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Genetic diversity of *Botrytis cinerea* isolates in different plant hosts and localities using ISSR molecular markers

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Abstract: *Botrytis cinerea* has a wide host range, the possibility of hiding plant infection from the initial stages, and causing severe damage (even up to 100%) to the host. Due to the chance of growth and activity of the pathogen in field, greenhouse, in laboratory, and even in cold storage conditions, its importance is increasing. The presence of pathogen in seedlings, mature plants, ripe fruits, and even storage conditions in different parts of Iran and its importance, we decided to investigate the genetic diversity of the isolates using the ISSR marker. For this purpose, 21 isolates including 16 isolates from the collection of the University of Tehran (isolated from seedling and leaf of cauliflower, grizzly lettuce, needled lettuce, purple basil, onion, tomato, strawberry and pomegranate) and five isolates collected from two strawberry greenhouses in Alborz province, were used. Ten ISSR primers were used to determine the genetic diversity. Analyzing the results by NTSYS software version 2.02e (based on SM and UPGMA clustering method) showed that the isolates fall into four fingerprinting groups. It was also observed that the resulting bands have 100% polymorphism (93.33% in one case). The calculated cophenetic coefficient for the data (0.89) confirmed the accuracy of the obtained dendrogram. Evidence suggests that there is high genetic diversity in *B. cinerea* isolates and also there is no relationship between host and geographical region.
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Key words: Gray mold, Greenhouse, strawberry, Cold room

INTRODUCTION

Botrytis cinerea is a fungus that belongs to Sclerotiniaceae (Helotiales, Ascomycota), which is commonly known as grey mold fungus (Polat et al. 2018a). The fungus destroys the tissue of living plants, and can grow and continue to live on the host plant even after the destruction (Naeimi & Zare 2013). *B. cinerea* can survive as mycelium or conidia for a long time and provide a source of subsequent infections (Reino et al. 2004). *B. cinerea* can live in both parasitic and saprophytic stages. In strawberry, the pathogen enters the bud at the beginning of flower formation, survives saprophytically, and remains there until the crop's maturation, shifts to the parasitic phase at the later stages of plant development and can cause tremendous damage (Naeimi & Zare 2013). Microconidia are mononuclear and rarely germinate *in vitro*, and play the role of male gametes in sexual reproduction (Dowling 2018). In general, *B. cinerea* attacks the leaves, flowers, fruits, and stems of numerous plants in the legumes, vegetables, ornamental plants, and fruits and causes scorching of leaves and flowers, stems and fruits rot, and even turns the fruit into a mummy form (Ebrahimzadeh & Abrinbana 2016). Other species of *Botrytis*, such as *B. pseudocinerea*, *B. caroliniana*, *B. fragariae*, and *B. mali* has also been identified as the causal agent of gray mold. However, *B. cinerea* is the most important among them (Decognet *et al.* 2006). The optimum temperature to grow the fungus is 20-25˚C, and the maximum growth temperature is 40˚C. However, the growth of the pathogen is possible at 0-10˚C in storage (Zhou et al. 2020). The presence of multinucleated conidia and vegetative adaptation lead to constant changes during the reproductive period of this fungus in field and laboratory conditions. Therefore, phenotypic and genotypic changes are widespread in this species (Younesi Bane et al. 2014). Several studies based on *B. cinerea* Bc-hch gene amplification, the presence of *Boty* and *Flipper* transportable elements, and vegetative compatibility

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groups (VCGs) have also revealed very high genetic diversity of this species (El Ghaouth et al. 1997, Fekete et al. 2012, Hu et al. 2018). Passing of time, due to observing the wide variation in *B. cinerea* isolates, and the interest of researchers to collect more accurate information about this fungus, they conducted studies using DNA-based markers. These markers are reproducible and show genetic differentiation within and between populations well (Younesi Bane et al. 2014).

