage cyst nematode revealed high (98 and more than 95 percent) similarity between the populations and the sequences of *ITS1-5.8S-ITS2* of rRNA gene of *H. cruciferae* deposited to NCBI. Since the ITS sequences of the Iranian populations of *H. cruciferae* showed high similarity to each other, only one sequence (yielded from K6 population) submitted to NCBI by Kp114545 accession number.

ITS-RFLP: The ITS-RFLP profile of all four populations digested with the enzymes (Table 4), displayed similar restriction sites. The resulted bands for four (*Mva* I, *BusR* I, *Alu* I, *Rsa* I) out of eight enzymes used by Subbotin *et al*. (2010) were identical with their results. Concerning the enzymes that were used for the first time in our study (Tables 4) only three enzymes namely *Pst* I (173, 340 and 512 bp), *Hin 61* (69, 174, 287 and 495 bp) and *Bsp* 1431(42, 355 and 628 bp) yielded RFLP patterns in the gene region (Figure 2).

No differences were observed among the RFLP-ITS-rDNA profiles of the four populations of *H. cruciferae* (Figure 2). ITS-RFLP is one of the reliable methods for analyzing the differences and similarities among populations and used frequently for identification of nematode populations (Subbotin *et al*., 2010; Baklava *et al*., 2015).

Table 6: *The restriction enzymes (in bold) used for the first time in present study for the ITS-RFLP of four populations of H. cruciferae.*

S.	Enzyme	S.	Enzyme	S.	Enzyme	S.	Enzyme
1	*Hinf* I (No cutting site)	5	*Hin*6 I (69, 174, 287, 495 bp)	10	*Hind* III (No cutting site	14	*Rsa* I (21, 130,321, 569 bp)
2	*Pst* I (173, 340, 512 bp)	6	*Pvu* I (No cutting site)	11	*Kpn* I (No cutting site)	15	*Mva* I (771, 270 bp)
3	*EcoR* I (No cutting site)	7	*Sa* II (No cutting site)	12	*BamH* I (No cutting site)	16	*BsuR* I (24,1070,325,522 bp)
4	*Xba* I (No cutting site)	8	*Msp* I (No cutting site)	13	*Alu* I	-	

