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Analysis of intraspecific genetic diversity in *Acidovorax citrulli* causing bacterial fruit blotch on cucurbits in Korea

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Abstract

Bacterial fruit blotch (BFB) caused by *Acidovorax citrulli* is a devastating disease found in many cucurbits cultivation fields. The genetic diversity for 29 strains of *A. citrulli* collected from various cucurbits in South Korea was determined by DNA fingerprinting with a pathogenicity test, multi locus analysis, Rep-PCR (repetitive sequence polymerase chain reaction), and URP (universal rice primers) PCR bands. Two distinct groups (Korean Clonal Complex, KCC1 and KCC2) in the population were identified based on group specific genetic variation in the multi locus phylogeny using six conserved loci and showed a very high similarity with DNA sequences for representative foreign groups [the group I (CC1-1 type) and the group II (CC2-5 type)] widely distributed worldwide, respectively. Additionally, in the case of *phaC*, a new genotype was found within each Korean group. The KCC1 was more heterogeneous compared to the KCC2. The KCC1 recovered mainly from melons and watermelons (ratio of 6 : 3) and 15 of the 20 KCC2 strains recovered from watermelons were dominant in the pathogen population. Accordingly, this study found that two distinct groups of differentiated *A. citrulli* exist in South Korea, genetically very similar to representative foreign groups, with a new genotype in each group resulting in their genetic diversity.

Keywords: *Acidovorax citrulli*, bacterial fruit blotch, genetic diversity, multi-locus, Rep-PCR

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Introduction

Bacterial fruit blotch (BFB) caused by *Acidovorax citrulli* (Schaad et al., 1978), is a devastating disease of many cucurbitaceae hosts (Burdman and Walcott, 2012). Since a highly aggressive strain was first reported from commercial watermelons at USA in 1989 (Somodi et al., 1991), BFB outbreaks

have occurred throughout the world. In Korea, the pathogen was first recovered from commercial watermelons at Cheonbuk province in 1991 (Song et al., 1991) and has since been reported from other cucurbit plants (Song et al., 2015). Like other bacterial diseases, use of chemical control against BFB is a little effective. The most effective strategy was known to use resistance cultivars (Wechter et al., 2011), the great genetic diversity of *A. citrulli* causes resistant cultivars to act restrictedly. Thus, the analysis information on the genetic diversity of *A. citrulli* containing the relationship within population and host's geographical origin can provide a very useful data base for developing resistant cultivars and for effective control strategy of the disease. Recently, many researchers have studied the genetic diversity of *A. citrulli* population by physiological, phenotypic characteristics, serology, biochemical reaction and pathogenicity tests (Walcott et al., 2004; Melo et al., 2014). Additionally, Song et al. (2015) also reported the existence of two groups in Korean *A. citrulli* population. However, these studies couldn't give enough information to understand genetic characteristics of Korean population. Therefore, the current study was tried to clarify the intraspecific genetic diversity within Korean *A. citrulli* population through multi-locus, repetitive-sequence-polymerase chain reaction (Rep-PCR) and universal rice primers (URP)-PCR analysis in order to obtain reliable data for developing resistance cultivars and the integrated control of bacterial fruit blotch in Korea.

Materials and Methods

Bacteria strains and DNA extraction

A total of 29 *A. citrulli* strains were collected from seeds, fruits, leaves and stems of melon, pumpkin, cucumber and watermelon plants in the cultivation fields of Korea from 2011 to 2014 (Table 1). All of these bacteria were stored in 15% sterile glycerol at - 80℃. The identification of all strains were performed through pathogenicity test by inoculation on watermelon seedlings and 16s rDNA sequencing (Song et al., 2015). Bacterial DNA was extracted using the DNeasy Tissue Kit (Qiagen Inc., USA).

Multi-locus analysis

For the multi-locus analysis, DNA sequences of six housekeeping genes; *adk* (437 bp), *glyA* (563 bp), *gltA* (487 bp), *pilT* (405 bp) obtained from our previous work (Song et al., 2015) and *ugpB* (452 bp) and *phaC* (479 bp) were selected and PCR products were performed using Primers of *ugpB* (ugpB1 & ugpB2) and *phaC* (phaCF & phaCR). Each PCR reaction was performed by mixture (Solgent Co., Ltd., Korea) under the PCR conditions described by Kang et al. (2002). All of the PCR amplicons were sequenced (Macrogen Ltd., Korea). The sequences were visualized and aligned with MEGA5 (Tamura et al., 2011) and also compared with foreign *A. citrulli* strains (ATCC29625; group I and 30002; group II). A maximum likelihood tree was constructed with the concatenated dataset of the four loci (*gltA, pilT, ugpB,* and *phaC*) as the DNA sequences for five loci (a*dk, gltA, glyA, pilT, ugpB*) of all Korean clonal complex (KCC)1 and KCC2 strains of *A. citrulli* were identical according to the group. *A. avenae* subsp. *cattleyae* were served as outgroups.

