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Development of a multiplex PCR assay based on the *pilA* gene sequences to detect different types of *Acidovorax citrulli*

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ABSTRACT

Bacterial fruit blotch (BFB) of cucurbits, caused by *Acidovorax citrulli*, is a major threat to commercial watermelon and melon production worldwide. At present, there are at least two genetically distinct sub-populations (group I and II) of *A. citrulli* that differ in host preference among cucurbit species and copper sensitivity. In this study, we analyzed the *pilA* gene sequences of 103 *A. citrulli* strains from China and other countries. Based on these data, we classified all tested *A. citrulli* strains into three types. The *pilA*-based type 1 strains in this study coincided with the previously established group I strains; while the type 2 strains coincided with group II strains. Ten strains that did not cluster with group I or II strains were classified into a new type, designated type 3. Based on differences in *pilA* sequences, we designed a multiplex PCR assay to distinguish the three *A. citrulli* pilus types. This multiplex PCR assay has proven to be viable for strain typing of 139 *A. citrulli* strains and for the detection of this pathogen in artificially inoculated seeds and leaves and naturally infected leaves and fruits. This assay proved to be rapid, accurate, reliable and applicable for early distinction of *A. citrulli* types associated with BFB epidemics. It may also inform the judicious and environmentally sound use of bactericides, especially copper-based compounds.

1. Introduction

Acidovorax citrulli causes bacterial fruit blotch (BFB), a devastating disease of cucurbitaceous plant species worldwide (Schaad et al., 2008). *A. citrulli* is seed borne and seed transmitted, and BFB outbreaks are primarily caused by contaminated seeds (Burdman and Walcott, 2012). BFB has the potential to cause substantial economic losses and is a major concern for both cucurbit fruit, seed and seedling producers.

Following identification of genetic variability among *A. citrulli* strains in 1991 (Somodi et al., 1991), considerable progress has been made in characterizing the pathogen's population structure. *A. citrulli* strains can be divided into at least two genetically distinguishable groups (I and II). Group I strains, isolated mainly from melon, are highly virulent on non-watermelon crops; while group II strains, isolated mainly from watermelon, are highly virulent to watermelon but mildly virulent on other cucurbit species (Walcott et al., 2004). In addition, there are significant differences between group 1 and group 2 strains in terms of type three secreted effector sequence, DNA fingerprinting, gas chromatography-fatty acid methyl ester (GC-FAME), carbon source utilization and the presence/absence of large DNA fragments (Walcott and Gitaitis, 2000; Walcott et al., 2004; Burdman et al., 2005; Feng et al., 2009; Yan et al., 2013; Eckshtain-Levi et al., 2016). In addition, group II strains are more sensitive to copper than the group I strains *in vitro*. Group I strains were sensitive to $CuSO_4$ at a concentration of 750 μg/mL, while group II strains were sensitive to 500 μg/mL $CuSO_4$ (Zhao et al., 2013). This suggests that the effective dose of copper for BFB management in the field differs depending on the *A. citrulli* group. The optimal copper concentration required to manage the disease could be significantly lower for BFB caused by group II strains compared to group I strains. Currently, foliar-applied copper-based compounds are widely used in melon and watermelon production fields, and excessive use has led to the emergence of copper-tolerant isolates and environmental pollution (Li et al., 2015). Accurately distinguishing *A. citrulli* groups during the early stages of outbreaks could improve the efficacy and environmental safety of copper-based compounds.

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Due to its speed, specificity and sensitivity, polymerase chain reaction (PCR) is well suited for distinguishing *A. citrulli* strains. Several PCR-based assays have been developed for the detection of *A. citrulli* in seeds (Hui et al., 2007; Bahar et al., 2008; Tian et al., 2013). Zhong et al. (2016) designed a specific primer pair, PL1/PL2, that produced a 332-bp PCR product only with DNA from group II *A. citrulli* strains. A PCR assay was developed to distinguish group I and II *A. citrulli* strains based on *Aave_2166*, a putative type III secretion effector gene (Zivanovic and Walcott, 2017). However, there are no reports of multiplex PCR assays that simultaneously distinguish group I and II strains. Compared to two simplex PCR assays, multiplex PCR is more rapid, less labor-intensive, and can detect complex infections simultaneously.

