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Pseudomonas amygdali (syn. *Pseudomonas savastanoi*) pv. *adzukicola* pv. nov., causal agent of bacterial stem rot of adzuki bean

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The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases as accessions LC651953–LC651965, LC652527–LC652532, LC662832 and LC662833.

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Abstract

Bacterial stem rot of adzuki bean (BSRA) was first recorded in Japan in 1979. The pathogen was identified as a new species, designated as "*Pseudomonas adzukicola*". However, due to the lack of a type strain, "*P. adzukicola*" has been recognized as invalid since 1980. In the 2000s, we obtained BSRA isolates and compared them with related *Pseudomonas* species. Inoculations with the BSRA isolates caused symptoms on adzuki bean, cowpea, hyacinth bean, and kidney bean. *Pseudomonas savastanoi* pv. *glycinea* (*Psag*) did not affect adzuki bean, whereas *P. savastanoi* pv. *phaseolicola* caused distinct symptoms on adzuki bean and had a different host range from the BSRA isolates. The BSRA isolates were similar to *Psag* in their bacteriological characteristics except that they utilized DL-α-alanine and L-histidine. Phylogenetic analyses based on four housekeeping genes suggested that the BSRA isolates were closely related genetically to *Psag*, belonging to *P. syringae* genomospecies 2 and *hrp* group IA. However, the rep-PCR results distinguished the BSRA isolates from *Psag* and the other *Pseudomonas* species. These results suggest that the BSRA agent is an independent taxon among genomospecies 2 bacteria and that the BSRA agent represents a new pathovar. Because *P. amygdali* is the oldest legitimate name of the genomospecies 2 bacteria, *P. savastanoi* should be treated as a synonym of *P. amygdali*. Therefore, we propose the name *P. amygdali* (syn. *P. savastanoi*) pv. *adzukicola* pv. nov. for the BSRA pathogen, as pathotype strain AZK-11 (SUPP2776, MAFF212478, ICMP24382).

Keywords Adzuki bean • Bacterial stem rot • *Pseudomonas amygdali* (syn. *Pseudomonas savastanoi*) pv. *adzukicola* pv. nov.

Introduction

Adzuki bean (*Vigna angularis* [Willd.] Ohwi and Ohashi) is an important annual fabaceous crop in Japan and eastern Asian countries. Sweet bean paste made from adzuki bean is an indispensable ingredient in traditional Japanese foods. Historically, adzuki beans were called "red diamonds" for their high market value (Kajiyama 1994). During the 2020 growing season, approximately 51,900 tons of adzuki beans were harvested over an area of 26,600 ha in Japan (Ministry of Agriculture, Forestry and Fisheries of Japan 2021).

Hokkaido, an island in northern Japan, is a major adzuki bean production area. Bacterial stem rot of adzuki bean (BSRA) (Fig. 1) was first reported in Hokkaido in the 1970s (Tanii and Baba 1979). The BSRA pathogen was recognized as a new species and designated as "*Pseudomonas adzukicola*" based on its host range and its bacteriological and serological properties (Tanii and Baba 1979); however, its type strain was neither designated nor preserved (Bradbury 1986). Therefore, the name "*P. adzukicola*" has been considered invalid since January 1, 1980, when the International Code of Nomenclature of Bacteria (1976 revision) (Lapage et al. 1975) was applied; the name was also not included in the Approved List of Bacterial Names (Skerman et al. 1980).

In the 2000s in Hokkaido, adzuki bean plants developed severe water-soaked or necrotic lesions on leaves, stems, and pods that were nearly identical to those caused by BSRA (Tanii and Baba 1979). To characterize the pathogen, we isolated bacteria from symptomatic plants in 2005, 2007, and 2009. Based on our preliminary examination (Todai et al. 2011), the pathogen appeared to be a member of the *P. syringae* (*Psy*) group bacteria (Anzai et al. 2000; Mulet et al. 2010), i.e., *Psy* sensu lato or *Psy* complex.

Psy group bacteria consists of nine genomospecies based on a genomic comparison using DNA–DNA hybridization (Gardan et al. 1999). Gardan et al. (1999)

101 placed *P. amygdali* (*Pa*) in genomospecies 2, indicating that it was the earliest synonym
102 of *P. ficuserectae* (*Pf*), *P. meliae* (*Pm*), and *P. savastanoi* (*Psa*). Additionally, Gomila et
103 al. (2017) used comparative genomics to confirm that *Pf*, *Pm*, and *Psa* are later
104 synonyms of *Pa*.

105 In this study, we applied a polyphasic approach to characterize and compare this
106 BSRA agent and strains of *Psy* group bacteria. The objective of this study was to provide
107 a full description of the bacterium that caused BSRA in Japan in the 2000s.

108

109 **Materials and methods**

110 **Bacterial strains**

111 Samples of diseased adzuki bean plants were collected in 12 fields in Hokkaido in 2005,
112 2007, and 2009. Bacteria were isolated from water-soaked lesions or necrotic lesions on
113 leaves, stems, and immature pods using a conventional streaking method on plates of
114 King's medium B (KB; King et al. 1954). The plates were incubated at 25 °C for 3 days
115 in the dark. The resultant translucent white colony was subjected to repeated single-
116 colony isolations to obtain a pure culture, and one isolate was selected for each adzuki
117 bean plant. Pathogenicity of the isolate on adzuki bean plant was tested and confirmed
118 as described in the next section. We designated 12 representative isolates with confirmed
119 pathogenicity in adzuki bean as BSRA isolates (Table 1), which were deposited in
120 Genebank, the Genetic Resources Center, National Agriculture and Food Research
121 Organization, Tsukuba, Ibaraki, Japan, (accession nos. MAFF 212478–212489). Isolate
122 AZK-11 was also deposited as ICMP24382 in the International Collection of
123 Microorganisms from Plants culture collection, Manaaki Whenua – Landcare Research,
124 Auckland, New Zealand. We also tested 18 reference strains including nomenspecies
125 and pathovars of *Psy* group bacteria including *Pa*, *Pf*, *Psa*, *Psy*, *P. tremae* (*Pt*), and *P.*

126 *viridiflava* (*Pv*) (Table 2). In this study, all species names except for *Pa*, which has not
127 been reported in Japan, are described in compliance with the *Common Names of Plant*
128 *Diseases in Japan* (2021.9 edition) (Anonymous 2021). All strains were grown at 28 °C
129 for 2–3 days on slants of modified potato–peptone–glucose agar (Nishiyama 1978),
130 which consisted of potato extract (200 g potato, 1000 ml DW), 5 g peptone, 3 g
131 $Na_2HPO_4 \cdot 12H_2O$, 0.5 g KH_2PO_4, and 15 g agar. The isolates were preserved in skim
132 milk solution (10 g skim milk and 1.5 g sodium glutamate in 100 ml DW) at −20 °C or
133 lyophilized. After the optical density of the bacterial water suspension was measured at
134 600 nm using a Nabi spectrophotometer (MicroDigital, Seongnam, Korea), the number
135 of colony forming units (cfu) was estimated preliminarily using the dilution method.
136 **Pathogenicity of BSRA isolates on several Fabaceae species (inoculation test 1)**
137 All 12 BSRA isolates (Table 1) and three reference strains (soybean bacterial blight
138 pathogen [*Psa* pv. *glycinea* strain SUPP211], kidney bean halo blight pathogen [*Psa*
139 pv. *phaseolicola* strain SUPP1139], and adzuki bean bacterial brown spot pathogen
140 [*Psy* pv. *syringae* strain 1-1]) were used to inoculate 10 Fabaceae species: adzuki bean
141 (*Vigna angularis* cv. Erimo-shozu), cowpea (*V. unguiculata* cv. Kegon-no-taki), mung
142 bean (*V. radiata*), broad bean (*Vicia faba* cv. Nintoku-issun), hyacinth bean (*Lablab*
143 *purpureus* cv. Akabana-fujimame), kidney bean (*Phaseolus vulgaris* cv. Taisho-
144 kintoki), pea (*Pisum sativum* cv. Hokkai-akabana), soybean (*Glycine max* cv. Otofuke-
145 osode), sword bean (*Canavalia gladiata* cv. Aka-natamame), and winged bean
146 (*Psophocarpus tetragonolobus*). All species except sword bean were grown in 10.5-cm
147 pots (three plants/pot) in a growth chamber (25 °C, ambient humidity, 16 h light/8 h
148 dark) until the first trifoliate leaves were produced. One sword bean plant was grown
149 in a 10.5-cm pot in the same way. For each species, three plants each were sprayed
150 until run off with a suspension of a strain (ca. 10^9 cfu/ml of sterile distilled water

151 [SDW] with 0.01% [v/v] Tween 80) that had been grown on KB agar plates for 2 days
152 at 28 °C. SDW with 0.01% (v/v) Tween 80 was used as a control. The plants were
153 maintained in the dark with high humidity at 25 °C for 48 h, then transferred to a
154 growth chamber (25 °C, ambient humidity, 16 h light/8 h dark). After 10 and 20 days,
155 the plants were examined for symptoms. Symptoms caused by each isolate and strain
156 were categorized as water-soaked lesions, necrotic lesions, dark brown lesions,
157 reddish-brown lesions, halos, stem rot, or bacterial ooze. Two experiments were
158 conducted.

