Genetic Structure Analysis of *Sclerotinia Sclerotiorum* (Lib.) de Bary Population from Different host plant species in North of Iran

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Abstract

The genetic diversity of Sclerotinia sclerotiorum populations recovered from different hosts were assessed using Simple Sequence Repeat fingerprinting. By using five SSR primers, 44 haploid groups detected among 65 isolates with 25 alleles polymorphism.

A high level of genetic diversity was observed at about 67.6% (with clonal fraction = 0.077 to 0.20) for regional populations were observed, and Shannon diversity index (Ho) for the whole regions found to be was 0.86 (Htot). Partition of total diversity (Htot) showed that 64% corresponded to a variation in diversity within S. sclerotiorum populations. The Nie's genetic identity illustrated that populations from Golestan province and wild plants were found to be the most diverse from the other province and agriculture plants. The variability found within closely related isolates of S. sclerotiorum demonstrate the effectiveness of SSR marker in identifying genetic diversity among S. sclerotiorum isolates.

Key words: microsatellites, *Sclerotinia Sclerotiorum*, Shannon diversity

Introduction

Sclerotinia Sclerotiorum (Lib.) de Bary is an important ascomycetous plant pathogenic fungus which has a wide geographic distribution and a diverse host range including many agronomic crops (1). Yield losses due to stem rot disease, are variable and sometimes reach to maximum level in susceptible plants (2)**.**This pathogen is the causal agent of sclerotinia stem rot in canola leading to serious losses in yield due to lodging and premature shattering of seedpods (3). Incidence of this disease in canola fields of Iran has ranged from 12.3% to 54.4% (4), and variation in MCGs and virulence and in such morphological characteristics as pigmentation of the mycelium of Iranian *S. sclerotiorum* populations have been reported, (5,6,7,8).

Sclerotinia sclerotiorum is a homothallic ascomycete fungus with a wide host range and geographical distribution. It is one of the most omnivorous plant pathogens (2). Sclerotia of *S. sclerotiorum* can survive for many years in soil (9). Soilborne sclerotia produce apothecia at the time when canola flowers, to produce airborne ascospores as primary inocula, which infect petals. Resistance sources to *S. sclerotiorum* in several crops has been identified (10,11). In Iran, the evaluation of tolerance in different lines and cultivars of rapeseed to sclerotinia stem rot has shown different levels of tolerance to the disease (12).

Control strategies must target a population instead of an individual if they are to be effective (13) and because in plant-pathogen interactions, development of new pathogenic races and the breaking down of resistance are limiting factors in resistance deployment against plant diseases then the pathogen's life history characteristics and evolutionary potential are major factors leading to the pathogen overcoming host resistance (14,15,16) 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 (13).

Two important factors for pathogen evolution are the reproductive/mating system and gene/genotype flow. The reproduction system will affect the distribution of alleles in a population. Sexual reproduction in out-crossing organisms will result in recombined genotypes, whereas in-breeding and clonal reproduction will fix the alleles within a clone. Out-crossing and recombination combine alleles from different sources into the same genetic background, resulting in different genotypes that may have a higher fitness (16). Furthermore, high levels of gene/genotype flow may facilitate the exchanging of genetic resources including isolates with novel virulence across a large geographical area (16).

Genetic variation among populations of *S. sclerotiorum* was assessed using two presupposed unrelated criteria, mycelial compatibility groups (MCG) and molecular markers (17). 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(18) and there are several published reports on the population genetic structure of *S. sclerotiorum* using different molecular markers (19,20,21,22,23,24,25,26,27,28,). All studies indicated that populations were genetically diverse and had a predominantly clonal component with occasional recombination within populations, as well as contemporary population diversification at a local scale.

Microsatellites are widely dispersed and evenly distributed in the genome of eukaryotes and have been used to study intraspecific genetic variability within populations (29,30). Microsatellite markers have high specificity, reproducibility, polymorphism and they are co-dominant markers (31). Recently, Sirjusingh and Kohn (2001) developed 23 microsatellite loci and two microsatellite-like polymorphic loci containing 2–10 alleles at each locus.

