SHORT COMMUNICATION

CLONAL OUTBREAKS OF BACTERIAL CANKER CAUSED BY *PSEUDOMONAS SYRINGAE* pv. *ACTINIDIAE* ON *ACTINIDIA CHINENSIS* AND *A. DELICIOSA* IN ITALY

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SUMMARY

A total of 28 representative *Pseudomonas syringae* pv. *actinidiae* strains isolated from all Italian regions (Emilia-Romagna, Latium, Piedmont, Veneto) where outbreaks of bacterial canker of kiwifruit (*Actinidia deliciosa*) and yellow kiwifruit (*A. chinensis*) were observed in 2008-2010, were assessed using repetitive-sequence PCR (rep-PCR) with ERIC and BOX primer sets and multilocus sequence typing (MLST) using *gapA*, *gltA, gyrB* and *rpoD* genes. The 2.3 kb sequences obtained from MLST were analyzed by means of mathematical-statistical tests to infer the gene polymorphism and the genetic structure of the strains. Both primer sets used in rep-PCR indicated an overall identity among all 28 *P.s.* pv. *actinidiae* strains irrespective of the host plant and cultivar from where they were isolated, as well as of the region or year of isolation. In addition, MLST revealed a low gene polymorphism. A clonal structure and neutral selection were inferred for the *P. s.* pv. *actinidiae* strains currently causing severe epidemics on *A. chinensis* and *A. deliciosa* in Italy. This indicates that they originated, most probably, from a single or very few introductions of latently infected kiwifruit propagative material, even though the possibility cannot be ruled out that cells of the pathogen already present in Italy may have mutated.

Key words: Repetitive-sequence PCR, BOX, ERIC, Multilocus sequence typing, bacterial epidemics.

Pseudomonas syringae pv. *actinidiae* (*Psa*), the causal agent of bacterial canker of kiwifruit (*Actinidia deliciosa*), was first reported from Japan (Takikawa *et al*., 1989), subsequently from South Korea and Italy (Koh *et al*., 1994; Scortichini, 1994). In both Asian countries, the pathogen causes severe epidemics greatly reducing the yield of kiwifruit (Koh *et al.*, 2003). A bacterial canker outbreak on *A. deliciosa* in central China (i.e. Shaanxi province) observed in 1990-1991, was reported ten years later (Liang *et al*., 2000), and another record came from Anhui province of southeast China (Li *et al*., 2004). In Italy, until 2007, *Psa* incited symptoms (i.e. leaf spotting, twig die-back) only sporadically in *A. deliciosa* cv. Hayward. However, from spring 2008 until now, substantial damages caused by this pathogen were observed on about 500 ha cultivated with yellow kiwifruit (*A. chinensis*) cvs Hort16A, JinTao and Soreli, mainly in the provinces of Latina and Roma (Latium, central Italy) (Ferrante and Scortichini, 2009, 2010). In the same areas, during 2009-2010, severe symptoms of bacterial canker were recorded also on *A. deliciosa* cv. Hayward. Moreover, in 2009 *Psa* was isolated in Emilia-Romagna from *A. chinensis* and, in 2010, disease outbreaks were observed in Piedmont and in the Viterbo province, both on *A. chinensis* and *A. deliciosa*. A single case of bacterial canker on *A. chinensis*, was observed in Veneto (northeast Italy). This pathogen was first isolated from *A. chinensis* in southwest China (Sichuan province) in 1989 (Wang *et al*., 1992), and later in southeast China (i.e. Anhui province) (Li *et al*., 2004).

It had been ascertained that the *Psa* population currently inciting severe outbreaks of bacterial canker on *A. chinensis* and *A. deliciosa* in central Italy is different from the *Psa* strains of the past, isolated in Japan, South Korea and Italy (Ferrante and Scortichini, 2010). To further investigate the population structure of this pathogen, we isolated or received *Psa* isolates from all the areas of Italy where symptoms of bacterial canker of kiwifruit and yellow kiwifruit were observed.

