DNA type analysis for differentiation of strains of *Xylophilus ampelinus* from Europe and Hokkaido, Japan

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Abstract

Strains of the bacterium *Xylophilus ampelinus* were collected from Europe and Hokkaido, Japan. Genomic fingerprints generated from a total of 43 strains revealed four DNA types (A–D) using the combined results of Rep-, ERIC-, and Box-PCR. Genetic variation was found among the strains examined; strains collected from Europe belonged to DNA types A or B and strains collected from Hokkaido belonged to DNA types C or D. However, strains belonging to each DNA type showed the same pathogenicity to grapevines (cultivar Kerner), and virulence was indistinguishable among these strains.

Key words: Bacterial blight of grapevine, DNA fingerprinting, genetic variation, *Xylophilus ampelinus*

Bacterial blight disease in grapevines (*Vitis vinifera*) is caused by *Xylophilus ampelinus* (Willems et al. 1987), a bacterium distributed throughout temperate regions worldwide, particularly in areas along the coast of the Mediterranean region (EPPO 2009). This bacterium is known to infect only *V. vinifera* and its hybrids (Panagopoulos 1987). Severe infection of susceptible cultivars can lead to major harvest losses. In 1940, Du Plessis (1940) reported harvest losses of 70% or more in South Africa. In France, serious damage from this bacterium has been reported since 1968, particularly for Alicante-Bouschet and Ugni Blanc vines in Charente and Grenache and Maccabeu vines in Languedoc (EPPO 1984). Additionally, a previous study showed that vines growing on their own roots in irrigated areas around Narbonne were severely affected (López et al. 1980).

In Japan, the first outbreak of the disease occurred in Hokkaido Prefecture, the northernmost island of Japan, in 2009 (Shinmura et al. 2012). Although the two major cultivars in Hokkaido, Kerner and Zweigeltrebe, were introduced from Germany and Austria, where the disease is seldom present, major damage occurred during this outbreak in 2009, even in the case of the fruits. No study has described the effects of differences in cultivars or climate on the occurrence of this disease; however, it has been shown to occur primarily in temperate regions. Thus, the occurrence of the disease in Hokkaido was surprising because Hokkaido is within a subarctic zone in which the average temperature is fairly low and freezing often occur in winter. In a previous study, *X. ampelinus* strains were shown to have high genomic similarity to each other; DNA-DNA hybridization values between some pairs of strains were approximately 100% (Willems et al. 1987). However, Manceau et al. (2000) analyzed a species-specific DNA marker in *X. ampelinus* and found genetic variation in the bacterium by using a primer set that was applicable to the strains collected from the

Greek cultivar ‘Sultana’. Although there have been no further reports regarding the genetic variation of *X. ampelinus*, we hypothesized that *X. ampelinus* strains isolated from Hokkaido might differ genetically from those commonly isolated in Europe.

Thus, the aim of this study was to investigate the genetic variation of strains of *X. ampelinus* isolated from Europe and Hokkaido by genetic classification of the bacterium using Rep-polymerase chain reaction (PCR) DNA fingerprint analyses.

The *X. ampelinus* strains used in this study are shown in Table 1. Strains isolated from France, Greece, and Spain were obtained from the French Collection of Plant-Associated Bacteria (CFBP) with permission from the Ministry of Agriculture, Forestry, and Fisheries of Japan (MAFF). The Japanese strains BB-1, BB-4, BB-9, and BB-15 were already isolated and were identified as *X. ampelinus* by analyzing the bacterial properties and 16S rDNA sequence (Shinmura et al. 2012) (Table 1). To prepare more *X. ampelinus* strains from Hokkaido, diseased leaves were collected from various locations within commercial fields in 2010, 2011, and 2013 (Table 1) and were homogenized with sterile distilled water (SDW) using a mortar and pestle. Each single colony that emerged 15 days after streaking on King’s medium B (EIKEN Chemical, Tochigi, Japan) at 25°C was isolated. The identity of each colony was determined using PCR with a specific primer set (XaTS1 and XaTS2 (Manceau et al. 2005)). Amplification conditions were as follows: initial denaturation at 94°C for 5 min; 40 cycles at 94°C for 30 s, 60°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 8 min (Manceau et al. 2005). Thirty-seven strains collected in Hokkaido and 10 representative Europe strains were identified as *X. ampelinus* by amplification of a 125-bp product. In eight strains, i.e., CFBP1926, CFBP5787, BB-1, BB-4, BB-5, BB-9, BB-15, and F-7, the sequence of 16s rDNA was analyzed via PCR using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r

