Genetic Diversity of *Pseudomonas syringae* pv. *actinidiae* Strains from Different Geographic Regions in China

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Accepted for publication 15 September 2018.

ABSTRACT

Pseudomonas syringae pv. *actinidiae* causes kiwifruit bacterial canker, with severe infection of the kiwifruit plant resulting in heavy economic losses. Little is known regarding the biodiversity and genetic variation of populations of *P. syringae* pv. *actinidiae* in China. A collection of 269 strains of *P. syringae* pv. *actinidiae* was identified from 300 isolates obtained from eight sampling sites in five provinces in China. The profiles of 50 strains of *P. syringae* pv. *actinidiae* and one strain of *P. syringae* pv. *actinidifoliorum* were characterized by Rep-, insertion sequences 50, and randomly amplified polymorphic DNA polymerase chain reaction (PCR). Discriminant analysis of principal coordinates, principal component analysis, and hierarchical cluster analysis were used to analyze the combined fingerprints of the different PCR assays. The results revealed that all isolates belonged to the Psal group, that strains of *P. syringae* pv. *actinidiae* from China have broad genetic variability that was related to source geographic region, and that Chinese strains can be readily differentiated from strains from France

but are very similar to those from Italy. Multilocus sequence typing of 24 representative isolates using the concatenated sequences of five housekeeping genes (*cts*, *gapA*, *gyrB*, *pfl*, and *rpoD*) demonstrated that strain Jzhy2 from China formed an independent clade compared with the other biovars, which possessed the *hopH1* effector gene but lacked the *hopA1* effector gene. A constellation analysis based on the presence or absence of the four loci coding for phytotoxins and a cluster analysis based on the 11 effector genes showed that strains from China formed two distinct clades. All of the strains, including K3 isolated in 1997 from Jeju, Korea, lacked the *cfl* gene coding for coronatine. In contrast, the *tox-argK* gene cluster coding for phaseolotoxin was detected in K3 and in the biovar 1 strains (K3, Kw30, and Psal92), and produced a false-positive amplicon for the *hopA1*-like gene in this study. To date, only one biovar (biovar 3) is represented by the strains of *P. syringae* pv. *actinidiae* from China, despite China being the center of origin for kiwifruit.

Kiwifruit (*Actinidia chinensis* Planchon and *A. deliciosa* Liang et Ferguson) is an important fruit crop, with primary production in China, Italy, and New Zealand (Vanneste 2017). *Pseudomonas syringae* pv. *actinidiae* is the causal agent of kiwifruit bacterial canker (KBC) and was first discovered in Japan in the early 1980s (Takikawa et al. 1989). There were earlier reports of a similar disease in California, United States (Opgenorth et al. 1983), but the causal bacterium was demonstrated to be different from that subsequently characterized in Japan (Takikawa et al. 1989). Within 10 years of identifying *P. syringae* pv. *actinidiae*, serious economic losses due to the disease were reported from South Korea (Koh et al. 1994). *P. syringae* pv. *actinidiae* was reported infecting kiwifruit in Italy and in China, where KBC was first reported in Hunan Province in 1985 and later reported in Anhui and Sichuan Provinces. Although the outbreak of KBC on *A. deliciosa* ‘Qinmei’ in Shaanxi

Province was first observed in 1991, it was not reported until 10 years later (Liang et al. 2000).

Despite a lack of prior knowledge concerning the target DNA sequences, polymerase chain reaction (PCR) amplification of the enterobacterial repetitive intergenic consensus (ERIC) sequences, Box elements (BOX, or repetitive sequence elements) and (GTG)₅ elements in bacterial genomes (collectively called Rep-PCR), randomly amplified polymorphic DNA (RAPD)-PCR, and insertion sequences 50 (IS50)-PCR are highly discriminating approaches for characterizing genetic relationships between strains and pathovars of *Pseudomonas* spp. (Weingart and Volksch 1997). The combination of Rep-PCR and IS50-PCR was previously demonstrated to provide high resolution genomic fingerprinting to discern intrapathovar diversity among strains of *P. syringae* pv. *actinidiae* (Mazzaglia et al. 2011).

There are four groups of *P. syringae* pv. *actinidiae* strains (Psal to -4) proposed based on multilocus sequence typing (MLST): Psal represents isolates from Japan and Italy; Psal2 represents isolates from Korea; Psal3 represents isolates from Italy, New Zealand, Chile, and China; and Psal4 represents isolates from New Zealand and Australia that exhibit low virulence (EPPO 2011). Moreover, four subpathovars or biovars (biovars 1 to 4) of *P. syringae* pv. *actinidiae* have been discriminated and named based on molecular characteristics, BOX-PCR electrophoretic profiles, the presence of type III secretion system effector protein genes (effector genes) *hopA1* and *avrD*, and virulence differences observed in the field. *P. syringae* pv. *actinidiae* strains in biovar 3 (found in Italy since 2008 and in New Zealand, Chile, and China) and biovar 4 (New Zealand) are separated from those isolated from past epidemics in Japan and Italy (biovar 1) or Korea (biovar 2) (Vanneste et al. 2013).

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Funding: This project was supported by the National Natural Science Foundation of China (31401831), Anhui Provincial Annual Key Project Foundation (1804a07020105), Anhui Provincial Natural Science Foundation (1408085QC62), Discipline Capacity Promotion at Anhui Agricultural University, China (XKTS 2013010), and Foreign Visiting Scholars of Anhui Higher Education Revitalization (gxfzD2016023).

R. He and P. Liu contributed equally to this work.

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures and three supplementary tables are published online.

Strains belonging to biovar 4 have been described as a new pathovar; namely, *P. syringae* pv. *actinidifoliorum* pv. nov. (Cunty et al. 2015b).

Although there have been several reports describing the occurrence of KBC in different provinces of China (Liang et al. 2000), the collection of strains of *P. syringae* pv. *actinidiae* from China is quite limited. Thus, the genetic diversity of populations of *P. syringae* pv. *actinidiae* from different geographic regions in China has not been characterized. However, recent availability of strains from China with associated genetic marker studies (Ciarroni et al. 2015; Cunty et al. 2015a) and the sequence of the genomes of *P. syringae* pv. *actinidiae* have provided some insight into the population biology and epidemiology of this important disease (Andersen et al. 2018; McCann et al. 2017), which is valuable in understanding how climate change may affect distribution of *P. syringae* pv. *actinidiae* in the future (Wang et al. 2018).

To gather more information on the genetic diversity of populations of *P. syringae* pv. *actinidiae* from different geographic regions in China, we collected 296 strains of *P. syringae* pv. *actinidiae* from 2012 to 2014. Of these strains, 51 (including *P. syringae* pv. *actinidifoliorum* strain 18804) were selected to study diversity and variation using Rep-, RAPD-, and IS50-PCR. Furthermore, five housekeeping genes (*cts*, *gapA*, *gyrB*, *pfk*, and *rpoD*) of 24 representative *P. syringae* pv. *actinidiae* strains were sequenced, and four loci for the coronatine or phaseolotoxin biosynthesis pathway and 11 effector protein genes were cloned and used to elucidate the phylogenetic relationships among strains of *P. syringae* pv. *actinidiae* worldwide.

MATERIALS AND METHODS

Collection of *P. syringae* pv. *actinidiae* strains. A collection of 269 strains of *P. syringae* pv. *actinidiae* was identified from 300 isolates obtained from eight sampling sites in five different kiwifruit-producing regions of China: Jiading District, in Shanghai municipality along the East Sea coastline; Yuexi and Jinzhai Counties in the Dabie mountain area of Anhui Province; Xiuwen County, in the southwest mountain area of Guizhou Province; Mianzhu and Pengzhou County, in the southwest basin of Sichuan Province; and Huxian and Weixian Counties, in the northwest plain of Shaanxi Province in China (Supplementary Fig. S1).