Sequences of the *pilA* gene vary among bacteria, even among those with similar phylogenetic relationships. Accordingly, *pilA* can be a potential candidate for detection and genotyping of bacteria (Alexander et al., 2004; Cehovin et al., 2011). PilA is a small (13–23 kDa) protein subunit and thousands of copies of PilA make up type IV pili (T4P). T4P are common hair-like appendages on bacterial cell surfaces. They mediate twitching motility, surface adhesion and colonization, biofilm formation, genetic material uptake, and virulence (Bieber et al., 1998; Zolghadr et al., 2010; Vignon et al., 2003). Bahar et al., (2009) reported that twitching motility was abolished in *A. citrulli* strain W1-A, a *pilA* mutant of the group II strain, W1. However, they failed to create a similar mutant in the group I strain, M6, because the *pilA* gene fragment could not be amplified with the same primer pair (Bahar et al., 2009). This result indicated substantial sequence differences for the *pilA* gene between group I and group II *A. citrulli* strains.

In order to differentiate the two types of *A. citrulli* strains, we analyzed *pilA* gene sequences from 103 *A. citrulli* strains. Phylogenetic analyses revealed three distinct *pilA* types. Based on *pilA* sequence differences, we developed and evaluated a multiplex PCR using *pilA* gene primers to simultaneously detect and differentiate the three types of *A. citrulli* strains.

2. Materials and methods

2.1. Bacterial strains and growth conditions

One hundred and thirty-nine *A. citrulli* strains were used in this study. Of these, 122 were collected from China and the other 17 were isolated from other countries (Supplementary Table 1). The identity of all *A. citrulli* strains was confirmed by PCR using the WFB1/WFB2 primer set (Table 1), as previously described (Walcott and Gitaitis, 2000). *A. citrulli* strains were routinely grown at 28 °C on King's medium B (King et al., 1954) amended with 100 μg/mL ampicilin.

Table 1
Sequences of oligonucleotide primers used in this study.

Primer pair	Sequences (5′ → 3′)	Amplicon size (bp)
WFB1	GACCAGCCACACTGGGAC	360 bp
WFB2	CTGCCGTACTCCAGCGAT	
65pilA-S	ACGGCTCATAGTGATTAGGG	858 bp
65pilA-A	GTGATGGATGGGCAGGTA	
A5pilA-S	AGCCTCATTCCAAGCAGA	825 bp
A5pilA-A	TGACGAAATTGTCACTCCC	
Tw6pilA-S	AGGTCGTCACCACCATCG	935 bp
Tw6pilA-A	TTCCAACAGTTTAGCAATCA	
BFB[a]	ATGAAGCGTACTGTTCAG	
BFB1	ACCATCGATATTAGCATC	348 bp
BFB2	TTAAGGAGCAAACGTGC	507 bp
BFB3	CAGAATCGAATCGTGCC	685 bp

[a] BFB/BFB1/BFB2/BFB3 are the multiplex PCR primers. BFB1, BFB2 and BFB3 are reverse primers, and their forward primer is BFB.