The aim of this research was to investigate the genetic structure within the 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

Naturally infected stems of different hosts were sampled from Golestan, Mazandaran and Gillan 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). Each isolate was purified by transferring the single hyphal tip onto the fresh medium, and generated sclerotia were stored at -20 ºC until use (5,7,8,21,32,33).

DNA Extraction

Isolates were grown on PDA and incubated at 21 to 24ºC in darkness for 5 days. Two plugs of the colony margin were transferred to 250 ml flasks containing 50 ml potato dextrose broth (PDB) and incubated at 21 to 24ºC in darkness for 2 days. Cultures were incubated for a further 4 days at 21 to 24ºC in darkness with shaking at 100 cycles min−1 in a rotary shaker. Mycelia were harvested by vacuum filtration, lyophilised and stored at −20ºC. Fifteen to 20 mg of dried mycelia were powdered and transferred to 1.5 Eppendorf tubes for DNA extraction according to Liu et al. (2000) with the following modifications: before precipitation

of DNA, an equal volume of phenol- chloroform- isoamyl alcohol (25:24:1) was added to the supernatant, which was then centrifuged at 12,000 rpm for 10 min. Also, the DNA pellet was washed twice with cold ethanol (70%), and then airdried at 37ºC for 20 min. The DNA pellet was dissolved in 30 μl of 1X TE (10 mM Tris-HCl, 1 mM EDTA) and stored at 5ºC (34).

Microsatellite genotyping
Microsatellite primers of five loci (12-2, 7-3, 106-4, 114-4 and 55-4) described by Sirjusingh and Kohn (2001), were tested in previous studies for polymorphism among 65 isolates of *S. sclerotiorum* representing the three regions sampled in Iran (5,8), In this study the population genetic structure were examined
Each PCR reaction was performed in a total volume of 50 μL containing 10-20ng of the purified DNA and the reaction buffer (100 μM each of dATP, dCTP, dGTP and dTTP, 200 nM of microsatellite rimer(CinaGen, Tehran) and 0.8 units of Taq polymerase, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl2, 50 mM KCl, 100 μg mL-1 gelatine, 0.05% tween 20 and 0.05% Nonidet P-40) . 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 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 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 (35).

Data analysis
The molecular size of each fragment was estimated using Photo-capt software (Image Analysis Software, Vilber Lourmat, France). 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 (36) was prepared and bootstrap analysis with 2000 samplings with the replacement was performed to generate a dendrogram of unweighted pair-group mean analysis (UPGMA; 37) using the Treecon-1.3b program (38).

Polymorphism Information Content (PIC).
The polymorphism information content determines the frequency of alleles polymorphism in the gene locus to study population**.** PIC, was calculated for each locus as follows:
$$PIC = 1 - \sum_{i=1}^{n} Pi^2$$

Where *pi* was The relative frequency of *i*th allels

Determination of Genotypic Diversity.
Isolates that have the same allele combination for each of the 5 loci were located in a haplotype group. Isolates present in a haplotype, are obtained from Clonal reproduction (Asexual reproduction or sexual reproduction of the type homothalism). Then Clonal fraction calculated for every three populations. Clonal fraction that is represents the frequency and Prevalence of colones in populations, calculated by *N-G/ N*. That *N* is population size and *G* is haplotypes size in population (39).

Shannon-Wiener's Index.
Shannon-Wiener's index, H0, was calculated for each population as follows:
$$H0 = -\sum (pi\ In\ pi)$$
Where *pi* was the frequency of *i*th haplotype (40)

Results

In previous study the diversity of 65 isolates of *S. Sclerotiorum* on different hosts representing three geographic populations from Iran, detected 44 haplotypes (5,8). In this study the results indicating low levels of clonality. Clones were also identified at the smallest scale of analysis within 7.7% in Golestan province to 20% in Mazandaran province (Table 2). All field populations, as the smallest scale of the analysis, were composed of more than one clone, indicating diversification within field populations. Number of clones per field and clone frequency within field populations varied depending on sample size (Table 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 1) . Therefore be said Locus of (AGAT)14(AAGC)4 with the Most of the alleles number identified and the highest PIC, could better than the rest of the locus used to identify the genetic distance samples.

