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Original scientific article

MOLECULAR TRACKING OF *Acidovorax citrulli*: UNVEILING PATHOGEN DYNAMICS AND BLOTCH DISEASE OUTBREAKS THROUGH SPECIFIC MARKERS

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Acidovorax citrulli (*Ac*), a gram-negative bacterium, is the causal agent of bacterial fruit blotch (BFB), which poses a significant threat to cucurbit crop production worldwide. Understanding the genetic diversity of *Ac* is crucial for identifying sources of resistance and implementing effective disease management strategies. In this study, we conducted the first genetic characterization of *Ac* strains collected in Turkey using Inter-Simple Sequence Repeat (ISSR) markers. These markers were selected based on repetitive domains mapped on the complete reference genome sequence of *Acidovorax citrulli* strain NWB SC196. The identity of the Turkish strains was confirmed through molecular (PCR) and serological (Immunofluorescence test and ELISA) methods, while the selected ISSRs, which exhibited similarity to flanked regions in the pathogen's whole genome sequence, were employed to assess the genetic diversity among *Ac* strains. We compared the profiles of Turkish strains with those of a collection of *Ac* strains from various countries, including the US, to explore a possible common origin. Specifically, we considered the dissemination of these strains through rootstocks used for grafted seedling production (*Cucurbita maxima* × *Cucurbita moschata*). The results demonstrated a shared

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genetic profile, suggesting a potential link between *Ac* strains collected in Turkey and foreign strains. The Mauve analysis, utilizing whole genome sequences of various *Ac* strains available in the NCBI database, displayed similar clustering patterns to those obtained using our selected molecular markers, confirming the discriminatory efficiency of our method. Based on the high discriminatory power of the selected markers, our proposed method offers a rapid and straightforward approach for genetic analysis of intraspecific variation and monitoring *Ac* gene flow across countries. The characterized strains and markers presented in this study serve as valuable resources and reference materials for further genetic investigations and tracking contamination sources associated with *Ac*.

Keywords: Acidovorax, bacterial blotch, *Cucurbitaceae,* genetic characterisation, molecular markers, whole genome comparison

INTRODUCTION

The genus *Acidovorax* contains species that show different pathogenic lifestyles on plants. Among these, *Acidovorax citrulli* (*Ac*), a gram-negative bacterium causing bacterial fruit blotch (BFB) of cucurbits, has been studied in detail (ROSENBERG *et al*., 2015). The disease was determined in the mid-1960s and then it became an important plant pathogen threatening crop production in the late 1980s due to large yield losses in commercial watermelon production fields at several locations in the United States (MAYNARD and HOPKINS, 1999). The *Cucurbitaceae* originated mainly from north-eastern Mexico and the southern United States (SMITH, 2006), and the rootstocks *Cucurbita maxima* × *Cucurbita moschata* have been widely used for grafted melon and watermelon production (KARAAĞAÇ and BALKAYA, 2013). Given that the disease is seed-borne, seeds are usually the primary source for BFB outbreaks, facilitating the *Ac* pervasive distribution through global seed trading. BFB diseases cause economic yield losses in cucurbit crops, including watermelon, melon, squash, pumpkin, and cucumber (BURDMAN and WALCOTT, 2012). Today, *Ac* is a serious plant pathogen affecting global cucurbit production, especially melon and watermelon. The great destructive damage of BFB and the lack of tools for managing the disease, including the unavailability of tolerant sources, is a serious threat for governmental regulatory agencies, but also the seed industry and commercial fruit producers (BURDMAN and WALCOTT, 2012; ZHAO and WALCOTT, 2018).

Ac strains have been categorised into two main groups (I and II) based on biochemical and genetic properties (FENG *et al*., 2009). Correspondingly, an additional third group was also identified which consists of only two strains weakly virulent pathogens on watermelon, melon, and squash seedlings (ECKSHTAIN-LEVI *et al*., 2014). Type III-secretion system playing role in the pathogenicity of *Ac* has been suggested as the reason for variable virulence of the strains (JOHNSON *et al*., 2011). Group I which comprises strains mostly isolated from melon and other non-watermelon cucurbits showed a range from moderately to highly aggressive levels of pathogenicity on various cucurbit crop species. Group II strains were the ones mainly isolated from watermelon, which shows high virulence, but are less virulent on other cucurbits (ZIVANOVIC and WALCOTT, 2017).

The symptoms of BFB include greasy-appearing, water-soaked dark, olive-green lesions or blotches that develop on the upper surface of fruit, which makes the fruit quality

unmarketable. The disease symptoms on the seedlings are water-soaked lesions on cotyledons and hypocotyl, which cause the collapse of the emerging seedlings. To distinguish the symptoms on leaves is not easy due to the different pathogenic behaviour of the strains, some of which do not lead to leaf fall, that this case causes the dissemination of inoculum sources. The stems, petioles and roots do not generally show disease symptoms (LATIN and HOPKINS, 1995). *Ac* was reported in the EPPO region (Turkey, Israel, Greece, Hungary, and Italy) and added to the EPPO A1 List (EPPO, 2021).