2.2. DNA sequencing, phylogenetic analyses and PCR primer design

The *pilA* sequences of 7 *A. citrulli* strains (AC00-1, ZJU, M6, W1, pslb97, tw6, and pslb65) were obtained from the National Center for Biotechnology Information (NCBI) and used to design 3 primer pairs (65pilA-S/65pilA-A, A5pilA- S/A5pilA-A and Tw6pilA-S/Tw6pilA-A) using Primer 5.0 (PREMIER Biosoft International) (Table 1). Using the 3 primer pairs, complete ORF sequences of *pilA* were generated for analysis of sequence diversity. Primers were synthesized by the Beijing Genomics Institute (BGI) (China). The *pilA* genes of 103 *A. citrulli* strains were amplified using KOD-Plus-Neo High Fidelity DNA Polymerase (TOYOBO, Shanghai, China) with a mixture of the 3 primer pairs. Each PCR reaction mixture contained 5 μl 10× KOD-Plus-Neo buffer, 5 μl of 2 mM dNTPs (0.2 mM each), 3 μl of 25 mM $MgSO_4$, 1.5 μl of 10 μM each primer, 1.5 μl of template DNA (*i.e.* different concentrations of bacterial cell suspensions or seed wash solution), 1 μl KOD-Plus-Neo (1.0 U/μl), and PCR-grade water for a total reaction volume of 50 μl. The thermocycler (BIO-RAD, USA) cycling conditions were 94 °C for 2 min, 30 cycles of 98 °C for 10 s, 54 °C for 30 s and 68 °C for 30 s, and 68 °C for 2 min. Five microliters of PCR product was separated by electrophoresis at 100 V for 45 min on a 1.5% agarose gel. Gels were stained with ethidium bromide and photographed using BIO-RAD Gel Doc™ XR + (Hercules, California, USA). The amplified products were purified using the MinElute Reaction Clean-up Kit (Qiagen, Beijing, China), and sequenced by Beijing BGI. Multiple alignment analyses of *pilA* sequences were conducted using DNAMAN V6 (Lynnon Corporation, USA), and phylogenetic analysis of the unique *pilA* DNA sequences was conducted using MEGA 7.0 software.

2.3. Primer design and construction of multiplex PCR detection assay

Oligonucleotide primers (BFB, BFB1, BFB2 and BFB3) were designed to detect the three types of *A. citrulli* strains with Primer 5.0. Primer sequences and PCR amplicon sizes are presented in Table 1. A multiplex PCR assay using the BFB primers was designed to detect the strains of different *A. citrulli* types. Each PCR reaction mixture contained 12.5 μl 2× PCR Mix (TaKaRa, China), 0.5 μl of each primer at 10 μM (BFB/BFB1/BFB2/BFB3), 1 μl template DNA, and sterilized deionized distilled water (sddH_2O) for a 25-μl reaction volume. The thermocycler (BIO-RAD, USA) reaction conditions were 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 54 °C for 30 s and 72 °C for 30 s, and then 72 °C for 2 min.

2.4. Sensitivity of multiplex PCR detection assay

Three representative isolates, pslb2 (type 1), pslbtw14 (type 2) and pslbtw6 (type 3) of *A. citrulli* were used to test the sensitivity of the PCR primers designed in this study. An overnight liquid culture of each *A. citrulli* isolated was adjusted to 0.1 OD_{600} (approximately 10^8 CFU/ml). A ten-fold dilution series (10^{-1} to 10^{-7}) in sddH_2O was used to assess PCR detection sensitivity. This experiment was repeated three times.

2.5. Detection of A. citrulli in artificially infected watermelon seeds

Six replications of 100 non-infected watermelon seeds (cv. Ruihong, $n = 24$) were inoculated separately by incubation for 20 min in a 200 ml cell suspension containing approximately 1×10^8 CFU/ml different types *A. citrulli* (pslb2, pslbtw14 and pslbtw6). Seeds were then air-dried overnight. An artificially inoculated seed was added to a 2 ml centrifuge tube containing 1.5 ml ddH_2O, and the tube was shaken at 180 rpm for 2 h at 28 °C (Zhao et al., 2009). Then, the supernatant was used as a template for detection, using the multiplex PCR detection assay. In each replicate, 10 seeds inoculated by each type of bacterial suspension were tested. This experiment was repeated three times.

2.6. Detection of A. citrulli in infected plant tissue

Two-week-old watermelon seedlings were spray-inoculated with cell suspensions of ~ 1×10^6 CFU/ml of three *A. citrulli* strains representing the three *pilA* types (type 1: pslb2, type 2 pslbtw14 and type 3 pslbtw6). Seven days after inoculation, 1 cm^2 leaf samples were excised from the junction of healthy and diseased tissues of leaves. Each leaf tissue sample was soaked in 75% alcohol for 2 min., and then rinsed 3 times with distilled water. Leaf tissue was put into a 2 ml centrifuge tube with 800 µl of sddH_2O and ground with a sterilized glass rod. The supernatant was used as template for PCR amplification using primers BFB/BFB1/BFB2/BFB3. The PCR conditions were as described above for the multiplex PCR assay. Five microliters of PCR product was separated by electrophoresis at 100 V for 45 min on a 1.5% agarose gel. In each replicate, 10 leaves inoculated by each type of bacterial suspension were tested. This experiment was repeated three times. 2 naturally infected melon leaves and 2 melon fruits from Hainan Province were tested as described above.

