Spanish Journal of Agricultural Research
18 (3), e1005, 12 pages (2020)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2020183-16398
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Genetic variability of *Colletotrichum lindemuthianum* isolates from Turkey and resistance of Turkish bean cultivars

Gulsum Palacioglu (Palacioglu, G)[1], Harun Bayraktar (Bayraktar, H)[1] and Goksel Ozer (Ozer, G)[2]

[1]Ankara University, Faculty of Agriculture, Dept. of Plant Protection, 06110, Ankara, Turkey. [2]Bolu Abant İzzet Baysal University, Faculty of Agriculture and Natural Sciences, Dept. of Plant Protection, 14030, Bolu, Turkey.

Abstract

Aim of study: To evaluate genetic variability and population structure of *C. lindemuthianum* isolates in Turkey and to record the reactions of some common bean cultivars to the pathogen isolates representing different genetic groups.

Area of study: : The study was performed in seven provinces of Turkey.

Material and methods: Genetic diversity of 91 *C. lindemuthianum* isolates obtained from different provinces of Turkey was characterized by 27 iPBS and 30 ISSR primers. Also, the resistance of 40 common bean cultivars was scored against three isolates representing different genetic groups.

Main results: The dendrogram based on the combined dataset of iPBS and ISSR markers classified the isolates into two main groups with a genetic similarity of 72%, which closely associated with the geographic distribution of the isolates. The dendrogram of Nei's genetic distances and Structure analysis supported the clustering of *C. lindemuthianum* isolates according to the geographical provinces. The results indicated that high level of genetic diversity (G_{ST}= 0.4) and low level of gene flow (N_M=0.748) exist among the populations. AMOVA analysis showed that 58.7% of total genetic variability resulted from genetic differences between the isolates within populations, while 41.29% was among populations. Four cultivars showed resistant reaction to three isolates, while the other cultivars were susceptible to at least one isolate.

Research highlights: The results indicated that iPBS and ISSR markers were reliable and effective tools for analyzing population structure of *C. lindemuthianum* and revealed high level of genetic and pathogenic diversity among pathogen populations in Turkey.

Additional key words: bean anthracnose; disease reaction; genetic diversity; iPBS retrotransposon; ISSR.

Abbreviations used: iPBS (inter-priming binding sites); ISSR (inter simple sequence repeat); LTR (long terminal repeat); PDA (potato dextrose agar); PIC (polymorphism information content); R (resistance interaction); S (susceptible interaction); UPGMA (unweighted pair-grouped method with arithmetic average).

Authors' contributions: iPBS (inter-priming binding sites); ISSR (inter simple sequence repeat); LTR (long terminal repeat); PDA (potato dextrose agar); PIC (polymorphism information content); R (resistance interaction); S (susceptible interaction); UPGMA (unweighted pair-grouped method with arithmetic average).

Citation: Palacioglu, G; Bayraktar, H; Ozer, G (2020). Genetic variability of *Colletotrichum lindemuthianum* isolates from Turkey and resistance of Turkish bean cultivars. Spanish Journal of Agricultural Research, Volume 18, Issue 3, e1005. https://doi.org/10.5424/sjar/2020183-16398

Received: 16 Jan 2020. **Accepted:** 27 Aug 2020.

Copyright © 2020 INIA. This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 International (CC-by 4.0) License.

Funding agencies/institutions	Project / Grant
Ankara University Scientific Research Projects Coordination Unit	18L0447010

Competing interests: The authors have declared that no competing interests exist.

Correspondence should be addressed to Harun Bayraktar: bayrakta@agri.ankara.edu.tr

Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important grain legumes for human consumption in many countries of the world. Turkey is a major bean producing country with a production of 630,347 t yr^{-1} and 239,000 t yr^{-1} as green and dry bean, respectively (http://faostat.fao.org/). Bean anthracnose caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara is a destructive fungal disease that restricts bean production worldwide (Kelly & Vallejo, 2004). The pathogen can infect all aerial parts of bean plants and causes typically reddish-brown lesions on pods, containing a mass of pinkish spores and surrounded by reddish brown to black ring borders. The pathogen survives on infected seeds and crop debris and

causes serious yield losses up to 100%, in especially where susceptible cultivars are grown under low temperature and high moisture conditions (Padder *et al.*, 2017). Seeds contaminated with the pathogen play an important role in the long distance distribution of the disease (Mohammed, 2013). The use of pathogen-free seed, crop rotation, fungicide treatments, and host resistance are suggested to control bean anthracnose in the disease management strategies (Mohammed, 2013). Thus, host plant resistance is the most reliable, economical and effective method for managing anthracnose disease on bean because chemicals have negative impacts on the human or environment, and the production of disease-free seed is difficult in developing countries (Meziadi *et al.*, 2016). Some bean varieties resistant to anthracnose have been reported, but high pathogenic variability within the pathogen populations is the primary obstacle in the breeding of resistance to the disease (Ishikawa *et al.*, 2008). To date, more than 247 races of the pathogen have been characterized all over the world (Martiniano-Souza *et al.*, 2017). The presence of many races causes the breakdown of resistance to the pathogen in cultivars carrying anthracnose resistance genes, identified by Co-symbol (Kelly & Vallejo, 2004). In Turkey, 39 races among 51 pathogen isolates were identified, 7 of which had no similarity with characterized races of *C. lindemuthianum*, suggesting that high level of pathogenic variability exists within the pathogen population (Madakbaş *et al.*, 2013). Also, differences were observed in the reactions of some Turkish common bean cultivars to anthracnose disease, and cv. Karaayşe was found as resistant (Madakbaş *et al.*, 2006).

Knowledge of the extent and patterns of genetic variation in the pathogen populations is indispensable for disease management and breeding strategies for resistance to the diseases. Molecular markers have been successfully used for understanding the evolutionary processes affecting pathogenic and genetic variation in the pathogen populations (Lima *et al.*, 2012; Debbi *et al.*, 2018). Comprehensive studies in different countries have been performed to evaluate genetic diversity and population structure of *C. lindemuthianum* by molecular approaches such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), random amplified microsatellites (RAMS), inter-retrotransposon amplified polymorphism (IRAP), and DNA sequencing of the internal transcribed spacer (ITS) region (Ansari *et al.*, 2004; Mahuku & Riascos 2004; Bardas *et al.*, 2007; Barcelos *et al.*, 2011; Mota *et al.*, 2016; Martiniano-Souza *et al.*, 2017). Inter-simple sequence repeats (ISSR) analysis is a reproducible and sensitive tool, which involves the amplification of DNA segment between adjacent, inversely oriented microsatellites regions. This method has been extensively used to characterize genetic diversity between populations of the fungal plant pathogens (Mahmodi *et al.*, 2014; Debbi *et al.*, 2018). The iPBSs (inter-priming binding sites) analysis, developed by Kalendar *et al.* (2010), is a PCR-based method for identifying conserved region of primer binding sites (PBS) among long terminal repeat (LTR) retrotransposon families. This method includes some advantages compared with other retrotransposon markers: iPBS markers is highly reproducible and allows the screening of diverse LTR sequences without the need for cloning and sequence knowledge. To date, the iPBS analysis has been widely applied to visualize genomic polymorphisms in a wide range of plant and animal species (Kalendar *et al.*, 2010). This marker system has been recently used in phylogenetic and genetic diversity studies on fungal pathogens (Özer & Bayraktar 2018; Ateş *et al.*, 2019), but not used to investigate genetic diversity of *C. lindemuthianum* populations. Turkey is a leading common bean producer in the world and anthracnose disease is common in its bean production areas, although little information is available about evolutionary variation and processes affecting genetic composition of *C. lindemuthianum* populations in Turkey (Canseven, 2008; Madakbaş *et al.*, 2013). Knowledge in this area could be useful in the selection of disease-resistant breeding materials and disease management. Therefore, the objective of this study was to evaluate genetic diversity and population structure of *C. lindemuthianum* isolates from major bean growing areas of Turkey and to record the reactions of 40 common bean cultivars to the pathogen isolates representing different genetic groups.

