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Original Contribution

ASSESSMENT OF GENETIC DIVERSITY AMONG *SCLEROTINIA SCLEROTIORUM* POPULATIONS IN CANOLA FIELDS BY REP-PCR

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ABSTRACT

The genetic diversity of *Sclerotinia sclerotiorum* populations recovered from canola was assessed using rep-PCR genomic fingerprinting. By using four rep-PCR primers, 1927 polymorphic bands out of a total of 2003 (96.2 %) were generated in 38 isolates of *S. sclerotiorum*. At the species level, Nei's gene diversity (*h*) was 0.233 and Shannon's index of diversity (*I*) was 0.376. Genetic diversity based on percentage of polymorphic bands ranged from 59.5% to 75.21%, demonstrated high level of genetic diversity. The cluster analysis based on UPGMA and Jaccard´s coefficient showed that most isolates from the same regions were grouped in the same cluster or a close cluster. The Nie's genetic identity illustrated that populations from Hashem Abad and Ali Abad were genetically close, while the population from Kalaleh was found to be the most diverse from the others. The variability found within closely related isolates of *S. sclerotiorum* demonstrate the effectiveness of rep-PCR marker in identifying genetic diversity among *S. sclerotiorum* isolates.

Key words: *Sclerotinia sclerotiorum*, MCG, rep-PCR, Nei's gene diversity

INTRODUCTION

The fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is a necrotrophic filamentous ascomycete plant pathogen with worldwide distribution that infects a large number of plants including crop species (1). This fungus can causes Sclerotinia stem rot on canola (oilseed rape, *Brassica napus* L.), which leads to serious losses in yield due to lodging and premature shattering of seedpods (2). Control of this disease is difficult because of broad host rang of its causal agent and long period survival of sclerotium, a pigmented resting structure of this pathogen, of it in soil (3).

Control strategies must target a population instead of an individual if they are to be effective. Thus, it should be focused more effort on understanding the genetic structure of fungal populations to understand how populations will evolve in response to different control strategies (4). Genetic variation among populations of *S. sclerotiorum* was assessed using two presupposed unrelated criteria, mycelial compatibility groups (MCG) and molecular markers (5). Mycelial (vegetative) compatibility grouping, the ability of two fungal strains hyphae to anastomose and form one integrated colony, in filamentous fungi are controlled by multiple loci (6). MCGs represent genetically distinct individuals, in other words they tend to be genetically isolated from each other (5). Of the molecular markers, DNA fingerprinting with Random amplified polymorphic DNA, RAPD (7), Inter simple sequence repeat, ISSR (8) and simple sequence repeat, SSR, (9) have revealed diversity in *S. sclerotiorum* genotypes.

Families of short interspersed repetitive elements are present in eubacteria, these are the repetitive extragenic palindromic (REP) elements, entrobacterial repetitive intergenic consensus (ERIC) sequences and the BOX element (10). Consensus primers to each of elements have been used in polymerase chain reactions to amplify regions between neighboring repetitive elements (11). This process called rep-PCR that has recently been applied with success in fingerprinting of several fungal genera (12, 13). The aim of the work presented here was to test the suitability of the rep-PCR as a tool for investigating

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genetic diversity among *S. sclerotirum* populations in canola.

MATERIAL AND METHODS

Sampling, culturing and testing of mycelial compatibility of *S. sclerotiorum* isolates

Naturally infected stems of canola were sampled from Golestan, Mazandaran and Western Azarbaijan provinces in 2005 and 2006. For isolation, sclerotia inside stems were surface sterilized by washing them for 1 min in 70% ethanol and 30% sodium hypochlorite, then rinsed three times with sterile distilled water. Finally, sclerotia were air dried on sterile filter paper for 10 min and placed on potato dextrose agar (PDA) plates. Plates were incubated in the dark at room temperature (20-22˚C). Hyphal tips were isolated on water agar (WA) and transferred to PDA plate. All mycelial cultures were maintained on PDA slants and stored at 4˚C for use throughout the study.

Mycelial compatibility testing was performed on PDA amended with 175 µl/L of McCormick's red food coloring as described previously (14). All pairings were scored after incubation in the dark at room temperature (20-22˚C) for 7 and 14 days and each pairing was performed twice.

