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Molecular fingerprinting of Botrytis *cinerea* population structure from different hosts

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Abstract
Botrytis cinerea (teleomorph: *Botryotinia fuckeliana*) causes gray mold disease on vegetable crops in greenhouses. Profound knowledge on pathogen diversity is necessary for efficiently disease management. In this study, forty-two *B. cinerea* isolates collected from 36 different greenhouses in Antalya province of Turkey were investigated. Twelve SRAP (sequence-related amplified polymorphism) and 18 ISSR (inter simple sequence repeat) primers producing high polymorphic fragments were used to genetic diversity of *B. cinerea* isolates infecting dill, basil, lettuce, bean, cucumber, tomato, pepper and eggplant. The unweighted pair-group method with arithmetic average analysis (UPGMA) was used to evaluate of combined ISSR and SRAP data showing a similarity range 0.15-0.90 among the isolates. Cophenetic correlation of the tree was high level (r=0.93). Interestingly, cluster analysis showed a divergent group consisting of lettuce isolates which were genetically different from the other isolates. On the other hand, transposable elements (*Flipper and Boty*) were detected among isolates from all the hosts. Isolates containing only the *Fliper* element were detected. The results showed that genetically characterized *B. cinerea* populations by a high level of genetic diversity were associated with genotype flow and the evolutionary potential of *B. cinerea*. In further studies, the newly tested molecular markers are useful and can be suggested for analyzing of genetic diversity and population structure of this pathogen on different hosts.

Keywords: Gray mold; Genetic diversity; Host differentiation; ISSR; SRAP; Transposable elements

Farklı konukçulardan elde edilen *Botrytis cinerea* populasyon yapısının moleküler tanılanması

Öz
Botrytis cinerea (teleomorph: *Botryotinia fuckeliana*) örtüaltı sebze yetiştiriciliğinde kurşuni küf hastalığı etmenidir. Patojende oluşan farklılıkların bilinmesi hastalıkla mücadelenin etkinliğini arttırmaktadır. Çalışmada, Türkiye'nin Antalya ilinde yer alan 36 farklı seradan 42 adet izolat kullanılmıştır. On iki SRAP (sequence-related amplified polymorphism) primer kombinasyonu ve 18 ISSR (inter simple sequence repeat) primeri dereotu, fesleğen, marul, fasulye, hıyar, domates, biber ve patlıcandan elde edilen *B. cinerea* izolatlarının genetik farklılıklarının belirlenmesinde oldukça yüksek polimorfizm sağlamışlardır. ISSR ve SRAP markırlardan elde edilen sonuçlar UPGMA (The unweighted pair-group method with arithmetic average analysis) analizine göre izolatlar arasında 0.15-0.90 oranında değişen benzerlik elde edilmiştir. Ayrıca, cophenetic correlation değeri r=0.93 ile oldukça yüksek bulunmuştur. Cluster analizi sonuçları değerlendirildiğinde marul izolatları diğer izolatlara göre oldukça uzak kümelenmiştir. Ayrıca, tüm izolatlar için tranpozabl elementler (*Flipper* ve *Boty*) araştırılmış ve sadece *Flipper* element tespit edilmiştir. Elde edilen genetik karakterizasyon sonuçlarına göre, *B. cinerea* populasyonunda oldukça yüksek seviyede genetik farklılıklar bulunmuştur. Bu durum, *B. cinerea*'nın evrimselleşme potensiyeli ve gen akışlarından kaynaklanabilir. Farklı konukçulardan elde edilen bu patojenin genetik farklılıklarının belirlenmesinde kullanılan moleküler markırlar, ileride yapılacak çalışmalara da ışık tutmaktadır.

Anahtar Kelimeler: Kurşuni küf; Genetik farklılık; Konukçu farklılığı; ISSR; SRAP; Transpozabl elementler

1. Introduction

Greenhouse cultivation is the most widespread style for horticultural crops with advantages worldwide (Jensen, 2002). In the world, China takes first place with 2 million 700 thousand hectares as protected agricultural land, South Korea, Spain, Japan and Turkey follow respectively (FAO, 2014). Mediterranean region of Turkey is unique area in the world due to the mild winter climatic conditions for greenhouse cultivation (Tüzel and Leonardi, 2010). About

250.000 da of Turkey's general greenhouse land is hosted in Antalya province that is around 35 percent of total greenhouse of Turkey capacity (TUIK, 2014). Diseases and pests are the most important factors limiting crop production in our country. On the other hand, use of over dose pesticide causes the environmental pollution, leads to damage on soil structure, disturb the natural balance in microflora, (Elad et al., 2007) and resistance to pesticides (Williamson et al., 2007; Sun et al., 2010; Shao et al., 2015). *B. cinerea* (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) causing gray mold disease is a polyphagous an airborne fungal pathogen attacking over 200 crop hosts worldwide. Pathogen limits vegetable cultivation in greenhouses and its control in fields is difficult. It has different modes of attack and survives as mycelia and/or conidia form for a long time (Williamson et al., 2007). In cultivation under greenhouse conditions, there is no available resistance variety to *B. cinerea*, yet. Therefore, effective control using chemicals is very important (Rosslenbroich and Stuebler, 2000; Sun et al., 2010). The pathogen is in the list of 'high-risk' category according to statement of Fungicide Resistance Action Committee (Brent and Hollomon, 1998; Angelini et al., 2012). Researchers have studied on its taxonomy and species for many years for effective disease management (Sun et al., 2010).

Molecular markers based on DNA have been introduced over the last two decades, which have revolutionized the entire scenario of biological sciences. Major DNA markers using for identification, diversity, taxonomy, relationship, fingerprinting and diagnostics were suggested (Datta et al., 2011; Baysal et al., 2013). In recent years, these markers as specific fragments of DNA that can be identified within the whole genome have been improved. SRAP molecular markers system also based on random amplification of coding regions in the genome. Its use in genetic diversity analysis is a simple, middle-yield, high-dominant total, repetitive way on genetic diversity of pathogens (Polat et al., 2014). ISSR markers allow a cost-effective detection and quantification of the pathogen (Schlötterer, 2004; Baysal et al., 2011). Transposons can have significant effects on distinctive phenotypic traits of phytopathogenic fungi. Two transposable elements, *Boty* and *Flipper*, are known to be associated with the ubiquitous fungus *B. cinerea* (Kecskeméti et al., 2014).

This study aimed to characterize and assess the fingerprinting of *B. cinerea* isolates from different host using combined dominant ISSR and co-dominant SRAP molecular markers. ISSR and SRAP markers have not been used before in any study related to genetic diversity of *B. cinerea* to evaluate of isolates collected from different hosts. Furthermore, transposable elements (*Flipper* and *Boty*) were detected among isolates from all the hosts.

