

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Pseudomonas syringae pv. actinidiae isolated from Actinidia chinensis Var. deliciosa in Northern Italy: genetic diversity and virulence

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1651509> since 2018-03-19T23:00:54Z

Published version:

DOI:10.1007/s10658-017-1267-9

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 ***Pseudomonas syringae* pv. *actinidiae* isolated from *Actinidia chinensis* var. *deliciosa* in**
2 **Northern Italy: genetic diversity and virulence**

3
4 Simona Prencipe^{1,2}, Maria Lodovica Gullino^{1,2}, Davide Spadaro^{1,2*}

5
6 ¹ DISAFA – Dipartimento di Scienze Agrarie, Forestali ed Alimentari, Università degli Studi di
7 Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy.

8 ² Centro di Competenza per l'Innovazione in campo agro-ambientale (AGROINNOVA), Università
9 degli Studi di Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy.

10

11 **Abstract**

12 Bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae*, is responsible for
13 significant economic losses, both in yield and quality. A collection of *P.s.* pv. *actinidiae* isolated in
14 Northern and Central Italy, during the first severe outbreak (2008-2010) and a few years afterwards
15 (2014), when the pathogen became endemic and established, was gathered. The genetic diversity
16 was evaluated with rep-PCR, RAPD-PCR, and MLSA of six housekeeping and effector genes. On
17 the same strains, the virulence was evaluated *in vitro* and *in vivo*, showing a higher disease index
18 for the strains isolated in 2014, compared with the strains of 2010. The molecular fingerprinting,
19 obtained by rep and RAPD analysis, revealed a high level of variability in the population of strains
20 of *P.s.* pv. *actinidiae* from Northern Italy. All the parameters considered – *Na*, *Ne*, *H*, *I*,
21 polymorphic loci, and AMOVA – showed a higher genetic diversity within the population of
22 Northern Italy isolated in 2014, compared to the older population. The study of the genetic diversity
23 and virulence permitted to show an increase of virulence and genetic diversity of the strains. The
24 fast and dramatic epidemics caused by *P. s.* pv. *actinidiae* could be an interesting model to study the
25 changes in the genetic diversity and virulence of a bacterial pathogen.

26

27 **Keywords:** bacterial canker, kiwifruit, pathogenicity, MLSA, rep-PCR, RAPD-PCR

28

29

30

31

32

33

34 *e-mail: davide.spadaro@unito.it

35 **Introduction**

36 The causal agent of bacterial canker on kiwifruit, *Pseudomonas syringae* pv. *actinidiae*, was first
37 isolated and described in Japan in 1984 (Takikawa *et al.*, 1989). In Italy, the first severe outbreak
38 occurred in 2008 (Balestra *et al.*, 2009). As reported by the European and Mediterranean Plant
39 Protection Organization (EPPO), the disease spread worldwide reaching France, Spain, Portugal,
40 Switzerland, New Zealand, Chile, Turkey and South Korea (EPPO, 2012). Bacterial canker causes
41 significant economic losses, both in yield at harvest and in reducing the postharvest quality, shelf
42 life, and susceptibility to postharvest rots (Prencipe *et al.*, 2016). Italy produces more kiwifruit than
43 any other country, apart from China, with 384,000 tons and 24,800 cultivated hectares (FAOSTAT,
44 2014). Currently, there are not chemical or biological products on the market able to completely
45 control *P. s.* pv. *actinidiae* on kiwifruit, but in the framework of integrated control strategies, copper
46 compounds alternated with resistance inducers could be used to develop new strategies to reduce
47 the disease development and spread (Monchiero *et al.*, 2015).

48 Different populations of *P. s.* pv. *actinidiae* have been described: Psa 1 or biovar 1, with the strains
49 responsible for the first occurrence of kiwifruit bacterial disease in Japan and Italy, producing
50 phaseolotoxin; Psa 2 or biovar 2, including the Korean population of Psa isolated in 1990,
51 producing coronatine; Psa 3 or biovar 3, grouped strains isolated in Europe, China, Chile and New
52 Zealand responsible for the current pandemic disease, which do not produce phaseolotoxin and
53 coronatine; Psa 4 or biovar 4 isolated in New Zealand, Australia and France, which do not produce
54 phaseolotoxin and coronatine (Cunty *et al.*, 2015). During 2010-2014, around 1,922 hectares of
55 kiwifruit were removed in the different Italian regions, due to the attack of the highly virulent
56 biovar 3.

57 The characterization of the population structure of the pathogen has been performed using
58 biological characteristics, as pathogenicity on *Actinidia* spp., and several molecular approaches,
59 including repetitive sequence PCR fingerprinting, with BOX and ERIC primers and multi-locus
60 Sequence Analysis (MLSA) of housekeeping genes (McCann *et al.*, 2013; Ferrante and Scortichini,
61 2015). Rep-PCR has been the most commonly used molecular method to analyse the diversity of
62 bacteria at subspecific level (Louws *et al.* 1994; Versalovic *et al.* 1994). On *P. s.* pv. *actinidiae*
63 MLSA of housekeeping genes revealed more variability than rep-PCR (Vanneste, 2013). Rep-PCR
64 and MLSA analyses are considered a good tool for biovar differentiation, but they are not able to
65 show the variability among strains of the same biovar (Scortichini *et al.*, 2012). Random amplified
66 polymorphic DNA (RAPD) was more resolute in discriminating intraspecific variability in bacterial
67 pathogens (Hartung *et al.*, 1993), also for *P.s.* pv. *actinidiae*, as reported by Mazzaglia *et al.* (2011).
68 Further information about genetic variability could be acquired through the analysis of the
69 sequences of effector genes involved in the Type III Secretion System (T3SS). The pathogenicity of

70 many Gram-negative bacteria, including *P.s. pv. actinidiae*, is linked to this system, that plays a
71 crucial role in the host-pathogen interaction. Genes encoding for this secretion system are called
72 *hrp/hrc* (hypersensitive response and pathogenicity, *hrp* conserved). T3SS is responsible to
73 translocate Avr and Hop (Hrp-dependent outer protein) effector proteins into host cells (Buttner *et*
74 *al.*, 2009).

75 The study of intra-pathovar diversity could help to understand the evolutionary process, at
76 subspecies level, that controls plant pathogen population, and to make inferences about adaptation
77 to the host and the environment (Burdon, 1993). After the first occurrence in 2010, few isolates of
78 the pathogen from Northern Italy have been investigated for their intra-pathovar variability
79 (Marcelletti and Scortichini, 2011).

80 The aim of this research was to investigate the genetic diversity and virulence variations of the
81 isolates of *P.s. pv. actinidiae* in the same geographical area at the time of the first epidemic
82 outbreak and four years afterwards, when the pathogen became endemic and established. The
83 identification was verified through a multidisciplinary approach, including molecular and host range
84 test. The intra-pathovar variability of *P.s. pv. actinidiae* was investigated in Northern Italy in 2010
85 and in 2014, with molecular typing using rep-PCR and RAPD-PCR. The pathogenicity and the
86 degree of virulence of the isolates were also evaluated. A MLSA of three effector genes *hopA1*,
87 *avrD1* and *hrpK1*, encoding three different T3SS secreted proteins, were chosen to investigate the
88 possible presence of polymorphic sites related to various functions of the T3SS.