These strains were isolated from diseased leaves or branches of kiwifruit and from three nonkiwifruit species (*Paulownia tomentosa*, *Setaira viridis*, and *Alternanthera philoxeroides*) and two species of leafhoppers (*Nephotettix bipunctatus* (Fabricius) and *Philagra hexamaculata* Schmidt) (Table 1). Samples collected from diseased plant tissue were cut with a sterile blade and suspended in sterile distilled water. The suspensions were diluted, streaked on nutrient-sucrose agar, and incubated at 28°C for 24 to 48 h in the dark (Mazzaglia et al. 2011). Pure cultures were obtained by transferring a single colony to King's B medium at 25 to 28°C for 10 to 12 h in the dark. The preliminary identification of all isolates was performed according to standard bacteriological techniques (Takikawa et al. 1989). Molecular confirmation was by PCR amplification of two sets of primers specific to *Pseudomonas syringae* pv. *actinidiae* using genomic DNA (gDNA), as described by Koh and Nou (2002) and Rees-George et al. (2010).

Biochemical characterization of isolates. The ability of the bacterial strain to induce a hypersensitive reaction (HR) when injected into tobacco plants was tested, as was the ability to rot potato, both following described protocols (Vanneste et al. 2013). Ice nucleation activity was determined as described by Lindow et al. (1978). The production of syringomycin was determined according to Gross and DeVay (1977). The absence of cytochrome C oxidase, a characteristic that differentiates *P. syringae* from other species of plant-pathogenic fluorescent *Pseudomonas* spp., was determined using Test Oxidase (Pro-Lab Diagnostics Inc., Round Rock, TX, USA). Production of levan and an arginine dihydrolase under

anaerobic conditions and the ability to hydrolyze esculin were determined using a previously described method (Lelliot et al. 1966).

Selection of additional strains and pathovars of *P. syringae* pv. *actinidiae*. In all, 51 strains of *P. syringae* pv. *actinidiae* from different geographic regions and 3 other pathovars of *Pseudomonas* spp. were used to determine phylogenetic relationships (Table 1). Of these strains, 37 were selected from the collection of 296 strains of *P. syringae* pv. *actinidiae* collected in China: 3 to 5 representative strains were selected from different sampling sites by the prereaction of different PCRs with the criteria of pattern similarity. A further 14 strains of *P. syringae* pv. *actinidiae* were selected from Italy (five strains), Spain (2 strains), France (2 strains), Portugal (1 strain), New Zealand (2 strains), Korea (1 strain), and Japan (1 strain). In addition, 25 characterized strains from New Zealand, Italy, Chile, Australia, Korea, Japan, and the United States previously deposited in the National Center for Biotechnology Information (NCBI) database were used to analyze the nucleotide sequences of the different housekeeping genes (Supplementary Table S1).

gDNA extraction. Bacterial Genomic DNA Isolation Kits (Norgen Biotek Corp., Thorold, ON, Canada) were used to obtain gDNA from freshly grown cultures of each strain. The quality of gDNA of each strain was checked using a Beckman-Coulter DU800 UV-Visible spectrophotometer (Beckman Coulter, Inc., Pasadena, CA, USA). The DNA concentration was adjusted to 50 ng/μl with Tris-EDTA buffer (Tris-HCl and EDTA, pH 8.0) and stored at -80°C.

Different PCR assays. Primers were selected for Rep-, IS50-, and RAPD-PCR assays and were synthesized by Sengen Co., Ltd. (Shanghai, P.R. China) (Table 2). All 296 isolates of *P. syringae* pv. *actinidiae* were screened against all primers. Three repetitive sequences primer sets—ERIC1R-ERIC2, BOXA1R, and (GTG)₅—were chosen for the Rep-PCR amplification (Mazzaglia et al. 2011). In addition, Primer IS50 complementary to the insertion sequence IS50 of Tn5 was used for strain typing. The Rep-PCR and IS50-PCR reactions were performed in a final volume of 25 μl containing 12.5 μl of 2× Taq PCR MasterMix (Aidlab Biotechnologies Co., Ltd., Beijing, China), 1 μl of template DNA, 1 μl of each primer (10 nmol/μl), and 10.5 μl of sterile distilled water. The Rep-PCR amplification was performed at 95°C for 7 min; followed by 30 cycles of a denaturation step of 94°C for 60 s, the annealing temperature of each primer for 60 s, and extension at 72°C for 8 min; and a final extension step at 72°C for 15 min. The IS50-PCR amplification conditions were as follows: an initial denaturation cycle of 95°C for 7 min; 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 5 min; and a final extension step of 72°C for 15 min.

Eight primers with arbitrary nucleotide sequences were selected from a panel of 160 RAPD primers screened to produce clear, reliable, and discriminable RAPD profiles; strains of *P. syringae* pv. *actinidiae* and *P. syringae* were tested according to previous protocols (Lee et al. 2005). Amplification was performed in a total volume of 20 μl containing 1 μl of each primer, 1 μl of the template DNA, 10 μl of 2× Taq PCR MasterMix, and 7 μl of sterile distilled water. The amplification consisted of an initial denaturation step of 95°C for 5 min; followed by 40 cycles of 94°C for 60 s, the annealing temperature of each primer for 60 s, and 72°C for 5 min; and a final extension step of 72°C for 15 min.

Amplification products (8 μl) were separated on a 1% agarose gel in 1× Tris-borate-EDTA buffer. All PCR amplifications were repeated twice to ensure reproducibility.

DNA fingerprints of the strains were visually inspected for similarity and were considered identical if the scored bands were at the same apparent migration distance, without consideration of variation in amplicon intensity. Amplified fragments of each strain were scored as 1 if present in all three runs but 0 if absent, even if absent in only one experimental run, which demonstrated that the fragment was not amplified reliably. The resulting matrix of 0 and 1

data representing amplicons from strains by primers using the Rep-, IS50-, and RAPD-PCR assays was subject to analysis. Discriminant analysis of principal coordinates (DAPC) (Jombart et al. 2010) was conducted according to the Wide Linear model, principal components analysis (PCA) was performed using covariances, and strain differences between countries for each principal component (PC) were explored based on least square means. Hierarchical cluster analysis was performed using the Ward method, and one-way analysis of variance (ANOVA) of PCs was performed with means separation using Duncan's new multiple-range method ($\alpha = 0.05$). Analyses were calculated using the software JMP for Windows (version Pro 14; SAS, Cary, NC, USA).