2. Materials and Methods

2.1. Survey studies

All plants infected with *Botrytis cinerea* were collected from the vegetable greenhouses during survey studies. Forty-two *B. cinerea* isolates were sampled representing from different districts Kumluca, Demre, Muratpaşa, Kepez, Aksu, Serik, and Alanya county of Antalya province in Turkey. Forty-two *B. cinerea* isolates infecting dill, basil, bean, cucumber, tomato, pepper, eggplant and lettuce were obtained from these different hosts. (Table 1). All samples were transferred to the laboratory in individual polyethylene bags to prevent cross contamination. Then, samples incubated in sterile petri dishes (PDA with 100 mgL^{-1} streptomycin sulphate) in incubator (23±1°C) to obtain abundant conidia. Single-spore was obtained from isolates and morphologically identified using microscope (Olympos BX 43). For molecular diagnostics, total genomic DNA was extracted from mycelium of *B. cinerea* using DNA isolation kit (Promega, Wizard Genomic DNA Purification Kit, Madison, US) according to the manufacturer's instructions. DNA quality (260/230 and 260/280 ratios) and concentration were checked by NanoDrop Spectrophotometer (Thermo Scientific-Waltham, Massachusetts). ITS1 and ITS4 universal primers (White et al., 1990) were used to identify of isolates for polymerase chain reaction (PCR). Each PCR reaction contained 1.5 µl DNA (50 nm) template DNA, each primer 1 µl (0.3 µM), 10 µl master (GeneAll, 2 X AmpMaster Taq) and 5 µl ddH_2O in a final reaction volume of 20 ml. An initial denaturation step at 95°C for 5 min was followed by 35 cycles of denaturation at 95°C

for 30 s, annealing at 58.5°C for 1 min and extension at 72°C for 1 min, with a final extension step of 72°C for 7 min. Amplified products were visualized by 2% high resolution agarose gel electrophoresis in 1xTAE buffer, stained ethidium bromide, and photographed under UV machine (ENDURO GDS Gel Documentation System). The ITS sequences of *B. cinerea* isolates were compared and confirmed using GenBank database of NCBI.

2.2. Molecular identification of pathogen isolates by molecular markers

2.2.1. ISSR markers

885, 890, 887, 886 and 880 (Baysal et al., 2009; Baysal et al., 2011; Baysal et al., 2013), 808, 809, 810, 812, 824, 825, 827, 829, 834, 835, 889, 731 and 112 (Polat et al., 2014; Ünlü et al., 2017) primers were used to analysis. Amplifications were carried out in reaction volumes of 15 µl containing 1µl DNA (50 nm) template DNA, 1µl dNTP (0.1 mM dNTPs), 1.5 µl $MgCl_2$ (2.5 mM $MgCl_2$), 0.2 µl Taq (0.6 U Taq DNA polymerase), 2 µl primer (0.3 µM primer),

Table 1. Samples list of *B. cinerea* collected host, location and GPS data (E and N)

E	N	Location	Host	Isolate ID	Another host in the same greenhouse
294111	4086863	Salur Village/Mavikent	Dill	D1	Pepper
402463	4049540	Elikesik Village/Alanya	Dill	D2	Pepper
532835	4238376	Topçular/Muratpaşa	Dill	D3	Dill
531435	4438171	Tarım District/Muratpaşa	Dill	D4	Dill
230532	4018238	Mazılca Mevkii/Demre	Basil	Ba1	Pepper
259167	4032816	Taçbaş Village/Kumluca	Basil	Ba2	Pepper
260539	4021570	Orta District, Seyrek Street/Mavikent	Basil	Ba3	Pepper
234747	4018260	Beymelek/Demre	Basil	Ba4	Pepper
233573	4016620	Beymelek/Demre	Bean	B1	Pepper
402598	4054128	Toslak Village/Kızılca District	Bean	B2	Pepper
259167	4032816	Taçbaş Village/Kumluca	Bean	B3	Pepper
258686	4022086	İncekum/Mavikent	Bean	B4	Pepper
259167	4032816	Taçbaş Village/Kumluca	Cucumber	C1	Pepper
255219	4026350	Kumluca	Cucumber	C2	Tomato
319390	4087312	Köseler District/Aşağı Kocayatak	Cucumber	C3	Pepper
768638	4017359	Güvercinlik-Akkent District/Demre	Cucumber	C4	Pepper
320243	4089662	Yukarı Kocayatak	Tomato	T1	Tomato
322078	4089786	Kayaburnu	Tomato	T2	Tomato
402598	4054128	Toslak Village/Kızılca District	Tomato	T3	Tomato
230988	4015897	Yaylakaya/Demre	Tomato	T4	Tomato
323051	4089345	Kayaburnu	Tomato	T5	Tomato
325346	4089693	Çandır/Serik	Tomato	T6	Tomato
306802	4092026	Aksu/Antalya	Tomato	T7	Tomato
245219	4020350	Kumluca	Tomato	T8	Tomato
325116	4089311	Çandır/Serik	Tomato	T9	Tomato
326548	4093570	Alacami Village/Çandır	Tomato	T10	Tomato
323051	4089345	Kayaburnu	Tomato	T11	Tomato
326548	4093570	Çandır/Serik	Pepper	P1	Pepper
320190	4088408	Çakallık/Köseler District/Aşağı Kocayatak	Pepper	P2	Pepper
768028	4018629	Köseler District/Aşağı Kocayatak	Pepper	P3	Pepper
322109	4089865	Kayaburnu	Pepper	P4	Pepper
769437	4017036	Yaylakaya/Demre	Pepper	P5	Pepper
230988	4015897	Yaylakaya/Demre	Pepper	P6	Pepper
321830	4089217	Kayaburnu	Eggplant	E1	Pepper
318476	4087008	Aşağı Kocayatak	Eggplant	E2	Eggplant
230983	4015889	Yaylakaya/Demre	Eggplant	E3	Eggplant
402474	4054114	Toslak Village, Kızılca District/Alanya	Eggplant	E4	Pepper
240219	4019050	Turunçova	Eggplant	E5	Eggplant
532835	4238376	Topçular/Muratpaşa	Lettuce	L1	Lettuce
531635	4439071	Tarım District/Muratpaşa	Lettuce	L2	Lettuce
59904	4475836	Topallı/Antalya	Lettuce	L3	Lettuce
395424	4465988	Gaziler Village/Kepez	Lettuce	L4	Lettuce

1.5 µl (1 X) PCR buffer and 5.8 µl ddH_2O. PCR reactions were performed under the following cycle program: initial denaturation step for 3 min at 94°C, followed by 35 cycles at 94°C for 45 s (denaturation), 48°C for 45 s (annealing) and 72°C for 90 s (extension), followed by a final extension step at 72°C for 10 min.