89

90 **Materials and methods**

91

92 **Microorganisms**

93 Forty strains of *P.s. pv. actinidiae* were used throughout this study. Thirty-three strains were
94 obtained from *Actinidia chinensis* var. *deliciosa* ‘Hayward’ Liang and Ferguson cultivated in four
95 areas of Northern Italy (Piedmont region: provinces of Vercelli (VC), Cuneo (CN), Torino (TO) and
96 Asti (AT)), during two years: twelve were isolated in 2010 and twenty-one in 2014. Five strains
97 from other Italian regions were also included: one isolated from *A. chinensis* var. *deliciosa*
98 ‘Hayward’ and one from *A. chinensis* ‘Hort16A’ (the reference strain of biovar 3 CFBP7286) in
99 Latina province (2008), one from *A. chinensis* ‘Hort16A’ in Rome province (2008), and two from
100 *A. chinensis* ‘Jin Tao’ and *A. chinensis* var. *deliciosa* ‘Hayward’ in Viterbo province (2010). Two
101 reference strains belonging to biovars 1 and 2 were also included: one isolated from Japan in 1984,
102 and one from Korea (Table 1).

103

104

105 **Isolation and pathovar identification**

106 *Isolation and identification.* Kiwifruit leaves with symptoms of bacterial canker, i.e. necrotic spots,
107 were washed in sterile water and macerated in Luria Bertani (LB) broth (Merck). The resulting
108 medium was spread on Pseudomonas Selective Agar plates with CFC supplement (Merck). After 48
109 hours incubation at 27°C, single Psa-like colonies were transferred to LB broth and grown on a
110 rotary shaker (120 rpm) at 27°C for 24 hours. DNA was extracted from 100 µl liquid medium using
111 Instagene Matrix (Biorad), according to manufacturer's instructions. PCR was carried out in a total
112 volume of 25 µl containing: 2.5 µl Buffer 10X, 0.5 µl MgCl₂, 0.75 µl dNTPs (10 mM), 1 µl each
113 primer (10 mM), 0.2 µl Taq DNA polymerase (Qiagen), and 40 ng template DNA.

114 *P.s. pv. actinidiae* strains were previously identified, through PCR technique described by Rees-
115 George *et al.* (2010) with primers PsaF1, PsaR2 and PsaF3 and PsaR4 and a further amplification of
116 the 16S rRNA region using primers 16F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and 16R1525
117 (5'-AAGGAGGTGATCCAGCCGCA-3'). The obtained amplicons were run on a 1% agarose gel
118 and compared with positive controls. The sequences obtained were compared with those deposited
119 in GenBank using BLAST programme. To validate the pathovar assignation, the analysis of rep-
120 PCR fingerprinting using BOX and REP primer set following Versalovic *et al.* (1994) and Louws *et*
121 *al.* (1994) and the duplex-PCR technique following Gallelli *et al.* (2011), were applied.

122
123 *Host range test.* A pathogenicity test was performed on lemon (*Citrus lemon*) to distinguish
124 between the pathovars *syringae* and *actinidiae* of *P. syringae* (Andolfi *et al.*, 2014). Fruits were
125 disinfected with sodium hypochlorite (1%) and rinsed with sterile distilled water. Ten µL of the
126 bacterial suspension was inoculated through sterile needle onto the wound. Inoculations with sterile
127 distilled water was used as negative controls. Fruits were kept at room temperature (20-23°C) and
128 pathogenicity was assessed 7 days after inoculation, according to Scortichini *et al.* (2003). The test
129 was carried out on two fruit per isolates and the result has occurred from ten sites each.

131 **Genetic diversity**

132 All the strains were studied by Random amplified polymorphic DNA PCR (RAPD-PCR) and
133 repetitive intergenic DNA sequence PCR (rep-PCR), in order to analyse the genetic diversity.
134 Furthermore, we investigated six genes through Multilocus Sequence Analysis (MLSA).

135
136 *Molecular fingerprinting using RAPD and rep-PCR.* Fingerprinting analysis on the isolates of *P.s.*
137 *pv. actinidiae* was performed by RAPD and rep-PCR using the primers listed in Table S1. RAPD-
138 PCR was carried out in a total volume of 25 µl containing: 2.5 µl Buffer 10 X, 0.5 µl MgCl₂, 0.75
139 µl dNTPs (10 mM), 1 µl each primer (10 mM), 0.2 µl of Taq DNA polymerase (Qiagen) and 20 ng

140 template DNA. PCR was performed with initial denaturation at 94°C for 1 min, followed by 40
141 cycles of 1 min at 94°C, 1 min at 36°C for annealing, and 2 min at 72°C for 1 min, with 7 min final
142 extension at 72°C. Rep-PCR with primer GTG₅ was performed following protocols by Versalovic
143 *et al.* (1994).

144 Fifteen µl of amplification products were separated by gel electrophoresis using TBE buffer with
145 2% agarose and 10 µl SYBRSafe® (Invitrogen) at 100 V/cm for 3 hours. Get Pilot 1 kb Plus Ladder
146 (100-10000 bp, Qiagen) was used as molecular marker. Gel profiles were visualized under UV
147 transilluminator using Quantity One program (BioRad Labs). Gel profiles were analysed to build up
148 a concatenated matrix. Cluster analysis was performed on the combined matrix of the fingerprinting
149 obtained from all the molecular markers. A dendrogram of similarity was obtained using an
150 unweight pair group method with arithmetic average (UPGMA) algorithm and Dice similarity index
151 (DC) with Past version 6.5. Branch robustness was evaluated using 1000 bootstrap replicates. Nei's
152 genetic diversity, effective number of alleles, percentage of polymorphic loci, and Shannon
153 information index were also calculated using GenAlex version 6.5 and PopGene version 1.32. The
154 genetic variation among the population was also evaluated by the analysis of molecular variance
155 (AMOVA) using GenAlex version 6.5. To analyse the relationships between results obtained from
156 fingerprinting analysis, a principal coordinates analysis (PCoA) was also performed using Past
157 version 6.5.

158

159 *Multilocus sequence analysis (MLSA)* On the same collection of strains, a MLSA was performed
160 using the primers listed in Table S2. Effector genes, *avrD1*, *hopA1* and *hrpK1* were chosen to allow
161 comparison of data obtained in previous studies about the population of Psa (Ferrante and
162 Scortichini, 2010; Vanneste *et al.*, 2011; Chapman *et al.*, 2012) and to evaluate the presence of
163 additional polymorphic sites. The housekeeping gene *cts* was sequenced to confirm the haplotype of
164 biovar 3. Furthermore, two housekeeping genes *pgi* and *gapA* genes were sequenced, following
165 Sarkar and Guttman (2004).

166 For the effector genes, the PCR programs were according to Ferrante *et al.* (2009) and Ferrante and
167 Scortichini (2010), but for *hrpK1* gene, the annealing temperature was modified to 67°C. For
168 housekeeping genes, PCR protocols were retrieved from Sarkar and Guttman (2004).