159 **Host range test of BSRA isolates (inoculation test 2)**

160 Two representative BSRA isolates (AZK-11 and 09Psa273) were tested to determine the
161 host range of the BSRA pathogen. We selected 27 species (24 herbaceous species [28
162 crop plants], 3 woody) among 12 families as test plants (Table S1). The 28 herbaceous
163 crop plants were grown from seed in 10.5-cm pots (1–3 seeds/pot) in a greenhouse (ca.
164 20–30 °C) for 4 weeks after sowing. The plants were inoculated by spraying as described
165 above or by pricking the leaf blade with a needle (23 G: diameter, 0.60 mm) through a
166 drop of bacterial suspension on the leaf (puncture method: Takikawa et al. 1989). The
167 plants were then placed in plastic boxes (74 × 44 × 35 cm) overnight to retain moisture
168 and then returned to the greenhouse. After 1 month, the plants were examined for
169 symptoms. Nursery stock of the woody Rosaceae species bungo Japanese apricot (cv.
170 Bungo-ume), peach (cv. Hakuho), and Sargent's cherry (cv. Ezo-yama-sakura) were also
171 grown in 30 cm pots (one plant per pot) for 2−3 years. In 2013, three young shoots of
172 these woody plants were inoculated using the puncture method, then maintained in the
173 greenhouse (ca. 20–30 °C) for 1 month, transferred outdoors, and grown from June to
174 September in 2013 in Naganuma (43°05N, 141°76E), Hokkaido, Japan. This experiment
175 was done once.

176 **Pathogenicity of BSRA isolates and reference bacterial strains on adzuki bean**
177 **(inoculation test 3)**
178 For detecting even weak virulence, leaves of adzuki bean cv. Erimo-shozu were
179 punctured as described above with the two representative BSRA isolates (AZK-11 and
180 09Psa273) or reference strains listed in Table 2, excluding *Pa*, *Psa* pv. *savastanoi*, and
181 *Pt*. Plants were kept in a growth chamber (25 °C, 100% relative humidity, in the dark)
182 for 48 h, then transferred to another growth chamber (25 °C, ambient humidity control,
183 16 h light/8 h dark) for 3 weeks. For each strain, symptoms observed around the
184 inoculation site were categorized as water-soaked lesion, small or large areas of necrosis,
185 or halo formation. Two experiments were conducted.
186 **Pathogenicity of BSRA pathogen and *Psa* pv. *phaseolicola* on immature pods of**
187 **adzuki bean and kidney bean (inoculation test 4)**
188 To clarify any differences in pathogenicity between BSRA pathogen and the kidney
189 bean halo blight pathogen *Psa* pv. *phaseolicola*, immature pods were removed from
190 adzuki bean or kidney bean plants then washed with tap water, and wiped with paper
191 towels sprayed with 70% ethanol. A suspension of BSRA isolate AZK-11 or *Psa* pv.
192 *phaseolicola* strain SUPP1139 were prepared as described above, then adjusted to ca.
193 10^9 cfu/ml, and used to puncture 10 sites on each of three immature pods from a plant
194 as described. SDW was used as control. The pods were then placed on a paper towel
195 wetted with SDW, covered with a polyethylene bag, and held at 25 °C in the dark. Pods
196 were examined for symptoms at 3 and 7 days post-inoculation (dpi). Two trials were
197 done.
198 **Morphological and bacteriological characteristics**
199 The morphology of BSRA isolate AZK-11 on a yeast–peptone (YP; 5 g yeast extract, 10
200 g peptone, 1000 ml DW, pH 6.8) agar (1.5% [w/v]) plate was examined after 3 days at

201 27 °C. Bacterial cells were stained with 2% (w/v) phosphotungstic acid solution for
202 observation with a JEM-1011 EM837513 transmission electron microscope (TEM)
203 (JEOL, Tokyo, Japan). Cells were stained with 0.25% (w/v) phenosafranine solution (25
204 mg phenosafranine in 1 ml 95% ethanol, diluted with 9 ml DW), then measured using a
205 light microscope. The flagella were counted as described by Tsuji and Takikawa (2018).
206 The bacteriological characteristics of the 12 BSRA isolates and 18 reference
207 strains were examined using the methods of Takikawa et al. (1989) and Suzuki et al.
208 (2003). Growth temperature in 1% (w/v) peptone broth was examined at 30 °C, 33 °C,
209 34 °C, and 35 °C. Liquefaction of sodium polypectate was tested as described by
210 Hildebrand (1971). The utilization of organic compounds as a sole carbon source was
211 determined using the modified medium of Ayers et al. (Anonymous 1957), consisting of
212 1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.03 g bromothymol blue, 15 g agar, and
213 1000 ml DW (pH 6.8–7.2), incorporating carbon sources at 0.1% (w/v). We tested 37
214 carbohydrates, 25 organic acids, and 22 amino acids. Any visible growth was scored as
215 positive. Except for gelatin liquefaction at 20 °C, bacterial strains were cultured at 25 °C
216 in all tests. Results were assessed after 2, 4, 7, 14, and 21 days. The tests were done once.

217 **DNA extraction**

218 Each isolate or strain was grown in 5 ml of YP broth for 2 days. The cultured bacterial
219 cells were collected by centrifugation at 18,700 × *g* for 1 min. The pellet was suspended
220 in Tris-EDTA buffer, consisting of 50 mM Tris-HCl and 40 mM EDTA (pH 8.0).
221 Genomic DNA was then extracted using the cetyltrimethylammonium bromide
222 procedure (Ausubel et al. 1987).

223 **Grouping based on *hrpZ* sequence**

224 Inoue and Takikawa (2006) classified 35 strains of the *Psy* group bacteria into five
225 groups (IA, IB, II, III, and IV) based on *hrpZ* sequences and developed five sets of

226 specific primers to distinguish these *hrp* groups. To identify *hrp* groups in this study, we
227 used the PCR analysis developed by Inoue and Takikawa (2006) using all 12 BSRA
228 isolates and all 18 reference strains and the primers listed in Table S2. The 25 µl reaction
229 mixture contained 1 µl template DNA, 0.2 µM of each primer, 100 µM dNTP mixture,
230 0.5 U HotStar Taq Plus DNA polymerase (Qiagen, Hilden, Germany), and 2.5 µl reaction
231 buffer and was run in a Dice TP650 system (Takara Bio, Kusatsu, Japan). The
232 thermocycling conditions consisted of 95 °C for 5 min; 30 cycles at 94 °C for 30 s, 60 °C
233 for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. PCR products
234 were separated in 1% agarose gels stained with GelRed (Biotium, Fremont, CA, USA)
235 or ethidium bromide and viewed using an ultraviolet light transilluminator. Two trials
236 were done.

237 **Fingerprinting by repetitive DNA sequence-based PCR (rep-PCR)**

238 To compare genetic diversity among *hrp* group IA strains including the BSRA isolates,
239 we used rep-PCR with primer sets BOXA1R (Versalovic et al. 1994) for repetitive DNA
240 sequences of the box A subunit of the BOX element of *Streptococcus pneumoniae*
241 (BOX), ERIC1R /ERIC2 (Versalovic et al. 1991) for enterobacterial repetitive intergenic
242 consensus (ERIC), and REP1R-I/REP2-I (Versalovic et al. 1991) for repetitive
243 extragenic palindromic sequence (REP) as described by Rademaker et al. (2004). It is
244 known that rep-PCR are particularly suitable for the rapid molecular characterization of
245 plant pathogenic bacteria, especially at the pathovar level (Louws et al. 1994). Table S2
246 lists the PCR primers used in this analysis. The thermocycling conditions were 95 °C
247 for 2 min; 35 cycles at 94 °C for 3 s, 92 °C for 30 s, and 50 °C for 1 min for BOX and
248 ERIC or 40 °C for 1 min for REP, then 65 °C for 8 min; and a final extension at 65 °C
249 for 8 min. All amplicons were electrophoresed with 1.5–2% agarose gels. Two trials
250 were done.

251 **16S rDNA sequence analysis**

252 The 16S rDNA from two representative BSRA isolates (AZK-11 and 09Psa273) and one
253 reference strain (*Psy* pv. *syringae* strain 1-1) were amplified and purified as described
254 by Takahashi et al. (2013) using primers in Table S2, then direct sequenced by BEX Co.
255 Ltd. (Tokyo, Japan). The obtained sequences were aligned using the GENETYX v10
256 program (GENETYX, http://www.genetyx.co.jp). Sequence data were deposited in the
257 DDBJ/EMBL/GenBank databases (accession LC651953, isolate AZK-11; LC651958,
258 isolate 09Psa273, strain 1-1). To compare the relationships among related *Psy* group
259 bacteria, the 16S rDNA sequences of 28 strains were obtained from the
260 DDBJ/EMBL/GenBank databases. Phylogenetic analysis was performed using MEGA
261 X software (Kumar et al. 2018). Phylogenetic trees were generated using the neighbor-
262 joining method and the Jukes–Cantor model, with *Pv* (CFBP2107^{T} = ICMP2848^{T})
263 sequences as the outgroup.