MLST of the housekeeping gene. To identify the biovar (MLST group) of the strains of *P. syringae* pv. *actinidiae* from the population in China, five housekeeping genes—*cts* (*gltA*), *gapA*, *gyrB*, *pfk*, and *rpoD*—that code for citrate synthase, glyceraldehyde-3-phosphate dehydrogenase, DNA gyrase B, phosphofructokinase, and σ factor 70, respectively, were cloned using a previously described method (Sarkar and Guttman 2004). The MLST was performed on 24 strains selected from each clade of the dendrogram based on the combined data of the 51 strains of *P. syringae* pv. *actinidiae* characterized using different PCR assays described in the previous section. Sequencing of the housekeeping genes was performed using the CEQ-DTCS Quick Start kit (Beckman Coulter, Inc.) on a Beckman-Coulter CEQ 8000 DNA

TABLE 1. Bacterial strains used in this study showing source, species or pathovar, geographic origin, year, and host from which they were isolated

Number	Strain name	Source	Sp. ^a	Host species, cultivar	Tissue ^b	Year ^c	Geographic origins	Orchard name	Collection ^d
1	Schy3	AHAU, China	Psa	<i>Actinidia chinensis</i> 'Hongyang'	Vine	2013	Penzhou, Sichuan, China	Agri-Bumper	This study
2	Schy4	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Vine	2013	Penzhou, Sichuan, China	Agri-Bumper	This study
3	Schy5	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Vine	2013	Penzhou, Sichuan, China	Agri-Bumper	This study
4	Schy9	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Vine	2013	Penzhou, Sichuan, China	Agri-Bumper	This study
5	Scht12	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Vine	2013	Penzhou, Sichuan, China	Agri-Bumper	This study
6	SH2	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Leaf	2013	Jiading, Shanghai, China	Agri-Tech	This study
7	SH8	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Leaf	2013	Jiading, Shanghai, China	Agri-Tech	This study
8	SH13	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Leaf	2013	Jiading, Shanghai, China	Agri-Tech	This study
9	SH18	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Leaf	2013	Jiading, Shanghai, China	Agri-Tech	This study
10	SH26	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Leaf	2013	Jiading, Shanghai, China	Agri-Tech	This study
11	GC31	AHAU, China	Psa	<i>A. deliciosa</i> 'Guichang'	Vine	2012	Xiuwen, Guizhou, China	Wangjiapu	This study
12	GC32	AHAU, China	Psa	<i>A. deliciosa</i> 'Guichang'	Vine	2012	Xiuwen, Guizhou, China	Wangjiapu	This study
13	GC40	AHAU, China	Psa	<i>A. deliciosa</i> 'Guichang'	Leaf	2012	Xiuwen, Guizhou, China	Wangjiapu	This study
14	GC41	AHAU, China	Psa	<i>A. deliciosa</i> 'Guichang'	Leaf	2012	Xiuwen, Guizhou, China	Wangjiapu	This study
15	GC42	AHAU, China	Psa	<i>A. deliciosa</i> 'Guichang'	Leaf	2012	Xiuwen, Guizhou, China	Wangjiapu	This study
16	WT2	AHAU, China	Psa	<i>Paulownia tomentosa</i>	Leaf	2013	Yuexi, Anhui, China	Zhubozhen	This study
17	WT3	AHAU, China	Psa	<i>P. tomentosa</i>	Leaf	2013	Yuexi, Anhui, China	Zhubozhen	This study
18	P1	AHAU, China	Psa	<i>Philagra hexamaculata</i>	Spume	2013	Yuexi, Anhui, China	Zhubozhen	This study
19	P2	AHAU, China	Psa	<i>P. hexamaculata</i>	Spume	2013	Yuexi, Anhui, China	Zhubozhen	This study
20	P3	AHAU, China	Psa	<i>P. hexamaculata</i>	Spume	2013	Yuexi, Anhui, China	Zhubozhen	This study
21	INS	AHAU, China	Psa	<i>Bothrogonia ferruginea</i>	Body	2012	Yuexi, Anhui, China	Zhubozhen	This study
22	JF8	AHAU, China	Psa	<i>A. chinensis</i> 'Jinfeng'	Leaf	2012	Yuexi, Anhui, China	Zhubozhen	This study
23	JF27	AHAU, China	Psa	<i>A. chinensis</i> 'Jinfeng'	Leaf	2012	Yuexi, Anhui, China	Zhubozhen	This study
24	Hwd1	AHAU, China	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	2012	Huxian, Shaanxi, China	Baixiaqu	This study
25	Hwd3	AHAU, China	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	2012	Huxian, Shaanxi, China	Baixiaqu	This study
26	Hwd4	AHAU, China	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	2012	Huxian, Shaanxi, China	Baixiaqu	This study
27	Hwd5	AHAU, China	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	2012	Huxian, Shaanxi, China	Baixiaqu	This study
28	Hwd6	AHAU, China	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	2012	Huxian, Shaanxi, China	Baixiaqu	This study
29	Jzhy2	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Leaf	2013	Jinzhai, Anhui, China	Tanghui	This study
30	Jzhy3	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Leaf	2013	Jinzhai, Anhui, China	Tanghui	This study
31	Jzhy6	AHAU, China	Psa	<i>A. chinensis</i> 'Hongyang'	Leaf	2013	Jinzhai, Anhui, China	Tanghui	This study
32	Jzzm1	AHAU, China	Psa	<i>A. chinensis</i> wild seedlings	Leaf	2013	Jinzhai, Anhui, China	Tanghui	This study
33	Jzzm2	AHAU, China	Psa	<i>A. chinensis</i> wild seedlings	Leaf	2013	Jinzhai, Anhui, China	Tanghui	This study
34	Jzmc1	AHAU, China	Psa	<i>Setaria viridis</i>	Leaf	2013	Jinzhai, Anhui, China	Tanghui	This study
35	Jzmc2	AHAU, China	Psa	<i>S. viridis</i>	Leaf	2013	Jinzhai, Anhui, China	Tanghui	This study
36	Jzgmcl	AHAU, China	Psa	<i>Alternanthera philoxeroides</i>	Leaf	2013	Jinzhai, Anhui, China	Tanghui	This study
37	Jzgmcl	AHAU, China	Psa	<i>A. philoxeroides</i>	Leaf	2013	Jinzhai, Anhui, China	Tanghui	This study
38	7285	CFBP	Psa	<i>Actinidia chinensis</i> 'Jintao'	Leaf	2008	Veneto, Italy	...	DAFNE, UT
39	7286	CFBP	Psa	<i>A. chinensis</i> 'Hort16A'	Leaf	2008	Latina, Italy	...	DAFNE, UT
40	LT19	Balestra	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	2008	Latina, Italy	...	DAFNE, UT
41	LT80	Balestra	Psa	<i>A. deliciosa</i> 'Hayward'	Vine	2008	Latina, Italy	...	DAFNE, UT
42	K3	Y. J. Koh	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	1997	Jeju, Korea	...	DAFNE, UT
43	Kw30	NCPBP 3740	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	1984	Shizuoka, Japan	...	DAFNE, UT
44	349	Balestra	Psa	<i>A. deliciosa</i> 'Summer'	Leaf	2010	Portugal	...	DAFNE, UT
45	3039B	A. Calzolari	Pss	<i>A. chinensis</i>	Leaf	2009	Romagna, Italy	...	DAFNE, UT
46	4254A	A. Calzolari	Pv	<i>A. chinensis</i>	Leaf	2009	Romagna, Italy	...	DAFNE, UT
47	2598	NCPBP	Pst	<i>Thea sinensis</i>	Leaf	1970	Japan	...	DAFNE, UT
48	Psa92	Balestra	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	1992	Latina, Italy	...	DAFNE, UT
49	827	Balestra	Psa	<i>A. chinensis</i> 'Jintao'	Leaf	2011	Galicia, Spain	...	DAFNE, UT
50	830	Balestra	Psa	<i>A. chinensis</i> 'Jintao'	Leaf	2011	Galicia, Spain	...	DAFNE, UT
51	18804	ICMP	Psa	<i>A. chinensis</i>	Leaf	2010	New Zealand	...	DAFNE, UT
52	18839	ICMP	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	2011	New Zealand	...	DAFNE, UT
53	1F	Anses	Psa	<i>A. chinensis</i> 'Jintao'	Leaf	2010	Aquitaine, France	...	DAFNE, UT
54	2F	Anses	Psa	<i>A. deliciosa</i> 'Hayward'	Leaf	2010	Rhone Alpes, France	...	DAFNE, UT

^a Species or pathovar: Psa = *Pseudomonas syringae* pv. *actinidiae*, Pss = *P. syringae* pv. *syringae*, Pst = *P. syringae* pv. *theae*, and Pv = *P. viridiflava*.

