Characterization of Pseudomonas syringae pv. actinidiae (Psa) isolated from France and assignment of Psa biovar 4 to a de novo pathovar: Pseudomonas syringae pv. actinidifoliorum pv. nov.

A. Cunty, F. Poliakoff, C. Rivoal, S. Cesbron, Marion Fischer-Le Saux, Christophe Lemaire, Marie-Agnès Jacques, C. Manceau, J. Vanneste

Plant Pathology (2015) **64**, 582–596 Doi: 10.1111/ppa.12297

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A. Cunty[abcde], F. Poliakoff[d], C. Rivoal[d], S. Cesbron[abc], M. Fischer-Le Saux[abc], C. Lemaire[abc], M. A. Jacques[abc], C. Manceau[d] and J. L. Vanneste[e]*

[a]*INRA, UMR1345 Institut de Recherche en Horticulture et Semences, Beaucouzé, F-49071, Beaucouzé;* [b]*Université d'Angers, UMR 1345 Institut de Recherche en Horticulture et Semences, SFR 4207 QUASAV, F-49045;* [c]*AgroCampus-Ouest, UMR1345 Institut de Recherche en Horticulture et Semences, F-49045;* [d]*Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail – Anses – Laboratoire de la santé des végétaux, Angers, France and* [e]*The New Zealand Institute for Plant & Food Research Ltd, Hamilton, New Zealand*

Since 2008, bacterial canker of kiwifruit (*Actinidia deliciosa* and *A. chinensis*) caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) has resulted in severe economic losses worldwide. Four biovars of Psa can be distinguished based on their biochemical, pathogenicity and molecular characteristics. Using a range of biochemical, molecular and pathogenicity assays, strains collected in France since the beginning of the outbreak in 2010 were found to be genotypically and phenotypically diverse, and to belong to biovar 3 or biovar 4. This is the first time that strains of biovar 4 have been isolated outside New Zealand or Australia. A multilocus sequence analysis based on four housekeeping genes (*gapA*, *gltA*, *gyrB* and *rpoD*) was performed on 72 strains representative of the French outbreak. All the strains fell into two phylogenetic groups: one clonal corresponding to biovar 3, and the other corresponding to biovar 4. This second phylogenetic group was polymorphic and could be divided into four lineages. A clonal genealogy performed with a coalescent approach did not reveal any common ancestor for the 72 Psa strains. Strains of biovar 4 are substantially different from those of the other biovars: they are less aggressive and cause only leaf spots whereas Psa biovars 1, 2 and 3 also cause canker and shoot die-back. Because of these pathogenic differences, which were supported by phenotypic, genetic and phylogenetic differences, it is proposed that Psa biovar 4 be renamed *Pseudomonas syringae* pv. *actinidifoliorum* pv. nov. Strain CFBP 8039 is designated as the pathotype strain.

Keywords: *Actinidia chinensis*, *Actinidia deliciosa*, bacterial canker of kiwifruit, multilocus sequence analysis, pathogenicity

Introduction

Outbreaks of *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit *Actinidia* spp., were first reported in Japan (1989), China (1992), Korea (1994) and Italy (1994) (Vanneste, 2013). More recent outbreaks in Italy (2009) and in other parts of Europe (2010), New Zealand (2010) and Chile (2010) are of greater economic importance than those previously documented (Vanneste, 2013).

Recently, strains of Psa isolated around the world over the last 30 years were grouped in four biovars based on biochemical, genetic and pathogenicity characteristics (Vanneste *et al.*, 2013). Multilocus sequence analysis (MLSA) and multilocus sequence typing (MLST) using housekeeping genes and effector gene sequences resulted in a similar grouping (Chapman *et al.*, 2012) to that described by Vanneste *et al.* (2013). Strains of biovar 1 produce phaseolotoxin and were initially isolated from Japan and Italy before 2008; strains of biovar 2 produce coronatine but not phaseolotoxin; strains of biovar 3 produce neither coronatine nor phaseolotoxin and are responsible for the most recent outbreaks (including the Italian outbreak of 2008). Recent studies suggest that strains of Psa biovar 3, responsible for the current global outbreak, may have originated in China between 10 and a few dozen years ago (Mazzaglia *et al.*, 2012; Butler *et al.*, 2013; McCann *et al.*, 2013). Strains of biovar 4 produce neither coronatine nor phaseolotoxin and are less aggressive than strains of other biovars (Vanneste *et al.*, 2013). They cause fewer necrotic spots on leaves than strains of biovar 3 and do not cause canker or shoot die-back (Vanneste *et al.*, 2013). Strains belonging to biovar 4 were previously isolated in Australia and New Zealand (Vanneste *et al.*, 2013).

*E-mail: Joel.Vanneste@plantandfood.co.nz

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Published online 7 November 2014

Plant Pathology

In France, Psa was detected for the first time in 2010 (Vanneste *et al.*, 2011). In 2011, the French Ministry of Agriculture requested a national survey to determine the area affected by Psa. This survey concerned first the orchards of *Actinidia chinensis*, then those of *Actinidia deliciosa* planted since 2006 with plants originating from Italy or New Zealand, and finally it was extended to all other orchards (Bourgouin & Fritsch, 2013). In 2012, the survey was extended to the nurseries and to a radius of 4 km around them. No extension of the epidemic was observed in 2012 (Bourgouin & Fritsch, 2013). However, the climatic conditions during spring 2013 favoured the spread of Psa and by the end of that year it was estimated that 10 to 15% of the orchards were affected (Bourgouin & Fritsch, 2013).

Psa has been registered by the European and Mediterranean Plant Protection Organization (EPPO) in the A2 list of pests recommended for regulation as quarantine pests (http://www.uab.cat/Document/526/300/patogens_vegetals_quarantena_2012.pdf). In November 2012, the Commission of the European Union (EU) ordered surveys and measures to limit the propagation of Psa in the EU member states. Pollen and plants originating from countries outside the EU must be accompanied by a phytosanitary certificate, inspected and, when appropriate, tested for the presence of Psa. Within the EU, a plant passport is required to certify that the plants are coming from a Psa-free area, and plants may also be inspected and tested for the presence of Psa. Presence of any biovar of Psa on a plant, including Psa biovar 4, which induces only leaf spots and does not lead to any economic loss (Vanneste *et al.*, 2013), lead to this plant being considered contaminated by Psa, and therefore subjected to all legal control measures including destruction of the plant material.

The initial aim of this study was to characterize the populations of Psa isolated from kiwifruit in France. The diversity observed between the strains analysed led to studying their phylogeny. Two hundred and eighty strains of Psa isolated from *Actinidia* spp. were characterized based on phenotypical traits (biochemical tests and pathogenicity assays) and genomic data (BOX PCR profiles). A representative sample of 72 of the strains isolated in this study and strains representing the four biovars of Psa were used for a multilocus sequence analysis using four housekeeping genes and for a clonal genealogy study.

Materials and methods

Bacterial isolation

Two hundred and eighty strains of Psa were isolated from leaves, canes, flower buds and roots of *Actinidia* species (*A. deliciosa*, *A. chinensis* or *A. arguta*) from different regions in France during the surveys conducted from 2010 to June 2013 (Table S1). The bacterial strains used for the genetic characterization are listed in Table 1.

When Psa strains were isolated from a plant with symptoms, tissues taken from the margins of necrotic lesions were macerated in sterile water and incubated for 20 min at room temperature. Aliquots of 100 μL were plated onto King's B medium supplemented with cycloheximide (0·018%) and boric acid (0·136%) (KBc-ba) and incubated at 25°C for 3 to 6 days. Psa-like colonies were purified twice on KBc-ba.

Bacterial characterization

Isolates were tested for cytochrome c oxidase activity using Test Oxydase (Pro-Lab Diagnostic), for production of levan on saccharose-rich medium, and for their ability to hydrolyse arginine and aesculin as described previously (Lelliott *et al.*, 1966). Production of a fluorescent pigment from bacterial colonies was observed on KBc-ba under ultraviolet light (λ = 560 nm). The potato test for presence of cell wall-degrading enzyme was performed as described by Vanneste *et al.* (2010). The hypersensitive response (HR) was monitored after injection of a bacterial suspension (10^8 colony-forming units (CFU) mL^{-1}) into tobacco leaves (*Nicotiana tabacum* 'Xanthi') (Vanneste *et al.*, 2013). The production of syringomycin was assessed according to Vanneste *et al.* (2013).

