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Research paper

# Characterization of *Botrytis cinerea* isolates collected on pepper in Southern Turkey by using molecular markers, fungicide resistance genes and virulence assay

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ABSTRACT

*Botrytis cinerea* is a polyphagous fungal pathogen causing gray mold disease. Moreover, it is one of the most destructive infections of small fruit crops such as pepper (*Capsicum annnum* L.). *C. sativum* is a species belonging to the *Solanaceae* family and Turkey is one of the main producers in the World. In the present work, aiming to obtain information useful for pest management, fifty *B. cinerea* isolates collected from Turkey and a reference isolate (B05.10) were characterized using molecular markers and fungicide resistance genes. Morphological and molecular (ITS1-ITS4) identification of *B. cinerea* isolates, the degree of virulence and mating types were determined. Since one or several allelic mutations in the histidine kinase (*Bos1*) and *β-tubulin* genes generally confer the resistance to fungicides, the sequences of these target genes were investigated in the selected isolates, which allowed the identification of two different haplotypes. Mating types were also determined by PCR assays using primer specific for MAT1–1 alpha gene (MAT1-1-1) and MAT1–2 HMG (MAT1-2-1) of *B. cinerea*. Twenty-two out of 50 isolates (44%) were MAT1–2, while 38% were MAT1–1. Interestingly, out of whole studied samples, 9 isolates (18%) were heterokaryotic or mixed colonies. In addition, cluster and population structure analyses identified five main groups and two genetic pools, respectively, underlining a good level of variability in the analysed panel.

The results highlighted the presence of remarkable genetic diversity in *B. cinerea* isolates collected in a crucial economical area for pepper cultivation in Turkey and the data will be beneficial in view of future gray mold disease management.

## 1. Introduction

Pepper (*Capsicum annnum* L.) is a species belonging to the *Solanaceae* family, which mainly grows under tropical and subtropical climates (Esbaugh, 1970). It is commercialized worldwide as spice or for fresh consumption (Onus, 2001) and Turkey is the third pepper producing country in the world (FAO, 2014). The Turkish province Antalyais is the biggest pepper producer of the country (~236.552 tons/year), covering 59% of the total greenhouse production in Turkey (TUIK, 2014). In this region, pest control is usually carried out using chemicals or through integrated pest management to avoid the development of resistance phenomenon.

*Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel), the causative agent of gray mold, is an airborne plant pathogen attacking over 200 crop-hosts worldwide. It is difficult to control because it has different strategies of infection, and has several hosts as inoculum sources. It can survive for a long time as mycelia and/or conidia (Williamson et al., 2007). Synthetic fungicides, particularly multisite fungicides, are often used for controlling the fungus (Rosslenbroich and Stuebler, 2000; Sun et al., 2010). Molecular markers, including transposable elements Boty and Flipper, and restriction fragment length polymorphisms (RFLPs) techniques were used to identify genetical diversity without clonal lineages for recombinational processes among *Botrytis cinerea* isolates (Giraud et al., 1997). The

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sampled population appeared to be composed by two subgroups, *transposa* (featuring both transposons) and *vacuma* (without either transposon). Conversely, a study carried on subpopulations, isolated in different greenhouses, pointed out a highly heterogeneous genetic diversity in *Botrytis cinerea*. Muñoz et al. (2002) observed that the populations of *B. cinerea*, isolated in Chile on grapevine and tomato, were genetically different without the emergence of important clonal lineages, not even in relation with fungicide selection pressure. On the contrary, Ma and Michailides (2005) studied the genetic structure of *B. cinerea* populations in California considering transposable elements and microsatellite primed (MP)-PCR. Interestingly, no differences between populations isolated from different hosts were observed. A significant genetic structure variation, depending on a restricted gene flow, was reported using microsatellite markers within *B. cinerea* group II on samples isolated from two natural host plants (Fournier and Giraud, 2008). Genetic differentiation on *B. cinerea* were identified using molecular markers and the variations were classified as group I (*Botrytis pseudocinerea*) and group II (*B. cinerea sensu stricto*) (Walker et al., 2011; Fekete et al., 2012). Additional studies carried on the genetic differentiation and diversity of *B. cinerea* populations pointed out correlations between hosts and locations (Rajaguru and Shaw, 2010; Muñoz et al., 2010). However, studies carried out in Tunisia on the genetic structure of *B. cinerea* populations did not show any correlation between the plant host and fungal differentiation. Interestingly, the studies were carried out using microsatellite markers on samples isolated on different hosts in various regions (Karchani-Balma et al., 2008).

The genome of *B. cinerea* has been sequenced and an intensive study of the molecular mechanisms has been initiated (Williamson et al., 2007; Amselem et al., 2011). In Chile, Muñoz et al. (2002), using RAPD primers, observed that the populations of *B. cinerea* on kiwifruit were clearly separated from populations isolated from symptomless tomato leaves. On the contrary, Ma and Michailides (2005) and Calpas et al. (2006) basing on clustering carried out through microsatellite markers observed a clear correspondence between the populations, which is in agreement with the results obtained by Karchani-Balma et al. (2008). They demonstrated that it was not possible to separate the populations sampled from the same host in different regions (Karchani-Balma et al., 2008). Similar results related to substantial genetic variability within the population of *B. cinerea* have been reported on grape cropped in Czech Republic (Váczy et al., 2008).

To assess the *B. cinerea* variability at molecular level they used dominant markers such as RFLP (Restriction Fragment Length Polymorphism) (Giraud et al., 1997; Asadollahi et al., 2013), RAPD (Random Amplified Polymorphic DNA) (Thompson and Latorre, 1999; Alfonso et al., 2000; Rigotti et al., 2002; Moyano et al., 2003; Kumari et al., 2014), AFLP (Amplified-Fragment Length Polymorphism) (Moyano et al., 2003) and ISSR (Inter Simple Sequence Repeats) (Bornet et al., 2002; González et al., 2009). Multilocus profiles, generated by codominant molecular markers, are highly suited to determine population structure and evolutionary biology in plant pathogenic fungi (Milgroom, 1996). SSR (Simple Sequence Repeats) markers, or microsatellite, were developed for *B. cinerea* and revealed a high level of polymorphism among isolates from various hosts, including grapevine (Fournier et al., 2002; Leyronas et al., 2015).