^b Isolation tissue.

^c Isolation year.

^d DAFNE = Department of Science and Technologies for Agriculture, Forestry, Nature and Energy.

sequencer according to the manufacturer's instructions. The sequences obtained from the housekeeping genes were submitted to the NCBI database for homologous alignment analysis (Table 3). The phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software MEGA 6.0 (Tamura et al. 2013) based on the minimum-evolution method with the nucleotide sequences to infer the genetic relationships between the tested strains of *P. syringae* pv. *actinidiae* and some known strains in the NCBI database: MAFF 212054, MAFF 212055, and MAFF 212056 (all biovar 5); 212130, 212132, and 212134 (all biovar 6); NCPPB 3739, NCPPB 3871, MAFF 302145, and MAFF 302143 (all biovar 1) from Japan and NCPPB 3873 from Italy (biovar 1); KACC 10584, KACC 10754, and KACC 10594 from Korea (all biovar 2); CRA-FRU 10.22 from Italy, Psa1A and Psa1B

from Chile, T11-0918, T10-04782, and T10-05454 from New Zealand (all biovar 3); T11-01369A from Australia and T10-05195, T10-05163, and T10-04976 from New Zealand (all biovar 4, *P. syringae* pv. *actinidifoliorum*) were used as the positive controls for each biovar.

Detection of phytotoxin and effector genes. Presence of phytotoxin gene clusters (the *tox-argK* gene cluster coding for phaseolotoxin and the *cfl* gene coding for coronatine) was performed for all 51 strains of *P. syringae* pv. *actinidiae* described above and was checked by PCR amplification, which was performed as described in previous studies (Bereswill et al. 1994; Sawada et al. 2002). The presence of 11 effector genes (*hopA1*, *hopH1*, *hopAF1*, *hopAA1-1*, *hopAA1-2*, *avrD1*, *hopB1*, *hopD1*, *hopAM1*, *hopAH1*, and *hopX1*) was checked for by PCR

TABLE 2. Primers used for the polymerase chain reaction (PCR) assays in this study and their efficiency for differentiation of strains of *Pseudomonas syringae* pv. *actinidiae* infecting kiwifruit

Method, primer ^a	Sequence	References	Annealing temperature (°C) ^b	Total bands amplified ^b	Polymorphic bands	Differential bands (%) ^c
Rep-PCR						
ERIC1R	5'-ATGTAAGCTCCTGGGGATTCA-3'	Weingart and Volksch 1997	52	11	4	63.6
ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'	...	...	...	...	...
BOXA1R	5'-CTACGGCAAGCGACGCTGACG-3'	...	53	10	5	50.0
(GTG) 5	5'-GTGGTGGTGGTGGTG-3'	...	45	11	8	27.3
RAPD-PCR						
OPA-13	5'-CAGCACCCAC-3'	Mazzaglia et al. 2011	45	10	3	70.0
A-24	5'-CTCCTGCTGTTG-3'	...	45	9	7	22.2
A-29	5'-GGTTCGGGAATG-3'	...	45	18	0	100
A-44	5'-GACGGTTCAAGC-3'	...	41	14	0	100
C-24	5'-CCTTGGCATCGG-3'	...	45	8	3	62.5
C-44	5'-CGCAGCCGAGAT-3'	...	45	13	3	76.9
OLD-F	5'-CACGATACATGGGCTTATGC-3'	...	45	14	1	92.9
OLD-R	5'-CTTTTCATCCACACTCCG-3'	...	45	13	1	92.3
IS50-PCR						
IS50	5'-GGTTCGGTTCAGGACGCTAC-3'	Weingart and Volksch 1997	45	14	10	28.6

^a ERIC = enterobacterial repetitive intergenic consensus, RAPD = random amplified polymorphic DNA, and IS50 = insertion sequences 50.

^b Total and polymorphic bands amplified for each primer refer to only the 51 strains of *Pseudomonas syringae* pv. *actinidiae*.

^c Percentages of the differential bands = [(total bands – polymorphic bands)/total bands] × 100%.

TABLE 3. Accession numbers of the five housekeeping genes *cts*, *gapA*, *gyrB*, *pfk*, and *rpoD* of the 24 representative strains of *Pseudomonas syringae* pv. *actinidiae* used in this study and deposited in the GenBank database of the National Center for Biotechnology Information

Number	Strain	<i>cts</i>	<i>gapA</i>	<i>gyrB</i>	<i>pfk</i>	<i>rpoD</i>
1	Schy9	KM896669	KP297246	KP297265	KP297284	KP297303
2	GC31	KM896670	KP297247	KP297266	KP297285	KP297304
3	SH8	KM896671	KP297248	KP297267	KP297286	KP297305
4	JF8	KM896672	KP297249	KP297268	KP297287	KP297306
5	INS	KM896673	KP297250	KP297269	KP297288	KP297307
6	P1	KM896674	KP297251	KP297270	KP297289	KP297308
7	Hwd3	KM896660	KP297252	KP297271	KP297290	KP297309
8	Jzzm2	KM896661	KP297253	KP297272	KP297291	KP297310
9	Jzhy2	KM896656	KP297254	KP297273	KP297292	KP297311
10	Jzhy14	KM896659	KP297255	KP297274	KP297293	KP297312
11	K3	KM896662	KP297256	KP297275	KP297294	KP297313
12	349	KM896663	KP297257	KP297276	KP297295	KP297314
13	830	KM896665	KP297258	KP297277	KP297296	KP297315
14	827	KM896664	KP297259	KP297278	KP297297	KP297316
15	1F	KM896666	KP297260	KP297279	KP297298	KP297317
16	18804	KM896668	KP297261	KP297280	KP297299	KP297318
17	Jzhy6	KM896657	KP297262	KP297281	KP297300	KP297319
18	Jzhy7	KM896658	KP297263	KP297282	KP297301	KP297320
19	Psa92	KM896667	KP297264	KP297283	KP297302	KP297321
20	Jzmc1	KJ855759	KP100046	KP100031	KP100036	KP100041
21	Jzgmc2	KJ855760	KP100047	KP100032	KP100037	KP100042
22	WT3	KJ855761	KP100048	KP100033	KP100038	KP100043
23	7285	KJ855762	KP100049	KP100034	KP100039	KP100044
24	18839	KJ855763	KP100050	KP100035	KP100040	KP100045

amplification using gDNA of 24 representative strains following the protocol described by Ferrante and Scortichini (2009, 2010, and 2011). Primers were synthesized by the Sengen Co., Ltd. (Table 4). A constellation diagram was constructed based on presence or absence data for the four loci coding phytotoxins and 11 effector genes based on the Ward method using JMP Software for Windows, version Pro 14.

RESULTS

Strains of *P. syringae* pv. *actinidiae* from different geographic regions in China. All 269 isolates collected had the biochemical characteristics of *P. syringae* pv. *actinidiae*; that is, they were not or were only slightly fluorescent on King's B medium, they induced an HR when infiltrated into tobacco plants but did not cause potato rot, they did not produce syringomycin or induce ice nucleation, and they did not have a cytochrome c oxidase or an arginine dehydrolase. They all produced levan.

Unique fragment produced by PCR. Most strains of *P. syringae* pv. *actinidiae* had a similar fingerprint pattern generated by Rep-PCR using primer BOXA1R, with the exception of the five strains GC31, GC32, GC40, GC41, and GC42 (Supplementary Figure S2) from Guizhou Province, which had a unique fragment of approximately 1,500 bp. In addition, strains K3 and Kw30 from Korea and Japan, respectively, possessed a unique fragment of approximately 300 bp, and strains K3, Kw30, and Psa92 from Italy lacked a polymorphic fragment of approximately 1,100 bp.

