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POPULATION GENETIC STRUCTURE ANALYSIS OF *SCLEROTINIA SCLEROTIORUM* (LIB.) DE BARY FROM DIFFERENT HOST PLANT SPECIES IN NORTHERN IRAN

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Abstract - The genetic structure of 65 *Sclerotinia sclerotiorum* isolates representing 52 field populations from three provinces in northern Iran were analyzed with Mycalial Compatibility Groupings (MCGs) and five polymorphic microsatellite loci. In total, 44 haplotypes were detected with 25 allele polymorphisms. A high level of genetic diversity was observed in about 67.6% (with clonal fraction = 0.077 to 0.20) of the regional populations studied and the Shannon diversity index (H_o) for the whole region was found to be 0.86 (H_{tot}). Partition of total diversity (H_T) showed that 64% corresponded to a variation in diversity within the *S. sclerotiorum* populations. By mycelial compatibility grouping (MCG) tests, the isolates were classified into 39 groups of which 26 MCGs were individual. Molecular and phenotypic analyses results of all of the isolates (except MCG4 and MCG23) were similar; however, the isolates in the MCG4 and MCG23 groups, with variable microsatellite haplotypes, were morphologically dissimilar. The results shown here were possibly due to high rates of outcrossing, as well as to the evolutionary potential within population of the pathogen in different locations in Iran. Nei's genetic identity showed that populations from Golestan province and wild plants were the most diverse.

Key words: Microsatellites, MCG, *Sclerotinia sclerotiorum*, genetic diversity

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is an important ascomycetous plant pathogenic fungus with wide geographic distribution and a diverse host range including many agronomic crops (Hartman, et al., 1999). Yield losses due to stem rot disease are variable and sometimes reach maximum level in susceptible plants (Purdy, 1979). This pathogen is the causal agent of sclerotinia stem rot in canola leading to serious losses in yield due to the lodging and premature shattering of seedpods (Gugel and Morrall, 1986).

Intraspecific variation in virulence (Marciano et al., 1983; Morral et al., 1972) and in such morphological characteristics as pigmentation of the mycelium, ascus, ascospore, sclerotial size and production (Boland and Smith, 1991; LeToumeau, 1979; Price et al., 1975; Purdy, 1955) have been reported in *S. sclerotiorum*. To identify the intraspecific variation of *S. sclerotiorum*, two presupposed unrelated criteria of mycelial compatibility groups (MCGs), and DNA fingerprinting have been employed (Kohn et al., 1990, 1991). Therefore, knowledge of the population genetic structure and evolutionary potential of the pathogen will provide insight into the most suitable breeding strategy for durable resistance (McDonald and Linde, 2002). The pathogens with higher evolutionary potential pose a greater risk of defeating resistance genes or counteracting other control methods such as applications of fungicides (McDonald and Linde, 2002). Two independent systems have been developed for the characterization of *S. sclerotiorum* genotypes; mycelial compatibility groups (MCGs) and DNA fingerprinting.

The mycelial compatibility-incompatibility grouping system is a useful method in studying the population dynamics of pathogenic and nonpathogenic isolates of fungi. This system is used for ascertaining the source of new races to a particular geographical area. The mycelial compatibility-incompatibility grouping system is an easy, quick, inexpensive, and macroscopic assay of the self/non-self recognition method and is determined using a side-by-side pairing system (Kohn et al., 1990). It has been suggested that MCGs represent genetically dissimilar individuals and each MCG is a particular genotype (Kohn et al., 1990). Infrequently, outbreeding in *S. sclerotiorum*, immigration of strains from other sites (Glass and Kuldau, 1992), genetic exchange, meiotic recombination (Carbone et al., 1999), mitotic recombination, transitory selection, selective neutrality, and diversifying selection (Kohli et al., 1992) are possible sources of the MCG diversity. DNA fingerprinting techniques can also be used to distinguish closely related fungal isolates. Southern hybridization of restriction-digested whole genomic DNA to cloned probe containing a 4.5 kb repeated dispersed element of nuclear DNA from *S. sclerotiorum* was used by Kohn et al. (1991), and this method was used in several subsequent studies (Kohn et al., 1995; Cubeta et al. 1997). Microsatellites are widely dispersed and evenly distributed in the genome of eukaryotes and have been used to study intraspecific genetic variability within populations (Gaggiotti et al., 1999; Sirjusingh and Kohn, 2001). Microsatellite markers have high specificity, reproducibility, polymorphism and they are co-dominant markers (Vanderkoornhuyse et al., 2001). Recently, Sirjusingh and Kohn (2001) developed 23 microsatellite loci and 2 microsatellite-like polymorphic loci containing 2-10 alleles at each locus.