3. Results

3.1. Sequencing and comparative analysis of the pilA gene

PCR amplicons were generated for all 103 *A. citrulli* strains using one of the three specific primer sets (65pilA-S/65pilA-A, A5pilA-S/A5pilA-A, Tw6pilA-S/Tw6pilA-A). DNA from 63.1% (65/103) of the *A. citrulli* strains yielded an 858 bp amplicon with the primer set 65pilA-S/65pilA-A and no amplicon with the other primer sets. A representative portion of the PCR results can be found in Supplementary image 1. DNA from 27.2% (28/103) of *A. citrulli* strains yielded an 825 bp amplicon by PCR with the primer set A5pilA-S/A5pilA-A. A representative portion of the PCR results can be found in Supplementary image 2. DNA from 10 *A. citrulli* strains yielded a 925 bp amplicon with the primer set Tw6pilA-S/Tw6pilA-A (Supplementary image 3). Following purification, all amplicons were sequenced. To ensure the accuracy of the sequencing results, each amplicon included 200 bp of upstream and downstream flanking sequences.

Sequence analysis revealed that all 103 *A. citrulli* strains could be classified into three types: based on the *pilA* gene sequences (Supplementary table 1). The most obvious difference among the three types was the ORF lengths. The numbers of nucleotides in the *pilA* genes for *A. citrulli* strains representing types 1, 2, and 3 were 522, 507 and 495 bp, respectively. The *pilA* gene sequences for strains within each type were almost identical except a few cases: the type 1 strain Fc247 had a transversion of A to T at the 473rd nucleotide; the type 2 strain pslbtw27 lacked nucleotides 371-373 (CAG) and had an A to G substitution at nucleotide position 431. All ten type 3 strains had identical *pilA* sequences. The aligned *pilA* gene sequences were used to create a phylogenetic tree which demonstrates the *pilA* sequence diversity between the three *A. citrulli* types (Fig. 1). The DNA sequence of three *pilA* types of *A. citrulli* were shown in Supplementary image 4.

Of the 65 type 1 strains, 60 were isolated from melon and 5 were recovered from watermelon in China (Inner Mongolia, Xinjiang, Shandong, Hebei, Hainan) and the United States. Twenty one of the type 2 strains were isolated from watermelon, 2 were recovered from melon and 5 came from other cucurbits) in China (Taiwan, Inner Mongolia, Xinjiang, Heilongjiang, Guangdong, Hainan, Beijing), the United States, Japan and India. All of the type 3 strains were isolated from watermelons in the different plots of the same village Daxing District of Beijing in 2005 (Supplementary table 1).

The sequencing results for the 10 type 3 strains in this study showed that the *pilA* gene was 495 base pairs long, which is different from the 522-bp and 507-bp amplicons obtained for type 1 and type 2 *pilA* genes, respectively. Therefore, we classified these 10 strains as type 3. BLAST analysis revealed significant differences in the *pilA* sequences among strains of types 1 2, and 3. The DNA sequence identity of *pilA* between types 3 and 1 was 55.81%, the identity between types 3 and 2 was 56.13%, and the identity between types 1 and 2 was 61.64%. Based on *pilA* sequence analysis, most of the type 1 and 2 strains were consistent with their placement in previously established groups I and II, respectively, except for the five strains (Fc380, pslb9, pslb19, pslbtw19, pslbtw20), which were classified into different groups based on the method employed (Supplementary table 1).

3.2. Design of specific primers

To rapidly and accurately distinguish the three *A. citrulli* types, a multiplex PCR assay was designed using the primers listed in Table 1. The same forward primer was used for amplification of all three types of strains, and the reverse primers were designed at positions unique to the specific types, to generate amplicons of different sizes. The reverse primers for type 1 and 2 *A. citrulli* strains were located within the *pilA* gene and when combined with the forward primer, the PCR assay generated 348-bp and 507-bp fragments, respectively. The reverse primer for type 3 was located downstream of the *pilA* gene and when combined with the forward primer, the PCR assay generated a 685-bp amplicon (Supplementary image 4).