The RAPD-PCR primer A-24 produced nine amplicons but the percentage of differential bands was only 22.2% and there was a distinctive 3,000-bp amplicon that was present only with strain 18804 and a second distinctive 1,800-bp amplicon with strains SH2, SH8, SH13, SH18, and SH26 from Shanghai; strains WT2 and WT3 from Yuexi County; and strains Jzhy2, Jzhy3, and Jzhy6 from Jinzhai County.

According to the amplicon pattern generated by IS50-PCR, the strains of *P. syringae* pv. *actinidiae* from Asia, including those from China, Korea, and Japan, had a similar pattern which was similar to the amplicon patterns for strains from Portugal and Italy, except for Psa92. However, the strains of *P. syringae* pv. *actinidiae* from Spain and France had a pattern similar to the strain of *P. syringae* pv. *actinidiae* with high virulence (*P. syringae* pv. *actinidiae* 18839) from New Zealand, and there was a distinctive pattern produced by strain *P. syringae* pv. *actinidifoliorum* 18804, a low-virulence isolate from New Zealand.

Different PCR components to differentiate populations of *P. syringae* pv. *actinidiae*. The amplification of the gDNA of the 54 selected bacterial strains (51 strains of *P. syringae* pv. *actinidiae*), followed by gel electrophoresis of the resulting PCR products, showed that polymorphic amplicons accounted for 34.5% of the 145 total amplicons assessed.

The percentage of differential bands in Rep-, RAPD-, and IS50-PCR was 47.0, 77.1, and 28.6%, respectively. This indicates that RAPD-PCR is the most efficient for the differentiation of populations of *P. syringae* pv. *actinidiae* but the stability of amplicons produced was not always consistent.

With Rep-PCR, primer ERIC1R-ERIC2, BOXA1R, and (GTG) 5 generated 11, 10, and 11 fragments, respectively, and the percentage of differential bands was 63.6, 50.0, and 27.3%, respectively, which indicated that ERIC1R-ERIC2 would be a more efficient primer for differentiating strains of *P. syringae* pv. *actinidiae* using Rep-PCR.

Genetic relationship of populations of *P. syringae* pv. *actinidiae* from China and other countries. In total, 145 discrete amplicons were generated with the 51 bacterial strains tested from eight countries (Japan, South Korea, Italy, France, Portugal, Spain, New Zealand, and China) using the three methods of PCR. The strains could be discriminated into distinct four groups—Psa1 from Japan and Italy; Psa2 from South Korea; Psa3 from Europe, New Zealand,

TABLE 4. Primer sets used in this study to detect effector and phytotoxin genes of *Pseudomonas syringae* pv. *actinidiae*

Gene, primer name	Primer sequence	Reference
Effector gene		
<i>hopAF1-F</i>	5'-CAAGCAGAAAGACGGCATC-3'	Ferrante and Scortichini 2009
<i>hopAF1-R</i>	5'-GCACACGCGACAGCAATG-3'	Ferrante and Scortichini 2009
<i>hopA1-F</i>	5'-CGGCAAGAGGTACGAGATTC-3'	Ferrante and Scortichini 2010
<i>hopA1-R</i>	5'-TTCAATGCCCTTAGCGGTGTG-3'	Ferrante and Scortichini 2010
<i>avrD1-F</i>	5'-CGATGGTTTCAAACATGTGG-3'	Ferrante and Scortichini 2010
<i>avrD1-R</i>	5'-TGTGCGTGACTGACAAGTGA-3'	Ferrante and Scortichini 2010
<i>hopB1-F</i>	5'-AGGCTATTATCCGCCAACCT-3'	Ferrante and Scortichini 2010
<i>hopB1-R</i>	5'-TCTTGCAACAGGATGCTCAC-3'	Ferrante and Scortichini 2010
<i>hopD1-F</i>	5'-GTTACTGAGCTGCCAGACC-3'	Ferrante and Scortichini 2010
<i>hopD1-R</i>	5'-TGGTGGCTACATGCAACAAT-3'	Ferrante and Scortichini 2010
<i>hopH1-F</i>	5'-CGTCTCGATATCCAGGCATC-3'	Ferrante and Scortichini 2011
<i>hopH1-R</i>	5'-TTCAGCTCGGATGGAGTTCT-3'	Ferrante and Scortichini 2011
<i>hopAA1-1-F</i>	5'-GATAAATGCGATTGCCGAT-3'	Ferrante and Scortichini 2011
<i>hopAA1-1-R</i>	5'-GAGCCTTGGGCTCTTTATCC-3'	Ferrante and Scortichini 2011
<i>hopAA1-2-F</i>	5'-TGCATTACCTGAGCACTTCG-3'	Ferrante and Scortichini 2011
<i>hopAA1-2-R</i>	5'-TGACTGTTTTTCGCCAGTTTG-3'	Ferrante and Scortichini 2011
<i>hopAM1-F</i>	5'-CCAAGTTAAATCGGCAGGAA-3'	Ferrante and Scortichini 2011
<i>hopAM1-R</i>	5'-TCAGGTCAACGCTATTTTCG-3'	Ferrante and Scortichini 2011
<i>hopAH1-F</i>	5'-AGTCCGACTTCAGGCTCAAA-3'	Ferrante and Scortichini 2011
<i>hopAH1-R</i>	5'-CGTCACGCTGTTTCAAGTTA-3'	Ferrante and Scortichini 2011
<i>hopX1-F</i>	5'-CTTGCCCGGCTACTTACTG-3'	Ferrante and Scortichini 2011
<i>hopX1-R</i>	5'-CGGACAAATGCATCATCAAG-3'	Ferrante and Scortichini 2011
Phaseolotoxin		
<i>PHA19</i>	5'-CGTCTGTAACCAAGTTGATCC-3'	Marques et al. 2000
<i>PHA95</i>	5'-GAATCCTTGAATGCGAAGGC-3'	Marques et al. 2000
<i>HM6</i>	5'-CGTGTCCGTGGATAAAAGC-3'	Marques et al. 2000
<i>HM13</i>	5'-GTTGAATTTCACTACCCG-3'	Marques et al. 2000
<i>OCTF-03</i>	5'-GACCGTCAAGGAAGAATTCGGGCGC-3'	Sawada et al. 2002
<i>OCTR</i>	5'-CGACCTTGTTGACCTCCCG-3'	Sawada et al. 2002
Coronatine		
<i>CFL-F</i>	5'-GGCGCTCCCTCGCACTT-3'	Bereswill et al. 1994
<i>CFL-R</i>	5'-GGTATTGGCGGGGGTGC-3'	Bereswill et al. 1994

and China; and Psa4 from New Zealand—of which the strains had been reassigned to a new pathovar, *P. syringae* pv. *actinidifoliorum*. All strains of *P. syringae* pv. *actinidiae* from China belong to Psa3. The results indicate that strain K3 from South Korea, which was thought to be a member of Psa2, has closer genetic affinity to Psa1 strain Kw30 from Japan (Fig. 1).

PCA revealed that a relatively small portion of the total variance contributed to the first two PCs (PC-1 = 28.1% and PC-2 = 16.7%). There were four distinctive subgroups (subgroups 1 to 4) in the worldwide group of Psa3 strains; three of the groups came from China (subgroups 1 to 3); strain 349 from Portugal; and strains 7285, 7286, LT19, and LT80 from Italy that were isolated in 2008 and were in the same subgroup 3 as strains from China, implying that they might have the same ancestor. The virulent strain 18839 from New Zealand was in subgroup 4, with strains 827 and 830 from Spain and strains F1 and F2 from France, indicating a close genetic relationship (Fig. 2A).