The catabolic activity on 95 substrates of the French Psa strains CFBP 7906, CFBP 7908, CFBP 7910, CFBP 7951, CFBP 8043 and CFBP 8161 was determined using two Biolog GN MicroPlates (Biolog) per bacterial strain, according to the manufacturer's instructions, with bacterial suspensions calibrated at an optical density (OD 600 nm) of 0·2 and 0·5. The colour changes were measured at 590 nm using a microplate spectrophotometer (μQuant, Bio-Tek) after 5 days of incubation at 24°C and the resulting data were corrected using the no-substrate control. The threshold value for a positive result was OD $\geq$ 0·350 at 590 nm. The ice nucleation activity (INA) of these six strains was determined according to Lindow *et al.* (1978); *P. syringae* pv. *syringae* CFBP 4702 and *P. syringae* pv. *tomato* CFBP 2212 were used as a positive and a negative control respectively. The motility of these strains was assessed on Moka medium (yeast extract 4 g, casamino acids 8 g, KH_2PO_4 2 g, $MgSO_4.7H_2O$ 0·3 g, Bacto agar 0·2% per litre) supplemented with 0·05% of 2-3-5 triphenyl tetrazolium chloride to follow the movement of the bacteria. A drop of 10 μL of a bacterial suspension (10^8 CFU mL^{-1}) was deposited on the centre of a Petri dish.

For molecular characterization, total DNA was extracted by boiling bacterial suspensions (10^6 CFU mL^{-1}) for 15 min, followed by a 10-min centrifugation at 10 000 ***g*** at 4°C. Three sets of primers (PsaF1/R2, Rees-George *et al.*, 2010; KN-F/KN-R, Koh & Nou, 2002; AvrDdpx-F/AvrDdpx-R, Gallelli *et al.*, 2011), yielding 280, 492 and 226 bp amplicons, respectively, were used to identify strains of Psa. The presence of the genes necessary for production of the toxin coronatine (*Cfl)* and of phaseolotoxin (*argK)* was determined using the primers CFLF/CFLR (Bereswill *et al.*, 1994) and ArgKF3/ArgKR (Templeton *et al.*, 2005), which yield 665 bp and 800 bp amplicons, respectively. BOX PCR was performed following the protocol published by Louws *et al.* (1994). The sequences of the primers used in this study are presented in Table S2. All PCR was conducted with a Veriti 96-well thermal cycler (Applied Biosystems). PCR products were separated by horizontal gel electrophoresis by loading 15 μL of the reaction in a 1·5% agarose gel in 1 × TBE buffer and staining with ethidium bromide (5 μg mL^{-1}). The DNA bands were visualized with Gel Doc XR+ BioradImager (Bio-Rad). To estimate the size of the amplicons, the 100 bp DNA molecular weight marker XIV (Roche Applied Science) was used. For the BOX PCR, the 1 kb Plus DNA ladder from Invitrogen was used.

Table 1 Strains of *Pseudomonas syringae* pv. *actinidiae* used in this study

Strain name	Host	Sample	Year of isolation	Country of isolation	Region of isolation	Fluorescence on KB[a]	Aesculin[b]	*avrD1*[c]	Biovar	Reference
CFBP 4909[PT]/ ICMP 9617	*Actinidia deliciosa*	Leaf/wood	1984	Japan	Shizuoka	–	–	+	1	Takikawa *et al.* (1989), Ferrante & Scortichini (2010)
CFBP 7287	*A. deliciosa*	Leaf/Wood	2008	Italy	Lazio	–	–	+	3	Balestra *et al.* (2009), Vanneste *et al.* (2013)
CFBP 7811/ 10627[d]	*Actinidia chinensis*	Wood	2010	New Zealand	Hawke's Bay	–	–	+	3	Vanneste *et al.* (2013)
CFBP 7812/ ICMP 19098	*A. chinensis*	Leaf	2010	New Zealand	Motueka	+	+	–	4	Vanneste *et al.* (2013)
CFBP 7901/ ICMP 18803	*A. chinensis*	Leaf	2010	New Zealand	Hawke's Bay	+	+	–	4	Chapman *et al.* (2012), this study
CFBP 7902/ ICMP 18804	*A. chinensis*	Leaf	2010	New Zealand	Bay of Plenty	+	+	–	4	Chapman *et al.* (2012), this study
CFBP 7903/ ICMP 18882	*A. chinensis*	Leaf	2010	New Zealand	Nelson	+	+	–	4	Chapman *et al.* (2012), this study
CFBP 7904/ ICMP 18883	*A. deliciosa*	Leaf	2010	New Zealand	Nelson	+	+	–	4	Chapman *et al.* (2012), this study
CFBP 7905/ ICMP 19074	*A. chinensis*	Leaf	2010	New Zealand	Bay of Plenty	+	+	–	4	Chapman *et al.* (2012), this study
CFBP 7906	*A. deliciosa*	Wood	2011	France	Rhône-Alpes	–	–	+	3	This study
CFBP 7907	*A. deliciosa*	Leaf	2011	France	Pays de la Loire	+	+	–	4	This study
CFBP 7908	*A. deliciosa*	Leaf	2011	France	Aquitaine	+	+	–	4	This study
CFBP 7909	*A. deliciosa*	Leaf	2012	France	Poitou-Charentes	+	+	–	4	This study
CFBP 7910	*A. deliciosa*	Leaf	2012	France	Aquitaine	–	–	+	3	This study
CFBP 7950/ ICMP 18806	*A. chinensis*	Leaf	2010	New Zealand	Bay of Plenty	+	+	–	4	Butler *et al.* (2013), this study
CFBP 7951/ ICMP 18807	*A. deliciosa*	Leaf	2010	New Zealand	Bay of Plenty	+	+	–	4	Butler *et al.* (2013), this study
CFBP 8025	*A. chinensis*	Leaf	2010	France	Aquitaine	–	–	+	3	This study
CFBP 8026	*A. deliciosa*	Leaf	2010	France	Rhône-Alpes	–	–	+	3	This study
CFBP 8027	*A. deliciosa*	Leaf	2010	France	Rhône-Alpes	–	–	+	3	Vanneste *et al.* (2011)
CFBP 8028	*A. deliciosa*	Leaf	2010	France	Rhone-Alpes	–	–	+	3	This study
CFBP 8029	*A. chinensis*	Wood	2010	France	Aquitaine	–	–	+	3	Vanneste *et al.*, (2011)
CFBP 8030	*A. chinensis*	Wood	2010	France	Aquitaine	–	–	+	3	Vanneste *et al.* (2011)
CFBP 8031	*A. deliciosa*	Wood	2011	France	Rhône-Alpes	–	–	+	3	This study
CFBP 8032	*A. chinensis*	Wood	2011	France	Aquitaine	–	–	+	3	This study
CFBP 8033	*A. chinensis*	Wood	2011	France	Aquitaine	–	–	+	3	This study
CFBP 8034	*A. deliciosa*	Wood	2011	France	Rhône-Alpes	–	–	+	3	This study
CFBP 8035	*A. deliciosa*	Wood	2011	France	Rhône-Alpes	–	–	+	3	This study
CFBP 8036	*A. deliciosa*	Wood	2011	France	Rhône-Alpes	–	–	+	3	This study
CFBP 8037	*A. deliciosa*	Leaf	2011	France	Aquitaine	–	–	+	3	This study

(continued)

Table 1 (continued)