Material and methods

Fungal material

Disease surveys were performed in Samsun, Tokat, Kastamonu, Çankırı, Zonguldak, Rize, Karabük provinces of Turkey during the growing seasons 2017 and 2018 (Fig. 1). The fields were chosen randomly and infected plant samples were collected from different areas of each field. Infected tissues were cut into small pieces (3-5 mm), surface sterilized in 1% NaOCl for 3 min, rinsed twice with sterile water and cultured on potato dextrose agar (PDA, Merck, Darmstadt, Germany) medium at 23°C with a 12-h dark/light cycle. Afterwards, all isolates were purified by single spore isolation technique and maintained on filter papers at 8°C or in Microbank tubes (Pro-Lab Diagnostics, UK) at –80°C in fungal culture collection of Department of Plant Protection, Faculty of Agriculture, Ankara University. All isolates were initially identified based on their conidial morphology such as size and shape of conidia, existence of setae and growth characteristics (Sutton, 1992). Also, morphological

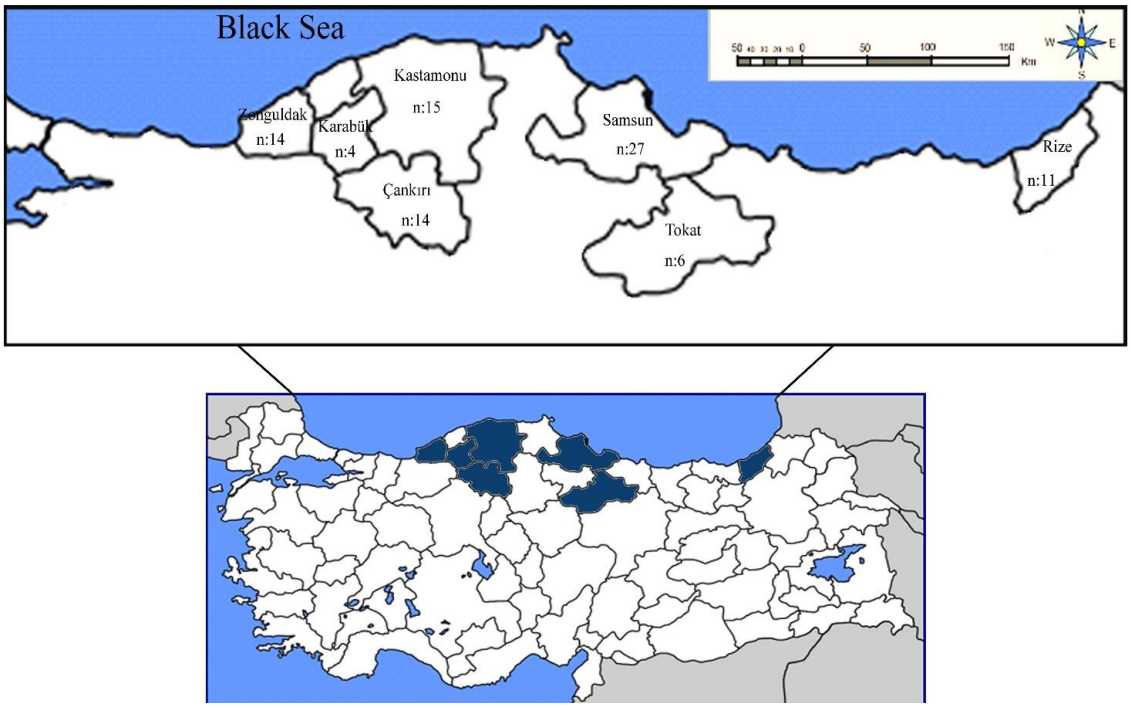

Figure 1. Geographical information and number of *Colletotrichum lindemuthianum* isolates from seven provinces of Turkey. n: number of isolates.

identification was confirmed with PCR assay by using species-specific primers ITS4 and ClF4 described by Chen *et al.* (2007).

DNA extraction

Genomic DNA was extracted according to DArT DNA isolation method (http://www.diversityarrays.com). Fungal mycelia were gently scraped from the surface of single spore cultures grown on PDA medium and transferred into 2 mL microcentrifuge tubes. The samples were suspended in 1 mL fresh buffer solution (125 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 0.8 M NaCl, 1% CTAB, 1% sarcosyl, 2% PVP-40 (K29-32), 0.5% sodium disulphite) and then incubated in a heating block at 65°C for 1h. The suspension was mixed vigorously with one volume of chloroform:isoamylalcohol (24:1) and centrifuged for 20 min at 10,000 g. The supernatant was precipitated with the same volume of ice cold isopropanol. The pellet was washed with 70% cold ethanol, air-dried and suspended in 50 µL of sterile water. DNA concentrations were determined spectrophotometrically and finally diluted to 20 ng μL^{-1}.

iPBS retrotransposon marker analysis

Twenty-seven iPBS retrotransposon primers designed by Kalendar *et al.* (2010) were used on initial screening tests. The primers that provided clear and reproducible band profiles were used to evaluate genetic diversity between all pathogen isolates. PCR reaction was performed in a total volume of 25 µL containing 1x Dream *Taq* Green buffer, 1 µM primer, 0.2 mM dNTPs, 1.5 U Dream *Taq* DNA polymerase 0.04 U *Pfu* DNA polymerase (Thermo Scientific). PCR amplification was carried out in a thermocycler programmed as follows: initial denaturation at 95°C for 3 min, 35 cycles of at 95°C for 15 sec, at annealing temperature for 1 min, and at 70°C for 1 min; and final extension at 72°C for 5 min (Table 1) (Andeden *et al.*, 2013). PCR products were separated by gel electrophoresis on a 1.4% agarose gel, stained with ethidium bromide and visualized under UV light. Gene Ruler plus 100 bp DNA ladder was used as molecular weight standards for electrophoresis (Thermo Scientific).

ISSR marker analysis

Thirty ISSR primers with di- or tri-nucleotide repeats designed by University of British Columbia were tested to select the primers, producing reliable and reproducible polymorphic band profiles between the pathogen isolates. PCR reaction was carried out in a total volume of 25 µL containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.8% Nonident P40, 0.2 mM dNTPs, 0.24 µM primer, 2.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Thermo Scientific). PCR conditions were performed as follows: 35 cycles of at 94°C for 30 s, at annealing temperature for 30 s (Table

Table 1. Characteristics of the primers used.