3.3. Multiplex PCR for differentiating the three pilA A. citrulli types

Using DNA from *A. citrulli* strains pslb2 (type 1), pslbtw14 (type 2) and pslbtw6 (type 3), PCR yielded expected bands with sizes of 348, 507 and 685 bp, respectively. When a mixture of DNA from strains of the three types were used as template, the three correct bands were generated (Fig. 2).

3.4. Sensitivity of the multiplex PCR assay

The sensitivity of the PCR assay was tested using strains representing the three *A. citrulli* types (pslb2, pslbtw14 and ps1btw6) at concentrations ranging from 10^1 to 10^8 CFU/ml. Cell suspensions of strains of each type yielded respective PCR amplicons at a concentration of 10^5 CFU/ml (Fig. 3).

3.5. Evaluation of multiplex PCR assay for detection of A. citrulli

Using the primer combination BFB/BFB1/BFB2/BFB3, cell suspensions from 139 strains of *A. citrulli* were amplified by PCR assay. As expected, using cell suspension from a single strain as template each PCR assay yielded one clear band, which is consistent with its *pilA* type.

The multiplex PCR assay revealed that only type 3 *A. citrulli* strains (pslbtw1-3 and pslbtw5-11) yielded the 685-bp fragment (Supplementary image 7). The other 129 strains grouped according to previous studies (Walcott et al., 2004; Feng et al., 2009), except for the six strains (Fc380, AAC200-23, pslb9, pslb19, pslbtw19, pslbtw20). Of the 129 strains, 82 were classified into type 1 (Supplementary image 5) and 47 were classified into type 2 (Supplementary image 6). The multiplex PCR assay was consistent with the results of *pilA* sequence analysis. While there were different grouping results for six strains, the results of this study are consistent with the results of previous studies (Walcott et al., 2004; Feng et al. 2009; Yan et al. 2013; Zhong et al., 2016). This indicates that the multiplex PCR described here is can be used to consistently distinguish genetically distinct *A. citrulli* groups.

3.6. Multiplex PCR detection of A. citrulli strains from artificially infected watermelon seeds

Seed washings from an artificially inoculated watermelon seed was used as a template for PCR amplification with the multiplex assay developed in this study. There was a single amplicon for all *pilA* types from watermelon seed washings, and respective band sizes were as expected (part of the test results can be found in Fig. 4). These results