Strain name	Host	Sample	Year of isolation	Country of isolation	Region of isolation	Fluorescence on KB[a]	Aesculin[b]	*avrD1*[c]	Biovar	Reference
CFBP 8038	*A. deliciosa*	Leaf	2011	France	Poitou-Charentes	+	+	–	4	This study
CFBP 8039	*A. deliciosa*	Leaf	2011	France	Aquitaine	+	+	–	4	This study
CFBP 8040	*A. deliciosa*	Leaf	2011	France	Pays de la Loire	+	+	–	4	This study
CFBP 8041	*A. deliciosa*	Leaf	2011	France	Pays de la Loire	+	+	–	4	This study
CFBP 8042	*A. deliciosa*	Leaf	2011	France	Pays de la Loire	+	+	–	4	This study
CFBP 8043	*A. deliciosa*	Leaf	2011	France	Pays de la Loire	+	+	–	4	This study
CFBP 8044/ ICMP 19440	*A. chinensis*	Leaf	2011	Australia	Victoria	+	+	–	4	Chapman *et al.* (2012), this study
CFBP 8045/ ICMP 19486	*A. chinensis*	Leaf	1990	Australia	Western Australia	+	+	–	4	Chapman *et al.* (2012), this study
CFBP 8046/ ICMP 19441	*A. chinensis*	Leaf	2011	Australia	Victoria	+	+	–	4	Chapman *et al.* (2012), this study
CFBP 8047	*A. deliciosa*	Leaf	2010	France	Rhône-Alpes	–	–	+	3	Vanneste *et al.* (2011)
CFBP 8048	*A. deliciosa*	Leaf	2011	France	Pays de la Loire	+	+	–	4	This study
CFBP 8049	*A. deliciosa*	Leaf	2011	France	Pays de la Loire	+	+	–	4	This study
CFBP 8050	*A. deliciosa*	Leaf	2011	France	Pays de la Loire	+	+	–	4	This study
CFBP 8051	*A. deliciosa*	Leaf	2011	France	Pays de la Loire	+	+	–	4	This study
CFBP 8052	*A. deliciosa*	Leaf	2011	France	Midi-Pyrénées	–	–	+	3	This study
CFBP 8053	*A. chinensis*	Leaf	2011	France	Midi Pyrénées	–	–	+	3	This study
CFBP 8054	*A. deliciosa*	Leaf	2011	France	Midi-Pyrénées	–	–	+	3	This study
CFBP 8055	*A. deliciosa*	Leaf	2011	France	Aquitaine	–	–	+	3	This study
CFBP 8056	*A. chinensis*	Wood	2012	France	Aquitaine	–	–	+	3	This study
CFBP 8057	*A. deliciosa*	Wood	2012	France	Rhône-Alpes	–	–	+	3	This study
CFBP 8058	*A. chinensis*	Wood	2012	France	Rhône-Alpes	–	–	+	3	This study
CFBP 8059	*A. deliciosa*	Wood	2012	France	Aquitaine	–	–	+	3	This study
CFBP 8060	*A. deliciosa*	Wood	2012	France	Aquitaine	–	–	+	3	This study
CFBP 8061	*A. deliciosa*	Wood	2012	France	Rhône-Alpes	–	–	+	3	This study
CFBP 8062	*Actinidia* sp.	Leaf	2012	France	Provence-Alpes-Côte d'Azur	–	–	+	3	This study
CFBP 8063	*A. chinensis*	Wood	2012	France	Aquitaine	–	–	+	3	This study
CFBP 8064	*A. deliciosa*	Leaf	2012	France	Poitou-Charentes	–	–	+	3	This study
CFBP 8065	*A. chinensis*	Leaf	2012	France	Aquitaine	–	–	+	3	This study
CFBP 8066	*A. deliciosa*	Leaf	2012	France	Midi-Pyrénées	–	–	+	3	This study
CFBP 8067	*A. deliciosa*	Leaf	2012	France	Pays de la Loire	+	+	–	4	This study
CFBP 8085	*A. deliciosa*	Leaf	2012	France	Pays de la Loire	+	+	–	4	This study
CFBP 8086	*A. deliciosa*	Leaf	2012	France	Pays de la Loire	+	+	–	4	This study
CFBP 8087	*A. deliciosa*	Wood	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8088	*A. deliciosa*	Wood	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8089	*A. deliciosa*	Wood	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8090	*A. deliciosa*	Wood	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8091	*A. deliciosa*	Wood	2013	France	Midi-Pyrénées	–	–	+	3	This study

(continued)

Table 1 (continued)

Strain name	Host	Sample	Year of isolation	Country of isolation	Region of isolation	Fluorescence on KB[a]	Aesculin[b]	*avrD1*[c]	Biovar	Reference
CFBP 8092	*A. deliciosa*	Wood	2013	France	Rhône-Alpes	–	–	+	3	This study
CFBP 8094	*A. deliciosa*	Wood	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8095	*A. deliciosa*	Wood	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8096	*A. deliciosa*	Wood	2013	France	Midi-Pyrénées	–	–	+	3	This study
CFBP 8097	*A. deliciosa*	Leaf	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8098	*A. chinensis*	Wood	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8099	*A. deliciosa*	Wood	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8100	*A. deliciosa*	Wood	2013	France	Midi-Pyrénées	–	–	+	3	This study
CFBP 8101	*A. deliciosa*	Leaf	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8102	*A. deliciosa*	Wood	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8103	*A. deliciosa*	Leaf	2013	France	Aquitaine	–	–	+	3	This study
CFBP 8104	*A. deliciosa*	Leaf	2013	France	Poitou-Charentes	+	+	–	4	This study
CFBP 8105	*A. deliciosa*	Leaf	2013	France	Pays de la Loire	+	+	–	4	This study
CFBP 8106	*A. deliciosa*	Leaf	2013	France	Pays de la Loire	+	+	–	4	This study
CFBP 8107	*A. deliciosa*	Leaf	2013	France	Aquitaine	+	+	–	4	This study
CFBP 8108	*A. deliciosa*	Leaf	2013	France	Poitou-Charentes	–	–	+	3	This study
CFBP 8109	*A. deliciosa*	Leaf	2013	France	Poitou-Charentes	–	–	+	3	This study
CFBP 8110	*A. deliciosa*	Leaf	2013	France	Poitou-Charentes	–	–	+	3	This study
CFBP 8160	*A. deliciosa*	Leaf	2013	France	Centre	+	+	–	4	This study
CFBP 8161	*A. deliciosa*	Leaf	2013	France	Centre	–	+	–	4	This study
ICMP 19071	*A. chinensis*		1997	Korea		–	–	+	2	Koh *et al.* (1994), Chapman *et al.* (2012)
NCPPB 3871	*A. deliciosa*	Leaf/wood	1992	Italy	Latina	–	–	+	1	Scortichini (1994), Ferrante & Scortichini (2010)

[PT]pathotype strain. CFBP: Collection Française de Bactéries associées aux Plantes; NCPPB: National Collection of Plant Pathogenic Bacteria; ICMP: International Collection of Microorganisms from Plants.
[a]+, fluorescence; –, no fluorescence.
[b]+, hydrolysis of aesculin; –, no hydrolysis of aesculin.
[c]+, presence of *avrD1* gene; –, absence of *avrD1* gene.
[d]Vanneste *et al.* (2013).

Pathogenicity assays

A leaf inoculation assay and a stem inoculation assay were conducted. In both assays, 24-h-old bacterial colonies were resuspended in sterile water to a concentration of 10^9 CFU mL^{-1}. In addition to the two reference strains CFBP 7811 (biovar 3) and CFBP 7812 (biovar 4) isolated in New Zealand, two strains of Psa biovar 3 (CFBP 7906 and CFBP 7910) and three strains of Psa biovar 4 (CFBP 7908, CFBP 8039 and CFBP 8043) isolated in France were used. Distilled water was used as a negative control. Leaf inoculation was performed by spraying until run-off with a suspension of the test bacterial strain on the abaxial side of six unwounded leaves of two 3-month-old *in vitro* cultures of *A. deliciosa* 'Hayward'. The plants were incubated in a climatic room at 20°C with relative humidity ranging from 80 to 90% and a 12 h photoperiod. Symptoms were monitored daily.

Stem inoculation was performed by wounding the internode between the two youngest fully developed leaves of six 3-month-old seedlings of *A. deliciosa* 'Hayward' and *A. chinensis* 'Hort16A' with a wooden toothpick previously dipped in the bacterial suspension. The symptoms were scored on a scale of 0 to 3: 0 corresponding to no symptoms, 1 to water soaking and necrosis around the wound site, 2 to necrosis along the inoculation point and 3 to stem collapse. The scores were averaged for each strain × cultivar combination to calculate a disease index (DI). Data were analysed with the nonparametric Kruskal–Wallis test (significance level of 5%) followed by the Steel–Dwass–Critchlow–Fligner multiple comparison test using XLSTAT 2011 software.

For the two inoculation methods, Koch's postulates were verified by characterizing the bacteria reisolated from the symptoms.

Housekeeping gene amplifications and sequencing

Four genes, *gapA*, *gltA* (also known as *cts*), *gyr*B and *rpo*D, which code for glyceraldehyde-3-phosphate dehydrogenase, citrate synthase, DNA gyrase B and sigma factor 70, respectively, were amplified using primers designed by Sarkar & Guttman (2004) and Hwang *et al.* (2005). In addition, a set of primers was designed using PRIMER3 (Rozen & Skaletsky, 2000) to amplify *gapA* from *P. viridiflava* CFBP 2107 (Table S2). PCRs were carried out in a final volume of 20 μL containing 1 U of Platinum *Taq* DNA polymerase (Invitrogen), 1× buffer, 2·5 mM $MgCl_2$, 200 μM dNTP, 0·2 μM of each primer and 1 μL of boiled extract. Reactions were performed on a Veriti 96-well thermal cycler (Applied Biosystems) using a thermal cycling programme of 3 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at Tm (Table S2) and 1 min at 72°C, and finished with 72°C for 5 min. The purity and yield of each amplicon were verified by loading 8 μL of the reaction product onto 1·5% agarose gel in 1 × TBE buffer. The two strands of the PCR products were sequenced by the Genoscreen Company (Lille, France).