However, the first four PCs (PC-1 to -4) accounted for 68.9% of the overall variance defining genetic relationships among strains of Psa3 populations worldwide. The plots of PC-1 versus PC-3 or PC-1 versus PC-4 show that strains from China have a broad genetic variability and plots of PC-2 versus PC-3 or PC-2 versus PC-4 show that the strains from China and strains from Europe and New Zealand have an independent evolutionary direction (Fig. 2B).

Furthermore, the ANOVA showed that significant differences were found for PC-2 and PC-4 but not for PC-1 or PC-3. Strains from China are genetically different from strains from France but are not different from strains from Italy (Supplementary Table S2).

A greater diversity in populations of Psa3 from China was found compared with that previously observed (Cunty et al. 2015a; Liu et al. 2016; McCann et al. 2017). Cluster analysis showed that the strains in clade I were from Xiuwen County in Guizhou Province (GC31-32 and GC40-42); the strains in clade II were from kiwifruit seedling rootstock (JZZM1-2) and two nonkiwifruit species, *S. viridis* (JZMC1-2) and *A. philloxeroides* (JZGMC1-2); the strains in clade III were primarily from kiwifruit cultivar Hongyang in Jinzhai County, Anhui Province (JZHY2, -3, and -6) and Jiading District in Shanghai municipality (SH2-3, -13, -18, and -28), and from *Paulownia tomentosa* near a kiwifruit orchard in Yuexi County, Anhui Province (WT2-3); and the strains in clade IV had a wide range of cultivar sources and geographic region origins, including Hongyang kiwifruit in Pengzhou County, Sichuan Province (G3-5, -8, and -12), kiwifruit cultivar Jinfeng (JF8 and J27) and two insect species, *Philagra hexamaculata* (P1-3) and *B. ferruginea* (IN5), from an orchard in Yuexi County, Anhui Province, and kiwifruit cultivar Hayward in Huxian County, Shaanxi Province (Hwd1 and -3 to -6). These results indicate that each province in China has unique and shared strains; indeed,

Fig. 1. Discriminant analysis of principal components (DAPC) of strains of *Pseudomonas syringae* pv. *actinidiae* collected from different countries based on a combined data set from different polymerase chain reaction (PCR) primers (Rep, insertion sequences 50, and random amplified polymorphic DNA PCR assays). Scatterplots show the first two canonical variables of the DAPC for the strains of *P. syringae* pv. *actinidiae* according to a linear model. Strain type groups are indicated by the double-lined circles, while the markers represent individual strains from different countries. Bacterial strains could be discriminated into four distinct groups: Psa1 (biovar 1) from Japan and Italy; Psa2 (biovar 2) from South Korea; Psa3 (biovar 3) from Europe, New Zealand, and China; and Psa4 (biovar 4) from New Zealand (the strains of this group had been reassigned as a new pathovar, *P. syringae* pv. *actinidifoliorum*). All strains of *P. syringae* pv. *actinidiae* from China belong to the Psa3 group. Strain K3 from South Korea has a closer genetic relationship with that of Psa1 from Japan.

strains from Anhui Province not only had indigenous strains in clade II but also shared strain types with Shanghai Sichuan, and Shaanxi Provinces in clade III and clade IV (Fig. 3).

MLST by housekeeping genes. Sequences of the five housekeeping genes *cts*, *gapA*, *gyrB*, *pfk*, and *rpoD* from the 24 representative strains were cloned and submitted to GenBank for homological alignment analysis. All 120 multilocus sequences were analyzed, deposited in the NCBI database, and assigned accession numbers (Table 3).

Fig. 2. Principal components analysis (PCA) of strains of *Pseudomonas syringae* pv. *actinidiae* collected from different countries based on a combined data set from polymerase chain reaction (PCR) assays using primers for Rep, insertion sequences 50, and random amplified polymorphic DNA PCR assays. Scatterplots show the first two principal components (PCs) of the PCA for the strains of Ps3 according to the covariance model. **A**, Strain subgroups (Subgr) are shown in different ellipses, with each marker representing individual strains. Strains were discriminated into four subgroups. **B**, The first two PCs had a relatively small contribution (44.8%) and the first four PCs accounted for just 68.9% of the total variance.

The MLST revealed that the genetic variability among the strains of *P. syringae* pv. *actinidiae* differed from that resulting from the different PCR assays. The phylogenetic tree constructed by the combined MLST data showed that the 24 strains of *P. syringae* pv. *actinidiae* had distinct multilocus sequence profiles but there was clustering of strains from different biovars or MLST groups (biovars 1 to 3, *P. syringae* pv. *actinidifoliorum*, and biovars 5 and 6), compared with the 25 previously characterized strains in the NCBI database used as positive controls for biovar IDs and strain DC3000 of *P. syringae* pv. *tomato*, which was used as an outgroup.

All of the strains of *P. syringae* pv. *actinidiae* from China, with the exception of strain Jzhy2, were in the same group as strain 7285 from Italy (cause of the KBC outbreak since 2008); strains 18839, T11-0918, T10-04782, and T10-05454 from New Zealand; strains 827 and 830 from Spain; strain 349 from Portugal; and strain 1F from France, all of which were classified as biovar 3 (Psa3). Only strain Jzhy2 from China was differentiated in an independent clade apart from the existing biovars or MLST groups. Interestingly, strains MAFF 212054, MAFF 212055, and MAFF 212056 (newly identified strains from Japan in biovar 5) had a closer genetic relationship to KACC 10584, KACC 10754, and KACC 10594 from Korea, all in biovar 2 (Psa2); and strains in biovar 6 were genetically closer to biovar 1. In contrast, K3 isolated in 1997 from Jeju, Korea clustered with strains Kw30, NCPPB 3739, NCPPB 3871, MAFF 302145, and MAFF 302143 from Japan and Psa92 from Italy and were classified as biovar 1 or Psa1 (Fig. 4).

Fig. 3. Constellation plot of strains of *Pseudomonas syringae* pv. *actinidiae* from China constructed by hierarchical cluster analysis using combined data from multiple polymerase chain reaction (PCR) fingerprinting with different primers for Rep, insertion sequences 50, and random amplified polymorphic DNA PCR assays. Strains in clade I were from Xiuwen County, Guizhou Province (GC31-32 and GC40-42); strains in clade II were from a seedling rootstock of kiwifruit (JZZM1-2) and two nonkiwifruit species, *Setaria viridis* (JZMC1-2) and *Alternanthera philoxeroides* (JZGMC1-2). Strains in clade III were primarily from Hongyang kiwifruit from Jinzhai County, Anhui Province (JZHY2, -3, and -6) and Jiading District in Shanghai municipality (SH2-3, -13, -18, and -28), and from *Paulownia tomentosa* growing near a kiwifruit orchard in Yuexi County, Anhui Province (WT2-3). Strains in clade IV had extensive cultivar sources with different geographic regions: from kiwifruit Hongyang in Pengzhou County, Sichuan Province (Schy3 to -5, -9, and -12); cultivar Jinfeng (JF8 and J27), and two insect species collected in kiwifruit orchards, *Phylagra hexamaculata* (P1-3) and *Bothrogonia ferruginea* (INS), in Yuexi County, Anhui Province; and kiwifruit cultivar Hayward in Huxian County, Shaanxi Province (Hwd1 and -3 to -6).

Strain 18804 of *P. syringae* pv. *actinidifoliorum* was clustered with T11-01369A from Australia and T10-05195, T10-05163, and T10-04976 from New Zealand, which had low virulence, and was in a clade distant from all strains in biovars 1 to 3 and biovars 5 and 6. Strain DC3000 of *P. syringae* pv. *tomato* was an outgroup.

