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Araştırma Makalesi (ResearchArticle)

Characterization and Haplotype Analysis of *Colletotrichum truncatum* in Greenhouse Tomato in Turkey

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Abstract: Anthracnose caused by *Colletotrichum truncatum* (Schwein.) Andrus and W.D. Moore, is an economically important disease of most tropical crops. In recent years, it has been reported that it is also pathogenic on tomatoes. In this study, the infected fruits were obtained from Antalya province in 2019. Isolates were purified by taking single spore. Conidia were measured as 22.5-32.5 × 3.75 µm. DNA isolation was carried out using the CTAB method. After the PCR amplification, the PCR product was run on agarose gel, visualized with a UV transilluminator, and sequenced. Phylogenetic analysis was conducted in MEGA 7. Based on morphological and phylogenetic analysis, the CT isolate was identified as *C. truncatum*. Pathogenicity tests were carried out using tomato leaves and cherry tomatoes. The inoculated leaves and tomatoes were incubated on a moist filter paper in climate chambers under 27 °C temperature and 12:12 h light-dark conditions. Acervuli were observed on infected tissues on the 7th day of inoculation. Haplotype, the number of haplotypes, and nucleotide diversity were analyzed by DnaSP 6.0 software. 8 haplotypes were determined according to the ITS sequence of 46 *C. truncatum* isolates from different countries. The median-joining network analysis of haplotypes was drawn using the NETWORK 10 program. It was determined that the CT isolate reported with this study from Turkey and the other reference isolates reported on tomatoes were in the H1 which is the most common haplotype.

Türkiye'de Sera Domatesinde *Colletotrichum truncatum'un* Karakterizasyonu ve Haplotip Analizi

Makale Bilgileri

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Anahtar Kelimeler

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Domates.

Öz: *C. truncatum*'un (Schwein.) Andrus and W.D. Moore neden olduğu antraknoz çoğu tropikal ürünün ekonomik olarak önemli bir hastalığıdır. Son yıllarda domateste de patojen olduğu rapor edilmiştir. Bu çalışmada, enfekteli meyveler 2019 yılında Antalya ilinden elde edilmiştir. İzolatlar tek spor alınarak saflaştırıldı. Konidiler 22.5-32.5 × 3.75 µm olarak ölçülmüştür. DNA izolasyonu CTAB metodu kullanılarak gerçekleştirildi. PCR amplifikasyonundan sonra, PCR ürünleri agaroz jelde koşturuldu, UV cihazı ile görüntülendi ve sekanslandı. Filogenetik analiz MEGA 7'de yapılmıştır. Morfolojik ve filogenetik analizlere dayanarak CT izolatı *C. truncatum* olarak tanımlanmıştır. Patojenite testleri domates yaprakları ve çeri domatesleri kullanılarak gerçekleştirildi. İnokule edilen yapraklar ve domatesler, 27 °C sıcaklık ve 12:12 saat aydınlık-karanlık koşulları altındaki iklim odasında nemli bir filtre kağıdı üzerinde inkübe edilmiştir. İnokülasyonun 7. gününde enfekteli dokularda acervuli gözlenmiştir. Haplotip, haplotip sayısı ve nükleotid çeşitliliği DnaSP 6.0 yazılımı ile analiz edilmiştir. Farklı ülkelerden 46 *C. truncatum* izolatının ITS sekansına göre 8 haplotip belirlenmiştir. Haplotiplerin

medyan birleştirme ağı analizi NETWORK 10 programı kullanılarak çizilmiştir. Türkiye'den bu çalışma ile rapor edilen CT izolatının ve domateste rapor edilen diğer referans izolatların en yaygın haplotip olan H1'de olduğu belirlenmiştir.

1. Introduction

Tomato is used both as fresh and as a raw material for various industrial products. Turkey is among the most important countries in tomato production and exports (FAO, 2020). In 2020, most of the total tomato production of 13 204.015 tons was obtained from Antalya, Bursa, and Manisa provinces, respectively 2 570.910, 1 335.430, and 1 123.684 tons. The province, with the highest greenhouse tomato production, is Antalya with 2 465.402 tons (TUIK, 2021).