(5′-TACGGYTACCTTGTTACGACTT-3′), and the strains were confirmed to be *X. ampelinus*. The 16S rDNA sequences of six strains from Hokkaido (BB-1, BB-4, BB-5, BB-9, BB-15 and F-7) were then deposited to the DNA Data Bank of Japan; the accession number of each strain are LC109307, LC109308, LC109305, LC109309, LC109310, and LC10906, respectively. Additionally, strains BB-5 and F-7 obtained in this study were deposited in the National Institute of Agrobiological Sciences Genebank; the MAFF accession numbers are listed in Table 1.

Because *X. ampelinus* was previously classified as *Xanthomonas ampelina*, the repetitive sequence-based PCR method previously used for determining the genetic variation of *Xanthomonas* was applied in this study (Adhikari et al. 2012; Gama et al. 2011; Kawaguchi 2014; Mondal and Mani 2009; Rademaker et al. 2005; Sahin et al. 2003;). Box-PCR, ERIC-PCR, and Rep-PCR were performed using the primer sets BOX and BOXA1R; ERIC, ERIC1R, and ERIC2; and REP, REP1R-I, and REP2-I reported by Versalovic et al. (1994), Hulton et al. (1991), and Martin et al. (1992), respectively. The Rep-PCR method used in this study was adapted from Louws et al. (1998). Bacterial cells from colonies grown on nutrient agar plates (Difco, Detroit, MI, USA) were picked and resuspended in 100 µL of SDW. The suspension was heated at 95°C for 10 min and then cooled on ice. The suspension was then centrifuged at 20,000 × *g* for 3 min, and the resulting supernatant was used as a template for PCR. PCR amplification was performed in a total volume of 25 µL containing 1 µL of DNA template, 2 µL of the primers, 12.5 µL of 2×Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), and 9.5 µL of SDW. The thermal cycler was programmed as follows: an initial denaturation step of 95°C for 14 min; 40 cycles of denaturation at 95°C for 30 s, annealing at either 40°C for 90 s for Rep-PCR or 50°C for 90 s for Box- and ERIC-PCR, and extension at 72°C for 90 s; and an additional extension at 72°C for 10

113 min. Amplified PCR products were separated on 2.0% (w/v) agarose gels prestained
114 with 10,000× GelRed (Wako, Tokyo, Japan), electrophoresed at 50 V/cm for 60 min,
115 and photographed under UV light. For confirmation of banding patterns, experiments
116 were repeated three times with independently prepared DNA samples.

117 Each DNA fragment with a distinct electrophoretic mobility was assigned a position
118 number and scored as either 1 or 0 depending on the presence or absence of the
119 fragment, respectively, at this position. The scores obtained from each PCR method
120 were combined for analysis. A dendrogram was constructed from similarity coefficient
121 data by the unweighted pair group method with arithmetic average (UPGMA) clustering
122 using DendroUPGMA (http://genomes.urv.cat/UPGMA/), applying the RMSD
123 coefficient with default parameters.

124 Four DNA types (A–D) were obtained from the combined results of Box-, ERIC-,
125 and Rep-PCR; three of seven, four of eight, and two of six DNA bands from Box-,
126 ERIC-, and Rep-PCR, respectively, were polymorphic (Fig. 1). Genetic variation was
127 found between strains collected from Europe and Hokkaido (Fig.2); strains collected
128 from Europe belonged to DNA types A or B, and strains collected from Hokkaido
129 belonged to other DNA types C or D (Table 1).