There are several reports concerning the population structure of *S. sclerotiorum* worldwide. Thirty-nine clones were identified among 66 isolates on canola (oilseed rape) in Canada from seven locations in Alberta, Saskatchewan and Manitoba (Kohli et al., 1992). Limited outcrossing among the *S. sclerotiorum* isolates was observed in North Carolina and California (Kohli and Kohn, 1998). Another study was focused on comparisons of the *S. sclerotiorum* populations collected from agricultural and wild populations in Norway (Kohn, 1995). It was found that there was genetic uniformity among populations on potato and canola; however, there was greater genetic diversity among wild populations. Sun et al. (2005) compared three *S. sclerotiorum* populations collected from across Europe, China and Canada. They found that genetic differentiation among and within populations was highly significant.

There is growing interest in oilseed rape and its production in Iran. Although *S. sclerotiorum* causes severe disease on many field and glasshouse grown crops all over the country, there is no report on analysis of the genetic variation in *S. sclerotiorum* in northern Iran. The aim of this research was to investigate the genetic structure within oilseed rape and the other hosts population of *S. sclerotiorum* in the Golestan, Mazandaran and Gillaun provinces of Iran based on molecular and morphological markers.

MATERIALS AND METHODS

Isolates

Isolates of *Sclerotinia sclerotiorum* were collected from 52 rapeseed, lettuce, bean, tomato, and cucumber fields in Gilan, Mazandaran and Golestan, northern provinces of Iran, during the 2006-2007 growth season. Samples were collected from infected plants and the sclerotia were removed from the each plant sample. Single sclerotium was selected as an isolate. The sclerotia were surface sterilized for 1 min in 70% ethanol or 2 min in 2.5% sodium hypochlorite, rinsed in sterile distilled water, plated on potato dextrose agar medium (PDA) and then incubated at 22 °C for two days. Each isolate was purified by transferring the single hyphal tip onto the fresh medium, and generated sclerotia were stored at -20°C until use (Atallah et al., 2004; Cubeta et al., 1997; Willets et al., 1980).

Mycelial Compatibility Group Determination

For evaluation of inter-form MCG variability, five

isolates from each field were paired together. 0.5cm-diameter mycelial plugs were obtained from the edge of two-day-old colonies on PDA. Three mycelial plugs were paired in 6.5 cm-diameter Petri dishes containing PDA amended with 75 µl of Wilton's red food coloring per liter of culture medium (Kohn et al., 2006). Petri dishes were incubated in the dark at 22°C for 14 days. Pairings of the 65 isolates were performed in a pyramidal design, where groups of 10 isolates were paired in all-pairwise combinations. Three replications were made for each pairing. Only incompatible isolates were paired subsequently. Compatible isolates were distinguished by the fusion of mycelia, without an accumulation of red dye in the fusion zone. Incompatible reactions produced a barrage recognized by an obvious red line on the bottom side of Petri dishes or by the formation of aerial mycelia along the barrage line. Pairings that yielded questionable reactions were repeated to ensure accurate results.

Temperature treatments

The radial growth of the *S. sclerotiorum* isolate was assessed at five different temperatures, including the optimum temperature (22°C), as well as 10 and 5°C below and above (12, 17, 27 and 32°C). PDA plates were inoculated with a 5 mm-diameter plug of the colonized agar and incubated in the dark at the various temperatures. Colony diameter was measured daily and the values averaged until the colony neared the edge of the dish. In a completely randomized design, four Petri dish replicates of each isolate were used four times. Data were analyzed by ANOVA using MSTAT-C program and mean values were compared at the $p \leq 0.05$ level using Duncan's multiple range test. The color of each colony was noted 21 days after the initial growth on PDA.

DNA extraction

DNA was extracted using the rapid mini-preparation method (Liu Don et al., 2000) with some modifications. This procedure included the following steps: (i) A small lump of the mycelia was added to a 1.5 ml Eppendorf tube containing 500 µl of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate) using a sterile toothpick, then the tube was left at room temperature for 10 min. (ii) After adding 150 µl of potassium acetate (pH 4.8; which was made of 60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml distilled water), the tube was vortexed briefly and spun at 10,000*g* for 1 min. (iii) The supernatant was transferred to another 1.5 ml Eppendorf tube and centrifuged again as described above. After transferring the supernatant to a new 1.5-ml Eppendorf tube, an equal volume of isopropyl alcohol was added. The tube was mixed by inversion briefly. (iv) The tube was spun at 10,000 *g* for 2 min, the supernatant was discarded, and the pellet was air-dried. Finally, the pellet of purified DNA was diluted in 1X TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) to a working concentration of 10–20 ng/µl and stored at 5°C (Hopwood et al., 1997).