2.2.2. SRAP markers

Twelve primer combination were created using 8 Em (reverse), 9 Me (forward) SRAP primers (Li and Quiros, 2001; Polat et al., 2014). Amplifications were carried out in reaction volumes of 15 µl containing 1 µl DNA (50 nm) template DNA, 1 µl dNTP (0.1 mM dNTPs), 1.5 µl $MgCl_2$ (2.5 mM $MgCl_2$), 0.2 µl Taq (0.6 U Taq DNA polymerase), 2 µl primer (0.3 µM primer), 1.5 µl (1 X) PCR buffer and 5.8 µl ddH2O. PCR reactions were performed under the following cycle program: initial denaturation step for 5 min at 94°C, followed by 5 cycles at 94°C for 1 min (denaturation), 35°C for 1 min (annealing) and 72°C for 1 min (extension), followed by 35 cycles at 94°C for 1 min (denaturation), 50°C for 1 min (annealing) and 72°C for 1 min (extension), followed by a final extension step at 72°C for 5 min.

2.3. Gel electrophoresis and data analysis

All PCR products were visualized by 2% high resolution agarose gel electrophoresis in 1xTAE buffer at 115 V for 3-4 h, stained ethidium bromide, and photographed under UV light (ENDURO GDS Gel Documentation System). Amplified bands from each primer were scored as present (1) or absent (0). The bands showing consistently amplifications were considered; smeared and weak bands were discarded from the analysis. Statistical analysis was carried out using the software PAST (Paleontological Statistics) (Hammer et al., 2001). The genetic similarity matrix, neighbor joining (NJ), principle component analysis (PCoA) and principal coordinate analysis (PCO) were constructed based on Dice's coefficient (Dice, 1945). On the other hand, Jaccard similarity index was determined (Jaccard, 1907). Polymorphism rates (Pr) were calculated using following formula. Pr= (number of polymorphic bands/total number of bands in that assay unit) x 100. Polymorphism information content (PIC) values were determined using following formula as described by Smith et al. (1997). PIC= 1-Σ fi^2, where fi^2 is the frequency of the i^{th} allele.

2.4. Detection of transposable elements 'Flipper' and 'Boty'

The primers (F300: 5'-GCA CAA AAC CTA CAG AAG A-3' and F1550: 5'-ATT CGT TTC TTG GAC TGT A-3') used for detection of the *Flipper* element amplify a 1250-bp product. The presence of the Boty element was tested using another pair of primers (BotyF4: 5'-CAG CTG CAG TAT ACT GGG GGA-3' and BotyR4: 5'-GGT GCT CAA AGT GTT ACG GGA G-3'), which amplify a 510 bp product (Ma and Michailides, 2005, Tanović et al., 2009). Amplifications were carried out in reaction volumes of 25 µl according to Ma and Michailides (2005). All PCR products were visualized by 2% high resolution agarose gel electrophoresis in 1xTAE buffer at 115 V for 3-4 h, stained EZ-vision one, and photographed under UV light (ENDURO GDS Gel Documentation System).

3. Results

After sporulation, *Botrytis cinerea* isolates collected from 36 vegetable greenhouses were incubated in sterile PDA petri dishes. Single-spore per isolate were developed, selected and morphologically characterized. Whole conidiophores and conidium were grape shape and conidia. Average conidiophore length was 648-2820 µm. Conidial structure was one cell with egg-shape hyaline. Formation of colonies was observed on PDA, after performing at 20°C under light. Aerial mycelium was produced. They were cottony, powdery, compact or radial pattern. Colonies were white, dirty white or greyish white in colour or hyaline then became light grey, dark grey to dark brown after 1 week. For molecular diagnosis of pathogen isolates, the complete ITS region of nuclear ribosomal DNA was sequenced using universal primers ITS1 and ITS4 (White et al., 1990). After morphological and molecular identification, forty-two single-spore isolates of *B. cinerea* from eight different hosts (dill, basil, bean, cucumber, tomato, pepper, eggplant and lettuce) were used to determine of their genetic variability (Table 1). The genetic diversity within

B. cinerea isolates was evaluated using selected ISSR and SRAP markers. For ISSR analysis, amplifications were successfully achieved with 14 primer pairs, and 9 primer combinations for SRAP analysis.

After screening eighteen ISSR primers, 14 primers produced polymorphic, well-resolved band fragments and only 4 primers (835, 890, 880 and 829) did not give any amplification. When a total of 14 ISSR primers were screened, 108 bands were scored. The number of bands scored per primer ranged from 4 (824) to 11 (827), with a mean of 7.71. Polymorphism rates were ranged from 50% (824) to 100% (812, 810, 808 and 889) (Table 2).

The PIC values for the 14 primers ranged from 0.35 (885) to 0.95 (809), with a mean of 0.68 (Table 2). PIC values were generally used in molecular studies as polymorphism score for a marker locus. As an estimate of the discriminatory power of a locus, PIC values were expressed not only the number of alleles, but also the relative frequencies of those alleles (Smith et al., 1997). PIC values ranged from 0 to 1. At a PIC of 0, the marker had only one allele. If a PIC value was greater than 0.7, it was considered to be highly informative. However, a PIC value of 0.44 was considered to be moderately informative. Markers with greater numbers of alleles tend to have higher PIC values, which were more informative (Hildebrand et al., 1992). Therefore, me3em15, me3em16, me4em11, me5em7, me6em15, me8em15, me9em12 and me10em2 primer combinations could be considered as informative in revealing the genetic diversity and determining genetic variation in isolates of *B. cinerea*. A total of 70 alleles were generated using the nine of twelve SRAP primer combinations. However, 3 primer combinations (me2em16, me1em13 and me2me9) did not give any amplification. The number of bands scored per primer ranged from 3 (me13em16 and me4em9) to 12 (me3em15), with a mean of 7.77. Polymorphism rates were found 100% (Table 3). The PIC values for the 9 primer combinations ranged from 0.67 (me4em9) to 0.98 (me10em2), with a mean of 0.84 (Table 3). Therefore, 112, 834, 887, 808, 825 and 809 markers were determined to be highly informative markers that could be considered due to its efficiency to determine the genetic diversity and variation in isolates of *B. cinerea*.

A similarity matrix was calculated using ISSR and SRAP data according to Dice's coefficient (Dice, 1945). Similarity dendrogram was constructed using UPGMA cluster analysis (Figure 1). Cophenetic correlation between ultrametric similarities of tree was found to be high (r=0.93), suggesting the cluster analysis that strongly represent the similarity matrix. Interpretation of the correlation coefficient matrix has been evaluated as follows: $r \geq 0.9$ is very good, $0.8 \leq r < 0.9$ is good, $0.7 \leq r < 0.8$ is poor and $r < 0.7$ is very poor (Aka-Kacar et al., 2005).

Cluster analysis (Figure 2), neighbor joining (Figure 3), multivariate PCoA (Figure 3a) and Principal Coornate Analysis (PCO) (Figure 3b) were used to investigate the relationships among isolates by using 14 ISSR and 9 SRAP markers. In the cluster analysis (Figure 1), isolates from lettuces were the most distinct genotypes from the others.