The partial sequences of the four housekeeping genes of the strains of Psa isolated in France were deposited on GenBank under the accession numbers KF937399 to KF937786.

MLSA and clonal genealogy

Sequence and phylogenetic analyses were performed using GENEIOUS v. 4.8.5 (Biomatters) and BIOEDIT programs (Hall, 1999). The sequences were concatenated according to the alphabetic order of the gene. The concatenated data set was 3159 bp long (*gapA* from bp 1 to 675, *gltA* from 676 to 1671, *gyrB* from 1672 to 2346, *rpoD* from 2347 to 3159).

A neighbour joining (NJ) tree was built with MEGA v. 5.1 using the Jukes–Cantor distance methods with the DNA sequences for the four housekeeping genes of Psa strains, six isolated in France, one in Japan, two in Italy, three in New Zealand and one in Australia (Table 1) and 10 type or pathotype strains of *Pseudomonas* representative of the genomospecies (G) defined by Gardan *et al.* (1999) (*P. syringae* pv. *syringae* CFBP 4702 for G1, *P. syringae* pv. *phaseolicola* CFBP 1390 for G2, *P. syringae* pv. *morsprunorum* CFBP 2351 and *P. syringae* pv. *tomato* CFBP 2212 for G3, *P. syringae* pv. *coronofasciens* CFBP 2216 for G4, *P. viridiflava* CFBP 2107 for G6, *P. syringae* pv. *tagetis* CFBP 1694 for G7, *P. syringae* pv. *theae* CFBP 2353 and *P. avellanae* CFBP 4060 for G8 and *P. cannabina* pv. *cannabina* CFBP 2341 for G9). The strain of *P. fluorescens* CFBP 2102 was used to root the tree.

Maximum likelihood (ML) trees were constructed with the sequence of each gene and with the concatenated sequences for Psa strains. The strain of *P. syringae* pv. *tomato* CFBP 2212 was used to root the trees. The model of evolution was determined using MODELTEST v. 3.7 with PAUP. The model scores were evaluated with the hierarchical likelihood ratio (hLRT) and the standard Akaike information criterion (AIC). Phylogenetic trees and bootstrap values were obtained for each gene and the concatenated data by the PHYML (Guindon *et al.*, 2010) method using TOPALI v. 2.5 (Milne *et al.*, 2009; available at http://www.topali.org/). Bootstrap analyses were done with 1000 replicates for NJ and ML analyses. The trees were visualized in MEGA v. 5.1. The phylogenetic congruence between the ML trees of each locus and the concatenated sequences was tested using the Shimodaira–Hasegawa test (Shimodaira & Hasegawa, 1999) implemented in the DNAML program from PHYLIP (Felsenstein, 2005).

In order to estimate the clonal genealogy of the four biovars of Psa, CLONALFRAME software (Didelot & Falush, 2007) was used. CLONALFRAME uses a coalescent-based Bayesian method to infer strain relationships. Three independent runs were performed on housekeeping gene sequences used for the MLSA. The parameter space was explored using a Markov chain Monte Carlo (MCMC) simulation of 1 000 000 iterations, out of which 500 000 were considered as burn-in. The interval genealogy sampling was set at 100, which ensured good independence between successive sampling.

Molecular data analysis

Rates of amino acid replacement over synonymous mutation K_a/K_s ratio were estimated using the Nei and Gojobori method (Nei & Gojobori, 1986) implemented in MEGA v. 5.1 (Tamura *et al.*, 2011). Estimations of recombination events were performed using a method based on seven different nonparametric detection algorithms implemented in RDP v. 3.38 (Martin *et al.*, 2005). Recombinant events were accepted when detected with at least three out of seven detection methods implemented in this software (Mhedbi-Hajri *et al.*, 2013).

Results

Isolation and characterization of strains of Psa isolated in France

From the 989 samples with symptoms received between 2010 and 2013, 280 strains with morphology similar to

that of Psa were isolated. They did not display cytochrome c oxidase or arginine dihydrolase activity. They produced levan, induced an HR on tobacco leaves but did not rot potato tissues. PCR performed with the primers developed by Rees-George *et al.* (2010) and by Koh & Nou (2002) generated amplicons of the expected size for Psa. None of the 280 strains produced a DNA fragment of the expected size using the primers designed to amplify the genes *cfl* and *argK*, nor did they produce syringomycin. Among the 280 strains, 248 did not hydrolyse aesculin and no fluorescent pigment was observed under UV light on King's B medium. Thus, these 248 strains had all the characteristics of strains of Psa biovar 3. The remaining 32 strains hydrolysed aesculin, were fluorescent on King's B medium with the exception of CFBP 8161, and did not produce a 226 bp DNA fragment with the primers designed to amplify *avrD1* (Gallelli *et al.*, 2011). These 32 strains, therefore, had all the characteristics of strains of Psa biovar 4. The origin of the strains is presented in Table S1.

The six strains of Psa biovar 3 and biovar 4 that were tested were motile and INA negative. The results of the two repetitions per strain of the Biolog plates were combined. Six carbon sources (Tween 40, Tween 80, *i*-erythritol, *β*-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, L-histidine and L-leucine) were catabolized by strains of biovar 4 and not those of biovar 3. In contrast, uridine was weakly used by strains of Psa biovar 3 but not by those of biovar 4 (Table S3).

The four biovars of Psa displayed different patterns when analysed by BOX PCR (Fig. 1). Strains of Psa biovar 1 and 2 displayed almost identical patterns with the presence of a band at 430 bp and the absence of a band of approximately 1300 bp. The strains of Psa biovar 2 presented an extra band of about 2500 bp. All 248 French strains identified as strains of biovar 3, presented a BOX PCR pattern similar to that of strains of Psa biovar 3 isolated from Italy (CFBP 7287) and New Zealand (CFBP 7811). All Psa biovar 4 strains presented extra bands of 500, 1500 and 2900 bp. The pattern of the 32 French strains identified as strains of biovar 4 was similar to the pattern of the CFBP 7812 strain of Psa biovar 4 isolated in New Zealand. However, one strain isolated in New Zealand (CFBP 7951) displayed a different pattern with three additional bands of 680, 780 and 950 bp.

Strains of Psa biovar 3 were isolated from leaves, wood, flower buds and roots of *A. deliciosa*, *A. chinensis* and *A. arguta*, mostly from orchards located in the main kiwifruit production areas in the southwest and southeast of France, which were planted in the last 10 to 20 years. In contrast, strains of Psa biovar 4 were isolated only from leaves of *A. deliciosa* mostly in older orchards from the northwest of France. These older orchards were planted in the 1970s and 1980s with plants mainly imported from New Zealand or with plants that were obtained from those vines (Table S1; Fig. 2).

Symptoms caused by the two biovars

When bacterial suspensions of strains of Psa biovar 3 and Psa biovar 4 were sprayed on to leaves of *A. delici-*

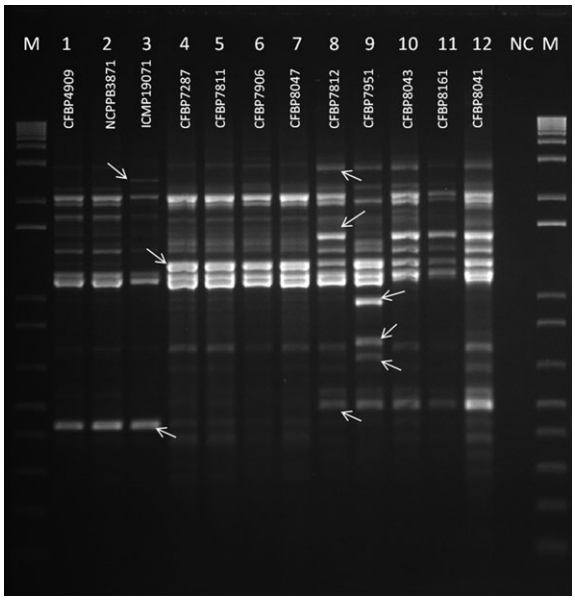

Figure 1 Agarose gel electrophoresis of DNA samples after BOX PCR. M, 1 kb Plus DNA ladder; lanes 1 and 2 *Pseudomonas syringae* pv. *actinidiae* (Psa) strains biovar 1; lane 3 Psa strain biovar 2; lanes 4, 5, 6 and 7 Psa strains biovar 3; lanes 8, 9, 10, 11 and 12 Psa strains biovar 4; NC, negative control (water). Arrows indicate bands that are differentially represented between the different biovars.