	Primers sequences (5'-3')	Ta (°C)	G+C (°C)	TB[a]	PB	PB(%)	PIC value per primer
ISSR primers							
$(AG)_8YC$	AGAGAGAGAGAGAGAGYC	54	52.8	7	4	57.1	0.27
$(GA)_8C$	GAGAGAGAGAGAGAGAC	52	52.9	9	8	88.8	0.23
$(AC)_8T$	ACACACACACACACACT	52	47.1	5	2	40,0	0.18
$(AC)_8YA$	ACACACACACACACACYA	56	47.2	5	4	80.0	0.19
$(GA)_9RY$	GAGAGAGAGAGAGAGAGARY	54	50.0	7	6	85.7	0.34
$(AG)_8T$	AGAGAGAGAGAGAGAGT	56	47.1	8	8	100.0	0.49
$DHB(CGA)_5$	DHBCGACGACGACGACGA	56	63.0	11	4	36.3	0.08
$YHY(GT)_7G$	YHYGTGTGTGTGTGTGTG	52	51.8	6	5	83.3	0.25
$BDB(ACA)_5$	BDBACAACAACAACAACA	56	37.1	4	1	25.0	0.12
Total				62	42		
Average				6.88	4.66	69.52	0.238
iPBS primers							
iPBS2077	CTCACGATGCCA	55	58	4	3	75.0	0.36
iPBS2219	GAACTTATGCCGATACCA	53	44	9	4	44.4	0.14
iPBS2399	AAACTGGCAACGGCGCCA	52	61	6	5	83.3	0.06
iPBS2221	ACCTAGCTCACGATGCCA	57	55	8	6	75.0	0.21
Total				27	18		
Average				6.75	4.5	69.4	0.192

[a] TB: number of total bands. PB: number of polymorphic bands. PPB: percentage of polymorphic bands. PIC: mean polymorphism information content.

1), and at 72°C for 2 min, ending with 1 cycle of 10 min at 72°C. PCR products were separated as mentioned above.

Disease reactions of common bean cultivars to the pathogen isolates

Forty common bean cultivars, commonly grown in Turkey were evaluated for their reactions to three isolates representing different genetic groups based on the combined data of iPBS and ISSR markers (Table 2). Seeds of each bean cultivar were disinfected with 1% NaOCl for 3 min, washed three times with water and sown directly into 15 cm in diameter plastic pots. The plants were incubated at 23°C for approximately two weeks until the first trifoliate leaves were fully expanded. Three replicate pots were used with five plants per pots.

To induce sporulation, the pathogen isolates were grown on M3 medium (10 g of sucrose, 20 g of agar, 2 g of KH_2PO_4, 1 g of $MgSO_4.7H_2O$, 6 g of peptone, 1 g of yeast extract agar, 1000 mL of distilled water) at 23°C for 15 days in the dark. Conidia were collected by adding 10 mL sterile distilled water to each petri dish and scraping the surface of cultures with a glass slide. Spore concentration of each isolate was adjusted to the final concentration of 1.2×10^6 spore mL^{-1} using a hemocytometer. Bean plants were sprayed to run off with 10 mL of the final inoculum suspension, covered with a polyethylene bag to maintain leaf wetness for 48 h and incubated at 20±1°C in growth room. Seven days after inoculation, disease evaluation was carried out according to 1-9 scale described by Schoonhoven & Pastor-Corrales (1987). Plants with disease reaction scores of 1–3 were considered resistant, whereas plants that were rated 4–9 were considered susceptible.

Data analysis

Reproducible bands were scored as present (1) or absent (0) for iPBS and ISSR markers. Genetic similarity matrix was constituted with Dice's coefficient of similarity created using the NTSYS-pc v.2.2 numerical taxonomy package program (Rohlf, 1998). The genetic similarity matrix was subjected to cluster analysis with an unweighted pair-grouped method with arithmetic average (UPGMA) to generate a dendrogram. Also, to compare the dendrograms formed using these marker systems, cophenetic

Table 2. Disease reaction of common bean cultivars to three *Colletotrichum lindemuthianum* isolates from different genetic groups.

Bean cultivars	Bean type	Isolate[a]		
		R11	S27	Z10
Asya	Green	S	S	S
Boncuk	Green	S	S	S
Fransız	Green	S	S	S
Gelincik	Green	S	S	S
Gina	Green	S	S	S
Java	Green	S	S	S
Karabacak	Green	S	S	S
Magnum	Green	S	S	S
Mina	Green	S	S	S
Miray	Green	S	S	S
Nazende	Green	S	S	S
Özayşe	Green	S	S	S
Perolar	Green	S	S	S
Sanımteni	Green	S	S	S
Sarıkız	Green	S	S	S
Sazova	Green	R	R	R
Seher yıldızı	Green	S	S	S
Sofia	Green	S	S	S
Tavil	Green	S	S	S
Volare	Green	S	S	S
Yalova 5	Green	S	S	S
Yalova 17	Green	S	S	S
Zeynebim	Green	S	S	S
40 günlük	Green	S	S	S
Belinay Sırık	Kidney	S	S	S
Buse Oturak	Kidney	S	R	S
Klas	Kidney	S	S	S
Selim	Kidney	S	S	S
Sembol	Kidney	S	S	S
Sırık barbunya	Kidney	S	S	S
Akdağ	Dry	S	R	R
Akın	Dry	R	R	R
Aras	Dry	S	S	S
Bulduk	Dry	S	S	S
Göynük 98	Dry	S	S	S
KaracaşeSir 90	Dry	R	R	R
Mecidiye	Dry	S	S	S
Önceler	Dry	R	R	S
Yakutiye	Dry	S	S	S
Zülbiye	Dry	R	R	R

[a] R: resistance interaction (score 1-3). S: susceptible interaction (score 4-9) based on the 1-9 scale of Schoonhoven & Pastor-Corrales (1987).

matrice values were calculated, and later compared by the Mantel test. The mean polymorphism information content (PIC) was calculated following the formula described by De Riek *et al.* (2001). The number of bands, percentage of polymorphic bands (PPB), Nei's gene diversity (h), Shannon's information index (I), total gene diversity (H_T), gene diversity within populations (H_S), genetic differentiation (G_{ST}), and gene flow (N_M) were calculated using POPGENE statistical software ver. 1.32 (Yeh *et al.*, 1999). Support for the population branches was estimated using the bootstrap procedure of TFPGA software ver. 1.3 based on 1,023 permutations. STRUCTURE v.2.2 was used to analyze genetic structure and population subdivision of *C. lindemuthianum* isolates based on the combined dataset (Pritchard *et al.*, 2000). Ten independent runs were performed for each number of clusters, K varying from 2 to 10. Each run was set to 50,000 iterations of Monte Carlo Markov Chain (MCMC) with a 5,000 burn-in period. The number of populations representing the best grouping was determined by ΔK as described by Evanno *et al.* (2005) using STRUCTURE HARVESTER. The analysis of molecular variance (AMOVA) and population pairwise FST were used to separate the variance between and within populations using the ARLEQUIN vers. 3.0 program (Excoffier *et al.*, 2005). Significance tests were calculated by performing 1,023 permutations at level $p<0.001$.