Table 1. Microsatellite and polymorphic loci* and Polymorphism Information Content for *Sclerotinia sclerotiorum*

Locus (GenBank Accession)	Repeat Motif	PIC	Primer sequence (5′-3′)	T_a (℃)	Size range (bp)	No. of alleles	Other species
12-2 AF377906	$(CA)_9$	0.499	CGATAATTTCCCCTCACTTGC GGAAGTCCTGATATCGTTGAGG	55	215-225	*2*	*No*
7-3 AF377909	$(GT)_{10}$	0.666	CCTGATATCGTTGAGGTCG ATTTCCCCTCACTTGCTCC	55	384-388	*4*	*No*
106-4 AF377921	$(CATA)_{25}$	0.816	TGCATCTCGATGCTTGAATC CCTGCAGGGAGAAACATCAC	55	491-571	*6*	*No*
114-4 AF377923	$(AGAT)_{14}(AAGC)_4$	0.866	GCTCCTGTATACCATGTCTTG GGACTTTCGGACATGATGAT	59	351-391	*8*	*Yes*
55-4 AF377918	$(TACA)_{10}$	0.745	GTTTTCGGTTGTGTGCTGG GCTCGTTCAAGCTCAGCAAG	59	173-221	*5*	*Yes*
Tottal						*25*	

T_a, annealing temperature

Table 2. Shannon diversity index and Clonal fraction of haplotypes *S. sclerotiorum* populations from different locations

Location	Haplotypes	Sample size	H_0	H_{avr}/H_{tot}	$H_{tot\text{-}}H_{avr}/H_{tot}$	*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	

In order to determine the genetic relationships among populations of fungal isolates, a separate matrix was used to the data obtained from the 25 polymorphic alleles (Table 1) and the fungal isolates were subsequently grouped by UPGMA cluster analysis. The dendrogram representing microsatellite marker polymorphisms among the fungal isolates is illustrated in. According to the dendrogram, nine major clusters can be defined among the 65 isolates (Fig. 1, published by barari et al., 2010,2012).

The genetic diversity of three populations from Golestan, Mazandaran and Gillan was evaluated using Shannon's index of diversity (Table 2). At the species level, Shannon's index of diversity ($H0$) was 1.509.

The population diversity for the isolates originating from Mazandaran province was the highest (H = 1.568) among the three populations. The order of genetic diversity based on Shannon's index of diversity was as follows (from highest to lowest): Mazandaran > Golestan > Gillan (Table 2).

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 those populations (Table 2).

Although a high level of diversity was observed between the 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.
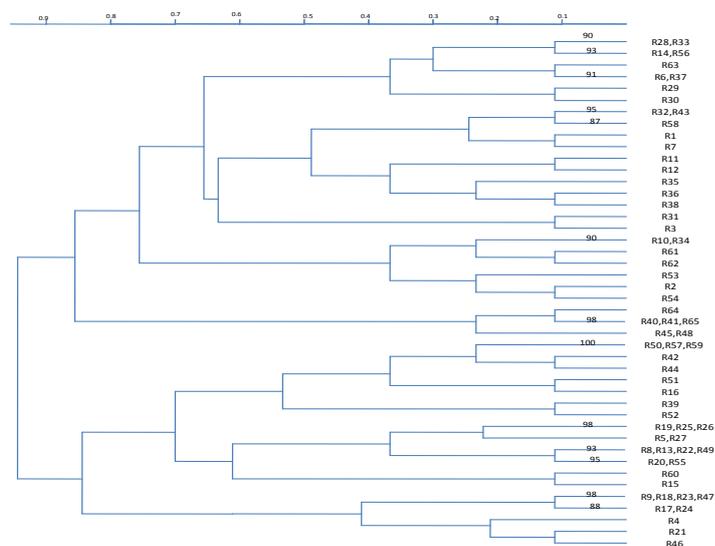

Fig.1. Unweighted pair-group mean analysis dendrogram of genetic distance among the 65 fungal isolates based on Nei's coefficients. The numbers given above the lines indicate the bootstrap values of 2000 replicates (published by barari et al., 2010,2012)

Discussion

In previous study the diversity of 65 isolates of *S. Sclerotiorum* on different hosts representing three geographic populations from Iran, were studied using five microsatellite loci that, we found microsatellite markers were very efficient in identifying genetic variation among the isolates, which these marker sets revealed polymorphism among the Iranian isolates (5,8).