Microsatellite primers, PCR conditions, separation of PCR products and data analysis

Five sets of microsatellite primers were used in this study, including $(AGAT)_{14}$, $(AAGC)_4$, $(CATA)_{25}$, $(CA)_9$, $(GT)_{10}$ and $(TACA)_{10}$ (Sirjusingh and Kohn, 2001). The PCR reaction mixture included 10-20 ng of the purified DNA and the reaction buffer (100 µM each of dATP, dCTP, dGTP and dTTP, 200 nM of microsatellite primer and 0.8 units of Taq polymerase, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 100 µg mL^{-1} gelatine, 0.05% tween 20 and 0.05% Nonidet P-40). The final reaction volume was adjusted to 50 µL with deionized H_2O. All of the reagents were obtained from Fermentas Inc., USA. Amplification was carried out using initial denaturation at 95°C for 8 min, followed by 35 cycles of primer annealing at 59°C (for all microsatellite primers) and extension at 72°C for 60 s, with a 5 min extension at 72°C on the final cycle. The PCR products were separated on a denaturating agarose gel (2.6% w/v). Gels were stained with ethidium bromide, visualized under UV light and digitally documented with the gel documentation UVP-V system. The gel was run at 90 W for 90 min (Nicholson et

al., 1997). All polymorphic alleles were identified from each microsatellite primer combination and bands representing alleles were scored as present (1) or absent (0). Nei's genetic distance matrix (Nei and Li, 1979) was prepared and bootstrap analysis with 2000 replications was performed to generate a dendrogram of unweighted pair-group mean analysis (UPGMA; Sokal and Michener, 1958) using the Treecon-1.3b program (Van de Peer and de Wachter, 1994).

Polymorphism Information Content (PIC)

The polymorphism information content determines the frequency of allele polymorphism in the gene locus to study population. PIC was calculated for each locus as follows:

$$PIC = 1-\sum^{n}_{i=1} pi^2$$

where *pi* was the relative frequency of *i*th alleles.

Determination of genotypic diversit

Isolates with the same allele combi[illegible] for each of the five loci were located in a haplotyp[illegible] group. Isolates present in a haplotype were obtained from clonal reproduction (asexual reproducti[illegible] or sexual reproduction of the typ[illegible] homothalism). Then the clonal fraction was calcula[illegible] for ev[illegible]y three populations. The clonal fracti[illegible] on re[illegible]s the frequency and prevalence [illegible] colon[illegible]s in populations, calculated from *N-G/ N*, wh[illegible] *N* is the population size and *G* is the haplotype size [illegible] the population (Zhan et al., 2002), and the genotypic diversity is calculated from *G/ N*.

Shannon-Wiener's Index

Shannon-Wiener's index, H_0, was calculated for each population as follows:

$$H_0=-\Sigma(pi \ In \ pi)$$

where *pi* was the frequency of *i*th haplotype (Shannon and Wiener, 1949)

RESULTS

Morphological variability and mycelial compatibility groups among S. sclerotiorum isolates

The growth rate of the isolates differed significantly. The isolates grew faster at 22°C, then at 27, 17 and 12°C, respectively. Isolates grew normally at 12, 17, 22 and 27°C, but weakly at 32°C. The colonies filled the 8-cm diameter Pet[illegible] dis[illegible]s in 3 days at 22°C. ANOVA for the grow[illegible]n rate dat[illegible] showed highly significant *P*-values [illegible]mon[illegible] the iso[illegible]ates at all temperature treatment[illegible] (Table 1). [illegible]h[illegible] color of the colonies on PDA grow[illegible] m[illegible]dium varied among the isolates. There we[illegible] three [illegible]ain [illegible]olony colors: brown to dark brown pig[illegible]entation[illegible] estricted to the region around the inoculati[illegible]n disc; beige pigmentation across the [illegible]ntire colony, a[illegible]nd white (no pigmentation) (Fig. 1). [illegible]Five isola[illegible]es (R28, R30, R33, R36 and R51) exhibited [illegible]own p[illegible]gmentation around the fungal discs and four [illegible]solates (R34, R38, R58 and R64) produced white [illegible]olonies without any pigment. The colony color of the rest of the isolates was beige (Table 1).

Mycelial compatible group variability among S. sclerotiorum isolates

Assessments of compatibility were based on mycelia continuity between the interacting colonies without formation of either a strip of thin mycelium or aerial mycelium, and the uniform distribution of sclerotia in the plate. Evaluation of compatibility was based on the failure of the two colonies to fuse, which was reflected by the formation of a strip of this mycelium or aerial mycelial at the interaction zone (Kohli et al., 1992). Mycelia incompatibility can also be indicated by the formation of a dark line along the interaction zone associated with the red food dye (Kohli et al., 1992). Thirty-nine MCGs were determined among the 65 studied isolates: 26 isolates were established as independent MCGs. The isolate that belonged to the independent MCGs was compatible only with itself. MCG4 and MCG18 consisted of eight and three isolates, respectively. MCG1, MCG5 and MCG 23 included four isolates and eight MCG included only two isolates. The

Table 1. MCGs, colony color and growth rate of *S. sclerotiorum* isolates at different temperatures after 2 days of growth.