Genetic variation in phytotoxin and effector genes. All of the tested strains, including K3 from Jeju, South Korea, lacked the *cfl* gene coding for coronatine. In contrast, the *tox-argK* gene cluster coding for phaseolotoxin was detected in strains K3 and Ps92 (Supplementary Table S3). This is the first report that at least some strains of *P. syringae* pv. *actinidiae* from South Korea have the *tox-argK* gene cluster coding for phaseolotoxin.

The constellation diagram showed that the strains from China formed two clades (clade 1 and clade 2); the strains from Europe and the virulent strain from New Zealand were in clade 2. However, the low-virulence strains from New Zealand were in an independent clade (clade 3), strains of which are now considered a new pathovar, *P. syringae* pv. *actinidifoliorum*; the biovar 6 strains from Japan were grouped in clade 4, closer to biovar 1; strains K3, Kw30, and Ps92 clustered in clade 5 (biovar 1); the strains from Japan grouped originally in biovar 5 grouped in clade 6, closer to biovar 2; and those strains in biovar 2 clustered in clade 7 (Fig. 5).

Strains K3, Kw30, and Ps92 in biovar 1 lacked both the amplified 444-bp fragment for effector gene *hopA1* and the 605-bp fragment for effector gene *hopH1*. Strain *P. syringae* pv.

actinidifoliorum 18804 (former biovar 4) produced an amplicon for *hopA1* but not for *hopH1*; and the remaining strains in biovar 3 had amplicons for both *hopA1* and *hopH1* genes, except for strains Jzhy2 and Jzhy6, which only produced amplicons for *hopH1* but lacked the *hopA1* gene (Supplementary Fig. S3).

It was notable that, of all the strains (Jzhy1 to -16) yielding an amplicon for *hopH1*, only five (Jzhy7, Jzhy13, Jzhy14, Jzhy15, and Jzhy16) yielded an amplicon for *hopA1*. Although the 16 strains were isolated from the same host cultivar (*Actinidia chinensis* 'Hongyang') in the same orchard (Jinzhai County, Anhui Province, China), 11 strains (Jzhy1 to -6 and Jzhy8 to -12) belonged to a new genotype that differed from the existing biovars or MLST groups according to the combination of the *hopA1* and *hopH1* effector genes.

With the exception of strain 18804, a common amplicon was found in the 24 representative strains screened for *hopA1*. Sequence analysis showed that strains K3, Kw30, and Ps92 had a longer amplicon (1,784 bp) compared with that produced for the *hopA1* sequence from other strains (701 bp).

DISCUSSION

PCR assay and MLST. The genetic variability and relationships among the strains of *P. syringae* pv. *actinidiae* characterized by the PCR assays differed slightly from that characterized by MLST: the PCR was more sensitive compared with the MLST in the discrimination of the *P. syringae* pv. *actinidiae* populations. According to the results of the PCR assay, the variation among strains of *P. syringae* pv. *actinidiae* from China was related to the geographic source location. Each of the geographic regions had unique strain variants of *P. syringae* pv. *actinidiae*; for example, strains in clade I were from Xiuwen County in Guizhou Province,

Fig. 4. Phylogenetic tree obtained from the combined sequence data of the five housekeeping genes *cts*, *gapA*, *gyrB*, *pfk*, and *rpoD*. The phylogenetic tree was constructed using MEGA 6.0 software and the minimum-evolution method. Bootstrap confidence values are indicated at each node and the scale bar indicate the genetic distance. The 25 known strains from the National Center for Biotechnology Information database were used as positive controls for biovar identity, and strain DC3000 of *Pseudomonas syringae* pv. *tomato* was used as an outgroup.

Fig. 5. Constellation plot of strains of *Pseudomonas syringae* pv. *actinidiae* from different countries constructed by hierarchical cluster analysis using the amplicon data for four loci coding phytotoxins and 11 effector genes. Note: The strains of *P. syringae* pv. *actinidiae* from China formed two distinct clades, clade 1 and clade 2, the European strains and virulent strain from New Zealand merged with clade 2; the low-virulence strains from New Zealand are transferred to a new pathovar, clade 3 (*P. syringae* pv. *actinidifoliorum*); and the new biovar 6 representing a strain from Japan was grouped in clade 4, closer to biovar 1; strains K3, Kw30, and Ps92 clustered as clade 5 (biovar 1); the remaining strains in biovar 5 from Japan formed clade 6, closer to biovar 2; and strains in biovar 2 clustered in clade 7.

clade II from Yuexi County in Anhui Province, clade III mainly from Jiading District in Shanghai, and clade IV mainly from Weixian in Shaanxi Province.

However, in some cases, RAPD-PCR has been reported to lack reproducibility (Tyler et al. 1997). Furthermore, with Rep-PCR, the same reaction performed at different laboratories may result in different profiles for the same or similar strains of *P. syringae* pv. *actinidiae* (Ferrante and Scortichini 2011; Mazzaglia et al. 2011). In our study, to ensure the reliability of the results, we selected reliable primers, used high-fidelity *Taq* polymerase, used a precise thermal cycler, and repeated the reactions to confirm reproducibility of amplification.

The RAPD method used in this investigation was able to discriminate strains of *P. syringae* pv. *actinidiae* at and below the level of biovar, and the discriminating power of this method has been confirmed by others (Bereswill et al. 1994; Koh et al. 2014; Mazzaglia et al. 2011). The RAPD method is widely used due to ease of operation, although reproducibility within and between laboratories has been questionable, perhaps due to the method's dependence on the type of *Taq* polymerase and thermal cycler used or other unidentified reasons (Van De Zande and Bijlsma 1995). On the other hand, in some situations, RAPDs can be reliable and reproducible. In our study, the method provided reproducible results to explore genetic variability when no sequence data were available for the genome in question.

Genetic diversity of populations of *P. syringae* pv. *actinidiae* in China. DAPC based on the combined PCR dataset demonstrated that the genetic variability in the populations of *P. syringae* pv. *actinidiae* from China was related to their geographic origin. Previously, and with the exception of strain Jzhy2 of *P. syringae* pv. *actinidiae*, only biovar 3 had been found in China (Ciarroni et al. 2015; Cuntly et al. 2015a; McCann et al. 2017). Although all of the strains of *P. syringae* pv. *actinidiae* from China belong to biovar 3, they have the broadest genetic diversity and can be divided into four distinct lineages (clades I to IV), as described in this study. Some of the *P. syringae* pv. *actinidiae* strains from China (Jzhy1 to -6 and Jzhy8 to -12) lacked the *hopA1* and *hopH1* effector genes. The presence of both of these genes is thought to be unique to strains of *P. syringae* pv. *actinidiae* causing epidemics of KBC on *A. chinensis* and *A. deliciosa* in Italy (Ferrante and Scortichini 2011) and New Zealand (Vanneste et al. 2013).

Sequence data from the five housekeeping genes uniquely identified strain Jzhy2 as biovar 3. Four of the housekeeping genes (*cts*, *gapA*, *gyrB*, and *pfk*) in strain Jzhy2 were identical to the other strains of biovar 3 but the sequence of *rpoD* in strain Jzhy2 had a 1-base change at five different positions of the locus sequence, resulting in an amino acid sequence change. Currently, we are conducting a genome sequencing approach of strains of *P. syringae* pv. *actinidiae* representative of the worldwide collection to reveal whether strain Jzhy2 belongs to a novel biovar of *P. syringae* pv. *actinidiae*.