The *Ac* genetic profiles have been extensively studied due to their pathogenic variation on different hosts (SILVA *et al*., 2016). Several molecular markers were developed thanks to the continuous advances in molecular biology, which have revolutionised our understanding of the evolution and organisation of genomes that allowed detection and analysis of genetic variation (ADHIKARI *et al*., 2017). They are powerful tools due to their effective discrimination of genotypic and phenotypic variation (GROVER and SHARMA, 2016). Nevertheless, the molecular markers should be reliable and, generate highly polymorphic bands in a rapid, simple, and cost-effective way. There are available highly polymorphic, high-throughput and cost-effective markers, such as Simple Sequence Repeat (SSR) and Single Nucleotide Polymorphism (SNP). Inter-Simple Sequence Repeat (ISSR) is also a method involving the amplification of two identical SSR regions and they produce polymorphism by PCR (AGRAWAL and SHRIVASTAVA, 2014). This technique is a simple and reliable marker system which has been proved on different organisms, especially plants, with highly reproducible results and abundant polymorphisms. In previous studies, ISSR markers allowed cost-efficient detection and represented as a genetic diversity tool of bacterial pathogens such as *Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas citri* pv. *malvacearum* (BAYSAL *et al*., 2011; KUMAR *et al*., 2018).

In the present study, we aimed to characterise a wide collection of *Ac* strains collected in Turkey. The genetic diversity of Turkish and other countries *Ac* strains, including the US, was then compared. We have also tried establishing the genetic panel to investigate whether any pathogen transmission which can be tracked on the pathogens collected from the propagation material is possible among these countries.

MATERIALS AND METHODS

Cultural, nutritional, physiological, serological and molecular identification

The bacteria strains were previously isolated from different commercial grafted seedlings (years between 2009 and 2012), showing typical symptoms on the foliage of watermelon and melon. These were the strains belonging to the culture collection for the pathogens in Adana Biological Control Research Institute, and Mugla Sitki Kocman University, Molecular Microbiology Unit, Mugla. Pathogenicity tests were performed for Turkish strains (21) by artificial inoculation of bacterial suspension (10^8 CFU ml^{-1}) to the rind of mature watermelon fruits, according to MIRIK *et al*. (2006). Bacteria were grown in King's B medium at 28°C for 48 h and a sterilized toothpick was dipped into a bacterial solution and then stabbed into the growing point of 2-week-old watermelon seedlings (modified from EPPO Diagnostic Standard PM 7/127 (2016). After inoculation of fruits and seedlings, the plant materials' high humidity condition was maintained under polyethylene bags for 48 h at 25°C. As a negative control, sterilized phosphate-buffered saline (PBS) was inoculated into the seedlings and fruits. The

typical symptoms appeared five days after inoculation with dark green water-soaked lesions on the foliage part of the seedlings and fruits. Negative control, PBS-inoculated plants, did not show any typical symptoms of BFB. To confirm Koch postulate results caused by the bacterial infection, the bacteria were re-isolated from inoculated seedlings. The colonies were performed on King's B medium, then DNA isolation was made according to DE BOER and WARD (1995), subjected to species-specific PCR according to WALCOTT and GITAITIS (2000) using WFB 1 (sense): 5'- GACCAGCCACACTGGGAC-3' and WFB 2 (anti-sense): 5'-CTGCCGTACTCCAGCGAT -3' primers. PCR products was visualized on gel electrophoresis according to SAMBROOK *et al*. (1989).

Table 1. The list of Acidovorax citrulli strains used in this study.

No	Code of strain	Source	Origin	No	Code of strain	Source	Origin
1	AU2	Melon	Australia	30	Ac_1	Grafted Seedling	Turkey
2	1_00	Watermelon	USA	31	Ac_2	Grafted Seedling	Turkey
3	92_300 (ATCC29625)	Watermelon	Korea	32	Ac_3_1	Grafted Seedling	Turkey
4	92_301	Watermelon	USA	33	Ac_4	Grafted Seedling	Turkey
5	92_305	Watermelon	USA	34	Ac_5	Grafted Seedling	Turkey
6	94_21	Watermelon	USA	35	Ac_6	Grafted Seedling	Turkey
7	94_39	Watermelon	USA	36	Ac_9	Grafted Seedling	Turkey
8	94_48	Watermelon	USA	37	Ac_11	Grafted Seedling	Turkey
9	98_17	Pumpkin	USA	38	Ac_13	Grafted Seedling	Turkey
10	99_5	Melon	USA	39	Ac_14	Grafted Seedling	Turkey
11	200_23	Watermelon	USA	40	Ac_16	Grafted Seedling	Turkey
12	200_30	Melon	USA	41	Ac_17	Grafted Seedling	Turkey
13	201_12	Watermelon	USA	42	Ac_18	Grafted Seedling	Turkey
14	202_66	Melon	Israel	43	Ac_19	Grafted Seedling	Turkey
15	203_57	Watermelon	USA	44	Ac_20	Grafted Seedling	Turkey
16	204_8	Watermelon	USA	45	Ac_21	Grafted Seedling	Turkey
17	205_22	Watermelon	USA	46	Ac_25	Grafted Seedling	Turkey
18	206_1	Watermelon	USA	47	Ac_27	Grafted Seedling	Turkey
19	206_2	Watermelon	USA	48	Ac_28	Grafted Seedling	Turkey
20	206_26	Watermelon	USA	49	Ac_K1	Grafted Seedling	Turkey
21	206_84	Unknown	China	50	Ac_K2	Grafted Seedling	Turkey
22	206_95	Unknown	China				
23	207_42	Watermelon	USA				
24	208_20	Watermelon	USA				
25	210_13	Unknown	USA				
26	210_7	Unknown	USA				
27	211_29	Unknown	Unknown				
28	211_36	Watermelon	USA				
29	211_76	Melon	Israel				