Extraction of genomic DNA

To obtain mycelial mat, isolates were grown in complete yeast medium broth containing 0.46 g KH_2PO_4, 1 g K_2HPO_4.3H2O. 0.5 g $MgSO_4.7H_2O$, 20 g D-glucose, 2 g yeast extract (Difco) and 2g Bacto-peptone (Difco) per liter, at room temperature for 3 days. The mycelium was harvested by vacuum filtration on sterile filter paper and stored in -70˚C quickly. Total genomic DNA of isolates was extracted as described by Safaie *et al*. (15) and quantified spectophotometrically at 260 nm.

Rep-PCR amplification and gel electrophoresis

Thirty-eight *S. sclerotiorum* isolates representative of 38 MCGs were selected for examining of genetic diversity. The used primer sets and their optimized annealing temperature were listed in **Table 2.** PCR amplifications were performed in a thermocycler (Eppendorf AG, Germany) at least twice in 25 µl volumes containing 40 ng of template DNA, 1 µM primer (CinaGen, Tehran), 200 µM each of four dNTPs, 1.5 units of *Taq* polymerase (CinaGen, Tehran), 2 mM $MgCl_2$ and 2.5 µl PCR buffer (50mM KCl, 10mM Tris-HCl, pH 9.0). The rep-PCR cycling condition was performed as follows: an initial denaturation for 2 min at 95˚C followed by 35 cycles of 1 min at 94˚C; 90s at annealing temperature, 2 min at 72˚C with a final extension for 8 min at 72˚C. Each PCR experiment was included a control lacking template DNA.

PCR products were resolved by electrophoresis at 1.5% agarose gel, for 3 h at 80 V in 1X TBE buffer (89 mM Tris, 89 mM boric acid, and 2mM EDTA) and followed by staining with ethidium bromide (0.1 µg μl^{-1}). Gel was photographed with Gel Documentation System (Vilbert, Lourmat, Marne La Vallee, France).

Data analysis

The molecular size of each fragment was estimated using Photo-capt software (Image Analysis Software, Vilber Lourmat, France). Banding patterns were scored as presence (1) or absence (0) of a band in each isolate for each primer. Similarity matrices were obtained by the unweighted pair group method using arithemetic averages (UPGMA) and Jaccard's coefficient. Clustering analysis was performed on data generated from each primer separately and on the combination data gained from each primer with the NTSYSpc2 program (16). The goodness of the dendrograms was assessed by bootstrap analysis with the WINBOOT program and 1000 repeated samplings with the replacement (17). The average number of alleles (*na*), the number of effective alleles (*ne*), Nei's gene diversity (*h*) and Shannon's index of diversity (*I*) were calculated using POPGENE 1.31 software (18).

RESULTS

Mycelial compatibility grouping

Twenty hundred and five isolates were obtained. From these, 64 isolates were selected for compatibility interaction and were mostly from Golestan province because canola is largely growing in this province **(Table 1)**. Among these tested isolates, 38 MCGs were identified (Karimi *et al*., unpublished data).

Table 1. *Sclerotinia sclerotiorum isolates grouped by name of isolates, location and MCG*