Since benzimidazole groups inhibit tubulin synthesis and separation of chromosomes in metaphase, resulting in the alteration of cell division (Delp, 1990), resistance to benzimidazole fungicides is usually related to specific point mutations in the $\beta$-tubulin gene, causing changes in amino acid sequences at the MBC-binding site (Leroux et al., 2002; Ma and Michailides, 2005). In *F. graminearum*, previous studies highlighted the existence of two homologous $\beta$-tubulins ($\beta 1$- and $\beta 2$-tubulin) and the resistance was associated to a single point mutation at codons 167 or 200 in the $\beta 2$-tubulin (Luo et al., 2009; Chen et al., 2009). This target gene was studied to follow benzimidazole resistance of *B. cinerea* isolates from different hosts, such as strawberry, tomato, pepper and eggplant in China (Chen et al., 2009). Since *B. cinerea* has no host specificity and infects a wide range of tissues, with significant genetic differentiation among isolates collected from different species (Ma and Michailides, 2005), genetic structure information could help in developing effective strategies for the disease control (Rigotti et al., 2002; McDonald and Linde, 2002).

The Sequence Related Amplified Polymorphism (SRAP) molecular markers system is a random amplification of coding regions in the genome. It is more reproducible and confident than RAPD. This approach was also extensively applied in genetic diversity analyses (Ferriol et al., 2003) and comparative genetics studies on different species (McDonald, 1997) including fungi (Baysal et al., 2009; Polat et al., 2014) and root knot nematodes (Devran and Baysal, 2012). SRAP is a PCR marker system combining simplicity, reliability and a moderate throughput ratio. Moreover, this technique is largely used in plant pathology to study genetic variability.

The Inter-Simple Sequence Repeat (ISSR) is a single primer amplifying DNA fragments between two SSR (Simple Sequence Repeat) regions oriented in opposite directions. Compared to AFLP, the ISSR markers analyses allow a cost-effective detection and quantification of the pathogen (Schlötterer, 2004). The works with ISSR demonstrated the hyper variable nature of ISSR markers and its potential for population studies, which was proved in plants (Martins-Lopes et al., 2007), insects (Soliani et al., 2010) and fungi (Polat et al., 2014). These markers were also suggested for the study of the implications related to resistance management on *Ceratitis capitata* (Beroiz et al., 2012).

Recent studies showed that mating type characterization can be done using PCR primer pairs to amplify the two mating type loci in *B. cinerea* (Amselem et al., 2011; De Miccolis et al., 2016; van Kan et al., 2010). This characterization was correlated with fungicide resistance of *B. cinerea* isolates. Moreover, frequency of the mating type allele was associated with unequal distribution of pathogen population (Kanetis et al., 2016)

Pepper is cultured in southern region of Turkey in unheated protected cultivation fields where gray mold is usually observed during the winter period. Growers alternate fungicides (benzimidazoles, dicarboximides, anilinopyrimidines and, to a lesser extent, *N*-phenylcarbamates) in a variable frequency depending on severity of the epidemy, which is mainly influenced by the climatic conditions. Since no information is available regarding the population structure of *B. cinerea* on pepper in Turkey, studies of target genes and molecular characterization (by using ISSR and SRAP markers) of a remarkable panel of isolates were carried out. This first information could provide the basis for investigating the sources and shifts in genetic diversity within *B. cinerea* isolates in the Country. In addition, *Bos1* and $\beta$-tubulin genes sequences were compared with structure population results. In summary, the goals of this study were to: (1) evaluate genetic diversity of *B. cinerea* collection isolated from pepper greenhouses in southern region of Turkey; (2) characterize the isolates using virulence assay, MAT types, *Bos1* and $\beta$-tubulin sequences and describe their population structure.

## 2. Material and method

### 2.1. Pathogen collection

Pepper plants infected with *Botrytis cinerea* were sampled from greenhouses collection. Samples were obtained from survey studies of the main producing area of pepper in Turkey. Fifty *B. cinerea* samples were isolated and analysed (Table 1, Fig. S1) and B05.10 isolate, provided from University of Wageningen (Netherland), was used as reference.

All isolates were transferred in individual polyethylene bags to prevent cross contamination and incubated in sterile petri dishes (PDA with 100 mg/l streptomycin sulphate) at 23 ± °C to allow an abundant production of conidia. After sporulation, single-spore per isolate were selected and characterized morphologically.