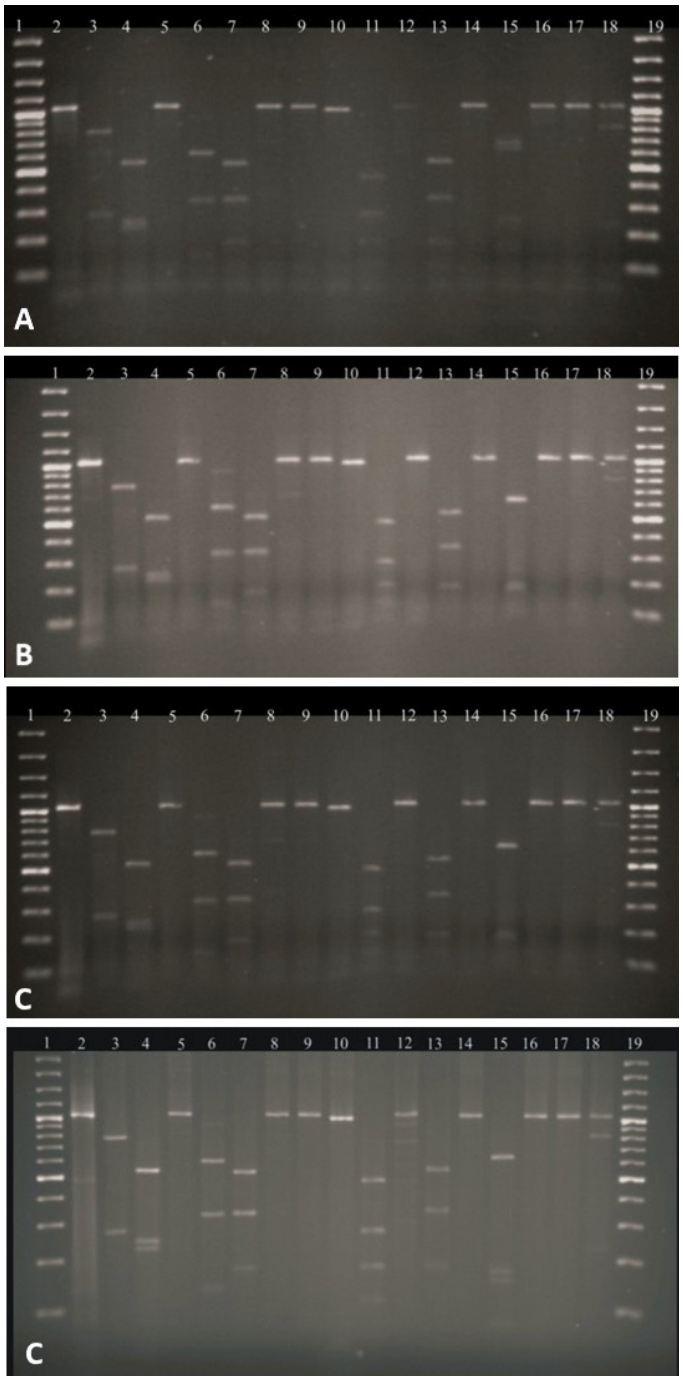

Figure 2: *RFLP-ITS-rDNA profiles of four populations of Heterodera cruciferae collected from Tabriz vegetables growing area against restriction enzymes. A: K6, B: K3, C: C6 and D: C3 (1: Ladder, 2: Intact PCR product, 3: Mva I, 4: Alu I, 5: Xba I, 6: Pst I, 7: Bsu R I, 8: Pvu I, 9: Sa II, 10: Hinf I, 11: Hin 6 I, 12: Eco RI, 13: Rsa I, 14: Bam H I, 15: Bsp 143 I, 16: Hind III, 17: Kpn I, 18: Msp I, 19: Ladder).*

Phylogeny based on ITS1-5.8S-ITS2 of rRNA

The phylogenetic tree (Figure 3) was generated based on the *ITS1-5.8S-ITS2* of rRNA sequence for the under studied populations and seven other sequences belonging to *H. cruciferae*, *H. carotae* and *H. urticae* used by Sasanelli *et al*. (2013). Similar to ITS-RFLP profiles, there are very low differences between different populations of *H. cruciferae* and even *H. carotae* and *H. urticae* populations.

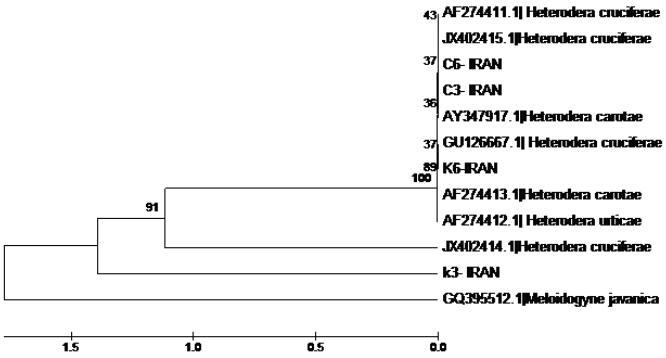

Figure 3: *Phylogenetic tree of Heterodera cruciferae as inferred from the ITS1-5.8S-ITS2 rRNA sequences of populations collected from Tabriz-Iran and other populations using the Maximum Likelihood. The bootstrap consensus tree inferred from 1000 replicates.*

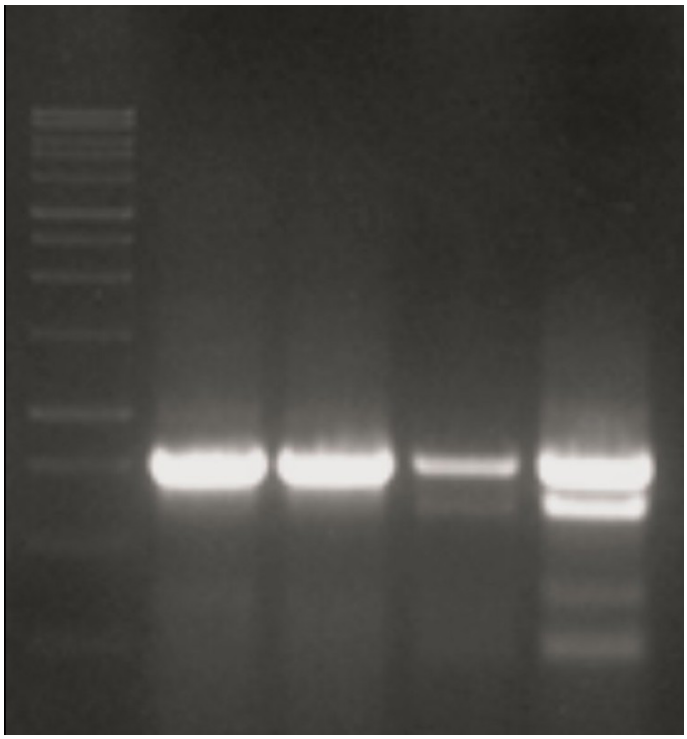

Figure 4: *The amplified D2-D3 of 28S rRNA region obtained from the studied populations of H. cruciferae using specific primers). (1: Ladder, 2: K6, 3: K3). (4: C6, 5: C3) after 3: K3.*

Heterodera carotae and *H. urticae* are morphologically distinguished from each other but their different populations could not be precisely differentiated using *ITS1-5.8S-ITS2* of rRNA gene sequence (Figure 3).