Name of isolate	Location	MCG
H1.4	Golestan (Hashem Abad)	1
H1.8	Golestan (Hashem Abad)	1
H1.9	Golestan (Hashem Abad)	1
H2.2	Golestan (Hashem Abad)	2
H2.3	Golestan (Hashem Abad)	2
H2.5	Golestan (Hashem Abad)	3
H3.3	Golestan (Hashem Abad)	3
H3.5	Golestan (Hashem Abad)	4
H3.6	Golestan (Hashem Abad)	4
H3.8	Golestan (Hashem Abad)	5
H4.4	Golestan (Hashem Abad)	5
H4.6	Golestan (Hashem Abad)	6
H4.9	Golestan (Hashem Abad)	6
H5.6	Golestan (Hashem Abad)	7
H5.7	Golestan (Hashem Abad)	7
H6.7	Golestan (Hashem Abad)	8
H7.10	Golestan (Hashem Abad)	8
H8.5	Golestan (Hashem Abad)	9
H8.9	Golestan (Hashem Abad)	9
H8.10	Golestan (Hashem Abad)	10
H9.3	Golestan (Hashem Abad)	10
H9.4	Golestan (Hashem Abad)	10
A1.4	Golestan (Ali Abad)	11
A1.6	Golestan (Ali Abad)	12
A2.1	Golestan (Ali Abad)	13
A2.6	Golestan (Ali Abad)	14
A3.2	Golestan (Ali Abad)	15
A3.8	Golestan (Ali Abad)	16
A4.3	Golestan (Ali Abad)	17
A4.9	Golestan (Ali Abad)	18
A5.1	Golestan (Ali Abad)	19
A5.8	Golestan (Ali Abad)	20
A7.5	Golestan (Ali Abad)	21
A7.7	Golestan (Ali Abad)	22
A8.6	Golestan (Ali Abad)	23
A8.9	Golestan (Ali Abad)	24
A9.5	Golestan (Ali Abad)	25
A9.8	Golestan (Ali Abad)	26
A10.3	Golestan (Ali Abad)	26
K1.4	Golestan (Kalaleh)	27
K1.11	Golestan (Kalaleh)	27
K2.5	Golestan (Kalaleh)	28
K2.6	Golestan (Kalaleh)	29
K2.7	Golestan (Kalaleh)	29
K2.9	Golestan (Kalaleh)	28
K3.8	Golestan (Kalaleh)	30
K3.9	Golestan (Kalaleh)	30
K4.7	Golestan (Kalaleh)	31
K5.6	Golestan (Kalaleh)	32
K5.8	Golestan (Kalaleh)	32
K6.6	Golestan (Kalaleh)	31
K8.6	Golestan (Kalaleh)	33
K9.1	Golestan (Kalaleh)	33
K9.2	Golestan (Kalaleh)	34
K9.9	Golestan (Kalaleh)	34
K10.1	Golestan (Kalaleh)	35
K10.10	Golestan (Kalaleh)	35
S-S Asli*	Golestan (Gorgan)	36
S-S 84*	Golestan (Gorgan)	36
A11*	Mazandaran (Ghaem Shahr)	37

Name of isolate	Location	MCG
A15*	Mazandaran (Ghaem Shahr)	37
B110*	Mazandaran (Ghaem Shahr)	37
Name of isolate	Location	MCG
Q1	Western Azarbaijan (Urmieh)	38
Q2	Western Azarbaijan (Urmieh)	38

*These isolates collected in 2005 and the others in 2006

Analysis of rep-PCR genomic fingerprints

Distinct and reproducible DNA fingerprint patterns were generated using four rep-PCR primers (REP 2-I, ERIC 1R, ERIC 2I and BOX 1AR). The primer ERIC 1R amplified twelve to 27 bands in the size ranging 0.3 to 4.3 kb **(Figure 1)**. The ERIC 2I generated six o 17 bands ranging in size 0.27 to 4.8 kb. The BOX 1AR amplified three to twelve bands per isolate which were 0.3 to 4.8 kb in size. The REP 2-I yielded nine to fourteen bands ranged in size 0.26 to 6 kb. A total of 2003 bands were amplified of which 1927 (96.2%) were polymorphic. Different primers revealed different levels of polymorphisms among the tested isolates. The highest number of bands was 765 with primer ERIC 1R, whereas primer BOX 1AR generated the minimum of 253 bands. The average number of bands per primer across the 38 isolates was 13.16. Cluster analysis of combined data revealed four clusters among the 38 isolates at 64 % similarity level **(Figure 2)**. Cluster A included 81.25% of Ali Abad isolates and three ones (Q1, A15, S-S Asli) from other regions. All isolates of Hashem Abad and 55.56% of Kalaleh isolates grouped into cluster B. Cluster C, D, E, F, G and H constituted of one isolate each. Jaccard similarity coefficient ranged from 0.28 to 0.88. Maximum similarity was found between isolates A2.1 and A2.6. At 80 % similarity level isolates placed into 31 clusters, indicating high level of genetic diversity among studied isolates of *S. sclerotiorum*. The cophentic correlation (r) was 0.96 indicating a good fit of the cluster analysis to the similarity data. Bootstrap values shown in dendrogram are more than 68% for branches formed between similarity levels 0.28 and 0.64 indicated the robustness of the dendrogram.