264 **Multilocus sequence analysis of *gap1*, *gltA*, *gyrB*, and *rpoD***

265 Partial DNA sequences of the *gap1*, *gltA*, *gyrB*, and *rpoD* housekeeping genes were
266 obtained from two representative BSRA isolates (AZK-11 and 09Psa273). The *gap1*,
267 *gltA*, *gyrB*, and *rpoD* sequences were amplified by PCR using methods described by
268 Tsuji and Takikawa (2018), Sarkar and Guttman (2004), Yamamoto et al. (1999), and
269 Maeda et al. (2006), respectively. Used primers are listed in Table S2. The amplified
270 PCR products were purified and cloned as described by Tsuji et al. (2017). Plasmids
271 containing the *gap1*, *gltA*, *gyrB*, and *rpoD* fragments were extracted using the miniprep
272 method described by Ausubel et al. (1987) and sequenced by BEX Co. Ltd. Sequence
273 data were deposited in the DDBJ/EMBL/GenBank databases (Table S3). As references,
274 the *gap1*, *gltA*, *gyrB*, and *rpoD* sequences of 28 strains were obtained from the
275 DDBJ/EMBL/GenBank databases; Table S3 lists their accession numbers. A

276 concatenated data set for *gap1*, *gltA*, *gyrB*, and *rpoD* sequences was used for multilocus
277 sequence analysis. Phylogenic analysis based on these housekeeping gene sequences
278 was performed as described for the 16S rDNA sequence analysis. The concatenated *Pv*
279 (ICMP2848^{T}) data set was used as an outgroup to construct the phylogenetic tree.
280

281 **Results**

282 **Disease symptoms**

283 In 2005, 2007, and 2009, symptoms typical of BSRA were observed in adzuki bean
284 fields in northern and eastern Hokkaido, Japan. In June of each year, small, circular
285 water-soaked spots and streaks on primary and first trifoliate leaves were found on
286 adzuki bean seedlings. The lesions turned into reddish-brown spots without halos and
287 gradually developed into necrotic lesions (Fig. 1a). In July, water-soaked stem lesions
288 and stem rot were observed at the flowering stage (Fig. 1b), which are distinctive
289 symptoms of BSRA. In August, we observed dark-greenish, water-soaked spots and
290 bacterial ooze on immature pods (Fig. 1c). Bacterial ooze exudates from petiole and
291 stem lesions were sometimes also observed. These symptoms are identical to the
292 description of BSRA provided by Tanii and Baba (1979). At a later stage of growth,
293 wedge-shaped necrotic lesions were observed on leaflets in severely infested fields (Fig.
294 1d); this symptom was not described by Tanii and Baba (1979). Among 12 BSRA
295 isolates, only isolate 09Psa289 was isolated from one such lesion.

296 **Pathogenicity of BSRA isolates on several Fabaceae species (inoculation test 1)**

297 Table 3 lists the results of pathogenicity tests using BSRA isolates on 10 Fabaceae
298 species. All tested BSRA isolates were virulent on adzuki bean. Water-soaked, reddish-
299 brown to dark brown spots and necrotic lesions were observed on inoculated adzuki
300 bean plant leaves at 10 dpi (Fig. S1a). Distinct halos were not observed around the

301 lesions. Leaflets wilted soon after symptoms were observed at the base of the leaflet.
302 Water-soaked red streaks and spots were also observed on petioles and stems (Fig. S1b).
303 If the nodes were affected, the lesions progressed to a stem rot. With nearly all tested
304 BSRA isolates, bacterial ooze exuded from lesions on the petioles and stems of adzuki
305 bean plant by 20 dpi (Fig. S1b), and these lesions were similar to those observed in the
306 field.

307 All BSRA isolates caused reddish-brown spots without halos on leaves of
308 hyacinth bean (Fig. S1c) and kidney bean (Fig. S1d) plants, but mainly caused dark
309 brown lesions on cowpea plant stems (Fig. S1e). Among the 12 isolates, only isolate
310 09Psa351 caused a small number of reddish-brown lesions on mung bean leaves. The
311 BSRA isolates caused no symptoms on broad bean, pea, soybean, sword bean, or winged
312 bean.

313 *Psa* pv. *glycinea* strain SUPP211, a pathogen causing soybean bacterial blight,
314 was virulent on kidney bean, soybean, and winged bean, but caused no symptoms on
315 adzuki bean. *Psa* pv. *phaseolicola* strain SUPP1139, a pathogen causing kidney bean
316 halo blight, was virulent on adzuki bean (Fig. S1f), hyacinth bean, kidney bean, and
317 winged bean. This strain induced discoloration or greenish-yellow halos around lesions
318 on adzuki bean or kidney bean leaves. *Psy* pv. *syringae* strain 1-1, a pathogen causing
319 adzuki bean bacterial brown spot, was virulent on adzuki bean (Fig. S1g), cowpea,
320 hyacinth bean, pea, and winged bean. This strain caused small water-soaked spots on
321 adzuki bean leaflet, similar to the initial symptoms induced by the BSRA isolates. The
322 lesions caused by *Psy* pv. *syringae* strain 1-1 gradually enlarged and developed into
323 light-brown necrotic flecks with distinct brown margins and discoloration around the
324 lesions. The central part of the necrotic area easily collapsed. No symptoms were
325 observed on stems. These symptoms caused by *Psy* pv. *syringae* strain 1-1 were identical

326 to the symptoms of bacterial brown spot on adzuki bean after natural infection in the
327 field (Tanii and Baba 1971) and differed distinctly from those of BSRA.

328 **Host range test of BSRA isolates (inoculation test 2)**

329 Table S1 lists the host range results. The tested BSRA isolates (AZK-11 and 09Psa273)
330 were pathogenic only on adzuki bean among the 27 tested species.

331 **Pathogenicity of BSRA isolates and reference bacterial strains on adzuki bean**
332 **(inoculation test 3)**

333 Table 4 lists the of pathogenicity results for BSRA isolates and reference strains on
334 adzuki bean plants. The two BSRA isolates (AZK-11 and 09Psa273), three strains of
335 *Psa* pv. *phaseolicola* (PP8172, SUPP191, and SUPP1139), and *Psy* pv. *syringae* strain
336 1-1 were pathogenic on adzuki bean. Symptoms that developed after puncturing were
337 almost identical to those after the spray inoculation (data not shown). *Psy* pv.
338 *coronafaciens* strain SUPP196 and *Psy* pv. *tabaci* strain SUPP278 rapidly induced
339 distinct yellow halos without water-soaked lesions around the inoculation sites on
340 adzuki bean leaves at 3–4 dpi, rapidly followed by light to dark brown necrosis around
341 inoculation sites (Fig. S1h, i). No other reference strains were pathogenic on adzuki bean.

342 **Pathogenicity of BSRA pathogen and *Psa* pv. *phaseolicola* on immature pods of**
343 **adzuki bean and kidney bean (inoculation test 4)**

344 At 3 dpi, BSRA isolate AZK-11 caused dark-greenish, water-soaked lesions on
345 immature adzuki bean and *Psa* pv. *phaseolicola* strain SUPP1139 (causing kidney bean
346 halo blight) also caused dark-greenish, water-soaked lesions on immature kidney bean
347 pods. They were identical to symptoms observed in infested fields (Fig. S2a, d). If the
348 adzuki bean pods were not fresh, the water-soaked lesions caused by the BSRA isolate
349 had often turned dark brown by 7 dpi (data not shown). At 7 dpi with *Psa* pv.
350 *phaseolicola*, lesions around the inoculation site on kidney bean pods had sometimes

351 turned a pale red (data not shown). The BSRA isolate also caused dark-greenish, water-
352 soaked lesions on kidney bean pods by 3 dpi. However, the water-soaked lesions
353 gradually became drier and lesions around inoculation sites had become reddish-brown
354 by 7 dpi (Fig. S2b). In contrast, brown lesions appeared on adzuki bean pods by 3 dpi
355 with *Psa* pv. *phaseolicola* (Fig. S2c).

356 **Morphological and bacteriological characteristics**

357 Colonies of BSRA isolate AZK-11 were creamy-white, circular, smooth, flat or slightly
358 convex, and 1–2 mm in diameter, with entire margins on YP agar. Cells were
359 nonsporulating, straight to slightly curved rods, 0.4–1.0 μm wide, 0.8–4.3 μm long
360 (average: 0.6 × 2.0 μm), and motile, with 1–6 polar flagella (Fig. S3).

361 All tested BSRA isolates were gram-negative and aerobic, formed translucent
362 white colonies, and produced small amounts of fluorescent pigment on KB agar. The
363 isolates oxidized glucose and grew at 33–34 °C but not at 35 °C. The tested BSRA
364 isolates were positive for levan production, tobacco hypersensitive reaction, reduction
365 of substances from sucrose, and catalase and urease activity. Negative reactions were
366 obtained in the following tests: oxidase activity, potato soft rot, arginine dihydrolase
367 activity, gluconate oxidation, Tween 80 hydrolysis, and nitrate reduction. All 12 BSRA
368 isolates multiplied in 0.3% NaCl concentration in 1% peptone water, and only 4 isolates
369 multiplied in 1% NaCl. The BSRA isolates utilized D-mannitol as their sole carbon
370 source. Eleven BSRA isolates utilized *myo*-inositol. All 12 were negative for utilization
371 of D-sorbitol, D-trehalose, D-tartaric acid, L-tartaric acid, *meso*-tartaric acid, DL-α-
372 alanine, β-alanine, L-histidine, and L-serine. These characteristics of the present BSRA
373 isolates, which differed from those of other tested *hrp* group IA strains and the bacterium
374 formerly identified as "*P. adzukicola*" (Tanii and Baba 1979), which is now invalid, are
375 summarized in Table 5. The bacteriological characteristics of reference strains in our

376 experiments were nearly consistent with the previous reports (Gardan et al. 1999; Ogimi
377 and Higuchi 1981; Psallidas and Panagopoulos 1975; Schaad et al. 2001; Tanii et al.
378 1976). Additional bacteriological characteristics of the 12 BSRA isolates are provided
379 in the description of the new pathovar.