There are many fungal pathogens that cause yield and quality losses by root, stem, flower, and fruit infections on tomatoes. *Colletotrichum* species can cause significant economic losses by affecting fruit production especially under field conditions, as well as causing significant damage in warehouses (Živković et al., 2010). Although the most common species on the tomato fruit is *C. coccodes*; *C. truncatum, C. gloeosporioides*, *C. acutatum*, *C. dematium*, *C. fioriniae,* and *C. nymphaeae* also cause fruit infections (Blancard, 2012; Víchová et al., 2012; Diao et al., 2014; Saini et al., 2017; Chechi et al., 2019). Other than fruit, they can cause serious infections on leaves, stems, and roots (Isaac, 1992; He et al., 2016; Belov et al., 2017). Root infections usually occur, when the inoculum level is too high or plants are stressed especially due to infection by other pathogens such as *Pyrenochaeta lycopersici* (Anonymous, 2017).

It has been stated that *C. fioriniae* and *C. gleosporioides* are also an entomopathogenic fungus, and in these species may be biovars that have acquired the ability to infect both insects and plants (Marcelino et al., 2008; 2009). These fungi, which can be found endophytically in the plant, can be used as a biological control agent in the control of insects (Marcelino et al., 2009). It has been reported that the *C. truncatum* is found endophytically in the plant (Ranathunge and Sandani, 2016). There is no literature on the presence of entomopathogenic biovars in the species.

Anthracnose caused by *C. truncatum* (Schwein) Andrus and W.D. Moore, is an economically important disease of most tropical crops (Cannon et al., 2012). This pathogen is the main pathogen especially in countries where chili pepper is grown (Rao et al., 2018). It has been reported as a pathogen in alfalfa in Turkey (Eken and Demirci, 2000). There are studies showing that there are races of the pathogen on lentils (Tullu et al., 2006). It is one of the important seed-borne diseases of soybean and chili pepper (Begum et al., 2007; Naveen et al., 2021). In recent years, it has been reported that it is also pathogenic on tomatoes (Diao et al., 2014; Saini et al., 2017; Villafana et al., 2018; Almaraz Sánchez et al., 2019). It also causes necrotic spots on the leaves (He et al., 2016) although it causes more fruit infections.

It is seed-borne (Naveen et al., 2021) and can also be found endophytically in non-host species (Ranathunge and Sandani, 2016). As a plant pathogenic fungus, it mostly infects dicotyledonous plants but has also been detected on *Cyperus rotundus* from monocotyledonous plants (Damm et al., 2009). It has also been reported to cause eye infections in humans (Shivaprakash et al., 2011).

C. truncatum has not been reported previously on tomatoes in Turkey. Currently, there are no tomato cultivars resistant to *C. truncatum*. In order to develop resistant cultivars, it is important to determine the pathogenic and genetic variation in the population. In this study, it is aimed to identify this pathogen, to perform pathogenicity tests and haplotype analysis.

2. Materials and methods

2.1. Isolation and morphological identification of pathogen

The infected fruits were obtained from Antalya province in 2019. Infected plant tissues were taken, surface-sterilized in 1% NaOCl for 1 min, dried between sterile blotting papers, and transferred to PDA medium. After the fungal sporulation was observed, conidia were spread onto the PDA medium by means of a sterile needle. Isolates were purified by transferring a single spore into PDA medium with sterile needles under a binocular microscope (Leica M165 C). Single spore isolates were maintained in agar slants at +4°C for further studies.

The measurements of 30 conidia obtained from an 8-days culture grown on PDA were performed with a 40X magnification using a Leica DM1000 light microscope. Morphological identification of the fungus was performed according to Damm et al., 2009.

2.2. Molecular identification

The acervuli and mycelia of the pathogen from the 12-days culture grown on PDA medium were scraped with a sterile scalpel and placed in a sterilized mortar. Fungal tissues were crushed by pouring liquid nitrogen into the mortar. DNA isolation was carried out using the CTAB method according to Lefort et al. (1998). The density and purity of DNA were measured by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

In the PCR reaction, 10 µM ITS4 and ITS5 primers (White et al. 1990), 50-100 ng DNA, 12.5 µl Thermo Dream Taq green mix was used, and the total volume was completed to 25 µl with PCR grade water. PCR amplification was performed using Thermocycler (Bio-Rad Thermal Cycler PTC-200). PCR conditions were 3 min at 95 °C, 35 cycles 30 s at 95 °C, annealing 30 s at 60 °C, elongation 60 s at 72 °C, and final elongation 10 min at 72 °C. PCR products were run on 1.5% agarose gel in 1X TBE buffer at 120 V current for 45 minutes and were visualized with a UV transilluminator. PCR product was purified and sequenced by BM Labs located in Ankara, Turkey. The sequence was subjected to BLAST analyze at NCBI gene bank and the accession number was taken.

