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Population Structure and Sexual Fertility of *Colletotrichum gloeosporioides* sensu lato from Citrus in Northern Iran

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ABSTRACT

Anthracnose caused by *Colletotrichum gloeosporioides* s. l. is one of the most important diseases of citrus in northern Iran. To study the genetic structure of *C. gloeosporioides* s. l. from citrus spp., infected samples were collected from three citrus cultivating regions of northern Iran, during the summer of 2009. Fifty one monoconidial isolates were used as the objective of REP- and BOX-PCR fingerprintings. Eight fingerprinting groups were observed in the constructed phenogram. The largest proportion (94.37%) of total genetic diversity (H_t) was attributed to diversity within populations (H_s). Estimates of Nei's genetic similarity and distances exhibited the high level of similarity among three populations. The value of gene flow, N_M= 8.4, indicates that there is low limitation to gene flow among these geographically distant populations, which make these groups genetically homogenous. The results indicate that the three geographic populations are not developing independently and can be part of a Mega-population. Forty three isolates were divided into 19 groups in the phenogram constructed by combination of morphological characteristics data. Estimation of correlation between morphologic and rep-PCR matrixes indicated a weak and non-significant correlation between morphology and rep-PCR haplotypes (r= 0.2, P= 0.992). Fifty one isolates were examined to clarify their sexual behavior. Eight isolates were identified as homothallic and 14 successful outcrosses were observed among self-sterile isolates.

Keywords: Anthracnose, Diversity, Gene flow, Perithecium.

INTRODUCTION

Anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. in Penz. (Teleomorph *Glomerella cingulata* (Stoneman) Spauld. and H. Schrenk) s. l. is one of the most important diseases of *Citrus* spp. in subtropical and temperate regions. Morphological studies were traditionally used to identify different species of *Colletotrichum* by using characters such as conidial and appressorial shape and size, presence or absence of setae, sclerotia and growth rate. These studies have also shown considerable diversity within *C. gloeosporioides* s. l. isolates from different hosts (Smith and Black, 1990; Gunnell and Gubler, 1992; Sutton, 1992; Talhinhas *et al.,* 2002). But, the wide range of variety in these characteristics together with the existence of isolates demonstrating mixed or intermediate characteristics make the exclusive use of morphological traits for separating different species or different groups in one species unreliable (Brown *et al.*, 1996; Photita *et al.,* 2005). Different genetic mechanisms are involved in

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Colletotrichum diversity. Sexual recombination is the most important mechanism in this process (Rodríguez-Guerra *et al.,* 2005). Edgerton (1914) reported successful outcross between two homothallic isolates of *G. cingulata*. According to his observation a comprehensive series of surveys were done to illustrate the genetic regulation of mating in *Glomerella* genus (Lucas *et al.,* 1944; Wheeler *et al*., 1948; Chilton and Wheeler 1949; Wheeler 1954). Based on the results of previous studies, Wheeler (1954) suggested a model to describe how mating was regulated genetically in *G. cingulata*, proposing that *G. cingulata* is basically homothallic and heterothallism in this fungus may arise as a result of mutations in genes involve in sexual reproduction. Recent molecular studies on *MAT1* locus in *G. cingulata* have demonstrated the existence of *MAT1-2* idiomorph in both mating pairs. This situation has never been observed in other filamentous ascomycetes (Vaillancourt *et al.*, 2000; Rodríguez-Guerra *et al.,* 2005; Garcia-Serrano *et al.,* 2008). However, sexual reproduction is one of the most important factors involved in genetic diversity of *G. cingulata*. Therefore, simultaneous study on sexual fertility, genetic diversity, and the relationship among *G. cingulata* isolates from different hosts and areas could be useful.

DNA fingerprinting is an important tool for detecting genetic diversity and clarifying relationship among *Colletotrichum* isolates from different hosts and regions. To determine the genetic structure of *C. gleoesporioides* s. l. isolates, from Trinidad and Mexico, Rampersad *et al.* (2013) studied the sequence of ACT, GPDH, β-TUB, and ITS gene regions of this pathogen. Results of their study showed a low level of genetic divergence between the two populations. They also reported that Mexican population, which had the most diversity, was the ancestral population. Multi-gene phylogeny used by Weir *et al*. (2012) to distinguish new species in *C. gloeosporioides* complex resulted in 22 species and one subspecies. Nguyen *et al.* (2009) studied the genetic structure of *C. gleoesporioides* populations obtained from coffee in northern and southern Vietnam using RAPD and MP-PCR. Their results showed high level of gene flow between populations. One of the genomic fingerprinting techniques used to elucidate DNA polymorphism in *Colletotrichum* spp. is repetitive-extragenic-palindromic polymerase chain reaction (rep-PCR). Suzuki *et al.* (2010) used rep-PCR to discriminate highly and weakly virulent isolates of *C. gloeosporioides* s. l. from strawberry using Box A1-r and ERIC-2 primers. In their study, isolates with high and weak virulence were separated into distinct groups but isolates from different hosts and regions were not discriminated. About 60 years ago, Petrak and Esfandiari (1941) reported *Citrus* anthracnose for the first time from Iran. At present, anthracnose is one of the most important diseases of *Citrus* in northern Iran.