Results

A total of 91 isolates were recovered from diseased plant samples, showing typical anthracnose symptoms on leaf, petioles, hypocotyl and pods. Based on morphological and cultural characters, all isolates were identified as *C. lindemuthianum*. Also, PCR assay was confirmed morphological identification of *C. lindemuthianum* isolates. Species-specific primer pair ITS4/ClF4 resulted in amplification of the expected 461-bp DNA fragment from all the isolates as described by Chen *et al.* (2007).

Thirty ISSR primers were used to reveal genetic variability among the populations of *C. lindemuthianum*, and nine primers produced interpretable and variable banding patterns (Table 1). These primers amplified 62 distinct bands, ranging in size from 0.5-3.5 kb. 67.7% of ISSR fragments were polymorphic, with an average of 4.66 polymorphic bands per primer (Fig. 2). The number of total bands scored per primer ranged from 4 ($BDB(ACA)_5$) to 11 ($DHB(CGA)_5$) with an average of 6.88 bands. The most informative primer was $(AG)_8T$, generating 8 (100%) polymorphic bands, while the least informative primer was BDB(ACA)5 with 25% polymorphism. The values of PIC for ISSR markers ranged from 0.08 (DHB(CGA)5) to 0.49 ($(AG)_8T$) with an average of 0.238. UPGMA cluster analysis based on Dice coefficients identified two major groups between the

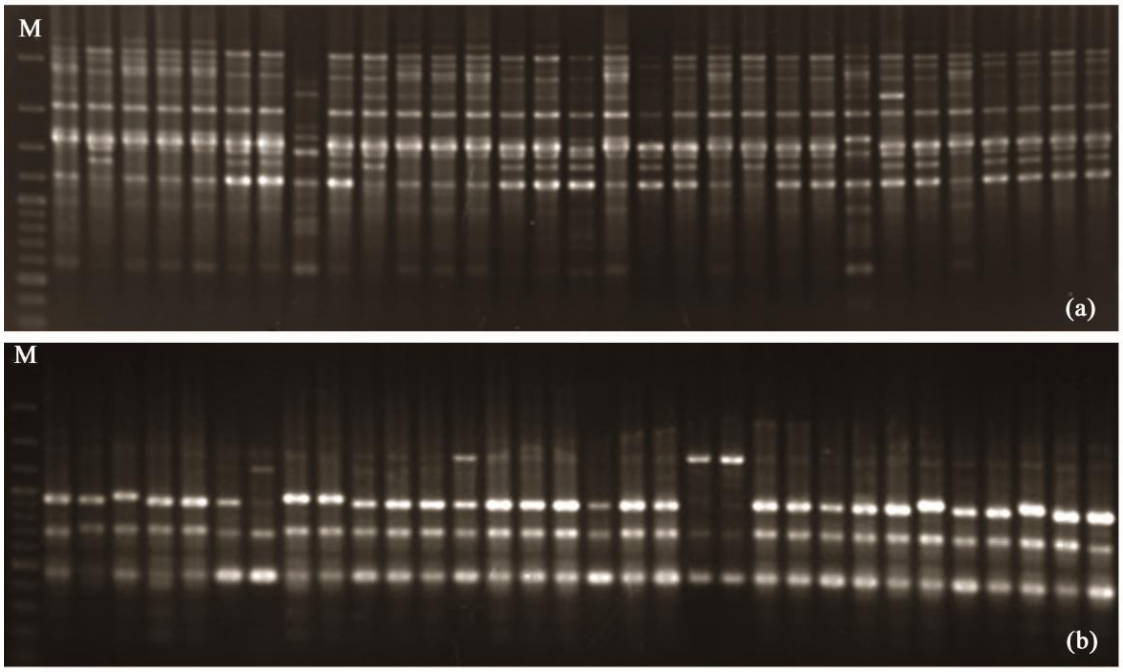

Figure 2. Representative banding patterns of the PCR product for (a) ISSR (YHY(GT)$_7$G) and (b) iPBS (iPBS2399) primers of different *Colletotrichum lindemuthianum* isolates. M: 100 bp DNA ladder.

pathogen isolates from different geographical provinces of Turkey at an arbitrary level of 69% similarity. Cluster I consisted of 19 isolates from Samsun as well as only one isolate (T6) from Tokat province. Cluster II was the largest group with 71 isolates, divided into two subgroups at approximately 90% similarity. All isolates from Rize province were separately grouped from the isolates from 6 provinces into cluster II.

Four of the 27 iPBS primers screened gave reproducible and clear DNA patterns (Table 1). The other primers did not produce any PCR product or provided low level of polymorphisms between the isolates. A total of 27 scorable bands was amplified with four primers, 18 of which were polymorphic (69% criterion) and ranged in size from 0.35 to 2.3 kb (Fig. 2). The number of average bands detected for each primer was 6.75. The primer iPBS2399 generated the greatest percent of polymorphism (83.3%). The mean PIC was 0.192. The dendrogram derived from iPBS data revealed two major clusters with a similarity of 78%. However, some differences were observed in the distribution of the isolates within the clusters compared with the clustering in the dendrogram of ISSR analysis. Cluster I consisted of two subgroups at 82% similarity and the isolates from Rize provinces were grouped with the isolates belonging to cluster I in the dendrogram of ISSR analysis. Cluster II separated two subgroups with a genetic similarity of about 89%.

UPGMA analysis of the combined dataset of iPBS and ISSR markers clustered the pathogen isolates into two major groups with a genetic similarity of 72%, each containing two subgroups (Fig. 3). The clustering of the isolates within groups in the combined dendrogram was more similar to the clustering based on ISSR data than iPBS data. The results indicated that there was a strong correlation between clustering in dendrogram and geographical origin of the isolates and the isolates from the same geographic province were more closely related to one another than isolates from different geographic provinces. Cluster I included 70.3% of the isolates from Samsun province and one isolate from Tokat province, likewise the dendrogram of ISSR analysis. Cluster II separated into two subgroups at approximately 87% similarity. All isolates from Rize province were grouped separately from the isolates, representing the other provinces. Cophenetic correlation efficient for dendrograms of ISSR and iPBS markers based on Mxcoph procedure was 0.96 and 0.87, respectively. The comparison of cophenetic matrix values by Mantel test showed a strong linear relationship (r= 0.73) between the dendrograms.