2.3. Pathogenicity tests

Pathogenicity tests were performed on moist filter paper with 3 replicates of Purdue 135 tomato variety obtained from the Gene Bank of the Czech Republic (https://grinczech.vurv.cz/gringlobal/search.aspx) and 5 replicates of cherry tomatoes. The leaves and tomatoes to be used in the tests were first washed with water and then surface disinfection was carried out with 70% alcohol. Spore suspension at a density of 1.2×10^6 conidia/ml was prepared using a hemocytometer from 9 days old fungus culture. 20 µl of this suspension was injected into leaves and tomatoes. 20 µl of water was injected into the leaves and tomatoes used as control. The inoculated leaves and tomatoes were incubated on a moist filter paper in climate chambers under 27 °C temperature and 12:12 h light-dark conditions (Torres-Calzada et al., 2018). The pathogen was re-isolated from infected tissues.

2.4. Phylogenetic and network analysis

The sequences of the isolates to be used in phylogenetic and network analysis were obtained from the NCBI gene bank. *Passalora fulva* was used as an out-group. Sequences were aligned in the MEGA7 program using ClustalW. The phylogenetic tree was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura 2+G) with bootstrap analysis 1500 replications (Kimura, 1980). Phylogenetic analysis was conducted in MEGA 7 (Kumar et al., 2018).

Haplotype, the number of haplotypes, and nucleotide diversity were calculated by DnaSP 6.0 software. The median-joining network analysis of haplotypes was drawn using NETWORK 10 program.

3. Results and Discussion

3.1. Morphologic identification

Conidia were hyaline, curved inward at the ends without septa. It was measured as 22.5-32.5 × 3.75 µm (x = 26.9-3.75 µm, n = 30). Brown setae were 2-5 septa. Culture, acervuli, conidia and setae of the pathogen on PDA medium are given in Figure 1.

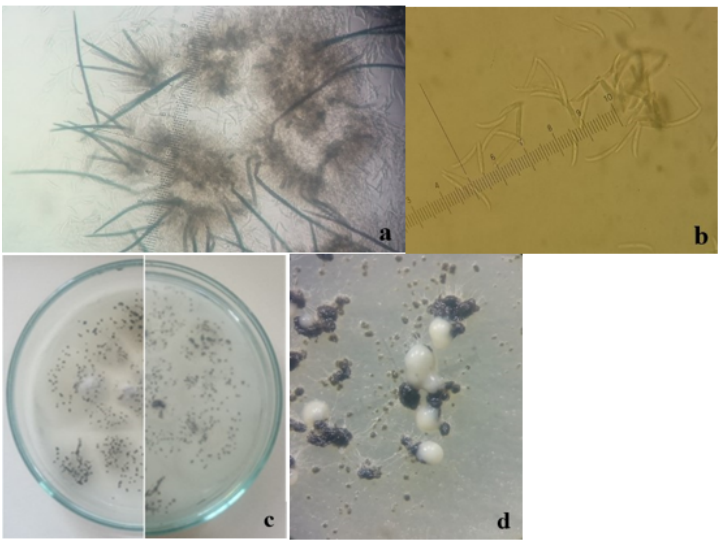

Figure 1. Setae (a), conidia (b), culture (c) and acervuli (d), of the fungus on the PDA medium.

3.2. Pathogenicity tests

In the pathogenicity tests were carried out on moist filter paper using tomato leaves and cherry tomatoes. Acervuli were observed on infected tissues on the 7th day of inoculation. Slightly inwardly curved conidia and setae of the pathogen were observed in the preparations made by taking the plant tissue (Figure 3). No symptoms were observed on the control fruits and leaves (Figures 2 and 3). CT isolate was evaluated as a pathogen.