Isolates	Host	Site	MCG s [a]	colony color [b]	Colony growth at different temperature (mm) [c]				
					12˚C	17˚C	22˚C	27˚C	32˚C
R4	Lettuce	Dashtenaz	1	Beige	7.0jk	12.5c	57.8jkl	43.8mno	2.4 ab
R19	Rapeseed	Amol	1	Beige	9.3mn	20.1c	70.0abcde	50.3q	6.9qrs
R25	Rapeseed	Rezvanshahr	1	Beige	9.6mn r	19.8c	60.9hij	50.1q	6.4pqr
R26	Rapeseed	Bandar Anzali	1	Beige	9.0m	20.0c	72.0abc	48.9pq	6.8 qr
R5	Lettuce	Kiakolla	2	Beige	4.0f	19.1c	59.0jkl	53.3qrs	8.uv
R27	Rapeseed	Bandar Anzali	2	Beige	4.4fg	5.5b	60.1ijk	57.1stu	8.8vw
R6	Lettuce	Kiakolla	3	Beige	6.8ij	14.1c	58.0jkl	50.5q	8.2 uv
R37	Rapeseed	Nokandeh	3	Beige	6.6ij	12.6c	60.1ijk	50.6q	7.6 tu
R8	Lettuce	Amol	4	Beige	6.5ij	13.8c	56.9jklm	40.5m	5.3jk
R13	Broad bean	Juibar	4	Beige	6.8ij	14.5c	57.8jkl	40.6m	4.8ij
R20	Rapeseed	Amol-hular	4	Beige	6.8ij	16.6c	58.[illegible]	39.5lm	5.0ijk
R22	Rapeseed	Babol	4	Beige	7.1jk	11.6c	5[illegible]9jklm	43.8mno	4.8 ij
R30	Rapeseed	Ghaemshahr	4	Brown	2.5cd	16.8c	68.0cdef	38.5klm	3.4 cd
R49	Rapeseed	Kordkoy	4	Beige	6.6ij	17.6c	68.1cdef	53.8qrs	7.6 tu
R55	Rapeseed	Semeskandeh	4	Beige	7.1jk	12.3c	6[illegible]6cdef	66.8wx	2.6 ab
R64	Rapeseed	Bayekolla	4	White	2.35cd	17.[illegible]c	6[illegible]ij	41.4mn	5.3jkl
R9	Lettuce	Amol	5	Beige	3.0de	[illegible].8c	43.1[illegible]	28.7ghi	1.9 ab
R18	Rapeseed	Amol	5	Beige	1.2b	[illegible].9b	60.2ijk	40.6m	4.8 ij
R23	Rapeseed	Chardangeh	5	Beige	0.9ab	[illegible]	60.[illegible]jk	41.0mn	5.4 kl
R47	Rapeseed	Behshahr	5	Beige	1.2b	6.8[illegible]	[illegible].8ijk	40.5mn	5.3jkl
R10	Broad bean	Kiakolla	6	White	11[illegible]	18.8c	59.0jkl	39.1lm	4.3 fg
R34	Rapeseed	Shirgah	6	White	1[illegible].5pq	19.8c	57.8jkl	40.6mn	4.3 fg
R14	Broad bean	Babol	7	Beige	7.0jk	13.1c	60.2ijk	50.3q	10.0 y
R56	Rapeseed	Semeskandeh	7	Beige	7.6k	[illegible].4c	61.1hij	48.6pq	10.0y
R17	Tomato	Juibar	8	Beige	3.7f	1[illegible].5c	43.1rst	31.6ij	6.4pq
R24	Rapeseed	Rezvanshahr	8	Beige	2.9de	16.8c	43.1rst	28.7ghi	1.9 ab
R21	Rapeseed	Babol	9	Beige	1.3b	11.1c	48.1pq	39.0lm	5.1 jk
R28	Rapeseed	Bandar anzali	10	Brown	7.4jk	14.4c	66.8efg	50.5q	5.4 lm
R33	Rapeseed	Juibar	10	Brown	7.4[illegible]	14.6c	67.2defg	50.3q	5.4 lm
R29	Rapeseed	Juibar	11	Beige	[illegible]c y	12.1c	63.0ghi	55.2s	9.1 xy
R31	Rapeseed	Juibar	12	Beige	1.2b	6.5b	42.5st	33.0ijk	8.1uv
R32	Rapeseed	Juibar	13	Beige	3.1de	7.6b	57.5jkl	44.6no	9.1xy