Genetic diversity estimates for each population are presented in Table 3. The highest percentage of polymorphic loci occurred in Samsun population (58.43%), while the percentage of polymorphic loci for the other populations changed from 12.36% (Karabük) to 48.31% (Tokat). Gene diversity (h) within the populations ranged from 0.046 (Karabük) to 0.217 (Samsun). Mean Shannon's information index (I) ranged from 0.069 to 0.321 among all populations. The total gene diversity (H_T) was 0.165±0.023 for all populations. The average gene diversity within populations (H_S) was 0.098±0.009, which accounted for 59.4% of the total genetic diversity. The proportion of diversity (G_{ST}) indicated that 0.4 of the total

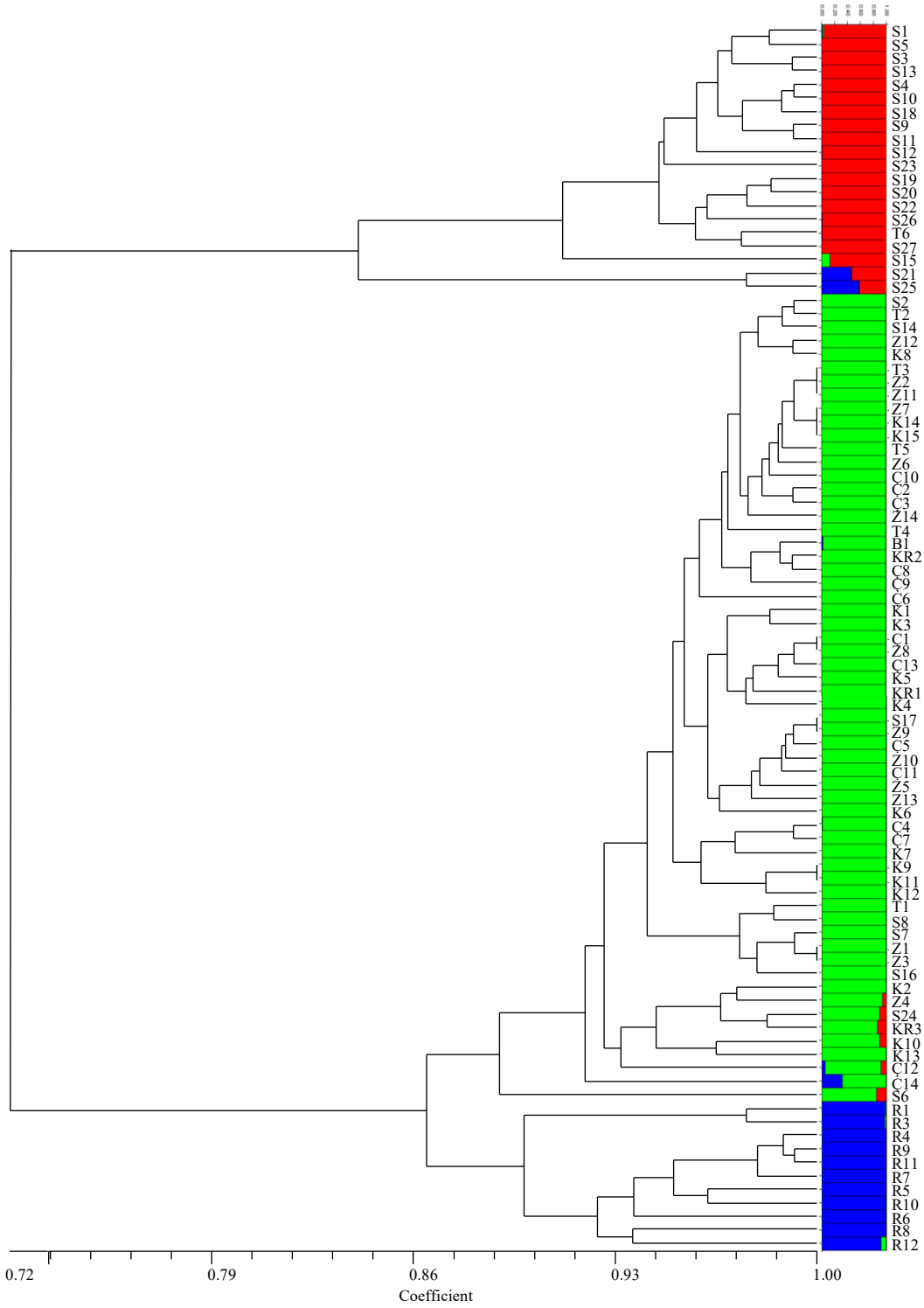

Figure 3. UPGMA clustering and population structure of *Colletotrichum lindemuthianum* isolates from seven provinces of Turkey. The isolates collected from different populations are separated by the initials: Samsun (S), Tokat (T), Kastamonu (K), Zonguldak (Z), Çankırı (Ç), Karabük (KR), and Rize (R).

genetic diversity was among the populations. The level of gene flow (N_M) was 0.748, demonstrating low levels of gene flow among all populations. The levels of population pairwise FST between different populations ranged from 0.6046 to -0.0060, suggesting the levels of Fst indices between Samsun and Rize provinces and the other provinces were significant ($p > 0.05$) (Table 4).

UPGMA analysis of Nei's genetic distances separated all populations into two main clusters in the dendrogram with 100% bootstrap support (Fig. 4). Samsun population

Table 3. Estimates of genetic diversity and distribution of gene diversity between the populations of *Colletotrichum lindemuthianum.*

Populations	N	PL, %	h	I
Samsun	27	58.43	0.217 ±0.207	0.321±0.296
Tokat	6	48.31	0.138±0.145	0.221±0.232
Kastamonu	15	26.97	0.071±0.135	0.114±0.205
Çankırı	14	21.35	0.054±0.118	0.087±0.182
Zonguldak	14	29.21	0.073±0.136	0.118±0.205
Karabük	4	12.36	0.046±0.124	0.069±0.186
Rize	11	28.09	0.091±0.159	0.140±0.236
All populations	H_T: 0.165±0.023	H_S: 0.098±0.009	G_{ST}: 0.400	N_M: 0.748

PL, percentage of polymorphic loci. h, Nei's gene diversity. I, Shannon's Information index. H_T: total gene diversity; H_S: gene diversity within populations; and G_{ST}: the proportion of total gene diversity found among populations, N_M: gene flow.

Table 4. Population pairwise difference (F_{ST}) between *Colletotrichum lindemuthianum* populations.

Populations	Samsun	Tokat	Kastamonu	Zonguldak	Çankırı	Karabük
Tokat	0.2709					
Kastamonu	0.4523*	0.0708				
Zonguldak	0.4943*	0.1085	0.0086			
Çankırı	0.4740*	0.0970	-0.0011	-0.0060		
Karabük	0.3947*	0.0332	0.0376	0.0111	0.0501	
Rize	0.3974*	0.3675*	0.5718*	0.6046*	0.5577*	0.5126*

*F_{ST} *p*-values are considered significant at $p<0.001$

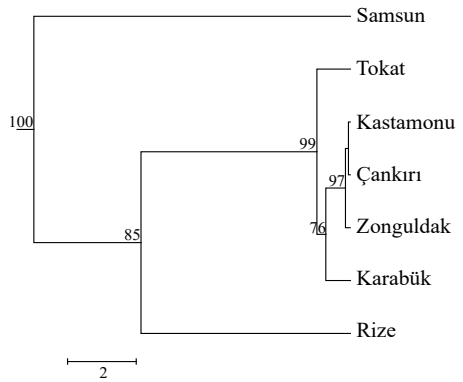

Figure 4. Dendrogram constructed for seven *Colletotrichum lindemuthianum* populations using Nei's genetic distance. Bootstrap values were calculated from 1,000 bootstraps.