Table 2. Diversity statistics for 14 inter simple sequence repeats (ISSR) primer studied in 42 *B. cinerea* isolates

No	Locus	Allel sizes (bp)	Ta	Pa	Pr (%)	PIC
1	112	400-500-700-1200-1400-2000-2500-900	8	7	88	0.76
2	731	400-500-600-900-1000-1500-800	7	6	86	0.43
3	834	500-600-800-900-1000-1100-1300-1500-2000	9	7	78	0.85
4	812	400-450-600-900-800-1000-1200-1500-1800	9	9	100	0.59
5	810	500-650-700-1100-1300	5	5	100	0.56
6	886	400-500-650-900-800-1000-1200-1300	8	6	75	0.66
7	887	400-500-800-900-1000-1200-1300-600-700	9	7	78	0.86
8	808	600-650-700-800-900-1000-1200-1500	8	8	100	0.75
9	885	500-600-700-900-1300-1500	6	5	83	0.35
10	889	550-700-800-900-1100-1200-1600	7	7	100	0.53
11	824	800-1300-1000-1600	4	2	50	0.83
12	825	400-550-750-900-1000-1200-1600	7	6	86	0.74
13	827	500-600-700-750-800-900-1000-1100-1300-1400-1500	11	10	91	0.69
14	809	400-800-900-350-500-600-1000-1100-1200-1500	10	7	70	0.95
		Total	108	92	-	-
		Mean	7.71	6.65	84.60	0.68

Ta: Total allel, Pa: Polimorphic allel, Pr: Polymorphism rates, PIC: Polymorphism information content

Table 3. Diversity statistics for 9 sequence related amplified polymorphism (SRAP) primer combinations studied in *B. cinerea* isolates from different locations and host plants

No	Locus	Allel sizes (bp)	Ta	Pa	Pr (%)	PIC
1	me3em15	150-180-250-300-400-500-600-800-950-1000-1200-1500	12	12	100	0.84
2	me3e16	500-800-1100	3	3	100	0.73
3	me4em9	220-450-1000	3	3	100	0.67
4	me4em11	350-400-500-600-700-1200-1300-1600-2400	9	9	100	0.89
5	me5em7	200-300-400-500-600-700-900-1000-1100-1200-1800	11	11	100	0.85
6	me6em15	150-180-300-600-700-800-1000-1300	8	8	100	0.84
7	me8em15	150-250-400-800-1000-1200-2000	7	7	100	0.83
8	me9em12	280-300-400-450-600-700-900-1300-1700	9	9	100	0.95
9	me10em2	250-150-300-500-700-800-900-1500	8	8	100	0.98
		Total	70	70	-	-
		Mean	7.77	7.77	100	0.84

Ta: Total allel, Pa: Polimorphic allel, Pr: Polymorphism rates, PIC: Polymorphism information content

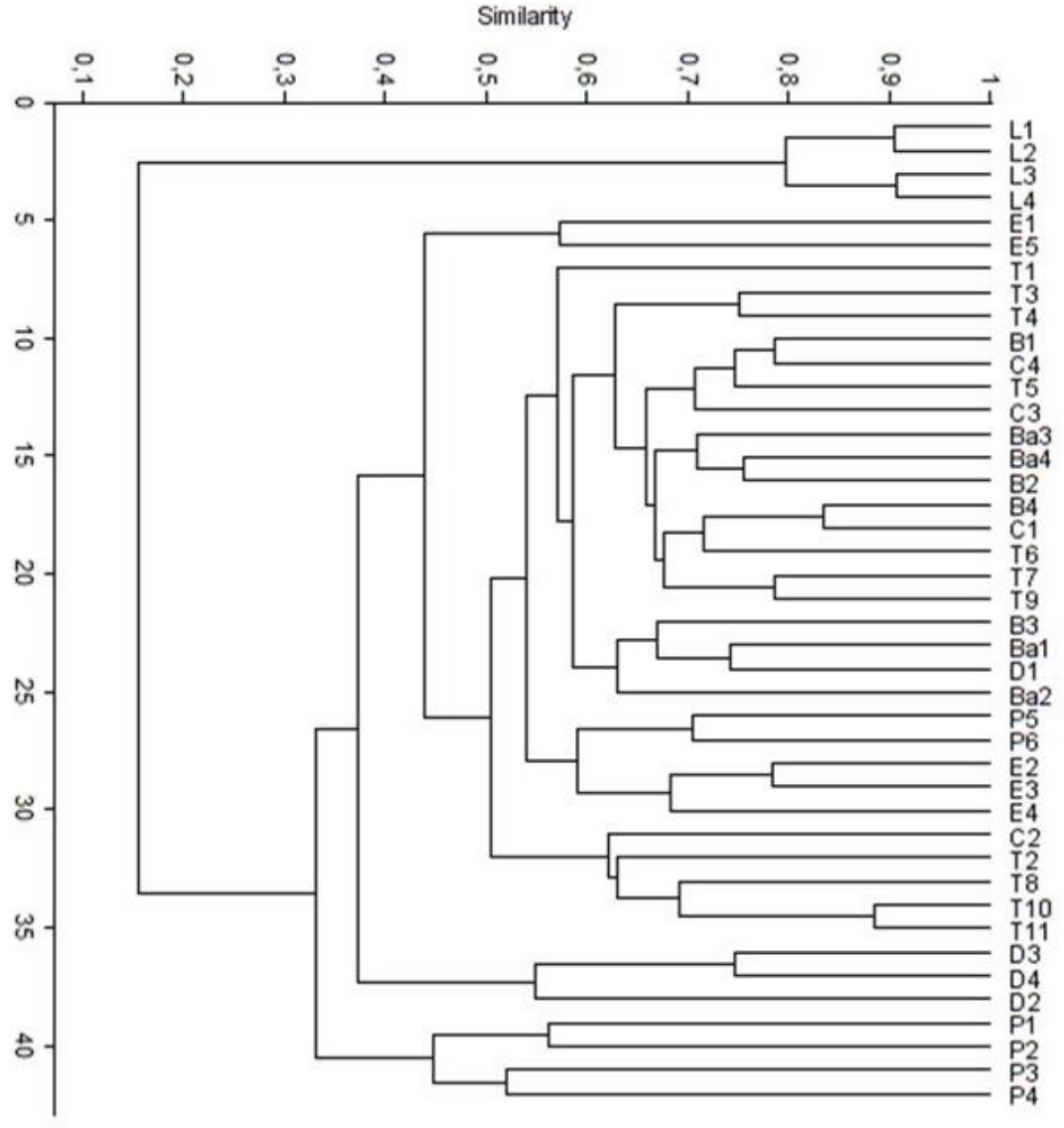


Figure 1. Dendrogram of *B. cinerea* isolates from different locations and host plants using UPGMA method obtained from ISSR and SRAP markers