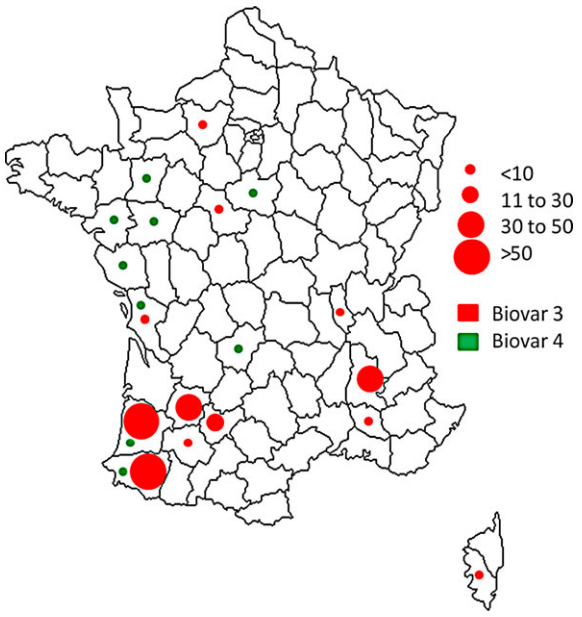

Figure 2 Distribution of *Pseudomonas syringae* pv. *actinidiae* (Psa) biovars 3 and 4 in the different departments of France from 2010 to June 2013. The circles indicate the number of orchards contaminated by Psa biovar 3 (in red) or biovar 4 (in green).

osa 'Hayward', similar symptoms developed of angular, dark brown spots, sometimes surrounded by a chlorotic halo and located between the veins (Fig. S1).

When stem-inoculated, the three Psa biovar 3 strains caused necrosis and collapse of the inoculated stems on *A. deliciosa* 'Hayward' and *A. chinensis* 'Hort16A' giving an average disease index (DI) of 2·5 to 3 (Fig. 3). In contrast, the DI of plants inoculated with the four strains of Psa biovar 4 was below 0·5, corresponding to no reaction or local water soaking at the inoculation point. DI differences between the two biovars were statistically significant ($P < 0{\cdot}05$). There were no significant differences among strains of the same biovar ($P > 0{\cdot}05$).

Haplotypic diversity measured by MLSA

Of the 280 strains isolated, 72 strains (50 of biovar 3 and 22 of biovar 4) representative of the different regions, hosts and years of isolation were selected for genetic analysis (Table 1).

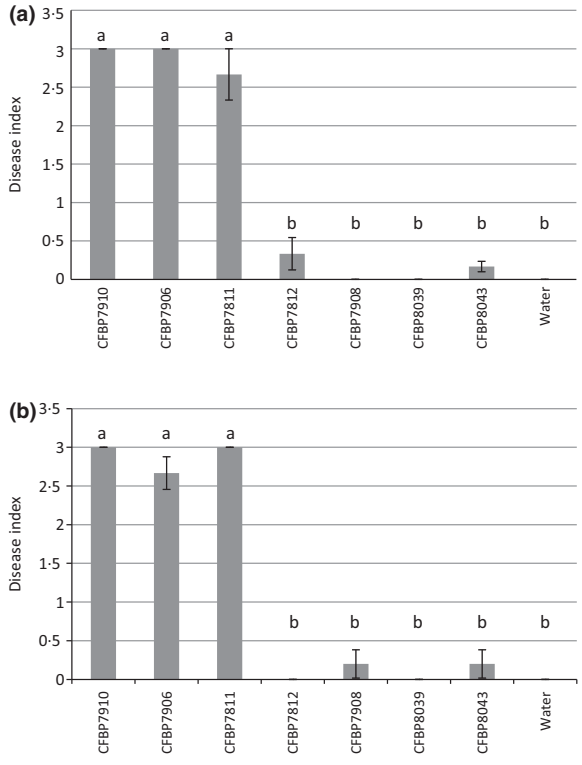

Figure 3 Pathogenicity of strains of *Pseudomonas syringae* pv. *actinidiae* biovar 3 (CFBP 7906, CFBP 7910, CFBP 7811) and biovar 4 (CFBP 7812, CFBP 7908, CFBP 8039, CFBP 8043) on seedlings of *Actinidia deliciosa* 'Hayward' 9 days after inoculation (a) and *A. chinensis* 'Hort16A' 8 days after inoculation (b). The disease index is proportional to the length of necrosis on the stem. Vertical bars represent standard errors of the means. Columns with the same letter are not significantly different ($P > 0{\cdot}05$) according to Kruskal–Wallis test.

All Psa strains, including those isolated from France, clustered within the phylotype that groups genomospecies 8 and 3 (Fig. 4). Strains of Psa biovar 3 isolated from France clustered with Psa biovar 3 strains isolated from New Zealand (CFBP 7811) and from Italy during the latest outbreak (CFBP 7287) (Figs 4 & 5). Strains of Psa biovar 4 isolated from France clustered with Psa biovar 4 strains isolated from New Zealand and Australia (Figs 4 & 5). *Pseudomonas syringae* pv. *theae* grouped with Psa biovars 1, 2 and 3 and not with Psa biovar 4.

All the strains of Psa examined by MLSA clustered in two groups, one corresponding to biovars 1, 2 and 3, and the other to biovar 4. This clustering is supported by medium to strong bootstrap values (>77%; Fig. 5). The phylogenetic trees built with the ML algorithm for each housekeeping gene (data not shown) were significantly congruent with that built using concatenated sequences (Fig. 5) as indicated by the Shimodaira–Hasegawa test ($P > 0{\cdot}05$; Table 2). The 72 strains isolated from France fitted into the clusters corresponding to either biovar 3 or biovar 4. The diversity within Psa biovar 4 strains was larger than that among the three other biovars, as revealed by the longer length of the internal branches. The cluster of Psa strains biovar 4 could be divided in four lineages (L): L1 consisted of strains isolated from New Zealand and Australia and one strain isolated from France (CFBP 8161); L2 contained only one strain (CFBP 8043) isolated from France; L3 contained only one strain (CFBP 7951) isolated from New Zealand; and L4 is made up of strains isolated only from France. No correlation was found between the geographical origin or the plant host and the lineages of strains of Psa biovar 4, but the majority of strains isolated from France were included in the L4 lineage.

Clonal genealogy of Psa strains

The three coalescent trees obtained by ClonalFrame were identical to each other and one of them is presented in Figure 6. The coalescent tree of the Psa strains revealed the same groups as that obtained with the phylogeny approach using the ML analysis (Fig. 6). The Psa strains were grouped in three clonal complexes: the first complex contains strains of Psa biovar 1, 2 and 3; the second contains strains of Psa biovar 4 L1 and L2; and the third contains strains of Psa biovar 4 L3 and L4.

According to ClonalFrame analysis, the common ancestor of the Psa biovar 1, 2 and 3 is as recent (coalescent unit of 0·45) as that of the Psa biovar 4 L1 and L2 (coalescent unit of 0·45), but more recent than that of the Psa biovar 4 L3 and L4 (coalescent unit of 0·71). The four lineages of Psa biovar 4 coalesced at the same time (coalescent unit of 1·30) as the common ancestor of the biovars 1, 2, 3 and 4 (coalescent unit of 1·30). No recent common ancestor was found for the four biovars.

