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1 # Genetic diversity of *Pseudomonas syringae* pv. *actinidiae* strains from different geographic
2 regions in China

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16 ## ABSTRACT

17 *Pseudomonas syringae* pv. *actinidiae* (Psa) causes kiwifruit bacterial canker (KBC), with severe
18 infection of the kiwifruit plant resulting in heavy economic losses. Little is known regarding the
19 biodiversity and genetic variation of populations of Psa in China. A collection of 269 strains of Psa
20 were identified from 300 isolates obtained from eight sampling sites in five provinces in China. The
21 profiles of 50 strains of Psa and one strain of *P. syringae* pv. *actinidifoliorum* (Psaf) were
22 characterized by Rep-, IS50-P, and RAPD-PCR. Discriminant analysis of principal coordinates

23 (DAPC), principal component analysis (PCA), and hierarchical cluster analysis were used to analyze
24 the combined fingerprints of the different PCR assays. The results revealed that all isolates belonged
25 to the Psa3 group, that strains of Psa from China have broad genetic variability that was related to
26 source geographic region, and that Chinese strains can be readily differentiated from strains from
27 France, but are very similar to those from Italy. Multilocus sequence typing (MLST) of 24
28 representative isolates using the concatenated sequences of five housekeeping genes (*cts*, *gapA*, *gyrB*,
29 *pfk*, and *rpoD*) demonstrated that strain Jzhy2 from China formed an independent clade compared to
30 the other biovars, which possessed the *hopH1* effector gene, but lacked the *hopA1* effector gene. A
31 constellation analysis based on the presence or absence of the four loci coding for phytotoxins and a
32 cluster analysis based on the eleven effector genes showed that strains from China formed two
33 distinct clades. All of the strains, including K3 isolated in 1997 from Jeju, Korea, lacked the *cfl* gene
34 coding for coronatine. In contrast, the *tox-argK* gene cluster coding for phaseolotoxin was detected
35 in K3 and in the Biovar1 strains (K3, Kw30, and Psa92), and produced a false positive amplicon for
36 the *hopAM1*-like gene in this study. To date, only one biovar (Biovar3) is represented by the strains
37 of Psa from China, despite China being the center of origin for the kiwifruit.

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40 Kiwifruit (*Actinidia chinensis* Planchon; *A. deliciosa* Liang et Ferguson) is an important fruit crop,
41 with primary production in China, Italy and New Zealand (Vanneste 2017). *P. syringae* pv. *actinidiae*
42 (Psa) is the causal agent of kiwifruit bacterial canker (KBC) and was first discovered in Japan in the
43 early 1980s (Takikawa et al., 1989). There were earlier reports of a similar disease in California,
44 USA (Opgenorth et al., 1983), but the causal bacterium was demonstrated to be different to that
45 subsequently characterized in Japan (Takikawa et al., 1989). Within ten years of identifying Psa,
46 serious economic losses due to the disease were reported from South Korea (Koh et al., 1994). Psa
47 was reported infecting kiwifruit in Italy, and in China, where KBC was first reported in Hunan
48 Province in 1985 and later reported in Anhui and Sichuan Provinces. Although the outbreak of KBC
49 on *A. deliciosa* 'Qinmei' in Shaanxi Province was first observed in 1991, it was not reported until ten
50 years later (Liang et al., 2000).

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

59 There are four groups (Psa1 to 4) of Psa strains proposed based on multilocus sequence typing
60 (MLST): Psa1 represents isolates from Japan and Italy; Psa2 represents isolates from Korea; Psa3

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61 isolates from Italy, New Zealand, Chile, and China; and Psa4 isolates from New Zealand and
62 Australia that exhibit low virulence (EPPO 2011). Moreover, four sub-pathovars or biovars
63 (Biovar1-4) of Psa have been discriminated and named based on molecular characteristics,
64 BOX-PCR electrophoretic profiles and the presence of type III secretion system effector protein
65 genes (abbr: effector genes) *hopA1* and *avrD*, and virulence differences observed in the field. Psa
66 strains in Biovar3 (found in Italy since 2008, in New Zealand, Chile, and China) and Biovar4 (New
67 Zealand) are separated from those isolated from past epidemics in Japan and Italy (Biovar1) or Korea
68 (Biovar2) (Vanneste et al., 2013). Strains belonging to Biovar 4 have been described as a new
69 pathovar, namely *P. syringae* pv. *actinidifoliorum* pv. nov (Psaf) (Cunty et al., 2015a).

70 Although there have been several reports describing the occurrence of KBC in different provinces
71 of China (Liang et al., 2000), the collection of strains of Psa from China is quite limited. Thus the
72 genetic diversity of populations of Psa from different geographic regions in China has not been
73 characterized. But recent availability of strains from China with associated genetic marker studies
74 (Ciarroni et al., 2015; Cunty et al., 2015b), and the sequence of the genomes of Psa have provided
75 some insight into the population biology and epidemiology of this important disease (McCann et al.,
76 2017; Andersen et al. 2018), which is valuable in understanding how climate change may affect
77 distribution of Psa in the future (Wang et al., 2018).

78 To gather more information on the genetic diversity of populations of Psa from different
79 geographic regions in China we collected 296 strains of Psa from 2012 to 2014. Of these strains, 51
80 (including Psaf strain 18804) were selected to study diversity and variation using Rep-, RAPD-, and
81 IS50-PCR. Furthermore, five housekeeping genes (*cts*, *gapA*, *gyrB*, *pfk*, and *rpoD*) of 24
82 representative Psa strains were sequenced, and four loci for the coronatine or phaseolotoxin

83 biosynthesis pathway and eleven effector protein genes were cloned and used to elucidate the

84 phylogenetic relationships among strains of Psa worldwide.

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87 **MATERIALS AND METHODS**

88 **Collection of Psa strains.** A collection of 269 strains of Psa was identified from 300 isolates
89 obtained from eight sampling sites in five different kiwifruit-producing regions of China: Jiading
90 District, in Shanghai municipality along the East Sea coastline; Yuexi and Jinzhai Counties in the
91 Dabie mountain area of Anhui Province; Xiuwen County, in the Southwest mountain area of
92 Guizhou Province; Mianzhu and Pengzhou County, in the Southwest basin of Sichuan Province; and
93 Huxian and Weixian Counties, in the Northwest plain of Shaanxi Province in China (Supplementary
94 Fig. 1).

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

106 **Biochemical characterization of isolates.** The ability of the bacterial strain to induce a

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107 hypersensitive reaction (HR) when injected into tobacco plants was tested, as was the ability to rot
108 potato, both following described protocols (Vanneste et al 2013). Ice nucleation activity was
109 determined as described by Lindow et al. (1978). The production of syringomycin was determined
110 according to Gross and DeVay (1977). The absence of cytochrome C oxidase, a characteristic that
111 differentiates *P. syringae* from other species of plant-pathogenic fluorescent Pseudomonas, was
112 determined using Test Oxidase (Pro-Lab Diagnostics Inc., Round Rock, TX, U.S.A). Production of
113 levan and an arginine dihydrolase under anaerobic conditions and the ability to hydrolyze esculin
114 were determined using a previously described method (Lelliot et al., 1966).

115 **Selection of additional strains and pathovars of Psa.** Fifty-one strains of Psa from different
116 geographic regions and three other pathovars of *Pseudomonas* species were used to determine
117 phylogenetic relationships (Table 1). Thirty-seven of the strains were selected from the collection of
118 296 strains of Psa collected in China: 3 to 5 representative strains were selected from different
119 sampling sites by the pre-reaction of different PCRs with the criteria of pattern similarity. A further
120 14 strains of Psa were selected from Italy (five strains), Spain (two strains), France (two strains),
121 Portugal (one strain), New Zealand (two strains), Korea (one strain), and Japan (one strain). In
122 addition, 25 characterized strains from New Zealand, Italy, Chile, Australia, Korea, Japan and the
123 USA previously deposited in the NCBI database were used to analyze the nucleotide sequences of
124 the different housekeeping genes (Supplementary Table 1).

125 **Genomic DNA extraction.** Bacterial Genomic DNA Isolation Kits (Norgen Biotek Corp., ON,
126 Canada) were used to obtain gDNA from freshly grown cultures of each strain. The quality of gDNA
127 of each strain was checked using a Beckman Coulter DU800 UV-Visible spectrophotometer

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128 (Beckman, Pasadena, CA, USA). The DNA concentration was adjusted to 50 ng/μL with Tris-EDTA
129 buffer (Tris-HCl and EDTA, pH 8.0) and stored at −80 °C.

130 **Different PCR assays.** Primers were selected for Rep-, IS50-, and RAPD-PCR assays and were
131 synthesized by Sengon Co., Ltd. (Shanghai, R. P. China) (Table 2). All 296 isolates of Psa were
132 screened against all primers. Three repetitive sequences primer sets, ERIC1R-ERIC2, BOXA1R and
133 (GTG)5 were chosen for the Rep-PCR amplification (Mazzaglia et al., 2011). In addition, Primer
134 IS50 complementary to the insertion sequence IS50 of Tn5 was used for strain typing. The Rep-PCR
135 and IS50-PCR reactions were performed in a final volume of 25 μL containing 12.5 μL of 2×Taq
136 PCR MasterMix (Aidlab Biotechnologies Co., Ltd., Beijing, China), 1 μL of template DNA, 1 μL of
137 each primer (10 nmol/μL), and 10.5 μL sterile distilled water (SDW). The Rep-PCR amplification
138 was performed at 95 °C for 7 min followed by 30 cycles of a denaturation step of 94 °C for 60 s, the
139 annealing temperature of each primer for 60 s, and extension at 72 °C for 8 min; and a final
140 extension step at 72 °C for 15 min. The IS50-PCR amplification conditions were as follows: an
141 initial denaturation cycle of 95 °C for 7 min; 40 cycles of denaturation at 94 °C for 1 min, annealing
142 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.