The objectives of this study were: (1) Comparing the genetic structures of *C. goleosporioides* s. l. field populations in northern Iran to determine the spatial distribution of genetic diversity on regional scales; (2) Clarifying the relationship between genetic diversity and morphological characteristics of *C. gloeosporioides* s. l. isolates from three different geographic regions of northern Iran, and (3) Studying the sexual compatibility between isolates from different areas and hosts.

MATERIALS AND METHODS

Fungal Isolates

Fifty one monoconidial *C. gloeosporioides* s. l. isolates were obtained from infected branches, leaves, and fruits of *Citrus* spp. (*C. sinensis* cv. Thamson Navel, *C. sinensis cv*. Sanguinella, *C. sinensis cv*. Valencia, *C. tangerine* and *C. unshiu)* in three main citrus cultivating regions (Ramsar, Sari, and Gorgan) of northern Iran, during the summer

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of 2009 (Figure 1 and Table 1). Ramsar, Sari, and Gorgan are in 36.90º N, 50.65º E, 36.56º N, 53.06º E, and 36.83º N, 54.43º E geographical coordinates, respectively. Sampling was performed in one orchard of each region. Infected tissues were collected from infected trees in each row, with ten meter distance between the trees, and placed in paper bags to transfer to the laboratory. Single spore isolates were maintained on desiccated sterile filter paper at -20ºC (Leung and Taga, 1988).

Cultural and Morphology Assessment

Morphological characters including the growth rate and size and shape of conidia and appressoria were measured for 43 out of 51 isolates (due to loosing eight isolates during the experiments). To measure the growth rate, monoconidial isolates were grown on PDA medium and incubated in darkness, at 27ºC. Three diameters for each of the colony replicates were measured daily for seven days and growth rate was measured by calculating the seven-day average of mean daily growth for each isolate. To measure the size of conidia, isolates were grown on PDA under 25ºC and dark condition. Fifty conidia of each isolate were measured in width and length under light microscope, after 14 days (Jagtap and Sontakke, 2009). To record the shape and size of appressoria, 10 µL of 10^6 conidial suspension of each isolate, which was prepared by using two-week-old harvested conidia from PDA plates, were placed on sterile glass slides. Glass slides were inserted in sterile Petri dishes containing wet sterile filter papers and incubated in darkness at 25ºC for 24 hours. Length and width of 50 appressoria of each isolate were measured under light microscope using 100X power oil immersion lens (Guetsky *et al.,* 2005).

Sexual Compatibility

Fifty one isolates were examined to test the sexual behavior (homothallic or heterothallic). To identify homothallic

Figure 1. Location of *Colletotrichum gloeosporioides* s. l. collection sites at provinces of Mazandaran and Golestan in northern Iran. The shaded area on the map represents the location of Mazandaran and Golestan within Iran. Colored circles show sites where three orchards were sampled intensively.

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Table 1. *Colletotrichum gloeosporioides* s. l. isolates used in this study.