The accuracy of the results and time and cost-effectiveness can be mentioned as the advantages of Inter Simple Sequences Repeat (ISSR) marker (El Ghaouth et al. 1997). This marker has been used to study the genetic diversity of *B. cinerea* isolates from various plants such as pepper, dill, tomato, lettuce, eggplant, basil, bean, and cucumber in Antalya, Turkey (Polat et al. 2018a,b). However, no study has yet been conducted using this marker for the genetic diversity of this species in Iran. All previous studies have used RAPD, SRAP, and most recently, SSR markers. The genetic diversity of *B. cinerea* isolated from various hosts has been studied in several parts of the world, such as: grapes (Campia 2014, Fournier & Girard 2018, Karchani-Balma et al. 2008, Walker et al. 2015, Zhou et al. 2020), strawberries (Isaza et al. 2019, Leyronas et al. 2015, Rasiukevičiūtė et al. 2018, Reino et al. 2004), blackberries (Karchani-Balma et al. 2008, Rasiukevičiūtė et al. 2018), raspberries (Asadollahi et al. 2013, Gogoi et al. 2020, Poczai et al. 2013, Zhou et al. 2020), nectarines (Diao et al. 2020), cucumbers (Leyronas et al. 2015), lettuces (Dowling 2018, Poczai et al. 2013), tomatoes (Diao et al. 2020, Leyronas et al. 2015, Poczai et al. 2013, Zhou et al. 2020), cherries (Diao et al. 2020) and beans (Leyronas et al. 2015). Start Codon Targeted (SCoT) polymorphism is also used in genetic diversity studies (Gogoi et al. 2020). This marker received much attention, immediately after its introduction by Clard and Mickel (2009), because of its advantages such as accessible design, high reproducibility, lack of specificity, convenient polymorphism, and dedicated band replication based on the protected region of the translation start codon (ATG) (Gogoi et al. 2020, Poczai et al. 2013). Nevertheless, SCoT based primers has only been used in 5% of genetic diversity studies using molecular markers. The uses of SCoT based primers are related to the study of genetic diversity or other cases on different plants (Poczai *et al*. 2013). For example, Gozin et al. (2004) studied the genetic relationships between Chinese diploid strawberry varieties using SCoT based primers. This primer have a higher polymorphism compared to ISSR (Guo-Xin et al. 2014), but the study of Gogoi et al. (2020) showed that SCoT and ISSR have the same efficiency in fingerprinting of genotypes (Gogoi *et al*. 2020). The infection of plants such as: pomegranate, strawberry, lettuce, onion, cauliflower, and tomato has been recently observed in greenhouses producing seedlings, and fruits and cold stores of these products in different regions of Iran.

With regards to economic importance of products such as strawberry, fancy lettuce, tomato and onion in the country, identifying the genetic diversity of the pathpgen, increase our knowledge to prevent or control the disease. Therefore, in this study, we investigate the genetic diversity of *B. cinerea* obtained from various plants in Alborz, Tehran, and Kurdistan provinces using ISSR primers.

MATERIALS AND METHODS

Sampling, fungal isolation

Sixteen *B. cinerea* isolates were received from the Fungal Collection of the University of Tehran, Department of Plant Pathology. Five isolates were collected from strawberry greenhouses in Alborz province (Tankaman and Nazarabad) during 2021. The characteristics of the isolates are shown in Table 1. Symptomatic stems, leaves and fruit with brown lesions were cultured on PDA medium for ten days (darkness, 25 ˚C) and purified by single spore isolation using water agar medium

DNA extraction

Purified fungal isolates were grown on Petri dishes containing potato dextrose agar, and colonies were transferred to flasks containing 50 ml potato dextrose broth and grown on an orbital shaker for two weeks at 100 rpm and 25˚C. Mycelium was submerged in liquid nitrogen and ground into a fine powder. Genomic DNA was extracted from the fine mycelial powder as described by Murray and Thompson (1980). DNA pellets were dissolved in 30µl of deionized sterile ddH2O and stored at -20°C.

ISSR amplification

Ten ISSR Primers (Table 2) were selected basis on their high PIC as described by (Polat et al. 2018a). PCR amplification was performed in a 25µl reaction volume. The PCR condition for ISSR was as follows: one initial denaturation at 94 ˚C for 2 min; followed by 45 cycles at 94 ˚C for 45 s annealing at 56 for 45 s, and extension at 72 ˚C for 2 min. A final 7 min extension was made at 72 ˚C. The amplified products were resolved on 0.8% agarose gel at 90 V cm^{-1} using TBE buffer and visualized under UV with Gel Doc.