Nutritional and physiological tests for *Ac* were carried out on our strains using the methods suggested by SCHAAD *et al.* (2001). Each culture was tested with conventional biochemical tests, including Gram staining, fluorescent pigment on King's B medium, growth at 41ºC, gelatin hydrolysis, reduction of nitrate, levan production, catalase, oxidase, arginine dihydrolase, glucose metabolism, and utilisation of sucrose, sorbitol and mannitol. Additionally, strains were also tested in view of hypersensitive response (HR) formation on six-week-old

tobacco (*Nicotiana benthamiana*) leaves and softening/rot on potato (SCHAAD *et al*., 2001). The serological characterisation was carried out by ELISA assay using *Ac*-specific antibody (Cat. No. SRA 14800, Loewe) and Immunofluorescence test according to manufacturers' instructions manual (Cat. No.07354, Loewe). Subsequently, 29 strains from several countries, kindly provided as DNA of the strains by Ronald R. Walcott (Prof. in University of Georgia, Athens, GA 30602), were added to the pathogen collection involving 21 strains from Turkey for the genetic characterization (Table 1).

Genetic marker studies on Ac strains

Total genomic DNA was extracted from 50 *Ac* strains (Table 1) collected from different cultivation fields in southern Turkey (21 strains, named Pool-1) and from different countries (DNA of 29 strains, named Pool-2) using a commercial DNA isolation kit (Promega, Wizard Genomic DNA Purification Kit, Madison, US), according to the manufacturer's instructions. The purity and quantity of the DNA extracts were assessed with a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). Starting from an ISSR panel previously used for bacterial characterisation (BAYSAL *et al*., 2011; KUMAR *et al*., 2018), we selected 12 markers showing *in silico* high homology to the complete reference genome sequence belonging to *Acidovorax citrulli* strain NWB SC196, considering their genome-specific repetitive domain regions. Seven out of twelve markers showed high polymorphism profiles and were used to characterize the *Ac* collection (Table S1). The final volume of 15 µl DNA was used as previously suggested by BAYSAL *et al*. (2011). The amplicons were separated using 1.5% agarose gel electrophoresis and stained with ethidium bromide. The gel with 15 µl reaction products was run for 5 h at 110 V. The visualization of the gel was done under ultraviolet light. The reproducibility of the DNA profiles was confirmed by repeating the PCR amplifications 3 times.

Data analysis

Each amplified band was a representative character or locus, and the polymorphic bands were analysed according to their visual scores, whether it is absent (0) or present (1). The bands showing consistent amplifications were considered, and weak/smeared bands were discarded using FAMD software (SCHLÜTER and HARRIS, 2006). The percentage of polymorphism (Pp) obtained with DNA amplification was determined according to the number of polymorphic loci relative to the total number of loci, regardless of allele frequency. Shannon's index (*I*) and Nei's genetic diversity (*He*) were also evaluated for each group by R/Vegan and R/poppr packages, respectively. The genetic distances across collections studied were evaluated through phylogenetic and Principal Coordinates (PCoA) analysis. Nei's distances (NEI, 1972) and Neighbour joining (NJ) algorithm were applied for cluster analysis using the R Core Team (2020). Bootstrap analysis was performed based on 1000 re-samplings. In addition, a heatmap based on the genetic diversity among strains from Turkey and all other strains was developed using the R/ggplot2 package. Finally, to infer the best population structuring of the studied collection, both Discriminant Analysis of Principal Components (DPAC) and structure analysis were carried out using the R/adegenet (JOMBART, 2008) and STRUCTURE software (PRITCHARD *et al*., 2000), respectively. In the STRUCTURE analysis, the most likely number of genetic groups (*K*) was performed following the procedure of EVANNO *et al*. (2005), which proposed an

ad hoc statistic, ΔK. The STRUCTURE analysis was carried out following the procedures reported by GARFÌ *et al*. (2013).

Genomic comparison studies

A total of 8 *Ac* (Acronym) whole genomic sequences from various countries available in the NCBI (National Center for Biotechnology Information) database were retrieved (Table 2). These sequences were then analysed using Mauve software from the Darling lab (DARLING *et al*., 2010). The objective of this study was to estimate the variation in the gene pool, aiming to identify common genomic regions that represent different subtype diversities based on the origin of the selected whole genome sequences of the strains. Additionally, collinear block analysis was conducted on these 8 genomes to observe a set of loci in different species. These loci, located on the chromosome, were investigated for conservation with the same order or placement in the genome.

Table 2. The list of Acidovorax citrulli strains of which sequences were retrieved from NCBI.

No	Code of strain	Source	Origin	Accession Nr.
1	NWB SC196 (Reference sequence)	Watermelon	South Korea	CP042323.1
2	NWB SC107	Watermelon	South Korea	CP042303.1
3	NWB SC074	Watermelon	South Korea	CP042302.1
4	HPP21-9-4B	Watermelon	South Korea	CP086060.1
5	HPP21-3-3B	Watermelon	South Korea	CP086023.1
6	KACC17005	Watermelon	South Korea	CP023687.1
7	AAC00-1	Unknown	USA	CP000512.1
8	M6	Melon	Israel	NZ_CP029373.1

RESULTS

Cultural, nutritional, physiological, serological and molecular identification

All strains showed general characteristic properties of the species *Ac* as Gram-negative, obligate aerobes, and did not produce fluorescent pigment on King's B medium (Figure S1). Bacterial colonies appeared typical shape as beige-tan coloured, round and non-mucoid. The strains induced HR on tobacco leaves (Figure S2) and did not produce potato soft rot. Regarding the remaining tests, the results were positive for oxidase (Figure S3) and growth at 41ºC. The strains used ethanol and D-glucose as carbon sources, and reduction of nitrate, mannitol, sucrose, arginine, and sorbitol were negative.