R43	Rapeseed	Bandarturkaman	13	Beige	3.3e	6.2b	57.1jklm	43.8mn0	8.7vw
R35	Rapeseed	Shirgah	14	Beige	13s	21.8c	51.3nop	49.1pq	3.6de
R36	Rapeseed	Arateh	15	Brown	7.6k	19.4c	73.0ab	50.9pqr	4.9 ij
R38	Rapeseed	Kordekhail-Sari	16	White	0.6a	3.1a	43.1rst	28.7ghi	0.9 a
R39	Rapeseed	Shast kalateh	17	Beige	5.9hi	19.4c	66.0efg	66.1wx	2 ab
R40	Rapeseed	Shast Kala[illegible]h	18	Beige	6.6ij	15.6c	69.0bcdef	50.6qr	4.3 fg
R41	Rapeseed	Kordk[illegible]	18	Beige	6.4ij	15.8c	67.1defg	50q	4.5ghi
R65	Rapeseed	Bay[illegible]olla	1[illegible]	Beige	6.8ij	19.9c	65.1fgh	53.3qrs	4.3 fg
R42	Rapeseed	Kordkoi	19	Beige	7.5k	20.1c	70.0abcde	51.0qr	1.3 a
R44	Rapeseed	K[illegible]dkoi-zare	20	Beige	1.6bc	8.4b	36u	31.1ij	4.5ghi
R45	Rapeseed	[illegible]ordkoi-zare	21	Beige	7.0j	14.0c	57.5jkl	50.3q	10.0 y
R48	Rapeseed	Beh[illegible]ahr	21	Beige	4.5fg	15.5c	59.0jkl	48pq	9.1 xy
R46	Rapeseed	B[illegible]shahr	22	Beige	4.6fg	5.4a	40.1	30.3i	6.3pq
R50	Rapeseed	[illegible]dkoi-K[illegible]	23	Beige	7.4jk	20.1c	54.5lmno	41.1mn	1.8 a
R57	Rapeseed	Hulla[illegible]	23	Beige	6.6ij p	12.3c	52.8mno	46.1no	3.4 cd
R58	Rapeseed	[illegible]ular	23	White	1.8bc	8.3b	44.4qrst	32.8ijk	4.6 hi
R59	Rapeseed	Hular	23	Beige	6.6ij	15.6c	51.1nop	43.3mno	4.3 fg
R51	Rapeseed	[illegible]dkoi-Kar.	24	Brown	4.0f	5.9c	54.5lmno	40.3m	3.5 de
R52	Rapeseed	Galugah	25	Beige	9.0m	18c	45.5qr	30.6hij	2.4 ab
R53	Rapeseed	Suteh	26	Beige	12.1qr	18.6c	43.1rst	31.6ij	6.4pq
R54	Rapeseed	Semeskandeh	27	Beige	10.0no	18.0c	65.0fgh	11.9ab	4.9 ij
R60	Rapeseed	Sari	28	Beige	11.6pq	19.8c	74.0a	60.1u	2.0 ab
R61	Rapeseed	Dashtenaz	29	Beige	2.0c	8.3b	44.5qrst	30.2i	4.6 hi
R62	Rapeseed	Dashtenaz	30	Beige	14.5u	20.5c	50.0op	33.1ij	9.4 y
R63	Rapeseed	Garakhail	31	Beige	11.3pq	20.6c	55.5klmn	36.8kl	5.6mn
R15	Wild sinapis	Juibar	32	Brown	6.4ij	15.6c	57.5jkl	38.5klm	4.8 ij
R16	Wild sinapis	Kordkoichardeh3	33	Brown	7.1j	19.1c	54.0lmno	41.1mn	4.3 fg
R1	Cucumber	Bahnamir	34	Brown	7.4jk	14.6c	59.0jkl	48.6pq	4.3 f
R2	Cucumber	Bahnamir	35	Beige	3.1de	14.4c	66.0efg	53.3qrs	4.5ghi
R3	Cucumber	Juibar	36	White	2.9de	21.8c	56.9jklm	30.3i	6.1pq
R7	Lettuce	Juibar	37	Brown	7.5jk	11.8c	43.1rst	11.5ab	4.9 ij
R11	Broad bean	Kiakolla	38	Beige	5.9hi	14.6c	52.3no	33.1ij	5.4 lm
R12	Broad bean	Kiakolla	39	Beige	3.3e	13.5c	59.2jkl	47.7pq	3.5 de
			LSD		1.287	1.902	2.055	2.820	0.845