380 **Grouping based on *hrpZ* sequence**

381 The 12 BSRA isolates and 10 reference strains (*Pa* type strain ICMP3918; *Psa* pv.
382 *glycinea* strains CHUK and SUPP211; *Psa* pv. *phaseolicola* strains PP8172, SUPP191,
383 and SUPP1139; *Psa* pv. *savastanoi* type strain and pathotype strain ICMP4352; *Psy* pv.
384 *morsprunorum* strain SUPP408; *Psy* pv. *myricae* strain SUPP166; and *Pt* type strain
385 SUPP449) yielded an amplicon of ca. 880 bp in PCR analysis using primers specific to
386 *hrp* group IA strains (Table 2, Fig. 2, data not shown for *Pa*, *Psa* pv. *savastanoi*, and *Pt*).
387 Using the same primers, *Psy* pv. *tabaci* strain SUPP278 yielded an amplicon of ca. 550
388 bp, presumably due to an internal deletion in the *hrpZ* gene of *Psy* pv. *tabaci* (Inoue and
389 Takikawa 2006; Taguchi et al. 2001). No amplicon was produced from the BSRA
390 isolates in PCR analysis using specific primers for the other *hrp* groups (IB, II, III, or
391 IV; data not shown). *Psy* pv. *syringae* strain 1-1, a pathogen of adzuki bean bacterial
392 brown spot, yielded an amplicon of ca. 750 bp in the PCR using the primer set specific
393 for *hrp* group III strains (Table 2). Two groups of adzuki bean pathogens, BSRA isolates
394 and *Psy* pv. *syringae* strain 1-1 belonged to distinct *hrp* groups (IA and III).

395 Four other groups of reference strains, *Pf* type strain SUPP1391 and *Psy* pv. *mori*
396 strain SUPP582, *Psy* pv. *maculicola* strain SUPP1331, *Psy* pv. *pisi* strain SUPP1662,
397 and *Psy* pv. *coronafaciens* strain SUPP196 yielded amplicons of sizes described by
398 Inoue and Takikawa (2006) in PCR analysis using primer sets specific for *hrp* groups
399 IB, II, III, and IV, respectively (Table 2). No amplicon from *Pv* strain SUPP113 was
400 produced by PCR using any primer sets specific for *hrp* groups (Table 2).

401 **Fingerprinting by rep-PCR**

402 Rep-PCR results obtained using the primer sets ERIC1R/2 (Fig. 3, data not shown for
403 *Pa*, *Psa* pv. *savastanoi*, or *Pt*), BOXA1R, and REP1R-I/2-I (Fig. S4, data not shown for
404 *Pa*, *Psa* pv. *savastanoi*, or *Pt*) revealed that all 12 BSRA isolates yielded identical DNA
405 fragment banding patterns, which differed from those of the other *hrp* group IA strains.
406 The reproducibility of the fingerprint for each rep-PCR was sufficiently confirmed.

407 **16S rDNA sequence analysis**

408 The 16S rDNA sequences of both representative BSRA isolates (AZK-11 and 09Psa273;
409 1371 bp) were completely identical. The BSRA isolates had 99.6–99.9% similarity with
410 other *hrp* group IA strains of *Psy* group bacteria for the 16S rDNA sequence. In the
411 phylogenetic tree based on 16S rDNA, BSRA isolates formed a sister clade within the
412 clade comprising *Psy* pathovars and related *Pseudomonas* species (Fig. S5), indicating
413 that the BSRA isolates belong to the *Psy* bacteria group.

414 **Multilocus sequence analysis of *gap1, gltA, gyrB,* and *rpoD***

415 The *gap1* (860 bp) and *gltA* (573 bp) nucleotide sequences were identical between BSRA
416 isolates AZK-11 and 09Psa273, but the isolates differed by 3 bp in *gyrB* (910 bp) and 1
417 bp in *rpoD* (804 bp). Sequences of the four housekeeping genes in the both BSRA
418 isolates were the closest to those of the seven *Psa* pv. *glycinea* reference strains
419 (ICMP2198^{PT}, B076, BR1, KN44, LN10, race4, and UnB647). The sequences of the
420 four housekeeping genes of the seven *Psa* pv. *glycinea* strains were identical. The *gap1*
421 and *gltA* sequences of both BSRA isolates were identical to those of the *Psa* pv. *glycinea*
422 strains. For *gyrB* sequences, isolates AZK-11 and 09Psa273 differed respectively by 1
423 and 2 bp from the *Psa* pv. *glycinea* strains. The *rpoD* sequences of isolate AZK-11 were
424 identical to those of the *Psa* pv. *glycinea* strains, whereas that of isolate 09Psa273
425 differed by 1 bp from those of the *Psa* pv. *glycinea* strains. In the phylogenetic tree based

426 on sequences of the four housekeeping genes, both BSRA isolates belonged to the same
427 clade as the *Psa* pv. *glycinea* strains (Fig. S6). These results were supported by the
428 phylogenetic tree based on the concatenated sequences of these four genes (Fig. 4; 3147
429 bp).
430

431 **Discussion**

432 The causal pathogen of BSRA was recently recognized as *Pseudomonas* sp. in Japan
433 (Anonymous 2021). To clarify the taxonomic position of this bacterium and to describe
434 it in detail, we compared its pathogenicity on various plant species and evaluated its
435 bacteriological characteristics and genetic traits using related *Psy* group bacterial species
436 and pathovars as reference strains.

437 BSRA isolates reproduced symptoms originally described by Tanii and Baba
438 (1979) in pathogenicity tests in adzuki bean plants; therefore, we identified the present
439 isolates as the authentic BSRA agent. The results of inoculation test 1 verified BSRA
440 isolates pathogenicity in cowpea, hyacinth bean, and kidney bean, but not in soybean,
441 as also reported by Tanii and Baba (1979). *Psa* pv. *phaseolicola*, a pathogen of kidney
442 bean halo blight (strain SUPP1139), and *Psy* pv. *syringae*, a pathogen of adzuki bean
443 bacterial brown spot (strain 1-1), were also pathogenic in adzuki bean. BSRA isolates
444 caused distinct water-soaked lesions, typical stem rot, and bacterial ooze on adzuki bean
445 stems, whereas *Psa* pv. *phaseolicola* strain SUPP1139 only slightly affected adzuki bean
446 stems. The results of the inoculation test 4 revealed that BSRA isolate AZK-11 caused
447 dark-greenish water-soaked lesions on immature adzuki bean pods, whereas *Psa* pv.
448 *phaseolicola* strain SUPP1139 rapidly developed brown lesions. Thus, we were able to
449 distinguish between adzuki bean pod lesions formed by the BSRA isolate and *Psa* pv.
450 *phaseolicola*, even though the water-soaked lesions caused by the BSRA pathogen often

451 turned dark brown over time. Adzuki bean leaf symptoms caused by *Psy* pv. *syringae*
452 strain 1-1 were also distinguishable from those caused by BSRA isolates. The results of
453 inoculation test 3 revealed that the pathogen causing oat halo blight (*Psy* pv.
454 *coronafaciens* strain SUPP196) and tobacco wild fire, (*Psy* pv. *tabaci* strain SUPP278)
455 caused symptoms on adzuki bean plants after puncture-inoculation, and induced distinct
456 halos that were not produced by the BSRA isolates. Thus, these five plant pathogens
457 including the BSRA agent are distinguishable by the symptoms caused on adzuki bean
458 plants. *Psa* pv. *glycinea* strains CHUK and SUPP211 that cause soybean bacterial blight
459 were not pathogenic on adzuki bean, and the BSRA isolates did not affect soybean.

460 Some bacteriological characteristics differed between the BSRA isolates and *Pa*,
461 *Psa* pv. *glycinea*, *Psa* pv. *phaseolicola*, *Psa*. pv. *savastanoi*, *Psy* pv. *morsprunorum*, *Psy*
462 pv. *myricae*, *Psy* pv. *tabaci*, and *Pt*, which were *hrp* group IA bacteria used as reference
463 strains. For example, *Pa*, *Psy* pv. *morsprunorum*, and *Pt* did not produce a fluorescent
464 pigment on KB agar; *Psa* pv. *savastanoi* was negative for levan production; *Psy* pv.
465 *myricae* and *Psy* pv. *tabaci* were positive for Tween 80 hydrolysis and utilized D-sorbitol,
466 L-tartaric acid, and *meso*-tartaric acid as sole carbon sources; and *Psa* pv. *phaseolicola*
467 utilized DL-α-alanine and L-serine, but not *myo*-inositol. The BSRA isolates were similar
468 to *Psa* pv. *glycinea* in most of the tested bacteriological characteristics, whereas the
469 BSRA isolates differed from *Psa* pv. *glycinea* in that they did not utilize DL-α-alanine
470 and L-histidine.

471 Both of the adzuki bean pathogens, the BSRA agent and *Psy* pv. *syringae*, belong
472 to distinct *hrp* groups, IA and III, respectively. In the phylogenetic tree based on *gap1*,
473 *gltA*, *gyrB*, and *rpoD*, both representative BSRA isolates and the *Psa* pv. *glycinea* strains
474 formed a single cluster, separated from other strains of *hrp* group IA and *Psy* group
475 bacteria. The rep-PCR results revealed a uniform banding pattern for BOX, ERIC, and

476 REP among the BSRA isolates, which was distinct from those of *Psa* pv. *glycinea* and
477 other strains in *hrp* group IA. These results indicate that the BSRA agent and *Psa* pv.
478 *glycinea* are more closely related than other *Psy* group bacteria and that the BSRA agent
479 is genetically distinct from *Psa* pv. *glycinea*.