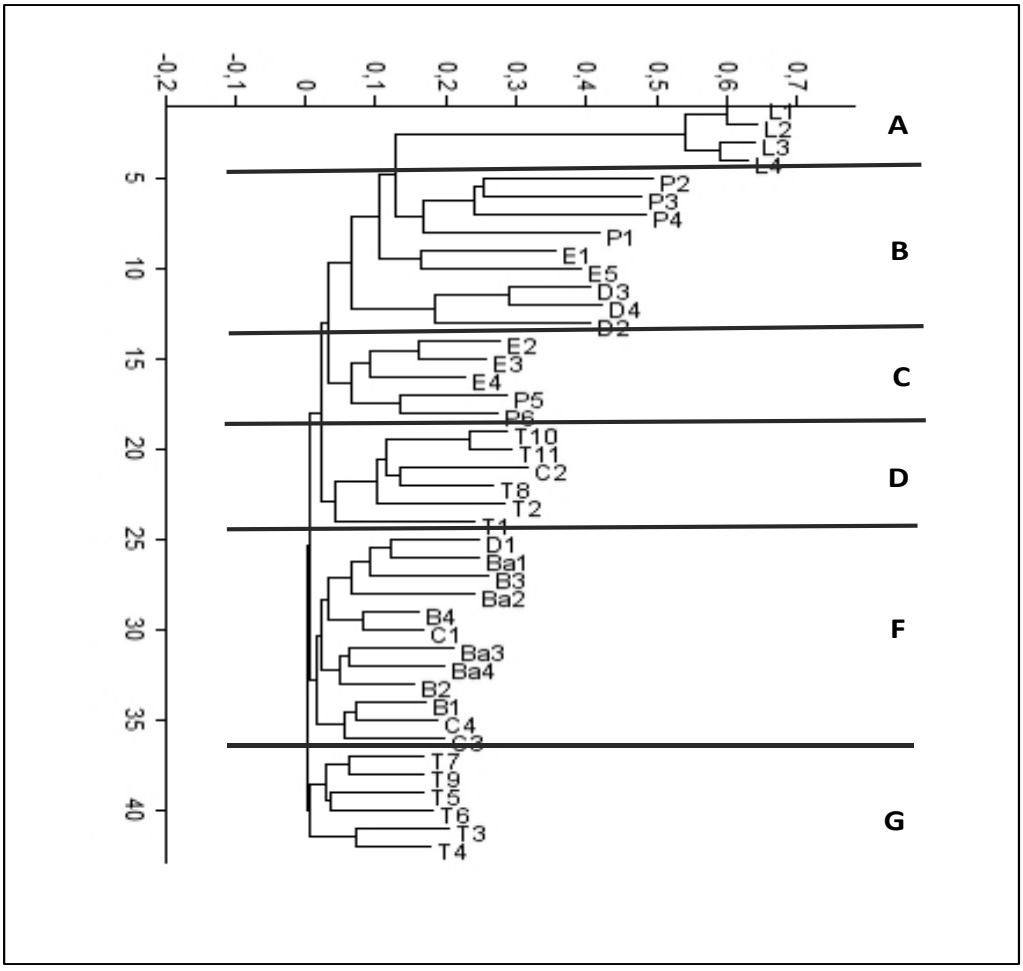
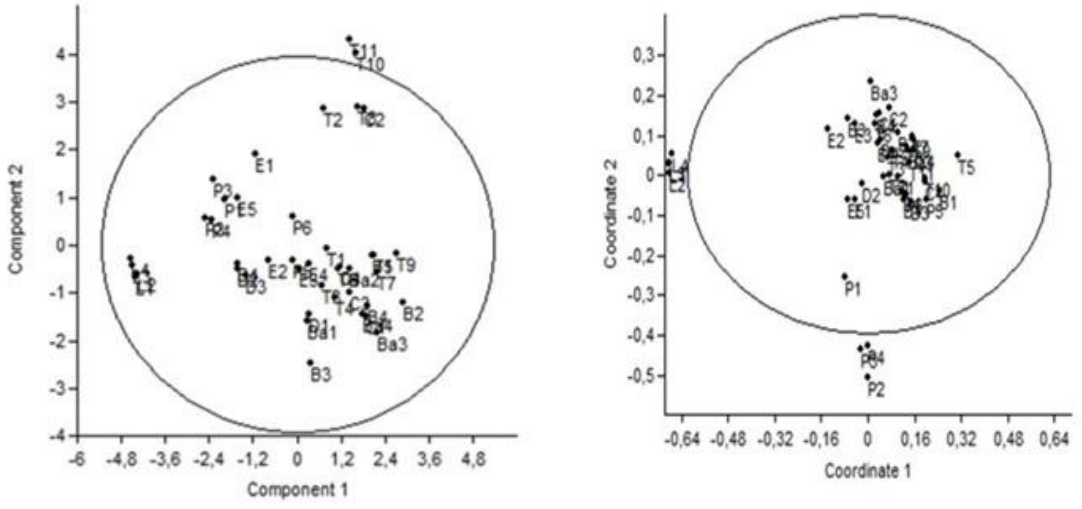


Figure 2. Neighbor joining of the *B. cinerea* isolates from different locations and host plants using UPGMA method obtained from ISSR and SRAP markers



a b

Figure 3. a) Principal Component Analysis (PCoA), b) Principal Coordinate Analysis (PCO) based on the Dice's genetic distances

However, L1-L2 and L3-L4 located within the same cluster were very similar to each other with 90-92%, respectively. In tomato isolates, T10 and T11 accessions showed divergence from the others with a high level of similarity (0.86).Neighbor joining analysis grouped all samples in six main clusters (Figure 2). Neighbor joining analysis has been proposed for reconstructing phylogenetic trees from evolutionary distance data. The principle of this method is to find pairs of neighbors that minimize the total branch length at each stage of clustering of neighbors (Saitou and Nei, 1987). As shown in Figure 2, clustering raised from A to G in the neighborhood. Therefore, isolates obtained lettuces (cluster A) were found the most distinct genotypes from the other isolates collected from vegetables. All of tomatoes isolates were took place cluster D and G. However, C2 isolate obtained from cucumber placed in the D cluster and involved in the same branch with T8 isolate.