169 PCR products were separated by gel electrophoresis using TAE buffer with 1% agarose and 1.5µl
170 SYBRSafe® (Invitrogen) at 85 V/cm for 40 minutes. Get Pilot Wide Range Ladder (200-4500 bp,
171 Qiagen) and 100 bp Ladder (100-600 bp, Qiagen) were used to compare the expected size of bands.
172 Bands were visualized under UV transilluminator using Quantity One program (BioRad Labs). The
173 PCR products were purified using QIAquick® PCR purification Kit (Qiagen) and sequenced in both
174 directions by Macrogen Inc. (The Netherlands).

175 Forward and reverse sequences for each gene were used to create a consensus sequence, using DNA
176 Baser programme (Heracle Biosoft S.R.L., Romania), for multi-alignment using CLUSTALW
177 through MEGA version 6. After cutting the trimmed regions and manual correction, dataset of 380,
178 887, 550, 518, 681 and 571 bp were obtained, respectively, for the genes *hopAI*, *hrpKI*, *avrDI*, *cts*,
179 *gapA* and *pgi*.

180 Single nucleotide polymorphisms (SNPs) were assessed on the aligned sequences, and one strain for
181 each haplotype and gene was deposited in GenBank.

182 Phylogenetic and molecular evolutionary analysis were generated by Neighbor-Joining algorithm
183 using MEGA version 6, branch robustness was evaluated using 1000 bootstrap replicates. GenBank
184 sequences from the National Center for Biotechnology Information (NCBI) were used as
185 references.

186

187 **Pathogenicity tests**

188 The isolates from Northern Italy were tested for their pathogenicity through *in vitro* and *in vivo*
189 assays and compared to those obtained for reference strains of biovars 1, 2 and 3. Before the tests,
190 the strains were inoculated in healthy plants and reisolated from necrotic spots to avoid the
191 virulence loss. Statistical analyses on the pathogenicity trials were realized by using IBM SPSS
192 statistics version 21 for variance analysis (one-way analysis of variance) using Duncan test $P \leq$
193 0.05. To assess the reliability of the *in vitro* pathogenicity assay, the data were compared with the
194 results of the *in vivo* trials and analysed with Student's t-test at 95% confidence level and
195 correlation coefficient (R^2). A disease index scale for the *in vitro* and *in vivo* tests is reported in
196 Figure 1. The symptoms observed (leaf spotting, necrotic area and water-soaked area) for the
197 isolates from Northern Italy were those typical of biovar 3.

198

199 *In vitro pathogenicity assay* Bacterial suspension was prepared after 24 hours growth in LB broth at
200 27°C on a rotary shaker (120 rpm). For *in vitro* assay, leaf discs (2 cm diameter) were prepared
201 from fourteen days-old leaves of *A. chinensis* var. *deliciosa* 'Hayward' (1-year old plant) and were
202 placed on 10 ml sterile water in not sealed three sector Petri dishes. The leaf disks were inoculated
203 with bacterial suspension (10^8 CFU/ml) at three equidistant points (three drops of 30 μ l each per
204 leaf disc). Control leaves were prepared similarly with sterile deionized water.

205 Disease index (0-5) was assigned to the symptoms observed on the disks kept at 20°C in the dark
206 for 12 days, corresponding to: 0=healthy leaves; 1= small necrotic spots or streaks (1-4% infected
207 area); 2= necrotic spots or larger veins (5-10%); 3= spots or converging necrotic areas (11-30%); 4=
208 converging necrotic areas ($\geq 50\%$); 5= completely necrotic leaves. The experiment was carried out

209 in three replicates for each strain (9 leaf discs per strain were scored). The pathogenicity assay was
210 performed twice.

211

212 *In vivo pathogenicity assay* The assay was performed in greenhouse on 1 year-old plants of *A.*
213 *chinensis* var. *deliciosa* ‘Hayward’ inoculated by directly spraying a bacterial suspension (10^8
214 CFU/ml) of each strain of *P.s.* pv. *actinidiae* onto kiwifruit leaves. Potted plants (30–40 cm high)
215 were inoculated on 25 March, 2014 and 24 September 2014 and kept in the greenhouse at
216 approximately 20 °C, RH 70-80% with natural daylight. After pathogen inoculation in the evening,
217 plants were covered with plastic film for 72 hours. Control plants were prepared similarly but
218 inoculated with sterile deionized water.

219 A disease index (D.I.), ranging from 0 to 5, was assigned, by observing 6 leaves per plant, 12 days
220 after inoculation: 0= no symptoms; 1= 1-4% infected area; 2= 5-10% infected; 3= 11-30% infected
221 area; 4= $\geq 50\%$ infected area; 5= completely necrotic. The classes of infection, used to describe the
222 severity of symptoms, were four: not pathogenic strain (NP; D.I. 0.0-0.4), low virulent strain (LV;
223 D.I. 0.5-1.9); moderate virulent strain (MV; D.I. 2.0-2.9); virulent strain (V; D.I. 3.0-3.9); highly
224 virulent strain (HV; D.I. 4.0-5.0). The experiment was performed with three replicates for each
225 strain (18 leaves per strain). The pathogenicity assay was carried out twice.

226

227

228 **Results**

229

230 **Pathogen identification**

231 Thirty-three bacterial isolates from symptomatic leaves of kiwifruit cultivated in Northern Italy
232 confirmed to belong to *P.s.* pv. *actinidiae* by amplification of two expected bands of 280 and 175
233 bp, after PCR reaction with two primer pairs of Rees-George *et al.* (2010) and sequencing of 16S
234 region (two sequences were deposited in GenBank with accession numbers KP794939 and
235 KP794940). The pathovar assignment was validated through duplex PCR with the amplification of
236 two PCR product for all strains and, typical fingerprinting profile using rep-PCR with ERIC primer
237 (Fig. 2). By considering the host range test, the populations did not induce any disease symptom on
238 the inoculated lemon fruits tested. Control fruits did not show any disease symptoms.

239

240 **Molecular characterization**

241 The molecular fingerprinting of strains of *P.s.* pv. *actinidiae* generated a band profile reproducible
242 in replicated experiment. Rep-PCR through ERIC primer produced a profile showing an identical
243 molecular pattern for all the Italian strains analysed (Psa 3) and allowed the differentiation of the

244 Korean strain –K2 (Psa 2) and the Japanese KW11 (Psa 1). UPGMA dendrogram divided the two
245 biovars from biovar 3 (Fig. 2). Rep-PCR through GTG₅ primer revealed also variability within
246 biovar 3, as shown in supplementary Figure S1.