Molecular diversity within and between biovars

The K_a/K_s ratio estimated for each gene of the four biovars is null for *gapA*, *gltA* and *gyrB* genes and equal to

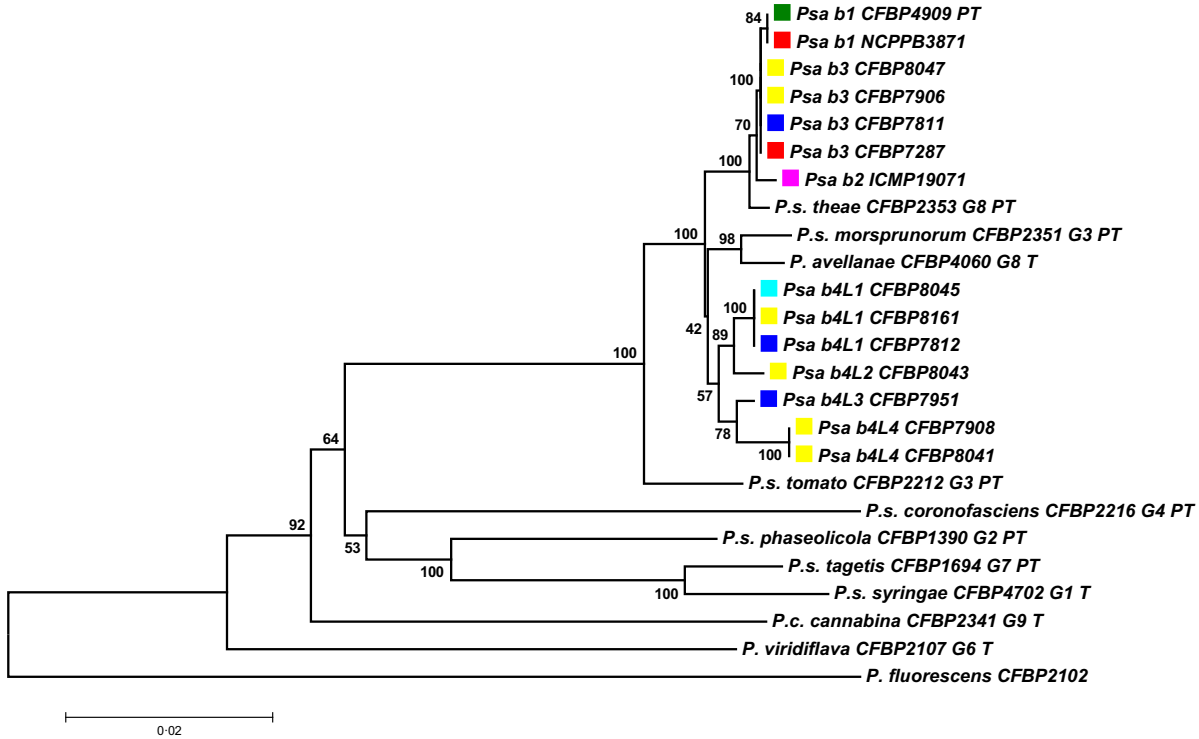

Figure 4 Neighbour joining tree constructed with the concatenated partial sequences of four housekeeping genes (*gapA*, *gltA*, *gyrB* and *rpoD*). Percentage of bootstrap scores obtained for 1000 replicates are indicated at each node. The labels G1 to G8 mentioned after the name of *Pseudomonas syringae* (Ps) pathovars indicate the genomospecies as defined by Gardan *et al.* (1999). T, type strain; PT, pathotype strain; b, biovar; L, lineage. The colours represent the geographical origin of the strains: dark blue, New Zealand; light blue, Australia; yellow, France; pink, Korea; red, Italy; and green, Japan. The scale bar represents the number of nucleotide substitutions per site.

0·123 for the *rpoD* gene, suggesting strong to moderate purifying selection for these genes. Combinations of primers designed by Sarkar & Guttman (2004) and by Hwang *et al.* (2005) were used in order to obtain longer partial DNA sequences of the housekeeping genes, expecting to increase the number of polymorphic sites. However, the value of the K_a/K_s ratio found for the housekeeping gene *rpoD* in this study was the same as that described by Chapman *et al.* (2012) who used shorter DNA sequences. No additional polymorphic sites were observed with the analysis of longer sequences for these four housekeeping genes.

The RDP program was used to detect possible recombination events. Only one out of the seven detection methods detected a possible recombinant event for the gene *gyrB*, so no significant recombination event was detected in the data set.

Discussion

A total of 280 strains isolated from *A. deliciosa* or *A. chinensis* from different regions of France between 2010 and June 2013 were identified as Psa biovar 3 or biovar 4. When the outbreak of bacterial canker of kiwifruit was reported in France, only one haplotype of Psa was detected in French orchards (Vanneste *et al.*, 2011), Psa biovar 3. In this study it is shown that strains of Psa biovar 3 and 4 have been present in France since at least 2010. This was confirmed by different methods including BOX PCR, which can be used to distinguish strains of biovar 3 from those of biovar 4 (Vanneste *et al.*, 2013). The BOX PCR pattern of the 32 strains of biovar 4 isolated in France was similar to that of the Psa biovar 4 strain CFBP 7812 isolated in New Zealand. However, a few differences were found compared with the Psa biovar 4 strain CFBP 7951 also isolated in New Zealand. Geographical populations could not be delineated by the BOX PCR study. The differences observed by Mazzaglia *et al.* (2011) using BOX PCR between strains of different geographic origin reflect that in that study each geographic location was represented by strains of only one biovar. Thus, the differences were due to the strains belonging to different biovars and not to their geographic origin.

MLSA, based on housekeeping genes, is a powerful tool to study phylogenetic relationships and species delineation of bacterial strains. The four housekeeping genes (*gapA*, *gltA*, *gyrB* and *rpoD*) selected by Hwang *et al.* (2005) have already been used to explore the diversity of *P. syringae* (Hwang *et al.*, 2005) and Psa (Ferrante & Scortichini, 2010; Chapman *et al.*, 2012). In this study this set of genes was used to perform MLSA on Psa strains and some type and pathotype strains representing the *P. syringae* genomospecies as defined by Gardan

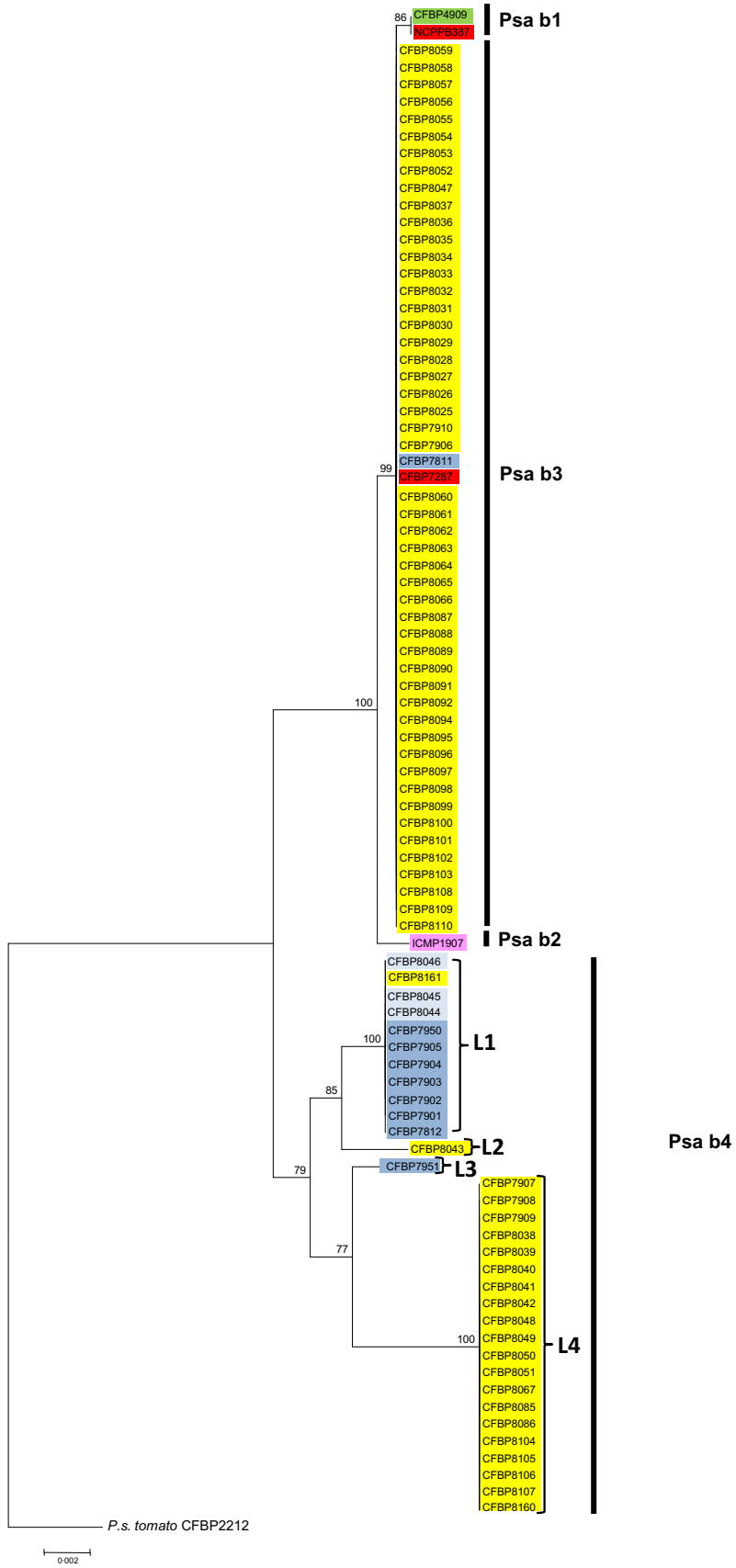

Figure 5 Maximum likelihood tree constructed with the concatenated partial sequences of four housekeeping genes (*gapA*, *gltA*, *gyrB* and *rpoD*) of 88 *Pseudomonas syringae* pv. *actinidiae* strains. Percentages of bootstrap scores obtained for 1000 replicates are indicated at each node. b, biovar; L, lineage. The colours represent the geographical origins of the strain: dark blue, New Zealand; light blue, Australia; yellow, France; pink, Korea; red, Italy; and green, Japan. The scale bar represents the number of nucleotide substitutions per site.