Notes:
MCGs, mycelial compatibility groups. Colors were recorded 21 days after sub culturing on PDA, The numbers represent the colony diameters (inoculation disc subtracted) of the isolates on PDA medium 2 days post-transfer on 8 cm plates. Mean values within a column followed by the same letter are not significantly different at the $P \leq 0.05$ level.

Table 2. Number of alleles recognized for each microsatellite marker set in the *S. sclerotiorum* population used in the present study compared with those from previous studies

Repeat motifs	PIC	Locus (Accessions No.)	Allele Number	Previous reports	Other species
$(CA)_9$	0.499	12-2 AF377906	2	4[a] 2[b]	No
$(GT)_{10}$	0.666	7-3 AF377909	4	5[a] 2[b]	No
$(CATA)_{25}$	0.816	106-4 AF377921	6	10[a] 3[b]	Yes
$(AGAT)_{14}(AAGC)_4$	0.866	114-4 AF377923	8	8[a] 4[b]	Yes
(TACA)10	0.754	55-4 AF377918	5	[b]	Yes

Notes: [a] Sirjusingh and Kohn, 2001 [b] Atallah et al. 2004

Table 3. Shannon diversity index and Clonal fraction of haplotypes *S. sclerotiorum* populations from different locations.

location	Haplotypes	Sample size	H_0	H_{avr}/H_{tot}	H_{tot}. H_{avr}/H_{tot}	*Clonal fraction*
Gilan	4	5	0.602			20%
Mazandaran	38	13	1.568			19.15%
Golestan	12	47	1.079			7.7%
Total	44	65	1.509	0.64	0.36	
	(a)		(b)	(c)		

remaining 26 isolates were compatible only with themselves (Table 1).

Genetic diversity among the S. sclerotiorum isolates

The microsatellite primers exhibited 25 clear polymorphic alleles from the 65 fungal samples. The number of polymorphic alleles per locus ranged from 2 to 8 (Table 2).

The (AGAT)14(AAGC)4 repeat motif revealed the highest number of polymorphic alleles (8) (Fig. 2) in the population while the primer combination with $(CA)_9$ repeat motif revealed the least (2). The polymorphism information content (PIC) for each locus calculated separately that was found out, ranging from 0.499 for $(CA)_9$ to 0.866 for $(AGAT)_{14}(AAGC)_4$ (Table 2) .

In order to determine the genetic relationships among populations of fungal isolates, a separate matrix was used for the data obtained from the 25 polymorphic alleles, and the fungal isolates were subsequently grouped by UPGMA cluster analysis. The dendrogram representing microsatellite marker polymorphisms among the fungal isolates is illustrated in Fig. 3.

The Shannon diversity index of haplotypes for the three regions (H_{tot}), ranged from 0.602 for Gillan province to 1.568 for Mazandaran province, with a mean of 1.083(H_{avr}). Overall, diversity was higher in the population from Mazandaran than in those from Gillan and Golestan. Partition of total diversity showed that 64% corresponded to a variation of diversity within *S. sclerotiorum* populations, while only 36% of diversity was responsible for variability among these populations (Table 3).

According to the dendrogram, nine major clusters can be defined among the 65 isolates. Although a high level of diversity was observed between the

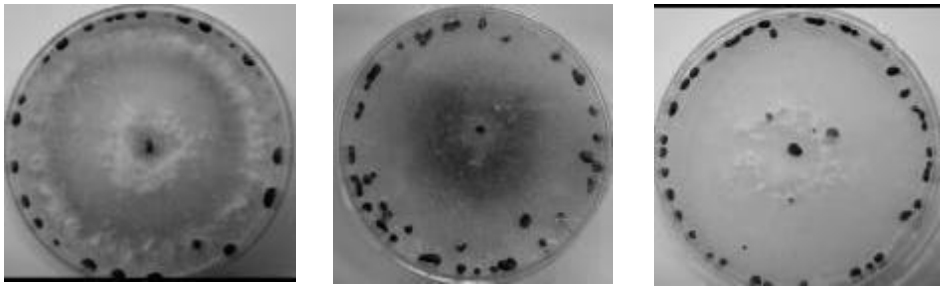

Fig. 1. The colony colors of *Sclerotinia sclerotiorum* isolates on PDA media, 3 weeks after culturing.(A) beige Pigmentation; (B) brown pigmentation; (c) no pigmentation (white).

Fig. 2. Microsatellite analysis of *Sclerotinia sclerotiorum* isolates amplified with the (CATA) primer. PCR amplicons were separated on a 2.6% agarose gel in 0.5 X TBE. Bands were stained with ethidium bromide and visualized on a UV-Transeluminator.

clusters, some isolates within clusters were identical for all the microsatellite markers. For instance, isolates R28 and R33 had similar alleles for all the microsatellite markers. Overall, 44 distinct isolates were identified among the 65 isolates based upon microsatellite alleles.