Data analysis

Amplified bands from each primer were scored as present (1) or absent (0). The information is input in an Excel sheet, and used as input data for ntedit software. Similarity matrix was estimated based on similarity coefficient. The obtained data was used to construct dendrogram by NTSYS software, version 2.20. Cluster analysis was based on the UPGMA

clustering method and the goodness of fit of the clustering to the data was calculated using the COPH procedure. Principal coordinate analysis (PCoA) was also done and the results were shown in a 3D view.

RESULTS

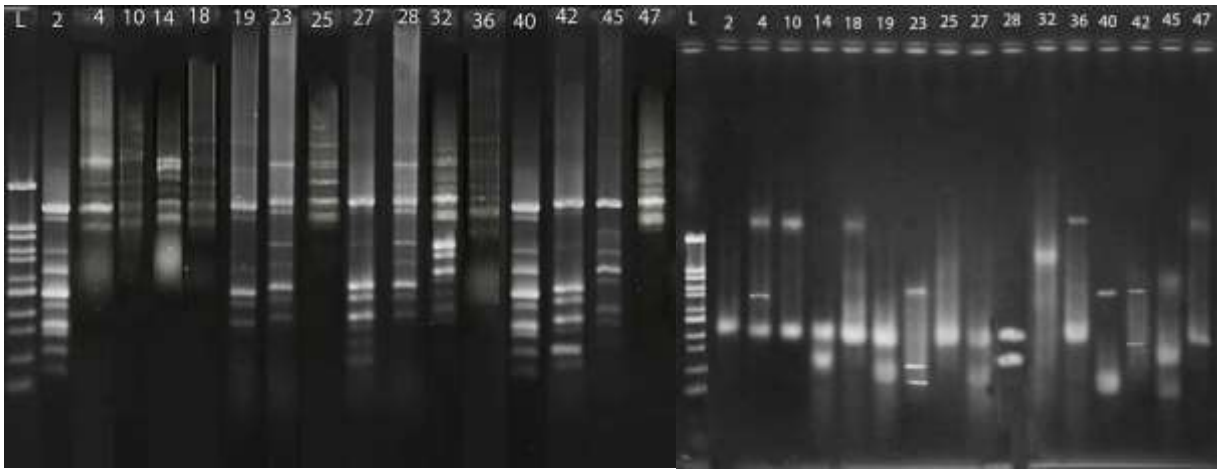
Ten ISSR Primers were used to amplify of loci in 21 *Botrytis cinerea* isolates (Fig. 1). The number of alleles, the number of polymorphic bands, and percentage of polymorphism were evaluated (Table 3). The maximum and the minimum number of bands belonged to primers 818 and 889, respectively. PCR results for two primers, 818 and 889, can be seen in Fig.1. All primers (except primer number 8 (93.33%)) showed 100% polymorphism Cluster analysis of isolates showed four groups containing isolates from different geographic regions and hosts (Fig. 2). Group 1 was the largest and included isolates of all regions and hosts (Hashtgerd strawberries (Alborz province), Sanandaj strawberries (Kurdistan province), Nazarabad tomatoes (Alborz province), Nazarabad onions (Alborz Province), Tehran strawberries (Tehran Province), Nazarabad Cauliflower, Grizzly Lettuce, Needled Lettuce and Purple Basil (Alborz Province) and Shiraz Pomegranate (Fars Province). The smallest group was group 2 (including one isolate from Tankaman strawberry (Alborz province). The remaining isolates, obtained from strawberries in Nazarabad and Tankaman, were placed in groups three and four. The cophenetic coefficient for the drawn dendrogram was 0.89. The obtained diagram, according to the information given to the software, is shown in Fig. 3. Principal component analysis was performed for the isolates. A three-dimensional view of the test results can be seen in Fig. 4.

Table 1. Isolates of *Botrytis cinerea* were used in this study.