The *Ac* strains showing pathogenicity was confirmed with Koch postulates on watermelon seedlings (Figure S4). All strains of Turkey showed typical symptoms of the pathogen on watermelon fruits (Figure S5). We did not apply Koch postulates on strains obtained from collection strains of Prof. Ronald R. Walcott due to sharing them as DNA material cause of official restrictions in transferring pathogen strains between countries. In accordance with our previous results, both serological (ELISA) and molecular (PCR) studies confirmed that all 21 strains isolated in Turkey belongs to *Ac* (Table S2, Figure S6, Figure S7).

Genetic marker studies on Ac strains and Data analysis

Tested ISSR markers (Table S1) showed remarkable polymorphic bands in accordance with their homology to the reference *Ac* strain (NWB SC196) genome available in the NCBI database (Table S3). Seven out of twelve selected primers, mapped on the *Ac* genome, amplified generating 63 well-resolved bands in the whole collection analysed (50 *Ac* strains), all of which were polymorphic. Amplicons sizes ranged from 150 to 2000 bp. Of the screened primers used to characterise *Ac* strains of interest, the most polymorphic loci were obtained from the ISSR primers ENEA34 [(ACC)6 CC] [polymorphism ratio of the markers (prm) 100%], while the least was the primer ISSR12+12b [(GA)8 YT] (prm 67%). The two groups (Pool-1 and Pool-2) of *Ac* exhibited the same pattern of low genetic diversity: the mean values of the Shannon index (*I*) and Nei's gene diversity (*He*) for the strains collected in Pool-1 were 0.36 and 0.24, respectively, while the strains coming from Pool-2 showed $I = 0.24$ and $He = 0.21$ (Table S4). Although the rate of genetic diversity was low, the NJ cluster analysis, based on the pairwise Nei's genetic distance matrix, among the *Ac* genotypes collected in Turkey (Pool-1) was able to highlight three main clusters (Figure 1a). Phylogenetic analysis was confirmed by PCoA, underlining a clear separation among the strains belonging to the three clusters (Figure 1b).

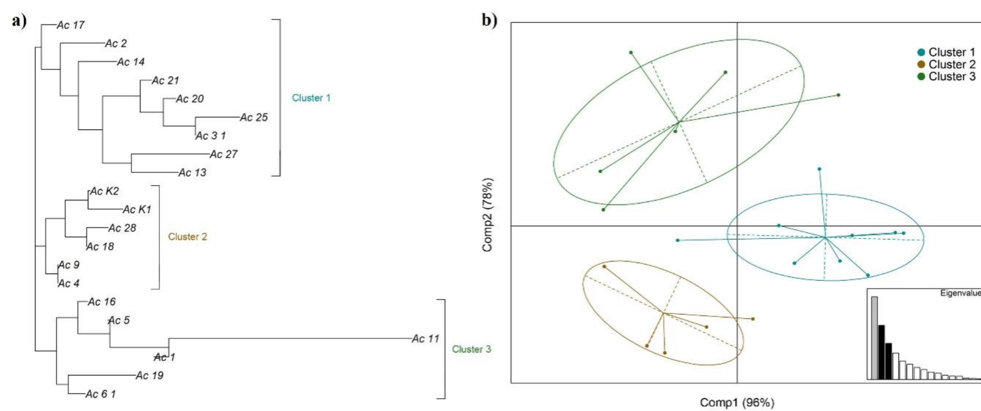

Figure 1. Phylogenetic (**a**) and PCoA (**b**) analysis of *Acidovorax citrulli* strains collected in Turkey by using ISSR markers.

To further characterize the genetic background of strains belonging to Turkey's group, we compared them to a wide collection of genotypes collected in several countries (Pool-2; Table 1). Cluster analysis split the strains in three main branches (Figure 2). Cluster A shared strains from Pool-1 (51%; 12) and the Pool-2 (49%; 11). Similarly, in cluster B, 57% (8) of strains were from Pool-1 and 43% (6) from Pool-2. In contrast, the last cluster (C) was mainly represented (92%)

by strains from Pool-2. PCoA analysis confirmed the genetic relationships shown by phylogenetic analysis (Figure S8). Indeed, some strains belonging to the two groups (Pool-1 and 2) represent two genetic groups with some overlap while the other side of panel which shows out of intersection show the strains with the different origins (Figure S8).