**Table 1**
Samples list of *B. cinerea* collected in Turkey, type of fruit and greenhouse, location and GPS data.

| Isolate ID | Type of fruit | Area (da) | Type of greenhouse | Location | GPS data |
|---|---|---|---|---|---|
| 2 | Capia | 2 | Glass | Cakallık/Köseler Mah./A.Kocayatak | 36319980 E, 4088087 N, 11 m |
| 3 | Capia | 2 | Plastic | Cakallık/Köseler Mah./A.Kocayatak | 36319688 E, 4087446 N, 8 m |
| | Capia | 3 | Plastic | Köseler Mah./A.Kocayatak | 36319316 E, 4087221 N, 25 m |
| 9 | Long | 1.5 | Glass | Y. Kocayatak | 36320238 E, 4089659 N, 16 m |
| 11 | Trilob | 2 | Glass | Y. Kocayatak | 36320473 E, 4089401 N, 18 m |
| 12 | Long | 2 | Glass | Y. Kocayatak | 36320504 E, 4089411 N, 17 m |
| 13 | Banana | 2 | Glass | Kayaburnu | 36322109 E, 4089865 N, 36 m |
| 15 | Banana | 3 | Glass | Kayaburnu | 36323051 E, 4089345 N, 25 m |
| 17 | Long | 2 | Plastic | Kayaburnu | 36321830 E, 4089217 N, 35 m |
| 18 | Capia | 2 | Glass | Konaklı/Alanya | 36402047 E, 4050450 N, 128 m |
| 20 | Capia | 2 | Plastic | Toslak köyü/Alanya | 36402474 E, 4054114 N, 135 m |
| 21 | Long | 1.5 | Plastic | Toslak köyü/Kızılca Mah. | 36402598 E, 4054128 N, 148 m |
| 22 | California | 2 | Glass | Toslak köyü/Alanya | 36403500 E, 4054678 N, 191 m |
| 23 | Capia | 2 | Plastic | Macarköyü/Gazipaşa | 36441232 E, 4010380 N, 65 m |
| 24 | California | 4 | Plastic | Aksu | 36 306802 E, 4092026 N, 40 m |
| 25 | Capia | 8 | Plastic | Salur Köyü/Mavikent | 36294111 E, 4086863 N, 58 m |
| 26 | Banana | 4 | Plastic | İncekum mevkii/Mavikent | 36258686 E, 4022086 N, 0 m |
| 27 | Banana | 3 | Plastic | İncekum mevkii/Mavikent | 36259638 E, 4021672 N, 0 m |
| 28 | Long | 2 | Plastic | Orta Mah. Şirket Sok. Seyrek Cad./Mavikent | 36260539 E, 4021570 N, 0 m |
| 29 | Long | 2 | Plastic | Orta Mah./Mavikent | 36260995E, 4,020,970 N, 0 m |
| 30 | Long | 2 | Plastic | Beykonak mevkii/Mavikent | 36,258,754 E, 4023375 N, 0 m |
| 31 | Banana | 4 | Plastic | Sarıcasu mevkii/Kumluca | 36256347 E, 4033202 N, 115 m |
| 33 | Banana | 3 | Plastic | Beşikçi Köy/Kemer Mah./Kumluca | 36261650 E, 4026899 N, 63 m |
| 34 | Long | 2 | Plastic | Taçbaş Köyü/Kumluca | 36259167 E, 4032816 N, 16 m |
| 35 | Capia | 2 | Plastic | Beymelek Mevkiii/Demre | 36 234747 E, 4018260 N, 3 m |
| 36 | Capia | 2 | Palstic | Beymelek Mevkiii/Demre | 36233573 E, 4016620 N, 0 m |
| 37 | Bell | 3 | Glass | Beymelek Mevkiii/Demre | 36232838 E, 4016458 N, 0 m |
| 38 | Capia | 1.5 | Glass | Beymelek Mevkiii/Demre | 36231565 E, 4016022 N, 0 m |
| 40 | Bell | 2 | Glass | Mazılca Mevkii/Demre | 36230532 E, 4018238 N, 18 m |
| 42 | Banana | 2 | Plastic | Köşkerler/Demre | 35767509 E, 4018905 N, 30 m |
| 43 | Trilob | 2 | Glass | Güvercinlik çıkmazı-Akkent Mah./Demre | 35768638 E, 4017359 N, 18 m |
| 45 | Bell | 2 | Glass | Yaylakaya/Demre | 36230988 E, 4015897 N, 5 m |
| 46 | Banana | 2 | Plastic | Yaylakaya/Demre | 36769437 E, 4017036 N, 7 m |
| 48 | Bell | 1.5 | Glass | Sura mevkii | 35765660 E, 4016065 N, 89 m |
| 49 | Banana | 2 | Plastic | Kınık Çavdır | 35710803 E, 4026513 N, 81 m |
| 51 | Banana | 2 | Plastic | Çavdır | 35710850 E, 4026590 N, 88 m |
| 52 | Long | 1.5 | Glass | Altınova | 36301827 E, 4091961 N, 87 m |
| 53 | Bell | 2 | Glass | Altınova | 36302798 E, 4089268 N, 62 m |
| 55 | Capia | 2 | Plastic | Çandır | 36325346 E, 4089693 N, 18 m |
| 57 | Capia | 8 | Plastic | Çandır | 36325411 E, 4090890 N, 18 m |
| 58 | Capia | 6.5 | Glass | Çandır | 36325832 E, 4092378 N, 18 m |
| 59 | Long | 1.5 | Glass | Çandır | 36326548 E, 4093570 N, 22 m |
| 60 | Long | 1.5 | Plastic | Alacami Köyü/Çandır | 36327385 E, 4096588 N, 75 m |
| 61 | Long | 2 | Plastic | Kumluca | 36257593 E, 4024884 N, 15 m |
| 66 | Bell | 2 | Glass | BATEM Kocayatak | 36319134 E, 4089225 N, 35 m |
| 68 | Capia | 3 | Glass | Beymelek/Demre | 36233451 E, 4016599 N, 18 m |
| 67 | Capia | 3 | Glass | Kumtepe mevkii, Mavikent/Kumluca | 36261412 E, 4022408 N, 12 m |
| 69 | Long | 2 | Glass | Gazipaşa | 36441290 E, 4010390 N, 60 m |
| 70 | Bell | 2 | Plastic | Elikesik Köyü/Alanya | 36402463 E, 4049540 N, 48 m |
| 71 | Bell | 2 | Plastic | Kayaburnu | 36321830 E, 4089217 N, 35 m |

Total genomic DNA was extracted from mycelial mass of all *B. cinerea* isolates using a DNA isolation kit (Promega, Madison, US) according to the manufacturer's instructions. DNA quality (260/230 and 260/280 ratios) and concentration was checked by NanoDrop Spectrophotometer (Thermo Scientific - Waltham, Massachusetts). Complete ITS region of nuclear ribosomal DNA was amplified as previously described (Paul, 2000), using universal primers ITS1 and ITS4 (White et al., 1990). Amplified products were visualized by 2% high resolution agarose gel electrophoresis in 1xTAE buffer, stained with ethidium bromide. The fragments obtained from isolates on petri dishes were also sequenced. The amplicons were cleaned up by enzymatic reactions (QIAquick PCR purification Kit, Qiagen, USA) and later sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, US). After a cleanup process performed by ZR Sequencing Clean Up Kit (Zymo Research, US), the samples were loaded on a 3130xl Genetic Analyzer (Applied Biosystems, CA, US) and analysed using Sequencing Analysis 5.3.1 software. Representative sequences for each ITS sequences obtained were aligned using ClustalW (Larkin et al., 2007) and queried against GenBank using BLAST.