With 28 representative strains causing current epidemics in Italy and with *Psa* strains isolated in the past (Takikawa *et al*., 1989; Scortichini, 1994), we performed repetitive-sequence PCR (rep-PCR) and multilocus sequence typing (MLST) analysis combined with appropriate statistical-mathematical tests to infer the population structure of the pathogen during the current epidemics of bacterial canker in Italy.

The *Psa* strains used in this study are listed in Table 1. For isolation, we applied routine techniques described elsewhere (Ferrante and Scortichini, 2009, 2010). For DNA extraction, for each strain, a loopful (*ca.* 2 mm in diameter) of a single colony that had been grown for 24 h on NSA at 25 to 27°C, was suspended in

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

Strain	Origin	Host-Cultivar	Year of isolation
CRA-FRU10.22	Latium - Latina	*A. chinensis* – Hort16A	2008
CRA-FRU8.52	Latium - Latina	*A. chinensis* – CK3	2008
CRA-FRU8.43	Latium - Latina	*A. chinensis* – Hort16A	2008
CRA-FRU8.57	Latium - Latina	*A. chinensis* – Hort16A	2009
CRA-FRU10.14	Latium - Latina	*A. chinensis* – JinTao	2009
CRA-FRU10.25	Latium - Latina	*A. chinensis* – Hort16A	2009
CRA-FRU11.47	Latium - Viterbo	*A. chinensis* – Hort16A	2010
CRA-FRU11.48	Latium - Viterbo	*A. deliciosa* - Hayward	2010
CRA-FRU11.502	Latium - Viterbo	*A. deliciosa* - Hayward	2010
CRA-FRU5.1	Latium - Latina	*A. deliciosa* - Hayward	2009
CRA-FRU8.69	Latium - Latina	*A. deliciosa* - Hayward	2009
CRA-FRU8.75	Latium - Latina	*A. deliciosa* - Hayward	2009
CRA-FRU10.24	Latium - Latina	*A. deliciosa* - Hayward	2009
CRA-FRU11.41	Latium - Roma	*A. deliciosa* - Hayward	2010
CRA-FRU12.52	Latium - Roma	*A. deliciosa* - Hayward	2010
4252 A.1	Emilia Romagna- Ravenna	*A. chinensis*	2009
4649.1	Emilia Romagna- Ravenna	*A. chinensis*	2009
TV 4175	Veneto - Treviso	*A. chinensis* – Hort16A	2010
231 a	Piedmont - Cuneo	*A. chinensis* – JinTao	2010
231 Ba	Piedmont - Cuneo	*A. chinensis* – JinTao	2010
284 a	Piedmont - Cuneo	*A. chinensis* – JinTao	2010
216 a	Piedmont - Cuneo	*A. deliciosa* - Hayward	2010
228 Ba	Piedmont - Cuneo	*A. deliciosa* - Hayward	2010
242 a	Piedmont - Cuneo	*A. deliciosa* - Hayward	2010
291 a	Piedmont - Cuneo	*A. deliciosa* - Hayward	2010
229 a	Piedmont - Cuneo	*A. deliciosa* - Hayward	2010
309 a	Piedmont - Vercelli	*A. deliciosa* - Hayward	2010
313 a	Piedmont - Asti	*A. deliciosa* - Hayward	2010

sterile saline solution 0.85% which was centrifuged at 12,000 *g* for 2 min. Then, the supernatant was discarded and the pellet was suspended in 100 µl of NaOH 0.05 M to an optical density corresponding to 1-2×10^8 CFU/ml. The suspension was placed in water at 95°C for 15 min, centrifuged at 12,000 *g* for 2 min and the supernatant was stored at -20°C for repetitive-sequence PCR (rep-PCR) analysis. BOXA1R and ERIC primer sets were synthesized by Primm (Milano, Italy). The rep-PCR method used was that of Louws *et al.* (1994). PCR amplifications were performed in duplicate, amplicons were separated by gel electrophoresis on 1.5% agarose (Seakem, USA) in 0.5X TAE buffer at 5 V/cm for 5 h, stained with ethidium bromide, visualized and photographed with a Bio-Rad Gel Doc XR+ apparatus (Hercules, USA).