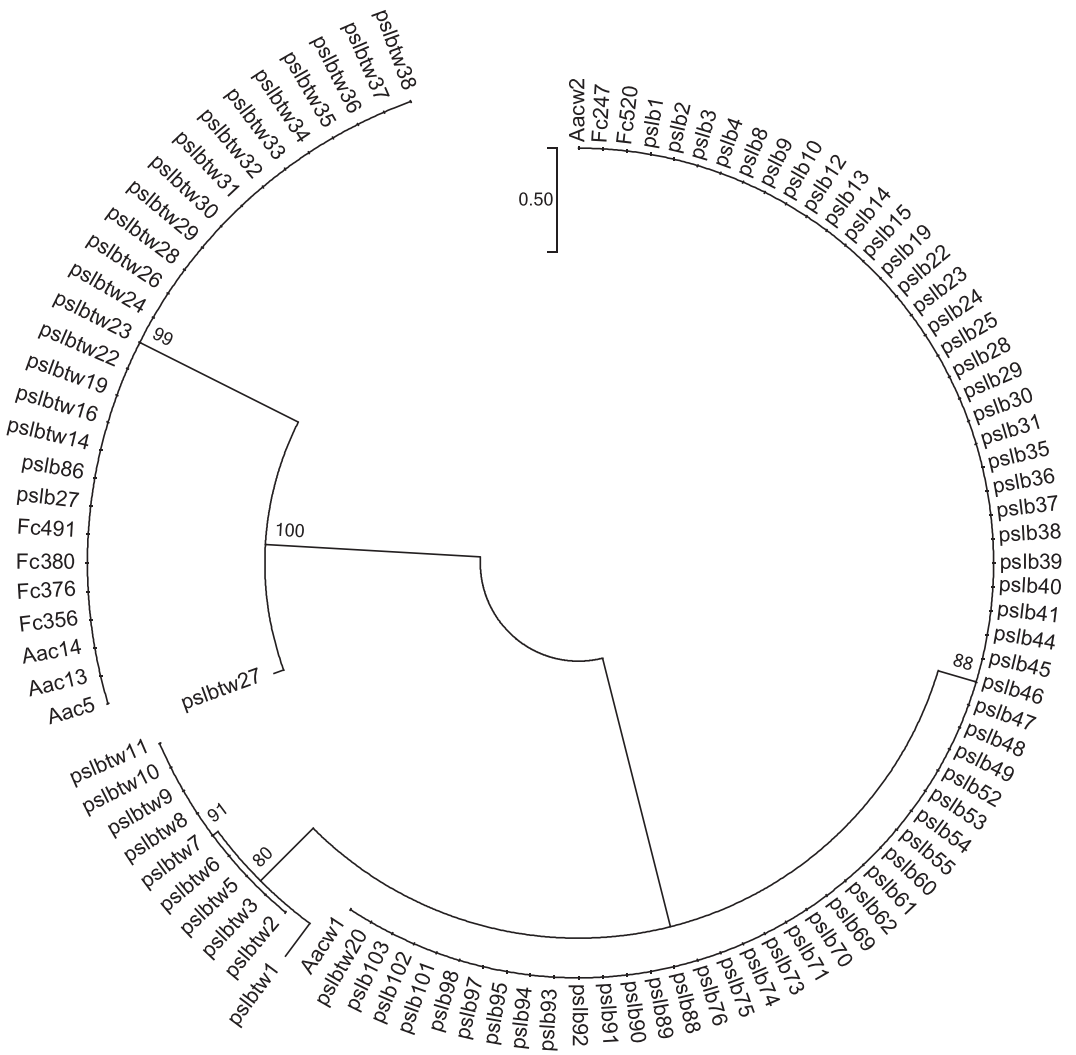

Fig. 1. Phylogenetic tree showing the relationships of 103 *A. citrulli* strains based on their *pilA* gene sequences using MEGA7. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 4.03568767 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Joseph, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 103 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 465 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

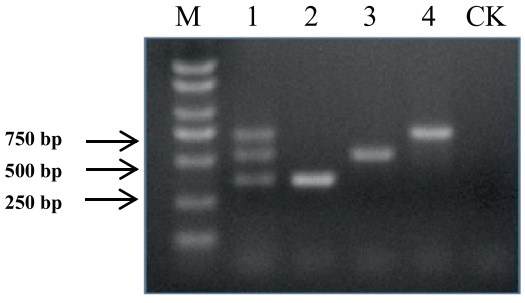

Fig. 2. PCR amplification of *A. citrulli* type 1, 2 and 3 strains using DNA from three types *A. citrulli* strains **as templates.**
M. DNA marker M2000 +; 1. mixture of pslb2, pslbtw14, and pslbtw6 DNA; 2. pslb2 DNA; 3. pslbtw14 DNA; 4. pslbtw6 DNA; CK. ddH_2O.

showed that the assay can be effectively used for detection of *A. citrulli* in single artificially infected seed without the influence of other impurities in the seed washings.

3.7. Detection of *A. citrulli* types in infected plant tissues

Leaf and fruit tissue grinding fluid of melon or watermelon (artificially and naturally infected) were used as templates for the multiplex PCR assay as described above. PCR products had expected sizes confirming that this method was applicable for direct detection of *A. citrulli* types in plant tissues. Part of the test results can be found in Fig. 5.