Table 2. *Sequence and optimized annealing temperature of rep-PCR primers used for the 38 Sclerotinia sclerotiorum isolates*

8	Sequence	Annealing temperature (˚C)	Reference
ERIC1R	5'-ATGTAAGCTCCTGGGGATTCAC-3'	45	Purkayastha *et al*., 2008
ERIC2I	5'-ATGTAAGTGACTGGGGTGAGCG-3'	51	Purkayastha *et al*., 2008
REP2-I	5'-ICGITTATCIGGCCTAC-3'	41	Purkayastha *et al*., 2008
BOX 1AR	5'-CTACGGCAAGGCGACGCTGACG-3'	66	Purkayastha *et al*., 2008

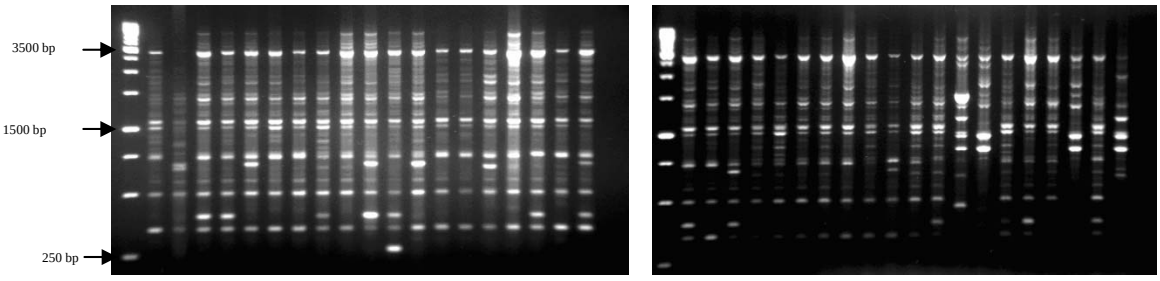

Figure 1. DNA fingerprinting of 38 selected isolates of *Sclerotinia. sclerotiorum* using ERIC IR primer. M: 1 Kb GeneRuler^{TM} size marker, Fermentas; NC: Negative Control

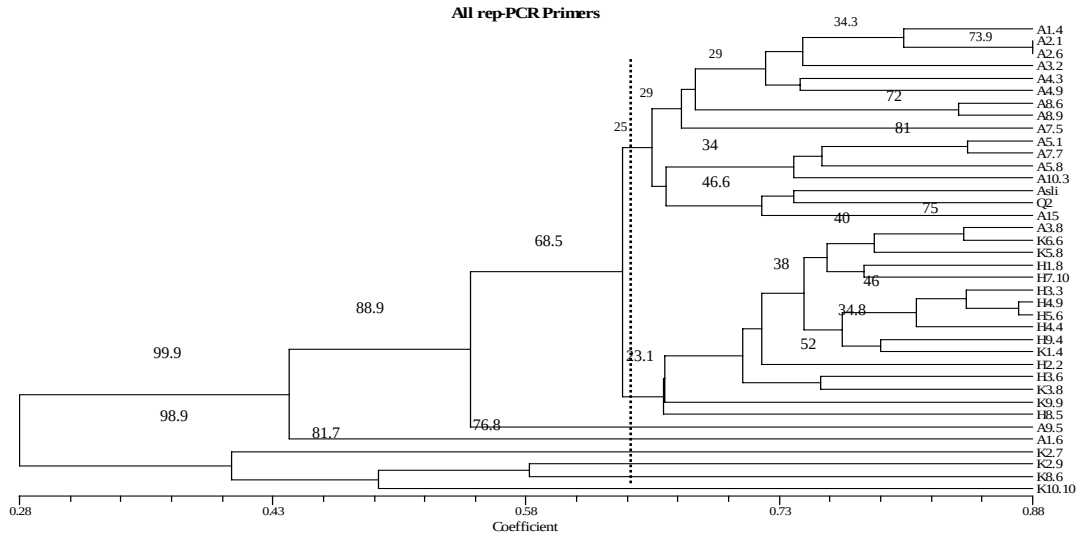

Figure 2. Jaccard/UPGMA cluster analysis of combined rep-PCR primers for 38 *Sclerotinia sclerotiorum* isolates, r = 0.96. Numbers on the main branches are the bootstrap values (%).