### 2.2. Virulence assays

The virulence of whole isolates was determined on fully expanded leaf, of Sera Demre-8 (susceptible) seedlings, inoculated with mycelia of *B. cinerea* isolates (50 isolates and B05.10 as reference). The experiment was repeated 3 times using three seedlings for each isolate. At blooming (20–30 cm high) plantlets were individually sprayed with spore suspension (carrot juice + 1% gelatin) of each isolate ($10^5$ spore/ml) until droplet runoff occurred. Inoculated plants were covered with polyethylene bags for 24 h, to maintain high relative humidity, and transferred in greenhouse. Ten days after inoculation, inoculated plants were examined and disease severity was recorded using a scale 0–4 (Delen et al., 1988; Güngör, 2006). Disease severity was evaluated according to Tawsend-Heuberger formula (Townsend and Heuberger, 1943). Data were evaluated using SAS (Statistic Analysis Program) and significant differences were calculated according to Duncan analysis.

### 2.3. β-tubulin and Bos1 gene analysis

For β-tubulin and *Bos1* gene analysis were used the primers Bcb-F/Bcb-R (Zhang et al., 2010) and BF4/BR4 (Ma et al., 2007), respectively. Amplifications were carried out in reaction volumes of 20 µl containing 1.5 µl genomic DNA (50 ng), 10 µl master mix (GeneAll, 2XAmpMaster Taq), 1 µl of each primer (0.3 µM primer) and 6.5 µl $ddH_2O$. An initial denaturation step at 94 °C for 3 min was followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 30 s and extension at 72 °C for 1 min, with a final extension step of 72 °C for 10 min. Amplified products were visualized by 2% high resolution agarose gel electrophoresis in 1xTAE buffer, stained with ethidium bromide. PCR amplification was performed three times for each isolate to avoid sequence mismatch. All amplicons, with expected size, were cloned into the pGEM-T Easy vector (Promega Co.) and sequenced by ABI PRISM 3130 Genetic Analyzer. To compare the amino acid sequences of *β-tubulin* and *Bos1* genes, DNA sequences were translated into amino acid sequence using standard code using the online tool ExPASy (Expert Protein Analysis System; http://web.expasy.org/translate/). The deduced amino acid sequences, from all fifty-one isolates, were then queried against the reference sequences (AAL37947.1 and AAB60307.1 for *Bos1* and β-tubulin, respectively) deposited in National Center for Biotechnology Information (NCBI) using blastp.

### 2.4. Mating type determination

MAT1–1 alpha and MAT1–2 HMG genes were studied using MATalpha5-MATalpha3 and HGM5-HGM3 primers respectively, to mating types within the analysed collection according to van Kan et al. (2010). PCR were carried out in reaction volumes of 20 µl containing 1 µl DNA (50 ng), 10 µl master mix (GeneAll, 2XAmpMaster Taq), 1 µl of each primer (0.3 µM primer) and 7 µl $ddH_2O$. PCR reactions were performed under the following cycle program: initial denaturation 4 min at 94 °C, followed by 32 cycles at 94 °C for 30 s, 58 °C for 90 s and 72 °C for 60 s, followed by a final extension step at 72 °C for 7 min. Amplified products were visualized by 2% high resolution agarose gel electrophoresis in 1xTAE buffer, stained with ethidium bromide. A 100-bp DNA ladder was used as molecular standard.

### 2.5. Molecular characterization of isolates by ISSR and SRAP markers

For genetic characterization of *B. cinerea* isolates ISSR (Inter-Simple Sequence Repeats) and SRAP (Sequence Related Amplified Polymorphism) markers were used. Eighteen ISSR markers (Table S1; Levi et al., 2005; Baysal et al., 2009; Baysal et al., 2011; Baysal et al., 2013; Polat et al., 2014) were selected. Amplifications were carried out in the reaction volumes of 15 µl containing 50 ng of DNA, 0.1 mM dNTPs, 2.5 mM $MgCl_2$, 0.3 µM of each primer. PCR reactions were performed under the following conditions: initial denaturation step for 3 min at 94 °C, followed by 35 cycles at 94 °C for 45 s, 48 °C for 45 s and 72 °C for 90 s (extension), followed by a final extension step at 72 °C for 10 min.

Eighteen primer combinations were created using 12 Em (reverse) and 11 Me (forward) SRAP primers (Table S1; Li and Quiros, 2001). Amplifications were performed as described above. PCR reactions were carried out using 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. All products were visualized by 2% high resolution agarose gel electrophoresis in 1xTAE buffer at 115 V for 4 h, stained with ethidium bromide. All PCR reactions were repeated twice. Amplified bands from each primer were scored as present (1) or absent (0). Only those bands that were consistently amplified were considered, whereas smeared and weak bands were discarded.

### 2.6. Data analysis

*B. cinerea* isolates were placed in genotypic clusters using STRUCTURE software (Pritchard et al., 2000) which employs a Bayesian clustering approach to identify distinct gene pools and to assign individuals to *K* populations based on the allele frequencies at each locus. The most likely number of genetic groups (*K*) was performed following the procedure of Evanno et al. (2005), which proposed an *ad hoc* statistic, Δ*K*. The STRUCTURE analysis was carried out following the procedures reported in Mercati et al., 2013. The 20 runs were averaged using CLUMPP (CLUster Matching and Permutation Program; (Jakobsson and Rosenberg, 2007) and shown in histograms using the program DISTRUCT (Rosenberg, 2004).

The pairwise genetic distances, for phylogenetic relationships among isolates, were estimated using Nei's (Nei, 1978) coefficient. The cluster analysis was performed according to the UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) algorithm and a dendrogram was generated using the R package adegenet (Jombart, 2008). Additional statistics were computed to estimate the grade of polymorphism among the isolates. The percentage of polymorphic loci, Shannon's Information index and the Nei's gene diversity within the collection analysed were evaluated using adegenet and GenAlex 6.5 (Peakall and Smouse, 2006).