Table 2 Results of the Shimodaira–Hasegawa test for congruence of tree topologies run on each of the maximum likelihood trees based on four housekeeping genes, individually and as concatenated (conc) sequences, of 88 strains of *Pseudomonas syringae* pv. *actinidiae*

	P-value				
Locus	*gapA*	*gltA*	*gyrB*	*rpoD*	conc
gapA		0·001	0·000	0·100	0·000
gltA	0·027		0·000	0·276	0·026
gyrB	0·011	0·000		0·284	0·015
rpoD	0·058	0·001	0·518		0·162
conc	0·494	0·528	0·598	0·890	

et al. (1999). The architecture of the NJ tree obtained (Fig. 4) was similar to the one described by Bull *et al.* (2011). Strains of Psa isolated in France clustered with strains belonging to genomospecies 8 and genomospecies 3 (G8 and G3) *sensu stricto* Gardan *et al.* (1999) and were closely related to other Psa strains of biovar 3 and 4. The structure of the tree and the localization of Psa strains within the tree were similar to results obtained by Chapman *et al.* (2012) with seven housekeeping genes. The strain of *P. syringae* pv. *theae* was more closely linked to Psa biovars 1, 2 and 3 strains than to the group of Psa biovar 4 strains. The strains of Psa biovar 4 were spread into groups different from those of strains of *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *avellanae*, *P. syringae* pv. *tomato* and Psa biovar 1, 2 and 3, but were included in the G3 + G8 complex described by Bull *et al.* (2011).

In this study MLSA allowed classification of Psa strains of biovar 4 isolated in France, New Zealand and Australia into four lineages. The diversity found within biovar 4 is greater than that described earlier (Butler *et al.*, 2013; McCann *et al.*, 2013). These authors showed that one strain isolated in New Zealand, CFBP 7951, did not cluster with the other New Zealand or Australian isolates. CFBP 7951 is the only strain in L3; it was called Psa NZ LV14 by McCann *et al.* (2013) and strains with DNA sequences for the four housekeeping genes (*gapA*, *gltA*, *gyrB* and *rpoD*) identical to that of CFBP 7951 were labelled PsHa by Butler *et al.* (2013). The phylogenetic relationships observed in this study did not show a correlation between the lineages of Psa biovar 4 and the geographical origin or the plant host. The fact that only strains isolated from France are found in L4 could simply be due to the limited number of strains isolated from New Zealand and Australia that were examined in this study.

This is the first account of Psa biovar 4 isolation in France and in Europe, and provides evidence that strains of Psa biovar 4 have been present in France since the beginning of the outbreak caused by strains of Psa biovar 3 in 2010. Leaf symptoms caused by strains of biovar 4 are identical to those caused by strains of biovar 3. Psa biovar 4 might have been present long before Psa biovar 3 was detected; however, any presence of biovar 4 in the old *A. deliciosa* orchards did not have an economic impact on kiwifruit production. Thus biovar 4 has not been detected until sampling for Psa detection was systematically conducted in the recent outbreak caused by strains of biovar 3 in 2010. In France, Psa biovar 4 was isolated only from leaves in old *A. deliciosa* orchards planted at the end of the 1970s with kiwifruit vines that originated from New Zealand. Strains of Psa biovar 4 could have been brought from New Zealand with these plants. Other kiwifruit vines from New Zealand were imported into Europe and the movement of plants between France and other European countries may have also helped to spread Psa biovar 4 within Europe; if this is the case, then the diversity within biovar 4 might even be larger than that described in this study. Alternatively, Psa biovar 4 might have host(s) other than kiwifruit, in which case it is possible that Psa biovar 4 originated in Europe or that it was introduced a long time ago or from a country other than New Zealand. Interestingly, Ferrante & Scortichini (2014) recently showed that, in contrast to strains of Psa biovars 1, 2 and 3, Psa strains of biovar 4 isolated in New Zealand (ICMP 18802, ICMP 18882, ICMP 18883) induce extensive symptoms on sour cherry (*Prunus cerasus*). That Psa biovar 4 might have been present in France and possibly Europe for a relatively long period of time is supported by the molecular diversity of strains of biovar 4 isolated in France. This work demonstrates that the biovar 4 Psa strains collected mainly in France, New Zealand and Australia structure into two clusters and four lineages as revealed by the NJ and ML trees (Figs 4 & 5).

Different studies conducted on a limited number of strains indicated that strains of Psa biovar 3 isolated from different parts of the world showed some differences in genes potentially related to pathogenicity; all strains isolated from Europe were similar (Mazzaglia *et al.*, 2012; Butler *et al.*, 2013; McCann *et al.*, 2013). The introduction of Psa biovar 3 into Europe has been a recent event and it is possible that a single strain was introduced and then spread throughout Europe. Based on genome sequence analyses, Mazzaglia *et al.* (2012), Butler *et al.* (2013) and McCann *et al.* (2013) concluded that strains of Psa biovar 3 had a Chinese common ancestor. In this study the clonal genealogy of strains of Psa has been estimated with ClonalFrame which has already been used for coalescent analysis of plant pathogenic bacteria (Mhedbi-Hajri *et al.*, 2013). The coalescent tree indicated that Psa biovars 1, 2 and 3 had a more recent common ancestor than they had with biovar 4. This study cannot reveal a common ancestor to the four biovars and suggests that other strains, not yet isolated, are involved in the evolution of *P. syringae* strains associated with kiwifruit.

All these results show important similarities and differences between Psa biovars 1, 2 and 3 and biovar 4: they infect the same host and cause similar symptoms on leaves. However, in contrast to strains of the other biovars, strains of biovar 4 do not induce shoot die-back or canker formation; they also have different biochemical properties. In Australia, strains of Psa biovar 4 were isolated 20 years ago and did not result in any noticeable