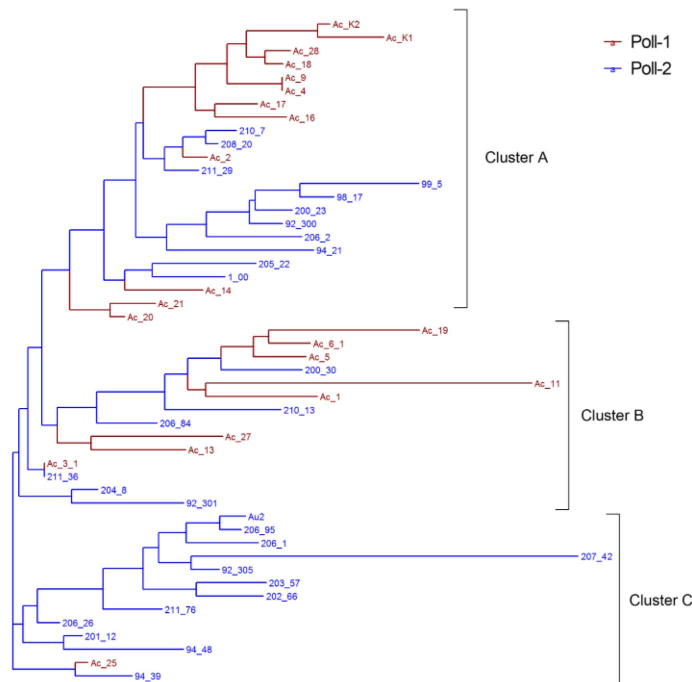

Figure 2. Neighbour joining (NJ) tree based on genetic diversity among *Acidovorax citrulli* strains belonging to the analysed whole panel. In red and blue, the strains belonging to Turkey (Pool-1) and other countries (Pool-2), respectively.

The heatmap developed through Nei's genetic distance (Figure 3a) highlighted a low genetic diversity across the collection studied, with 207_42, Ac_11 and Ac_19 are the most different strains. This evidence was confirmed by both DAPC (Figure 3b) and STRUCTURE (Figure 3c) analysis, showing two and three main gene pools (*K*), respectively, but with a common genetic background. DAPC analysis shows an overlap between pool 1 and pool 2, which could be attributed as the admixture and third gene pool consistent with STRUCTURE. The strains collected in Turkey (Pool-1) were characterized primarily by K2 (pool dark blue), while K1 (light blue) was mainly represented in the strains belonging to Pool-2. K3 (orange) was

common between the two groups (Figure 3c, Table S5). However, a high rate of admixture (42%) was observed (Table S5).

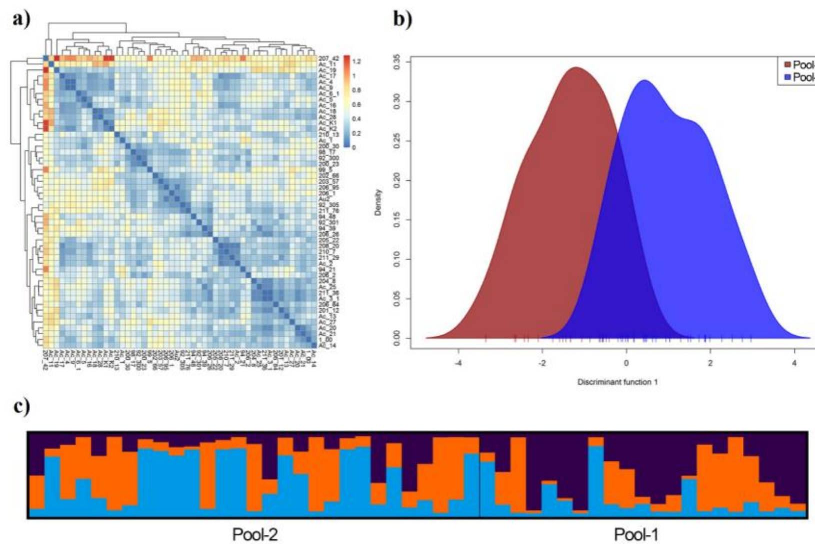

Figure 3. Genetic distance (**a**) and structure population evaluation by DAPC (**b**) and STRUCTURE (**c**) of Turkey (Pool-1) and other countries' strains (Pool-2).

Genomic comparison studies

The Mauve analysis revealed a genomic comparison among 8 different *Ac* strains, highlighting the presence of common regions in the bacterial genome (Figure 4)(Supplementary data 1). Furthermore, the output from the collinear genome comparison analysis displayed 3 main clusters (A, B, C) with sub-clusters that exhibited variation based on the origins of the subtypes (Figure 5)(Supplementary data 1). Similar clustering patterns were observed when using our selected molecular markers to test *Ac* strains. Subtypes with the same origin were grouped within the same sub-cluster, which consisted of 3 main branches (Figure 2 and 5). These findings indicate that *Ac* strains exhibit similar genetic diversity, as evidenced by both molecular markers and genomic comparison, in relation to their geographical distribution. Additionally, the phylogenetic tree constructed by NCBI demonstrated the presence of 3 main groups (A, B, C) in Figure 6, further validating our results.

These results suggest that our markers allow for the discrimination of *Ac* strains at the intra-specific level, although there is still a wide range of genetic variation to explore within the common regions of the *Ac* genome.