Finally, the genetic similarities among *B. cinerea* samples were assessed through principal coordinate analysis (PCoA) using the GenAlEx (Peakall and Smouse, 2012). The genetic diversity was also represented in a heatmap using the heatmap.2 procedure of the R/Bioconductor gplots package (Chen et al., 2010).

## 3. Results

In the present study a multidisciplinary approach was used to whole characterize, for the first time, a remarkable collection of *B. cinerea* isolates collected in Antalya, which is a Southern region of Turkey (Table 1; Fig. S1) largely known to be the most important economical area for pepper production. In particular, fifty isolates and a reference strain (B05.10) were investigated through a morphological and molecular approach.

### 3.1. Morphological and ITS analyses

The mycelium was branched, septate, hyaline to brown. Conidiophores arise directly from the mycelia or from sclerotia. They were straight, septate and often branched at the apex dichotomously or trichotomously. Conidiophores and conidium were grape shape. The average length of the conidiophores was between 650 and 2823 µm. Conidial structure was celled with hyaline egg-shape (Fig. 1). Colonies formation was observed on potato dextrose agar, at 20 °C under light. Aerial myceliums were cottony, powdery with both compact and radial pattern. Colonies were characterized by a white, dirty white, grayish white or hyaline colour, which became light gray or dark gray to dark brown after 1 week.

The representative ITS sequences, obtained from isolates, were visualized by agarose gel (Fig. S2). The sequences obtained from particularly from isolate samples displaying typical colony types on petri dishes were blasted to GenBank (accession number: gb|KX074008.1|) (Fig. S3), confirming their homology to *B. cinerea* reference sequences.

### 3.2. Virulence assays

Virulence assays are in agreement with our observations recorded under natural conditions in protected fields. The virulence assay pointed out that all the isolates tested where characterized by a virulence degree that ranged from 1.00 to 2.93 (Table S2). Among all the isolates the sample 71 was the one with the lowest virulence (1.00), whereas with the isolates 9 and 40 were the most virulent showing

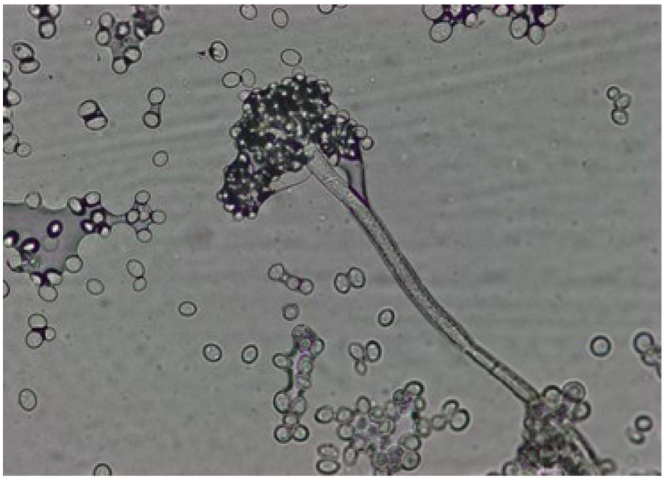

**Fig. 1.** Microscopic observation on *B. cinerea*; conidial structure was one cell with egg-shape hyaline.

infection values of 2.88 and 2.93, respectively. Reference isolate (B05.10) pointed out a virulence value of 2.11 which was higher than 70% of analysed samples.

### 3.3. Mutations in target genes

Two target genes, *Bos1* and *β-tubulin*, involved in the fungicide resistance mechanism were investigated in all isolates collected. To determine the presence or absence of mutations in genes encoding target proteins for iprodione, the *Bos1* gene was sequenced using BF4/BR4 primers pair. The sequencing allowed verifying the presence of a null mutation at amino acid position 1040. This point mutation is located in the kinase core domain of C-terminal region. Sequences examination showed that all isolates had identical amino acid sequence, with the codon CGA (Arginine - Arg/R), underlining the putative sensitive of the analysed isolates to iprodione.

The fragment containing the benzimidazole box in the *β*-tubulin gene, corresponding to codons 198-200, was investigated. Interestingly, a punctual allelic change at amino acid position 198 (E198A) was found. In detail, 44 strains had a glutamic acid (E) at position 198 (GAG), whereas the remaining strains (including B05.10) had alanine (Ala/A; GCG) (Table S3).

In summary, on the basis of the *Bos1* and *β*-tubulin gene sequences, two different haplotypes were identified (Table S3; Fig. 2).

### 3.4. Mating type determination

In the investigated samples, 31 (62%) out of 50 isolates amplified by using HGM5-HGM3 primer pairs, showed the amplicon (1050 bp) for MAT1-2-1, while 19 (56%) out of 50 studied isolates amplified for MAT1-2-1 (expected size 1100 bp). Out of whole studied samples, 8 isolates (16%) showed amplicon with both MAT1-1-1 and MAT1-2-1 (Table S4, Fig. S4a and Fig. 4b).

### 3.5. Genetic relationships among B. cinerea isolates collected

To evaluate the genetic diversity within the studied *B. cinerea* collection ISSR and SRAP markers were used.

The genetic structure and the likely number of genetic groups (*K*) within the whole collection were investigated by the software STRUCTURE. The Δ*K* evaluation statistic proposed by Evanno et al. (2005) revealed a clear optimum for $K = 2$. The isolates included in each groups are shown in Fig. 2. Thirty-nine out of 51 strains (about 75%) were assigned to a cluster at $K = 2$ by using a > 70% threshold for group classification. Interestingly, except for three isolates (samples 30, 43 and 52) the samples belonging to pool 2 (green coloured) pointed out a membership degree ranging from 0.704 to 0.992 (Table S5) and most of the strains (80%) highlighted a 90% of membership. On the contrary, in the pool 1 (gray coloured) 54% had a value > 0.700 and only 8 out of 23 isolates (39%) showed a membership value higher than 90% (Fig. 2; Table S5).

