Phylogenetic Relationships of *Pseudomonas syringae* pv. *syringae* Isolates Associated with Bacterial Inflorescence Rot in Grapevine

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Abstract

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Pseudomonas syringae pv. *syringae* causes extensive yield losses in wine-grape production in some Australian cool-climate vineyards. Putative *P. syringae* pv. *syringae* isolates from infected grapevines within a range of vineyards were genotyped using RNA polymerase β -subunit (*rpoB*) and multilocus sequence typing (MLST) using primers for glyceraldehyde-3-phosphate dehydrogenase (*gapA*), citrate synthase (*gltA*), DNA gyrase B (*gyrB*), and σ factor 70 (*rpoD*). The isolates were also evaluated for pathogenicity by inoculation of detached grapevine leaves. The isolates were grouped by MLST data into two well-supported clades, each containing a mixture of pathogenic and

nonpathogenic grapevine isolates, indicating that *P. syringae* pv. *syringae* in Australian vineyards is genetically diverse. Each clade also contained *P. syringae* pv. *syringae* from nongrape hosts pathogenic to grapevine, demonstrating a lack of host specificity and possible potential for cross-infection of grape and other horticultural crops. Furthermore, the isolation of pathogenic *P. syringae* pv. *syringae* isolates from grapevine sucker shoots suggests that sucker shoots may allow overwintering of the pathogen. The vineyard quarantine status of *P. syringae* pv. *syringae* may need to be reconsidered, due to its easy dispersal through pruning equipment.

In the past decade, wine grape (*Vitis vinifera*) crops grown in the Tumbarumba viticultural region of southeastern New South Wales have been affected by a new bacterial disease. Grapevine symptoms included leaf spots, necrotic lesions on leaf blades and shoots, and loss of inflorescences early in the season. The disease, “bacterial inflorescence rot” (BIR), is caused by bacterium *Pseudomonas syringae* pv. *syringae* (Whitelaw-Weckert et al. 2011). The Tumbarumba region has a cool to moderate climate and is prone to spring frosts (Bureau of Meteorology 2012), so that overhead water sprinkler systems are commonly used to prevent frost damage. It has been proposed that the promotion of humid foliar microclimates by the overhead sprinkler systems may be responsible for some of the grapevine symptoms (Whitelaw-Weckert et al. 2011).

The first report of *P. syringae* foliar symptoms on grapevine was from Argentina, which showed necrotic lesions on petioles, shoots, leaf blades, rachis, and tendrils (Klingner et al. 1976). Several similar reports on grapevine have followed, with reports of bark necrosis (Cugusi et al. 1986), bacteriosis (Samedov et al. 1988), and angular leaf lesions (Hall et al. 2002) caused by *P. syringae*. In these cases, no loss of inflorescences and fruit yield were detailed, and *P. syringae* was considered to be a weak pathogen with little economic impact.

The first observation of bacterial leaf spot (BLS) on wine-grape cultivars in Australian vineyards was reported after heavy rains in the northern Adelaide Hills in October 2000 (Hall et al. 2002). Bacterial “ooze” was observed on necrotic tissue of ‘Verdelho’ grape and *P. syringae* (pv. unspecified) isolates were collected and deposited in the Australian Collection of Plant Pathogenic Bacteria/New South Wales (NSW) Industry and Investment Culture Collection as DAR73915 and DAR75241. In the following season, further infections were confirmed, with increased severity, spreading to three other nearby South Australian cool wine-growing regions and affecting ‘Cabernet Sauvignon’, ‘Viognier’, ‘Merlot’, ‘Sauvignon Blanc’, and ‘Chardonnay’. Although *P. syringae* was recovered from lesions on these vines, there was no reported effect on inflorescences or loss of crop (Hall et al. 2002).

The first account of the detrimental effects of *P. syringae* on wine-grape yield was not recorded until 2006 (Whitelaw-Weckert et al. 2011). Foliar symptoms similar to those of the Adelaide Hills region were described but with the added loss of inflorescences (BIR) and, thus, yield loss in vineyards across the Tumbarumba region. *P. syringae* pv. *syringae* was identified from necrotic inflorescences, bacterial ooze, and necrotic spots and lesions on leaves and shoots (Whitelaw-Weckert et al. 2011). Recently, BIR was also reported on *V. vinifera* in the Sistan region of Iran (Abkhoo 2015).

P. syringae pv. *syringae* also causes significant economic damage to nonviticultural crops in Australia. It causes bacterial necrosis of mango (Golzar and Cother 2008), leading to necrosis of the panicle and reduced fruit set (Young 2008). It also causes bacterial blight in 40% of Australian field pea crops, with up to 75% yield loss in susceptible cultivars (Richardson and Hollaway 2011). Considerable variation in host range may occur between and within the *P. syringae* pathovars (Sawada et al. 1999). For example, *P. syringae* pv. *tomato* causes a hypersensitivity response (HR) in *Arabidopsis* and tomato but not bean, whereas *P. syringae* pv. *phaseolicola* can cause HR in bean and *Arabidopsis* but not tomato (Feil et al. 2005).

Phylogenetic analysis using polymerase chain reaction (PCR) fingerprinting methods has played an important role in demonstrating hierarchical clustering of bacterial pathogens, including *P. syringae* (Gardan et al. 1999; Hwang et al. 2005; Sarkar et al. 2006; Sawada et al. 1999; Whitelaw-Weckert et al. 2011; Yamamoto et al. 2000).

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Recently, phylogenetic analysis using multilocus sequence typing (MLST) has become an integral tool in bacterial evolution analysis studies. MLST involves the concatenation of a number of core genome sequences that are ubiquitous among all strains of a bacterial species, and which are essential for the survival of the organism (Hwang et al. 2005). These housekeeping genes are chosen on the basis of being less prone to horizontal gene transfer and providing insight into the evolutionary history of bacteria (Hacker and Carniel 2001). This has been demonstrated by Sarkar and Guttman (2004), who determined the evolutionary history of 60 *P. syringae* isolates using seven housekeeping genes such as aconitate hydratase B, phosphofructokinase, phosphoglucosomerase, and others. Hwang et al. (2005) later refined the number of housekeeping genes to four (citrate synthase, glyceraldehydes-3-phosphate dehydrogenase, DNA gyrase B, and σ factor 70) in an effort to obtain similar results at a lower cost. The sampling of diverse genomes within a phylogenetic framework can reveal general evolutionary trends indicative of changes in lifestyle. This also allows for the identification of genetic changes that allow differentiation between populations that have recently undergone host range shifts (Sarkar et al. 2006).