Isolates	Year isolated	Host	Location	Symptoms
RO21	2009	*Citrus unshiu*	Ramsar	Leaf spot
RO22	2009	*Citrus unshiu*	Ramsar	Lesion of branch
RO33	2009	*Citrus unshiu*	Ramsar	Fruit spot
RV42	2009	*Citrus sinensis* cv. Valencia	Ramsar	Lesion of branch
RT51	2009	*Citrus sinensis* cv. Thamson Navel	Ramsar	Leaf spot
RT73	2009	*Citrus sinensis* cv. Thamson Navel	Ramsar	Fruit spot
RY82	2009	*Citrus tangerine*	Ramsar	Lesion of branch
RY83	2009	*Citrus tangerine*	Ramsar	Fruit spot
RY92	2009	*Citrus tangerine*	Ramsar	Lesion of branch
RY93	2009	*Citrus tangerine*	Ramsar	Fruit spot
RY102	2009	*Citrus tangerine*	Ramsar	Lesion of branch
RO111	2009	*Citrus unshiu*	Ramsar	Leaf spot
RO121	2009	*Citrus unshiu*	Ramsar	Leaf spot
RO122	2009	*Citrus unshiu*	Ramsar	Lesion of branch
RT162	2009	*Citrus sinensis* cv. Thamson Navel	Ramsar	Lesion of branch
RY172	2009	*Citrus tangerine*	Ramsar	Lesion of branch
RT191	2009	*Citrus sinensis* cv. Thamson Navel	Ramsar	Leaf spot
RT201	2009	*Citrus sinensis* cv. Thamson Navel	Ramsar	Leaf spot
ST61	2009	*Citrus sinensis* cv. Thamson Navel	Sari	Leaf spot
ST72	2009	*Citrus sinensis* cv. Thamson Navel	Sari	Lesion of branch
ST81	2009	*Citrus sinensis* cv. Thamson Navel	Sari	Leaf spot
SK92	2009	*Citrus sinensis* cv. Sanguinella	Sari	Lesion of branch
ST161	2009	*Citrus sinensis* cv. Thamson Navel	Sari	Leaf spot
ST172	2009	*Citrus sinensis* cv. Thamson Navel	Sari	Lesion of branch
ST182	2009	*Citrus sinensis* cv. Thamson Navel	Sari	Lesion of branch
ST191	2009	*Citrus sinensis* cv. Thamson Navel	Sari	Leaf spot
ST192	2009	*Citrus sinensis* cv. Thamson Navel	Sari	Lesion of branch
SK202	2009	*Citrus sinensis* cv. Sanguinella	Sari	Lesion of branch
SK212	2009	*Citrus sinensis* cv. Sanguinella	Sari	Lesion of branch
GT12	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Lesion of branch
GT41	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT42	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Lesion of branch
GK251	2009	*Citrus sinensis* cv. Sanguinella	Gorgan	Leaf spot
GT261	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT262	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Lesion of branch
GT451	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT511	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT541	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT561	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT581	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT631	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT641	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT651	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT272	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Lesion of branch
GT311	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT321	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT352	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Lesion of branch
GK391	2009	*Citrus sinensis* cv. Sanguinella	Gorgan	Leaf spot
GT431	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot
GT441	2009	*Citrus sinensis* cv. Thamson Navel	Gorgan	Leaf spot

isolates, all isolates were grown on YPSS (Yeast Powder-Soluble Starch agar) culture medium (Emerson, 1958) under 21±1ºC, 12-hour photoperiod, for 21-30 days. Homothallic isolates were identified and crossed in all possible combinations to test sexual compatibility. Also, self-sterile isolates were randomly selected for paired crosses. Two individuals, which crossed successfully and produced abundant

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perithecia, were chosen as representatives and all other isolates were crossed with them. All crosses were performed on Petri dishes containing 15 mL YPSS culture medium under 21±1ºC, 12-hour photoperiod for 21-30 days.

DNA Extraction

Fungal cultures were grown in 60 mL of Potato Dextrose Broth (PDB) for five days at 27ºC on a rotary shaker at 120 rpm. Total DNA was extracted from freeze-dried mycelium by using Core-One^{TM} Plant Genomic DNA Isolation Kit (Corebio, South Korea) according to manufacturer's instruction. The DNA was dissolved in 60 µL deionized water and stored at -20ºC. To use in PCR reaction, DNA was diluted to a final concentration of 10 ng μL^{-1}.

Rep-PCR Amplification

Two types of primers i.e. Rep1RI: (5'-IIIICGICGICATCIGGC-3') and Rep2I: (5'-ICGICTTATCIGGCCTAC-3') and BOX: 1A–1R (5'–CTACGGCAAGGCGA CGCTGACG-3') were used (Versalovic *et al.,* 1991). Amplifications were carried out in 20 µL PCR mixture containing 10-100 ng of genomic DNA, 1X PCR buffer, 2 mM $MgCl_2$, one unit of *Taq* DNA polymerase (Cinnagen, Iran), 0.2 mM of each dNTP and 1 µM of each primer. PCR reactions were performed in a Biometra T1 Thermal cycler. The amplification cycle was started with initial denaturing at 95ºC for 5 minutes, followed by 35 cycles of denaturing at 94ºC for 3 seconds and 92ºC for 30 seconds, annealing at 39ºC for 1 minute (for Rep primers) and 49ºC (for Box primer), extension at 72ºC for 7 minutes and a final extension of 72ºC for 10 minutes. PCR products were resolved by electrophoresis on 1.25% agarose gel in 1X TBE buffer and stained with ethidium bromide. DNA banding patterns were visualized under UV light and photographed by using the Gel-Documentation system, IMAGO (B and L). Amplified DNA fragments sizes were estimated by comparison with 100 bp molecular marker Gene ruler^{TM} DNA Ladder Mix.