C2 isolate from cucumbers and T8 isolate from tomatoes were grown in the same greenhouse. All of isolates from the eggplants and peppers were in B and C cluster. While D2, D3 and D4 isolates obtained from dills have been in the cluster B, isolate D1 is in the cluster F with Ba1 at the same branch. D3 and D4 isolates from dill in greenhouses, D1 and D2 isolates were from dill in the peppers with the same greenhouses. Moreover, D1 and Ba1 isolates were obtained from nearby regions. On the other hand, all of isolates from basils and beans were in the cluster F. The isolate from pepper was obtained from same fields where bean and cucumber had cultivated before, this case was not valid only for isolate C2. PCoA and PCO, scattered plot reporting the first two components, describing all analyzed isolates are given in Figure 3. The main two coordinated analysis explained 80 and 70% variability, respectively. PCoA and PCO analysis were used with together due to PCO was necessarily inferior to PCoA. Because each point was exactly placed where it ought to be in PCoA, whereas in PCO each point was only approximated based on a best-fit model of the dissimilarities (Podani and Miklos, 2002). Likewise, results of PCO were shown dissimilarities of isolates from different hosts. As seen in NJ and dendrogram, all isolates of lettuce placed in the furthest point in the diagram of PCO analyse. On the other hand, isolate P2, P3 and P4 were outside of the ring. Although isolate P1 was in same cluster with other samples that it placed near to border line. P5 and P6 isolates were in same cluster. This result can be associated with close regions of collected samples that P2 and P3 isolates has derived from Kayaburnu which is close distriction to Kocayatak. Moreover, P1 isolate was derived from Serik which is near to these regions. These results show isolate P5 and P6 from Demre were genetically different according to regions.

Finally, the genetic diversity among isolates was also visualized by Jaccard analysis. Jaccard similarity and distance indices values ranged from 0 to 0.65 (Figure 4). Their ranged from 0 (no overlap) to 1 (complete congruence). While selecting convenient evaluation method, Jaccard is numerically sensitive to mismatch when there is reasonably strong overlap. Dice values are high for the same pair of segmentations. The Jaccard distance, which measures dissimilarity between sample sets, is complementary tool to the Jaccard coefficient (Levandowsky and Winter, 1971). While Dice's coefficient gives attention to bands showing matches, Jaccard approaches has importance to determine of differentiation (Carriço et al., 2005). Transposable elements (*Flipper* and *Boty*) were detected among isolates from all the hosts. The presence or absence of two transposable elements was tested in every strain using the PCR reaction. *Flipper* transposable element was determined with 1250 bp product in all of isolates, while none of the isolates amplified the expected 510 bp product corresponding to the *Boty* element.

4. Discussion

B. cinerea and its sexual form *B. fuckeliana* Whetzel comprises 22 species and one hybrid (Yohalem et al., 2003). Recent studies have shown *B. cinerea* that a remarkable genetic differences and morphological variability are present (van Der Vlugt-Bergmans et al., 1993; Diolez et al., 1995; Mirzaei et al., 2007). Its classification based on serological method (Jarvis, 1977) besides morphological and cultural characteristics has not provided contributions to taxonomy in this genus (Jarvis, 1980). For last decades, DNA-based molecular techniques have become