247 One example of RAPD-PCR using OPB13 primer is reported in Fig. 3. The concatenated matrix of
248 the fingerprinting of the strains of *P.s. pv. actinidiae* was used to generate a dataset, and a
249 dendrogram of similarity (Fig. 4) was obtained by using Dice coefficient and UPGMA clustering
250 algorithm. The co-phenetic correlation coefficient obtained was 0.9474, well supporting the
251 dendrogram resulting from matrix used. The dendrogram showed a cluster of the strains of *P.s. pv.*
252 *actinidiae* belonging to biovar 3, which was significantly separated from the two reference strains
253 of biovar 1 and biovar 2 (Fig. 4). The Japanese strain (biovar 1) and the Korean one (biovar 2) were
254 separated at a similarity level of 0.70 supported by a high bootstrap value (99) and separated from
255 Italian strains at similarity level 0.54 (bootstrap value 83). Among the Italian strains, all belonging
256 to the biovar 3, two main clusters, with a Dice coefficient (DC) of 0.46, supported by a high
257 bootstrap value (100), were generated: Cluster I included all the strains from Northern Italy, and
258 Cluster II included all the strains from Central Italy. Furthermore, the strains from Northern Italy
259 could be divided in two sub-clusters well supported by bootstrap values, according to the year of
260 isolation: sub-cluster Ia (bootstrap: 89), with all the strains isolated in 2014, and sub-cluster Ib
261 (bootstrap: 98), with all the strains isolated in 2010.

262 The high bootstrap value and the length of the branches, measured with the Dice coefficient (DC),
263 showed a great variability of the strains isolated in 2014. Sub-cluster Ia showed a DC ranging from
264 0.68 (between strains RC1 and RC2/RC4) to 0.90 (between strains RL1 and RL2), with a mean DC
265 in the sub-cluster of 0.79.

266 Within sub-cluster Ib, the strains of 2010 showed on average a higher similarity compared with the
267 strains of 2014. The DC ranged from 0.75 (between 38/10 and 36/10c1) to 0.92 (between 41/11 and
268 39/10), with a mean DC in the sub-cluster of 0.89.

269

270 **Genetic diversity and principal coordinates analysis**

271 Genetic diversity parameters of the three populations of *P.s. pv. actinidiae* are shown in Table 2.
272 The highest diversity was observed for the population of *P.s. pv. actinidiae* isolated from Northern
273 Italy in 2014, with the highest number of alleles ($N_e=1.38$), gene diversity ($H=0.23$), and Shannon
274 index ($I=0.35$), and 71.79% of polymorphic loci.

275 The genetic distance (Table S3) was 0.207 between the populations of Northern Italy of 2014 and
276 2010, 0.278 between the population of Northern Italy of 2014 and the Central Italy population, and
277 0.317 between the population of Northern Italy of 2010 and the Central Italy population. A high
278 variability of the three populations of *P.s. pv. actinidiae* was shown by the value of the total genetic

279 diversity ($H_T = 0.321$), while H_S within the population was 0.199. The G_{ST} value (35%) showed
280 the proportion of genetic diversity linked to the number of individuals of the population. The
281 AMOVA was used to analyse the population structure (Table S4), showing that 58% of the
282 variation was within the population, while 42% was among the populations.

283 The scatter diagram of the principal coordinate analysis (Fig. 5) showed three distinct groups, two
284 for the populations of *P. s. pv. actinidiae* from Northern Italy and one heterogeneous group
285 including the strains from Central Italy and the strains of biovar 1 and 2. The Japanese and the
286 Korean strain showed to be more clearly separated in the dendrogram (Fig.4). These strains were
287 grouped with strains of Central Italy because the PCoA analysis visualization reduces the dimension
288 of complex data matrix into a bidimensional space. The first coordinate explained 25.67% of the
289 total variability (eigenvalue 1.07), while the second one explained 24.83% (eigenvalue 0.87),
290 therefore the two coordinates could explain over half of the variance.

291

292 **Multilocus sequence analysis (MLSA)**

293 A DNA sequence dataset, with partial sequences of *cts*, *avrDI*, *hopAI* and *hrpKI* was built.
294 Phylogenetic analysis was performed for each gene sequence dataset. The partial gene sequences of
295 *pgi* and *gapA* were 100% identical for all the isolates considered (data not shown).

296 Figures S2a and S2b show two major clades for *cts* and *avrDI* genes. For *cts* gene, two clades are
297 shown (Figure S2a; bootstrap value: 88%): clade I, including all Italian isolates, with two cytosines
298 in position 251 and 431, and a clade II, including the Japanese and Korean isolates, with a thymine
299 in position 251 and an adenine in position 431. The same clustering, supported by 64% bootstrap
300 value, is shown for *avrDI* gene (Figure S2b), with all Italian isolates with two adenines in position
301 43 and 528, and the Japanese and Korean isolates, with a cytosine in position 43 and a guanine in
302 position 528.

303 Figures S3a and S3b show the Neighbour-Joining analysis of *hrpKI* and *hopAI* genes. Sequences
304 alignment showed very low sequence diversity, but six single nucleotide polymorphisms (SNPs)
305 occurred in four strains isolated in Northern Italy in 2014: two in *hopAI* and four in *hrpKI*. Among
306 the SNPs in *hopAI* and *hrpKI*, two resulted in amino acid variation, while four were silent (Table
307 3). Amino acid variation occurred from isoleucine (ATC) to phenylalanine (TTC) in strain PSA1 at
308 position 107 and from serine (AGC) to threonine (ACC) at position 82 for RL5 strain. For the
309 Japanese and Korean strains, *hopAI* gene was not amplified.

310

311

312

313

314 Pathogenicity tests

315 In both pathogenicity tests, one *in vitro* and another *in vivo*, the first symptoms, necrotic spots, were
316 observed six days after inoculation of *P.s. pv. actinidiae* on the leaves. Uninoculated control
317 remained healthy. -

318 By considering the results of the two Northern Italy populations *in vivo*, the strains of 2014 showed
319 a higher variability in the virulence (D.I. ranging from 0.59 to 3.93), compared to the strains of
320 2010 (D.I. ranging from 0.71 to 3.21). The strains with the highest virulence were five isolated in
321 2014 (QV2; D.I.: 3.93; QV3: 3.57; QV4: 3.09; RL4: 3.93; and PSA1: 3.21), and one isolate of 2010
322 (41/11: 3.21). The isolates were divided in three categories according to their virulence *in vivo*
323 (Figure S4) and the strains of 2014 confirmed on average a higher virulence: 24% were virulent
324 (V), 48% moderately virulent (MV), and 29% low virulent (LV). The strains of 2010, divided in the
325 same three categories, showed a different sharing: 9% virulent, 17% moderately virulent, and 75%
326 low virulent.

327 The correlation coefficient (R^2) between the two disease indexes was 0.87, showing a high
328 reliability of the *in vitro* test compared to the *in vivo* test. In addition, the Student's t-test applied to
329 both assays showed no significant differences ($P \geq 0.05$). In the *in vitro* test, the strains of *P.s. pv.*
330 *actinidiae* isolated in 2014 were significantly more virulent ($P \leq 0.05$), with a higher disease index
331 (average D.I.: 1.80; Table 1), than the strains isolated in 2010 (average D.I.: 1.18). The same
332 tendency was observed in the *in vivo* test (Table 1), where the strains of 2014 showed an average
333 D.I. of 2.27, higher compared to the average D.I. (1.57) of the strains of 2010 ($P \leq 0.05$).