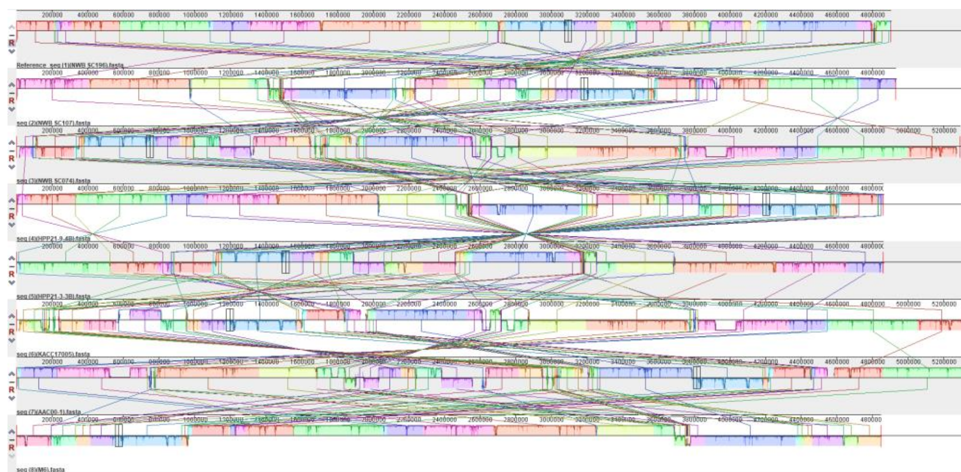

Figure 4. Mauve analysis output of 8 different *Acidovorax citrulli* strains with available whole genome sequences which retrieved from NCBI.

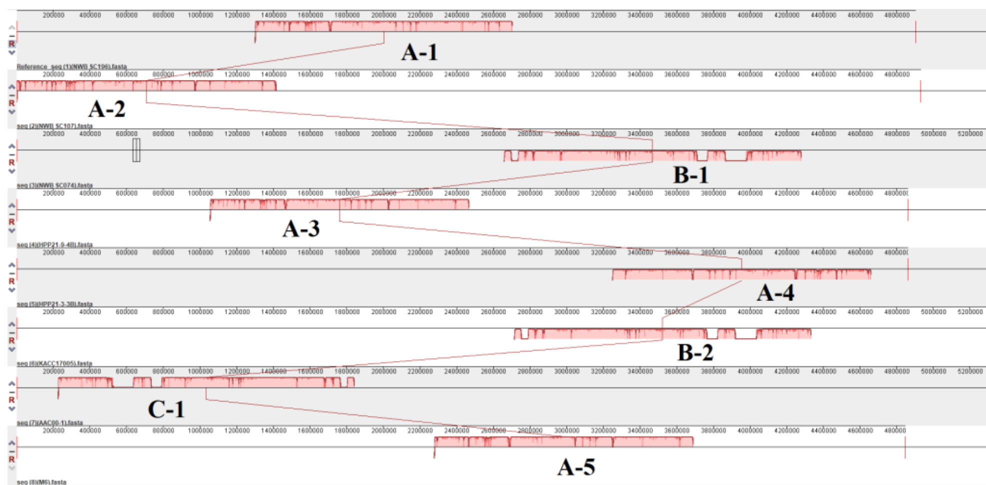

Figure 5. Collinear block analyses output based on Mauve alignment which shows rearrangement of genes existing inverted repeat and small-single copy. The sequence given on the top of the figure (A-1) is reference sequence retrieved from NCBI (Accession Nr: CP042323.1). The figure depicted formation of 3 various genome landscape.

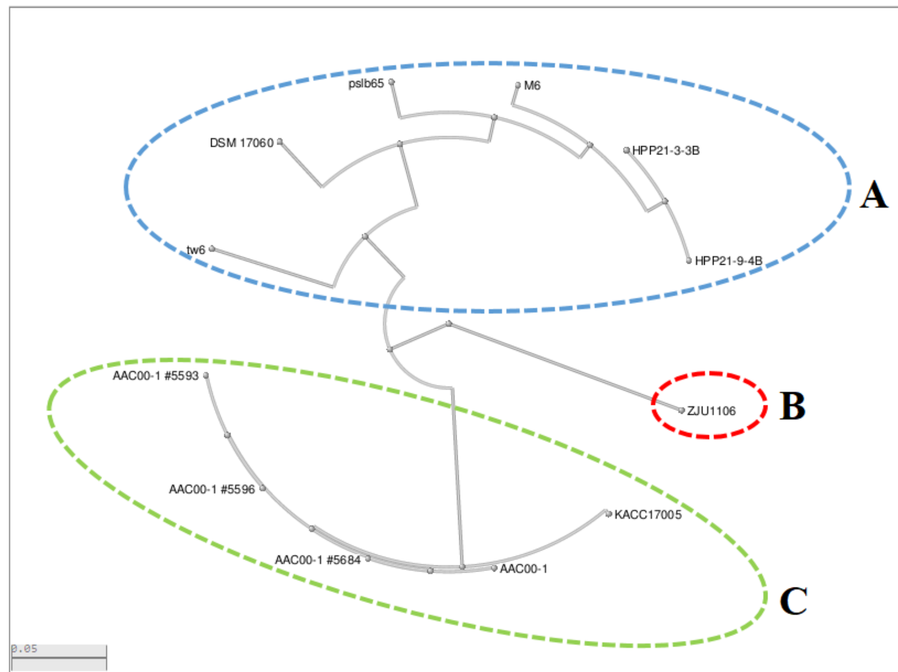

Figure 6. Phylogenetic tree constructed by NCBI shows existence of 3 main groups (A, B, C) (https://www.ncbi.nlm.nih.gov/genome/?term=txid80869[Organism:exp]).

DISCUSSION

Bacterial fruit blotch (BFB) disease causes yield losses in melon and watermelon, affecting a wide range of hosts within the *Cucurbitaceae* family (BURDMAN and WALCOTT, 2012). Previous studies by BURDMAN *et al*. (2005) revealed the existence of two distinct groups within *Acidovorax citrulli* (*Ac*), aligning with the findings that a specific strain is associated with watermelon (Group II) while other strains are linked to non-watermelon cucurbits (Group I). In this study, the Turkish strains were confirmed to be *Ac* strains through cultural, nutritional, physiological, serological, and molecular characterization using species-specific PCR analysis, as described by WALCOTT and GITAITIS (2000).