Isolate	**Host**	**Plant tissue**	**Location**	**Accession number**
2	*Fragaria annussa* Duchesne	Fruit	Hashtgerd (Alborz province)	
4	*Solanum Lycopersicum* L.	Seedlings	Karaj (Alborz Province)	
10	*Allium cepa* L.	Seedlings	Nazarabad (Alborz Province)	
14	*Fragaria annussa* Duchesne	Refrigerated fruit	Tehran (Tehran Province)	ABRIIC 10358
18	*Lactuca sativa* var. lingifolia asterales	Seedlings	Nazarabad (Alborz Province)	
19	*Cichorium endivia* var. crispum	Seedling	Nazarabad (Alborz Province)	
23	*Brassica oleraceae* var. botrytis L.	Seedlings	Nazarabad (Alborz Province)	
25	*Cichorium endivia* var. crispum	Seedling	Nazarabad (Alborz Province)	
27	*Ocimum basilicum* L.	Seedlings	Nazarabad (Alborz Province)	
28	*Ocimum basilicum* L.	Seedlings	Nazarabad (Alborz Province)	
32	*Punica granatum* L.	Refrigerated fruit	Neyriz (Fars Province)	ABRIIC 10357
36	*Fragaria annussa* Duchesne	Leaf	Sanandaj (Kurdistan Province)	
40	*Fragaria annussa* Duchesne	Refrigerated fruit	Sanandaj (Kurdistan Province)	
42	*Fragaria annussa* Duchesne	Fruit	Sanandaj (Kurdistan Province)	
45	*Fragaria annussa* Duchesne	Fruit	Sanandaj (Kurdistan Province)	
47	*Fragaria annussa* Duchesne	Leaf	Sanandaj (Kurdistan Province)	
A	*Fragaria annussa* Duchesne	Fruit	Tankaman (Alborz Province)	
B	*Fragaria annussa* Duchesne	Fruit	Tankaman (Alborz Province)	
C	*Fragaria annussa* Duchesne	Leaf	Nazarabad (Alborz Province)	
D	*Fragaria annussa* Duchesne	Strawberry	Nazarabad (Alborz Province)	
E	*Fragaria annussa* Duchesne	Strawberry	Nazarabad (Alborz Province)	

Table 2. The ISSRs primers were used in this study.

Primer	Sequence	Complete sequence (5'-3')	Annealing temperature	Reference
808	(AG)8C	AGAGAGAGAGAGAGAGC	50	Polat at al. 2018a
818	(CA)8G	CACACACACACACACAG	50	Polat at al. 2018a
809	(AG)8G	AGAGAGAGAGAGAGAGG	50	Polat at al. 2018a
B	(AG)8TG	AGAGAGAGAGAGAGAGTG	52	Polat at al. 2018a
C	(AG)8CG	AGAGAGAGAGAGAGAGCG	54	Polat at al. 2018a
D	(AG)8	AGAGAGAGAGAGAGAG	46	Polat at al. 2018a
AA3	(AG)8TA	AGAGAGAGAGAGAGAGTA	50	Polat at al. 2018a
ISSR8	(GA)8GG	GAGAGAGAGAGAGAGAGG	54	Polat at al. 2018a
ISSR9	GAGG(AG)6G	GAGGAGAGAGAGAGAGG	53	Polat at al. 2018a
889	AGTCGTAGT(AC)7	AGTCGTAGTACACACACACACAC	61	Polat at al. 2018a



A B

Fig. 1. PCR products of 21 *Botrytis cinerea* isolates (16 isolates (2, 4, 10, 14, 18, 19, 23, 25, 27, 28, 32, 36, 40, 42, 45 and 47) obtained from the collection of the University of Tehran and five isolates (A-E) collected in this research on 1% Agarose gel with ISSR primers A) primer 818, B) primer 889 by using 100 bp DNA ladder

Table 3. Data related to the total number of bands, polymorphic bands, and polymorphic coefficients for used primers in this study.