Statistical Analysis

Morphological and molecular data were separately analyzed by using cluster analysis. Morphological similarity between two isolates was calculated based on general similarity coefficient of Gower (1971). Phenogram of morphological characteristics was generated using Unweighted Pair-Group Method Arithmetic Average (UPGMA) in NTSYS-pc version 2.1 (Rholf, 1998).

The presence and absence of each fragment in DNA fingerprinting patterns was scored as 1 and 0, respectively. The similarity matrices were constructed with the Similarity for Qualitative Data Program (SIMQUAL) in the NTSYS-pc. The similarity was estimated based on Dice's coefficient and phenogram was generated by using UPGMA in NTSYS-pc. The correlation between morphology and rep-PCR patterns was calculated by comparing morphology and rep-PCR similarity matrices based on Mantel test, using the Matrix Comparison Program (MXCOMP) option of NTSYS-pc.

Genetic diversity parameters including percentage of polymorphic loci, gene diversity (h), genetic distance, gene diversity within populations (H_S), total gene diversity (H_T), genetic differentiation (G_{ST}), and gene flow (N_M) were calculated among populations of *C. gloeosporioides* s. l. by using Popgene version 1.31 (Yeh *et al.,* 1999). Phenogram of Nei and Li (1979) genetic distances was constructed based on UPGMA in NTSYS-pc to show the relationships among three populations. To partition the total molecular variance within and between populations, Analysis of Molecular Variance (AMOVA) was performed by the program Genalex version 6.1 (Excoffier *et al.,* 1992), using 99 permutations.

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RESULTS

Cultural and Conidial Morphology

Forty three *C. gloeosporioides* s. l. isolates were separated into 19 groups designated as A to S, in phenogram constructed based on morphological characteristics (Figure 2). Groups A, C, D, E, F, L and O consisted of six, ten, two, six, two, three, and two isolates, respectively, and other groups consisted of a single isolate. Isolates were not separated into clusters according to their geographic origins and host origins.

Rep-PCR Analysis

Two types of rep-PCR primers were used to amplify DNA of 51 *C. gloeosporioides* sensu lato isolates. Fifty six scorable DNA bands were amplified from genome of all isolates (Figures 3 and 4). REP and Box primers yielded 25 and 31 DNA bands ranging in size from 535 to 2,215 bp and 666 to 3,625 bp, respectively. Eight fingerprinting groups, designated as A to H in the phenogram, were generated from combined rep- and BOX-PCR data of all three populations (Figure 5). Among the haplotypes, 94.11% belonged to groups A, B, D, and E (mainly group A), while each of 4 other groups consisted of a single haplotype. Isolates were not separated into clusters according to their geographic and host origins. The estimation of correlation between morphologic and rep-PCR matrixes by using a simple Mantel test revealed that there was a weak and non-significant correlation between morphology and rep-PCR haplotypes (r= 0.2, P= 0.992).

Genetic Structure of *C. gloeosporioides* s. l. Populations

Percentage of polymorphic loci in three populations ranged from 67.86 to 87.5% with an average of 80.36%. The highest percentage of polymorphic loci was seen in Ramsar population while Sari population