Rep-PCR and URP-PCR

DNA fingerprinting was conducted using Rep-PCR primers; ERIC1R/ERIC2 and BOXA1R (Louws et al., 1994) and URP-PCR primers; URP2, URP5, URP6, and URP8 from Kang et al. (2002). Conditions for PCR amplification reactions

were performed as described by Kang et al. (2002). RAPD bands were scored from the agarose gel and recorded as present (1) or absent (0) and assembled into a data matrix. The combined six results were analyzed with NTSYS (Exter Biological Software), and dendrograms were generated using the unweighted pair group method with average (UPGMA).

Table 1. Host, geographic origin and multi-locus genotypes of *Acidovorax citrulli* strains isolated from cucurbit cultivation fields in Korea.

Strain No.	Year	Hosts	Geographic origin	Multi-locus genotype		Path ogenicity[w]	References
				Group	phaC		
11246	2012	melon	Goksung, Jeonnam	KCC1	G	+	Song et al., 2015
11247	2012	melon	Goksung, Jeonnam	KCC1	A	+	〃
11248	2012	melon	Goksung, Jeonnam	KCC1	G	+	〃
14194	2014	melon	Anseong, Gyeonggi	KCC1	G	+	〃
14201	2014	melon	Gwangju City	KCC1	A	+	〃
14202	2014	melon	Anseong, Gyeonggi	KCC1	G	+	〃
12091	2012	watermelon	Buyeo, Chungnam	KCC1	A	+	〃
12158	2012	watermelon	Nonsan , Chungnam	KCC1	G	+	〃
14222	2014	watermelon	Yicheon, Gyeonggi	KCC1	A	+	〃
11171	2011	cucumber	Nonsan , Chungnam	KCC2	G	+	〃
12170	2012	cucumber	Nonsan , Chungnam	KCC2	G	+	〃
13217	2013	cucumber	Buyeo, Chungnam	KCC2	G	+	〃
13255	2013	cucumber	Youngam, Jeonnam	KCC2	G	+	〃
14236	2014	melon	Goksung, Jeonnam	KCC2	G	+	〃
17913	2012	pumpkin seed	Busan City	KCC2	G	+	〃
11147	2011	watermelon	Gochang, Jeonbuk	KCC2	A	+	〃
11162	2011	watermelon	Buyeo, Chungnam	KCC2	G	+	〃
11163	2011	watermelon	Buyeo, Chungnam	KCC2	G	+	〃
11164	2011	watermelon	Nonsan , Chungnam	KCC2	G	+	〃
11165	2011	watermelon	Nonsan , Chungnam	KCC2	G	+	〃
11201	2012	watermelon	Jinju, Gyeongnam	KCC2	A	+	〃
11251	2012	watermelon	Suwon, Gyeonggi	KCC2	A	+	〃
11259	2012	watermelon	Hamyang, Gyeongnam	KCC2	G	+	〃
12089	2012	watermelon	Buyeo, Chungnam	KCC2	G	+	〃
12090	2012	watermelon	Buyeo, Chungnam	KCC2	G	+	〃
13034	2013	watermelon	Anseong, Gyeonggi	KCC2	A	+	〃
13211	2013	watermelon	Youngam, Jeonnam	KCC2	G	+	〃
17000	2012	watermelon	Buyeo, Chungnam	KCC2	G	+	〃
17912	2012	watermelon	Andong , Gyeongbuk	KCC2	A	+	Willems et al., 1992
29625[x]	1978	watermelon	USA, Georgia	KCC1	nd	nd	Feng et al., 2009
19822[y]	1997	rice	Japan	OG	nd	nd	Schaada et al., 2008
33619[z]	nd	Orchid	USA	OG	nd	nd	