MLST has been utilized in an attempt to solve the complex relationships between *P. syringae* pathovars and host range. Several studies have used MLST to detail and clarify whether host range and pathovar can be expressed phylogenetically (Clarke et al. 2010; Hwang et al. 2005; Martín-Sanz et al. 2013; Sarkar and Guttman 2004; Sarkar et al. 2006). Although the selection pressures determining host range may be similar across *P. syringae* pathovars, the sequencing of *P. syringae* pv. *syringae* genomes across host species has not been sufficiently deep to uncover trends indicating evolutionary differentiation among groups (Lindeberg et al. 2009). The fact that numerous plant species play host to *P. syringae* pv. *syringae* provides an extra challenge for determining evolutionary differentiation, especially in hosts such as grapevine.

The objectives of this project were to (i) use phylogenetic and molecular techniques to identify *P. syringae* pv. *syringae* isolates collected from diseased grapevines with symptoms of BIR and BLS, (ii) use MLST to compare pathogenic and nonpathogenic *P. syringae* pv. *syringae* isolates from the same regions and from other hosts, and (iii) investigate the relationships between the genetic patterns and the virulence of the isolates from grape and other host species.

Materials and Methods

Four vineyards affected by BIR, three in the Riverina region (Tumbarumba, NSW) and one in the Southern Tablelands (Murrumbateman, NSW), were sampled between September and October 2006, 2011, and 2013. In addition, grapevine samples were obtained from BIR-affected vineyards in the Coonawarra, South Australia (SA) and Piper's River, Tasmania (TAS). Samples were also collected from a vineyard with BLS but no BIR in the Macedon Ranges region (Hanging Rock, Victoria [VIC]), and from apparently healthy grapevines in Victorian vineyards: Glenlofty in the Pyrenees and Hallston in Gippsland. Leaves, shoots, and rachis were collected with ethanol-sterilized equipment, placed into Zip-Lock polyethylene bags, and kept at 4°C until bacterial isolation.

Bacterial isolates from culture collections. *P. syringae* pv. *morsprunorum* (DAR33419) and *P. syringae* pv. *syringae* (DAR72042 and DAR73915) were obtained from the NSW Industry and Investment Culture Collection (Orange, Australia). *P. syringae* isolates BRIP34823, BRIP38670, BRIP34831, BRIP34899, BRIP38817, BRIP34832, BRIP34805, BRIP38811, and BRIP34803 were obtained from the Department of Agriculture, Fisheries and Forestry, Queensland. *P. syringae* isolates DAR82449, DAR82450, DAR82451, DAR82432, and DAR8453 were obtained from Dr. Thomas Hill, Colorado State University.

Isolation of *P. syringae*. *P. syringae* was isolated from leaves, shoots, and inflorescences of grapevines with apparent BIR symptoms. Plant tissues were rinsed with tap water, surface sterilized with sodium hypochlorite solution (1% available chloride) for 3 min, and then rinsed in three washes of sterile deionized water (SDW). The sterilized tissue was then aseptically cut into approximately 5-by-5-mm

pieces, placed on *Pseudomonas* selective CCF (PS) agar (Oxoid) and incubated in darkness at 25°C for up to 3 days. *Pseudomonas* colonies were then subcultured onto nutrient agar and PS agar to isolate pure colonies. Pure colonies were tested for gram stain, fluorescence under UV light (254 nm), and oxidase production (Lelliott and Stead 1987).

Identification of *P. syringae*. Motile gram-negative, fluorescent, oxidase-negative rod-shaped bacteria were selected and maintained on King's B (KB) agar containing peptone at 20 g/liter, MgSO₄ · 7H₂O at 1.5 g/liter, K₂HPO₄ at 1.5 g/liter, 10 ml of glycerol, and agar bacteriological No. 1 (Oxoid) at 15 g/liter at 25°C in darkness. Isolates were tested under the levan and oxidase reaction, potato soft rot, arginine dihydrolyase activity, and tobacco leaf HR (LOPAT) testing scheme (Lelliott and Stead 1987). Pure *P. syringae* cultures were stored at -80°C in nutrient broth containing 30% (vol/vol) glycerol. Tests involving 2-keto gluconate production, nitrate reduction, and production of acid from sucrose were also conducted, as previously described by Lelliott and Stead (1987). Pathovars *P. syringae* pv. *syringae* (from grapevine, cowpea, and stonefruit), *P. syringae* pv. *maculicola*, *P. syringae* pv. *striafaciens*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *mori*, and *P. syringae* pv. *tabaci* were also used to confirm LOPAT identification of *P. syringae* among pathovars.

Koch's postulates. A representative strain of *P. syringae* pv. *syringae* (DAR82161) from Tumbarumba was tested for its ability to cause disease (spreading necrotic leaf lesions) on leaves on four live potted grapevines (Chardonnay and 'Shiraz'). Prior to inoculation, two leaves per plant were removed and checked for the absence of *P. syringae* pv. *syringae* infection in asymptomatic or healthy-looking plants by surface disinfecting with sodium hypochlorite (1% available chloride) for 1 min, macerating in a mortar and pestle with 5 to 10 ml of sterile phosphate-buffered saline (pH 7), serially diluting, spreading (100 µl) over PS agar, and incubating at 25°C in darkness. After demonstrating the absence of endogenous *P. syringae* pv. *syringae* in the potted *V. vinifera* Chardonnay and Shiraz plants, the leaves were spray inoculated with a fine mist suspension (1 × 10⁸ CFU/ml) of *P. syringae* pv. *syringae* isolate DAR82161 (obtained originally from a necrotic grapevine rachis, Tumbarumba) in SDW until visible run-off. The plants were then enclosed in clear plastic bags to maintain humidity at >99%, and arranged in randomized complete blocks in a glasshouse maintained at 25 and 15°C during day and night, respectively. Potted vines were watered to field capacity twice weekly. Disease development was monitored over 21 days and leaf samples collected at the end of the experiment, at which time leaves were surface sterilized, plated onto PS agar, and incubated under the same conditions as described above.