4. Discussion

In this study, we characterized *A. citrulli* strains using *pilA* gene sequences. PilA is the major structural subunit of T4P and is commonly used in the genetic characterization of bacterial species. Kus et al. (2004) classified *Pseudomonas aeruginosa* into five phylogenetic groups based on differences in pilin gene sequences. Similarly, Luke-Marshall et al. (2011) divided the human pathogen *Moraxella catharralis* into two groups based on differences in the DNA sequence of the *pilA* gene. Herein, the *pilA* sequences from 7 *A. citrulli* strains (tw6, AC00-1, ZJU, M6, W1, pslb97, and pslb65) were analyzed and we found that they only shared 67.93% identity, which allowed for the classification of *A.*

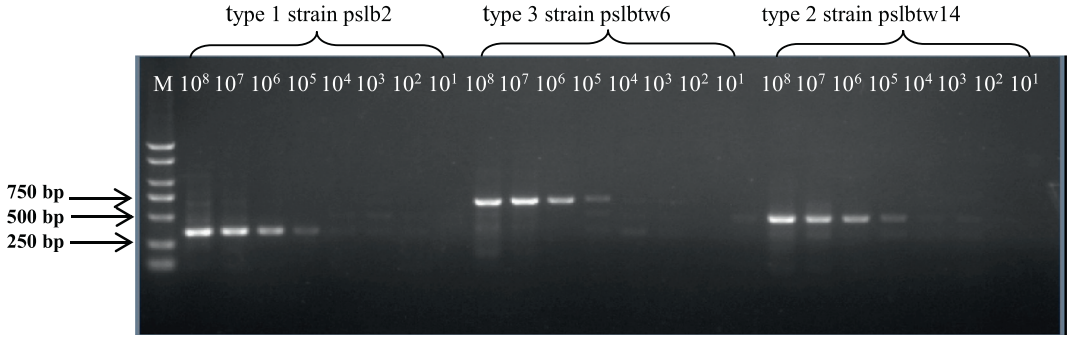

Fig. 3. Results of a PCR assay using ten-fold serial dilutions of cell suspensions of *A. citrulli* strains representing the three *pilA* types. DNA marker M2000+; the concentrations of three strains is 10^8–10^{10}CFU/ml, respectively.

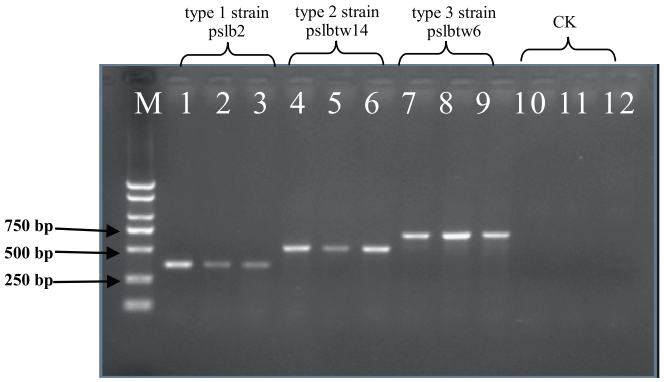

Fig. 4. Results of multiplex PCR assays for three *A. citrulli* types using artificially infected watermelon seeds. Lane M. DNA marker M2000+; 1–3. artificial inoculated watermelon seeds by type I strain pslb2; 4–6. artificial inoculated watermelon seeds by type II strain pslbtw14; 7–9. artificial inoculated watermelon seeds by type III strain pslbtw6 ; 10–11. health seeds control; 12. ddH_2O.

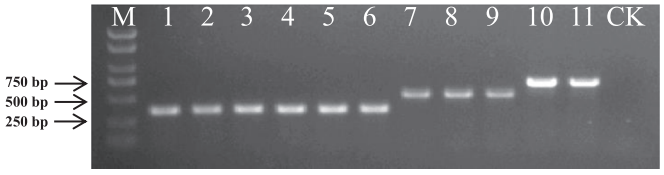

Fig. 5. Results of multiplex PCR assays simultaneously detecting and differentiating *A. citrulli* types in infected watermelon and melon fruit and leaf tissues. Lanes: M. DNA marker M2000+; 1–2. artificial inoculated melon leaves by type I strain pslb2; 3–4. natural infected melon leaves from Hainan Province; 5–6. natural infected melon fruits from Hainan Province; 7–9. artificial inoculated watermelon leaves by type II strain, pslbtw14; 10–11. artificial inoculated watermelon leaves by type III strain, pslbtw6; CK. ddH_2O.