143 Eight primers with arbitrary nucleotide sequence were selected from a panel of 160 RAPD primers
144 screened to produce clear, reliable, and discriminable RAPD profiles; strains of Psa and *P. syringae*
145 were tested according to previous protocol (Lee et al., 2005). Amplification was performed in a total
146 volume of 20 μL containing 1 μL of each primer, 1 μL of the template DNA, 10 μL 2×Taq PCR
147 MasterMix and 7 μL of sterile distilled water. The amplification were an initial denaturation step of
148 95 °C for 5 min followed by 40 cycles of 94 °C for 60 s, the annealing temperature of each primer
149 for 60 s, 72 °C for 5 min, and a final extension step of 72 °C for 15 min.

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

152 DNA fingerprints of the strains were visually inspected for similarity and were considered
153 identical if the scored bands were at the same apparent migration distance, without consideration of
154 variation in amplicon intensity. Amplified fragments of each strain were scored as 1 if present in all
155 three runs, but 0 if absent, even if absent in only one experiment run, which demonstrated that the
156 fragment was not amplified reliably. The resulting matrix of 0 and 1 data representing amplicons
157 from strains by primers using the Rep-, IS50-, and RAPD-PCR assays was subject to analysis.
158 Discriminant analysis of principal coordinates (DAPC, Jombart et al., 2010) was conducted
159 according to the Wide Linear model, principal components analysis (PCA) was performed using
160 covariances, and strain differences between countries for each principal component were explored
161 based on least square means, Hierarchical cluster analysis was performed using the Ward method,
162 and one-way analysis of variance (ANOVA) of principal components was performed with means
163 separation using Duncan's new multiple range method ($\alpha = 0.05$). Analyses were calculated using the
164 Software JMP Version Pro 14 for Windows (Cary, North Carolina, U.S.A).

165 **Multilocus sequence typing of the housekeeping gene.** To identify the biovar (MLST group) of
166 the strains of Psa from the population in China, five housekeeping genes, *cts* (*gltA*), *gapA*, *gyrB*, *pfk*
167 and *rpoD*, that code for citrate synthase, glyceraldehyde-3-phosphate dehydrogenase, DNA gyrase B,
168 phosphofructokinase and sigma factor 70, respectively, were cloned using a previously described
169 method (Sarkar and Guttman, 2004). The MLST was performed on 24 strains selected from each
170 clade of the dendrogram based on the combined data of the 51 strains of Psa characterized using
171 different PCR assays described in the previous section. Sequencing of the housekeeping genes was

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172 performed using the CEQ-DTCS Quick Start kit (Beckman Coulter, Inc., Pasadena, CA, USA) on a
173 Beckman-Coulter CEQ 8000 DNA sequencer according to the manufacturer's instructions. The
174 sequences obtained from the housekeeping genes were submitted to the NCBI database for
175 homologous alignment analysis (Table 3). The phylogenic trees were constructed using the
176 Molecular Evolutionary Genetics Analysis (MEGA) software MEGA 6.0 (Tamura et al., 2013) based
177 on the Minimum Evolution method with the nucleotide sequences to infer the genetic relationships
178 between the tested strains of Psa and some known strains in the NCBI database: MAFF 212054,
179 MAFF 212055, and MAFF 212056 (all Biovar5), 212130, 212132, and 212134 (all Biovar6),
180 NCPPB 3739, NCPPB 3871, MAFF 302145, and MAFF 302143 (all Biovar1) from Japan and
181 NCPPB 3873 from Italy (Biovar1); KACC 10584, KACC 10754 and KACC 10594 from Korea (all
182 Biovar2); CRA-FRU 10.22 from Italy, Psa1A and Psa1B from Chile, T11-0918, T10-04782 and
183 T10-05454 from New Zealand (all Biovar3); T11-01369A from Australia, and T10-05195,
184 T10-05163 and T10-04976 from New Zealand (all Biovar4, Pasf) were used as the positive controls
185 for each biovar.

186 **Detection of phytotoxin and effector genes.** Presence of phytotoxin gene clusters (the *tox-argK*
187 gene cluster coding for phaseolotoxin and the *cfl* gene coding for coronatine) was performed for all
188 51 strains of Psa described above and was checked by PCR amplification, which was performed as
189 described in previous studies (Bereswill et al., 1994; Sawada et al., 2002). The presence of eleven
190 effector genes (*hopA1, hopH1*, *hopAF1*, *hopAA1-1*, *hopAA1-2*, *avrD1*, *hopB1*, *hopD1*, *hopAM1*,
191 *hopAH1*, and *hopX1*) was checked for by PCR amplification using gDNA of 24 representative strains
192 following the protocol described by Ferrante and Scortichini (2009, 2010, and 2011). Primers were
193 synthesized by the Sengon Co., Ltd. (Table 4). A constellation diagram was constructed based on

194 presence or absence data for the four loci coding phytotoxins and eleven effector genes based on the

195 Ward method using JMP Software Version Pro 14 for Windows.

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197 **RESULTS**

198 **Strains of Psa from different geographic regions in China.** All 269 isolates collected had the
199 biochemical characteristics of *P. syringae* pv. *actinidiae*, i.e., they were not, or were only slightly
200 fluorescent on King's B medium, they induced an HR reaction when infiltrated into tobacco plants,
201 but did not cause potato rot, and did not produce syringomycin, nor induce ice nucleation, and did
202 not have a cytochrome c oxidase or an arginine dehydrolase. They all produced levan.

203 **Unique fragment produced by PCR.** Most strains of Psa had a similar fingerprint pattern
204 generated by Rep-PCR using primer BOXA1R, with the exception of the five strains GC31, GC32,
205 GC40, GC41, and GC42 (lane 11, 12, 13, 14 and 15, Supplementary Fig. 2 A) from Guizhou
206 province, which had a unique fragment of approximately 1500 bp. In addition, strains K3 and Kw30
207 (lane 42 and lane 43) from Korea and Japan, respectively, possessed a unique fragment of
208 approximately 300 bp, and strains K3, Kw30, and Psa92 (lane 48) from Italy lacked a polymorphic
209 fragment of approximately 1100 bp.

210 The RAPD-PCR primer A-24 produced 9 amplicons, but the percentage of differential bands was
211 only 22.2% and there was a distinctive 3000-bp amplicon that was present only with strain 18804
212 (lane 51, Supplementary Fig. 2 B) and a second distinctive 1800 bp amplicon with strains SH2, SH8,
213 SH13, SH18 and SH26 (lane 6, 7, 8, 9 and 10, respectively) from Shanghai, strains WT2 and WT3
214 (lane 16 and 17, respectively) from Yuexi County and strains Jzhy2, Jzhy3 and Jzhy6 (lane 29, 30
215 and 31, respectively) from Jinzhai County (Fig. S 2 b).

216 According to the amplicon pattern generated by IS50-PCR, the strains of Psa from Asia, including

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217 those from China, Korea and Japan had a similar pattern which was similar to the amplicon patterns
218 for strains from Portugal and Italy, except for Psa92 (lane 48, Supplementary Fig. 2 C). However, the
219 strains of Psa from Spain and France had a pattern similar to the strain of Psa with high virulence,
220 Psa 18839 (lane 52), from New Zealand, and there was a distinctive pattern produced by strain Psaf
221 18804 (lane 51), a low virulence isolate from New Zealand.

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

226 The percentage of differential bands in Rep-, RAPD- and IS50- PCR was 47.0%, 77.1%, and
227 28.6%, respectively. This indicates that RAPD-PCR is the most efficient for the differentiation of
228 populations of Psa, but the stability of amplicons produced was not always consistent.

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

233 **Genetic relationship of populations of Psa from China and other countries.** A total of 145
234 discrete amplicons were generated with the 51 bacterial strains tested from eight countries (Japan,
235 South Korea, Italy, France, Portugal, Spain, New Zealand and China) using the three methods of
236 PCR. The strains could be discriminated into distinct four groups: Psa1 from Japan and Italy, Psa2
237 from South Korea, Psa3 from Europe, New Zealand and China, and Psa4 from New Zealand, of

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238 which the strains had been reassigned to a new pathovar Psaf. All the strains of Psa from China
239 belong to Psa3. The results indicate that strain K3 from South Korea, which was thought to be a
240 member of Psa2, has closer genetic affinity to Psa1 strain Kw30 from Japan (Fig. 1).

241 PCA revealed that a relatively small portion of the total variance contributed to the first two
242 principal components (PC-1, 28.1%; PC-2, 16.7%). There were four distinctive subgroups (Subgr1-4)
243 in the worldwide group of Psa3 strains, three of the groups came from China (Subgr1-3), strain 349
244 from Portugal and strains 7285, 7286, LT19, and LT80 from Italy that were isolated in 2008 and were
245 in the same Subgr3 as strains from China, implying they might have the same ancestor. The virulent
246 strain 18839 from New Zealand was in Subgr4, with strains 827 and 830 from Spain, and strains F1
247 and F2 from France, indicating a close genetic relationship (Fig 2 a).