Primer's name	Maximum no. of bands	Number of polymorphic bands	Polymorphism (%)
808	14	14	100
818	19	15	100
809	18	18	100
B	17	17	100
C	16	16	100
D	9	9	100
AA3	16	16	100
ISSR8	15	14	93/33
ISSR9	17	17	100
889	11	11	100

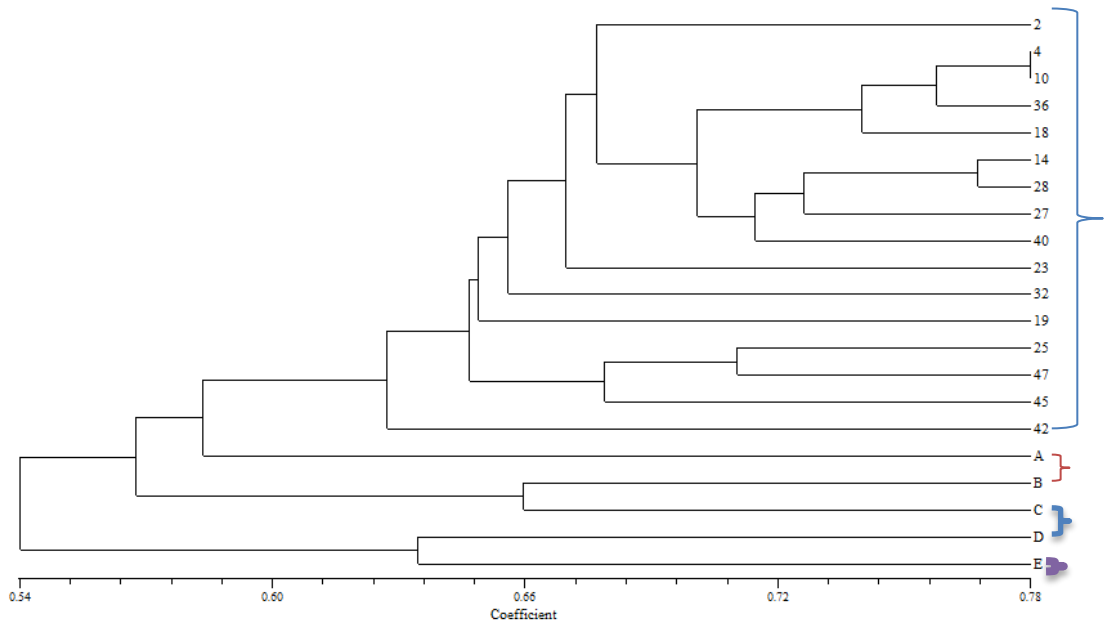


Fig. 2. Clustering of the 21 *Botrytis cinerea* isolates studied. Dendrogram was drawn based on similarity coefficient and UPGMA clustering method by NTSYS software version 2.02e software.