Using combined ISSR and SRAP markers, a total of 169 and 143 bands, with a mean of private bands of 24%, were observed in pool 1 and pool 2, respectively (Table 2). However, the two genetic pools exhibited a moderate level of genetic diversity, as highlighted by the Nei's (0.286 *vs*. 0.205) and the Shannon's Information Index (0.440 *vs*. 0.323) (Table 2).

To investigate the relationships among isolates both Cluster analysis and multivariate PCoA were carried on 18 ISSR and 18 SRAP markers. Phylogenetic analysis grouped all samples in five main clusters (Fig. 3A). Excepted for sample 30, 43 and 52, which are linked to cluster II, cluster I included all the isolates belonging to pool 1 identified by STRUCTURE analysis (Fig. 2). The cluster analysis highlighted that discrimination among samples with same origin is quite difficult. However, the population structure analysis was able to separate the samples based on their genetic background. As previously mentioned samples 30, 43 and 52 are not grouped in the cluster I. It is probably due to their mixture in the genetic pools (Fig. 2; Table S5). The bootstraps among clusters, ranging from 93 to 99%, should avoid misclassifications. The results conserning the PCoA analysis (a scattered plot reporting the first two components and describing all isolates analysed) is reported in Fig. 3B. The main two coordinates explained

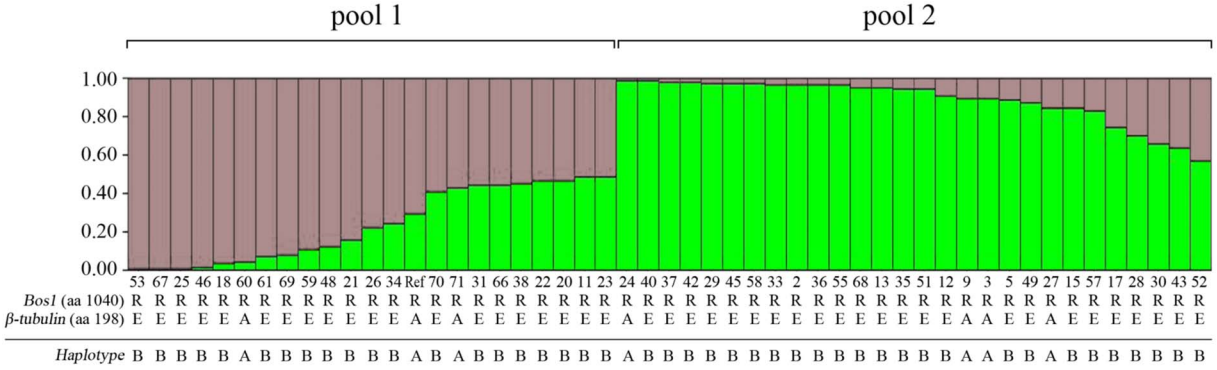


**Fig. 2.** Hierarchical organisation of genetic relatedness of *B. cinerea* collection analysed using STRUCTURE software. Each colour represents one genetic pool (pool 1 = gray; pool 2 = green) and the length of the coloured segment shows the estimated membership proportion of each sample to designed group. Each individual is represented by a vertical line partitioned into *K* coloured segments. In the figure are also indicated the haplotypes for *Bos1* and *β-tubulin* genes. Ref = reference (B05.10). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Genetic diversity for the two gene pools recognized through population structure analysis.

| Gene pool | $N_b$ | $N_{pb}$ | Polymorphic loci (%) | h | I |
|---|---|---|---|---|---|
| Pool 1 | 169 | 37 | 93.89 | 0.286 | 0.440 |
| Pool 2 | 143 | 11 | 76.11 | 0.205 | 0.323 |

Nb = No. of different bands.
Npb = No. of Bands Unique to a Single Population.
h = Nei's genetic diversity.
I = Shannon's Information Index.

40% (Coord 1) and 20% (Coord 2) of total variability. Similarly, in cluster analysis the samples were grouped based on their genetic pool previously identified in the population structure (Fig. 3B; gray and green coloured ellipses). In addition, the five clusters were also clearly distinguible, with cluster I located in the two right quadrants (Fig. 3B).

Finally, the genetic diversity among isolates was also visualized through a heatmap, displaying both the clustering of samples and the dissimilarity among genotypes (Fig. 4). As already suggested by the previous analyses, this analysis confirmed that the analysed panel was splitted in two main genetic clusters/pools. In addition, the heatmap underlined a remarkable level of genetic diversity among isolates (Fig. 4) even if it was not possible to correlate their geographic origins with the clustering.

## 4. Discussion

The introduction of modern fungicides with specific mechanism of action is causing the phenomenon of resistance in fungal populations, especially in *B. cinerea* which could be considered a high risk pathogen (Brent and Hollomon, 2007). Consequently, it is discouraged to repeatedly use fungicide with similar targets within one growing season (Leroux et al., 2002). Nevertheless, in the last 30 years the chemical control of *B. cinerea* was mainly achieved using systemic fungicides (*e.g.* benzimidazole fungicide carbendazim) with single modes of action, which were often mixed with protective fungicides such as chlorothalonil and pyrimethanil (Sun et al., 2010).

Nowadays, the fungicide resistance in *B. cinerea*, infecting protected pepper fields, is widespread in the southern Turkey as well as in other contries. It is mainly due to its high genetic variability, owing to its abundant sporulation and polycyclic nature, joined to the high concentrations of sprays required for gray mold control (Cai et al., 2015). Many pathogens, including *Monilinia fructicola* (Chen et al., 2013), *Penicillium digitatum* (Sánchez-Torres and Tuset, 2011), *Sclerotinia homeocarpa* (Ok et al., 2011) showed single isolates with multiple resistance to fungicides, but none pathogen appears to be as adaptable to environmental stresses and fungicide pressure as *B. cinerea* affecting pepper fields, which exhibited multi-fungicide-resistant phenotypes mainly due to the stepwise accumulation of single resistances.