DISCUSSION

The genetic diversity of 65 isolates of *S. sclerotiorum* on different hosts representing three geographic populations from Iran were studied using five microsatellite loci. In the present study, we found that microsatellite markers were very efficient in identifying genetic variation among the isolates, although we employed only five of the 25 marker sets described previously (Sirjusingh and Kohn, 2001) that had revealed polymorphisms among the Iranian isolates. When we compared our findings with those by Atallah et al. (2004), we found one more allele at $(TACA)_{10}$, two more alleles at $(GT)_{10}$, three more at $(CATA)_{25}$ and four more at $(AGAT)_{14}$ $(AAGC)_4$ loci. In contrast, Sexton and Howlett (2004) identified more alleles at five microsatellite loci than reported by Atallah et al. (2004).

We found 44 different clones (haplotypes) among the 65 isolates representing the population, indicating a high rate of variability in the region ($\hat{G}$/N%=66.7%). On average this is similar to that found in Australian populations ($\hat{G}$/N%=36 to 80% (Sexton and Howlett, 2004); $\hat{G}$/N%=28 to 68% (Sexton et al., 2006)) by analyzing eight microsatellite loci, and Atallah et al. (2004) showed that 92% of the variability among 167 isolates was found within subpopulations in the Columbia basin of Washington State. The genotypic diversity obtained in Turkey (63%) (Mert-Türk et al., 2007) was also similar to the current study.

Although a high level of variation between clusters was observed, some of the isolates within a cluster were similar at all marker microsatellites. In 50% of the isolates, *S. sclerotiorum* divided into 9 large clusters, which indicates the diversity in these isolates is high. Based on this cluster, the population of the Golestan province completely separated from the other populations (Gilan and Mazandaran). In addition, the *Sclerotia* wild population isolated from wild mustard were placed in separate haplotype and mycelia compatibility groups. According to the semi-arid conditions of Golestan province compared to the temperate climates (Mazandaran and Gilan) this phenomenon was predictable and in accordance with the evolutionary history of population haplotypes (Carbone and Kohn, 2001), which indicated that isolates may

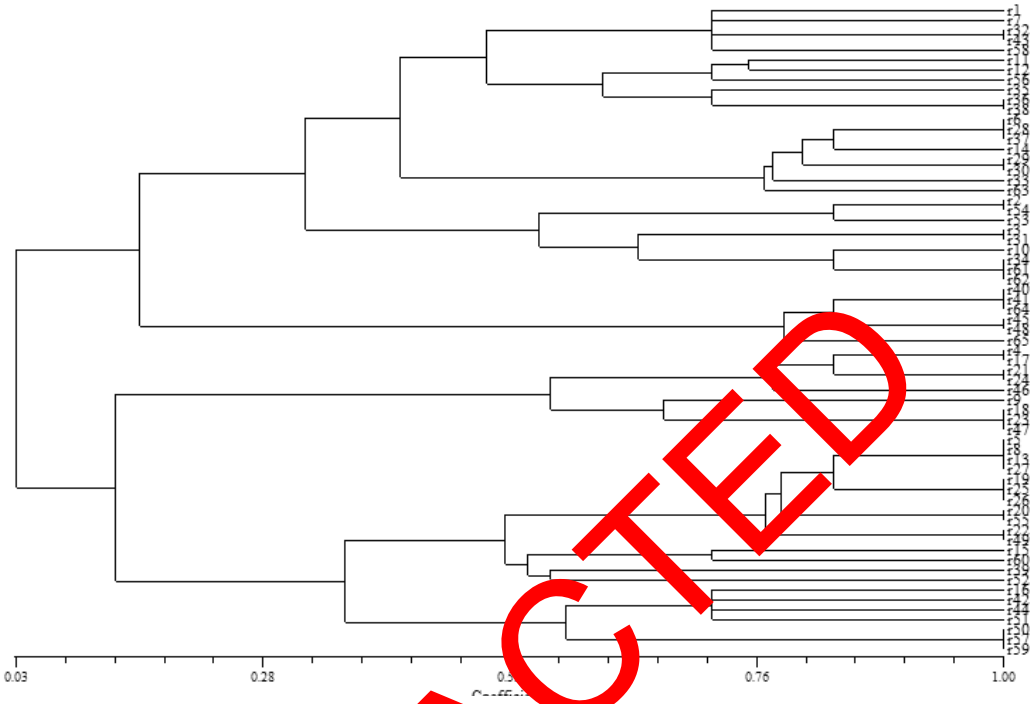

Fig. 3. Unweighted pair-group mean analysis dendrogram of genetic distance among the 65 fungal isolates based on Nei's coefficients. The numbers given above the lines indicate the bootstrap values of 2000 replicates.

be grouped into subtropical, temperate, wild and two relatively recently evolved temperate subtropical populations. This geographic clustering infers that ecological conditions may contribute to adaptations associated with growth temperature range, light intensity, or sclerotial germinization requirements.