248 However, the first four principal components (PC-1-4) accounted for 68.9% of the overall variance
249 defining genetic relationships among strains of Psa3 populations worldwide. The plots of PC-1 vs.
250 PC-3 or PC-1 vs. PC-4 show that strains from China have a broad genetic variability and plots of
251 PC-2 vs. PC-3 or PC-2 vs. PC-4 show that the strains from China and strains from Europe and New
252 Zealand have an independent evolutionary direction (Fig 2 b). Furthermore, the ANOVA showed that
253 significant differences were found for components 2 and 4, but not for components 1 or 3. Strains
254 from China are genetically different to strains from France, but are not different to strains from Italy
255 (Supplementary Table 2).

256 A greater diversity in populations of Psa3 from China was found compared to that previously
257 observed (Cunty et al., 2015b; Liu et al., 2016; MaCann et al., 2017). Cluster analysis showed that
258 the strains in Clade I were from Xiuwen County in Guizhou Province (GC31-32, GC40-42); the
259 strains in Clade II were from kiwifruit seedling rootstock (JZZM1-2), two non-kiwifruit species, *S.*

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260 *viridis* (JZMC1-2) and *A. philoxeroides* (JZGMC1-2); the strains in Clade III were primarily from
261 kiwifruit 'Hongyang' in Jinzhai County, Anhui Province (JZHY2, 3 and 6) and Jiading District in
262 Shanghai municipality (SH2-3, 13, 18 and 28), and from *P. tomentosa* near a kiwifruit orchard in
263 Yuexi County, Anhui Province (WT2-3); and the strains in Clade IV had a wide range of cultivar
264 sources and geographic region origins including kiwifruit 'Hongyang' in Pengzhou County, Sichuan
265 Province (G3-5, 8 and 12), kiwifruit 'Jinfeng' (JF8, 27) and two insect species, *P. hexamaculata*
266 (P1-3) and *B. ferruginea* (IN5) from an orchard in Yuexi County, Anhui Province, and kiwifruit
267 'Hayward' in Huxian County, Shaanxi Province (Hwd1, 3-6). These results indicate that each
268 Province in China has unique and shared strains; indeed, strains from Anhui province not only has
269 indigenous strains in Clade II, but also shared strain types with Shanghai Sichuan, and Shaanxi
270 provinces in Clade III and Clade IV (Fig. 3).

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

275 The MLST revealed that the genetic variability among the strains of Psa differed to that resulting
276 from the differentPCR assays. The phylogenic tree constructed by the combined MLST data showed
277 that the 24 strains of Psa had distinct multilocus sequence profiles but there was clustering of strains
278 from different biovars or MLST groups (Biovar 1-3, Psaf, and Biovar 5-6), compared with the 25
279 previously characterized strains in the NCBI database used as positive controls for biovar IDs and
280 strain DC3000 of *P. s.* pv. *tomato*, which was used as an outgroup (Supplementary Table 1).

281 All of the strains of Psa from China, with the exception of strain Jzhy2, were in the same group as

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282 strain 7285 from Italy (cause of the KBC outbreak since 2008), strains 18839, T11-0918, T10-04782,
283 and T10-05454 from New Zealand, strains 827 and 830 from Spain, strain 349 from Portugal, and
284 strain 1F from France, all of which were classified as Biovar 3 (Psa3). Only strain Jzhy2 from China
285 was differentiated in an independent clade apart from the existing biovars or MLST groups.
286 Interestingly, strains MAFF 212054, MAFF 212055, and MAFF 212056 (newly identified strains
287 from Japan in Biovar 5), had a closer genetic relationship to KACC 10584, KACC 10754, and
288 KACC 10594 from Korea, all in Biovar2 (Psa2); and strains in Biovar 6 were genetically closer to
289 Biovar1. In contrast, K3 isolated in 1997 from Jeju, Korea clustered with strains Kw30, NCPPB
290 3739, NCPPB 3871, MAFF 302145 and MAFF 302143 from Japan and Psa92 from Italy and were
291 classified as Biovar 1 or Psa1 (Fig. 4).

292 Strain 18804 of Psaf was clustered with T11-01369A from Australia and T10-05195, T10-05163
293 and T10-04976 from New Zealand, which had low virulence and was in a clade distant to all strains
294 in Biovar1-3 and Biovar5-6. Strain DC3000 of *P. s*. pv. *tomato* was an outgroup.

295 **Genetic variation in phytotoxin and effector genes.** All of the tested strains, including K3 from
296 Jeju, South Korea lacked the *cfl* gene coding for coronatine. In contrast, the *tox-argK* gene cluster
297 coding for phaseolotoxin was detected in strains K3 and Psa92 (Supplementary Table 3). This is the
298 first report that at least some strains of Psa from South Korea have the *tox-argK* gene cluster coding
299 for phaseolotoxin.

300 The constellation diagram showed that the strains from China formed two clades, Clade 1 and
301 Clade 2; the strains from Europe and the virulent strain from New Zealand were in Clade 2. But the
302 low-virulence strains from New Zealand were in an independent clade, Clade3, strains of which are
303 now considered a new pathovar, Psaf; the Biovar 6 strains from Japan were grouped in Clade 4,

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304 closer to Biovar 1; strains K3, Kw30 and Psa92 clustered in Clade 5 (Biovar 1); the strains from
305 Japan grouped originally in Biovar 5 grouped in Clade 6, closer to Biovar 2 and those strains in
306 Biovar 2 clustered in Clade 7 (Fig. 5).

307 Strain K3, Kw30, and Psa92 in Biovar1 lacked both the amplified 444 bp fragment for effector
308 gene *hopA1* and the 605 bp fragment for effector gene *hopH1*. Strain Psaf 18804 (former Biovar 4)
309 produced an amplicon for *hopA1* but not for *hopH1*; and the remaining strains in Biovar 3 had
310 amplicons for both *hopA1* and *hopH1* genes, except for strains Jzhy2 and Jzhy6, which only
311 produced amplicons for *hopH1,* but lacked the *hopA1* gene (Supplementary Fig. 3 A, C).

312 It was notable that of all the strains (Jzhy1-16) yielding an amplicon for *hopH1*, only five (Jzhy7,
313 Jzhy13, Jzhy14, Jzhy15, and Jzhy16) yielded an amplicon for *hopA1*. Although the 16 strains were
314 isolated from the same host cultivar (*A. chinensis* 'Hongyang') in the same orchard (Jinzhai County,
315 Anhui Province, China), eleven strains (Jzhy1-6, Jzhy8-12) belonged to a new genotype that differed
316 from the existing biovars or MLST groups according to the combination of the *hopA1* and *hopH1*
317 effector genes (Supplementary Fig. 3 B, D).

318 With the exception of strain 18804, a common amplicon was found in the 24 representative strains
319 screened for *hopAM1*. Sequence analysis showed that strain K3, Kw30 and Psa92 had a longer
320 amplicon (1784 bp) compared to that produced for the *hopAM1* sequence from other strains (701 bp,
321 Supplementary Fig. 3 E, F).

322

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323 **DISCUSSION**

324 **PCR assay and MLST.** The genetic variability and relationships among the strains of Psa
325 characterised by the PCR assays differed slightly to that charcaterised by MLST: the PCR was more
326 sensitive compared to the MLST in the discrimination of the Psa populations. According to the
327 results of the PCR assay, the variation among strains of Psa from China was related to the geographic
328 source location. Each of the geographic regions had unique strain variants of Psa, for example,
329 strains in Clade I were from Xiuwen county in Guizhou Province, in Clade II from Yuexi County in
330 Anhui Province, in Clade III mainly from Jiading District in Shanghai, and in Clade IV the strains
331 were mainly from Weixian in Shaanxi Province.

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

338 The RAPD method used in this investigation was able to discriminate strains of Psa at and below
339 the level of Biovar, and the discriminating power of this method has been confirmed by others
340 (Bereswill et al., 1994; Mazzaglia et al., 2011; Koh et al., 2014). The RAPD method is widely used
341 due to ease of operation, although reproducibility within and between laboratories has been
342 questionable, perhaps due to the method's dependence on the type of *Taq* polymerase and thermal
343 cycler used, or other unidentified reasons (Louis and Bijlsma, 1995). On the other hand, in some
344 situations RAPDs can be reliable and reproducible. In our study the method provided reproducible

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345 results to explore genetic variability when no sequence data were available for the genome in
346 question.

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

356 Sequence data from the five housekeeping genes uniquely identified strain Jzhy2 as Biovar3. Four
357 of the housekeeping genes (*cts*, *gapA*, *gyrB*, and *pfk*) in strain Jzhy2 were identical to the other
358 strains of Biovar3, but the sequence of *rpoD* in strain Jzhy2 had one-base change at five different
359 positions of the locus sequence resulting in an amino acid sequence change. Currently, we are
360 conducting a genome sequencing approach of strains of Psa representative of the world-wide
361 collection to reveal whether strain Jzhy2 belongs to a novel biovar of Psa.