KCC, Korean clonal complex; OG, Outgroup; A, adenine; G, guanine; nd, not determined.
[w] All strains were shown virulence activities.
[x] ATCC29625 Multilocus sequence typing (MLST) clonal complex (CC) and sequence type assignments.
[y]ATCC19822 *A. avenae* subsp. *avenae*.
[z]ATCC33619 *A. avenae* subsp. *cattleyae.*

Results and Discussion

Multi-locus relationship analysis

Upon multi-locus relationship analysis using 1,823 bp of the total base sequence of the genes which included *gltA, pilT, ugpB,* and *phaC, A. citrulli* were classified into two groups; KCC1 and KCC2 within the same clade apart from *A. avenae* subsp. *avenae* and *A.* subsp. *cattaleyae* (Fig. 1).

In this study, the allelic profile analysis using the base sequence of the genes including *adk, gltA, glyA, pilT, ugpB* except

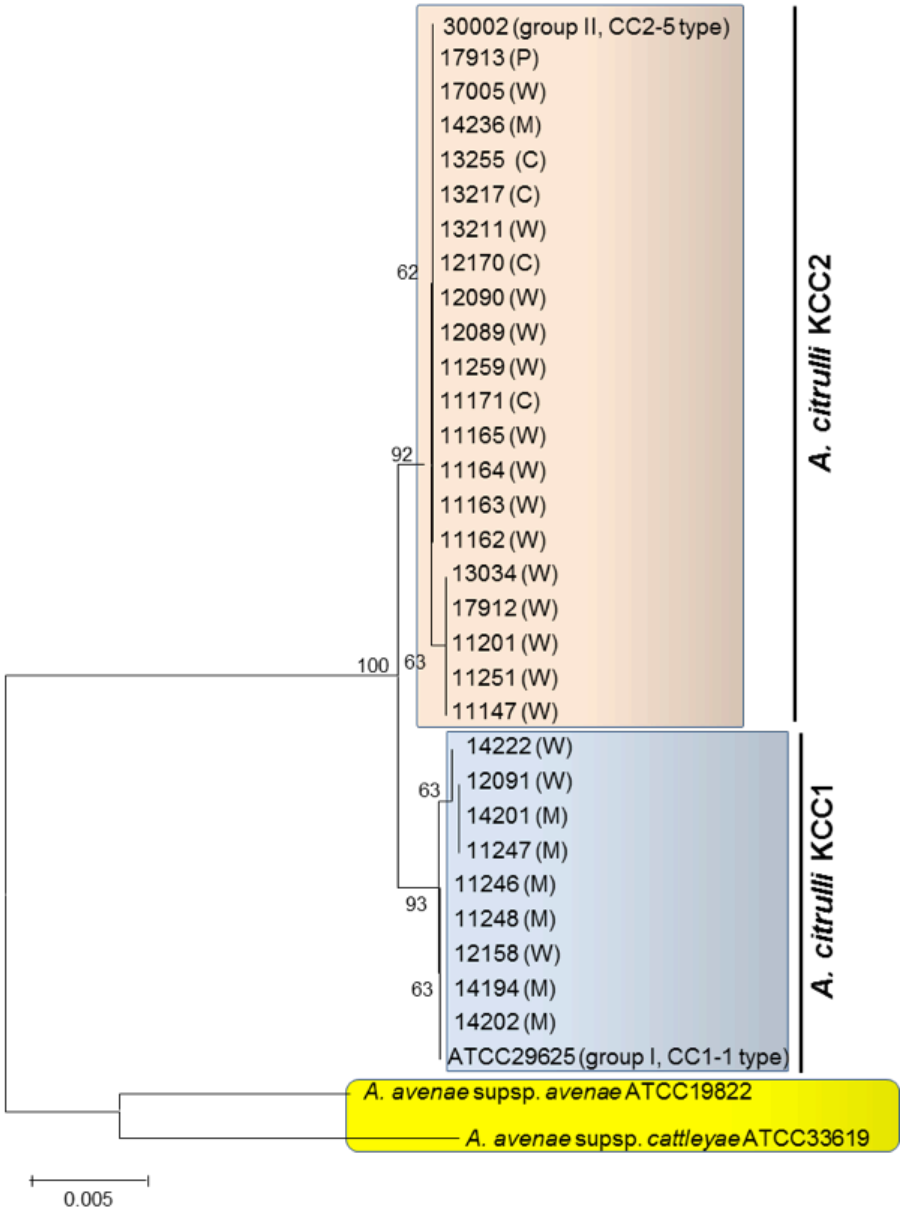


Fig. 1. Maximum likelihood phylogeny of 29 Korean strains of *Acidovorax citrulli* with two foreign strains; *A. citrulli* CC1 and CC2 types (Feng et al., 2009) and two *Acidovorax* spp. as the outgroup. The tree was constructed from 1,823 bp using four genes: *pilT*, *gltA, ugpB* and *phaC*. Bootstrap values with 1,000 replicates for the major divisions of *A. citrulli* from the outgroups. C, cucumber; M, melon; W, watermelon; P, pumpkin.