Grapevine pathogenicity leaf test assay. The grapevine pathogenicity leaf test assay (GPLTA) was carried out as described by Cohen et al. (1999), with minor changes. Briefly, healthy leaves were detached from *V. vinifera* Chardonnay plants (grown under greenhouse conditions) and were surface sterilized in 1% available chloride solution containing TWEEN 80 detergent (Sigma) at 100 µl/liter for 3 min, followed by four washes in sterile distilled water. Leaf discs (12 mm in diameter) were aseptically cut with a number 8 cork borer (12 mm in diameter) and placed abaxial side up on 1% agar. For inoculations, 50-µl drops of test bacteria were spot inoculated onto leaf discs at concentrations of approximately 1 × 10⁸ CFU/ml and incubated at 25°C for up to 7 days in a moist sealed bag at relative humidity of >99% in an Intellus Control System Incubator (Percival). Isolates were considered pathogenic to grapevine if typical necrotic symptoms (i.e., appearance of brown lesions at the point of inoculation) appeared within 2 days and were observed over 7 days.

RNA polymerase β -subunit gene analysis. Cultures grown on KB agar for 48 h in darkness were used for DNA extraction, using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. The RNA polymerase β -subunit gene (*rpoB*) was used for pathovar identification by PCR. The primers used for *rpoB* (Table 1) have been previously shown to amplify a 1,247-bp fragment from *Pseudomonas* spp. (Ait-tayeb et al. 2005). PCR reactions (25 µl) were carried out using a BioRad C1000 Thermal Cycler

with GoTaq Green polymerase (Promega) according to the manufacturer's instructions, with a final primer concentration of 0.3 μ M and approximately 100 ng of template DNA. Cycle conditions were 94°C for 3 min; then, 40 cycles of 94°C for 45 s 55°C for 1 min, and 72°C for 90 s; with a final extension step of 72°C for 10 min.

MLST. The four housekeeping genes used for MLST were *gapA*, encoding glyceraldehyde-3-phosphate dehydrogenase; *gltA*, encoding citrate synthase (also known as *cts*); *gyrB*, encoding DNA gyrase B; and *rpoD*, encoding σ factor 70. The MLST protocol was carried out essentially as described by Hwang et al. (2005). PCR was carried out as described above, with the following cycle conditions: 94°C for 2 min, 60°C for 1 min, and 72°C for 1 min for 36 cycles, using primers outlined in Table 1.

PCR products were purified using a PCR Purification Kit (Qiagen) following the manufacturer's instructions and quantified on a 1% agarose gel stained with ethidium bromide. The purified products were sequenced by the Australian Genome Research Facility. Sequence data were analyzed using MEGA5 software and aligned using CLUSTAL W. A BLAST search was conducted on trimmed *rpoB* sequences to confirm identity and to determine the pathovar. *P. syringae* pv. *morsprunorum* (DAR33419) and *P. syringae* pv. *syringae* (DAR72042) were also used in this study as negative and positive controls, respectively, and *P. fragi* ST128 (isolated from a grapevine, Hanging Rock, VIC) was used as the outgroup. Separate phylogenetic trees were generated using neighbor-joining (NJ; *rpoB* and MLST data) and unweighted pair-group method with arithmetic mean (UPGMA; *rpoB* data) with Jukes-Cantor corrected distances (Jukes and Cantor 1969; Kumar et al. 2004) and statistical confidence for sequence groups determined using a bootstrap test with 1,000 pseudoreplicates (Felsenstein 1985).

Accession numbers. Sequence data obtained in this study were deposited into GenBank (Supplementary Table S1).

Results

In order to investigate the genetic diversity of *P. syringae* pv. *syringae* isolates present in Australian vineyards affected by BIR, isolates were collected from symptomatic diseased grapevines (Fig. 1). Symptoms of grapevine BIR or BLS included leaf spots with yellow chlorotic haloes (Fig. 1A), necrotic lesions on shoots (Fig. 1B), bacterial ooze and abscised flowers in inflorescences (Fig. 1C), and death of inflorescences (Fig. 1D). Abscission of the necrotic inflorescences occurred in most cases, resulting in full loss of grape bunches. Once established in a vineyard, the severity of these symptoms generally progressed over the following seasons.

P. syringae was isolated from all surface-sterilized diseased grapevine tissues with BIR and BLS symptoms. Bacterial ooze emerging from the diseased plant tissue and from surfaced-sterilized plant tissue on PS agar consisted of pure cultures of motile oxidase-negative, fluorescent gram-negative bacilli, 0.5 to 1.0 μ m wide and 2.0 to 3.0 μ m long. Colonies produced yellow pigment on PS and KB agar

that fluoresced blue under UV light (254 nm; data not shown). These features are consistent with those of *P. syringae*.

The *P. syringae* isolates were then further characterized using the LOPAT testing regime, which enables the separation of plant saprophytic pseudomonads from pathogenic pseudomonads (Lelliott and Stead 1987). All the *P. syringae* isolates were positive for levan-type colonies on sucrose agar but were negative for oxidase reaction, potato soft rot, and arginine dihydrolyase activity (Lelliott and Stead 1987; Lelliott et al. 1966). Nonpathogenic grapevine *P. syringae* pv. *syringae* isolates (DAR82449, DAR82450, DAR82451, and DAR82452) were unable to cause tobacco leaf HR (Table 2).

Further characterization involved tests for 2-keto gluconate production, nitrate reduction, and acid production from sucrose. The results indicate that most isolates of *P. syringae* were unable to produce 2-keto gluconate from gluconate or to reduce nitrate but were positive for acid production from sucrose. In contrast, one isolate (BRIP34823, *P. syringae* pv. *syringae* from cowpea) was able to produce 2-keto gluconate from gluconate and to reduce nitrate. These results further verified the identification of most of the isolates as LOPAT group 1a *P. syringae* from grapevine (Lelliott and Stead 1987). Interestingly, DAR82445, DAR82447, DAR82443, and DAR82162, isolated from grapevine sucker shoots (latent buds that

Fig. 1. A, Riesling with symptoms of dark leaf spots with yellow chlorotic haloes, Tumbarumba, NSW. B, Riesling petiole with longitudinal necrotic lesions (Tumbarumba, NSW). C, Riesling inflorescence affected by bacterial inflorescence rot; bacterial ooze (arrows) and abscised flowers (Tumbarumba, NSW). D, Necrotic rachis on Cabernet Sauvignon (from field grapevine, Coonawarra, SA). Images supplied by L. Quirk (C), DPI NSW, and N. Scarlett (deceased), Rathbone Wine Group, Melbourne, VIC. (D).