362 Wild kiwifruit infected with Psa has not been found in China, except for the one occurrence near
363 the KBC-diseased orchard described in the current study. But a strain of Psa was isolated from wild
364 growing plants of *A. arguta* and *A. kolomikta* in Japan in 1987-1988 (Ushiyama et al., 1992). Thus
365 only one biovar of Psa has been characterized in China: Biovar 3, despite China being recognized as
366 center of diversity and thus of origin for most species of kiwifruit (Ferguson and Huang, 2007). This

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367 result may be irreconcilable with the prevailing expectation that more biovars of Psa should be
368 present in China.

369 **Genetic variation among the global populations of Psa.** Environmental variables other than the
370 host can be expected to influence variability of a plant pathogen (Morris et al., 2009), and producing
371 a few cultivars at a high density can enhance the risk of an epidemic or pandemic of KBC caused by
372 a one or a few specific bacterial pathovars (McCann et al., 2013). The genetic variation in Psa makes
373 transport of host material a possible source of novel strains in new areas, and phytosanitary
374 regulation of the species a priorioty for consideration. Thus, exchange of kiwifruit plant material has
375 resulted in transport of the pathogen and resulting epidemics of KBC in Italy (Balestra et al., 2018)
376 and South Korea (Koh et al., 2014). Furthermore, some strains isolated from orchards in Japan in
377 1987 and 1988 where streptomycin had been routinely applied were found to be streptomycin
378 resistant (Nakajima et al., 1995), and copper-resistant strains of Psa were isolated from orchards
379 where copper-based bactericides are used to control bacterial canker caused by Psa in Japan
380 (Nakajima et al., 2002), Italy (Petriccione et al., 2017), and New Zealand (Colombi et al., 2017).
381 Indeed, these issues regarding the variability and adaptability of Psa have raised questions on the
382 reliability of long-term strategies to control KBC solely with copper compounds or antibiotics, and
383 have clear ramifications for transport of plant material between kiwifruit production areas.

384 Diversity can be related to the geographic origin of a pathogen. Analysis of multiple loci variable
385 number of tandem repeats analysis (MLVA) in Psa showed broad genetic variability, but different
386 strains were also retrievable in Japan and Korea (Ciarroni et al., 2015). Compared to strains collected
387 in 2010, results from rep-PCR and RAPD analysis revealed a high level of variability in the
388 population of Psa collected from northern Italy in 2014. Indeed, at least two biovars (Biovar 3 and

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389 Psaf Biovar 4) of Psa had been found in New Zealand (Vanneste et al., 2013), France (County et al.,
390 2014; 2015b), Spain (Abelleira et al., 2015); and Biovar 1, 3, 5, 6 and Psaf (Biovar 4) were found in
391 Japan (Sawada et al., 2014; 2015; 2016 and 2017); Biovar 1, 2 and 3 in Korea (Koh et al., 2014;
392 McCann et al., 2017), and Biovar 1 and Biovar 3 in Italy (Ferrante and Scortichini, 2015). But all of
393 the strains of Psa from China were grouped in Biovar3 by MLST, with the exception of strain Jzhy2.
394 The coexistence of many types of Psa in both Japan and Korea suggested that they are the origin and
395 center of diversity of Psa, rather than China (McCann et al., 2017). The identification of new strains
396 in Japan (Sawada et al., 2016; 2017) and Italy (Marcelletti et al., 2011) supports the assertion that
397 variants may continue to emerge and cause local epidemics and possibly pandemics (Vanneste 2017).
398 However, all strains from New Zealand share several single nucleotide polymorphisms (SNPs) that
399 distinguish them from all other strains of Psa. Similarly, all strains of Psa that caused the epidemic in
400 Italy form a distinct clonal group, and those from Chile form a third group (Butler et al., 2013).
401 MLVA and DAPC revealed that strains isolated in Chile, China, and New Zealand are genetically
402 distinct from strains of Psa isolated in France and in Italy, which appear to be closely related at the
403 genetic level (Cunty et al., 2015b). Another MLVA based on 13 variable number tandem repeat
404 (VNTR) loci indicated that strains of Psa from Italy showed very limited diversity, with strains from
405 China having broader genetic variability, but those from Japan and Korea being genetically the most
406 heterogeneous group (Ciarroni et al., 2015).

407 In this study, the strains from Japan (MAFF 212054, MAFF 212055, and MAFF 212056) that
408 were clustered as the novel Biovar 5 also clustered with strains of Biovar 2 on the phylogenic tree
409 constructed using the combined sequence data of the five housekeeping gene, although the reported
410 strains of Biovar 5 had neither the coronatine nor the phaseolotoxin biosynthetic genes conserved in

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411 Biovar 2 (Sawada et al., 2014). Thus the strains of Biovar 5 causing KBC in Japan were most closely
412 related to Biovar 2 according to the average nucleotide identity (ANI) assay (Fujikawa and Sawada,
413 2016). Strains of Biovar 3 also were isolated in Japan, but among 22 strains, 14 possessed Pac_ICE1,
414 the remaining 8 had no homologs of Pac_ICE1, Pac_ICE2, or Pac_ICE3 (Sawada et al., 2015).
415 Recently, another novel biovar (Biovar 6) of Psa causing KBC in Japan was found, and that pathogen
416 did not possess *hopH1*, *hopH3* or *hopZ5* genes, yet produced both phaseolotoxin and coronatine
417 (Sawada et al., 2016).

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

428 Strains that cluster in the same clade likely have a common origin, and a number of strains from
429 different geographic regions in the same cluster demonstrate that they should have common ancestry.
430 Nevertheless, long-term and long-distance transmission of strains of Psa is due primarily to the
431 introduction of scions, seedling rootstocks, and pollen of kiwifruit. The strain types of Psa present in
432 different continents was not consistent with the geographic locations of Europe, Asia or Oceania due

433 to the complex routes for transmission of Psa that have resulted in the introduction and
434 reintroduction of the pathogen worldwide where kiwifruit is cultivated. Migration from kiwifruit to
435 non-kiwifruit species or transmission by insects may also have had a profound effect on the observed
436 genetic variation of Psa (Liu et al., 2016).
437

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447

448

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TABLE 1 Bacterial strains used in this study with their source, geographic origin, and host from which they were isolated.

No.	Strain name	Source	Species /pathovar[a]	Hostspecies/cultivar	Isolation tissue	Isolation year	Geographic origins	Orchard name	Collection
1	Schy3	AHAU, China	Psa	*Actinidiachinensis*'Hongyang'	Vine	2013	Penzhou, Sichuan, CN	Agri-Bumper	This study
2	Schy4	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Vine	2013	Penzhou, Sichuan, CN	Agri-Bumper	This study
3	Schy5	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Vine	2013	Penzhou, Sichuan, CN	Agri-Bumper	This study
4	Schy9	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Vine	2013	Penzhou, Sichuan, CN	Agri-Bumper	This study
5	Scht12	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Vine	2013	Penzhou, Sichuan, CN	Agri-Bumper	This study
6	SH2	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Leaf	2013	Jiading, Shanghai, CN	Agri-Tech	This study
7	SH8	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Leaf	2013	Jiading, Shanghai, CN	Agri-Tech	This study
8	SH13	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Leaf	2013	Jiading, Shanghai, CN	Agri-Tech	This study
9	SH18	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Leaf	2013	Jiading, Shanghai, CN	Agri-Tech	This study

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10	SH26	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Leaf	2013	Jiading, Shanghai, CN	Agri-Tech	This study
11	GC31	AHAU, China	Psa	*A.deliciosa* 'Guichang'	Vine	2012	Xiuwen, Guizhou, CN	Wangjiapu	This study
12	GC32	AHAU, China	Psa	*A.deliciosa* 'Guichang'	Vine	2012	Xiuwen, Guizhou, CN	Wangjiapu	This study
13	GC40	AHAU, China	Psa	*A.deliciosa* 'Guichang'	Leaf	2012	Xiuwen, Guizhou, CN	Wangjiapu	This study
14	GC41	AHAU, China	Psa	*A.deliciosa* 'Guichang'	Leaf	2012	Xiuwen, Guizhou, CN	Wangjiapu	This study
15	GC42	AHAU, China	Psa	*A.deliciosa* 'Guichang'	Leaf	2012	Xiuwen, Guizhou, CN	Wangjiapu	This study
16	WT2	AHAU, China	Psa	*Paulownia tomentosa*	Leaf	2013	Yuexi, Anhui, CN	Zhubozhen	This study
17	WT3	AHAU, China	Psa	*Paulownia tomentosa*	Leaf	2013	Yuexi, Anhui, CN	Zhubozhen	This study
18	P1	AHAU, China	Psa	*Philagra hexamaculata*	Spume	2013	Yuexi, Anhui, CN	Zhubozhen	This study
19	P2	AHAU, China	Psa	*Philagra hexamaculata*	Spume	2013	Yuexi, Anhui, CN	Zhubozhen	This study
20	P3	AHAU, China	Psa	*Philagra hexamaculata*	Spume	2013	Yuexi, Anhui, CN	Zhubozhen	This study
21	INS	AHAU, China	Psa	*Bothrogonia ferruginea*	Body	2012	Yuexi, Anhui, CN	Zhubozhen	This study
22	JF8	AHAU, China	Psa	*A.chinensis* 'Jinfeng'	Leaf	2012	Yuexi, Anhui, CN	Zhubozhen	This study