phaC presented the existence of species-specific and group-specific base mutation in 7 sites of KCC1 and KCC2 groups in 29 Korean *A. citrulli* strains with foreign isolates (Table 2). The 30th bases were cytosine (C), thymine (T) in *adk*; 439th, 442th, and 451th bases were C, guanine (G), adenine (A) and G, A, C in *gltA*; 452th were A and G in *glyA*; 94th were C and T in *pilT*; and 280th were G and C in *ugpB* in the strains of KCC1 and KCC2 groups, respectively (Table 2). DNA sequences of *gltA, pilT,* and *ugpB* genes for CC1-1 type shared 86% of CC1 group (group I) and CC2-5 type shared 82% of the CC2 group (group II); foreign *A. citrulli* strains used in studies of Feng et al. (2009) that showed the consistency with Korean strains of KCC1 and KCC2 group, respectively. On the other hand, no group-specific mutation occurred in the base sequence of *phaC* gene, while the 84th base appeared randomly with 20 : 9 ratio in the individual strains of G to A, demonstrating another mutation within each group in Korean *A. citrulli* population (Table 1). DNA sequences for CC1-1 type (ATCC29625) and CC2-5 type (30002), foreign *A. citrulli* strains that showed the consistency with G types of KCC1 and KCC2 group having only G at the 84th base.

DNA fingerprinting Rep-PCR and URP-PCR

According to the cluster analysis using DNA fingerprinting data with these results, we have confirmed two clusters forming 29 unique haplotypes from 29 strains (Fig. 2). 65% of similarity was shown between the clusters. Cluster 1 included 11 recovered strains from watermelon and melon, and cluster 2 included 18 recovered strains from watermelon, melon, cucumber, and pumpkin, which were consistent with the classification results of KCC1 and KCC2 groups by multi-locus analysis. When dividing by two clades of isolated strains from watermelon and melon, 72% of similarity was shown within the group of KCC1 cluster. KCC2 group showed 81% similarity, which was higher than KCC1 group; isolated hosts did develop the cluster but not clade.

Consequently, KCC1 group showed higher isolation rate in the melon than in the watermelon unlike the strains in KCC2 group. 103 bands derived from comprehensive results of both Rep-PCR and URP-PCR, 6 were monomorphic and 97 were polymorphic (Supplementary Fig. 1). Therefore, we could secure the fingerprinting data with more reliable genetic diversity from the results of Rep-PCR and URP-PCR than those from multi-locus phylogenetic analysis. Meanwhile, the strains in KCC2 group which were predominant in the watermelon showed higher occurrence rate within the group of *A. citrulli* species than the KCC1 group. They were widely recovered from various cultivation regions of the cucurbits in Gyeongbook,

Table 2. Comparison of DNA variation sites for six genes of Korean KCC1 and KCC2 groups with two foreign groups of Acidovorax citrulli.

Group	Gene (bp)[y]					
	gltA (439, 442, 451)	*ugpB* (280)	*pilT* (94)	*adk* (30)	*glyA* (452)	*phaC* (84)
Group I [z]	C, G, A	G	C	nd	nd	G
KCC1	C, G, A	G	C	C	A	A or G
Group II [z]	G, A, C	C	T	nd	nd	G
KCC2	G, A, C	C	T	T	G	A or G

KCC, Korean clonal complex; A, adenine; C, cytocine; G, guanine; T, thymine; nd, not determined.
[y] DNA variation sites within each gene. DNA sequences of *adk, glyA, gltA, pilT* were obtained from results of Song et al. (2015).
[z] group I (CC1-1 type); ATCC29625 and group II (CC2-5 type); 30002 (Feng et al., 2009, Walcott et al., 2004). DNA sequences for *gltA, pilT, ugpB* and *phaC* of *A. citrulli* strains are 30081; EU928015, EU928415, EU928623, and EU928313, and 30001; EU928015, EU928426, EU928634, and EU928322.