We found 44 different clones (haplotypes) among the 65 isolates (5,8) representing the population indicating a high rate of variability in the region (S/N%=66.7%). which on average is similar to that found in Australian populations (S/N%=36 to 80%, Sexton and Howlett 2004; S/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%) (41) 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 markers microsatellites.In 50% of isolates *S. Sclerotiorum* divided into 9 large clusters that represent the diversity in these isolates is high. Based on this cluster, the population of the Golestan province separated from the other populations (except with the east of Mazandaran), In addition, Sclerotinia wild population isolated from wild sinapis completely, were placed in separate haplotype. According to the semi-arid conditions of Golestan province than in temperate climates (Mazandaran and Gilan) this phenomenon was predictable that was in accordance with the evolutionary history of population haplotypes (25), which indicated that isolates may 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 vernalization requirements.

Cluster analysis showed that shared microsatellite haplotypes among populations between east of Mazandaran and Golestan indicate considerable gene flow that it in New Zealand populations and some other areas have also been reported (42). Shared haplotypes among populations either indicate exchange of haplotypes among populations and/or the populations represent the same founder population. There are several mechanisms by which haplotypes can be exchanged among neighbouring countries/ fields. Transporting contaminated soil and fertilizing with manure from animals fed infected plants are two common ways of spreading fungal inocula in the form of sclerotia or mycelium from field to field (43). Irrigation is also other way for spreading of Sclerotinia species among fields. Sclerotia remained viable for at least ten to 21 days in flowing water (43). Infected seeds potentially could be a source of infection, although this method of transmission has not been proven. Migration (exchange of haplotypes) among populations through the movement of sclerotia with soil over large distances, can link different fields into one genetic neighbourhood. Furthermore, airborne ascospores, which are the most common source of infection within fields (43), also provide a mechanism for long-distance dispersal of haplotypes among populations. Shared haplotypes among populations could also be the result of sexual reproduction, i.e. inbreeding or out-crossing among similar genotypes. Resultant ascospores would be genetically similar and appear clonal. because ascospores can be dispersed over long distances (32), it is not surprising to find shared clonal genotypes among several regions. Shared microsatellite haplotypes among populations were also found within Australia and North America (23,24,21).

Clonal fractions of populations from Iran (ranging from 0.077 in Golestan to 0.20 in Mazandaran) and it from California (0.12) (19) is similar and confirm that most populations have a small clonal fraction.

Shannon's index of diversity were highest for the isolates originating from Mazandaran compared with other regions. This indicates that these isolates revealed more polymorphisms than those from the other provinces. Therefore, the potential for the emergence of isolates resistant to fungicides and pathogenic on different canola cultivars is possible in this site. In populations of *S. sclerotiorum* diversity can generates by factors, such as immigration, mutation and diversifying selection (28). Furthermore, the observation of high genetic

diversity among *S. sclerotiorum* isolates can be due to sexual reproduction by outcrossing (21). Although *S. sclerotiorum* is a homothallic fungi but it is possible that microconidia, produced by it which unable to germinate (44), act as spermatia similar to reports in closely related genera (45).

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 favour 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.

CONCLUSION

The isolates were collected from fields in a geographical region of Iran where diverse crops are also cultivated. Given the high genetic diversity of this fungus, regards to control Sclerotinia stem rot integration of disease management systems, combining biological, cultural methods and chemical should be applied.

In conclusion, this is the original report on the genetic variation within a population of *S. sclerotiorum* in Iran.

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