In addition, a relatively high level of sexual reproduction in populations of the fungus could be suggested, even if the occurrence of sexuality is still not clear. Using PCR detection, mating type of isolates of *B. cinerea* was determined (van Kan et al., 2010; Wessels et al., 2013; Kanetis et al., 2016; De Miccolis et al., 2016). To the best of our knowledge, in isolates collected from Turkey, there are no studies focused on both mating types. Moreover, sexual structures of the fungus in Turkey have never been observed in cropped fields. The present study has shown that there is not a significant difference in the proportion of MAT1-1 and MAT1-2 populations obtained from greenhouses-grown peppers in Turkey. On the other hand, some of isolates have both MAT1-2 and MAT1-1 mating type (Table S4, Fig. S4), suggesting that they were heterokariyotic at MAT1 locus (Kumari et al., 2014). Similar results were also documented in other studies (Wessels et al., 2013; Kumari et al., 2014). These cases are also observed in nature and are important source of genetic variation in *B. cinerea* (Faretra et al., 1988).

The studies of multi-fungicide resistant “superstrains” could highlight potential weaknesses in common resistance management strategies (Fernández-Ortuño et al., 2015). Therefore, the present work was focused on the investigation of the genetic background and pathotypes of *B. cinerea* isolates belonging to an important collection sampled in the south of Turkey. The study aims to achieve useful information that will allow to improve the pest management and the reduction of post-harvest infection on pepper.

The chosen molecular markers (ISSR and SRAP) were already used to evaluate the genetic diversity in other pathogens, such as *Vibrio cholerae* (Kumar et al., 2007), *Fusarium oxysporum* f.sp. *lycopersici* (Baysal et al., 2009), *Clavibacter michiganensis* subsp. *michiganesis* (Baysal et al., 2011), *Pseudoperonospora cubensis* (Polat et al., 2012), *Fusarium oxysporum* f.sp. *lycopersici*, *F. oxysporum* f. sp. *melongenea* (Baysal et al., 2013) and to detect isolates with mutations associated to fungicide resistance (Ziogas et al., 2009). Structure population analysis split the investigated samples into two main genetic pools. In each pool, there are samples belonging to both identified haplotypes without a

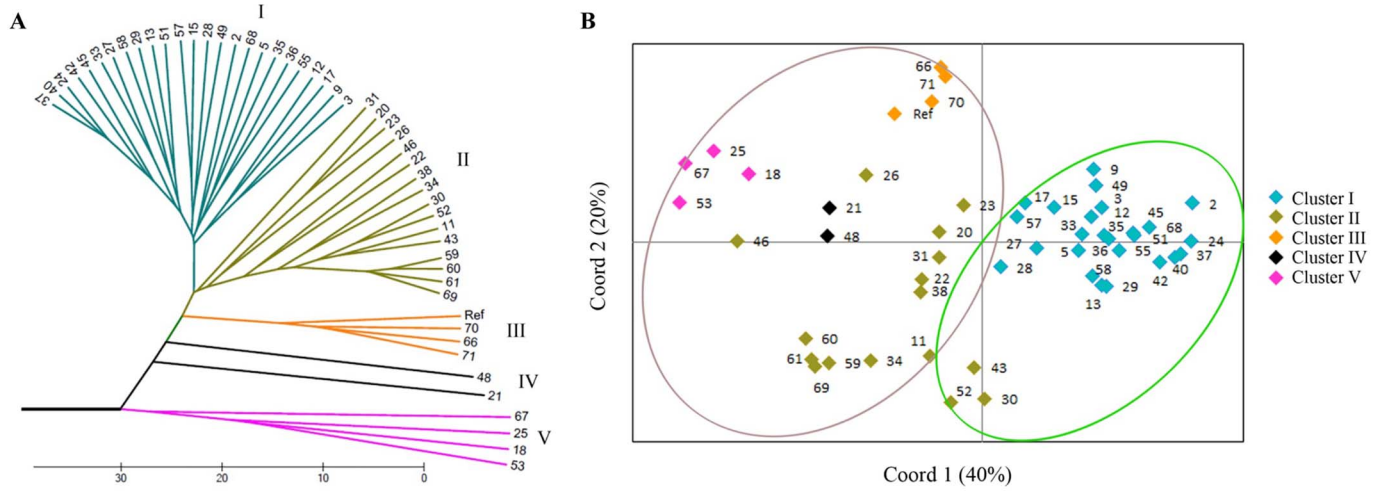


**Fig. 3.** A. Dendrogram of genetic relationships among 50 isolates collected from Turkey and the reference generated with Nei's coefficient (Nei, 1978) and UPGMA cluster analysis. Different colours represent the strains belonging to distinct genetic groups (cluster I, II, III, IV and V). Ref = reference (B05.10). B. Principal Component Analysis (PCoA) based on the Nei's genetic distances. Two ellipses showed gene pools identified in the population structure (Fig. 2). The five clusters represent the strains belonging to distinct genetic groups identified in the phylogenetic analysis. Ref = reference (B05.10).