Gyeonggi, Gyeongnam, Jeonnam, Jeonbuk, and Chungnam, etc.

Accordingly, using both multi-locus analysis and Rep-PCR and URP-PCR analysis, we have revealed that two groups of genetically differentiated *A. citrulli* existed in Korea, very similar to two foreign groups widely distributed worldwide, with a variety of genotypes in each group resulting in the genetic diversity and this is very meaningful. Upon further comparison analysis on the pathogenicity differences using various hosts of these strains and on the features of the responses against various control agents, it is considered to be the very useful data in the development of effective resistance cultivars and for the establishment of the disease control system against BFB due to *A. citrulli*.

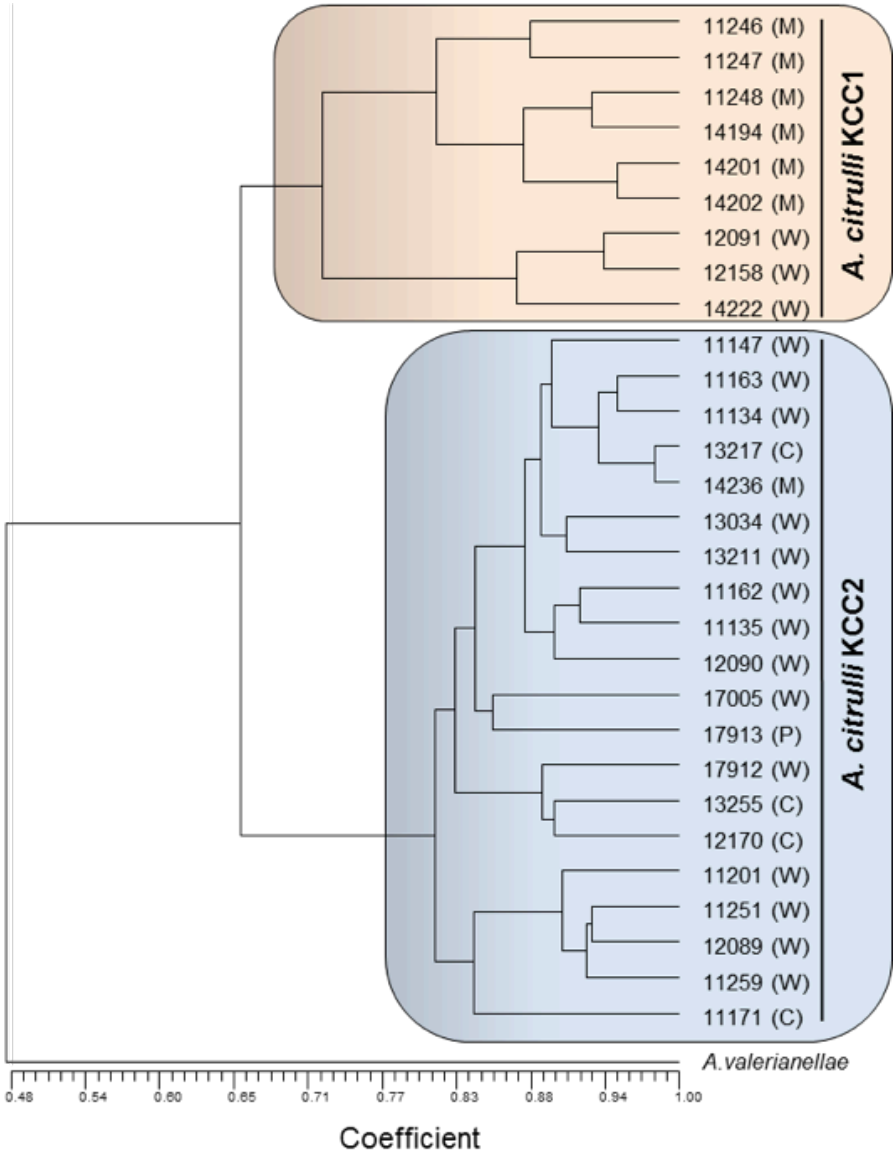
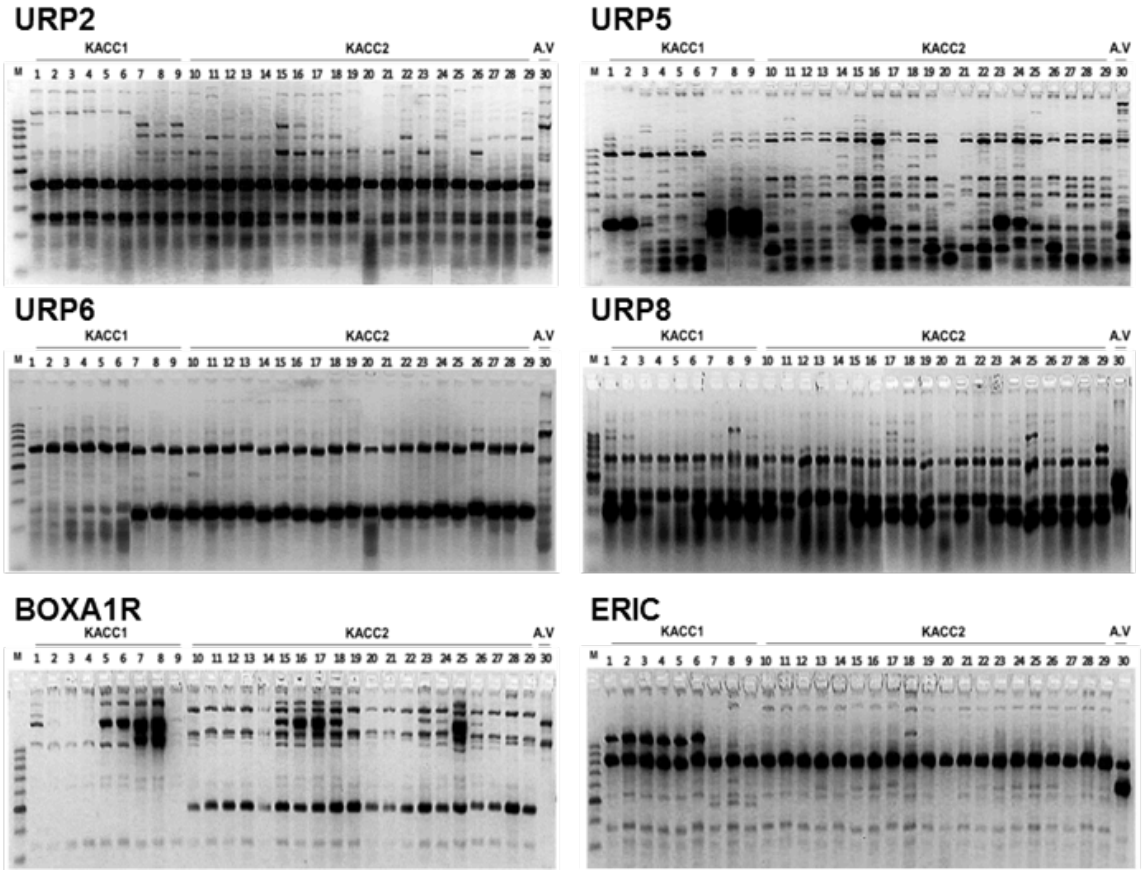