480 Tanii and Baba (1979) reported "*P. adzukicola*," which is an invalid name, as the
481 BSRA causal agent, indicating that it differed from *Psa* pv. *glycinea*, *Psa* pv.
482 *phaseolicola*, and *Psy* pv. *syringae* in its bacteriological characteristics, host range, and
483 serological reaction. Our host range and bacteriological results for the present BSRA
484 isolates were nearly identical to those described by Tanii and Baba (1979), except for
485 the degree of NaCl tolerance and utilization of D-trehalose, β-alanine, and D-tartaric acid.
486 Tanii and Baba (1979) reported that "*P. adzukicola*" was tolerant to 0.2–0.5% NaCl; the
487 present BSRA isolates grew at 0.3–1.0% NaCl concentration, but their NaCl tolerance
488 appeared to be low. Although it is impossible to directly compare the present BSRA
489 isolates with "*P. adzukicola*" due to the lack of an available strain, the current BSRA
490 agent appears to be identical to "*P. adzukicola*".

491 Our polyphasic approach clearly distinguished the present BSRA isolates from
492 other strains of *Psy* group bacteria. Although the current BSRA agent seems to be most
493 closely related to *Psa* pv. *glycinea* based on its bacteriological characteristics and the
494 multilocus sequence analysis, the BSRA agent appears to be an independent taxon
495 among *Psy* group bacteria due to its pathogenicity and genetic differences according to
496 our rep-PCR results. Therefore, we conclude that the BSRA agent should be recognized
497 as a new pathovar among *Psy* group bacteria. Gardan et al. (1992) proposed that *Psy*
498 subsp. *savastanoi* should be elevated to the species level, as *Pseudomonas savastanoi*,
499 and that this species should include three pathovars (*P. savastanoi* pv. *glycinea*, *P.*
500 *savastanoi* pv. *phaseolicola*, and *P. savastanoi* pv. *savastanoi*) based on the results of

501 DNA–DNA hybridization studies and their bacteriological characteristics. Gardan et al.
502 (1999) later reported nine genomospecies of *Psy* group bacteria based on genomic
503 comparison using DNA–DNA hybridization and that *Psa* was placed in genomospecies
504 2, as a synonym of *Pa*, as well as *Pf* and *Pm*. Among *Psy* group bacteria, *hrp* groups IA,
505 which includes *Psa* pathovars, and IB correspond to genomospecies 2 (Inoue and
506 Takikawa 2006). Phylogenetic analysis using the *rpoD* gene sequence (Parkinson et al.
507 2011) and comparative genomics analysis (Gomila et al. 2017) have also confirmed that
508 *Pa*, *Pf*, *Pm*, and *Psa* are members of genomospecies 2, which are called phylogroup 3
509 and phylogenomic branch VI, respectively, and Gomila et al. (2017) also confirmed that
510 *Pf*, *Pm*, and *Psa* are later synonyms of *Pa*. Because *Pa* is the oldest legitimate name for
511 genomospecies 2, Harmon et al. (2018) concluded that the correct name for species
512 included in genomospecies 2 should be *Pa* and that the new pathovar and other
513 organisms in genomospecies 2 should be referred to as *Pa*. Our results indicate that the
514 BSRA agent is most closely related to *Psa* pv. *glycinea* strains among *hrp* group IA
515 strains, including *Pa*. Therefore, we conclude that the BSRA agent is also a member of
516 genomospecies 2, described by Gardan et al. (1999). We also conclude that *Psa* does not
517 consist of *Psa* pv. *glycinea*, *Psa* pv. *phaseolicola*, and *Psa* pv. *savastanoi* for the
518 following reasons: (1) Many of the pathovars to be related to *Psa* pv. *savastanoi*, i.e.,
519 pathogens of bacterial gall of tree, are not included in *Psa*. (2) Because many researchers
520 suggest that *Psa* is a synonym for *Pa* (Gardan et al. 1999; Gomila et al. 2017; Harmon
521 et al. 2018) as described above, their species names should be united. (3) Among *Psy*
522 genomospecies 2 bacteria, the oldest and valid species name is *Pa* but not *Psa*, as
523 described above. Hence, we consider that the name *Psa* is inappropriate, and also that
524 *Psa* pv. *glycinea* and *Psa* pv. *phaseolicola* currently recognized to belong to *Psa* should
525 be transferred to *Pa*. However, the focus of our present study was a taxonomic of the

526 BSRA pathogen, we do not propose here that the scientific name of *Psa* should be
527 changed. For these reasons, we propose that the BSRA agent should be recognized as a
528 new pathovar of *Pa*, and that it is named *Pseudomonas amygdali* (syn. *Pseudomonas*
529 *savastanoi*) pv. *adzukicola* pv. nov. We designate strain AZK-11 (accessions SUPP2776,
530 MAFF212478, ICMP24382) as the pathotype strain.

531 **Description of *Pseudomonas amygdali* (syn. *Pseudomonas savastanoi*) pv. *adzukicola***
532 **pv. nov.**

533 *Pseudomonas amygdali* (syn. *Pseudomonas savastanoi*) pv. *adzukicola* pv. nov.
534 (adzukicola, inhabiting adzuki bean [*Vigna angularis*]). Gram-negative aerobic
535 bacterium. Cells are nonsporulating, straight to slightly curved rods, 0.4–1.0 μm wide,
536 0.8–4.3 μm long (average: 0.6 × 2.0 μm), and motile, with 1–6 polar flagella. Colonies
537 on yeast peptone agar plates are creamy-white in color, circular, with smooth surfaces,
538 flat or slightly convex, and 1–2 mm in diameter, with an entire margin. Translucent white
539 colonies form on King's medium B agar plates and produce small amounts of fluorescent
540 pigment. Strains are negative for oxidase activity, potato soft rot, and arginine
541 dihydrolase activity and positive for levan production and tobacco hypersensitive
542 reaction. The maximum growth temperature is 33–34 °C. Polyhydroxybutyrate granules
543 are not accumulated. Glucose is metabolized oxidatively, and alkali is produced in
544 purple milk. The strains are positive for the reduction of substances from sucrose, for
545 catalase and urease activity and negative for gluconate oxidation; Tween 80, esculin,
546 arbutin, and starch hydrolysis; nitrate reduction; lecithinase, pectate lyase,
547 phenylalanine deaminase, and tyrosinase activity; pectolysis at pH 5, 7, and 8.5; gelatin
548 liquefaction; nitrate respiration; indole, 3-keto-lactose and H_2S production; casein
549 decomposition; growth factor requirement; and coronatine-like activity on potato slices.
550 NaCl tolerance is low, with a maximum at 0.3–1.0% concentration in 1% peptone water.

551 Strains are positive for utilization of D-glucose, D-fructose, D-galactose, D-mannose, L-
552 arabinose, D-xylose, D-ribose, sucrose, D-raffinose, glycerol, D-arabitol, D-mannitol,
553 acetate, citrate, fumarate, gluconate, L-malate, quinic acid, succinate, triacetin, L-
554 asparagine, L-aspartate, L-glutamate, L-glutamine, and trigonelline. *Myo*-inositol
555 utilization differs among strains. Xylan, malonate, and D-saccharate utilization are
556 weekly positive. Colonies of strains grow little on media containing poly-galacturonate
557 and without a color change. Strains are negative for the utilization of D-arabinose, L-
558 rhamnose, L-sorbose, D-cellobiose, lactose, maltose, melibiose, D-trehalose, D-
559 melezitose, ethanol, ethylene glycol, *meso*-erythritol, adonitol, L-arabitol, dulcitol, D-
560 sorbitol, dextrin, alginate, glycogen, starch, α-methyl- D-glucoside, salicin, *n*-butyrate,
561 capric acid, formate, glutarate, glycerate, glycolate, hippurate, DL-lactate, maleate,
562 mesaconic acid, propionate, sebacic acid, D-tartaric acid, L-tartaric acid, *meso*-tartaric
563 acid, DL-α-alanine, β-alanine, L-arginine, DL-homoserine, DL-phenylalanine, glycine, L-
564 histidine, L-isoleucine, L-leucine, L-lysine, L-ornithine, sarcosine, L-serine, L-tyrosine,
565 L-tryptophan, L-valine, and betaine. Pathogenic to *Vigna angularis* from natural
566 infection and to *V. unguiculata*, *Phaseolus vulgaris*, and *Lablab purpureus* after
567 inoculation. The pathotype strain is AZK-11 (SUPP2776, MAFF212478, ICMP24382).
568

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Declarations

Conflicts of interest

The authors declare that they have no conflict of interest.

Human and animal rights

This article does not contain any studies with human participants or animals performed by any of the authors.