D2-D3 of 28S rRNA

The amplified fragment of *D2-D3* of 28S rRNA

region showed identical size in all the four populations (Figure 4). The similarity between the sequences was more than 95 percent, the length of sequence of *D2-D3* of 28S rRNA obtained from the populations was 750 bp that was deposited to GenBank under Kp114546 accession number.

Phylogenetic tree of the *D2-D3* of 28S rRNA sequence was created and it was found that all the four Iranian populations of the cabbage cyst nematode were clustered at same clade and there was no intraspecific polymorphism among *H. cruciferae* populations but other populations of the cabbage cyst nematode reported by Sasanelli *et al*. (2013) and the outgroup were clustered in different groups (Figure 5).

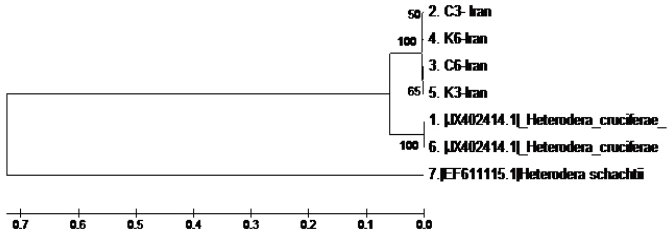

Figure 5: *Phylogenetic tree as inferred from D2-D3 of 28S rRNA sequences of H.cruciferae populations collected from Tabriz-Iran and other populations, using the Maximum Likelihood method. The bootstrap consensus tree inferred from 1000 replicates.*

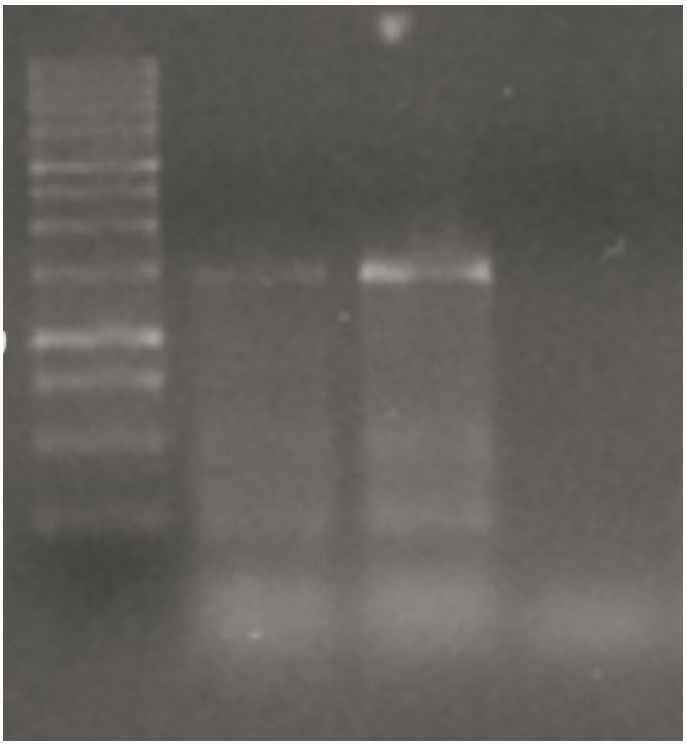

Figure 6: *The amplified hsp 90 gene obtained from the studied populations of Heterodera cruciferae using specific primers). (1: Ladder, 2: K6, 3: K3).*

hsp 90 gene

The *hsp* 90 gene was amplified and it was revealed that the length of *hsp* 90 gene in *H. cruciferae* was about 1250 bp. Since the primers used in the PCR was degenerated, amplification of the region was only completed in two (K6 and K3) populations. The PCR product profile of both populations was found to be similar and sequences of the two populations had 96 percent similarity in alignment (Fig. 6). The partial sequence lenght of the *hsp 90* gene of Iranian populations of *H. cruciferae* was 826 bp and was submitted to NCBI under Kp114552 accession number.

As there was no previous record of *hsp* 90 gene for the *H. cruciferae* in NCBI, creating of phylogenetic tree by using the *hsp* 90 gene sequences was not possiable.