130 Pathogenicity tests were performed as described by Sevillano et al. (2014) with the
131 CFBP1926, CFBP5787, BB-5, and F-7 strains, which were classified into DNA
132 fingerprint groups A, B, C, and D, respectively. The strains were shake-cultured in 100
133 mL of nutrient broth (1% w/v beef extract, 1% w/v polypeptone, 0.5% w/v NaCl, pH
134 7.0) at 120 rpm and 25°C until they reached a concentration of 10^8 cells/mL. Cells were
135 harvested from 10 mL of culture by centrifugation (2,500 × *g*), suspended in 1 mL of
136 20% (v/v) glycerol, and adjusted to a concentration of approximately 10^9 cells/mL.
137 Healthy leaves from three-year-old potted grapevines (cultivar Kerner) growing in a

138 greenhouse (five leaves per strain) were placed onto the surface of Petri dishes (9-cm
139 diameter) containing 2.0% (w/v) water agar media with the abaxial side of the leaves
140 turned upward. A small wound was made in the center of three veins of each leaf with a
141 hypodermic needle, and 10 μL of the bacterial suspension was applied directly to the
142 injury. A negative control was run in parallel using a 20% (v/v) glycerol solution. Petri
143 dishes were sealed with Parafilm and incubated at room temperature (20–24°C) with a
144 12-h photoperiod until necrosis was visible (7–8 days). Virulence was quantified using
145 an electrolyte leakage assay. The leaf pieces inoculated with the strains from each DNA
146 type (three fragments per leaf) were obtained from the inoculation point using a cork
147 borer (0.5-cm diameter). Fifteen leaf fragments were then transferred to a 50-mL
148 conical tube containing 10 mL of SDW. Samples were vortexed for 5 s and then
149 incubated for 10 min at room temperature. This water solution was transferred to
150 another clean tube, and electrolyte leakage was measured using a conductivity meter
151 DS-14 (HORIBA, Kyoto, Japan). These experiments were performed three times.
152 All representative strains belonging to DNA types A-D caused necrosis on the
153 leaves, and the degree of electrolyte leakage did not significantly differ among the types
154 (Tukey-Kramer test, $p < 0.05$; Fig. 3, 4). This finding is supported by a previous report
155 that cultivar-specific strains and races have never been discovered in this bacterium
156 (EFSA 2014).
157 *Xylophilus ampelinus* is a plant pathogenic bacterium classified in a taxonomic
158 group with a single species and genus and is known to be pathogenic only to European
159 grapevines (*V. vinifera*) and their hybrids (Panagopoulos 1987). This species of
160 grapevine is not widely used in Japan, and importation of these cultivars occurred
161 relatively recently, in the 1980s. Therefore, the bacterial strains that caused the disease
162 in Hokkaido were considered to have been introduced from Europe. Suzaki and Sato

(2014) also surmised that the 2012 outbreak of the disease in Akita Prefecture, soon after the 2009 outbreak in Hokkaido, was related to a latent infection rather than to propagation and expansion from Hokkaido. However, in this study, we observed genetic polymorphism between strains from Hokkaido and Europe (France, Greece, and Spain).

In Hokkaido, expansion of the wine industry has occurred with Kerner, Seibel 13053, Zweigeltrebe, and Muller-Thurgau cultivars, which are tolerant to cold climate conditions. Kerner and Zweigeltrebe were crossbred in Germany and Austria, respectively, where the disease has not been reported. Although Seibel 13053 was bred in France, this cultivar is commonly grown in North America, New Zealand, and England; however, the disease has not been reported in these countries. Therefore, it is possible that the disease has been suppressed by environmental factors or other factors in these countries and that the cultivars were already infected latently by certain DNA types (i.e., C and D) prior to being imported into Hokkaido. This hypothesis is supported by the results of the pathogenicity analysis in the present study; each DNA type of the bacterial strain was shown to be pathogenic for cultivar Kerner (Fig. 3), which was bred in Germany. However, the European strains in this study were mainly collected in the 1980s; thus, it is also possible that the strains had mutated after a long time. If the present strains in Europe were found to be of DNA type C or D, the transmission processes would be simpler because such a finding would suggest that the Hokkaido strains were introduced from Europe in recent years. In contrast, if the European strains did not show alterations even now, the Hokkaido strains could be considered to be introduced from other countries or hereditary changes would be expected to have occurred for adaptation to the unique cold climate in Hokkaido. Additionally, the possibility of latent infection of the native Japanese strains cannot be excluded.