334

335 Discussion

336 In a collection of *P.s. pv. actinidiae* strains isolated in two Italian regions, Piedmont (Northern
337 Italy) and Latium (Central Italy), during the first severe outbreak in 2010 and after four years
338 (2014), species, pathovar and biovar were assigned through different methods including rep-PCR
339 using BOX and ERIC primers and duplex PCR. The molecular fingerprinting, in accordance with
340 Ferrante and Scortichini (2010) and Mazzaglia *et al.* 2011, permitted to generate unique and
341 repeatable genetic patterns for the strains of *P.s. pv. actinidiae*, discriminating the Italian strains
342 (biovar 3) from the Asian strains (biovars 1 and 2). The analysis on Northern Italy strains showed
343 that the use of rep-PCR through ERIC produced the same pattern profile for all the strains of *P.s.*
344 *pv. actinidiae*, as already reported in the analysis of isolates from Latium and from other Italian
345 regions (Marcelletti and Scortichini, 2011).

346 The molecular fingerprinting, obtained by the concatenated matrix of rep-PCR through GTG₅
347 primer and RAPD PCR analysis, revealed a high level of variability in the population of strains of
348 *P.s. pv. actinidiae* from Northern Italy, according to Mazzaglia *et al.* (2011) for the analysis of Psa

349 Italian strains. These techniques enabled also to differentiate the population of Italian *P.s. pv.*
350 *actinidiae* both depending on geographical origin and year of isolation. In particular, strains from
351 Northern Italy clustered together (Cluster I), while the strains from Central Italy formed another
352 group (Cluster II). The effect of the geographical origin on the genetic diversity of the isolates was
353 already reported by several authors. Genetic diversity was also associated to the year of isolation
354 and the strains were divided in two major clades. This diversity could be related to the pressure of
355 different weather conditions at the collection site, which could influence the structure of bacterial
356 population (Scortichini, 2005; Kolliker *et al.*, 2006).

357 Furthermore, compared to the initial clonal population of 2008-2010 present in Italy, *P.s. pv.*
358 *actinidiae* isolated in 2014 showed a higher variability, also supported by a lower similarity index.
359 These results were also confirmed by the results from *Na*, *Ne*, *H*, *I* and polymorphic loci, that
360 attributed the highest diversity within population to the strains isolated in Northern Italy in 2014.
361 The AMOVA also showed the highest percentage of genetic variance within the population of
362 Northern Italy isolated in 2014. An intra-pathovar diversity was also demonstrated for other
363 bacterial pathogens, as reported by Picard *et al.* (2008) for *Xantomonas axonopodis pv. allii* and
364 Giovanardi *et al.* (2016) for *X. arboricala pv. pruni*.

365 The highest genetic identity was shown between the two populations of Northern Italy (0.813).
366 This identity could be attributed to the limited geographic area of isolation, as reported for other
367 *Pseudomonas* species (Sisto *et al.*, 2007).

368 Intra-pathovar variability was also shown for the virulence. Few papers considered the *in vivo*
369 virulence of the strains, mostly focusing on the different biovars of *P. syringae pv. actinidiae* (Koh
370 *et al.*, 2014). We demonstrated, through *in vivo* pathogenicity test, the occurrence of different levels
371 of virulence in both Northern Italy populations. A higher disease index was shown for strains
372 isolated in 2014, compared with strains of 2010. There was no correlation between the
373 fingerprinting clusters and the virulence groups (Figure S5), as already reported for other bacterial
374 pathovars (Picard *et al.*, 2008; Giovanardi *et al.*, 2015).

375 Moreover, a new *in vitro* test was developed. The time after inoculation was optimized and the
376 highest correlation with *in vivo* test was found at 12 days after inoculation. The *in vitro* test could be
377 used for virulence evaluation to speed up the *in vivo* virulence assessment, showing a good
378 correlation coefficient.

379 A MLSA of some effector genes belonging to the T3SS, a conserved secretion system involved in
380 pathogenicity (Lindeberg *et al.*, 2012), was performed to evaluate if the variability in virulence
381 observed could be associated with mutations in these genes. The analysis revealed only four SNPs
382 for the strains of Psa isolated in 2014, while for the strains of 2010 no mutations were observed.

383 Among the SNPs, only two resulted in different amino acidic sequence. Thus, no positive
384 correlation was found between SNPs and virulence levels.

385 The mutations found in the genes of virulence could be related to the process of genetic
386 differentiation, by considering that these classes of genes are evolving at a faster rate than the
387 genome as a whole (Remenant *et al.*, 2010). The few SNPs found in the effector genes are not
388 sufficient to justify the different levels of virulence, as the pathogenicity in *P.s. pv. actinidiae* is
389 controlled by several effector genes (Marcelletti *et al.*, 2011). By comparing the whole genome of
390 two strains of *P. aeruginosa* with different virulence, Lee *et al.* (2006) found that the pathogenicity
391 is the result of a large pool of pathogenicity-related genes.

392 In conclusion, our study confirms that the biovar can be differentiated generating unique
393 fingerprinting patterns by rep-PCR. RAPD technique showed an increased level of resolution in
394 evaluating the genetic diversity within the biovar, at least with the set of primers used in this study.
395 Through this multidisciplinary approach, we can state that a significant diversity occurs within the
396 strains of *P.s. pv. actinidiae* from Northern Italy, both in genetics and in virulence. The study of the
397 genetic diversity and virulence of the isolates of *P.s. pv. actinidiae* in the same geographical area at
398 the first epidemic outbreak and four years afterwards, when the pathogen became endemic and
399 established, highlighted an increase of average virulence of the strains and a higher level of genetic
400 diversity. All the parameters considered – *Na*, *Ne*, *H*, *I*, polymorphic loci, and AMOVA – showed a
401 higher genetic diversity within the population of Northern Italy isolated in 2014, compared to the
402 older population. This constitutes a proof to the hypothesis that the initial epidemics started from a
403 uniform population (Shapiro, 2016).

404 As previously demonstrated, *P. s. pv. actinidiae* has an overall clonal population structure, but the
405 genomes carry a marked signature of recombination within the pathovar (McCann *et al.*, 2013). *P.*
406 *s. pv. actinidiae* has a high multiplication rate (Choi *et al.*, 2014), that permits to rapidly introduce
407 polymorphisms, confirmed also by the SNPs found in the effector genes, and to modify the genetic
408 structure of the population in a relatively short time span (Schuenzel *et al.*, 2005).

409 The fast and dramatic epidemics caused by *P. s. pv. actinidiae* could become an interesting model
410 to study the changes of pathogen genetic diversity, but further investigation through genome
411 sequencing and comparative genome analysis could clarify the higher intraspecific variability
412 within the same biovar.

413

414 **Acknowledgments**

415 The Authors wish to thank the financial support of Piedmont Region in the framework of the project
416 “PRO.ACT.IN.” (PSR FEASR 2007/2013, European Fund for Rural Development, Measure 124,
417 Action 1). The Authors gratefully acknowledge Dr. Luca Nari and Dr. Graziano Vittone from

418 Agrion, and the growers for their support in sampling, Dr. Giorgio Balestra (Università della
419 Tuscia) for providing the Central Italy and Asian strains, and Dr. Fabiano Sillo (University of
420 Turin) for the support in molecular data analysis.