Table 1. Primers used for *rpoB* and multilocus sequence typing (MLST)

Primer ^a	Sequence	Function	Authors
LAPSp	TGGCCGAGAACCAGTTCCGCG	Pathovar identification	Ait-tayeb et al. 2005
LAPS27ps	CGGCTTCGTCCAGCTTGTTCA	Pathovar identification	Ait-tayeb et al. 2005
gapA+264p	CCGGCSGARCTGCCSTGG	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
gapA+312s	CGARTGCACSGGBCTSTTCACC	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
gapA-874ps	GTGTGRTTGGCRTC GAARATCGA	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
gltA+174p	CCTCBTGCAGTTCGAAGATCACC	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
gltA+513s	CTGRTCGCC AAGATGCCGAC	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
gltA-1130s	CGAAGATCACGGTGAACATGCTGG	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
gltA-1192p	CTTGTA VGGRCYGGAGAGCATTTTC	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
gyrB+271ps	CBGCRGCVGARGTSATCATGAC	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
gyrB-1022ps	TTGTCYTTGGTCTGSGAGCTGAA	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
rpoD+174p	AGGTGGAAGACATCATCCGCATG	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
rpoD+364s	YGAAGGCGARATYGRAATCG	MLST	Sarkar and Guttman 2004; Hwang et al. 2005
rpoD-1222ps	CCGATGTTGCCCTTCCTGGATCAG	MLST	Sarkar and Guttman 2004; Hwang et al. 2005

^a Forward-strand primer (+), reverse-strand primer (–), PCR primer (p), and sequencing primer (s).

sprout from the crown, the basal region of the trunk slightly below and above the soil level, of the grapevine trunk) in BIR-affected Tumburumba vineyards had results for LOPAT, 2-keto gluconate production, nitrate reduction, and acid production from sucrose identical to those of the *P. syringae* pv. *syringae* pathogens isolated from infected rachi in the same vineyards (Table 2).

To confirm that *P. syringae* pv. *syringae* was responsible for the disease development on grapevine, Koch's postulates were tested by inoculating leaves of potted *V. vinifera* Chardonnay and Shiraz plants with a representative strain of *P. syringae* pv. *syringae* (DAR82161) and maintaining the leaves under humid conditions. Leaf lesions developed similarly to those observed in vineyard-infected material, with dark necrotic spots with yellow chlorotic haloes appearing on the leaves within 48 h postinoculation (Fig. 2A). These lesions progressed along the veins to noninoculated regions (Fig. 2A), until the leaves senesced (Fig. 2B). After 3 weeks postinoculation, tissue samples were collected and *P. syringae* pv. *syringae* was recovered from the leaves as described above. No *P. syringae* was isolated from the noninoculated plants, thus confirming Koch's postulates.

The remainder of the *P. syringae* isolates collected were also assessed for their ability to cause necrosis on detached grapevine leaf discs using the GPLTA. All *P. syringae* pv. *syringae* isolates obtained from grapevines displaying BIR or BLS produced necrosis

in the GPLTA whereas grapevine isolates from healthy vineyards (i.e., DAR82449, DAR82450, DAR82451, and DAR82452) did not. Interestingly, some *P. syringae* pv. *syringae* isolates from non-grape hosts (e.g., DAR72042 from apple leaf spot, Batlow, NSW; BRIP34823 isolated from cowpea showing leaf necrosis; and BRIP38670, BRIP38817, and BRIP34899 from stone fruit trees with canker) gave positive GPLTA results. Of the non-*syringae*

Fig. 2. Symptoms of *Pseudomonas syringae* pv. *syringae* infection on leaves from potted grapevines used to test Koch's postulates. **A**, Potted Shiraz leaf showing dark bacterial leaf spots with yellow chlorotic haloes after 48 h post spray inoculation. **B**, Leaf senescence on potted Chardonnay grapevine 4 days post spray inoculation with *P. syringae* pv. *syringae*.

Table 2. Levan and oxidase reaction, potato soft rot, arginine dihydrolyase activity, and tobacco leaf hypersensitivity response identification of *Pseudomonas syringae*^a

Isolate	Lev	Ox	Pot	Arg	Tob	2KG	NR	AFS	<i>P. syringae</i> pv. ^b
DAR33419	+	—	—	—	+	—	—	+	<i>morsprunorum</i>
DAR72042	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR73915	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR77819	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR77820	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82159	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82160	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82161	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82162	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82165	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82166	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82169	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82170	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82171	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82440	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82441	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82442	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82443	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82444	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82445	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82446	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82447	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82448	+	—	—	—	+	—	—	+	<i>syringae</i>
DAR82449	+	—	—	—	—	—	—	+	<i>syringae</i>
DAR82450	+	—	—	—	—	—	—	+	<i>syringae</i>
DAR82451	+	—	—	—	—	—	—	+	<i>syringae</i>
DAR82452	+	—	—	—	—	—	—	+	<i>syringae</i>
DAR82453	+	—	—	—	+	—	—	+	<i>syringae</i>
BRIP34803	+	—	—	—	+	—	—	+	<i>tabaci</i>
BRIP34805	+	—	—	—	+	—	—	+	<i>mori</i>
BRIP34823	+	—	—	—	+	+	+	+	<i>syringae</i>
BRIP34831	+	—	—	—	+	—	—	+	<i>syringae</i>
BRIP34832	+	—	—	—	+	—	—	+	<i>strifaciens</i>
BRIP34899	+	—	—	—	+	—	—	+	<i>syringae</i>
BRIP38670	+	—	—	—	+	—	—	+	<i>syringae</i>
BRIP38811	+	—	—	—	+	—	—	+	<i>phaseolicola</i>
BRIP38817	+	—	—	—	+	—	—	+	<i>maculicola</i>
ST128	+	—	—	—	—	—	—	—	<i>fragi</i>

^a Lev = levan like colonies, Ox = oxidase reaction, Pot = potato rot assay, Arg = arginine dihydrolyase activity, Tob = tobacco leaf hypersensitivity reaction, 2KG = 2-keto gluconate production from gluconate, NR = nitrate reduction, and AFS = acid from sucrose.