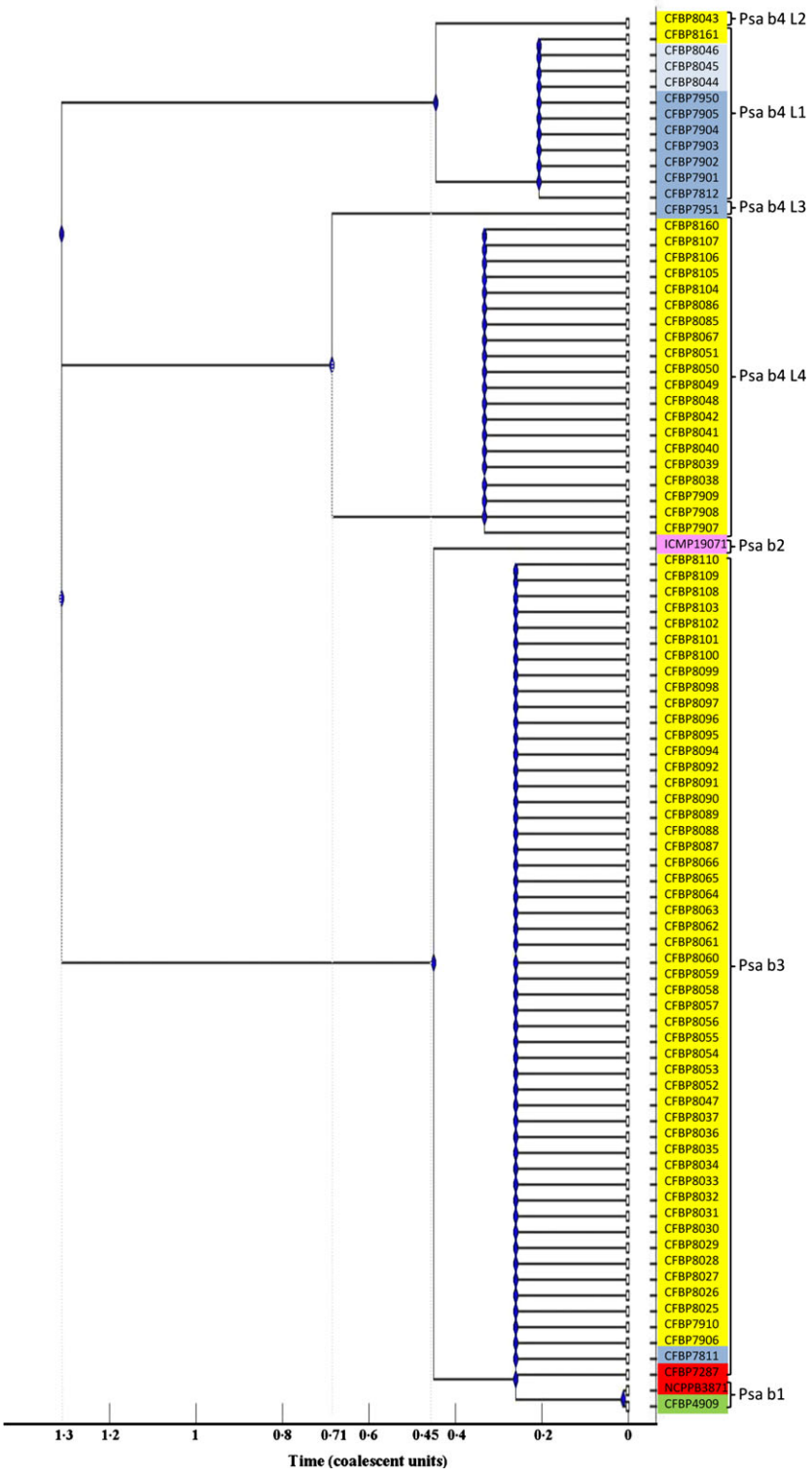

Figure 6 Coalescent tree showing the clonal genealogy of strains of the four biovars of *Pseudomonas syringae* pv. *actinidiae* (Psa) based on partial sequences of four housekeeping genes (*gapA*, *gltA*, *rpoD* and *gyrB*). b, biovar; L, lineage. The colours represent the geographical origins of the strain: dark blue, New Zealand; light blue, Australia; yellow, France; pink, Korea; red, Italy; and green, Japan.

loss of productivity (EPPO, 2011). Strains of Psa biovar 4 isolated in New Zealand were most probably present long before strains of Psa biovar 3 were detected (Vanneste *et al.*, 2013). No loss of productivity has been reported in New Zealand due to the presence of Psa biovar 4 (Vanneste *et al.*, 2013). It is crucial to distinguish strains of Psa biovar 4 from Psa biovar 1, 2 and 3 as these two groups of strains cause two distinct diseases on the same host plant and the management of the two diseases requires different control measures. The legislation written to protect kiwifruit orchards from the devastating disease caused by Psa biovars 1, 2 or 3 can

force kiwifruit producers to take drastic measures, such as the removal of the orchard. Such measures are disproportionate to the economic consequences induced by strains of Psa biovar 4.

In other pathosystems there are examples of closely related pathogenic bacteria that cause different symptoms on the same host range; they are distinguished by a different pathovar name in agreement with the official definition of a pathovar: 'The term *pathovar* is used to refer to a strain or set of strains with the same or similar characteristics, differentiated at infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts' (http://www.isppweb.org/about_tppb_naming.asp). On rice, *Xanthomonas oryzae* pv. *oryzae* (Xoo), the causal agent of bacterial blight is vascular, while *Xanthomons oryzae* pv. *oryzicola* (Xoc), the causal agent of bacterial leaf streak is not (Niño-Liu *et al.*, 2006). On Brassicaceae, *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot, is vascular but *X. campestris* pv. *raphani* causes only bacterial leaf spot (Fargier & Manceau, 2007). In the case of *P. syringae* pathogenic to kiwifruit, two groups, genetically distinct with only one able to cause systemic infection, are called *P. syingae* pv. *actinidiae*. Ferrante & Scortichini (2014) proposed to remove Psa biovar 4 from the pathovar *actinidiae*, which would still contain strains of Psa biovars 1, 2 and 3. Moreover, the phylogenetic study of strains of Psa revealed that Psa biovars 1, 2 and 3 strains are genetically related to each other and close to *P. syringae* pv. *theae*, whereas Psa biovar 4 strains grouped separately. That these two different pathogens share the same name is confusing. Therefore, the authors propose to keep the name *Pseudomonas syringae* pv. *actinidiae* for the strains that cause leaf spot and vascular infection, these being the strains of Psa biovars 1, 2 and 3, in accordance with the description provided by Takikawa *et al.* (1989); and it is proposed to allocate the biovar 4 strains, which cause only leaf spot, to a new pathovar: *Pseudomonas syringae* pv. *actinidifoliorum* pv. nov.

Description of *Pseudomonas syringae* pv. *actinidifoliorum* pv. nov

Pseudomonas syringae pv. *actinidifoliorum* (ac.ti.ni.di.fo.li.o' rum. N. L. fem. n. Actinidia, systematic name of the kiwifruit plant, L. neut. plur. gen. foliorum of the leaves, N. L. neut. plur. gen. n. *actinidifoliorum* referring to the isolation of strains of the kiwifruit leaves).

Colonies of *Pseudomonas syringae* pv. *actinidifoliorum* cultivated on nutrient agar plates are round, convex, glistening, viscous, translucent and white. The bacteria are Gram-negative rods, aerobic, motile, can produce fluorescent pigment on King's B medium and do not have the genes coding coronatine (*cfl*) and phaseolotoxin (*argK*). Positive reactions are: levan production, tobacco hypersensitive reaction and hydrolysis of aesculine. Negative reactions are: cytochrome oxidase, arginine hydrolase, potato rot test, ice nucleation activity. *Pseudomonas syringae* pv. *actinidifoliorum* uses: Tween 40, Tween 80, L-arabinose, D-arabitol, *i*-erythritol, D-fructose, D-galactose, α-D-glucose, *m*-inositol, D-mannitol, D-mannose, D-sorbitol, sucrose, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, β-hydroxybutyric acid, β-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-histidine, L-leucine, L-proline, L-pyroglutamic acid, L-serine, L-threonine, γ-aminobutyric acid, inosine and glycerol as a carbon source. Formic acid, malonic acid, L-ornithine and uridine are only used weakly as a carbon source. The following carbon sources are not used by *P. syringae* pv. *actinidifoliorum*: α-cyclodextrin, dextrin, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, D-cellobiose, L-fucose, gentiobiose, α-D-lactose, lactulose, maltose, D-melibiose, β-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-trehalose, turanose, xylitol, α-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenlyacetic acid, *p*-hydroxyphenlyacetic acid, itaconic acid, α-ketovaleric acid, D,L-lactic acid, sebacic acid, glucuronamide, glycyl-L-aspartic acid, hydroxy-L-proline, L-phenylalanine, D-serine, D,L-camitine, urocanic acid, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, D,L,α-glycerol phosphate, α-D-glucose-1-phosphate and D-glucose-6-phosphate.

Pseudomonas syringae pv. *actinidifoliorum* is a plant pathogen that cause leaf spots on *A. chinensis* and *A. deliciosa*. The pathotype strain is CFBP 8039.

Acknowledgements

The authors wish to thank the French International Office of the Kiwi (BIK), SCAAP kiwifruits de France, Anses, France and The New Zealand Institute for Plant & Food Research Limited, Ruakura Research Centre, New Zealand, for financial support. The authors also thank Janet Yu and Deirdre Cornish for their help during pathogen test interpretation, Corinne Audusseau and Sandrine Paillard for the isolation of the French strains, Sophie Bonneau and Karine Durand for their help during the MLSA, and Perrine Portier, Martial Briand and Géraldine Taghouti at the French Collection of Plant-associated Bacteria (CIRM-CFBP) for providing and maintaining bacterial strains. A. C. is supported by a fellowship provided by Anses and Région Pays de la Loire, France.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 Leaf symptoms caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) on *Actinidia deliciosa* 'Hayward' 20 days after inoculation with Psa biovar 4 strain CFBP 7812 (a) and with Psa biovar 3 strain CFBP 7906 (b).

Table S1 Origin of the strains of *Pseudomonas syringae* pv. *actinidiae* isolated in France from *Actinidia* spp. from 2010 to June 2013.

Table S2 List of PCR primers used in this study.

Table S3 Utilization of 95 carbon sources by six French strains of *Pseudomonas syringae* pv. *actinidiae* (Psa) using Biolog GN MicroPlate (Biolog). Grey highlight indicates carbon sources that distinguish Psa biovar 3 (b3) from Psa biovar 4 (b4).