Figure 2. Controls and tomatoes inoculated with *C. Truncatum*.

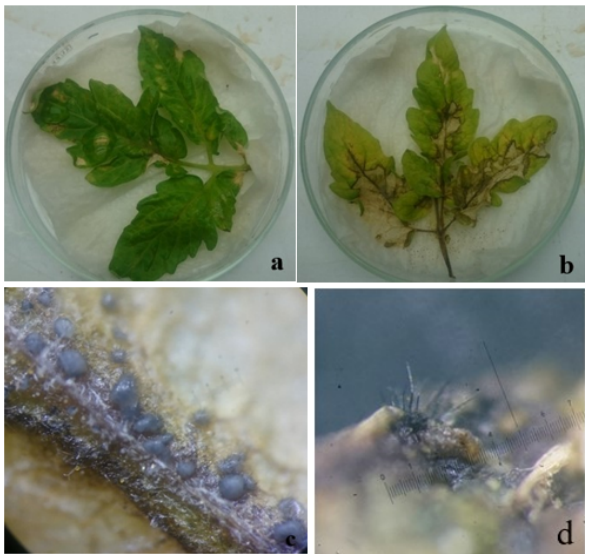

Figure 3. Control (a), symptom of *C. truncatum* on leaf on the moist filter paper (b) and acervuli on the infected leaf (c-d).

3.3. Phylogenetic and network analysis

The accession number (MW453147) of the CT isolate was taken from the NCBI gene bank. The CT isolate showed similarity with the *C. truncatum* isolates in the NCBI gene bank. In addition, in the phylogenetic analysis performed with other *Colletotrichum* species, the pathogen was clustered together with the *C. truncatum* isolate (Figure 4). Information about the *Colletotrichum* species used in the study are given in Table 1.

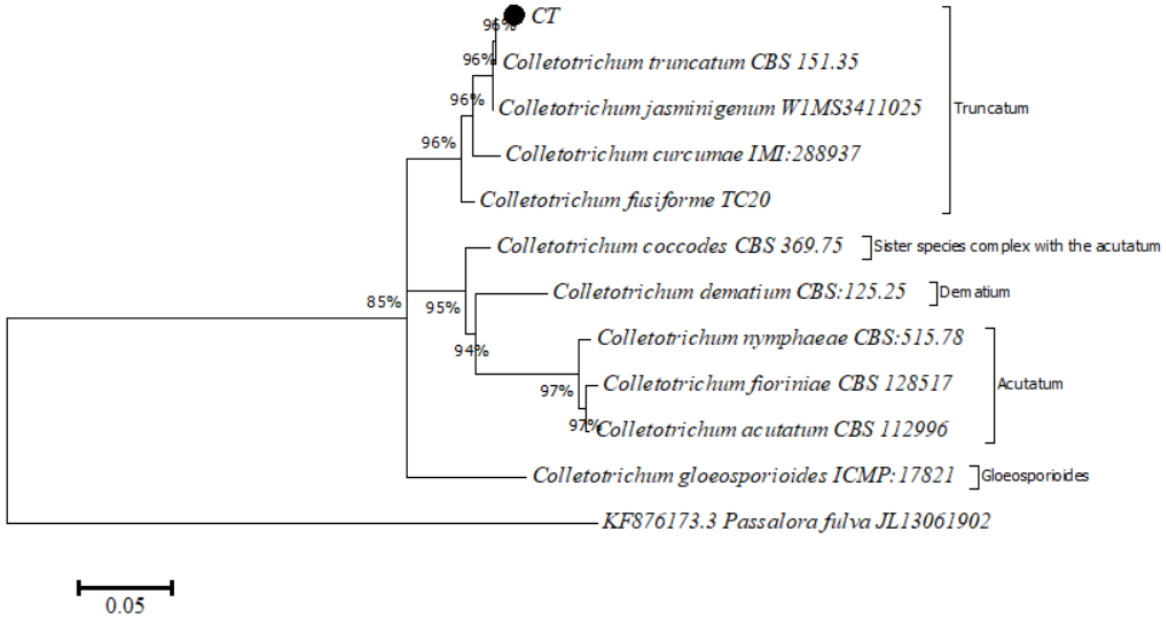

Figure 4. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (G). Evolutionary analysis was conducted in MEGA 7; bootstrap values higher than 50% are shown on branches.