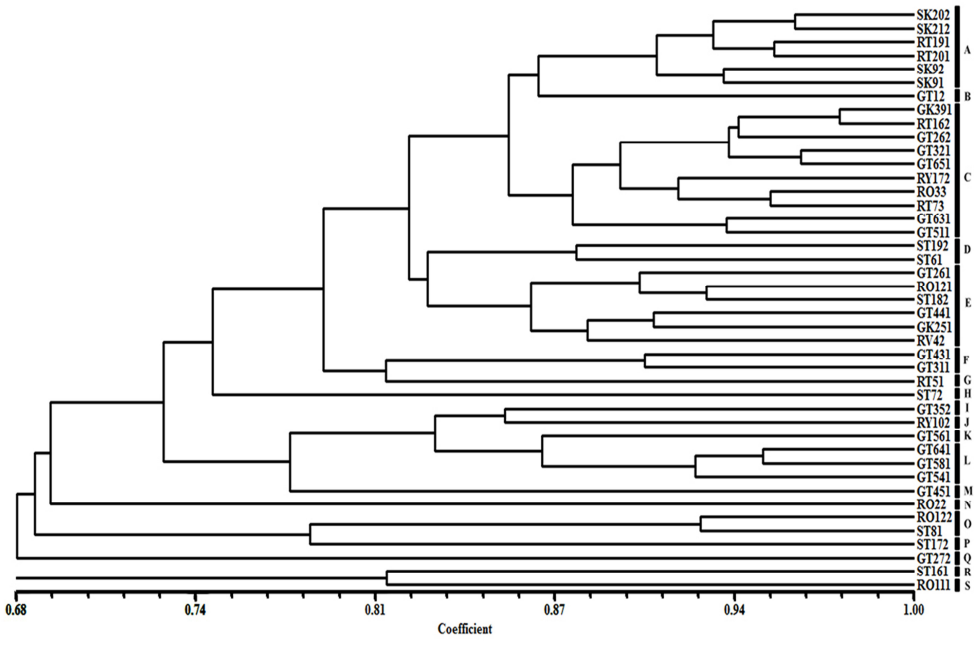

Figure 2. Phenogram constructed by Unweighted Pair Group Method with Arithmetic average (UPGMA) based on combined data for morphologic characteristics indicating relationships among *Colletotrichum gloeosporioides* s. l. isolates used. Defined groups designated as A to S are indicated on the right.

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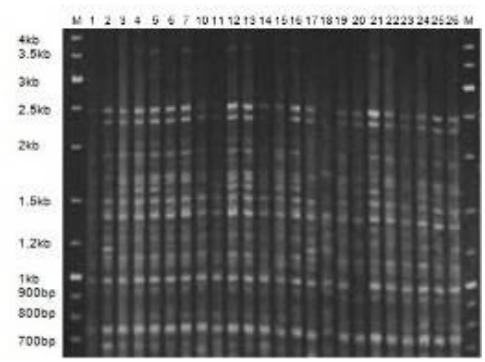

Figure 3. Rep-PCR FingerPrint (FP) profiles among the *Colletotrichum gloeosporioides* s. l. isolates on 1.25% agarose gel. The isolates were analyzed using the box A1-1R primer (BOX-PCR). Lanes M: Gene ruler TM DNA Ladder Mix, and Lanes 1 to 24: Isolates RT73, SK212, GT431, RO33, GT581, GT631,RT162, ST192, RV42, RT191, GK251, RY92, ST161, RT201, RT51, Ro11, Ry82, SK92, ST72, GT651, GT451, GT262, GT311, GT561.

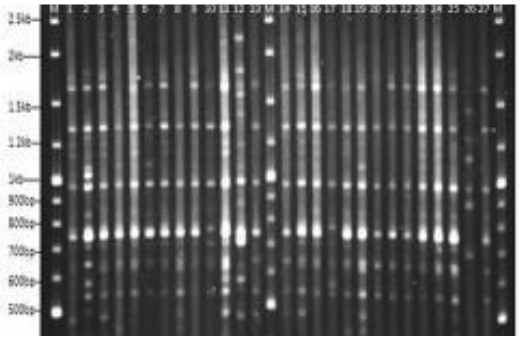

Figure 4. Rep-PCR FingerPrint (FP) profiles among the *Colletotrichum gloeosporioides* s. l. isolates on 1.25% agarose gel. The isolates were analyzed using the Rep primer (Rep-PCR). Lanes M: Gene ruler TM DNA Ladder Mix, and Lanes 1 to 27: Isolates GT651, Sk202, GT641, ST81, ST161, RY102, GT352, RY93, GT321, GT41, ST61, RT51, RY82, GK391, SK91, RO121, GT451, ST182, GT262, GT261, GT272, Gt541, GT431, ST82, ST192.