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23	JF27	AHAU, China	Psa	*A.chinensis* 'Jinfeng'	Leaf	2012	Yuexi, Anhui, CN	Zhubozhen	This study
24	Hwd1	AHAU, China	Psa	*A.deliciosa* 'Hayward'	Leaf	2012	Huxian, Shaanxi, CN	Baixiaqu	This study
25	Hwd3	AHAU, China	Psa	*A.deliciosa* 'Hayward'	Leaf	2012	Huxian, Shaanxi, CN	Baixiaqu	This study
26	Hwd4	AHAU, China	Psa	*A.deliciosa* 'Hayward'	Leaf	2012	Huxian, Shaanxi, CN	Baixiaqu	This study
27	Hwd5	AHAU, China	Psa	*A.deliciosa* 'Hayward'	Leaf	2012	Huxian, Shaanxi, CN	Baixiaqu	This study
28	Hwd6	AHAU, China	Psa	*A.deliciosa* 'Hayward'	Leaf	2012	Huxian, Shaanxi, CN	Baixiaqu	This study
29	Jzhy2	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Leaf	2013	Jinzhai, Anhui, CN	Tanghui	This study
30	Jzhy3	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Leaf	2013	Jinzhai, Anhui, CN	Tanghui	This study
31	Jzhy6	AHAU, China	Psa	*A.chinensis* 'Hongyang'	Leaf	2013	Jinzhai, Anhui, CN	Tanghui	This study
32	Jzzm1	AHAU, China	Psa	*A.chinensis* wild seedlings	Leaf	2013	Jinzhai, Anhui, CN	Tanghui	This study
33	Jzzm2	AHAU, China	Psa	*A.chinensis* wild seedlings	Leaf	2013	Jinzhai, Anhui, CN	Tanghui	This study
34	Jzmc1	AHAU, China	Psa	*Setaira viridis*	Leaf	2013	Jinzhai, Anhui, CN	Tanghui	This study
35	Jzmc2	AHAU, China	Psa	*Setaira viridis*	Leaf	2013	Jinzhai, Anhui, CN	Tanghui	This study

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36	Jzgmc1	AHAU, China	Psa	*Alternanthera philoxeroides*	Leaf	2013	Jinzhai, Anhui, CN	Tanghui	This study
37	Jzgmc2	AHAU, China	Psa	*Alternanthera philoxeroides*	Leaf	2013	Jinzhai, Anhui, CN	Tanghui	This study
38	7285	CFBP	Psa	*A.chinensis* ‘Jintao’	Leaf	2008	Veneto, Italy		DAFNE,UT
39	7286	CFBP	Psa	*A.chinensis* ‘Hort16A’	Leaf	2008	Latina, Italy		DAFNE,UT
40	LT19	Balestra	Psa	*A.deliciosa* ‘Hayward’	Leaf	2008	Latina, Italy		DAFNE,UT
41	LT80	Balestra	Psa	*A.deliciosa* ‘Hayward’	Vine	2008	Latina, Italy		DAFNE,UT
42	K3	Y. J. Koh	Psa	*A.deliciosa* ‘Hayward’	Leaf	1997	Jeju, Korea		DAFNE,UT
43	Kw30	NCPPB 3740	Psa	*A.deliciosa* ‘Hayward’	Leaf	1984	Shizuoka, Japan		DAFNE,UT
44	349	Balestra	Psa	*A.deliciosa* ‘Summer’	Leaf	2010	Portugal		DAFNE,UT
45	3039B	A. Calzolari	Pss	*A.chinensis*	Leaf	2009	Romagna, Italy		DAFNE,UT
46	4254A	A. Calzolari	Pv	*A.chinensis*	Leaf	2009	Romagna, Italy		DAFNE,UT
47	2598	NCPPB	Pst	*Thea sinensis*	Leaf	1970	Japan		DAFNE,UT
48	Psa92	Balestra	Psa	*A.deliciosa* ‘Hayward’	Leaf	1992	Latina, Italy		DAFNE,UT

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49	827	Balestra	Psa	*A.chinensis* 'Jintao'	Leaf	2011	Galicia, Spain	DAFNE,UT
50	830	Balestra	Psa	*A.chinensis* 'Jintao'	Leaf	2011	Galicia, Spain	DAFNE,UT
51	18804	ICMP	Psa	*A.chinensis*	Leaf	2010	New Zealand	DAFNE,UT
52	18839	ICMP	Psa	*A.deliciosa* 'Hayward'	Leaf	2011	New Zealand	DAFNE,UT
53	1F	Anses	Psa	*A.chinensis* 'Jintao'	Leaf	2010	Aquitaine, France	DAFNE,UT
54	2F	Anses	Psa	*A.deliciosa* 'Hayward'	Leaf	2010	Rhone Alpes,France	DAFNE,UT

[a]Psa = *Pseudomonas syringae pv. actinidiae*; Pss = *P. syringae* pv. *syringae*; Pst = *P. syringae* pv. *theae*; Pv = *P. viridiflava.*

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

Method	Primer	Sequence	References	[a]Anneal temperature(℃)	Total bands amplified[a]	Polymorphic bands	[b]Percentages of differential bands[b]
Rep-PCR	ERIC1R	5'-ATGTAAGCTCCTGGGGATTCA-3'	Weingart and Volksch, 1997	52	11	4	63.6
	ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'					
	BOXA1R	5'-CTACGGCAAGGCGACGCTGACG-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'-CTTTTCATCCACACACTCCG-3'		45	13	1	92.3
IS50-PCR	IS50	5'-GGTTCCGTTCAGGACGCTAC-3'	Weingart and Volksch, 1997	45	14	10	28.6

[a]The total and polymorphic bands amplified for each primer refer to only the 51 strains of *Pseudomonas syringae* pv. *actinidiae.*
[b]Percentages of the differential bands ＝ [(Total bands － Polymorphic bands) / Total bands] × 100 %.

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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 NCBI.

No.	Strain	*cts*	*gapA*	*gyrB*	*pfk*	*rpoD*
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

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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	hopAF1-F	5'-CAAGCAGAAAGACGGCATC-3'	Ferrante et al., 2009
	hopAF1-R	5'-GCACACGCGACAGCAATG-3'	
	hopA1-F	5'-CGGCAAGAGGTACGAGATTC-3'	Ferrante et al., 2010
	hopA1-R	5'-TTCAATGCCTTTAGCGTGTG-3'	
	avrD1-F	5'-CGATGGTTTCAAACATGTGG-3'	
	avrD1-R	5'-TGTGCGTGACTGACAAGTGA-3'	
	hopB1-F	5'-AGGCTATTATCCGCCAACCT-3'	
	hopB1-R	5'-TCTTGCAACAGGATGCTCAC-3'	
	hopD1-F	5'-GTTACTGAGCTCGCCAGACC-3'	
	hopD1-R	5'-TGGTGGCTACATGCAACAAT-3'	
	hopH1-F	5'-CGTCTCGATATCCAGGCATC-3'	Ferrante et al., 2011
	hopH1-R	5'-TTCAGCTCGGATGGAGTTCT-3'	
	hopAA1-1-F	5'-GATAAATGCGATTGCCGATT-3'	
	hopAA1-1-R	5'-GAGCCTTGGGCTCTTTATCC-3'	
	hopAA1-2-F	5'-TGCATTACCTGAGCACTTCG-3'	
	hopAA1-2- R	5'-TGACTGTTTTCGCCAGTTTG-3'	
	hopAM1-F	5'-CCAAGTTAAATCGGCAGGAA-3'	
	hopAM1-R	5'-TCAGGTCAACGCTATTTTCG-3'	
	hopAH1-F	5'-AGTCCGACTTCAGGCTCAAA-3'	
	hopAH1-R	5'-CGTCACGCTGTTCGAAGTTA-3'	
	hopX1-F	5'-CTTTGCCCGGCTACTTACTG-3'	
	hopX1-R	5'-CGGACAAATGCATCATCAAG-3'	
Phaseolotoxin	PHA19	5'-CGTCTGTAACCAGTTGATCC-3'	Marques et al., 2000
	PHA95	5'-GAATCCTTGAATGCGAAGGC-3'	
	HM6	5'-CGTGTCCGTGGATAAAAGC-3'	
	HM13	5'-GTTGAATTTCACTACCCG-3'	
	OCTF-03	5'-GACCGTCAAGGAAGAATTCGGGCGC-3'	Sawada et al., 2002
	OCTR	5'-CGACCTTGTTGACCTCCCG-3'	
Coronatine	CFL-F	5'-GGCGCTCCCTCGCACTT-3'	Bereswill et al., 1994
	CFL-R	5'-GGTATTGGCGGGGGTGC-3'	

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Fig. 1. Discriminant Analysis of Principal Coordinates (DAPC) of strains of Psa collected from different countries based on a combined data set from different PCR primers (Rep-, IS50- and RAPD-PCR assays). The scatterplots show the first two canonical variables of the DAPC for the strains of Psa according to a linear model. Strain type groups are indicated by the double-lined color circles, while the markers represent individual strains from different countries. The bacterial strains could be discriminated into four distinct groups, i.e. Psa1 (Biovar1) from Japan and Italy, Psa2 (Biovar2) from South Korea, Psa3 (Biovar3) from Europe, New Zealand and China, and Psa4 (Biovar4) from New Zealand (the strains of this group had been reassigned as a new pathovar, Psaf), all the strains of Psa from China belong to the Psa3 group. The strain K3 from South Korea has a closer genetic relationship with that of Psa1 from Japan.