References

Anonymous (1957) Manual of microbiological methods. McGraw-Hill, New York

Anonymous (2021) Common names of plant diseases in Japan (in Japanese), 9th edn. The Phytopathological Society of Japan, Tokyo

Anzai Y, Kim H, Park JY, Wakabayashi H, Oyaizu H (2000) Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. Int J Syst Evol Microbiol 50:1563–1589

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) (1987) Current protocols in molecular biology, vol 1. Wiley, New York

Bradbury JF (1986) Guide to plant pathogenic bacteria. CAB International Mycological Institute, Kew

Gardan L, Bollet C, Abu Ghorrah M, Grimont F, Grimont PAD (1992) DNA relatedness among the pathovar strains of *Pseudomonas syringae* subsp. *savastanoi* Janse (1982) and proposal of *Pseudomonas savastanoi* sp. nov. Int J Syst Bacteriol 42:606–612

Gardan L, Shafik H, Belouin S, Broch R, Grimont F, Grimont PAD (1999) DNA

601 relatedness among the pathovar of *Pseudomonas syringae* and description of
602 *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and
603 Dowson 1959). Int J Syst Bacteriol 49:469–478
604 Gomila M, Busquets A, Mulet M, Garcia-Valdés E, Lalucat J (2017) Clarification of
605 taxonomic status within the *Pseudomonas syringae* species group based on a
606 phylogenomic analysis. Front Microbiol 8:2422
607 Harmon CL, Timilsina S, Bonkowski J (2018) Bacterial gall of *Loropetalum chinense*
608 caused by *Pseudomonas amygdali* pv. *loropetali* pv. nov. Plant Dis 102:799–806
609 Hildebrand AC (1971) Pectate and pectin gels for differentiation of *Pseudomonas* sp.
610 and other bacterial plant pathogens. Phytopathology 61:1430–1436
611 Inoue Y, Takikawa Y (2006) The *hrpZ* and *hrpA* genes are variable, and useful for
612 grouping *Pseudomonas syringae* bacteria. J Gen Plant Pathol 72:26–33
613 Kajiyama T (1994) The red diamonds volume 1 (in Japanese). Shueisha, Tokyo
614 King EO, Ward MK, Raney DE (1954) Two simple media for the demonstration of
615 pyocyanin and fluorescein. J Lab Clin Med 44:301–307
616 Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular
617 evolutionary genetics analysis across computing platforms. Mol Biol Evol
618 35:1547–1549
619 Lapage SP, Sneath PHA, Lessel EF, Skerman VBD, Seeliger HPR, Clark WA (1975)
620 International code of nomenclature of bacteria. Bacteriological code, 1976 revision.
621 American Society for Microbiology, Washington DC
622 Louws FJ, Fulbright DW, Stephens CT, de Bruijn FJ (1994) Specific genomic
623 fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and
624 strains generated with repetitive sequences and PCR. Appl Environ Microbiol
625 60:2286–2295

Maeda Y, Shinohara H, Kiba A, Ohnishi K, Furuya N, Kawamura Y, Ezaki T, Vandamme P, Tsushima S, Hikichi Y (2006) Phylogenic study and multiplex PCR detection of *Burkholderia plantarii, Burkholderia glumae* and *Burkholderia gladioli* using *gyrB* and *rpoD* sequences. Int J Syst Evol Microbiol 56:1031–1038

Ministry of Agriculture, Forestry and Fisheries of Japan (2021) Statistics of agriculture, forestry and fisheries. Ministry of Agriculture, Forestry and Fisheries. https://www.maff.go.jp/j/tokei/kekka_gaiyou/tokutei_sakumotu/r2/syukaku_mame/index.html (in Japanese). Cited 28 Sep 2021

Mulet M, Lalucat J, García-Valdés E (2010) DNA sequence-based analysis of the *Pseudomonas* species. Environ Microbiol 12:1513–1530

Nishiyama K (1978) The tentative plan of simple identification method of plant pathogenic bacteria (in Japanese). Plant Prot 32:283–288

Ogimi C, Higuchi H (1981) Bacterial gall of yamamomo (*Myrica rubra* S. et Z.) caused by *Pseudomonas syringae* pv. *Myricae* pv. nov. (in Japanese). Ann Phytopath Soc Japan 47:443–448

Parkinson N, Bryant R, Bew J, Elphinstone J (2011) Rapid phylogenetic identification of member of the *Pseudomonas syringae* species complex using the *rpoD* locus. Plant Pathol 60:338–344

Psallidas PG, Panagopoulos CG (1975) A new bacteriosis of almond caused by *Pseudomonas amygdali* sp. nov. Ann Inst Phytopathol Benaki 11:94–108

Rademaker JLW, Louws FJ, Versalovic J, de Bruijn FJ (2004) Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. In: Kowalchuk GA, de Brujin FJ, Head IM, Akkermans ADL, van Elsas JD (eds) Molecular microbial ecology manual, 2nd edn. Kluwer, Dordrecht, pp 1–33

Sarkar SF, Guttman DS (2004) Evolution of the core genome of *Pseudomonas syringae*,

651 a highly clonal, endemic plant pathogen. Appl Environ Microbiol 70:1999–2012
652 Schaad NW, Jones JB, Chun W (eds) (2001) Laboratory guide for the identification of
653 plant pathogenic bacteria, 3rd edn. APS Press, St Paul
654 Skerman VBD, McGowan V, Sneath PHA (1980) Approved lists of bacterial names. Int
655 J Syst Bacteriol 30:225–420
656 Suzuki A, Togawa M, Ohta K, Takikawa Y (2003) Occurrence of white top of pea caused
657 by a new strain of *Pseudomonas syringae* pv. *pisi*. Plant Dis 87:1404–1410
658 Taguchi F, Tanaka R, Kinoshita S, Ichinose Y, Imura Y, Andi S, Toyoda K, Shiraishi T,
659 Yamada T (2001) $Harpin_{psta}$ from *Pseudomonas syringae* pv. *tabaci* is defective and
660 deficient in its expression and HR-inducing activity. J Gen Plant Pathol 67:116–
661 123
662 Takahashi F, Ogiso H, Fujinaga M, Ishiyama Y, Inoue Y, Shirakawa T, Takikawa Y
663 (2013) First report of bacterial blight of crucifers caused by *Pseudomonas*
664 *cannabina* pv. *alisalensis* in Japan. J Gen Plant Pathol 79:260–269
665 Takikawa Y, Serizawa S, Ichikawa T, Tsuyumu S, Goto M (1989) *Pseudomonas syringae*
666 pv. *actinidiae* pv. nov.: the causal bacterium canker of kiwifruit in Japan. Ann
667 Phytopath Soc Jpn 55:437–444
668 Tanii A, Baba T (1971) Bacterial plant diseases in Hokkaido 1. Bacterial brown spot of
669 adzuki bean caused by *Pseudomonas syringae* van Hall (in Japanese). Bull
670 Hokkaido Pref Agric Exp Stn 23:90–97
671 Tanii A, Baba T (1979) Bacterial stem rot of adzuki bean (*Phaseolus radiatus* var. *aurea*
672 Prain) caused by *Pseudomonas adzukicola* A. Tanii et T. Baba nov. sp. Bull
673 Hokkaido Pref Agric Exp Stn 42:29–42
674 Tanii A, Takakuwa M, Baba T, Takita T (1976) Studies on halo bright of Beans
675 (*Phaseolus vulgaris*) caused by *Pseudomonas phaseolicola* (Burkholder) Dowson

(in Japanese). Misc Publ Hokkaido Prefect Tokachi Agric Exp Stn 6:1–60

Todai T, Takahashi F, Yasuoka S, Takikawa Y (2011) Classification of the pathogen of bacterial stem rot of adzuki bean (abstract in Japanese). Jpn J Phytopathol 77:246–247

Tsuji M, Ohta K, Tanaka K, Takikawa Y (2017) Comparison among Japanese isolates of *Pseudomonas savastanoi* pv. *savastanoi*, causal agent of olive knot disease. J Gen Plant Pathol 83:152–161

Tsuji M, Takikawa Y (2018) *Pseudomonas syringae* pv. *alliifistulosi* pv. nov., the causal agent of bacterial leaf spot of onions. J Gen Plant Pathol 84:343–358

Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucl Acids Res 19:6823–6831

Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25–40

Yamamoto S, Bouvet PJM, Harayama S (1999) Phylogenetic structures of the genes *Acinetobacter* based on *gyrB* sequences: comparison with the grouping by DNA–DNA hybridization. Int J Syst Bacteriol 49:87–95

696 **Figure legends**

697 **Fig. 1** Field symptoms of bacterial stem rot of adzuki bean (BSRA) in Hokkaido, Japan.
698 **a** Reddish-brown spots and necrotic lesion on primary leaf. **b** Water-soaked lesion and
699 rot on stem. **c** Water-soaked spots on immature pods. **d** Wedge-shaped necrotic lesions
700 on leaflets

701 **Fig. 2** Detection of *hrpZ* amplified by PCR with primer set for *hrp* group IA. Lanes M,
702 Hi-Lo DNA Marker (Bionexus); 1, AZK-11; 2, AZB-21; 3, 09Psa251; 4, 09Psa258; 5,
703 09Psa263; 6, 09Psa273; 7, 09Psa275; 8, 09Psa279; 9, 09Psa289; 10, 09Psa351; 11,
704 09Psa355; 12, 09Psa361; 13, *Pseudomonas savastanoi* (*Psa*) pv. *glycinea* SUPP211; 14,
705 *Psa* pv. *glycinea* CHUK; 15, *Psa* pv. *phaseolicola* SUPP1139; 16, *Psa* pv. *phaseolicola*
706 SUPP191; 17, *Psa* pv. *phaseolicola* PP8172; 18, *P. syringae* (*Psy*) pv. *morsprunorum*
707 SUPP408; 19, *Psy* pv. *tabaci* SUPP278; 20, *Psy* pv. *myricae* SUPP166. One microliter
708 of each sample was electrophoresed in 1% agarose gel at 100 V

709 **Fig. 3** DNA fragment-banding patterns obtained through rep-PCR (ERIC). Lanes M, Hi-
710 Lo DNA Marker (Bionexus); 1, AZK-11; 2, AZB-21; 3, 09Psa251; 4, 09Psa258; 5,
711 09Psa263; 6, 09Psa273; 7, 09Psa275; 8, 09Psa279; 9, 09Psa289; 10, 09Psa351; 11,
712 09Psa355; 12, 09Psa361; 13, *Pseudomonas savastanoi* (*Psa*) pv. *glycinea* SUPP211; 14,
713 *Psa* pv. *glycinea* CHUK; 15, *Psa* pv. *phaseolicola* SUPP1139; 16, *Psa* pv. *phaseolicola*
714 SUPP191; 17, *Psa* pv. *phaseolicola* PP8172; 18, *P. syringae* (*Psy*) pv. *morsprunorum*
715 SUPP408; 19, *Psy* pv. *tabaci* SUPP278; 20, *Psy* pv. *myricae* SUPP166. Three
716 microliters of each sample were electrophoresed in 2% agarose gel at 50 V