Therefore, it may be possible to determine the history of the isolated strains and the transmission process in Hokkaido by further detection in areas where the disease has not yet been reported and by surveying the current European strains, although recent strains have not been stocked in CFBP.

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Figure Legends

Fig. 1. Analysis of DNA types of *Xylophilus ampelinus* by DNA fingerprinting using repetitive sequence-based PCR. DNA type A, strain CFBP1926; DNA type B, strain CFBP5787; DNA type C, strain BB-5; and DNA type D, strain F-7.

Fig. 2. Dendrogram constructed by UPGMA for DNA types of *Xylophilus ampelinus*.

Fig. 3. Necrotic activity of strains of *Xylophilus ampelinus* DNA types quantified using an electrolyte leakage assay. Control: 20% glycerol solution; DNA type A, strain CFBP1926; B, strain CFBP5787; C, strain BB-5; D, strain F-7. Error bars indicate 95% confidence intervals. Bars marked with the same letter do not differ at $P = 0.05$

Fig. 4. Necrotic lesion formation on leaves by each DNA type of *Xylophilus ampelinus*. A: 20% glycerol solution; B: DNA type A, strain CFBP1926; C: DNA type B, strain CFBP5787; D: DNA type C, BB-5; E: DNA type D, strain F-7.

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Table 1 List of *Xylophilus ampelinus* strains used in this study

Strain	Cultivar	Location	Isolation year	DNA type
CFBP 1192	Sultana	Greece, Crete	1966	A
CFBP 1313	Ugni Blanc	France, Oléron	1971	A
CFBP 1796	Ugni Blanc	France, Charente Maritime	1975	A
CFBP 1800	Alicante	France, Aude Les Craboulets	1976	A
CFBP 1833	Grenache	France, Aude Les Craboulets	1976	A
CFBP 1841	Ugni Blanc	France, Ardéche Saint Reméze	1977	A
CFBP 1926	Grenache	Spain, Tosos	1978	A
CFBP 2059	Rupestris du Lot	France, Vaucluse Collet du Bray	1981	A
CFBP 4977	Clairette	France, Drôme Vercheny Le Haut	1999	B
CFBP 5787	Ugni Blanc	France, Charente Saint Preuil	2001	B
T-1	Müller-Thurgau	Hokkaido, Urausu	2011	C
T-2	Kerner	Hokkaido, Urausu	2011	C
T-3	Lemberger	Hokkaido, Urausu	2011	C
BB-1	Lemberger	Hokkaido, Urausu	2010	C
BB-2	Zweigeltrebe	Hokkaido, Urausu	2010	D
T-4	Pino Blanc	Hokkaido, Urausu	2011	C
T-5	Muskat Ottonel	Hokkaido, Urausu	2011	C
BB-4	Lemberger	Hokkaido, Yoichi	2010	C
BB-5 (MAFF212143)	Kerner	Hokkaido, Yoichi	2010	C
BB-9	Zweigeltrebe	Hokkaido, Yoichi	2010	C
BB-21	Zweigeltrebe	Hokkaido, Yoichi	2010	C
BB-13	Cabernet Cubin	Hokkaido, Yoichi	2010	C
Y-4	Müller-Thurgau	Hokkaido, Yoichi	2011	C
Y-8	Bacchus	Hokkaido, Yoichi	2011	C
N-1	Morino-Muskat	Hokkaido, Niki	2011	D
N-2	Zweigeltrebe	Hokkaido, Niki	2011	C
N-3	Kerner	Hokkaido, Niki	2011	C
N-6	Zalagyongye	Hokkaido, Niki	2011	C
TM-1	Zalagyongye	Hokkaido, Otobe	2011	C
TM-2	Merlot	Hokkaido, Otobe	2011	C
OK-1	Müller-Thurgau	Hokkaido, Okushiri	2011	D
OK-2	Merlot	Hokkaido, Okushiri	2011	C
OK-3	Chardonnay	Hokkaido, Okushiri	2011	D
OK-4	Pinot Gris	Hokkaido, Okushiri	2011	C
OK-5	Zweigeltrebe	Hokkaido, Okushiri	2011	C
OK-8	Seibel 13053	Hokkaido, Okushiri	2011	C
OK-10	Kerner	Hokkaido, Okushiri	2011	C
OK-11	Pinot Noir	Hokkaido, Okushiri	2011	C
BB-15	Seibel 10076	Hokkaido, Furano	2010	C
F-1	Zweigeltrebe	Hokkaido, Furano	2011	C
F-2	Kerner	Hokkaido, Furano	2011	C
F-3	Cabernet Sauvignon	Hokkaido, Furano	2011	C
F-4	Zalagyongye	Hokkaido, Furano	2011	C
F-5	Irsai Oliver	Hokkaido, Furano	2011	C
F-6	Seibel 5279	Hokkaido, Furano	2011	C
F-7 (MAFF212144)	Seibel 13053	Hokkaido, Furano	2011	D
F-12	New York Muscat	Hokkaido, Furano	2013	C