421

422 **Compliance with Ethical Standards**

- 423 • There are no potential conflicts of interest.
- 424 • The research does not involve human participants nor animals.
- 425 • The research does not involve informed consent.

426

427

428 **References**

429

430 Andolfi, A, Ferrante, P, Petriccione, M, Cimmino, A, Evidente, A, & Scortichini, M. (2014).
431 Production of phytotoxic metabolites by *Pseudomonas syringae* pv. *actinidiae*, the causal
432 agent of bacterial canker of kiwifruit. *Journal of Plant Pathology*, *96*, 169-176.

433 Balestra, G.M., Mazzaglia, A., Quattrucci, A., Renzi, M., & Rossetti, A. (2009). Occurrence of
434 *Pseudomonas syringae* pv. *actinidiae* in Jin Tao kiwi plants in Italy. *Phytopathologia*
435 *Mediterranea*, *48*, 299-301.

436 Burdon, J.J. (1993). Genetic variation in pathogen populations and its implications for adaptation to
437 host resistance. In T. Jacobs and J. E. Parlevliet (Eds.), *Durability of disease resistance* pp.
438 41–56. Dordrecht: Kluwer.

439 Buttner, D., & Sheng Yang, H. (2009). Type III Protein Secretion in Plant Pathogenic Bacteria.
440 *Plant Physiology*, *150*, 1656–1664.

441 Chapman, J.R., Taylor, R.K., Weir, B.S., Romberg, M.K., Vanneste, J.L., Luck, J., & Alexander,
442 B.J.R. (2012). Phylogenetic relationships among global populations of *Pseudomonas*
443 *syringae* pv. *actinidiae*. *Phytopathology*, *102*, 1034-1044.

444 Choi, E.J., Lee, Y.S., Kim, G.H., Koh, Y.J., & Jung, J.S. (2014). Phenotypic characteristics of
445 *Pseudomonas syringae* pv. *actinidiae* strains from different geographic origins. *Korean*
446 *Journal of Microbiology*, *50*, 245-248.

447 Cuntly, A., Poliakoff, F., Rivoal, C., Cesbron, S., Fischer-Le Saux, M., & Lemaire C. (2015).
448 Characterization of *Pseudomonas syringae* pv. *actinidiae* (psa) isolated from France and
449 assignment of psa biovar 4 to a de novo pathovar: *Pseudomonas syringae* pv. *actinidifoliorum*
450 pv. nov. *Plant Pathology*, *64*, 582–596.

451 EPPO, (2012). *Pseudomonas syringae* pv. *actinidiae*. Bacterial canker of kiwifruit. Available
452 [http://www.eppo.int/QUARANTINE/Alert_List/bacteria/P_syringae_pv_actinidiae.htm].
453 Accessed on December 13, 2016.

454 FAOSTAT, 2014. [<http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QC/E>]. Accessed on May
455 4, 2017.

456 Ferrante, P., Clarke, C.R., Cavanaugh, C.A., Michelmore, R.W., Buonauro, R., & Vinatzer, B.
457 (2009). Contributions of the effector gene hopQ1-1 to differences in host range between
458 *Pseudomonas syringae* pv. *phaseolicola* and *P. syringae* pv. *tabaci*. *Molecular Plant*
459 *Pathology*, *10*, 837–842.

460 Ferrante, P., & Scortichini, M. (2010). Molecular and phenotypic features of *Pseudomonas*
461 *syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow
462 kiwifruit (*Actinidia chinensis*) in central Italy. *Plant Pathology*, *59*, 954–962.

- 463 Ferrante, P., & Scortichini, M. (2015). Redefining the global populations of *Pseudomonas syringae*
464 pv. *actinidiae* based on pathogenic, molecular and phenotypic characteristics. *Plant*
465 *Pathology*, *64*, 51–62.
- 466 Gallelli, A., L'Aurora, A., & Loreti, S. (2011). Gene sequence analysis for the molecular detection
467 of *Pseudomonas syringae* pv. *actinidiae*: developing diagnostic protocols. *Journal of Plant*
468 *Pathology*, *93*, 425–435.
- 469 Giovanardi, D., Bonneau, S., Gironde, S., Fischer-Le Saux, M., Manceau, C., & Stefani, E. (2015).
470 Morphological and genotypic features of *Xanthomonas arboricola* pv. *juglandis* populations
471 from walnut groves in Romagna region, Italy. *European Journal of Plant Pathology*, *145*, 1–
472 16.
- 473 Giovanardi, D., Dallai, D., & Stefani, E. (2016). Population features of *Xanthomonas arboricola*
474 pv. *pruni* from *Prunus* spp. orchards in northern Italy. *European Journal of Plant Pathology*,
475 <http://dx.doi.org/10.1007/s10658-016-1040-5>.
- 476 Hartung, J.S., Daniel, J.F., & Pruvost, O.P. (1993). Detection of *Xanthomonas campestris* pv. *citri*
477 by the polymerase chain reaction method. *Applied and Environmental Microbiology*, *59*,
478 1143-1148.
- 479 Koh, H.S., Kim, G.H., Lee, Y.S., Koh, Y.J., & Jung, J.S. (2014). Molecular Characteristics of
480 *Pseudomonas syringae* pv. *actinidiae* strains isolated in Korea and a multiplex PCR assay for
481 haplotype differentiation. *The Plant Pathology Journal*, *30*, 96–101.
- 482 Kolliker, R., Kraehenbuehl, R., Boller, B., & Widmer, F. (2006). Genetic diversity and
483 pathogenicity of the grass pathogen *Xanthomonas translucens* pv. *graminis*. *Systematic and*
484 *Applied Microbiology*, *29*, 109–119.
- 485 Lee, D.G., Urbach, J.M., Wu, G., Liberati, N.T., Feinbaum, R.L., Miyata, S., & Ausubel, F.M.
486 (2006). Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial.
487 *Genome Biology*, *7*, R90.
- 488 Lindeberg, M., Cunnac, S., & Collmer, A. (2012). *Pseudomonas syringae* type III effector
489 repertoires: last words in endless arguments. *Trends in Microbiology*, *20*, 199-208.
- 490 Louws, F.J., Fulbright, D.W., Stephems, C.T., & De Bruijn, F.J. (1994). Specific genomic
491 fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains
492 generated with repetitive sequence and PCR. *Applied and Environmental Microbiology*, *60*,
493 2286–2295.
- 494 Marcelletti, S., Ferrante, P., Petriccione, M., Firrao, G., & Scortichini, M. (2011). *Pseudomonas*
495 *syringae* pv. *actinidiae* draft genomes comparison reveal strain-specific features involved in
496 adaptation and virulence to *Actinidia* species. *PLoS ONE*, *6*, e27297.