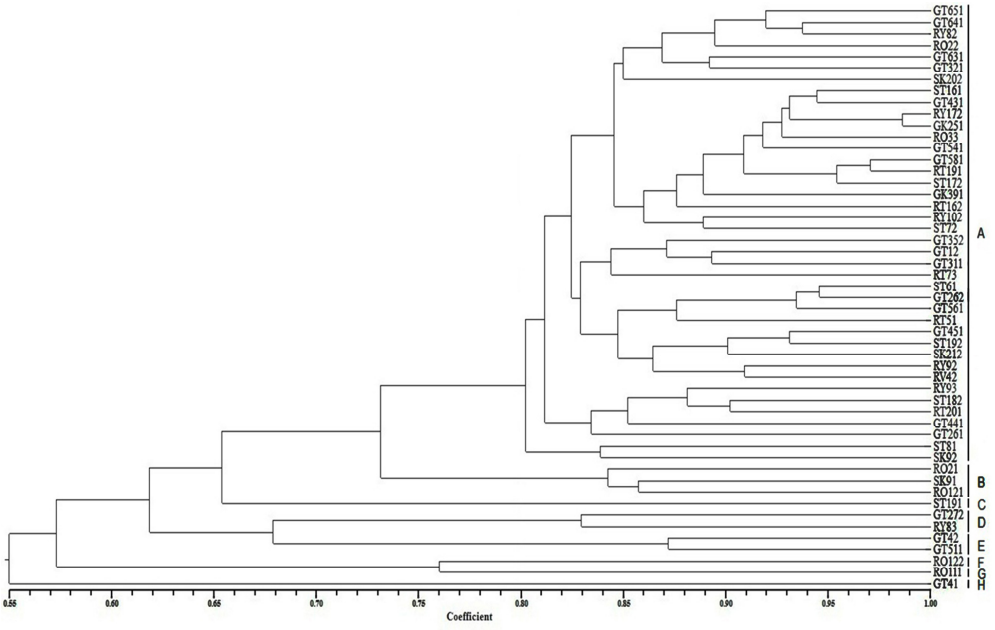

Figure 5. Dendrogram constructed by Unweighted Pair Group Method with Arithmetic average (UPGMA) based on combined data for Rep-PCR and BOX-PCR, indicating relationships among *Colletotrichum gloeosporioides* s. l. isolates obtained from three populations in northern Iran. Defined groups designates as A to H are indicated on the right.

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showed the lowest polymorphism. The value of gene diversity (h) among all populations ranged from 0.192 to 0.243. The highest value of *h* was observed in Ramsar population while Sari population exhibited the lowest value of this parameter.

The total gene diversity (H_t) for all populations of *C. gloeosporioides* s. l. and the average of gene diversity within populations (H_s) was 0.236 and 0.223, respectively. The largest proportion (94.37%) of total genetic diversity was attributed to diversity within populations (H_s) compared to 5.63% among them. The value of genetic differentiation (G_{ST}) was 0.06 over all loci. The value of gene flow (N_M) for all populations was 8.4. Nei's genetic distances ranged from 0.016 to 0.034 (Table 2). The highest similarity (lowest diversity) was observed between populations of Gorgan and Ramsar, while populations of Sari and Gorgan exhibited the lowest similarity (Table 3).

Results of AMOVA exhibited a nearly similar pattern of population differentiation. Most of the genetic variation was distributed among individuals within populations (98%) compared to 2% among populations (Table 4).

Sexual Compatibility Status

Isolates SK91, RO111, RY172, RO122, GT41, GT42, GT352, and GT272 were identified as homothallic. Self-fertility in each of homothallic isolates was proved, producing mono-ascosporic cultures. Thirty initial crosses were made randomly to find a successful outcross among self-sterile isolates. Isolates RT51 and RT162, which mated successfully, were chosen as

Table 2. Genetic diversity estimates within and among the three populations of *Colletotrichum gloeosporioides* s. l.[a]

Percentage of polymorphic loci	I	*H*	*N*	Geographic region
85.71	0.371	0.235	21	Gorgan
67.86	0.303	0.192	12	Sari
87.50	0.382	0.243	18	Ramsar
80.36	0.352	0.223	51	Total
G_{ST}: 0.056	H_T: 0.236±0.23			
N_M: 8.4	H_S: 0.223±0.19			

[a] *N*: Number of isolates; *I*: Shannon's Information index (Lewontin 1972); *H:* Nei's (1973) gene diversity; *GST*: The proportion of total gene diversity found among populations; N_M: Total gene flow among populations; *HT*: Total gene diversity, and *HS*: Gene diversity within populations; and.

Table 3. Genetic distance between pairwise combinations of *Colletotrichum gloeosporioides* s. l. populations.