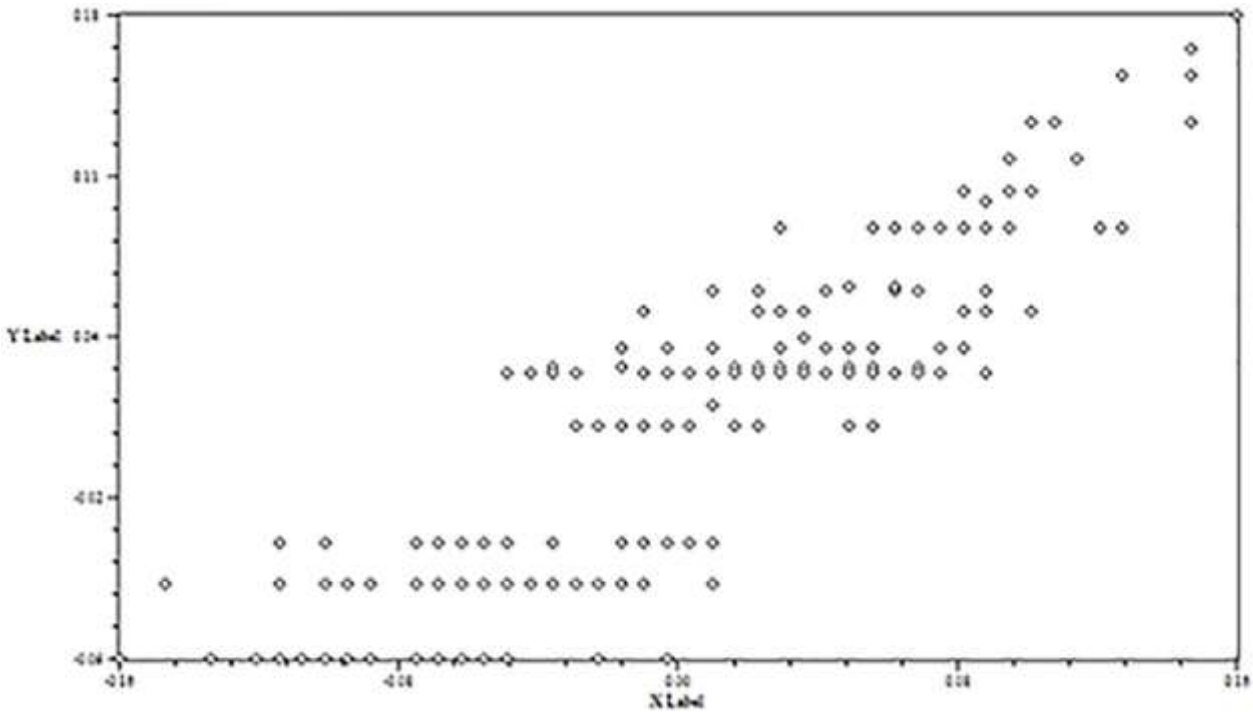


Fig. 3. Schematic view of cophenetic coefficient analysis of 21 *Botrytis cinerea* isolates. Chart were drown based on data of this study by NTSYS version 2.02e software.

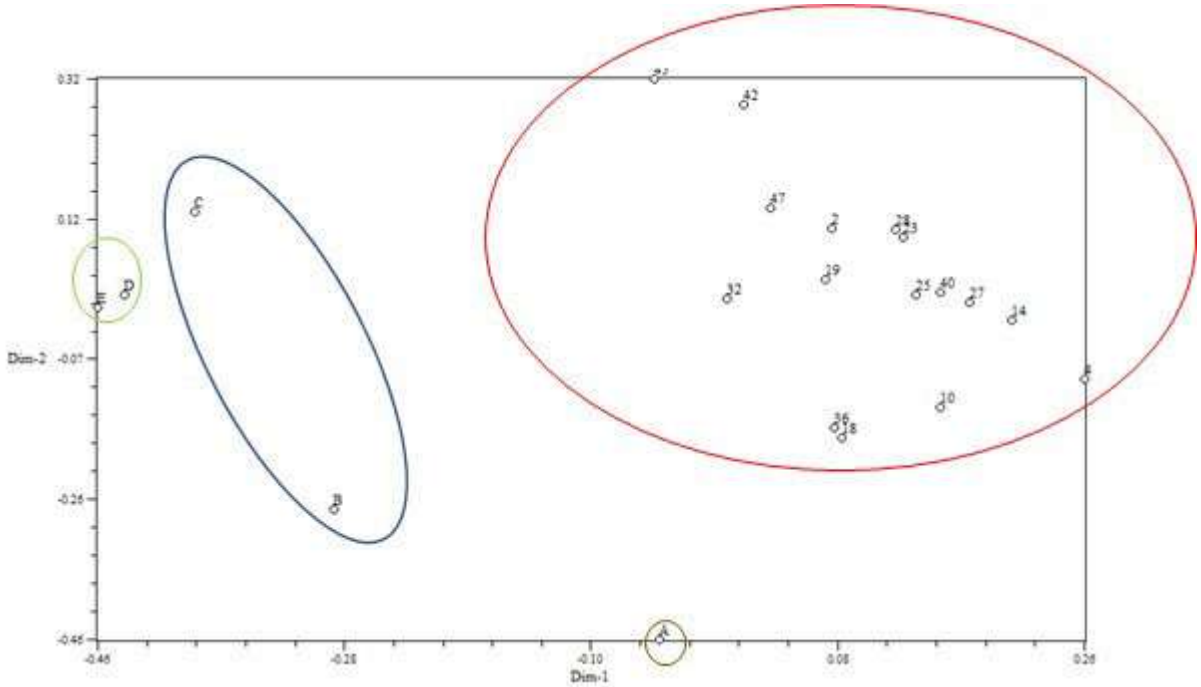


Fig. 4. 3D view of principal component analysis (PCoA) of 21 *Botrytis cinerea* isolates studied in this research by NTSYS version 2.02e software.