The genetic diversity of three populations from Ali Abad, Hashem Abad and Kalaleh was evaluated using Nei's gene diversity and Shannon's index of diversity **(Table 3)**. At the species level, Nei's gene diversity (*h*) was 0.233 and Shannon's index of diversity (*I*) was 0.376. At the population level, the percentage of polymorphic bands (*PPB*), the average number of alleles (*na*) and the number of effective alleles (*ne*) of three populations were different. The population diversity for the isolates originating from Kalaleh was the highest (*PPB* = 75.21, *h* = 0.291, *I* = 0.429) among the three populations. The order of genetic diversity based on Nei's gene diversity and Shannon's index of diversity was as follows (from highest to lowest): Kalaleh > Ali Abad > Hashem Abad. Genetic diversity based on percentage of polymorphic bands ranged from 59.5% to 75.21% in three populations, demonstrated high level of genetic diversity.

Table 3. *Summary of statistical data for genetic diversity of studied Sclerotinia sclerotiorum isolates*

Population	Sample size	Polymorphic loci	*PPB*	*Na*	*Ne*	*H*	*I*
Ali Abad	16	72	59.5	1.595	1.276	0.170	0.265
Hashem Abad	10	45	37.19	1.372	1.206	0.123	0.188
Kalaleh	9	91	75.21	1.750	1.516	0.291	0.429
Mean	11.66	69.33	57.30	1.572	1.333	0.195	0.294
Total	35	118	97.52	1.975	1.361	0.233	0.376
SD				0.156	0.301	0.148	0.192

PPB, percentage of polymorphic bands; *na*, number of alleles; *ne*, number of effective alleles; *h*, Nei's gene diversity; *I*, Shannon's information index; *SD*, standard deviation

The Nei's genetic distance of all selected isolates (ranging from 0.066 to 0.089) were very small and the genetic identity (ranging from 0.915 to 0.936) was high **(Table 4)**. The genetic identity between Hashem Abad and Ali Abad was the highest and indicated that the populations from these two regions were most similar. The population from Kalaleh was the most distant from the others.

Table 4. *Nei's unbiased measures of genetic identity and genetic distance*

Population	Ali Abad	Hashem Abad	Kalaleh
Ali Abad	—	0.936	0.915
Hashem Abad	0.066	—	0.927
Kalaleh	0.089	0.076	—

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

DISCUSSION

Rep-PCR marker was used to determine the genetic diversity among *S. sclerotiorum* isolates on canola in north of Iran. Rep-PCR genomic fingerprinting with primers ERIC, BOX and REP had the capacity to discriminate *S. sclerotiorum* isolates and revealed high level of genetic diversity among isolates, this is in accordance with those obtained in other studies (19-21). In populations of *S. sclerotiorum* diversity can generates by factors, such as immigration, mutation and diversifying selection (22). Furthermore, the observation of high genetic diversity among *S. sclerotiorum* isolates can be due to sexual reproduction by outcrossing (Atallah *et al*., 2004). Although *S. sclerotiorum* is a homothallic fungi but it is possible that microconidia, produced by it which unable to germinate (23), act as spermatia similar to reports in closely related genera (24).

The high similarity between Ali Abad and Hashem Abad populations indicates considerable gene flow (19) and it shows these two populations may originate from one ancestor. This gene flow may be occurring via natural dispersal mechanisms involving ascospores, but may be facilitated by the inadvertent movement of sclerotia by farm machinery (25).

Nei's gene diversity and Shannon's index of diversity were highest for the isolates originating from Kalaleh compared with other regions. This indicates that isolates from Klaleh revealed more polymorphisms than those from the other regions. Therefore, the potential for the emergence of isolates resistant to fungicides and pathogenic on different canola cultivars is possible in Kalaleh. Cluster analysis showed that K2.7, K2.9, K8.6 and K10.10 belonged to Kalaleh (44.45% isolates of Kalaleh) constructed four clusters by self, suggests that new genotypes are evolving in this region. The emergence of new genotypes could be a phylogeographic process (26) or, could be associated with increased cropping of canola (27), leading to movement of *S. sclerotiorum* onto this crop from several host plants.

In conclusion regards to high genetic variation among *S. sclerotiorum* isolates to control Sclerotinia stem rot of canola integration of disease management systems, combining biological, chemical and cultural methods should be applied.

The results of the present investigation demonstrate the effectiveness of rep-PCR genomic fingerprinting in identifying genetic diversity differences among *S. sclerotiorum* isolates. The major advantages of this technique are its simplicity, the universality of PCR primers and tolerance of a wider range of DNA concentrations in generating reproducible results.

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