clustered separately from the other populations in dendrogram. The populations of Tokat, Kastamonu, Zonguldak, Çankırı and Karabük were clustered together at the level of 0.028 genetic distance, while Rize population formed a distinct cluster with genetic distance of 0.12. STRUCTURE analysis generated similar population clustering patterns to those revealed in the dendrogram (Fig. 3). The highest magnitude of ΔK was found with K = 3 (LnP(K) = -1102.9) and therefore three genetic clusters were detected. This result was related to the geographical distribution of the isolates. Cluster I consisted of the isolates obtained from mostly Samsun provinces, while the isolates

from Rize province belonged to cluster 3. The other 60 isolates from different provinces were placed in cluster II. AMOVA analysis revealed highly significant differences among the provinces, contributing 40.6% of the total genetic variation (Table 5). The 59.4% of variation existed within the individual populations.

The three isolates (R11, Z10, and S27) of *C. lindemuthianum*, representing different populations in the dendrogram derived from the combined data of ISSR and iPBS markers, were evaluated for their virulence on 40 common bean cultivars under controlled conditions (Table 2). Three isolates were highly pathogenic on all common bean cultivars tested with a few exception and caused typical anthracnose symptoms. The isolates, R11 and Z10 caused the mean disease severity ratings of 6.9 and 6.7, respectively, while the isolate S27 was 5.8. The cultivars Sazova, Zülbiye, Akın and Karacaşehir 90 were resistant to three isolates with a disease reaction of < 3 according to 1-9 scale of Schoonhoven & Pastor-Corrales (1987). Also, cv. Buse oturak showed resistant reaction to the isolate S27, while it was regarded as susceptible to the other isolates. The cvs. Önceler and Akdağ were only susceptible to the isolates Z10 and S27, respectively. The other common bean cultivars were found to be susceptible to the three isolates.

Discussion

Anthracnose disease can cause significant economic losses in bean growing areas of Turkey, when environmental conditions are favorable for disease development, and especially in the fields where infected seed material is used (Madakbaş *et al.*, 2006, 2013). However, little information is available on genotypic variability and population structure of *C. lindemuthianum* in Turkey. This study aimed to evaluate genetic variation between the populations of *C. lindemuthianum* to contribute more information for breeding schemes and management strategies to bean anthracnose. To date, extensive studies using different molecular markers have been performed to analyze genetic variability between the populations of *C. lindemuthianum* (Barcelos *et al.*, 2011; Mota *et al.*, 2016; Martiniano-Souza *et al.*, 2017). The iPBS analysis has been used for the first time to clarify intraspecific variability between the isolates of *C. lindemuthianum.* The results indicated that ISSR and iPBS-retrotransposon markers are useful molecular tools for analyzing genetic polymorphisms of *C. lindemuthianum* and revealed high level of genetic variability between the pathogen isolates in agreement with the previous studies, showing that *C. lindemuthianum* is a highly diverse pathogen (Ishikawa *et al.*, 2008; Barcelos *et al.*, 2011; Martiniano-Souza *et al.*, 2017). The mean number of total and polymorphic bands for both markers indicated similar levels of genetic polymorphisms between the isolates. Nine and four of ISSR and iPBS primers screened, respectively, amplified clear and polymorphic bands between the isolates. The other markers were assumed to not be conserved within this fungi species. This observation was in agreement with the results of a previous study, indicating that 9 of 82 primers used to analyze genetic variability of *F. oxysporum* f. sp. *cumini* isolates were informative (Özer & Bayraktar, 2018). However, the mean PIC, an important index that shows the efficiency and discrimination power of the primer, was higher with ISSR than with iPBS markers. The high values of PIC indicated that there was high level of genetic variability between the pathogen isolates and the primers used were effective in analyzing this genetic variation. This high value of PIC was also observed in the study of Ateş *et al.* (2019), who found high levels of genetic variation with a mean PIC value of 0.16 among isolates of *Fusarium oxysporum* using iPBS markers. The cophenetic correlation efficient between the various dendrograms based on Mantel test was highly significant, suggesting a strong linear relationship between the dendrograms. Similar correlation between dendrograms was reported by Bardas *et al.* (2009) analyzing populations of *C. lindemuthianum* in Greece using RAM, RAPD and ERIC–BOX PCR markers. The correlation between cophenetic matrice values obtained from RAM and RAPD dendrograms was highly significant (r = 0.71), while the correlation between ERIC–BOX and RAM markers was found as r = 0.58, ($p < 0.05$) based on the results of Mantel test. The dendrogram of the combined dataset from iPBS and ISSR markers revealed two major genetic clusters among the pathogen isolates at ~ 72% similarity. The isolates belonging to the same geographic

Table 5. Analysis of molecular variance (AMOVA) for *Colletotrichum lindemuthianum* populations based on sampling provinces.

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation	Fst	*p*-value [a]
Among populations	6	328.888	3.919	41.29		
Within populations	85	473.492	5.570	58.70	0.412	<0.0000
Total	91	802.380	9.489			

[a] Probability of a larger value obtained by chance, determined by 1,023 permutations of the data set.

origin were observed to be more closely related to one another. Similar distribution of genetic diversity between *C. lindemuthianum* isolates has been reported by other researchers using different molecular markers. Canseven (2008) evaluated genetic variability between 47 isolates of *C. lindemuthianum* from Turkey using Rep-PCR, RAMS and RAPD marker systems and grouped the pathogen isolates into 5, 2 and 3 clusters in dendrogram, respectively. However, the clustering based on these markers failed to reveal a relationship between pathogenic and geographical variability of isolates. Similarly, Souza *et al.* (2010) clustered the pathogen populations into five distinct groups with similarity coefficients, ranging from 0.43 to 1.00. Also, an average of bands per primer was 4.57, similar to those of iPBS and ISSR markers in our study. With UPGMA analysis of RAM and ERIC–BOX PCR markers, Bardas *et al.* (2009) reported the presence of two main groups with 80% genetic dissimilarity and three main groups with 68% genetic dissimilarity among the populations of *C. lindemuthianum* from three bean growing areas of Greece, respectively.