For MLST analysis, fragments of *gapA*, *gltA, gyrB* and *rpoD* genes were amplified from genomic DNA of *Psa* strains and extracted using the alkaline lysis method. Gene fragments were amplified and sequenced with primers described by Sarkar and Guttman (2004). PCR was carried out in a total volume of 25 µl containing 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% TritonX-100, pH 9), 50 pmol of each primer, 1.25 U GoTaq DNA Polymerase (Promega, USA), 0.2 mM of each dNTP (Promega, USA), 2 mM $MgCl_2$ and 50 ng DNA. All PCR reactions were performed in a BioRad thermal cycler at the following conditions: denaturation at 95°C for 5 min, 30 sec of annealing at 52°C, 72°C, 62°C and 76°C for *gapA*, *gltA, gyrB* and *rpoD,* respectively, extension at 72°C for 1 min for 35 cycles followed by a final extension at 72°C for 5 min. PCR products were custom sequenced by Primm (Milano, Italy).

Sequences were edited with Geneious 4.7.4 (http://www.geneious.com/) and aligned using ClustalW 1.83 (http://www.ebi.ac.uk/tools/clustalw2/). All ambiguous and terminal sequences were trimmed before data analysis. Each gene fragment was analysed independently. Analyses were performed also with the concatenated sequences. The Tajima's D genetic diversity and population divergence test (Tajima, 1989) and the Fu and Li's D tests for neutrality of mutations (Fu and Li, 1993) were estimated using the DnaSP software (Librado and Rozas, 2009) and applied to each gene fragments and, collectively, to *Psa* strains isolated from *A. deliciosa* and *A. chinensis*. To assess the clonality of the strains, the standardised index of association I_A was estimated using START2, a web tool available at http://www.pubmlst.org/, to test the null hypothesis of linkage equilibrium for multilocus data (Jolley *et al*., 2001). *P* values, calculated using both the parametric and the Monte Carlo methods, were used to discriminate the significance of I_A.

Table 2. Genetic variability of *Pseudomonas syringae* pv. *actinidiae* strains obtained with multilocus sequence typing.

Gene/Host	Length (bp)	% G+C	Base frequency A/C/G/T	% of polymorphic sites	Haplotype diversity (Hd)
gapA	634	60,60	0,216/0,342/0,264/0,178	0,79	0,270
gltA	556	58,00	0,225/0,332/0,248/0,195	0,72	0,373
gyrB	610	54,70	0,249/0,275/0,272/0,204	1,80	0,849
rpoD	521	59,40	0,210/0,301/0,293/0,196	0,38	0,320
Concatenated	2321	58,20	0,225/0,313/0,269/0,193	0,95	0,910
A. chinensis	2321	58,20	0,225/0,313/0,269/0,193	0,60	0,922
A. deliciosa	2321	58,20	0,225/0,313/0,269/0,193	0,09	0,700

Rep-PCR fingerprint performed with BOX and ERIC primer sets showed that the *Psa* strains currently causing severe epidemics of bacterial canker in northern (Piedmont, Veneto and Emilia-Romagna) and central Italy (Latium) are all identical or very similar among them. In fact, this typing could not discriminate the strains either when coming from *A. chinensis* or *A. deliciosa* or when the year or the areas of isolation of the strains were compared (Fig. 1 and 2). In fact, both primers yielded highly similar patterns for the strains tested. MLST provided 2.3 kb of gene sequences with which the assessment of gene polymorphism, the selection and linkage disequilibrium tests were performed. A low genetic variability among *Psa* strains was observed also following assessment of gene polymorphism. In fact, the percentage of the polymorphic sites among the genes varied from 0.38