Table 1. Isolates used in molecular characterization studies

Species complexes	*Colletotrichum* species	Isolates	Accession numbers
Truncatum	*Colletotrichum truncatum*	CT	MW453147
	Colletotrichum truncatum	CBS_151.35	GU227862
	Colletotrichum curcumae	IMI:288937	GU227893.1
	Colletotrichum fusiforme	TC20	MT318539.1
	Colletotrichum jasminigenum	W1MS3411025	KC172075.1
Dematium	*Colletotrichum dematium*	CBS:125.25	GU227819
Sister species complex with the acutatum species complex	*Colletotrichum coccodes*	CBS_369.75	HM171679
Gloeosporioides	*Colletotrichum gloeosporioides*	ICMP:17821	JX010152
Acutatum	*Colletotrichum_fioriniae_*	CBS_128517	JQ948292
	*Colletotrichum_nymphaeae_*	CBS:515.78	JQ948197
	Colletotrichum_acutatum	CBS_112996	JQ005776

In the previous phylogenetic analysis studies (Cannon et al., 2012; Jayawardena et al., 2016), the species complexes including the *Colletotrichum* species are given in Table 1. In phylogenetic analysis, it is seen that *C. jasminigenum* is the molecularly closest species to the *C. truncatum* (Figure 4). This species is only reported on the *Jasminium sambac* in Vietnam. Its conidia are similar to the slightly inward curved conidia of the *C. truncatum*. It does not form setae in PDA medium (Wikee et

al., 2011). In this study, it was observed that CT isolate formed setae in PDA medium. It was found to be compatible with *C. truncatum* in terms of the shapes and sizes of conidia and setae (Damm et al., 2009). Based on morphological and phylogenetic analysis, the CT isolate was identified as *C. truncatum*. All of the *C. truncatum* isolates reported on tomatoes were found to be in the H1 haplotype group in the network analysis. The accession numbers of the isolates used in the analysis are given in Table 2. 65% of the isolates were in the H1 haplotype. After H1, the second most common haplotype was determined to be H2. This haplotype includes isolates from Nepal, Brazil, the USA, India, Denmark (Figure 5, Table 2). H3 Bangladesh, H4 and H5 Mexico, H6, H7, and H8 haplotypes contain unique isolates reported from Brazil. No relationship could be determined between the geographic distribution of the isolates and haplotypes.

8 haplotypes were determined in the median-joining network analysis performed according to the ITS sequence of 46 *C. truncatum* isolates from different countries (Figure 5). Haplotype diversity was Hd: 0.5440. Nucleotide diversity was Pi: 0,00136. It was determined that Fu and Li's D (FLD: -2,90185) and Fu and Li's F (FLF: -2,78932) test results were statistically significant ($P < 0.05$).