717 **Fig. 4** Neighbor-joining tree constructed using the Jukes–Cantor model based on
718 concatenated sequences of the *gap1*, *gltA*, *gyrB*, and *rpoD* (3147 bp) of the
719 representative BSRA isolates and related bacterial species and pathovars. T, type strain
720 of the species; PT, pathotype strain of the pathovars. Table S3 lists accession numbers

721 of sequences from the DDBJ/EMBL/GenBank databases. Numbers at nodes are
722 bootstrap values (≥50%) expressed as percentages of 1000 replicates. Bar: 0.01
723 substitutions per nucleotide position
724

Table 1 Strains of bacterial stem rot used in this study

Strain[a]	Accession[b]	Collection information				
		Cultivar	Plant part	Location	Collector	Year
AZK-11	MAFF212478	Syumari	Leaf	Hokkaido, Japan	S. Yasuoka	2007
AZB-21	MAFF212479	Erimo-shozu	Immature pod	Hokkaido, Japan	S. Yasuoka	2005
09Psa251	MAFF212480	Kita-no-otome	Immature pod	Hokkaido, Japan	T. Todai	2009
09Psa258	MAFF212481	Erimo-shozu	Leaf	Hokkaido, Japan	T. Todai	2009
09Psa263	MAFF212482	Syumari	Immature pod	Hokkaido, Japan	T. Todai	2009
09Psa273	MAFF212483	Syumari	Stem	Hokkaido, Japan	T. Todai	2009
09Psa275	MAFF212484	Syumari	Immature pod	Hokkaido, Japan	T. Todai	2009
09Psa279	MAFF212485	Syumari	Stem	Hokkaido, Japan	T. Todai	2009
09Psa289	MAFF212486	Syumari	Leaf	Hokkaido, Japan	T. Todai	2009
09Psa351	MAFF212487	Erimo-shozu	Immature pod	Hokkaido, Japan	T. Todai	2009
09Psa355	MAFF212488	Syumari	Immature pod	Hokkaido, Japan	T. Todai	2009
09Psa361	MAFF212489	Syumari	Stem	Hokkaido, Japan	T. Todai	2009

[a] Most of strain were isolated from water-soaked lesions, but strain 09Psa289 was isolated from wedge-shaped necrotic lesion on a leaf. Two strains AZK-11 and 09Psa273 were preserved in the Shizuoka University Plant Pathology (SUPP) culture collection as SUPP2776 and in International Collection of Microorganisms from Plants (ICMP) as ICMP24382. Strain is also preserved in SUPP culture collection as SUPP2775.

[b] MAFF: Ministry of Agriculture, Forestry and Fisheries

Table 2 Reference strains used in this study

Taxon	Strain[a]	Other strain number[a]	Host of origin	*hrp* group[b]
Pseudomonas amygdali	ICMP3918[T]		Almond	IA
P. ficuserectae	SUPP1391[T]	L-7[T]	Wild fig	IB
P. savastanoi pv. *savastanoi*	ICMP4352[T, PT]		Olive	IA
P. savastanoi pv. *phaseolicola*	PP8172		Kidney bean	IA
P. savastanoi pv. *phaseolicola*	SUPP191	KZ2w	Kudzu	IA
P. savastanoi* pv. *phaseolicola	**SUPP1139**	**BQH-1**	Kidney bean	IA
P. savastanoi pv. *glycinea*	CHUK		Soybean	IA
P. savastanoi* pv. *glycinea	**SUPP211**	**daizu8101**	Soybean	IA
P. syringae pv. *coronafaciens*	SUPP196	AVPCO8101	Oat	IV
P. syringae pv. *maculicola*	SUPP1331	90S-4	Cauliflower	II
P. syringae pv. *mori*	SUPP582	mori8601	Mulberry	IB
P. syringae pv. *morsprunorum*	SUPP408	U7805	Mume	IA
P. syringae pv. *myricae*	SUPP166	yamamomo801	Wax myrtle	IA
P. syringae pv. *pisi*	SUPP1662	PP105	Pea	III
P. syringae* pv. *syringae	**1-1**	**SUPP2781, MAFF212490**	Adzuki bean	III
P. syringae pv. *tabaci*	SUPP278	Pt7364	Tobacco	IA
P. tremae	SUPP449[T]	TO1[T]	*Trema orientalis*	IA
P. viridiflava	SUPP113	hakusai801	Chinese cabbage	-

Bold indicates strains used in pathogenicity tests in 10 Fabaceae plants (Table 3)

[a] *ICMP* International Collection of Microorganisms from Plants, *SUPP* Shizuoka University Plant Pathology, *MAFF* Ministry of Agriculture, Forestry and Fisheries, *T* type strain of the species, *PT* pathotype strain of the pathovar

[b] Result of PCR analysis performed in this study using group-specific primers based on *hrpZ* gene sequences (Inoue and Takikawa 2006)

Table 3 Pathogenicity of BSRA isolates and three reference strains in 10 Fabaceae plants (inoculation test 1)

Plant species	BSRA isolates (n=12)		Reference strains					
			Psag		Psap		Psys	
	L	S	L	S	L	S	L	S
Adzuki bean (*Vigna angularis*)	DB, N, RB, WS	BO, RB, RT, WS	-	-	DB, WS	WS	RB, N	-
Broad bean (*Vicia faba*)	-	-	-	-	-	-	-	-
Cowpea (*Vigna unguiculata*)	DB, RB, WS	DB, WS	-	-	-	-	RB, N	-
Hyacinth bean (*Lablab purpureus*)	RB, WS	RB, WS	-	-	RB, WS	-	RB	-
Kidney bean (*Phaseolus vulgaris*)	RB, WS	RB	RB	-	DB, H, RB, WS	RB	-	-
Mung bean (*Vigna radiata*)	RB (1/12)	-	-	-	-	-	-	-
Pea (*Pisum sativum*)	-	-	-	-	-	-	-	WS
Soybean (*Glycine max*)	-	-	DB, H, WS	WS	-	-	-	-
Sword bean (*Canavalia gladiata*)	-	-	-	-	-	-	-	-
Winged bean (*Psophocarpus tetragonolobus*)	-	-	RB	-	DB	-	DB, H	-

The BSRA isolates results did not vary among isolates, except for pathogenicity in mung bean, which had only reddish-brown spots caused by isolate 09Psa351

Reference strains: Psag, *Pseudomonas savastanoi* pv. *glycinea* SUPP211; Psap, *P. savastanoi* pv. *phaseolicola* SUPP1139; Psys, *P. syringae* pv. *syringae* 1-1

L leaves, *S* stems, *BO* bacterial ooze, *DB* dark brown lesions, *H* halos, *N* necrotic lesions, *RB* reddish-brown lesions, *RT* rot, *WS* water-soaked lesions, - non-pathogenic

Table 4 Pathogenicity of BSRA isolates and reference strains in adzuki bean plants (inoculation test 3)

Taxon	Strains	Symptom
BSRA isolate	AZK-11	LN, SN, WS
BSRA isolate	09Psa273	LN, SN, WS
Pseudomonas ficuserectae	SUPP1391	-
P. savastanoi pv. *glycinea*	CHUK	-
P. savastanoi pv. *glycinea*	SUPP211	-
P. savastanoi pv. *phaseolicola*	PP8172	SN, WS
P. savastanoi pv. *phaseolicola*	SUPP191	SN, WS
P. savastanoi pv. *phaseolicola*	SUPP1139	SN, WS
P. syringae pv. *coronafaciens*	SUPP196	H, SN
P. syringae pv. *maculicola*	SUPP1331	-
P. syringae pv. *mori*	SUPP582	-
P. syringae pv. *morsprunorum*	SUPP408	-
P. syringae pv. *myricae*	SUPP166	-
P. syringae pv. *pisi*	SUPP1662	-
P. syringae pv. *syringae*	1-1	SN
P. syringae pv. *tabaci*	SUPP278	H, SN
P. viridiflava	SUPP113	-

H halo formation, *LN* large area of necrosis, *SN* small area of necrosis, *WS* water-soaked lesion, - no symptoms except for pinpoint necrosis

Table 5 Comparison of bacteriological characteristics among the examined BSRA isolates, "*Pseudomonas adzukicola*", and *hrp* group IA strains of *P. syringae* bacteria