^b Pathovar as determined from *rpoB* sequencing.

pathovars, *P. syringae* pv. *maculicola* (BRIP38817 from diseased cabbage), *P. syringae* pv. *striafaciens* (BRIP34832 isolated from oat with leaf spot), and *P. syringae* pv. *tabaci* (BRIP34803 from soybean with leaf spot) gave positive GPLTA results, whereas BRIP38811 (pv. *phaseolicola* isolated from bean with leaf spot), BRIP34805 (pv. *mori* from white mulberry), and DAR33419 (pv. *morsprunorum* from a wild cherry leaf) were negative for GPLTA but were positive for the tobacco leaf HR (Table 3).

rpoB gene sequencing was used as the final step in *P. syringae* pathovar identification. When purified *rpoB* PCR products from all isolates were sequenced and BLAST searches conducted, grapevine *P. syringae* isolates were identified as *P. syringae* pv. *syringae*, with 100% similarity to *P. syringae* pv. *syringae* B728a (accession number CP000075) (Table 3). The *rpoB* sequence of the representative grapevine *P. syringae* pv. *syringae* BIR isolate, DAR82448 from Tumbarumba, was identical to the sequence for a leaf spot isolate (DAR73915) originally collected from an Adelaide Hills vineyard in 2000. It was also identical with *P. syringae* pv. *syringae* isolates from three Tumbarumba vineyards associated with loss of crop yield

in the field due to inflorescence necrosis: DAR82171 from a diseased cane, DAR82169 from an infected rachis, and DAR8244 and DAR82446 from sucker shoots. In contrast, some known BIR *P. syringae* pv. *syringae* isolates differed by 3 to 7 nucleotides (nt) (of 426 nt) from DAR82448, showing that this group of pathogens include some genetically relatively dissimilar *P. syringae* pv. *syringae* isolates.

The *rpoB* sequences were used to produce a phylogenetic tree inferred using the NJ method, which showed *P. syringae* pv. *syringae* to be separated from the other *P. syringae* pathovars, albeit with low bootstrap support (42%). The *rpoB* sequences of seven of the Tumbarumba grapevine isolates clustered with the Adelaide Hills isolate (DAR73915), with 87% bootstrap support (Fig. 3). The NJ method was found to be superior to UPGMA for generating a useful phylogenetic tree. The evolutionary distances in the UPGMA tree were not well expressed due to high levels of heterogeneity and weak bootstrap support (data not shown).

MLST has been used to produce phylogenetic trees with higher discriminatory power, resulting in clearer assumptions on evolutionary

Table 3. Characteristics of isolates of *Pseudomonas syringae* pv. *syringae* from Australian vineyards with symptoms of bacterial inflorescence rot^a

Isolate ^b	Host	Host symptom	Host origin ^c	GLPTA ^d	<i>rpoB</i> (nt) ^e
Pss DAR72042	<i>Malus × domestica</i> (apple) Royal Gala	Leaf spot	Batlow, BLS, 1997	+	4
Pss DAR73915	<i>Vitis vinifera</i> , unspecified cultivar	Leaf spot	Adelaide Hills, BLS, 2000	+	0
Pss DAR77819	<i>V. vinifera</i> Sauvignon Blanc	Necrotic rachis	Tumbarumba, BIR, 2006	+	3
Pss DAR77820	<i>V. vinifera</i> Sauvignon Blanc	Shriveled berry	Tumbarumba, BIR, 2006	+	3
Pss DAR82159	<i>V. vinifera</i> Pinot Noir	Shriveled berry	Tumbarumba, BIR, 2011	+	3
Pss DAR82160	<i>V. vinifera</i> Pinot Noir	Cane necrotic lesion	Tumbarumba, BIR, 2011	+	1
Pss DAR82161	<i>V. vinifera</i> Pinot Noir	Necrotic rachis	Tumbarumba, BIR, 2011	+	1
Pss DAR82162	<i>V. vinifera</i> Pinot Noir	Sucker shoot necrotic lesion	Tumbarumba, BIR, 2011	+	7
Pss DAR82165	<i>V. vinifera</i> Pinot Noir	Shoot necrotic lesion	Tumbarumba, BIR, 2011	+	4
Pss DAR82166	<i>V. vinifera</i> Pinot Noir	Leaf spot	Tumbarumba, BIR, 2011	+	4
Pss DAR82169	<i>V. vinifera</i> Pinot Noir	Necrotic rachis	Tumbarumba, BIR 2006	+	0
Pss DAR82170	<i>V. vinifera</i> Riesling	Shoot necrotic lesion	Tumbarumba, BIR, 2006	+	5
Pss DAR82171	<i>V. vinifera</i> Pinot Noir	Cane necrotic lesion	Tumbarumba, BIR, 2011	+	0
Pss DAR82440	<i>V. vinifera</i> Pinot Noir	Shriveled berry	Murrumbateman, BIR, 2013	+	6
Pss DAR82441	<i>V. vinifera</i> Chardonnay	Leaf spot	Hanging Rock, BLS, 2013	+	4
Pss DAR82442	<i>V. vinifera</i> Pinot Noir	Necrotic rachis	Piper's River, BIR, 2014	+	7
Pss DAR82443	<i>V. vinifera</i> Pinot Noir	Sucker cane necrotic lesion	Tumbarumba, BIR, 2013	+	5
Pss DAR82444	<i>V. vinifera</i> Pinot Noir	Sucker shoot necrotic lesion	Tumbarumba, BIR, 2013	+	0
Pss DAR82445	<i>V. vinifera</i> Pinot Noir	Leaf spot	Tumbarumba, BIR, 2013	+	4
Pss DAR82446	<i>V. vinifera</i> Pinot Noir	Sucker shoot necrotic rachis	Tumbarumba, BIR, 2013	+	0
Pss DAR82447	<i>V. vinifera</i> Pinot Noir	Sucker shoot necrotic rachis	Tumbarumba, BIR, 2013	+	4
Pss DAR82448	<i>V. vinifera</i> Pinot Noir	Necrotic rachis	Tumbarumba, BIR, 2013	+	
Pss DAR82449	<i>V. vinifera</i> frost affected Chardonnay	Shoot lesion	Glenlofty, ND, 2007	–	7
Pss DAR82450	<i>V. vinifera</i> frost affected Chardonnay	Shoot lesion	Glenlofty, ND, 2007	–	7
Pss DAR82451	<i>V. vinifera</i> Chardonnay	Healthy shoot	Hallston, ND, 2007	–	3
Pss DAR82452	<i>V. vinifera</i> Chardonnay	Healthy shoot	Hallston, ND, 2007	–	7
Pss DAR82453	<i>V. vinifera</i> Cabernet Sauvignon	Necrotic rachis	Coonawarra, BIR, 2010	+	9
Pss BRIP34823	<i>Vigna unguiculata</i> subsp. <i>unguiculata</i> (cow pea)	Leaf spot	Toowoomba, BLS, 1971	+	4
Pss BRIP34831	<i>Prunus americana</i> (American plum)	Canker	Stanthorpe, C, 1981	–	7
Pss BRIP34899	<i>P. persica</i> (peach)	Canker	Not specified (VIC), C, 1971	+	7
Pss BRIP38670	<i>P. americana</i> (American plum)	Canker	Mount Tully, C, 1972	+	6
Pmo BRIP34805	<i>Morus alba</i> L. (white mulberry)	Not specified	Stanthorpe, 1980	–	22 (Pmo)
Psm BRIP38817	<i>Brassica oleracea</i> var. <i>capitata</i> (L.) Alef. (cabbage)	Not specified	Marcoola Beach, 1978	+	12 (Psm)
Psm DAR33419	<i>P. avium</i> (wild cherry)	Leaf	Armidale, 1975	–	23 (Psm)
Psp BRIP38811	<i>Phaseolus vulgaris</i> (common bean)	Bean spot	Benora Point, 1977	–	20 (Psp)
Pstab BRIP34803	<i>Glycine max</i> (L.) Merr. (soybean)	Not specified	Not specified (VIC), 1980	+	20 (Pstab)
Pstr BRIP34832	<i>Avena</i> spp. (oat)	Not specified	Wyaga, 1981	+	19 (Pstr)
<i>P. fragi</i> (ST128)	<i>Vitis vinifera</i> Sauvignon Blanc	Cane bacterial ooze	Hanging Rock, 2013	–	37 (<i>P. fragi</i>)