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278 Fig. 1

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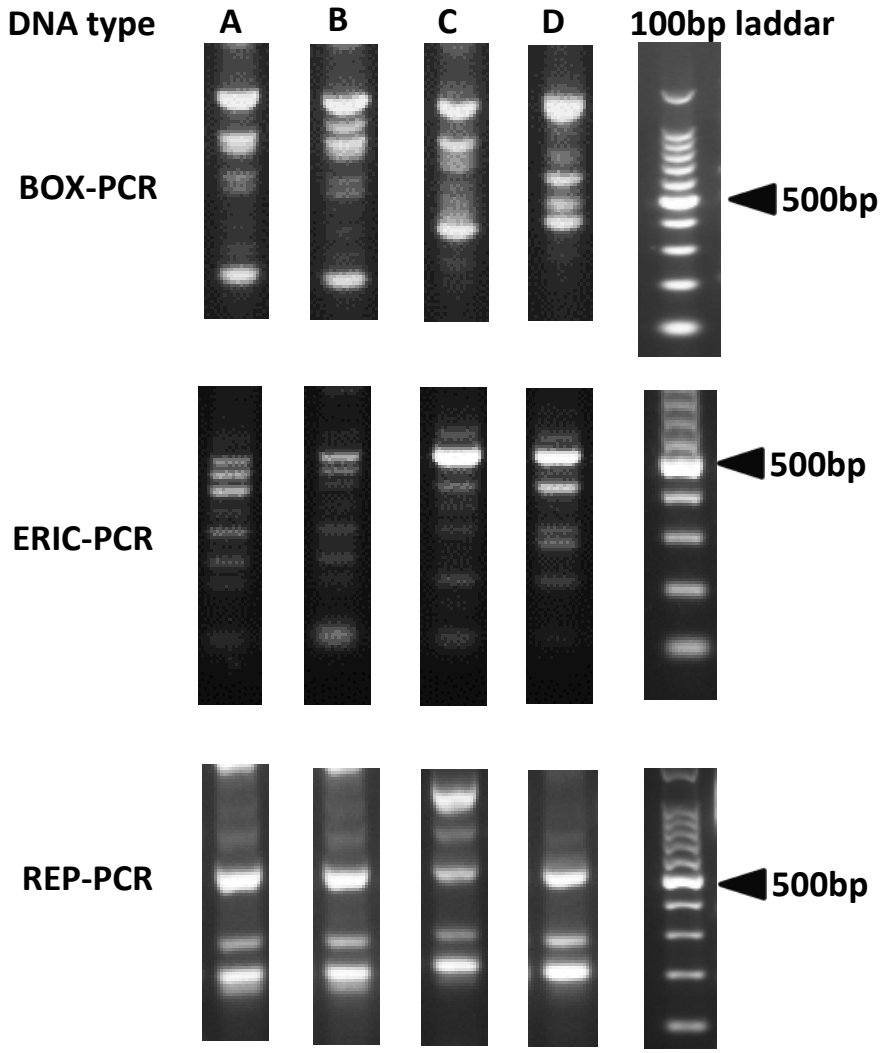