- 497 Marcelletti, S., & Scortichini, M. (2011). Clonal outbreaks of bacterial canker caused by
498 *Pseudomonas syringae* pv. *actinidiae* on *Actinidia chinensis* and *A. deliciosa* in Italy.
499 *Journal of Plant Pathology*, *93*, 479-483.
- 500 Mazzaglia, A., Renzi, M., & Balestra, G.M. (2011). Comparison and utilization of different PCR-
501 based approaches for molecular typing of *Pseudomonas syringae* pv. *actinidiae* strains from
502 Italy. *Canadian Journal of Plant Pathology* *33*, 8–18.
- 503 McCann, H.C., Bertels, F., Erik, H., Rikkerink, A., Fiers, M., Lu, A., Rees-George, J., Andersen,
504 M.T., Gleave, A.P., Haubold, B., Wohlers, M.W., Guttman, D.S., Wang, P.W., Vanneste, J.,
505 Rainey, P.B., & Templeton, M.D. (2013). Genomic analysis of the kiwifruit pathogen
506 *Pseudomonas syringae* pv. *actinidiae* provides insight into the origins of an emergent plant
507 disease. *PLoS Pathogens*, *9*, e1003503.
- 508 Monchiero, M., Gullino, M.L., Pugliese, M., Spadaro, D., & Garibaldi, A. (2015). Efficacy of
509 different chemical and biological products in the control of *Pseudomonas syringae* pv.
510 *actinidiae* on kiwifruit. *Australasian Plant Pathology*, *44*, 13-23.
- 511 Picard, Y., Roumagnac, P., Legrand, D., Humeau, L., Robène-Soustrade, I., Chiroleu, F., Gagnevin,
512 L., & Pruvost, O. (2008). Polyphasic characterization of *Xanthomonas axonopodis allii*
513 associated with outbreaks of bacterial blight on three *Allium* species in the Mascarene
514 archipelago. *Phytopathology*, *98*, 919-925.
- 515 Prencipe, S., Nari, L., Vittone, G., Gullino, M.L., & Spadaro, D. (2016). Effect of bacterial canker
516 caused by *Pseudomonas syringae* pv. *actinidiae* on postharvest quality and rots of kiwifruit
517 ‘Hayward’. *Postharvest Biology and Technology*, *113*, 119-124.
- 518 Rees-George, J., Vanneste, J.L., Cornish, D.A., Pushparajah, I.P.S., Yu, J., Templeton, M.D., &
519 Everett, K.R. (2010). Detection of *Pseudomonas syringae* pv. *actinidiae* using polymerase
520 chain reaction (PCR) primers based on the 16S–23S rDNA intertranscribed spacer region and
521 comparison with PCR primers based on other gene regions. *Plant Pathology*, *59*, 453-464.
- 522 Remenant, B., Coupat-Goutaland, B., Guidot, A., Cellier, G., Wicker, E., Allen, C., Fegan, M.,
523 Pruvost, O., Elbaz, M., Calteau, A., Salvignol, G., Mornico, D., Mangenot, S., Barbe, V.,
524 Médigue, C., & Prior, P. (2010). Genomes of three tomato pathogens within the *Ralstonia*
525 *solanacearum* species complex reveal significant evolutionary divergence. *BMC Genomics*,
526 *11*, 379.
- 527 Sarkar, S.F., & Guttman, D.S. (2004). Evolution of the core genome of *Pseudomonas syringae*, a
528 highly clonal, endemic plant pathogen. *Applied and Environmental Microbiology*, *70*, 1999-
529 2010.

- 530 Schuenzel, E.L., Scally, M., Stouthamer, R., & Nunney, L. (2005). A multigene phylogenetic study
531 of clonal diversity and divergence in North American strains of the plant pathogen *Xylella*
532 *fastidiosa*. *Applied and Environmental Microbiology*, *71*, 3832-3829.
- 533 Scortichini, M. (2005). The population structure of some plant pathogenic bacteria: An ecological
534 and adaptive perspective. *Journal of Plant Pathology*, *87*, 5-12.
- 535 Scortichini, M., Marchesi, U., Dettori, M.T., & Rossi, M.P. (2003). Genetic diversity, presence of
536 the syrB gene, host preference and virulence of *Pseudomonas syringae* pv. *syringae* strains
537 from woody and herbaceous host plants. *Plant Pathology* *52*, 277–286.
- 538 Scortichini, M., Marcelletti, S., Ferrante, P., Petriccione, M., & Firrao, G. (2012). *Pseudomonas*
539 *syringae* pv. *actinidiae*: a re-emerging, multi-faceted, pandemic pathogen. *Molecular Plant*
540 *Pathology*, *13*, 631-640.
- 541 Shapiro, B.J. (2016). How clonal are bacteria over time? *Current Opinion in Microbiology*, *31*,
542 116-123.
- 543 Sisto, A., Cipriani, M.G., Tegli, S., Cerboneschi, M., Stea, G., & Santtili, E. (2007). Genetic
544 characterization by fluorescent AFLP of *Pseudomonas savastanoi* pv. *savastanoi* strains
545 isolated from different host species. *Plant Pathology*, *56*, 366–372.
- 546 Takikawa, Y., Serizawa, S., Ichikawa, T., Tsuyumu, S., & Goto, M. (1989). *Pseudomonas syringae*
547 pv. *actinidiae* pv. nov.: the causal bacterium of canker of kiwifruit in Japan. *Annals of the*
548 *Phytopathological Society of Japan*, *55*, 437–444.
- 549 Vanneste, J.L., Cornish, D.A., Yu, J., Audusseau Paillard, S., Rivoal, C., Poliakoff, F. (2011).
550 Presence of the effector gene hopAl in strains of *Pseudomonas syringae* pv. *actinidiae*
551 isolated from France and Italy. *New Zealand Plant Protection*, *64*, 252–8.
- 552 Vanneste, J.L. (2013). Recent progress on detecting, understanding and controlling *Pseudomonas*
553 *syringae* pv. *actinidiae*: a short review. *New Zealand Journal of Plant Protection*, *66*, 170-
554 177.
- 555 Versalovic, J., Schneider, M., De Bruijn, F.J., & Lupski, J.R. (1994). Genomic fingerprinting of
556 bacteria using repetitive sequence based polymerase chain reaction. *Methods in Molecular*
557 *Cell Biology*, *5*, 25–40.

Table 1 – Strain name, cultivar, geographical location, year of isolation and results of the pathogenicity assay *in vitro* and *in vivo* for the *P. s. pv. actinidiae* strains used in this study.