Cabbage cyst nematodes host plants are mostly in Brassicaceae and Lamiaceae families. *Brassica oleracea, Chenopodium album, Raphanus sativus*, *Coriandrum sativum* and *Sisymbrium loeselii* as main cultivated cruciferous species in the vegetable farmlands of Tabriz naturally and annually infected by *H. cruciferae* (Khanzad-Bonab and Jabbari, 2019). Tabriz vegetable farmland are under cultivation of different vegetables for many years. For this long time, the nematode has enough time to establish itself on the crops as a pathogen. Because of the availability of different host plants and weeds belonging to the two above mentioned families in this region as well as cultivation of a so long time, it was thought that especial and certain differentiations of the nematode might have been occurred among various host plants. But, the results of this study showed similarity of morphological, morphometrical and molecular characters between the main and dominant populations of the nematode on cabbages. The populations of *H. cruciferae* were not clustered in a single clade based on *ITS1-5.8S-ITS2* rRNA sequences. PCR-ITS-RFLP results showed that all four studied populations are similar to each other and to PCR-ITS-RFLP profiles generated by effect of 16 restriction enzymes on *H. cruciferae* populations, too. Because these results may relate to the selected gene region for studies which is not appropriate for diagnosis of differences among the populations or may be related to high similarity of the genome region in *H. crucifeare*, *H. carotae* and *H. urtica* species as well. Mehaline *et al*. (2020) showed that

while *H. cruciferae* and *H. carotae* are very close species to each other. On the other hand, the results show different of ITS1, ITS2 regions sequences capacity to making distinguish between different populations of the nematodes, are different (Subbotin *et al.*, 2001; Mehaline *et al.*, 2020). On the other hand, researchers like Escobar *et al.* (2018) mention that ITS rRNA and *CO*I partial sequences do not have potential to make differentiation between *H. cruciferae* and *H. carotae* and host range is very crucial for the species separation.

D2-D3 of 28S rRNA sequences also is frequently used, mostly at species level, by some researchers for molecular studies in different groups of nematodes (Van Den Berg *et al.*, 2016; Wang *et al.*, 2013; Skantar *et al.*, 2012; Douda *et al.*, 2010). However, it used at a species level in different *Globodera pallida* and *G. rostochiensis* populations by Douda *et al.* (2010). The results of this study revealed that the ability of the gene for differentiation between populations is far better than *ITS1-5.8S-ITS2* region. It could be due to shorter length and less variability in D2-D3 28SrRNA of the gene region, in current study all the four populations made a separate clade compared to the populations reported from other localities based on the region sequence and it confirmed that all populations are same.

Skantar and Karta (2004) used *hsp* 90 gene sequence for molecular characterization and phylogenetic evaluation of some nematodes including *H. glycines*. Skantar *et al.* (2012) also used the gene for molecular identification of different populations of *Hetrodera. zeae*. In current study he results of *hsp* 90 sequences were similar to those of *D2-D3* of 28S rRNA. Since there is not any similar study on cabbage cyst nematode using this part of nematode genome, it was not possible to do any more comparison.

Sequence-based methods may involve analyses of nucleotide sequence either the nuclear DNA, mitochondrial DNA (mtDNA) or the whole genome of nematodes. The rDNA and mitochondrial cytochrome c oxidase subunit I (*CO*I) genes are preferred by most studies on nematode (Derycke *et al.*, 2010; Van Megen *et al.*, 2009). Mitochondrial genes have great ability to diagnose different populations and races of cyst nematodes (Subbotin *et al.*, 2010). Subbotin *et al.* (2015) noticed the different populations of *Longidorus* discordance in phylogenetic relationships inferred from the ITS1 rRNA and *COX*- I gene sequence datasets. Toktay *et al.* (2022) recently used *COX* -I and rDNA-ITS region sequences for identification of cabbage cyst nematode in Nigde province and Turkish populations were separated from each other using *ITS* and *COX* -I genes sequences however, except one of the studied populations which showed *rRNA-ITS* sequences similarity with populations from Italy, Algeria, the Netherlands and Russia, the other studied populations were similar to Iranian *H. cruciferae* populations. Escobar *et al.* (2018) by working on *H. carotae* and *H. cruciferae* populations also confirmed the ability of *COX* -I gene for the population's differentiation. While the results of current study confirm similarity in all four populations of *H. cruciferae* in Tabriz farmlands, it sounds that the ability of different sequences to making differentiation between populations is not a fix and it can show different capability upon to species. Morphometrical and morphological data in all cases are crucial and should be the main and important part of any identification.

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Novelty Statement

Novelty of the research are:

- First study about identification of different population of the nematode based on traditional and molecular methods.
- Identification of some restriction enzymes cutting sites on the nematode.
- Identification of partially sequence of hsp 90 gene in *H. cruciferae*

Conflict of interest

The author has declared no conflict of interest.

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