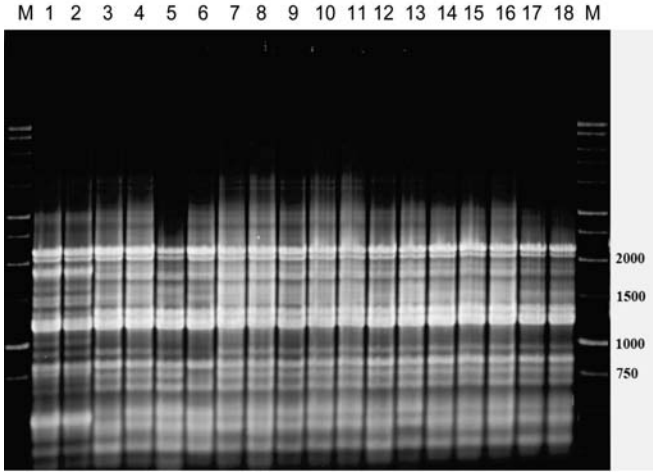

Fig. 1. Representative repetitive BOX-PCR fingerprinting patterns from genomic DNA of *Pseudomonas syringae* pv. *actinidiae* strains obtained from *Actinidia chinensis* and *A. deliciosa* in different areas of Italy during epidemics of 2008-2010 compared with strains of the same pathogen isolated in Italy in 1992. M: molecular size marker (1 kb DNA ladder; Promega, Madison, WI, USA); the size of the bands are indicated in base pairs. Lane 1: NCPPB 3871 (*A. deliciosa*); lane 2: NCPPB 3873 (*A. deliciosa*); lane 3: CRA-FRU 10.22 (Latina, 2008, *A. chinensis*); lane 4: 4251.A.1 (Ravenna, 2008, *A. chinensis*); lane 5: 4649.1 (Ravenna, 2008, *A. chinensis*); lane 6: CRA-FRU 10.25 (Latina, 2009, *A. chinensis*); lane 7: CRA-FRU 10.24 (Latina, 2009, *A. deliciosa*); lane 8: CRA-FRU 11.50 (Viterbo, 2010, *A. deliciosa*); lane 9: CRA-FRU 11.48 (Viterbo, 2010, *A. deliciosa*); lane 10: CRA-FRU 11.47 (Viterbo, 2010, *A. chinensis*); lane 11: TV4175 (Treviso, 2010, *A. chinensis*); lane 12: CRA-FRU 12.52 (Roma, 2010, *A. deliciosa*); lane 13: CRA-FRU 11.41 (Roma, 2010, *A. deliciosa*); lane 14: CRA-FRU 12.53 (Latina, 2010, *A. chinensis*); lane 15: CRA-FRU 309a (Vercelli, 2010, *A. chinensis*); lane 16: 231a (Cuneo, 2010, *A. chinensis*); lane 17: 228b (Cuneo, 2010, *A. deliciosa*); lane 18: 229a (Cuneo, 2010, *A. deliciosa*).