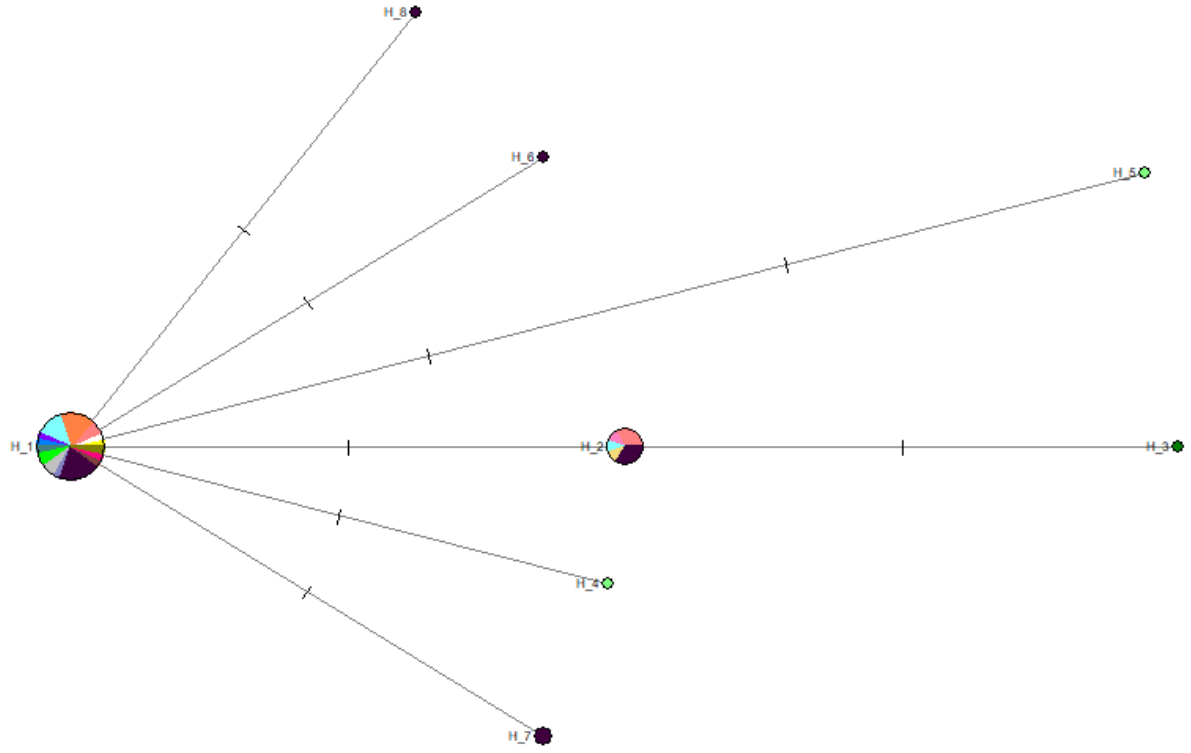

Figure 5. Network analysis of the *C. truncatum*. Each country is represented by a different colour; the size of the circle and circle slices indicates the number of isolates in the haplotype; Turkey ( ),USA ( ), Brazil ( ), China ( ), Trinidad and Tobago ( ), India ( ), Laos ( ), Indonesia ( ), Pakistan ( ), Mexico ( ), Denmark ( ), Burkina Faso ( ), Thailand ( ), Martinique ( ), Israel ( ), Sudan ( ), Nepal ( ), Bangladesh ( ).

Currently, 14 species complexes of *Colletotrichum* fungi are recognized (da Silva et al., 2020). The closest species complexes to the truncatum species complex are gloeosporioides and boninense (Cannon et al., 2012). Boninense species complex, which contains a large number of *Colletotrichum* species, which can be identified only by ITS region, is accepted as the sister species complex of the truncatum species complex (Cannon et al., 2012). The conidium morphology of the *C. truncatum* species is quite different from the gloeosporioides and boninense species complexes to which it is phylogenetically close (Damm et al., 2012).

The haplotypes found at the terminal in network analysis are new haplotypes that have recently been formed. Older haplotypes are found more inland in network analysis (Posada and Crandall, 2001).

Younger haplotypes show more limited geographic association, while older haplotypes show a wider geographic distribution (Carbone and Kohn, 2004). Accordingly, it can be said that terminal haplotypes are younger than the H1 haplotype. In network analysis, it is seen that the H3 haplotype originates from the H2 haplotype. In terms of geographical location, it can be said that the H3 haplotype originates from Nepal and India, which are included in the H2 haplotype (Katoch et al., 2016).

Table 2. *C. truncatum* isolates used in network analysis

Haplotype	Frequency	Accession numbers and country
Hap_1	30	**MW453147, Turkey; KC460308, China**; GU228254, USA; **GU227862, USA**; **MG822829, Trinidad and Tobago**; **MG822828, Trinidad and Tobago**; **MG822827, Trinidad and Tobago**; JF749808, Trinidad and Tobago; HQ287583, Trinidad and Tobago; **KY399773, India**; GU227886, India; GU227880, India; HM231266, India; GU227889, Laos; GU227891, Martinique; GU227879, Indonesia; GU227885, Mexico; HM562707, Mexico; DQ454016, Thailand; DQ454028, Thailand; GU227871, Burkina Faso; KJ614299, Brazil; KJ614302, Brazil; KJ614311, Brazil; KJ614334, Brazil; GU227864, Brazil; GU227892, Brazil; GU227868, Israel; GU227874, Sudan; GU227872, Pakistan
Hap_2	9	GU227863, USA; GU227865, USA; GU227866, USA GU227867, Denmark; GU227878, India; GU227890, Nepal; KJ614306, Brazil; KJ614321, Brazil; KJ614328, Brazil
Hap_3	1	GU227882, Bangladesh
Hap_4	1	HM439289, Mexico
Hap_5	1	HM450132, Mexico
Hap_6	1	KJ614293, Brazil
Hap_7	2	KJ614313, Brazil; KJ614314, Brazil
Hap_8	1	KJ614319, Brazil