Although we did not test for outcrossing, environmental conditions may facilitate outcrossing. The isolates were collected from fields in a geographical region of Iran where diverse crops are also cultivated. The environmental conditions in the region may favor sexual recombination within *S. sclerotiorum*. Oilseed rape is a new crop to this area and the high level of polymorphism may reflect the movement of *S. sclerotiorum* onto this crop from several wild plant hosts.

Clonal fractions of populations from Iran (ranging from 0.077 to 0.20) and from California (0.12) (Malvárez et al. 2007) are similar and confirm that most populations have a small clonal fraction.

Shannon's index of diversity was highest for the isolates from Mazandaran, which had 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 Mazandaran.

Mycelial compatibility grouping, a phenotypic marker system controlled by multiple loci, was often associated with groups of identical or closely related microsatellite haplotypes (these results support the hypothesis that isolates within a single MCG and sharing the same microsatellite alleles may be clonal) except for MCG4 and MCG23 whose microsatellites showed polymorphisms between members of these two groups. For instance, MCG23 included 4 isolates (R50, R57, R58, and R59) that were all compatible when paired on PDA media. R50, R57 and

R59 shared identical microsatellite alleles; R58 differed from the three other isolates by four microsatellites and clustered closely with different isolates in the UPGMA dendrogram (Fig. 3). We confirmed the pairing of the isolates belonging to this MCG in two further replications, as the molecular data did not agree with the pairing. .

It may be indicated that isolates within a MCG tend to separate and form independent groups. The existence of unique MCGs in a sampling area suggests that new MCGs and thereby new genotypes are evolving. The emergence of new genotypes could indicate that MCGs could be adapted to specific field microclimates. In addition, the evolving of new genotypes may be associated with increased cropping of canola in such areas (Hambleton et al., 2002), leading to movement of *S. sclerotiorum* onto this crop from several other host plants, or due to an ascogenous system in sexual reproduction, indicating a sexual population in contrast to the clonal population structure as indicated by other studies (Kohli et al., 1992; Cubeta et al., 1997).

However, given the non-clonal nature of MCG4 and MCG23, it will be important to examine a large number of additional isolates to confirm this hypothesis. This could explain why members of one MCG change in one or more microsatellite genetic groups over time. The colony diameters of the isolates belonging to the same MCG were statistically significant in agreement with each other at all temperature points. In addition, the growth of all isolates belonging to MCG23 (R50, R57, and R59) was similar at all temperature points, except for R58 (Table 1). More variation in the mean growth rate was observed in isolates of MCG4. We presume that the variation in coloration is correlated with genetic differences within the population. The colony colors of the isolates within the same MCGs were similar, except for MCG4 and MCG23. MCG23 consisted of four isolates; the color of isolate R58 was dominantly white, while the rest were beige (Table 1).

Carpogenic germination of *S. sclerotiorum* sclerotia is affected greatly by soil moisture and temperature. Hao et al. (2003) and Clarkson et al. (2004) reported that apothecium production by *S. sclerotiorum* is largely confined to a 10-15°C soil temperature and a -0.03 or -0.07 MPa soil moisture. Stem rot symptoms caused by *S. sclerotiorum* in northern Iran can be observed in mid-May but predominantly occur in early June. The average air temperature of the last 3 years was 14.2°C in April and 20.4°C in May. The rainfall for the same time period was 46.5 mm in April and 21.6 mm in May, and relative humidity 82 and 79.3 percent, respectively (data from the State Meteorological Station based in Ghaemshahr). It has been reported that 15-25°C air temperature and high humidity (optimum >95% RH; but also possible as low as 50-60%) are required for ascospore germination (Clarkson et al., 2004). Although we did not test for outcrossing, environmental conditions may facilitate outcrossing.

CONCLUSION

The isolates were collected from fields in a geographical region of Iran where diverse crops are cultivated. Given the high genetic diversity of this fungus, we suggest that the environmental conditions in the region may favor sexual recombination within *S. sclerotiorum*. Oilseed rape is a new crop to this area and the high level of polymorphism may reflect the movement of *S. sclerotiorum* onto this crop from several wild plant hosts. This is an original report on genetic variation within a population of *S. sclerotiorum* in Iran presenting molecular data and morphological characters demonstrating that the population is genetically diverse.

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