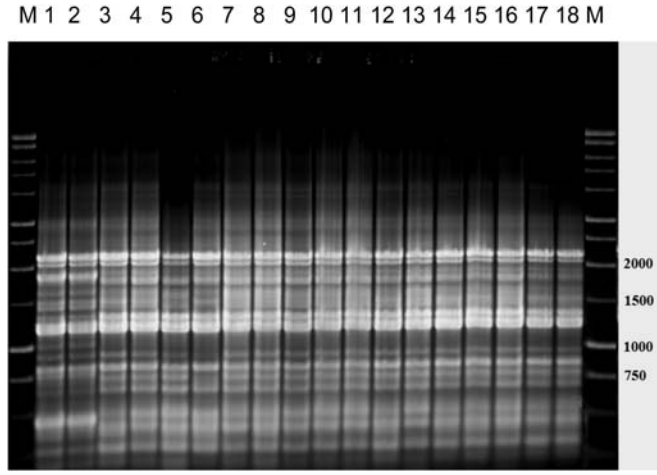

Fig. 2. Representative repetitive ERIC-PCR fingerprinting patterns from genomic DNA of *Pseudomonas syringae* pv. *actinidiae* strains obtained from *Actinidia chinensis* and *A. deliciosa* in different areas of Italy during epidemics of 2008-2010 compared with strains of the same pathogen isolated in Italy in 1992. M: molecular size marker (1 kb DNA ladder; Promega, Madison, WI, USA); the size of the bands are indicated in base pairs. Lane 1: NCPPB 3871 (*A. deliciosa*); lane 2: NCPPB 3873 (*A. deliciosa*); lane 3: CRA-FRU 10.22 (Latina, 2008, *A. chinensis*); lane 4: 4251.A.1 (Ravenna, 2008, *A. chinensis*); lane 5: 4649.1 (Ravenna, 2008, *A. chinensis*); lane 6: CRA-FRU 10.25 (Latina, 2009, *A. chinensis*); lane 7: CRA-FRU 10.24 (Latina, 2009, *A. deliciosa*); lane 8: CRA-FRU 11.50 (Viterbo, 2010, *A. deliciosa*); lane 9: CRA-FRU 11.48 (Viterbo, 2010, *A. deliciosa*); lane 10: CRA-FRU 11.47 (Viterbo, 2010, *A. chinensis*); lane 11: TV4175 (Treviso, 2010, *A. chinensis*); lane 12: CRA-FRU 12.52 (Roma, 2010, *A. deliciosa*); lane 13: CRA-FRU 11.41 (Roma, 2010, *A. deliciosa*); lane 14: CRA-FRU 12.53 (Latina, 2010, *A. chinensis*); lane 15: CRA-FRU 309a (Vercelli, 2010, *A. chinensis*); lane 16: 231a (Cuneo, 2010, *A. chinensis*); lane 17: 228b (Cuneo, 2010, *A. deliciosa*); lane 18: 229a (Cuneo, 2010, *A. deliciosa*).

Table 3. Selection tests performed with *Pseudomonas syringae* pv. *actinidiae* strains upon multilocus sequence typing.

Gene/host	Tajima Test	*p* value	Fu and Li Test	*p* value
gapA	-1,5710	0,0500	-1,4400	0,1637
gltA	-0,7040	0,2760	-0,9340	0,3020
gyrB	-1,5063	0,2010	-0,8701	0,1070
rpoD	-0,2937	0,4440	-0,8154	0,4750
Concatenated	-1,1545	0,1250	-1,1609	0,1320
A. chinensis	-0,6511	0,2770	-0,2398	0,4460
A. deliciosa	-0,9726	0,4450	-0,9726	0,4740

to 1.80% (Table 2). The selection tests applied to the four genes and to the concatenated data revealed that the *Psa* strains are currently under neutral selection. In fact, both the Tajima and the Fu and Li values were significantly negative. The same results were obtained when the tests were applied taking into account the host plants from where the strains originated (Table 3). The index of association I_A, was always significantly higher than 0 (i.e. 0.21) when tested with the classical approach *sensu* Maynard-Smith *et al.* (1993), indicating linkage disequilibrium and a clonal structure for these strains.

Taken together, the data obtained from rep-PCR and MLST gene polymorphism strongly indicated that all *Psa* strains isolated in Italy from *A. chinensis* and *A. deliciosa* during 2008-2010 are highly similar, thus confirming and widening previous assessments of the strains isolated from Latium (Ferrante and Scortichini, 2010). Such *Psa* population(s) is (are) diverse from the others inciting severe damages to *A. deliciosa* in Japan and South Korea (Koh *et al*., 2003) as well as from the population found in Latium in 1992.

The *Psa* strains currently causing outbreaks of bacterial canker in Italy have a clonal structure. This indicates that they originated, most probably, from a single or very few introductions of latently infected kiwifruit propagative material, even though it cannot be ruled out the possibility that cells of the pathogen already present in Italy might have mutated. The clonality of the pathogen can be explained by the recent and very rapid dissemination of a highly virulent population of *Psa* capable of colonizing and infecting both *A. chinensis* and *A. deliciosa* in the most important areas of kiwifruit cultivation in Italy. Moreover, such a population is displaying neutral selection, which indicated that it has currently reached an adaptive equilibrium (i.e. capability of colonization and infection) within its ecological niches (i.e. *A. chinensis* and *A. deliciosa*).

Finally, the *Psa* strains NCPPB 3871 and NCPPB 3873, isolated in Italy in 1992 (Scortichini, 1994), were claimed to not belong to this pathovar (Mazzaglia *et al*., 2010; Rees-George *et al.*, 2010). However, in our hands as well as in those or other scientists (U. Mazzucchi, P. Minardi and E. Stefani, personal communication) identification of both NCPPB cultures resulted in genuine *Psa*.

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