Genetic estimates of population structure led to a better clarification of genetic variation within and between the populations of *C. lindemuthianum* from different geographical origin of Turkey. A positive correlation was found between geographical provinces and the clustering of the isolates. Samsun and Rize isolates clustered separately from those of the other provinces. Similarly, structure analysis revealed three clusters, supporting the geographical distribution of the pathogen isolates into the dendrogram (the highest value of ΔK was obtained for with K = 3). This result was in agreement with that obtained by Ansari *et al.* (2004), observing some associations between genetic diversity and geographical areas of *C. lindemuthianum*. AMOVA analyses and G_{ST} value both revealed a relatively high level (40%) of genetic diversity among the populations. Approximately 59.4% of total gene diversity could be attributed to differences within the populations. This difference within the populations indicated that genetic recombinations in pathogen reproduction may play an important role in the population evolution of *C. lindemuthianum* in Turkey. Conidial anastomosis between different strains contributes to high level of genetic diversity within the populations (Ishikawa *et al.*, 2012). Also, host-pathogen co-evoluation within the geographic areas, cultivated widely local bean genotypes may have led to high level of gene diversity within the populations. This result is in agreement with the study of Madakbaş *et al.* (2013), who reported that the presence of many races within populations of *C. lindemuthianum* in Turkey was associated with long term selection of new pathotypes under the pressure of local gene pool and environmental conditions and also, seed exchange between different provinces contributed to the distribution of pathogenic variability. Similar distribution of genetic diversity between the populations was reported by Sicard *et al.* (1997) and Souza *et al.* (2010), attributing 58.46%, and 69.03% of the total variability to differences between populations, respectively. Different results were reported by Damasceno e Silva *et al.* (2007), who indicated 3.94% and 96.06% of the genetic variability being among and within regions in Brazil. The level of gene flow (N_m) was estimated to be 0.7482 among all populations, demonstrating low levels of gen flow between different provinces. These results can be explained by the low level of seed exchange between the regions, the more widespread cultivation of local varieties and the agricultural practices in each region. Also, the levels of genetic diversity observed between the isolates within the same population may be attributed to sexual recombination, parasexual cycle, mutation and transposons, which play a role in the life cycle of the pathogen (Ishikawa *et al.*, 2012; Padder *et al.*, 2017).

The use of host plant resistance is the most economically and effective management means control to anthracnose disease and several dominant resistance genes have been described in common beans (Kelly & Vallejo, 2004; Meziadi *et al.*, 2016). However, the development of resistant cultivars is very difficult due to high pathogenic and genetic variability of *C. lindemuthianum* (Ishikawa *et al.*, 2008). Thus, many studies have been performed to evaluate the response of common bean cultivars or genotypes to different pathogenic groups of *C. lindemuthianum* and some bean varieties have been recorded as resistant to anthracnose (Bardas *et al.*, 2007). The present study has evaluated resistance reactions of common bean cultivars against the pathogen isolates representing different genotypic groups. The pathogenicity assays revealed significant differences in the reactions of common bean cultivars. Four cultivars (Sazova, Zülbiye, Akın and Karacaşehir 90) were resistant and the other cultivars susceptible to at least one isolates. Madakbaş *et al.* (2006) evaluated 16 fresh bean cultivars to 5 isolates under greenhouse and laboratory conditions. Karaayşe cultivar was resistant to all isolates under greenhouse condition, but susceptible under laboratory condition, while cvs. Sazova and Nassua were resistant to one isolate under greenhouse condition. Also, four cultivars (Gina, Volare, Yalova 5, Yalova 17) used in this study were regarded as susceptible to all isolates. These results supported that host response of common bean cultivars is variable depending on the pathogen isolates tested and inoculation conditions. Similar findings were reported by Keterew *et al.* (2018), who screened 36 common bean genotypes against seven virulent isolates in Ethiopia. Seven genotypes were resistant to all isolates and the other genotypes responded susceptible or highly susceptible to the virulent isolates.

In conclusion, the present study represented the most detailed study to investigate genetic diversity of *C. lindemuthianum* in Turkey by combining iPBS and ISSR

data. Both markers were reliable and effective tools for analyzing genetic diversity between the pathogen isolates. The results showed that *C. lindemuthianum* possessed high level of genetic polymorphism and clustered into two major groups. Our results also indicated the presence of a relatively high level of genetic diversity and low level of gene flow between the populations. Estimates of genetic diversity showed a strong correlation with geographical origin of the isolates. Also, four common bean cultivars were evaluated as resistant to different genotypic groups of the pathogen. Thus, future studies should be conducted in order to characterize the anthracnose resistance gene in these bean cultivars, to ensure the integration of these genes into breeding programs and to provide more detailed information on pathogen biology in Turkey. This information will contribute to the improvement of breeding strategies, selection of resistance genes and bean anthracnose management.

Acknowledgements

This research was a part of Ph. D. thesis of G. Palacıoğlu. The authors are thankful to Ankara University Scientific Research Projects Coordination Unit for financial support.

References

Andeden EE, Baloch FS, Derya M, Kilian B, Özkan H, 2013. iPBS-Retrotransposons-based genetic diversity and relationship among wild annual Cicer species. J Plant Biochem Biotechnol 22 (4): 453-466. https://doi.org/10.1007/s13562-012-0175-5

Ansari KI, Palacios N, Araya C, Langin T, Egan D, Doohan FM, 2004. Pathogenic and genetic variability among *Colletotrichum lindemuthianum* isolates of different geographic origins. Plant Pathol 53 (5): 635-642. https://doi.org/10.1111/j.0032-0862.2004.01057.x

Ateş D, Altinok HH, Ozkuru E, Ferik F, Erdogmus S, Can C, Tanyolac MB, 2019. Population structure and linkage disequilibrium in a large collection of *Fusarium oxysporum* strains analysed through iPBS markers. J Phytopathol 167: 576-590. https://doi.org/10.1111/jph.12848

Barcelos QL, Souza EA, Silva KD, 2011. Vegetative compatibility and genetic analysis of *Colletotrichum lindemuthianum* isolates from Brazil. Gen Mol Res 10 (1): 230-242. https://doi.org/10.4238/vol10-1gmr907

Bardas GA, Koutita O, Tzavella-Klonari K, 2007. Geographical distribution, pathotype characterization, and molecular diversity of *Colletotrichum lindemuthianum* in Greece and resistance of Greek bean cultivars. Plant Dis 91 (11): 1379-1385. https://doi.org/10.1094/PDIS-91-11-1379

Bardas GA, Koutita O, Tzavella-Klonari K, 2009. Molecular diversity and assessment of biological characteristics of Greek *Colletotrichum lindemuthianum* populations. J Phytopathol 157 (5): 311-318. https://doi.org/10.1111/j.1439-0434.2008.01477.x

Canseven SK, 2008. Virulence and genetic diversity of *Colletotrichum lindemuthianum* isolates collected from common bean varieties of Turkey. M.Sc. Thesis, Boğaziçi University, Turkey.