* Isolates reported on tomato are written in bold.

Negative values in Fu and Li's F test indicate that the population is expanding. The fact that the test results are statistically significant indicates that the polymorphism in the population is not random, it occurs as a result of selection pressure.

The wide host range of the *C. turuncatum* is indicative of selection pressure on different hosts. It is thought that genetic diversity analysis of populations with limited hosts from different parts of the world will provide useful information on the evolutionary behaviour and the formation of effective management strategies based on host resistance (Katoch et al., 2016).

Phylogenetic analysis performed with *C. turuncatum* from different countries have shown that high of gene flow occur between geographically different populations of the pathogen (Katoch et al., 2016). It has been determined that the pathogen forms perithecium in sexual reproduction (Armstrong and Banniza, 2006). Gene flow and genetic diversity are higher in fungi that reproduce both sexually and asexually (Carbone and Kohn, 2004). For these reason, it can be said that the *C. turuncatum* has a high evolutionary potential.

In the network analysis of *C. truncatum* isolates from different hosts and different geographic regions, based on the ITS region, it has been reported that the H1 is more dominant among 11 haplotypes (Katoch et al., 2016). In the haplotype analysis performed using GAPDH, HIS3 and ITS regions, it was determined that *C. truncatum* has 27 haplotypes. These haplotypes formed three groups. Although there is a certain grouping in network analysis, no relationship was determined between the geographic distribution and haplotypes of this pathogen (Rogério et al., 2017). In this study, similar to the study of Katoch et al. (2016), the *C. truncatum* isolates reported on tomato were included in H1, the most dominant haplotype. Mutations in the ITS region have led to the emergence of new haplotypes. Since the gene flow is a high pathogen, a geographic relationship cannot be determined in network analysis performed based on single and multiple gene regions.

It has been stated that some wild lentil cultivars can be used in the development of resistant plants against *C. truncatum* races on Lentil (Tullu et al., 2006). Similarly, studies are needed to

determine the wild tomato variety(s) that can be used as a source of resistance against this pathogen on tomato.

In this study, it was determined that CT isolate infects tomato leaves and fruits. Conducting more extensive pathogenicity tests using other hosts of the pathogen will enable the determination of pathogenic variation in Turkey.

In cross inoculation tests with *C. truncatum* isolates obtained from papaya, pepper and physic nut, it was determined that the isolates infect the fruits and leaves of all three hosts. Although the most aggressive isolates were obtained from pepper; isolates, more aggressive on papaya and physic nut which alternative hosts, were also reported (Torres-Calzada et al., 2018). It has been hypothesized that the pathogen transitions from its main host pepper to its alternative host. The detection of more aggressive isolates of the pathogen on the alternative host shows that if the population of these aggressive isolates increases over time, the pathogen may cause significant economic losses on alternative hosts and become the main pathogen of these plants.

4. Conclusion

In this study, the CT isolate obtained from greenhouse tomato was identified as *C. truncatum* based on morphological and phylogenetic analysis. This isolate caused leaf and fruit infection on tomato. With this study, *C. truncatum* is reported for the first time on tomato in Turkey. Phylogenetic analysis and haplotype analysis were carried out using ITS region. Phylogenetic analysis showed that the closest species to *C. truncatum* was to be the *C. jasminigenum*. In the haplotype analysis a total of 8 haplotypes were identified. It has been determined that Turkish and other reference isolates reported on tomato were in the H1 which the most common haplotype. Isolates of the pathogen that may be more aggressive on alternative hosts have been reported. This pathogen can migrate to alternative hosts in Turkey, and the dominance of more aggressive isolates in the population may cause significant yield losses.

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