^a Comparison of *P. syringae* pv. *syringae* *rpoB* sequences with that of the known Tumbarumba bacterial inflorescence rot isolate, DAR82448.

^b Pss: *P. syringae* pv. *syringae*; Pma: *P. syringae* pv. *maculicola*; Pstr: *P. syringae* pv. *striafaciens*; Psm: *P. syringae* pv. *morsprunorum*; Pmo: *P. syringae* pv. *mori*; Pph: *P. syringae* pv. *phaseolicola*; and Pstab: *P. syringae* pv. *tabaci*.

^c BIR = bacterial inflorescence rot affected vineyard, BLS = bacterial leaf spot affected vineyard, ND = vineyard not affected by leaf spot or bacterial inflorescence rot, and C = canker.

^d Grapevine pathogenicity leaf test assay: positive (+) or negative (–) for necrosis on leaf disc.

^e Number of *rpoB* nucleotides (nt) different from DAR82448 (out of 426 nt).

one nonpathogenic), peach (pathogenic), and grapevine (one pathogenic and one nonpathogenic). Clade 3 contains other pathovars tested in this study (including pvs. *tabai*, *mori*, *morsprunorum*, *maculicola*, *striafaciens*, and *phaseolicola*; Fig. 4).

Distribution in Australian vineyards. This study has used phylogenetic and molecular techniques to investigate the genetic diversity of *P. syringae* pv. *syringae* isolates from diseased vines of wine-grape cultivars in Australian vineyards. Included were isolates from grapevines displaying symptoms of BIR or BLS, alone or in combination, within eight vineyards situated in six different viticultural regions across Australia. *P. syringae* pv. *syringae* was isolated from diseased grapevines in the following cool-climate vineyards

Fig. 3. Phylogenetic relationships between *Pseudomonas* spp. based on *rpoB* sequence. The evolutionary relatedness was inferred using the Neighbor-Joining method. (Saitou and Nei 1987). Isolates that are pathogenic on grapevine leaves are shaded. Pss: *P. syringae* pv. *syringae*; Pma: *P. syringae* pv. *maculicola*; Pstr: *P. syringae* pv. *striafaciens*; Psm: *P. syringae* pv. *morsprunorum*; Pmo: *P. syringae* pv. *mori*; Php: *P. syringae* pv. *phaseolicola*; and Pstab: *P. syringae* pv. *tabaci*. Asterisk (*) indicates that these sequence data were downloaded from GenBank and not tested for grapevine pathogenicity. Numbers on nodes are bootstrap values, the frequency (%) with which a cluster appeared in a bootstrap test of 1,000 runs.

affected by BIR: three in Tumbarumba, NSW; one in Murrumbateman, Southern Tablelands, NSW; one in Piper's River, TAS; and one in Coonawarra, SA. We also isolated *P. syringae* pv. *syringae* from diseased grapevines in a cool-climate vineyard with BLS symptoms only (Hanging Rock, Macedon Ranges, VIC) and a culture collection isolate from an Adelaide Hills, SA cool-climate vineyard with BLS (Hall et al. 2002). Finally, we isolated nonpathogenic *P. syringae* pv. *syringae* from apparently healthy grapevines in cool-climate Victorian vineyards not affected by BIR at Glenlofty, Pyrenees and at Hallston, Gippsland (Fig. 5).

Hall et al. (2002) originally reported *P. syringae* as a weak pathogen of grapevines in the Adelaide Hills region. In that investigation, *P. syringae* pv. *syringae* caused no yield loss, although it did cause increasingly severe foliar symptoms in subsequent seasons. A table-grape vineyard in Mildura, VIC, watered with overhead water sprinklers, also recorded symptoms of *P. syringae* BIR in 1998, 2001, 2004, and 2014 on 'Sultana' and 'Red Globe' grape (C. Skyllas, Victorian Department of Environment and Primary Industries, personal communication). Bacterial isolates from the Mildura table-grape vineyard could not be obtained for our study and,

Fig. 4. Phylogenetic relationships between *Pseudomonas* spp. based on *gapA*, *gltA*, *gyrB*, and *rpoD* concatenated multilocus sequence typing data. Evolutionary relatedness was inferred using the neighbor-joining method (Saitou and Nei 1987). Isolates that are pathogenic on grapevine leaves are shaded. Pss: *P. syringae* pv. *syringae*; Pma: *P. syringae* pv. *maculicola*; Pstr: *P. syringae* pv. *strifaciens*; Psm: *P. syringae* pv. *morsprunorum*; Pmo: *P. syringae* pv. *mori*; Php: *P. syringae* pv. *phaseolicola*; and Pstab: *P. syringae* pv. *tabaci*. Numbers on nodes are bootstrap values, the frequency (%) with which a cluster appeared in a bootstrap test of 1,000 runs.

therefore, could not be compared with the cool-climate wine-grape Australian isolates of *P. syringae* pv. *syringae*. However, the collection dates indicate that symptoms of BIR caused by *P. syringae* may predate the report of Hall et al. (2002).