	D1	D2	Ba1	Ba2	Ba3	Ba4	B1	B2	B3	B4	C1	C2	C3	C4	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	P1	P2	P3	P4	P5	P6	E1	E2	E3	E4	E5	L1	L2	L3	L4	D3
D1	1,00																																								
D2	0,23	1,00																																							
Ba1	0,59	0,26	1,00																																						
Ba2	0,40	0,21	0,53	1,00																																					
Ba3	0,36	0,19	0,35	0,39	1,00																																				
Ba4	0,48	0,29	0,42	0,37	0,55	1,00																																			
B1	0,51	0,25	0,40	0,37	0,47	0,56	1,00																																		
B2	0,50	0,26	0,46	0,53	0,54	0,60	0,61	1,00																																	
B3	0,49	0,22	0,52	0,45	0,47	0,36	0,33	0,43	1,00																																
B4	0,44	0,29	0,48	0,47	0,55	0,54	0,50	0,56	0,52	1,00																															
C1	0,41	0,28	0,49	0,48	0,52	0,51	0,47	0,53	0,53	0,71	1,00																														
C2	0,29	0,14	0,31	0,39	0,39	0,31	0,33	0,37	0,31	0,42	0,40	1,00																													
C3	0,48	0,24	0,45	0,43	0,39	0,49	0,58	0,52	0,42	0,51	0,56	0,34	1,00																												
C4	0,51	0,28	0,48	0,40	0,38	0,44	0,65	0,49	0,37	0,52	0,51	0,34	0,56	1,00																											
T1	0,39	0,19	0,34	0,35	0,41	0,37	0,42	0,45	0,37	0,37	0,39	0,24	0,37	0,45	1,00																										
T2	0,28	0,18	0,29	0,34	0,34	0,30	0,36	0,36	0,25	0,39	0,34	0,44	0,32	0,39	0,44	1,00																									
T3	0,39	0,24	0,39	0,40	0,43	0,42	0,42	0,44	0,38	0,48	0,43	0,23	0,43	0,43	0,45	0,45	1,00																								
T4	0,39	0,28	0,37	0,41	0,46	0,50	0,48	0,49	0,40	0,44	0,51	0,23	0,43	0,43	0,41	0,34	0,60	1,00																							
T5	0,44	0,33	0,35	0,33	0,46	0,54	0,65	0,53	0,29	0,50	0,49	0,32	0,49	0,55	0,43	0,35	0,43	0,55	1,00																						
T6	0,33	0,34	0,32	0,37	0,48	0,42	0,43	0,44	0,40	0,57	0,54	0,31	0,39	0,47	0,38	0,39	0,45	0,51	0,56	1,00																					
T7	0,38	0,26	0,40	0,43	0,48	0,44	0,53	0,54	0,38	0,49	0,52	0,35	0,43	0,45	0,41	0,32	0,40	0,47	0,58	0,53	1,00																				
T8	0,30	0,24	0,29	0,36	0,38	0,35	0,41	0,39	0,28	0,47	0,42	0,52	0,32	0,39	0,36	0,44	0,31	0,37	0,46	0,42	0,41	1,00																			
T9	0,38	0,27	0,36	0,46	0,45	0,47	0,50	0,60	0,42	0,50	0,47	0,36	0,48	0,39	0,41	0,36	0,44	0,52	0,56	0,55	0,65	0,45	1,00																		
T10	0,27	0,18	0,29	0,35	0,32	0,35	0,43	0,42	0,25	0,37	0,34	0,43	0,35	0,37	0,37	0,49	0,34	0,34	0,41	0,36	0,38	0,51	0,42	1,00																	
T11	0,24	0,20	0,25	0,36	0,29	0,31	0,39	0,40	0,23	0,35	0,35	0,42	0,33	0,33	0,33	0,45	0,30	0,35	0,38	0,36	0,42	0,55	0,44	0,79	1,00																
P1	0,27	0,15	0,32	0,27	0,22	0,18	0,25	0,23	0,28	0,23	0,28	0,22	0,23	0,26	0,22	0,25	0,24	0,24	0,19	0,25	0,26	0,26	0,25	0,27	0,26	1,00															
P2	0,26	0,17	0,25	0,15	0,12	0,20	0,27	0,19	0,14	0,16	0,15	0,10	0,21	0,23	0,18	0,17	0,21	0,23	0,23	0,15	0,19	0,16	0,20	0,17	0,15	0,39	1,00													
P3	0,21	0,14	0,15	0,20	0,14	0,22	0,31	0,22	0,14	0,18	0,17	0,11	0,16	0,23	0,26	0,23	0,19	0,23	0,21	0,21	0,21	0,22	0,20	0,25	0,26	0,24	0,36	1,00												
P4	0,23	0,09	0,25	0,21	0,13	0,19	0,24	0,19	0,19	0,21	0,20	0,14	0,21	0,27	0,22	0,21	0,20	0,16	0,16	0,18	0,17	0,15	0,19	0,19	0,14	0,23	0,32	0,35	1,00											
P5	0,36	0,16	0,34	0,32	0,29	0,34	0,42	0,37	0,29	0,35	0,33	0,22	0,41	0,41	0,44	0,29	0,37	0,37	0,39	0,29	0,40	0,26	0,39	0,26	0,25	0,19	0,19	0,20	0,30	1,00										
P6	0,33	0,18	0,33	0,38	0,33	0,31	0,40	0,33	0,32	0,34	0,32	0,28	0,37	0,38	0,40	0,36	0,33	0,33	0,38	0,32	0,42	0,34	0,36	0,32	0,33	0,25	0,22	0,20	0,26	0,54	1,00									
E1	0,25	0,22	0,23	0,30	0,21	0,26	0,28	0,31	0,24	0,36	0,32	0,29	0,38	0,32	0,24	0,33	0,27	0,24	0,26	0,33	0,23	0,28	0,28	0,36	0,37	0,21	0,18	0,22	0,22	0,27	0,32	1,00								
E2	0,26	0,23	0,28	0,38	0,35	0,28	0,31	0,34	0,38	0,42	0,44	0,26	0,39	0,37	0,32	0,29	0,33	0,39	0,28	0,45	0,38	0,31	0,38	0,28	0,31	0,26	0,12	0,18	0,17	0,37	0,33	0,44	1,00							
E3	0,32	0,20	0,31	0,43	0,40	0,28	0,37	0,36	0,42	0,44	0,41	0,32	0,43	0,41	0,31	0,33	0,36	0,38	0,34	0,43	0,43	0,33	0,38	0,27	0,31	0,24	0,14	0,15	0,17	0,37	0,53	0,38	0,64	1,00						
E4	0,33	0,23	0,41	0,41	0,35	0,30	0,39	0,42	0,38	0,44	0,44	0,27	0,46	0,54	0,43	0,34	0,37	0,37	0,39	0,43	0,49	0,31	0,42	0,32	0,35	0,24	0,16	0,16	0,19	0,46	0,46	0,40	0,49	0,55	1,00					
E5	0,21	0,23	0,24	0,28	0,19	0,26	0,28	0,27	0,24	0,31	0,32	0,21	0,30	0,28	0,19	0,27	0,24	0,26	0,22	0,27	0,27	0,24	0,26	0,26	0,30	0,23	0,14	0,24	0,16	0,21	0,28	0,40	0,37	0,30	0,34	1,00				
L1	0,08	0,10	0,10	0,09	0,14	0,07	0,03	0,10	0,15	0,14	0,13	0,06	0,06	0,05	0,09	0,09	0,10	0,10	0,00	0,15	0,07	0,04	0,05	0,01	0,03	0,11	0,06	0,05	0,05	0,02	0,06	0,12	0,21	0,16	0,11	0,09	1,00			
L2	0,08	0,10	0,11	0,11	0,13	0,07	0,03	0,11	0,17	0,15	0,13	0,05	0,06	0,05	0,09	0,07	0,11	0,11	0,00	0,13	0,06	0,05	0,06	0,03	0,04	0,11	0,06	0,08	0,06	0,02	0,04	0,12	0,22	0,14	0,14	0,11	0,82	1,00		
L3	0,07	0,09	0,10	0,12	0,14	0,07	0,03	0,10	0,16	0,14	0,13	0,08	0,06	0,05	0,07	0,08	0,10	0,10	0,00	0,14	0,08	0,06	0,07	0,03	0,05	0,13	0,05	0,07	0,05	0,02	0,05	0,11	0,22	0,17	0,11	0,13	0,67	0,70	1,00	
L4	0,07	0,09	0,11	0,12	0,16	0,07	0,03	0,10	0,20	0,15	0,13	0,11	0,06	0,04	0,08	0,09	0,09	0,09	0,00	0,13	0,08	0,10	0,08	0,04	0,06	0,12	0,05	0,09	0,05	0,02	0,07	0,14	0,23	0,17	0,12	0,14	0,63	0,65	0,83	1,00
D3	0,35	0,35	0,26	0,18	0,19	0,28	0,28	0,26	0,20	0,24	0,23	0,14	0,29	0,35	0,27	0,16	0,22	0,24	0,35	0,25	0,32	0,24	0,27	0,16	0,15	0,16	0,16	0,12	0,11	0,24	0,21	0,20	0,19	0,21	0,27	0,13	0,02	0,02	0,02	0,02	1,00
D4	0,27	0,40	0,26	0,20	0,19	0,23	0,27	0,21	0,18	0,26	0,24	0,14	0,24	0,34	0,23	0,19	0,18	0,20	0,32	0,23	0,26	0,24	0,22	0,16	0,14	0,18	0,13	0,10	0,10	0,21	0,21	0,19	0,19	0,22	0,24	0,16	0,02	0,02	0,02	0,02	0,59

Figure 4. Jaccard analyze of the *B. cinerea* isolates from different locations and host plants

increasingly popular and important to detection of genetic differences. Within molecular tools, gene markers are also effective, and they identify an abundance of genetic linkage between identifiable locations within a chromosome which is able to be repeated for verification (Doveri et al., 2008). Molecular characterization and understanding of the population structure of pathogenic fungi are important for improvement of effective disease-control strategies (Malvick and Percich, 1998).

In previous studies, genetic diversity of *B. cinerea* and the level of resistance to the fungicides were investigated using molecular markers. Moreover, the resistance to the fungicide fenhexamid level of the pathogen from different hosts (grape, tomato, cucumber, onion, strawberry, gerbera and rose) were analyzed using molecular markers in Tunisia (Dorsaf Ben and Hamada, 2005). Ma and Michailides (2005) have investigated population sensitivity to the hydroxyanilide, fenhexamid fungicides of *B. cinerea* using transposable elements, DNA fingerprinting generated by microsatellite primed–polymerase chain reaction (MP-PCR), and were tested on 234 isolates collected from fig, grape, kiwifruit,

pea, and squash in California. *Botrytis cinerea* is a common species and inhabited on a wide range of host plants as a parasite or saprophyte (Domsch et al., 1993). Improvement of resistance variety to disease offers excellent perspectives for improved disease control. However, breeding for resistance variety against *B. cinerea* has been difficult in most crops except tomato. Recently, there has been substantial advance in conventional breeding for grey mold resistance in tomato (ten Have et al., 2007). Wild genotype *S. habrochaites* (LYC4) was used for resistance to *B. cinerea* in to *S. lycopersicum* (Finkers et al., 2007). However, up to now, there is no stated any tomato cultivars exhibiting resistance to gray mold in greenhouses (Ingram and Meister, 2006).