146x102mm (300 x 300 DPI)

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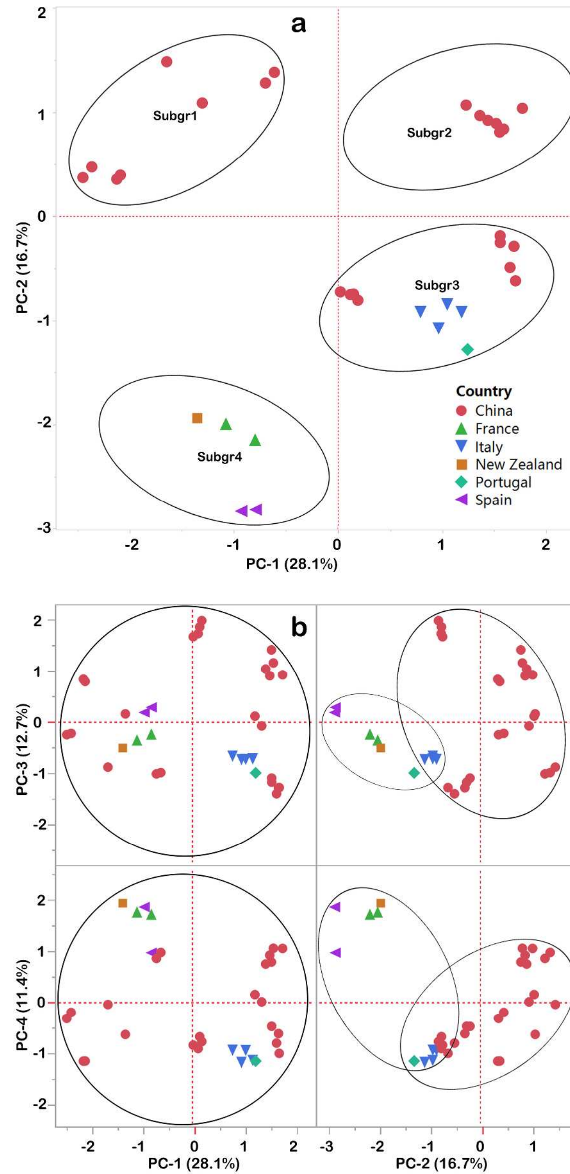

Fig. 2. Principal Components Analysis (PCA) of strains of Psa collected from different countries based on a combined data set from PCR assays using primers for Rep-, IS50- and RAPD-PCR assays. The scatterplots show the first two principal components of the PCA for the strains of Psa3 according to the covariance model. Strain subgroups are shown by different marker colors and ellipses (a), with each marker represent individual strains. The strains were discriminated into four subgroups. The first two principal components had a relatively small contribution (44.8%) and the first four principal components accounted for just 68.9% of the total variance (b).

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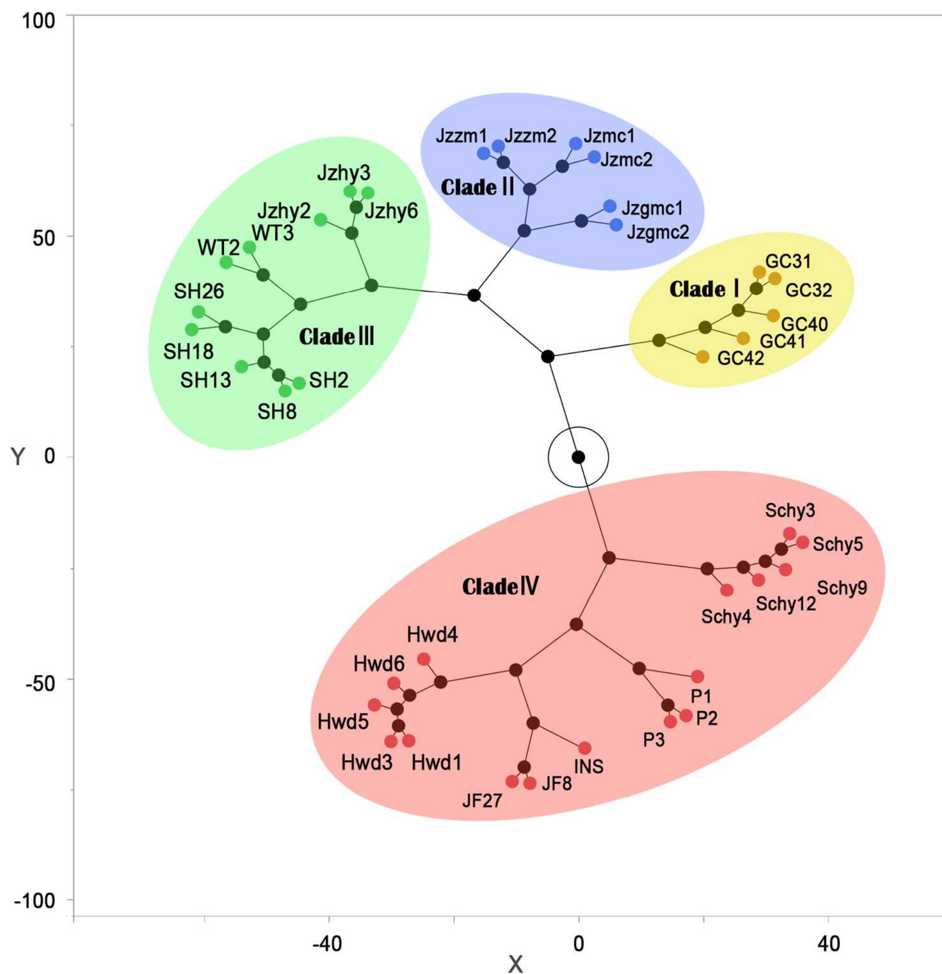

Fig. 3. Constellation plot of Chinese Psa3 strains constructed by hierarchical cluster analysis using a combined data set of the multiplex PCR fingerprint.┼ The strains in Clade I were only from Xiuwen County Guizhou Province (GC31-32, GC40-42); the strains in Clade II were from a seedling rootstock of kiwifruit (JZZM1-2), two nonkiwifruit plants *Setaira viridis* (JZMC1-2) and *Alternanthera philoxeroides* (JZGMC1-2). the strains in Clade III were mainly from 'Hongyang' kiwifruit at Jinzhai County Anhui Province (JZHY2, 3, 6) and Jiading District Shanghai municipality (SH2-3, 13, 18, 28), and from *Paulownia tomentosa* near kiwifruit orchard at Yuexi County Anhui Province(WT2-3). the strains in Clade IV had extensive cultivar sources with such different geographic regions from kiwifruit 'Hongyang' in Pengzhou County, Sichuan Province (Schy3-5, 9, 12), 'Jinfeng' kiwifruit (JF8, 27) and two insects species collected in kiwifruit orchards, *Philagra hexamaculata* (P1-3) and *Bothrogonia ferruginea* (INS) in Yuexi County Anhui Province, and kiwifruit 'Hayward' in Huxian County Shaanxi Province (Hwd1, 3-6).┼

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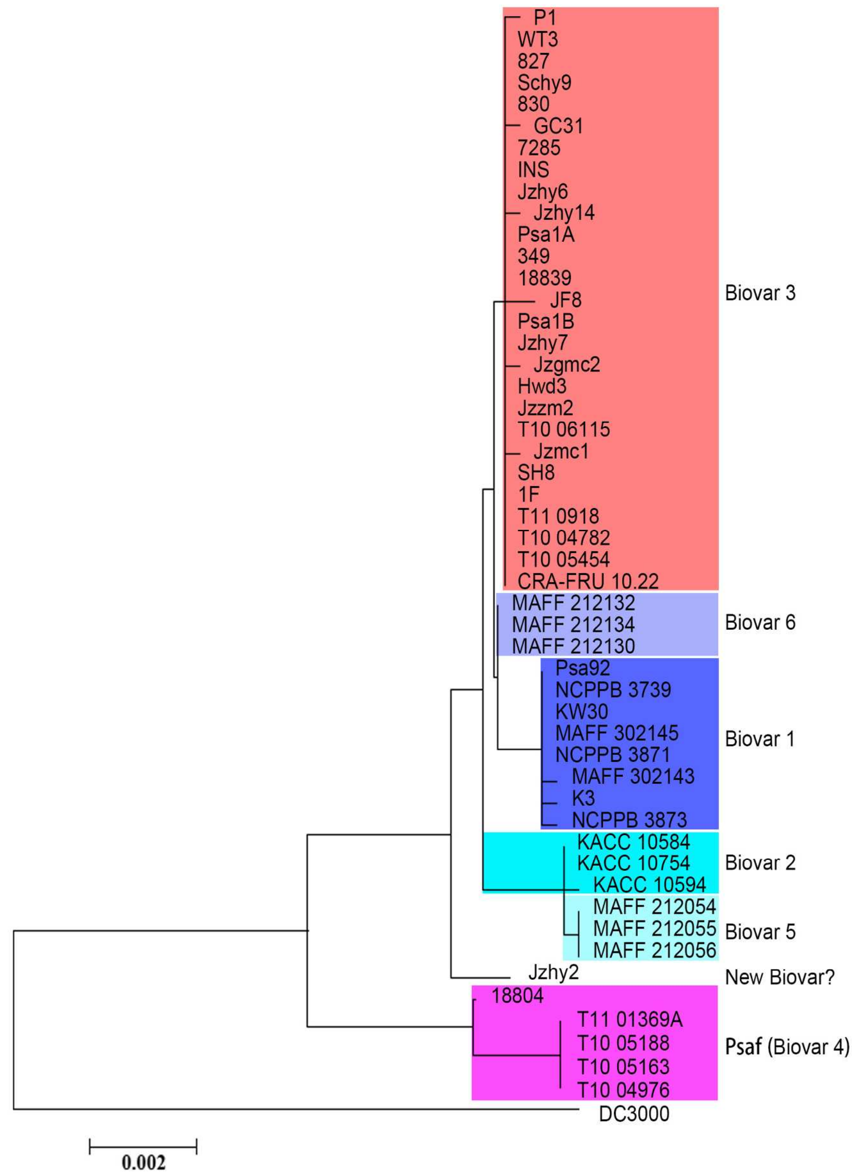