Our genetic studies demonstrated intraspecific genetic variation based on genome fragments detected through molecular markers with electrophoresis. Analysis of *Ac* strains using ISSR markers revealed significant levels of polymorphism (Figure 1, 2, and 3). The data indicated a genetic linkage between two distinct strain pools: Pool-1 (Turkish strains) and Pool-2 (widespread strains globally). To our knowledge, the selected ISSR markers utilized in this study successfully distinguished among *Ac* strains. Given that this pathogen is listed in the EPPO A1 List (2021), the findings related to genetic diversity obtained with the selected markers are

significant for monitoring and preventing further spread of the bacterium across cultivation regions within countries. Watermelon and melon are economically important crops worldwide, including Turkey. Thus, after investigating the selected markers that produced polymorphic bands in the extensive *Ac* collection, we aimed to explore the reasons for the distinct genetic profiles observed among *Ac* strains based on their origins.

Interestingly, the ISSR marker sequences generated fragments that matched with various regions in the entire genome of the pathogen, based on the *Ac* reference genome sequence (strain NWB SC196). This may explain the amplification of the markers (Table S3) and the resulting polymorphism associated with genetic diversity. Consequently, we propose that these markers could be employed for tracking pathogen discrimination and dissemination within regions, particularly in cases of possible transmission through infected propagation materials. Similar to previous studies on *C. michiganensis* subsp. *michiganensis*, the selected ISSR markers have proven effective in discriminating pathogen strains based on regions and inoculum sources (BAYSAL *et al*., 2011). The method suggested by BAYSAL *et al*. (2011) has also been integrated with an automated PCR system, enabling rapid detection of genetic diversity among *C. michiganensis* subsp. *michiganensis* strains (BAEYEN *et al*., 2016). Therefore, the panel system presented here could potentially be applied to develop an automated system for *Acidovorax* strains as well.

Although most of the ISSR markers were mainly used for plant genetics studies, with the help of whole genome sequences, new optimised ISSR markers can be designed and used to discriminate bacterial variability. ISSR regions are present among SSR regions that has been suggested to have a role in enhancing antigenic variance of the pathogen related to host response (MRÁZEK *et al*., 2007). In our study, detected genetic diversity among collected strains can be associated with antigenic variance of the strains depending on host preference and geographical differences. The ISSR markers employed in this study could be considered for tracking of the strains but also antigenic variance comparison among the origin of the strains. Because the rare presence of SSR regions, and ISSR regions depending on the dissemination of SSR region, in prokaryotes has a potential to deactivate or alter the genes playing role in virulence as suggested by ROCHA and BLANCHARD (2002). Our ISSR markers could integrate into genomic regions encoding proteins alternating patterns related to host-microbe interaction, which varies depending on pathogenic origin and evolutionary process (MRÁZEK, 2006). Our study pointed to possible functional differences among *Acidovorax* strains according to possible strong mutation pressures that could be correlated with a genetic polymorphism on *Ac* strains and a variety of possible selective forces occurring in the nature (FIELD and WILLS, 1998).

Microsatellites are extremely high mutable regions associated with the point mutations in coding and non-coding genes. Their mutation ratio ranges from 10^{-6} to 10^{-2} events per locus per microbial generation. These rates affect the variance of features in next generations (JARNE and LAGODA, 1996). As ISSR is a region related to microsatellites, the selected primers could help to estimate mutable regions in the genome of pathogenic microorganisms. The diversity obtained by ISSR markers could be considered as the panel related to the diversification of the strains according to mutable regions.

Recently, *Ac* has disseminated in many regions of the world through the cultivation of contaminated commercial cucurbit seeds and seedlings. Despite the economic importance of the

disease, there is still much to learn about *Ac* pathogenesis. The genome sequences of many strains are now available in NCBI GenBank as well as the optimisation of molecular manipulation and inoculation methods have prompted fundamental studies and allowed advances towards to understand the *Ac* pathogenicity more in detail. Even though multi locus sequence typing provides genetic diversity data by allelic differentiation considering at least seven housekeeping genes which is a replicable and reliable method for evolutionary characterisation of bacterial pathogens (FENG *et al*., 2009), it does not ensure intraspecific variation like ISSR markers.

Initially, *Ac* strains were thought to comprise a homogeneous population, but the studies showed a genetic variability within *Ac* strains (BURDMAN and WALCOTT, 2012). Drastically, a previous study has suggested no discrimination with REP, ERIC and BOX PCR (MELO *et al*., 2014) according to repetitive sequence depending on geographical or host origin relationships in contrast to our findings due to the higher efficiency of our selected ISSR markers.