Fig. 2. Cluster analysis of *Acidovorax citrulli* strains and *A. valerianella* 12066 based on DNA fingerprint profiles generated by polymerase chain reaction (PCR) amplification of repetitive elements and universal rice primers (URP) primers. Distance matrix data was generated using Dice's (1945) coefficient of similarity and the dendrogram was constructed using the unweighted pairwise group method with arithmetic mean (UPGMA) algorithm. C, cucumber; M, melon; W, watermelon; P, pumpkin.



Supplementary Fig 1. Representative DNA fingerprinting by four universal rice primers-polymerase chain reaction (URP-PCR) and two repetitive elements amplification of Acidovorax citrulli strains. M; 100 bp plus DNA ladder, Lane 1; 11246, 2; 11247, 3; 11248, 4; 14194, 5; 14201, 6; 14202, 7; 12091, 8; 12158, 9; 14222, 10; 11147, 11; 11162, 12; 11163, 13; 11164, 14; 11165, 15; 11201, 16; 11251, 17; 11259, 18; 12089, 19; 12090, 20; 13034, 21; 13211, 22; 17005, 23; 17912, 24; 13255, 25; 11171, 26; 12170, 27; 13217, 28; 14236, 29; 17913 of A. citrulli and 30; A. valerianella 12066.

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