citrulli strains into three types based on nucleotide differences of *pilA*. Most of the published studies report that *A. citrulli* strains belong to two groups. However, based on the analysis of 11 the type III secretion system (T3SS) effector genes, DNA hybridization, DNA fingerprinting, multilocus sequence typing (MLST) and toxicity analysis, Eckshtain-Levi et al. (2014) found a third group comprised of strains from India (ZUM4000 and ZUM4001). The *pilA* type1 and type 2 strains in this study are consistent with the previously established group I and II strains, respectively, except for the five strains (Fc380, pslb9, pslb19, pslbtw19, pslbtw20). The grouping results of the five strains were inconsistent, based on different classification methods. Eckshtain-Levi et al. (2014) believed that errors in sequencing may be responsible for the difference in grouping results of Fc380 using MLST and PFGE classification methods. The *pilA* type 3 included 10 strains and represents a new group. These 10 strains were previously classified into different groups. Yan et al. (2013) classified them into group I using pulsed field gel electrophoresis (PFGE), and into group II using MLST, while Zhong et al. (2016) classified them into group I using primers PL1/PL2. In order to analyze the types of *pilA* sequences of ZUM4000 and ZUM4100, three pairs of primers (65pilA-S/65pilA-A, A5pilA-S/A5pilA-A and Tw6pilA-S/Tw6pilA-A) were used in an attempt to amplify the *pilA* gene. Under the conditions tested, no amplicons were obtained, suggesting that either a highly divergent *pilA* sequence exists, the *pilA* sequence is in a different genomic context, or *pilA* is not present. Regardless of the scenario, a fourth group appears to exist.

The two previously established *A. citrulli* groups show differences in host range, pathogenicity, copper sensitivity, type three secreted effector sequence, DNA fingerprinting, GC-FAME, carbon source utilization and the presence/absence of large DNA fragments (Zhao et al., 2013; Walcott and Gitaitis, 2000; Eckshtain-Levi et al., 2014). Therefore, it is of practical significance to quickly and accurately distinguish strains of *A. citrulli* for the rational use of copper-based compounds for effective BFB management. At present, there are few tools/assays to differentiate *A. citrulli* at the sub-specific level. To our knowledge, the multiplex PCR assay reported here is the first to simultaneously detect and differentiate three of the four types of *A. citrulli*. In this study, the reproducibility and sensitivity of the multiplex PCR were confirmed by testing a large number of samples. Compared with the PL1/PL2 primers that can only detect group II strains, the multiplex PCR assay reported here differentiated the three known *pilA* types simultaneously. Compared with MLST, PFGE, and GC-FAME analyses, this method saves time and labor and requires less equipment and money. Contaminated seeds can be important vehicles for long distance transmission of pathogens, and usually serve as the primary source of BFB outbreaks (Walcott and Gitaitis, 2000). Hence, a rapid, reliable, and sensitive seed-detection assay is required to limit the spread of BFB. Direct detection of *A. citrulli* in infected leaves and fruits is technically straightforward and effective for detecting and distinguishing *A. citrulli* types, because it eliminates *A. citrulli* isolation from infected tissues and DNA preparation. This assay has great potential for integrated pest management through early detection of BFB outbreaks.

Unfortunately, the analyses of the multiplex PCR assay developed here has some shortcomings. For example, only artificially inoculated seeds were tested; so the assay must be evaluated and optimized for naturally infected seeds with varying levels of contamination. In addition, our study found that *Acidovorax avenae* subsp. *avenae* strains yielded the 345-bp fragment as same as *A. citrulli* type 1 strains using the multiplex PCR assay. Therefore, this method cannot distinguish *A. citrulli* from other species of *Acidovorax*. In order to accurately identify the level of subpopulations in plant tissue, it is necessary to combine this technique with PCR using *A. citrulli* specific primers in this assay. Although *A. citrulli* can be classified into three categories based on differences of the *pilA* gene, the differences in biological characteristics between the three types of strains are not clear. According to the result

of Zhao et al. (2013), the copper sensitivity of both type 3 and type 1 strains was 750 μg/mL, with no significant difference. It is indicated that type 3 cannot be distinguished from type 1 strains by the sensitivity of copper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.02.003.

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