On the variety of the strains and their pathogenicity, the studies on ATCC29625 type strain obtained from watermelon indicated that some strains do not cause hypersensitive response (HR) in tobacco, and they have less virulence on watermelon compared to other tested strains on the same host (SOMODI *et al*., 1991). Furthermore, the strains causing BFB in cantaloupe melon had appeared to have a remarkably virulent attitude on melon but less on *Cucumis myriocarpus* Naudin in Queensland, Australia (O'BRIEN and MARTIN, 1999). This prominent diversity, confirmed by WALCOTT *et al*. (2004), has suggested the existence of two groups identified in a wide range of cucurbitaceous species. Strains belonging to Group I have been classified as moderately virulent on cantaloupe melon, pumpkin, and zucchini. Group II has been more virulent on watermelon than other cucurbit plants in the US (WALCOTT *et al*., 2004). Another study has also acknowledged the diversity involving Group I with the studies based on Repetitive Sequence-based PCR (rep-PCR) with the low variability that confirmed the results of substrate utilisation analyses and the 23S rDNA partial sequence analysis in Brazil (SILVA *et al*., 2016). The existence of two groups was also announced in Israel (BURDMAN *et al*., 2005). In our study, the marker employed (ACC)6 CC) with 100% polymorphism on collected strains showed accordance with the findings of ZIVANOVIC and WALCOTT (2017) due to the discriminatory role of the putative type III secretion effector gene, Aave_2166. This gene region matched with the sequence of our tested ISSR marker (ACC)6 CC), showing a higher polymorphism ratio (Table S3). Additionally, whole genome comparison among the strains have indicated existence of 3 main clusters (A, B, C) which could be associated with the group formation of *Ac* strains (Figure 5). In our study, the similar pool formation using the selected molecular markers also incorporated with three different groups which has proved the efficiency of the molecular markers (Figure 2). Also, we assume that the biotic and abiotic stress could cause spontaneous mutations which enforces the genomic rearrangement on some specific regions in bacterial genome (Figure 5). Collinear analysis carried out on 8 available genomes revealed the existence of 3 main groups in *Ac* strains, which is accordance with the results obtained with tested markers. These findings suggested to have remarkable discriminative potential of our markers employed in this study.

Our findings suggested that we were able to discriminate these strains by selected markers tested in this study. *Ac* strains isolated from various locations had diversity in view of substrate

utilisation profiles, which were in accordance with their pathogenicity in Australia and the United States (WALCOTT *et al*., 2004). The differences in virulence of *Ac* strains were also observed within tested 41 strains on melon and watermelon seedlings, plants, and fruits in Brazil (OLIVEIRA *et al*., 2007). We have also determined the genetic diversity at the intraspecific level within the same gene pools depending on their geographical location (Table 1, Figure 2). Given the scarce studies carried out on the genetic diversity of Turkish *Ac* strains isolated from melon and watermelon, new studies are necessary to understand the intraspecific genetic diversity of new pathogenic strains. Our study aimed to contribute for improving molecular markers to determine intraspecific variation of *Ac*. Additionally, the rootstocks (*C. maxima* × *C. moschata*) have been widely used for grafted seedlings in Turkey. The findings indicated that rootstock-seeds should also be tested for *Ac* infections to prevent transmission of the pathogen by propagation materials. We believe that our selected markers can be used for tracking *Ac* infection and transmission sources for further studies. Furthermore, the selected ISSR markers could contribute for improving SCAR markers and SYBR Green labelled real-time PCR probes for determining intraspecific variation within *Acidovorax citrulli* strains.

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MOLEKULARNO PRAĆENJE *Acidovorax citrulli*: OTKRIVANJE DINAMIKA PATOGENA I IZBIJANJA BOLESTI PREKO SPECIFIČNIH MARKERA

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Izvod

Acidovorax citrulli (*Ac*), gram-negativna bakterija, je uzročnik bakterijske mrlje voća (BFB), koja predstavlja značajnu pretnju za proizvodnju useva tikvice širom sveta. Razumevanje genetskog diverziteta *Ac* je ključno za identifikaciju izvora rezistencije i primenu efikasnih strategija upravljanja bolestima. U ovoj studiji, sproveli smo prvu genetsku karakterizaciju *Ac* sojeva prikupljenih u Turskoj koristeći ISSR. Ovi markeri su odabrani na osnovu repetitivnih domena mapiranih na kompletnoj referentnoj sekvenci genoma *Acidovorak citrulli* soja NVB SC196. Identitet turskih sojeva je potvrđen molekularnim (PCR) i serološkim (test imunofluorescencije i ELISA) metodama, dok su odabrani ISSR, koji su pokazali sličnost sa bočnim regionima u celoj sekvenci genoma patogena, korišćeni za procenu genetske raznolikosti među *Ac* sojeva. Uporedili smo profile turskih sojeva sa onima iz kolekcije *Ac* sojeva iz različitih zemalja, uključujući SAD, da bismo istražili moguće zajedničko poreklo. Konkretno, razmatrali smo širenje ovih sojeva kroz podloge koje se koriste za proizvodnju kalemljenih sadnica (*Cucurbita maxima* × *Cucurbita moschata*). Rezultati su pokazali zajednički genetski profil, što ukazuje na potencijalnu vezu između *Ac* sojeva prikupljenih u Turskoj i stranih sojeva. Mauve analiza, koristeći čitave sekvence genoma različitih *Ac* sojeva dostupnih u bazi podataka NCBI, pokazala je slične obrasce grupisanja onima dobijenim korišćenjem naših odabranih molekularnih markera, potvrđujući diskriminatornu efikasnost naše metode. Na osnovu velike diskriminatorne moći odabranih markera, naš predloženi metod nudi brz i direktan pristup za genetsku analizu intraspecifičnih varijacija i praćenje protoka *Ac* gena u zemljama. Karakterizovani sojevi i markeri predstavljeni u ovoj studiji služe kao vredni resursi i referentni materijali za dalja genetska istraživanja i praćenje izvora kontaminacije povezanih sa *Ac*.

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