Ramsar	Sari	Gorgan	Populations
		0.0	Gorgan
	0.0	0.035	Sari
0.0	0.026	0.017	Ramsar

Table 4. The AMOVA results for the three populations of *Colletotrichum gloeosporioides* s. l.[a]

%	*Est Var*	*MS*	*SS*	*df*	Source
2%	0.119	8.754	17.508	2	Among Pops
98%	6.667	6.774	325.159	48	Within Pops
100%	6.893		342.667	50	Total

%: Percentage of Molecular Variance; *Est Var:* Estimated Variance; *MS*: Mean Square; *SS*: Sums of Squares, and *df*: Degrees of freedom,

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representatives and all other self-fertile and self-sterile isolates were crossed with them. Also, all homothallic isolates were crossed with each other to identify possible successful mating. Twelve isolates mated successfully with isolate RT51 while only one isolate (ST72) mated with isolate RT162. All isolates which mated successfully with RT51 were sexual incompatible with RT162. Also, isolate ST72 was sexually incompatible with isolate RT51, but compatible with 12 mentioned isolates. All other self-sterile isolates were sexually incompatible with both RT51 and RT162 and did not mate with them. For each of the successful combinations, single ascospore was isolated and tested for its self-fertility. None of these single ascospore cultures were self-fertile. None of the self-fertile isolates crossed successfully with isolates RT51 and RT162 and also other seven self-fertile isolates.

DISCUSSION

The most important citrus producing areas of northern Iran are distributed in Mazandaran and Golestan provinces. *Colletotrichum gloeosporioides* s. l., the causal agent of anthracnose is an important pathogen of citrus orchards in northern Iran and causes reduction in yield annually (unpublished data). In order to choose the best management strategy, study on genetic structure of disease causal agent is necessary (Bayraktar, 2010). The development of different molecular markers and using them for the detection of genetic structure gives the better point of view about genetic relationships among different populations of the organisms (Kubik *et al.,* 2009). Morphological studies were done in order to detect any relationship between fungal isolates morphology with geographic and host regions. The results showed that isolates with similar morphological characteristics were distributed in different geographic regions and host species. In other words, these characteristics could not separate *C. gloeosporioides* s. l. isolates into distinct groups according to the sampling areas and hosts. Sanders and Korsten (2003) studied the different morphological characteristics and showed that none of these characteristics, except spore length, could separate *C. gloeosporioides* s. l. isolates from mango and avocado into distinct groups. They also revealed that isolates from mango producing areas had higher growth rate.

The regional separation of isolates was not observed in the dendrogram constructed by using rep-PCR data because there was probably evidence of admixture among all the three regions isolates. The separation according to host species was not also observed in this dendrogram. Also, a low correlation was detected between rep-PCR and morphological grouping of isolates. The evidence of high level of gene flow was detected among the isolates of the three regions. Different mechanisms that enable gene flow may act separately or in combination. Some of these mechanisms are: (1) Exchanging of infected saplings and fruit; (2) Alternate hosts which allow certain genotypes to survive and spread across the area, and (3) Vegetative compatibility (McDermott and McDonald, 1993; Milgroom, 1996; McDonald and Linde, 2002; Milgroom and Peever, 2003). The main citrus producing areas of Iran covering 104 thousand hectares are located in the north part of the country. Proper circumstances including moderate and highly humid weather together with extensive forests containing different genus of *C. gloeosporioides* s. l. hosts could help the pathogen to survive and spread across the area (unpublished data). Moreover, Khansarei Atigh *et al*. (2010) reported the existence of vegetative compatibility among isolates of these three regions. High levels of vegetative compatibility along with existence of alternate hosts and prevalent transmission of saplings and fruit among these three regions can support the high level of gene flow detected in this study.

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The estimated low level of genetic differentiation and high levels of Nei's genetic similarity among populations could be explained by gene flow. The existence of sexual reproduction can exchange alleles among populations. Sexual reproduction among isolates of different regions, which was observed in this study, introduces new alleles to each region. Although the selection omits some of these new recombined genotypes, the selected genotypes spread across the area and increase the frequency of new alleles. The N_M values of > 8 indicate that there is low limitation to gene flow among these geographically distant populations. Therefore, new genotypes can transmit among the three populations and cause more genetic similarity in these three populations. Consistent with our results, Rampersad (2013) detected high level of gene flow and genetic similarity between two groups of *C. gloeosporioides* s. l. isolates from papaya in Trinidad. Nguyen *et al.* (2009) also reported high gene flow between northern and southern populations of *C. gleoesporioides* obtained from coffee in Vietnam.