DISCUSSION

Botrytis cinerea can infect a large group of host plants worldwide. Although some species, like *B. pseudocinerea*, *B. caroliniana*, *B. fragariae*, and *B. mal* were mentioned as the causal agent of this destructive disease in Iran (Mirzaei et al. 2008), *B. cinerea* is the most important one due to extend prevalence and damage.

Botrytis cinerea isolates show a very high level of diversity (Polat et al. 2018a, b). However, there are not several studies on genetic diversity among isolates obtained from a different host, in different areas, in Iran. So, we decide to find out genetic diversity, using ISSR markers.

Results showed that all primers were amplified and showed high polymorphism. One of the criteria for genetic diversity is the amount of polymorphism between isolates. The results of this study showed that all primers formed utterly different bands. The polymorphism coefficient was calculated 100% according to the amplified and polymorphic bands. The polymorphism coefficient for only one band from ISSR8 primer in all samples, was 93.33%. These results indicate that the primers used were able to separate the isolates. The dendrogram obtained from the data shows that the primers could divide the isolates into four groups.

On the other hand, the cophenetic coefficient (r = 0.89), indicates that the drawing method of the dendrogram is correct. All isolates from the University of Tehran were grouped together, regardless the sampling area and host type. The second group contained only one of the isolates obtained from Tankaman of Alborz province. Interestingly, even though the two isolates obtained from Nazarabad were settled in the fourth group, one of the isolates in the mentioned place was placed in a separate group (third group) next to the isolates from Tankaman of Alborz province. Dendrogram results indicate that isolates obtained from a crop in two regions and samples isolated from various plants in close geographical areas may be genetically similar.

Also, the isolated obtained from different products in a region with an extended geographical distance (pomegranate of Fars province) can be classified in a group with other isolates. The placement of isolates obtained from two different lettuce cultivars in a group was consistent with the results of Polat et al. (2018a) research, the various cultures of pepper obtained from greenhouses were grouped together. Another study that confirmed such results was conducted by Asadollahi et al. (2013) in northeastern Hungary using SSR primers. They showed that isolates obtained from two crops in one year could be placed in the same group. Another issue in the present study, as several studies had shown, is placing strawberry samples (isolated from one region or different regions) in different groups. For example, the study survey by Polat et al. (2018b), using the ISSR primer, provided evidence that isolates detached from a product may be located in different dendrogram groups. This study divided isolates obtained from dill into two groups due to different sampling locations, and pepper isolates had similar conditions. The only difference was that the two pepper isolates, which were detached from the others, were the same in terms of the sampling area. A study by Rasquicit et al. (2018) using SSR on strawberries in Germany also showed that it is even possible to place two isolates of a single host cultivar in two separate groups.

It should be noted that the cultivars planted in sample locations (Tankaman and Nazarabad) greenhouses, were different. A recent study in China on nectarine, tomato, and cherry using SSR also grouped samples of a host in various regions into separate groups (Diao et al.2020). Another aspect of this study, which confirms the present study's data, is that the isolates obtained from cucumber, tomato, and eggplant are in the same group. These samples were collected from areas close to each other. In this study, since the greenhouse strawberry in Hashtgerd received its seedlings from Kurdistan province, the observed similarity is well justified.

On the other hand, the uniformity of fungal isolated from grizzly lettuce, coniferous lettuce, basil, onion, and tomato and the proximity of Tehran and Alborz provinces, justifies the results.