Characterization of population structure is important to development of disease-control strategies. Classification of *Botrytis* genus is largely based on morphological and cultural characteristics (Hennebert, 1973; Jarvis, 1977). Merely, many *Botrytis* species are morphologically similar and growth conditions of pathogen that significantly influence variation (Beever and Weeds, 2004). Although morphological characters are so far suggested in identification of *Botrytis cinerea*, in recent years molecular markers have been suggested (White et al., 1990; Rigotti et al., 2002). In our study, the complete ITS (Internal Transcribed Spacer) region of nuclear ribosomal DNA was sequenced as previously described (Paul, 2000) and used to molecular diagnosis using universal primers ITS1 and ITS4 (White et al., 1990). ITS1 and ITS4 primers have been suggested to identify of *B. cinerea* (Kaur, 2015). Mirzaei et al. (2007) have carried out taxonomical studies using specific primers on the genus *Botrytis* and these primers were also effective in discrimination of isolates from roses in greenhouses at central regions of Iran (Khazaeli et al., 2010). In assessing of the *B. cinerea* population variability at molecular level, they also used dominant markers such as RAPD, AFLP and ISSR. In Korea, analysis of genetic variation in *B. cinerea* isolates derived from 9 different host (cucumber, gerbera, ginseng, grape, kiwi, pear, tomato and strawberry) were investigated using 29 decaprimers RAPD markers on 29 isolates of *B. cinerea* isolated from table grapes and other crops in Chile (Choi et al., 1998). Any single primer was not reported to understand differentiation of *B. cinerea*'s the host or geographical origin (Thompson and Latorre, 1999). In another study, RAPD molecular marker were performed on 34 fungal strains isolated from strawberry and other host plants to detect polymorphism (Rigotti et al., 2002). Fourty-four isolates of *B. cinerea* collected from six greenhouses were analyzed by RAPD and AFLP to determine the genetic relationships of *B. cinerea* populations in Almería, Spain. Although polymorphisms were more frequently detected per primer with AFLP than with RAPD, polymorphisms obtained with RAPD were more frequently per loci than AFLP (Moyano et al., 2003). Seventy nine isolates of *B. cinerea* from different host plants and different locations of India and Nepal have been checked to understand of their genetic variability on the basis of geographical regions with defined groups according to cluster analysis based on RAPD markers (Kumari et al., 2014). RAPD and ISSR molecular markers were used to analyze isolates from grapes and other fruits to compare the genetic polymorphism and evolutionary relationships between the two groups (Group A and Group B). Group A contained strains producing conidiospores quickly and numerously, group B were isolates which could hardly produce conidiospores on PDA. Using RAPD-PCR method, E17 primer could amplify a 600-700 bp band in all of the tested isolates and this primer could be used and suggested as molecular marker to follow of *B. cinerea*'s genetic diversity. ISSR cluster analysis demonstrated genetic differences among strains from two groups (Cui, 2013). Multilocus profiles generated by co-dominant molecular markers are highly suited to determine population structure and evolutionary biology in plant pathogenic fungi (Milgroom, 1996). SSR markers were also developed for *B. cinerea* and revealed a high level of polymorphism among isolates from various hosts, including grapevine (Fournier et al., 2002). Leyronas (2015) used SSR marker to determinate of genetic differences on isolates from tomatoes and lettuces. Obtained results suggest an absence of clear host specialization of *B. cinerea* on tomato and lettuce that similar results have been reported by Choi et al. (2003), Kumari et al. (2014) and Asadollahi et al. (2013). Most of genetic studies in *Botrytis* genus have been carried out on *B. cinerea*. Choi et al. (2003)

reported high level of genetic variation among population that could be caused by heterokaryosis among preexisting molecular phenotypes. MP-PCR data set were consistent with absence of sexual recombination in sampled populations of this pathogen (Ma and Michailides, 2005). Asadollahi et al. (2013) suggest the occurrence of host-specific, sympatric divergence of generalist phytoparasites in perennial hosts.

In our study, *B. cinerea* isolates from some vegetables crops cultivated in greenhouses were characterized using combined dominant ISSR and co-dominant SRAP molecular markers. We show that genetic diversity in these vegetable isolates is high and all of them were identical. Host specificity of *B. cinerea* from different hosts has been affected with several parameters depending on phenology of the hosts, spores migration (Asadollahi et al., 2013). Isenegger et al. (2008)'s results highlighted the potential threat of host resistance breakdown as a result of considerable genetic diversity, genotype flow and the evolutionary potential of *B. cinerea*. On the other hand, the sudden change of fungal population observed following fungicide treatment supports the hypothesis that a change of the *B. cinerea* population in the air, in the form of vegetative spores, could result in an abrupt change of *B. cinerea* populations on hosts. However, eventual host preferences of *B. cinerea* variants may also play a role (Asadollahi et al., 2013).

To the best of our knowledge, there is no study which focused on transposable elements in isolates collected from Turkey. The present study has shown that *Flipper* type is common in all *B. cinerea* population obtained from greenhouses-grown vegetable in Turkey. On the other hand, none of isolates has *Boty* type. Many studies have examined, *Boty* transposable elements were detected in Europe America and Australia, while *Flipper* transposable elements alone have been isolated only in southern and Eastern Europe, Tunisia and Bangladesh (Vercesi et al., 2014). Under the present experimental conditions, this subgroup was dominant. More studies are needed to determine if this means that *Boty and Flipper* is invading the *vacuma* group, or these strains belong to another subpopulation of *B. cinerea* (Tanović et al., 2009).

5. Conclusion

The rapidly genetic changes on pathogen population can be associated with a result of different fungicide applications leading to changes in host preferences of *B. cinerea* enforcing formation of variants in our cultivation fields. However further studies based on specific gene sequences and tracking on mutations are necessary to understand the major reason for these genetic diversity on pathogen at our region. As a pioneer study, to the best of our knowledge, there is no study related to genetic discrimination of *B. cinerea* isolates using ISSR and SRAP molecular markers on *B. cinerea*. We employed different ISSR and SRAP markers to study the population genetics of *B. cinerea* isolates from different hosts for major districts in Turkey. Moreover, transposable elements (*Flipper* and *Boty*) were detected among isolates from all the hosts, and *Boty* transposable elements have never been observed in greenhouse vegetable production in Turkey.

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