Analysis of population genetic structure indicates high levels of gene diversity among populations of *C. gloeosporioides* s. l. infected citrus in northern Iran. Gene diversity is not affected by recombination because the frequency of alleles may be equal in fungus which has sexual reproduction with other fungus reproduce clonally (McDonald, 1997). The highest level of gene diversity is observed in isolates which are collected at or near the species original center or in the areas where the population is older (McDonald, 1997). The origin of citrus is in the part of southeast Asia boarded by northeast India (Andrews, 1961). The first place in northern Iran where citrus plants were cultivated 300 years ago was near Ramsar (unpublished data). In this study, the highest level of gene diversity was observed in Ramsar population which is the oldest citrus cultivating area in northern Iran. Consistent with our result, Rampersad *et al.* (2013) reported Mexican population of *C. gleoesporioides* s. l. as the ancestral population because of the higher genetic diversity than Trinidad population. Weeds *et al*. (2003) reported that *C. gloeosporioides* s. l. isolates from *Stylosanthes* spp. showed higher levels of genetic diversity when they were isolated from host species origin or where the host species naturalized.

High level of $H_{s,}$, which was supported by the result of AMOVA test, showed that most of the genetic diversity belonged to within populations. All these results, together with low genetic differentiation among the three geographic populations and results of Nei's genetic similarity and distance, indicate that *C. gloeosporioides* s. l. populations are not developing independently and the fungus has homogenous population structure across northern Iran. The results also suggest that these three populations can be part of the Mega genetic population. Abang *et al*. (2006) studied the pathogenic and genetic variability among *C. gloeosporioides* s. l. isolates from different yam hosts in the agroecological zones in Nigeria and reported results similar to this study.

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بررسی ساختار جمعیت ها و باوری جنسی قارچ sensu lato *Colletotrichum gloeosporioides* به دست آمده از درختان مرکبات در شمال ایران

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چکیده

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آنتراکنوز مرکبات با عامل *Colletotrichum gloeosporioides* sensu lato یکی از بیماری های خسارت‌زای مرکبات در شمال ایران است. به منظور بررسی ساختار ژنتیکی جمعیت های قارچ، طی تابستان سال ۱۳۸۷، نمونه‌برداری از سه باغ مرکبات در شهرستان‌های رامسر، ساری و گرگان انجام شد. پنجاه و یک جدایه تک اسپور، با استفاده از نشانگر مولکولی rep-PCR و دو آغازگر REP و BOX مورد مطالعه قرار گرفتند. در دندروگرام رسم شده براساس الگوی انگشت‌نگاری DNA، جدایه‌ها در هشت گروه قرار گرفتند. بررسی پارامترهای مربوط به ژنتیک جمعیت در میان سه جمعیت جغرافیایی، با استفاده از نرم افزار Popgene version 1.31 انجام گردید. نتایج نشان دادند که٪ ۹۴/۳۷ از تنوع ژنتیکی کل (H_t) ناشی از تنوع ژنتیکی در درون جمعیت‌ها (H_s) است. بررسی شباهت ژنتیکی بر مبنای ضریب تشابه Nie، سطح تشابه بالایی را در میان سه جمعیت مورد مطالعه نشان داد. وجود سطح بالایی از جریان ژنی (N_M) (8.4 = نشان دهنده‌ی وجود جریان ژنی فعال در میان سه جمعیت می‌باشد که سطح بالای تنوع ژنتیکی در درون جمعیت‌ها، شباهت ژنتیکی زیاد در میان جمعیت‌ها و عدم تفکیک این سه جمعیت جغرافیایی از یکدیگر را توجیه می‌کند. نتایج نشان دادند که جمعیت‌ها به طور مستقل توسعه نیافته‌اند و تعامل موجود میان آنها سبب شده تا جمعیت های این قارچ در شمال ایران تا حدی همگن باشد و در حقیقت یک جمعیت بزرگ را تشکیل دهند. در دندروگرام رسم شده براساس مجموعه خصوصیات مورفولوژیکی، جدایه ها در نوزده گروه قرار گرفتند. بررسی همبستگی میان ماتریکس‌های مورفولوژیک و rep-PCR نشان داد که همبستگی میان هاپلوتیپ های حاصل از rep-PCR و مورفولوژی بسیار ضعیف است (r=0.2, P=0.992). بررسی باروری جنسی در پنجاه و یک جدایه نشان داد که هشت جدایه خودبارور و بقیه خودنابارور بودند. در میان جدایه‌های خودنابارور، چهارده تلاقی موفق دگر باروری مشاهده گردید.

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