Wild kiwifruit infected with *P. syringae* pv. *actinidiae* has not been found in China, except for the one occurrence near the KBC-diseased orchard described in the current study. However, a strain of *P. syringae* pv. *actinidiae* was isolated from wild growing plants of *A. arguta* and *A. kolomikta* in Japan in 1987 to 1988 (Ushiyama et al. 1992). Thus, only one biovar of *P. syringae* pv. *actinidiae* has been characterized in China (biovar 3), despite China being recognized as a center of diversity and, thus, of origin for most species of kiwifruit (Ferguson and Huang 2007). This result may be irreconcilable with the prevailing expectation that more biovars of *P. syringae* pv. *actinidiae* should be present in China.

Genetic variation among the global populations of *P. syringae* pv. *actinidiae*. Environmental variables other than the host can be expected to influence variability of a plant pathogen (Morris et al. 2009), and producing a few cultivars at a high density can enhance the risk of an epidemic or pandemic of KBC caused by a one or a few specific bacterial pathovars (McCann et al. 2013). The genetic

variation in *P. syringae* pv. *actinidiae* makes transport of host material a possible source of novel strains in new areas, and phytosanitary regulation of the species a priority for consideration. Thus, exchange of kiwifruit plant material has resulted in transport of the pathogen and resulting epidemics of KBC in Italy (Balestra et al. 2018) and South Korea (Koh et al. 2014). Furthermore, some strains isolated from orchards in Japan in 1987 and 1988 where streptomycin had been routinely applied were found to be streptomycin resistant (Nakajima et al. 1995), and copper-resistant strains of *P. syringae* pv. *actinidiae* were isolated from orchards where copper-based bactericides are used to control bacterial canker caused by *P. syringae* pv. *actinidiae* in Japan (Nakajima et al. 2002), Italy (Petriccione et al. 2017), and New Zealand (Colombi et al. 2017). Indeed, these issues regarding the variability and adaptability of *P. syringae* pv. *actinidiae* have raised questions regarding the reliability of long-term strategies to control KBC solely with copper compounds or antibiotics, and have clear ramifications for transport of plant material between kiwifruit production areas.

Diversity can be related to the geographic origin of a pathogen. Analysis of multiple loci variable number of tandem repeats analysis (MLVA) in *P. syringae* pv. *actinidiae* showed broad genetic variability but different strains were also retrievable in Japan and Korea (Ciarroni et al. 2015). Compared with strains collected in 2010, results from Rep-PCR and RAPD analysis revealed a high level of variability in the population of *P. syringae* pv. *actinidiae* collected from northern Italy in 2014. Indeed, at least two biovars (biovar 3 and *P. syringae* pv. *actinidifoliorum* biovar 4) of *P. syringae* pv. *actinidiae* had been found in New Zealand (Vanneste et al. 2013), France (Cuntly et al. 2015a), and Spain (Abelleira et al. 2015); biovars 1, 3, 5, 6, and *P. syringae* pv. *actinidifoliorum* (biovar 4) were found in Japan (Sawada et al. 2014; 2015, 2016, 2017); biovars 1, 2, and 3 were found in Korea (Koh et al. 2014; McCann et al. 2017); and biovars 1 and 3 were found in Italy (Ferrante and Scortichini 2015). However, all of the strains of *P. syringae* pv. *actinidiae* from China were grouped in biovar 3 by MLST, with the exception of strain Jzhy2. The coexistence of many types of *P. syringae* pv. *actinidiae* in both Japan and Korea suggested that they are the origin and center of diversity of *P. syringae* pv. *actinidiae*, rather than China (McCann et al. 2017). The identification of new strains in Japan (Sawada et al. 2016, 2017) and Italy (Marcelletti et al. 2011) supports the assertion that variants may continue to emerge and cause local epidemics and, possibly, pandemics (Vanneste 2017). However, all strains from New Zealand share several single-nucleotide polymorphisms that distinguish them from all other strains of *P. syringae* pv. *actinidiae*. Similarly, all strains of *P. syringae* pv. *actinidiae* that caused the epidemic in Italy form a distinct clonal group, and those from Chile form a third group (Butler et al. 2013). MLVA and DAPC revealed that strains isolated in Chile, China, and New Zealand are genetically distinct from strains of *P. syringae* pv. *actinidiae* isolated in France and in Italy, which appear to be closely related at the genetic level (Cuntly et al. 2015a). Another MLVA based on 13 variable number tandem repeat loci indicated that strains of *P. syringae* pv. *actinidiae* from Italy showed very limited diversity, with strains from China having broader genetic variability and those from Japan and Korea being genetically the most heterogeneous group (Ciarroni et al. 2015).

In this study, the strains from Japan (MAFF 212054, MAFF 212055, and MAFF 212056) that were clustered as the novel biovar 5 also clustered with strains of biovar 2 on the phylogenetic tree constructed using the combined sequence data of the five housekeeping genes, although the reported strains of biovar 5 had neither the coronatine nor the phaseolotoxin biosynthetic genes conserved in biovar 2 (Sawada et al. 2014). Thus, the strains of biovar 5 causing KBC in Japan were most closely related to biovar 2 according to the average nucleotide identity assay (Fujikawa and Sawada 2016). Strains of biovar 3 also were isolated in Japan but, among 22 strains, 14 possessed Pac_ICE1 and the remaining 8 had no homologs of Pac_ICE1, Pac_ICE2, or Pac_ICE3 (Sawada et al.

2015). Recently, another novel biovar (biovar 6) of *P. syringae* pv. *actinidiae* causing KBC in Japan was found, and that pathogen did not possess *hopH1*, *hopH3*, or *hopZ5* genes yet produced both phaseolotoxin and coronatine (Sawada et al. 2016).

Moreover, we found that strain K3 from Jeju, Korea (thought to be *P. syringae* pv. *actinidiae* biovar 2) had the *tox-argK* gene cluster coding for phaseolotoxin, lacked the *cfl* gene coding for coronatine, and clustered in biovar 1 with strains from Japan (Kw30, NCPPB 3739, and MAFF 302145) and Italy (Psa92 and NCPPB 3873) based on MLST of housekeeping genes and the phytotoxin and effector genes. This observation supports prior results based on sequenced genomes of *P. syringae* pv. *actinidiae* that strain K3 from South Korea grouped with the Japanese isolates in biovar 1 (McCann et al. 2017). It should be emphasized that clades 1 to 7 described in this study used presence of phytotoxin and effector genes to discriminate biovars, and strains of biovar 3 clustered in clades 1 and 2, which are different from the pandemic and nonpandemic clades previously proposed by genome sequencing analysis (McCann et al. 2017).

Strains that cluster in the same clade likely have a common origin, and a number of strains from different geographic regions in the same cluster demonstrate that they should have common ancestry. Nevertheless, long-term and long-distance transmission of strains of *P. syringae* pv. *actinidiae* is due primarily to the introduction of scions, seedling rootstocks, and pollen of kiwifruit. The strain types of *P. syringae* pv. *actinidiae* present in different continents was not consistent with the geographic locations of Europe, Asia, or Oceania due to the complex routes for transmission of *P. syringae* pv. *actinidiae* that have resulted in the introduction and reintroduction of the pathogen worldwide where kiwifruit is cultivated. Migration from kiwifruit to nonkiwifruit species or transmission by insects may also have had a profound effect on the observed genetic variation of *P. syringae* pv. *actinidiae* (Liu et al. 2016).

ACKNOWLEDGMENTS

We thank Y. Bing (Tangsheng Kiwifruit Company Ltd., Jinzhai County, Anhui, China) and Z. Chao (EF, AHU, Hefei, China) for their helpful assistance in field sampling, and C. Watkins (Cornell University, Ithaca, NY, USA) for helpful suggestions and language revisions of the article.

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