Chen YY, Conner RL, Gillard CL, Boland GJ, Babcock C, Chang KF, Hwang SF, Balasubramanian PM, 2007. A specific and sensitive method for the detection of *Colletotrichum lindemuthianum* in dry bean tissue. Plant Dis 91 (10): 1271-1276. https://doi.org/10.1094/PDIS-91-10-1271

Damasceno e Silva KJ, De Souza EA, Ishikawa FH, 2007. Characterization of *Colletotrichum lindemuthianum* isolates from the state of Minas Gerais, Brazil. J Phytopathol 155 (4): 241-247. https://doi.org/10.1111/j.1439-0434.2007.01226.x

De Riek J, Calsyn E, Everaert I, Van Bockstaele E, De Loose M, 2001. AFLP based alternatives for the assessment of distinctness, uniformity and stability of sugar beet varieties. Theor Appl Gen 103 (8): 1254-1265. https://doi.org/10.1007/s001220100710

Debbi A, Boureghda H, Monte E, Hermosa R, 2018. Distribution and genetic variability of *Fusarium oxysporum* associated with tomato diseases in Algeria and a biocontrol strategy with ındigenous *Trichoderma* spp. Front Microbiol 9: 282. https://doi.org/10.3389/fmicb.2018.00282

Evanno G, Regnaut S, Goudet J, 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14 (8): 2611-2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Excoffier L, Laval G, Schneider S, 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. University of Berne, Switzerland. https://doi.org/10.1177/117693430500100003

Ishikawa F, Souza E, Davide L, 2008. Genetic variability within isolates of *Colletotrichum lindemuthianum* belonging to race 65 from the state of Minas Gerais, Brazil. Biologia 63 (2): 156-161. https://doi.org/10.2478/s11756-008-0039-6

Ishikawa FH, Souza EA, Shoji JY, Connolly L, Freitag M, Read ND, Roca MG, 2012. Heterokaryon incompatibility is suppressed following conidial anastomosis tube fusion in a fungal plant pathogen. PloS one 7 (2): e31175. https://doi.org/10.1371/journal.pone.0031175

Kalendar R, Antonius K, Smýkal P, Schulman AH, 2010. iPBS: a universal method for DNA fingerprinting and

retrotransposon isolation. Theor Appl Gen 121: 1419-1430. https://doi.org/10.1007/s00122-010-1398-2

Kelly JD, Vallejo VA, 2004. A comprehensive review of the major genes conditioning resistance to anthracnose in common bean. HortScience 39: 1196-1207. https://doi.org/10.21273/HORTSCI.39.6.1196

Keterew Y, Dawit W, Selvaraj T, Lencho A, 2018. Physiological characterization and evaluation of common bean genotypes against anthracnose (*Colletotrichum lindemuthianum* (Sacc. and Magnus) Lams-Scriber) races in West Shewa, Ethiopia. Int J Life Sci 6 (3): 744-754.

Lima JS, Figueiredo JG, Gomes RG, Stringari D, Goulin EH, Adamoski D, Kava-Cordeiro V, Galli-Terasawa LV, Glienke C, 2012. Genetic diversity of *Colletotrichum* spp. an endophytic fungi in a medicinal plant, Brazilian pepper tree. ISRN Microbiology https://doi.org/10.5402/2012/215716

Madakbaş SY, Dolar FS, Bayraktar H, Ellialtıoğlu Ş, 2006. Determination of strains causing the disease of anthracnose (*Colletotrichum lindemuthianum* (Sacc. & Magnus) Lambs. Scrib.) seen in areas where grown fresh bean in the Middle Blacksea region and research on determination of the condition resistance of disease of some bean variates. VI. Vegetable Agr. Symp., Kahramanmaraş Sütçü İmam Univ., Turkey, pp: 138-142.

Madakbaş SY, Ergin M, Bekar NK, 2013. Identification and characterization of anthracnose (*Colletotrichum lindemuthianum* (Sacc. & Magnus) Lambs. Scrib.) isolates from Turkey using differential set cultivars. J Food Agr Environ 11 (2): 385-392.

Mahmodi F, Kadir JB, Puteh A, Pourdad SS, Nasehi A, Soleimani N, 2014. Genetic diversity and differentiation of *Colletotrichum* spp. isolates associated with Leguminosae using multigene loci, RAPD and ISSR. Plant Pathol J 30 (1): 10-24. https://doi.org/10.5423/PPJ.OA.05.2013.0054

Mahuku GS, Riascos JJ, 2004. Virulence and molecular diversity within *Colletotrichum lindemuthianum* isolates from Andean and Mesoamerican bean varieties and regions. Eur J Plant Pathol 110 (3): 253-263. https://doi.org/10.1023/B:EJPP.0000019795.18984.74

Martiniano-Souza MC, Gonçalves-Vidigal MC, Lacanallo GF, Costa AF, Vidigal Filho PS, Dartibale GB, Coêlho M, Calvi AC, Felipin-Azevedo R, 2017. Genetıc variability of *Colletotrichum lindemuthianum* by sequencing ITS regions. Annu Rep Bean Improv Coop 60: 9-10.

Meziadi C, Richard MM, Derquennes A, Thareau V, Blanchet S, Gratias A, Pflieger S, Geffroy V, 2016. Development of molecular markers linked to disease resistance genes in common bean based on whole genome sequence. Plant Sci 242: 351-357. https://doi.org/10.1016/j.plantsci.2015.09.006

Mohammed A, 2013. An overview of distribution, biology and the management of common bean anthracnose. J Plant Pathol Microbiol 4 (8): 1-6.

Mota SF, Barcelos QL, Dias MA, Souza EA, 2016. Variability of *Colletotrichum* spp. in common bean. Gen Mol Res 15 (2): gmr.15027176. https://doi.org/10.4238/gmr.15027176

Özer G, Bayraktar H, 2018. Genetic diversity of *Fusarium oxysporum* f. sp. *cumini* isolates analyzed by vegetative compatibility, sequences analysis of the rDNA IGS region and iPBS retrotransposon markers. J Plant Pathol 100 (2): 225-232. https://doi.org/10.1007/s42161-018-0063-5

Padder BA, Sharma PN, Awale HE, Kelly JD, 2017. *Colletotrichum lindemuthianum*, the causal agent of bean anthracnose. J Plant Pathol 99 (2): 317-330.

Pritchard JK, Stephens M, Donnelly P, 2000. Inference of population structure using multilocus genotype data. Genetics 155 (2): 945-959.

Rohlf FI, 1998. NTSYS-pc. Numerical taxonomy and multivariate analysis system, version 2.0. Applied Biostatistics, NY, USA.

Schoonhoven AV, Pastor-Corrales MA, 1987. Standard system for the evaluation of bean germplasm. Centro Internacional de Agricultura Tropical (CIAT), Apartado Areo 6713, Cali, Colombia. 56 pp. https://agris.fao.org/agris-search/search.do?recordID=QT880000288

Sicard D, Michalakis Y, Dron M, Neema C, 1997. Genetic diversity and pathogenic variation of *Colletotrichum lindemuthianum* in the three centers of diversity of its host, *Phaseolus vulgaris*. Phytopathology 87 (8): 807-813. https://doi.org/10.1094/PHYTO.1997.87.8.807

Souza EA, Camargo Jr OA, Pinto JM, 2010. Sexual recombination in *Colletotrichum lindemuthianum* occurs on a fine scale. Gen Mol Res 9 (3): 1759-1769. https://doi.org/10.4238/vol9-3gmr863

Sutton BC, 1992. The Genus Glomerella and its anamorph Colletotrichum. In: Colletotrichum: biology, pathology, and control; Bailey, JA & Jeger MJ (eds). CAB Int, Wallingford, pp. 1-26.

Yeh FC, Yang RC, Boyle TB, Ye ZH, Mao JX, 1999. POPGENE version 1.32, the user-friendly shareware for population genetic analysis. Mol Biol & Biotechnol Centr, Univ. Alberta, Canada.