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282 Fig. 2

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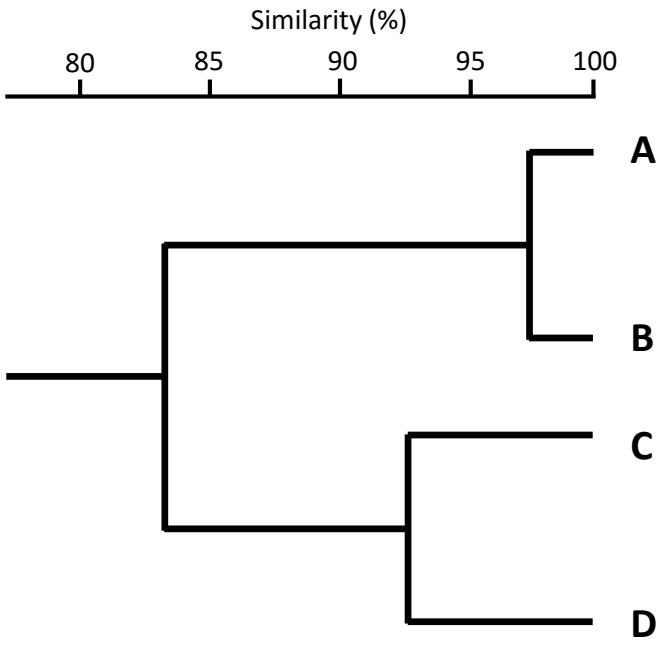

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286 Fig. 3

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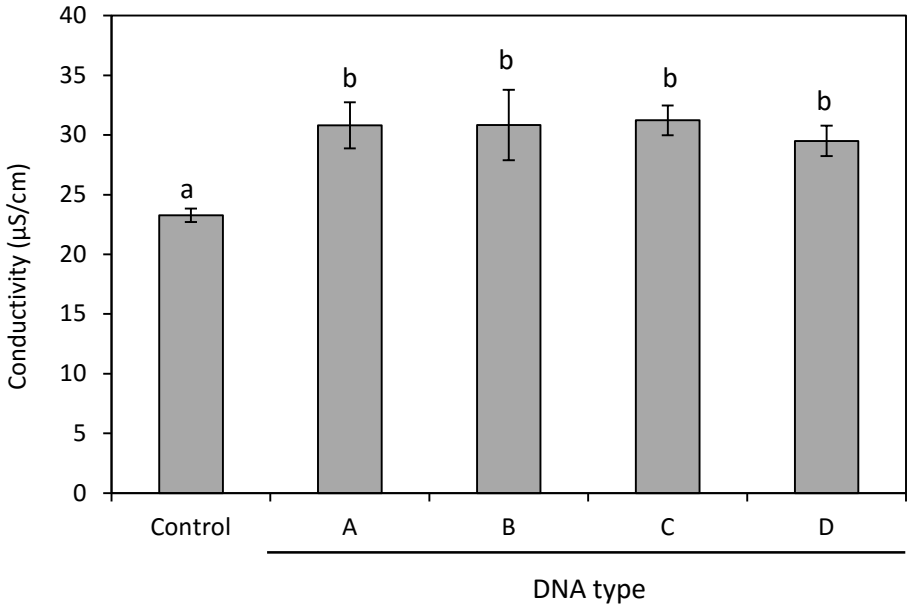

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Fig. 4