Fig. 4 The phylogenetic tree obtained from the combined sequence data of the five housekeeping genes *cts, gapA, gyrB, pfk*, and *rpoD*. The phylogenetic tree was constructed using MEGA 6.0 software and the Minimum Evolution method. Bootstrap confidence values are indicated at each node and the scale bar indicate the genetic distance. The sequences of the 24 representative strains are presented in Table 3. In addition, the 25 known strains from the NCBI database were used as positive controls for biovar identity, and the strain DC3000 of *P. s*. pv. *tomato* was used as an outgroup.

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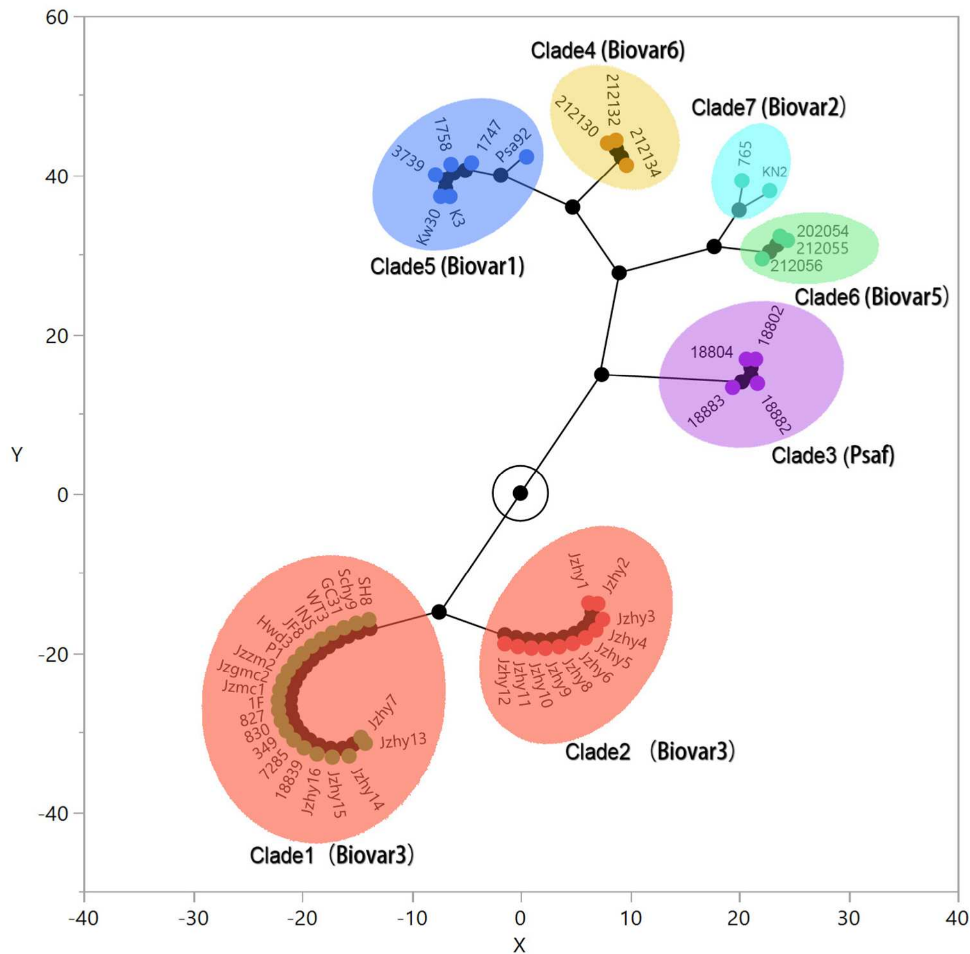

Fig. 5 A constellation plot of strains of Psa from different countries constructed by hierarchical cluster analysis using the amplicon data for four loci coding phytotoxins and eleven effector genes. ┼ Note: The strains of Psa from China formed two distinct clades, Clade 1 and Clade 2, the European strains 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 (Psaf); and the new Biovar 6 representing a strain from Japan was grouped in Clade 4, closer to Biovar 1; strains K3, Kw30, and Psa92 clustered as Clade 5 (Biovar1); the remaining strains in Biovar5 from Japan formed Clade 6, closer to Biovar 2 and strains in Biovar 2 clustered in Clade 7.┬

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SUPPLEMENTARY TABLE 1. Nucleotide sequences of the five housekeeping genes of some known strains from the database of NCBI that were used in this study.

No.	Strain name	Source	Pathovar/types	Hostspecies/cultivar	Isolation tissue	Isolation year	Geographic origin
1.	NCPPB 3873	NCBI	Psa/Biovar1	*A.deliciosa* 'Hayward'	Leaf	1992	Latina, Italy
2.	Wa1 (MAFF 302145)	NCBI	Psa/Biovar1	*A. deliciosa*	Leaf	1988	Wakayama, Japan
3.	Kw4 (MAFF 302143)	NCBI	Psa/Biovar1	*A. deliciosa*	Leaf	1987	Kanagawa, Japan
4.	Kw11 (NCPPB 3739)	NCBI	Psa/Biovar1	*A.deliciosa* 'Hayward'	Leaf	1984	Shizuoka, Japan
5.	Kw30 (NCPPB 3740)	NCBI	Psa/Biovar1	*A.deliciosa* 'Hayward'	Leaf	1984	Shizuoka, Japan
6.	KACC 10584	NCBI	Psa/Biovar2	*A. chinensis*	Leaf	1997	Jeonnam, Korea
7.	KACC 10754	NCBI	Psa/Biovar2	*A. chinensis*	Leaf	1997	Jeonnam, Korea
8.	KACC 10594	NCBI	Psa/Biovar2	*A. chinensis*	Leaf	1998	Jeonnam, Korea
9.	T10-04782	NCBI	Psa/Biovar3	*A. chinensis*	Leaf	2010	New Zealand
10.	T10-05454	NCBI	Psa/Biovar3	*A. deliciosa*	Leaf	2010	New Zealand
11.	T11-0918	NCBI	Psa/Biovar3	*A. deliciosa*	Leaf	2011	New Zealand

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12.	CRA-FRU 10.22	NCBI	Psa/Biovar3	*A. chinensis* 'Hort16A'	Leaf	2008	Latina, Italy
13.	Psa1B	NCBI	Psa/Biovar3	*A. deliciosa*	Leaf	2010	Maule, Chile
14.	Psa1A	NCBI	Psa/Biovar3	*A. deliciosa*	Leaf	2010	Maule, Chile
15.	T11-01369A	NCBI	Psa/Biovar4(Psaf)	*A. chinensis*	Leaf	2011	Victoria, Australia
16.	T10-05195	NCBI	Psa/Biovar4(Psaf)	*A. deliciosa*	Leaf	2010	Nelson, New Zealand
17.	T10-05163	NCBI	Psa/Biovar4(Psaf)	*A. chinensis*	Leaf	2010	Hastings, New Zealand
18.	T10-04976	NCBI	Psa/Biovar4(Psaf)	*A. chinensis*	Leaf	2010	Pongakawa, New Zealand
19.	MAFF 212054	NCBI	Psa/Biovar5	*A. chinensis* 'Hort16A'	Leaf	2012	Saga, Japan
20.	MAFF 212055	NCBI	Psa/Biovar5	*A. chinensis* 'Hort16A'	Leaf	2012	Saga, Japan
21.	MAFF 212056	NCBI	Psa/Biovar5	*A. chinensis* 'Hort16A'	Leaf	2012	Saga, Japan
22.	212130	NCBI	Psa/Biovar6	*A. deliciosa* 'Hayward'	branches	2015	Nagano, Japan
23.	212132	NCBI	Psa/Biovar6	*A. deliciosa* 'Hayward'	branches	2015	Nagano, Japan
24.	212134	NCBI	Psa/Biovar6	*A. deliciosa* 'Hayward'	branches	2015	Nagano, Japan
25.	DC3000	NCBI	*P. s.* pv. *tomato*	*L. esculentum*	Leaf	2003	USA

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SUPPLEMENTARY TABLE 2. Analysis of variance of the least square means and means separation to detect differences between countries for each principal component of the PCA.