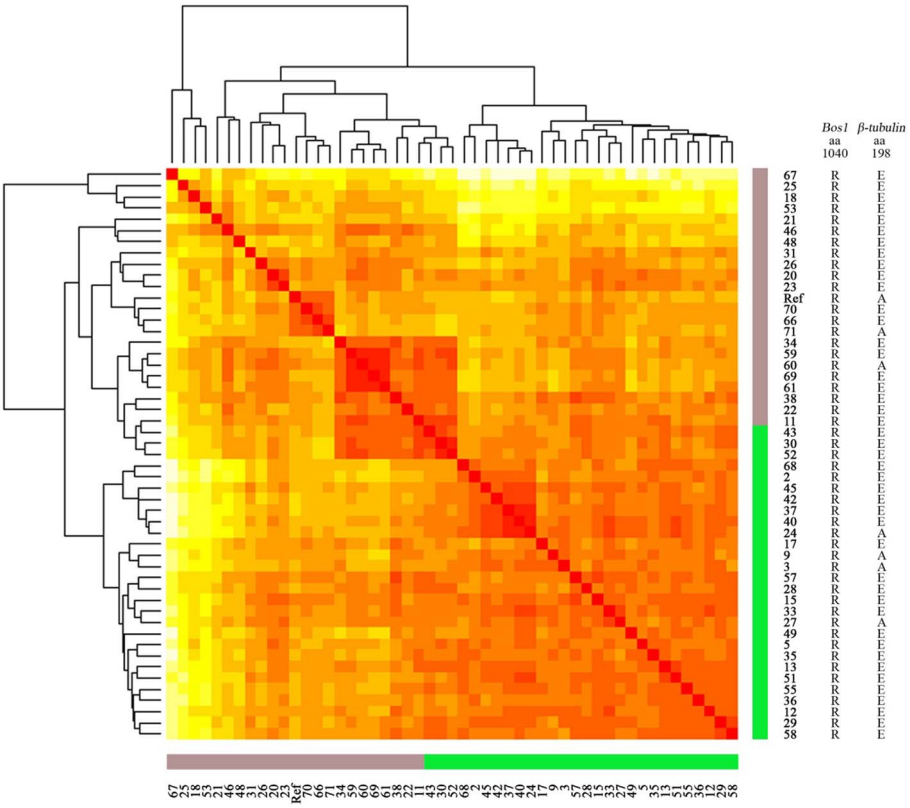


**Fig. 4.** Genetic diversity among analysed isolates visualized through a heatmap. Colour gradient display the dissimilarity among genotypes: red indicates the most similar samples, while white shows the lowest genetic similarity. Gray and green blocks represent the two gene pools identified in the population structure analysis (Fig. 2). In the figure are also indicated the haplotypes for *Bos1* and *β-tubulin* genes. Ref = reference (B05.10). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clear relationship between their genetic structure and β-tubulin profiles. Despite the moderate genetic diversity both pools were clearly separated into two groups also by the phylogenetic and PCoA studies, even if the hierarchical organisation didn't reflect the geographical dissemination. Between them, we identified two different haplotypes with different behaviour to some fungicides. The widespread prevalence of two main haplotypes indicates that they have been probably selected within the last decade owing to pest management.

In Antalya region, evolutionary forces other than selection could act on *B. cinerea* populations by means of the host species or by fungicide applications. Among all evolutionary forces, host selection might play a pivotal role in shaping pathogen population structure (Leung et al., 1993). However, this is apparently not the case of the *B. cinerea* populations studied since the pathogen is not host-specialized, infecting at least 235 hosts in temperate regions (MacFarlane, 1968). Occurrence of pathogenic migration could be assumed based on our findings, since our results suggested that there is no correlation between geographical dissemination of pathogen and the differentiation of sub-populations. Therefore, the observed pathogenic migration could be related to the management of disease control at a regional scale.

The importance of specific amino acids substitution for the fungicide resistance mechanisms is well known in *B. cinerea*. Previous studies showed that a mutation at amino acid sequence in position 365 of the class III histidine kinase gene (*Bos1*) is involved in the resistance to dicarboximide (Ma et al., 2007). The frameshift mutation G143A in cytochrome *b* (*cytb*) confers high resistance to pyraclostrobin, a fungicide belonging to pyrazole group. In the 3-keto reductase gene (*erg27*) a single point mutation at amino acid position 412 from a phenylalanine to serine (F412S) is implicated in the resistance to SBI fungicide group (Fernández-Ortuño et al., 2015). The resistance to carbendazim and other benzimidazole fungicides in *B. cinerea* is conferred by one or several allelic mutations in the *β-tubulin* (Liu et al., 2013). In a recent work (Cai et al., 2015) a mutation in position 233 (M233I) in the β-tubulin confers resistance to Zoxamide. Since benomyl and procymidone, which lead to high level mutation on pathogens (Zhang et al., 2010; Lamberth et al., 2012; Lamberth et al., 2013; Oshima et al., 2002; Cui et al., 2002; Ma et al., 2007; Fillinger et al., 2012), have been recently banned in Turkey by Ministry of Food, Agriculture and Livestocks (Anonymous, 2011), we have investigated structural shifting by spraying of benzimidazole and dicarboximide fungicides, used to *B. cinerea* management in fields. In most cases, amino acid substitutions in the β-tubulin explain the resistance to benzimidazoles in various pathogens including *B. cinerea* (Young and Slawecki, 2005) and the two most commonly reported mutations are at codon 198 and 200. In our study, the target genes sequencing of isolates with the highest virulence, belonging to the two different haplotypes, underlined that a mutation in the β-tubulin sequence (aa 198) seems to be an indicator for haplotype variation. Our results are in agreement to previous studies, underling that the glutamic acid at position 198 of the β-tubulin protein is one of the key factors for fungicide resistance in *B. cinerea* (Cai et al., 2015).

To our knowledge there is a lack of information about the fungicide resistance behaviour of *B. cinerea* and its genetic diversity on pepper in Turkey. Therefore, we highlighted a putative resistance formation to benzimidazole groups in Turkey by β-tubulin gene analysis (Zhang et al., 2010; Malandrakis et al., 2011). On the other hand, no mutation to dicarboxmide group fungicides using *Bos1* gene analysis was identified (Cui et al., 2002; Ma et al., 2007; Fillinger et al., 2012). Results could indicate a shift of the endemic population toward fungicide resistance dominance and a genetic differentiation of pathogens, which could be mainly dependent by the excessive benzimidazole applications in our region.

In summary, the present work is a pioneer study carried out in Turkey on *B. cinerea* isolates from pepper, which represent an economical important crop in the Country. Our findings clearly showed genetic diversity of analysed *B. cinerea* population and its distribution within two main genetic pools by using population structure and two different haplotypes, studying the fungicide resistance genes. We can hypothesize that the genetic background of *B. cinerea* population collected in the Southern Turkey evolves in response to different control

strategies. The monitoring data obtained in this study is original for Turkey; these results should be taken into account when considering disease management.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.02.019.

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