Infection of grapevine. The observed symptoms produced by *P. syringae* pv. *syringae* on grapevine (necrotic lesions on leaf tissue, shoots, and inflorescences) are in agreement with those previously reported for BIR by Whitelaw-Weckert et al. (2011) and Abkhoo (2015). *P. syringae* pv. *syringae*, which has an extensive plant host range, can be widely distributed on plant surfaces, water, and soil. Following heavy spring rains, the bacterium spreads across wet plant surfaces on shoots and inflorescences and through the leaf stomata (Melotto et al. 2006). In grapevine, *P. syringae* pv. *syringae* infection starts in the leaves (BLS), followed by systemic movement of bacteria to the bunch rachis (BIR) (Whitelaw-Weckert et al. 2011). BIR of immature grapevine inflorescences shows similarities to a disease of immature fruit blossoms by *P. syringae* pv. *syringae* in other woody fruit trees: apple (Mansvelt and Hattingh 1989), lychee (Alfrose et al. 2014), mango (Cazorla et al. 1998; Golzar and Cother 2008; Young 2008); pear (Mansvelt and Hattingh 1987; Moragrega et al. 2003), and stone fruit (Little et al. 1998).

Because *P. syringae* pv. *syringae* is a relatively new pathogen to the wine industry, symptoms on grapevine may be misidentified as other pathological or physiological conditions. Some symptoms of BIR may have previously been attributed to *Botrytis cinerea*, which causes gray mold of grape bunches. *B. cinerea* can infect inflorescences early in the season, causing necrosis (Keller et al. 2003). Similarly, *P. syringae* pv. *syringae* infection also induces necrosis in inflorescences but with the additional symptom of occasional visible bacterial ooze emerging from the plant tissues. Underreporting may also have been caused by the misdiagnosis of *P. syringae* pv. *syringae* as a physiological disorder. Prior to the Whitelaw-Weckert et al. (2011) study, symptoms of bacterial inflorescence rot in Tumbarumba were attributed to physiological causes: inflorescence necrosis and bunch-stem necrosis. Inflorescence necrosis and bunch-stem necrosis are induced by ammonium toxicity (Keller and Koblet 1995), and their symptoms include rachis or pedicel lesions and inflorescence or bunch abscission (Capps and Wolf 2000). The symptoms are similar to those of bacterial inflorescence rot, except that the bacterial disease causes the additional symptom of water-soaked appearance and bacterial ooze consisting of pure cultures of *P. syringae* pv.

syringae (Abkhoo 2015; Whitelaw-Weckert et al. 2011). The similarity of these symptoms may have led to *P. syringae* pv. *syringae* infections being underreported within the wine industry. Future investigations should include surveying inflorescences early in the season and comparing bunch numbers at harvest within vineyards containing known *P. syringae* pv. *syringae* infection.

Phenotypic identification. Phenotype-based methods for identifying *P. syringae* such as LOPAT can produce variable results depending on pathovar, host origin, or the nature of the bacterium itself (Lelliott and Stead 1987). The nonpathogenic grapevine *P. syringae* pv. *syringae* isolates (DAR82449, DAR82450, DAR82451, and DAR82452) were unable to cause tobacco leaf HR. Because the LOPAT protocol was devised for pathogenic bacteria, the protocol appears to have successfully differentiated between the pathogenic and nonpathogenic strains in our collection. This is consistent with the findings of Diallo et al. (2012), who demonstrated that their environmental *P. syringae* isolates were unable to cause HR in tobacco. The HR is the result of a cell death program used by plants to combat infecting bacteria. It is initiated by plant resistance proteins following recognition of effectors secreted into the plant cell by the invading bacteria to suppress host defenses. However, HR does not occur unless the bacteria have a functional type III secretion system (T3SS), which is encoded by the *hrp* and *hrc* genes (He 1996). The HR-negative isolates identified by Diallo et al. (2012) lacked at least one gene in the canonical *hrp/hrc* locus or the associated conserved effector locus which prevents them from initiating HR on tobacco. Further studies are required to investigate whether the nonpathogenic grapevine *P. syringae* pv. *syringae* isolates identified in the current study also contain mutations in the *hrp/hrc* locus.

Molecular characterization of isolates. The present study used *rpoB* sequence typing and MLST analysis to confirm the identity of the DAR73915 isolate, originally collected by Hall et al. (2002) from the Adelaide Hills, as *P. syringae* pv. *syringae*. Interestingly, five *P. syringae* pv. *syringae* isolates collected from diseased grapevines in Tumbarumba, NSW were found to have *rpoB* sequences identical to the DAR73915 Adelaide Hills isolate. Because the *rpoB* gene has been established as a reliable marker for bacterial strain identification with high resolution for phylogenetic applications (Ait-tayeb et al. 2005; Mollet et al. 1997), these results indicate that the Adelaide Hills and Tumbarumba *P. syringae* pv. *syringae* isolates may have originated from the same source.

This study also used MLST data from four genes (*gapA*, *gltA*, *gyrB*, and *rpoD*) to characterize the core genome of *P. syringae* pv. *syringae* isolates collected from grapevines in Australian vineyards. The grape *P. syringae* pv. *syringae* isolates were grouped by MLST into two separate clades with excellent bootstrap support. Each clade contained a mixture of pathogenic (to grapevine) and nonpathogenic isolates. The clades also contained nongrape *P. syringae* pv. *syringae* hosts and a mixture of pathogenic (to grapevine) and nonpathogenic strains, indicating that *P. syringae* pv. *syringae* isolates in Australian vineyards are genetically diverse.

Pathogenicity. Not only did molecularly similar *P. syringae* pv. *syringae* isolates differ in pathogenicity toward grapevine but *P. syringae* pv. *syringae* isolates pathogenic to grapevine also had significantly different *rpoB* and MLST sequences. In addition, some *P. syringae* pv. *syringae* isolates from nongrape hosts were positive for GPLTA pathogenicity testing on grapevine leaf discs, indicating a lack of host specificity and a potential source for cross-infection of grape from other horticultural crops, although further studies are required to investigate whether these nongrape isolates can cause grapevine BIR. These results are consistent with the results of Najafi and Taghavi (2014), who reported that *P. syringae* pv. *syringae* isolates obtained from diseased tissues of *Prunus* spp., beet, pear, quince, oat, millet, wheat, barley, and rice were all pathogenic to peach seedlings, regardless of the original host or position within a phylogenetic tree. Martín-Sanz et al. (2013) also showed that the core genome of *P. syringae* (by MLST) was only weakly associated with the pathovar designation and the plant host from which the bacteria were isolated. Pathogenicity and the preferred plant host are less