Country	Component1	Component2	Component3	Component4
Portugal	1.2462 a	-1.2812 bc	-0.9982 a	-1.1530 c
Italy	1.0002 a	-0.9388 ab	-0.7094 a	-1.0456 c
China	-0.0085 a	0.4529 a	0.1216 a	-0.0777 bc
Spain	-0.8565 a	-2.8237 c	0.2301 a	1.4108 ab
France	-0.9336 a	-2.0698 c	-0.3060 a	1.7293 a
New Zealand	-1.3528 a	-1.9337 c	-0.5124 a	1.9303 a
P value	0.4583	<0.001	0.5503	<0.001
LSD	3.0540	1.4130	2.0640	1.4880

Mean values in each column followed by different lower-case letters are significantly different based on Duncan's new multiple range test ($\alpha = 0.05$).

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SUPPLEMENTARY TABLE 3. The four loci coding for phytotoxins and the eleven effector genes in the 24 strains of *Pseudomonas syringae* pv. *actinidiae* screened for in this study.

No	Strain	Phaseolotoxin			Coronatine	Type III secretion system effector protein genes										
	name	*PHA*	*HM*	*OCT*	*CFL*	*hopH1*	*hopA1*	*hopAF1*	*hopAA1-1*	*hopAA1-2*	*avrD1*	*hopB1*	*hopD1*	*hopAM1*	*hopAH1*	*hopX1*
1.	Jzhy1	−[a]	−	−	−	+	−	−	+	+	+	+	+	+	+	−
2.	Jzhy2	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−
3.	Jzhy3	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−
4.	Jzhy4	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−
5.	Jzhy5	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−
6.	Jzhy6	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−
7.	Jzhy7	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
8.	Jzhy8	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−
9.	Jzhy9	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−
10.	Jzhy10	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−
11.	Jzhy11	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−
12.	Jzhy12	−	−	−	−	+	−	−	+	+	+	+	+	+	+	−
13.	Jzhy13	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
14.	Jzhy14	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
15.	Jzhy15	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
16.	Jzhy16	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−

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17.	18839	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
18.	7285	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
19.	349	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
20.	830	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
21.	827	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
22.	1F	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
23.	Jzmc1	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
24.	Jzgmc2	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
25.	Jzzm2	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
26.	P1	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
27.	HWD3	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
28.	JF8	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
29.	INS	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
30.	WT3	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
31.	GC31	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
32.	Schy9	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
33.	SH8	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−
34.	K3	+	+	+	−	−	−	−	+	+	+	+	+	−	+	+
35.	Psa92	+	+	+	−	−	−	+	+	+	+	+	+	−	+	+
36.	Kw30	+	+	+	−	−	−	−	+	+	+	+	+	−	+	+
37.	18804	−	−	−	−	−	+	+	+	+	−	+	−	−	+	−
38.	3739	+	+	+	−	−	−	−	+	+	+	+	+	−	+	+
39.	1758	+	+	+	−	−	−	−	+	+	+	+	+	−	+	+

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40.	1747	+	+	+	−	−	−	−	+	+	+	+	+	−	+	+
41.	765	−	−	−	−	−	−	−	+	+	+	+	+	−	+	+
42.	KN2	−	−	−	+	−	−	−	+	+	+	+	+	−	+	+
43.	18802	−	−	−	−	−	+	+	+	+	−	+	−	−	+	−
44.	18882	−	−	−	−	−	+	+	+	+	−	+	−	−	+	−
45.	18883	−	−	−	−	−	+	+	+	+	−	+	−	−	+	−
46.	212054	−	−	−	−	−	+	+	+	+	+	+	+	−	+	+
47.	212055	−	−	−	−	−	+	+	+	+	+	+	+	−	+	+
48.	212056	−	−	−	−	−	+	+	+	+	+	+	+	−	+	+
49.	212130	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+
50.	212132	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+
51.	212134	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+

[a]The symbol ‘—’ indicated the absence of the gene in the strain and the symbol ‘＋’ indicated the presence of the gene in the strain.

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Supplementary Fig. 1 Sampling sites for the strains of Pseudomonas syringae pv. actinidiae collected in China in this study; there were five different areas representing typical kiwi-cultivating climates in China. The five representative areas are indicated by brown overlay: the East Sea coastline in Shanghai municipality, the Mid-east plain of Anhui Province, the Southwest mountain area of Guizhou Province, the Southwest basin of Sichuan Province and the Northwest plain of Shaanxi Province. The black markers indicate the sampling sites: Jiading District in Shanghai, Jinzhai and Yuexi County in Anhui province, Meixian and Huxian County in Shaanxi province, Pengzhou and Mianzhu County in Sichuan province, and Xiuwen County inof Guizhou province.

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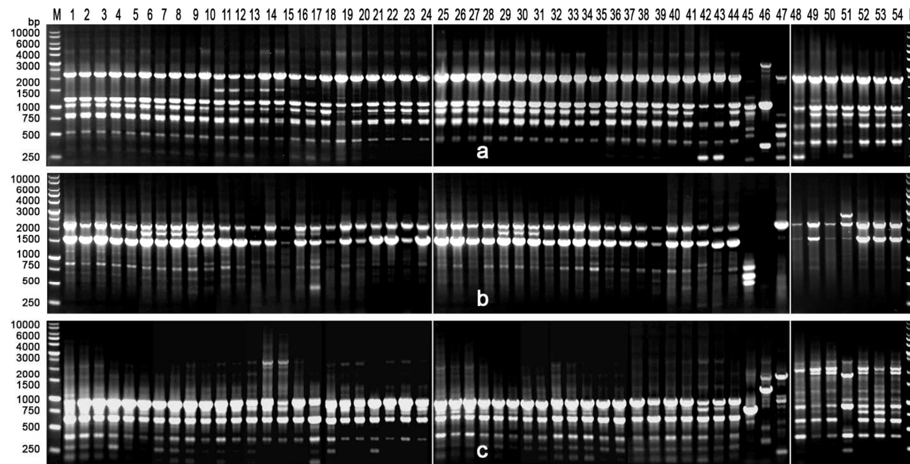

Supplementary Fig. 2 Multiple PCR fingerprinting patterns from genomic DNA of 51 strains of *Pseudomonas syringae* pv. *actinidiae* (Psa) and 3 related pathovars of *P. syringae*. Panels are as follows: Rep-PCR with primer BOX A1R (a), RAPD-PCR with primer A-24 (b) and IS50-PCR with primer IS50 (c). A 10 μL amount of each of the PCR products was loaded onto a 1.2% agarose gel with ethidium bromide. The amplicon profiles for the strains of Psa were from China (Lanes 1-37), Italy (Lanes 38-41 and 48), Korea (Lane 42), Japan (Lane 43), Portugal (Lane 44), Spain (Lanes 49 and 50), New Zealand (Lanes 51 and 52) and France (Lanes 53 and 54); *P. syringae* pv. *syringae* strain 3039B (Lane 45), *P. viridiflava* strain 4254A (Lane 46) and *P. syringae* pv. *theae* strain 2598 (Lane 47) are shown. The left and right lanes labeled M show the DNA molecular size marker (1-kb ladder; Aidlab Biotechnologies Co., Ltd., Beijing, China). Sizes are indicated in base pairs.

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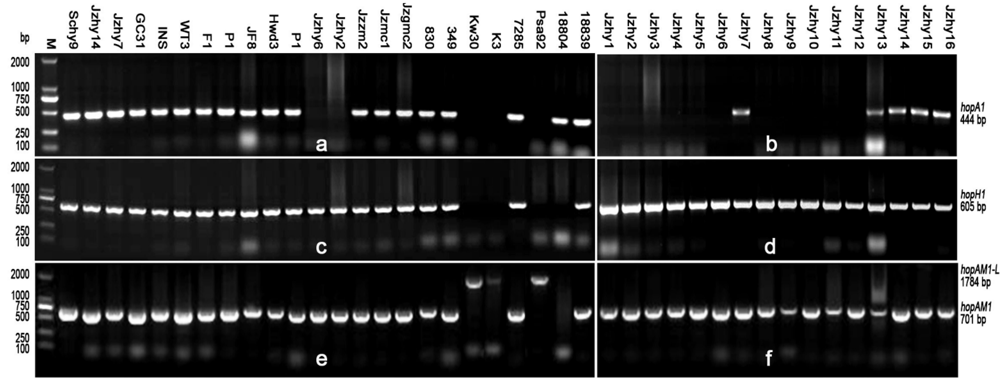

Supplementary Fig. 3 The effector genes detected by specific primers using PCR and agarose gel electrophoresis that were used to differentiate the strains of 24 isolates of *Pseudomonas syringae* pv. *actinidiae*. Panel a and b: A 444-bp fragment of the effector gene *hopA1*; Panel c and d: A 605-bp fragment of the effector gene *hopH1*. Strain Kw30, K3, and Psa92 belonged to Biovar 1 and lacked both *hopA1* and *hopH1* genes; all of the strains in Biovar3 had both *hopA1* and *hopH1* genes; Biovar 4 strain 18804 (Psaf) had *hopA1* but lacked *hopH1*. Newly identified strains (Jzhy1-6 and Jzhy8-12) were found among isolations of Psa from China that lacked *hopA1* but had *hopH1*. Panel c and d: All strains in Biovar3 had the *hopAM1* gene (701 bp), but that in Biovar 4 (Psaf) did not. Strain Kw30, K3, and Psa92 in Biovar 1 yielded a fragment that was 1784 bp (*hopAM1-L*).

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