Characteristics	BSRA isolates (n=12)	Pad (n=15)[a]	Pa	Psag (n=2)	Psapb (n=2)	Psapk	Psas	Psymo	Psymy	Psyt	Pt
Fluorescent pigment production	+	+	-	+	+	+	+	-	+	+	-
Levan production	+	+	+	+	+	+	-	+	+	+	-
Oxidase activity	-	-	-	-	-	-	-	-	-	-	-
Potato soft rot	-	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase activity	-	-	-	-	-	-	-	-	-	-	-
Tobacco hypersensitive reaction	+	+	nt	+	+	+	nt	+	+	+	nt
Esculin hydrolysis	-	-	-	-	-	-	-	-	-	+	-
Arbutin hydrolysis	-	-	-	-	-	-	-	-	-	+	-
Tween 80 hydrolysis	-	-	+	-	+	-	w+	-	+	+	+
Litmus milk	K	K	K	K	K	K	K	K	K	KD	K
Tyrosinase	-	-	-	-	-	-	-	-	+	-	-
Utilization of											
myo -Inositol	+ (11/12)	nt	+	+	-	-	+	-	+	+	w+
D-Mannitol	+	+	w+	+	-	+	+	+	+	+	-
D-Sorbitol	-	-	+	-	-	-	-	+	+	+	-
D-Trehalose	-	+	-	-	-	-	-	-	-	-	-
D-Tartaric acid	-	+	w+[b]	-	-	-	-	-	+	-	+[b]
L-Tartaric acid	-	-	-	-	-	-	+	+	+	+	-
meso -Tartaric acid	-	nt	-	-	-	-	-	w+	+	+	-
DL-α-Alanine	-	nt	-	+	+	+	+	+	+	+	+
β-Alanine	-	+	-	-	-	-	-	-	-	+	-
L-Histidine	-	nt	-	+	-	-	-	+	+	w+	-
L-Serine	-	nt	-	-	+	+	+	-	+	+	-

Results did not vary among the examined BSRA isolates except for *myo*-inositol utilization, for which 09Psa351 was negative

Reference strains: Pad, "*P. adzukicola*" (now invalid); Pa, *P. amygdali* ICMP3918^{T}; Psag, *P. savastanoi* (*Psa*) pv. *glycinea* CHUK and SUPP211; Psapb, *Psa* pv. *phaseolicola* PP8172 and SUPP1139 (isolated from kidney bean); Psapk, *Psa* pv. *phaseolicola* SUPP191 (kudzu); Psas, *Psa* pv. *savastanoi* ICMP4352$^{T, PT}$; Psymo, *P. syringae* (*Psy*) pv. *morsprunorum* SUPP408; Psymy, *Psy* pv. *myricae* SUPP166; Psyt, *Psy* pv. *tabaci* SUPP278; Pt, *P. tremae* SUPP449^{T} (T: type strain of the species; PT:

pathotype strain of the pathovar)

+ Positive, *w*+ weakly positive, weak reaction or later reaction than positive strain, - negative, *nt* not tested, *D* digestion, *K* alkalinization

[a] Results from Tanii and Baba (1979)

[b] Growth but no color change to blue in media

Fig. 1

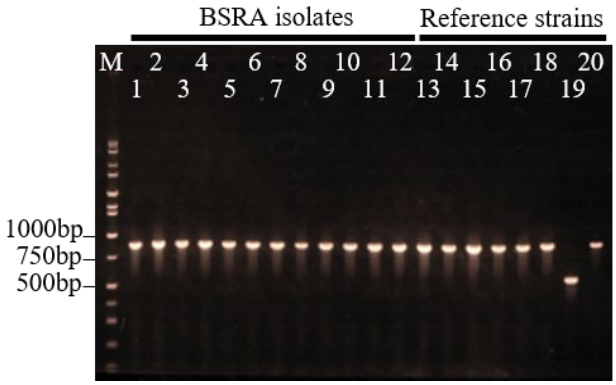
BSRA isolates
Reference strains
M 2 4 6 8 10 12 14 16 18 20
1 3 5 7 9 11 13 15 17 19
1000bp
750bp
500bp

Fig. 2

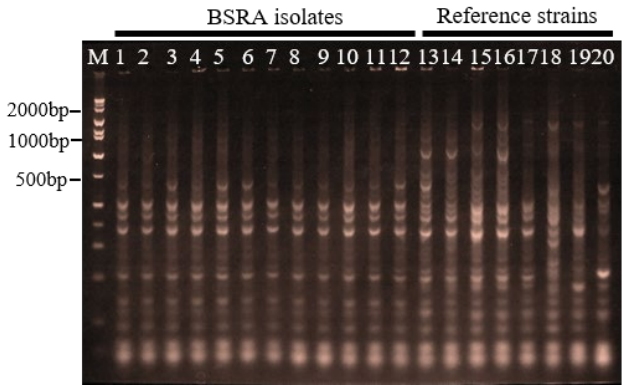
BSRA isolates
Reference strains
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
2000bp
1000bp
500bp

Fig. 3

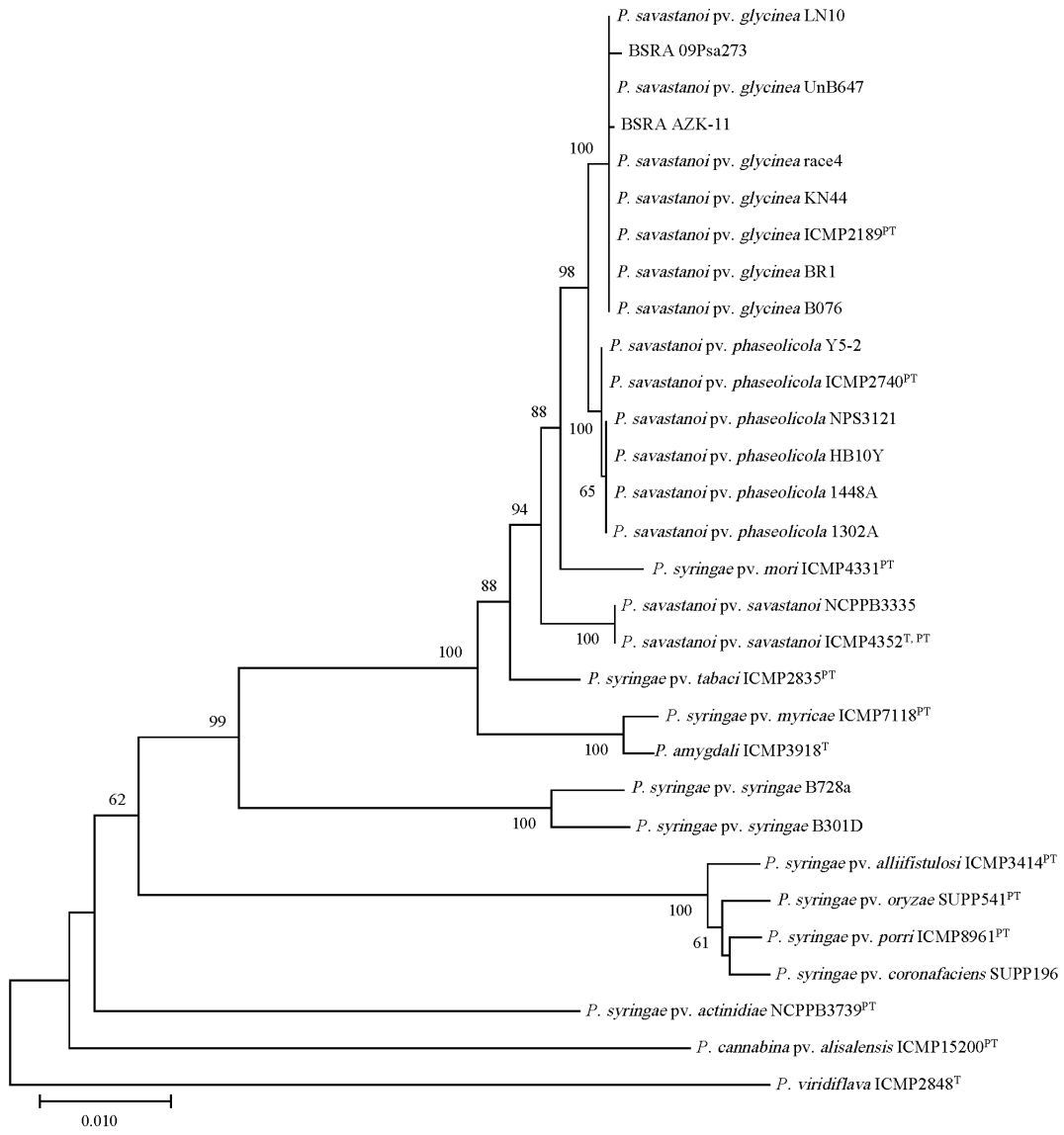
P. savastanoi pv. glycinea LN10
BSRA 09Psa273
P. savastanoi pv. glycinea UnB647
BSRA AZK-11
P. savastanoi pv. glycinea race4
P. savastanoi pv. glycinea KN44
P. savastanoi pv. glycinea ICMP2189PT
P. savastanoi pv. glycinea BR1
P. savastanoi pv. glycinea B076
P. savastanoi pv. phaseolicola Y5-2
P. savastanoi pv. phaseolicola ICMP2740PT
P. savastanoi pv. phaseolicola NPS3121
P. savastanoi pv. phaseolicola HB10Y
P. savastanoi pv. phaseolicola 1448A
P. savastanoi pv. phaseolicola 1302A
P. syringae pv. mori ICMP4331PT
P. savastanoi pv. savastanoi NCPPB3335
P. savastanoi pv. savastanoi ICMP4352T, PT
P. syringae pv. tabaci ICMP2835PT
P. syringae pv. myricae ICMP7118PT
P. amygdali ICMP3918T
P. syringae pv. syringae B728a
P. syringae pv. syringae B301D
P. syringae pv. alliifistulosi ICMP3414PT
P. syringae pv. oryzae SUPP541PT
P. syringae pv. porri ICMP8961PT
P. syringae pv. coronafaciens SUPP196
P. syringae pv. actinidiae NCPPB3739PT
P. cannabina pv. alisalensis ICMP15200PT
P. viridiflava ICMP2848T
100
98
88
100
65
94
88
100
100
99
100
62
100
100
61
0.010

Fig. 4