Fig. 5. *Pseudomonas syringae* pv. *syringae* isolated from eight cool-climate Australian grape vineyards plus one warm climate table-grape region with overhead watering system. □: *P. syringae* pv. *syringae* from diseased grapevines within cool-climate vineyards affected by bacterial inflorescence rot; (1) Tumbarumba, NSW; (2) Murrumbateman in the Southern Tablelands, NSW; (3) Piper's River, TAS; (4) Coonawarra, SA. Δ: *P. syringae* pv. *syringae* from cool-climate vineyards with grapevine bacterial leaf spot symptoms only; (5) Adelaide Hills, SA; and (6) Hanging Rock in the Macedon Ranges, VIC. ○: *P. syringae* pv. *syringae* from apparently healthy grapevines in Victorian cool-climate vineyards not affected by bacterial inflorescence rot; (7) Glenlochy in the Pyrenees, VIC; (8) Hallston in Gippsland, VIC. ☆: *P. syringae* pv. *syringae* from Red Globe and Sultana table grape in warm climate region Sunraysia, VIC (9) (reported by Victoria Department of Environment and Primary Industries Diagnostics Service, 16 November 1998, but not included in the current phylogenetic studies).

likely to be directed by the core genome (e.g., as characterized by RAPD and MLST) than by the “flexible genome”, which consists of genes encoding proteins responsible for adaptation to specific niches, evolving through horizontal exchange (Hwang et al. 2005). Further investigations are required to investigate horizontal transfer of genes encoding pathogenicity within the flexible genome of grapevine-pathogenic *P. syringae* pv. *syringae*.

Source of inoculum. The *rpoB* and MLST sequences of *P. syringae* pv. *syringae* isolate DAR82446, which was isolated from a necrotic rachis on a grapevine sucker shoot in a Tumbarumba vineyard, were identical to those of the pathogenic *P. syringae* pv. *syringae* isolates causing BIR within the same vineyard. Because DAR82446 was also positive for the GPLTA, this implies that grapevine sucker shoots may allow overwintering of pathogenic *P. syringae* pv. *syringae*. Similarly, *P. avellanae*, the causal agent of hazelnut bacterial canker, infects sucker shoots on hazelnut trees, and it has been suggested that latently infected sucker shoots used for propagation may have been the main vehicle for widespread dispersal of the pathogen in Italy (Scortichini 2002). In many fruit crops, *P. syringae* can also overwinter in buds, tissue around leaf scars, and at the margin of necroses and cankers (Bultreys and Kaluzna 2010). In addition, because *P. syringae* pv. *syringae* has been isolated from weeds (*Geranium* spp. and *Malva* spp.) within Californian stone fruit orchards affected by bacterial canker (Little et al. 1998), it is possible that *P. syringae* pv. *syringae* may overwinter on weeds within vineyards. The role of other plant species within the vineyard as potential sources of *P. syringae* pv. *syringae* inoculum and the progression of colonization within the grapevine host needs to be investigated.

Dispersion. The results of the current study demonstrate the general spread of *P. syringae* pv. *syringae* across cool-climate Australian vineyards. This is also supported by the phylogenetic data demonstrating similarity between isolates of *P. syringae* pv. *syringae* from grapevine tissue originating from separate vineyards (e.g., DAR82440 from Murrumbateman, NSW and DAR82442 from Piper’s River, TAS). *P. syringae* may be dispersed by many mechanisms, including agricultural tools used in pruning and harvesting (Carroll et al. 2010). Hall et al. (2002) previously showed that BLS symptoms and disease severity increased in vineyards in subsequent seasons, and that transmission to other nearby vineyards may result. This suggests that the shared use of contaminated pruning equipment within and between vineyards may have resulted in disease spread (Lamichhane et al. 2014). This mode of transmission has also been observed with *P. syringae* from cherry (Carroll et al. 2010).

P. syringae may also be dispersed by soil particles (Hollaway and Bretag 1997), honey bees (Pattemore et al. 2014), insects and mammals (Bashan 1986), water sources (Morris et al. 2008), and precipitation from clouds (Clarke et al. 2010; Monteil et al. 2014; Morris et al. 2008, 2013). Interestingly, *P. syringae* pv. *syringae* infections of grapevines in cool-climate regions of Australia are often associated with heavy spring rains (Hall et al. 2002; Whitelaw-Weckert et al. 2011). Similarly, Hirano et al. (1996) demonstrated an association between bacterial populations and rainfall on bean cultivars. A comprehensive review conducted by Morris et al. (2013) indicated that the water cycle and rainfall are important for *P. syringae* pv. *syringae* movement through the environment. However, because *P. syringae* from water sources such as rainfall and snowmelt have been reported to lack essential T3SS effectors for virulence (Mohr et al. 2008), and humidity has been demonstrated to increase bacterial motility in bean (Leben et al. 1970), it is more likely that the increased disease in vineyards with heavy spring rain and overhead water sprinklers is caused by the increased humidity caused by these water sources. It will be important to determine whether the combination of high humidity and unclean pruning equipment plays a major role in motility and dispersal of this pathogen.

Conclusions

The results from this investigation provide the foundations for an improved understanding of the genetic structure and diversity of grapevine pathogen *P. syringae* pv. *syringae*. We have demonstrated

that infection of Australian grapevines with pathogenic *P. syringae* pv. *syringae* occurs in at least six cool-climate viticultural regions plus one warmer region with overhead water spray irrigation. It is clear that genetically and pathogenically distinct strain groups of *P. syringae* pv. *syringae* can be isolated from grapevines, and that genetically distinct strain groups of *P. syringae* pv. *syringae* from other plant hosts may infect grapevine.

On the basis of this study, we conclude that the presence of *P. syringae* pv. *syringae* in Australian cool-climate vineyards may pose a threat to the Australian wine industry. Damage caused by *P. syringae* pv. *syringae* can lead to severe economic losses. Furthermore, some isolates of *P. syringae* pv. *syringae* lack host specificity and, therefore, may be transmitted from one crop to another. The isolation of pathogenic *P. syringae* pv. *syringae* from grapevine sucker shoots also suggests that sucker shoots allow overwintering of the pathogen. The vineyard quarantine status of *P. syringae* pv. *syringae* may need to be reconsidered, due to its easy dispersal through pruning and other equipment. Appropriate quarantine measures and sterilization of pruning machinery are highly recommended in susceptible vineyards.

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