Part of the results of Polat et al.(2018b) and the study of Walker et al. (2015), who worked on the genetic diversity of the different *B. cinerea* isolates from different hosts in France using SSR, confirm the correct placement of isolates from Tankaman and Nazarabad strawberries in two separate groups. However, the proximity of the two sampling places may cast doubt on the obtained results. Nevertheless, the results of Hu et al. (2018) using SSR on strawberries in South Carolina based on high variability between isolates obtained from close fields and even isolates from different organs of a flower, another confirmation of the results of the present research. Earlier, Hamada & Ben Ahmed (2005) and Karchami-Bala et al. (2008) examined strawberry-derived isolates in separate groups by examining different hosts in Tunisia.

Given the results and the location of isolates separated from cold storage seedlings and fruits or strawberry fruits and plants, there is no relationship between genetic diversity and the stage of the plant from which the fungus was derived. On the other hand, observing the isolates of different hosts in a group and placing the isolates of one host in two separate groups confirms the dependence of genetic diversity on the location or type of host. Studies by Wakeie et al. (2008), Rajagros & Shaw (2010), Campia (2014), and Isaza et al. (2019) (all examined genetic diversity in different parts of the world using SSR), confirm the independence of gene diversity at the sampling site.

Finally, the outcomes of this research show that despite high genetic diversity of *B. cinerea* isolates, which had studied in this project, genetic diversity is not affected by the host and geographical region, and there is no relation between them.

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تنوع ژنتیکی جدایه‌های *Botrytis cinerea* از گیاهان میزبان و مناطق مختلف با استفاده از آغازگرهای ISSR

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چکیده : قارچ *Botrytis cinerea*، به دلیل داشتن دامنهٔ میزبانی گسترده، امکان آلوده کردن پنهان گیاه از مراحل ابتدایی و وارد کردن خسارت بالا (حتی تا ۱۰۰٪) به میزبان، از اهمیت بسیار بالایی برخوردار است. از طرف دیگر، امکان رشد و فعالیت این قارچ در شرایط مزرعه، گلخانه، آزمایشگاه و حتی سردخانه اهمیت آن را دو چندان می‌کند. با توجه به جداسازی این قارچ از نشاء، گیاه کامل، میوهٔ رسیده و حتی انبارشده در مناطق مختلف کشور، تنوع ژنتیکی جدایه‌های به دست آمده با استفاده از آغازگرهای ISSR مورد بررسی قرار گرفت و به این منظور، ۲۱ جدایه شامل ۱۶ جدایهٔ دریافت‌شده از کلکسیون دانشگاه تهران (جدایه‌ها از گیاهچه و برگ پیاز، ریحان بنفش، کاهو سوزنی، کاهو گریزلی،گل کلم و گوجه‌فرنگی در نظرآباد، میوهٔ توت‌فرنگی تازه/سردخانه‌ای در تهران، سنندج و هشتگرد و میوهٔ انار سردخانه‌ای در نیریز) و پنج جدایه حاصل از نمونه‌برداری (زمستان ۱۳۹۹) از دو گلخانهٔ توت‌فرنگی در نظرآباد و تنکمان (استان البرز) مورد استفاده قرار گرفتند. تکثیر DNA ژنومی نمونه‌ها با استفاده از ۱۰ آغازگر ISSR و بررسی نتایج با استفاده از نرم‌افزار NTSYS نسخهٔ 2.02e (بر اساس ضریب تشابه SM و روش خوشه‌بندی UPGMA) نشان داد که جدایه-ها در چهار گروه انگشت‌نگاری متفاوت قرار می‌گیرند. همچنین مشاهده شد که باندهای حاصل از تکثیر دارای چندشکلی ۱۰۰٪ (فقط در یک مورد ۹۳/۳۳) هستند. ضریب کوفنتیک محاسبه شده برای داده‌ها (۰/۷۷) درستی دندروگرام رسم‌شده را تأیید کرد. شواهد موجود، همراستا با سایر مطالعات، حاکی از آن است تنوع ژنتیکی جدایه‌های *B. cinerea* در ایران بالا بوده و تحت تأثیر میزبان یا منطقهٔ جغرافیایی قرار ندارد.

کلمات کلیدی: کپک خاکستری، گلخانه، توت‌فرنگی، سردخانه

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