Strain	Cultivar	Geographical location*	Year	Pathogenicity assay						
				<i>in vitro</i> D.I.			<i>in vivo</i> D.I.			Class infection
				Mean	±SD		Mean	±SD		
RC1	Hayward	Italy - Piedmont (TO)	2014	1.50	± 0.55	ae**	2.14	± 1.11	be**	MV***
RC2	Hayward	Italy - Piedmont (TO)	2014	1.67	± 0.52	af	2.02	± 1.14	ad	MV
RC4	Hayward	Italy - Piedmont (TO)	2014	1.33	± 1.37	ad	1.79	± 0.99	ad	LV
RC6	Hayward	Italy - Piedmont (TO)	2014	1.83	± 0.98	af	2.02	± 0.84	ad	MV
RL1	Hayward	Italy - Piedmont (CN)	2014	1.17	± 0.98	ad	1.19	± 0.98	a	LV
RL2	Hayward	Italy - Piedmont (CN)	2014	0.33	± 0.52	a	0.59	± 0.70	ac	LV
RL4	Hayward	Italy - Piedmont (CN)	2014	3.50	± 0.55	g	3.93	± 0.39	g	V
RL5	Hayward	Italy - Piedmont (CN)	2014	2.00	± 2.10	bg	2.62	± 1.08	cg	MV
CC1	Hayward	Italy - Piedmont (TO)	2014	1.67	± 0.52	af	2.26	± 1.05	bf	MV
CC2	Hayward	Italy - Piedmont (TO)	2014	1.67	± 1.86	af	2.29	± 1.16	cg	MV
CC5	Hayward	Italy - Piedmont (TO)	2014	1.17	± 0.75	ad	2.14	± 1.28	be	MV
CC6	Hayward	Italy - Piedmont (TO)	2014	0.83	± 0.41	ab	0.71	± 0.90	ab	LV
PSA1	Hayward	Italy - Piedmont (CN)	2014	3.17	± 1.03	dg	3.21	± 0.99	eg	V
PSA3	Hayward	Italy - Piedmont (CN)	2014	0.83	± 0.41	ab	0.84	± 0.84	ab	LV
PSA8	Hayward	Italy - Piedmont (CN)	2014	1.00	± 1.55	ac	1.66	± 1.08	ad	LV
PSA9	Hayward	Italy - Piedmont (CN)	2014	2.00	± 1.10	bf	2.62	± 0.98	cg	MV
QV2	Hayward	Italy - Piedmont (CN)	2014	2.83	± 2.04	eg	3.93	± 0.60	g	V
QV3	Hayward	Italy - Piedmont (CN)	2014	2.50	± 0.55	cg	3.57	± 0.90	fg	V
QV4	Hayward	Italy - Piedmont (CN)	2014	2.17	± 0.75	bg	3.09	± 1.67	dg	V
QV5	Hayward	Italy - Piedmont (CN)	2014	1.50	± 0.84	ae	2.14	± 1.43	be	MV
BA3	Hayward	Italy - Piedmont (VC)	2014	3.00	± 0.63	fg	2.86	± 1.36	dg	MV
38/10	Hayward	Italy - Piedmont (CN)	2010	0.83	± 0.41	ab	0.84	± 0.54	ab	LV
310	Hayward	Italy - Piedmont (VC)	2010	1.00	± 0.89	ad	0.95	± 1.08	ab	LV
74/10	Hayward	Italy - Piedmont (CN)	2010	1.17	± 0.75	ad	1.31	± 0.84	ac	LV
41/11	Hayward	Italy - Piedmont (CN)	2010	2.33	± 0.52	cg	3.21	± 1.17	eg	V
229	Hayward	Italy - Piedmont (CN)	2010	1.33	± 2.07	ad	2.26	± 0.54	bf	MV
309	Hayward	Italy - Piedmont (VC)	2010	0.83	± 1.17	ab	1.19	± 0.59	ac	LV
39/10	Hayward	Italy - Piedmont (CN)	2010	1.17	± 1.17	ad	1.79	± 2.01	ae	LV
36/10 c1	Hayward	Italy - Piedmont (AT)	2010	1.67	± 0.52	af	1.91	± 1.61	ae	LV
34/10	Hayward	Italy - Piedmont (CN)	2010	1.33	± 0.52	ad	2.26	± 1.23	bf	MV
314	Hayward	Italy - Piedmont (CN)	2010	1.00	± 0.89	ac	1.19	± 1.16	ac	LV
313	Hayward	Italy - Piedmont (CN)	2010	0.67	± 1.03	ab	0.71	± 0.90	ab	LV
36/10 c2	Hayward	Italy - Piedmont (CN)	2010	0.83	± 0.98	ab	1.19	± 1.16	ac	LV
CFBP7286	Hort16A	Italy - Latium (LT)	2008	3.00	0.00	-	3.83	0.63	-	V
K2	-	Korea - Jeonnam	unknown	1.17	0.75	-	2.68	0.40	-	MV
KW11	-	Japan - Shizuoka	1984	3.33	0.52	-	3.67	0.94	-	V
VT439	Jin Tao	Italy - Latium (VT)	2010	1.67	0.82	-	-	-	-	
VT511	Hayward	Italy - Latium (VT)	2010	1.00	0.89	-	-	-	-	
LT23	Hayward	Italy - Latium (LT)	2008	2.00	0.63	-	-	-	-	
RM310	Hort16A	Italy - Latium (RM)	2008	2.83	0.41	-	-	-	-	

563
564 * Letters in brackets indicate the Italian province of isolation: AT stands for Asti; CN for Cuneo; LT for
565 Latina; RM for Roma; TO for Torino; VC for Vercelli; VT for Viterbo.
566 ** Values in the same column followed by the same letter are not statistically different by Duncan's multiple
567 range test ($P \leq 0.05$)
568 *** Severity of symptoms in vivo: low virulent strain (LV; D.I. 0.5-1.9); moderate virulent strain (MV; D.I.
569 2.0-2.9); virulent strain (V; D.I. 3.0-3.9)

570 **Table 2-** Genetic diversity parameters of the three populations of *P. s. pv. actinidiae* considered in
571 this study.

<i>Population</i>	<i>N. strains</i>	<i>Na</i>	<i>Ne</i>	<i>H</i>	<i>I</i>	<i>PL (%)</i>
Piedmont 2014	21	1.72 ± 0.029	1.38 ± 0.023	0.23 ± 0.012	0.35 ± 0.017	71.79
Piedmont 2010	12	1.54 ± 0.033	1.23 ± 0.019	0.15 ± 0.011	0.24 ± 0.016	54.70
Latium	5	1.47 ± 0.033	1.28 ± 0.022	0.17 ± 0.013	0.26 ± 0.019	46.58
Mean (± SE)	-	1.58 ± 0.031	1.26 ± 0.021	0.18 ± 0.012	0.28 ± 0.017	62.39

572

573 Na: observed number of alleles; Ne: effective number of alleles; H: Nei's genetic diversity; I:
574 Shannon information index; PL: Polymorphic loci (%).

575

576 **Table 3** - Polymorphic sites on four strains of *P.s. pv. actinidiae* isolated in 2014. Bases and triplet
 577 with the corresponding amino acids codification for references are indicated in italics, variant are
 578 indicated in regular type, whereas different amino acids are indicated in bold. K stands for lysine, I
 579 for isoleucine, F for phenylalanine, P for proline, G for glycine, S for serine, and T for threonine.

Strain	<i>HopAI</i>			<i>HrpK1</i>				
	Accession Number	320	321	Accession Number	31	121	246	625
Reference 313 (2010)	KU984444	<i>G</i>	<i>A</i>		<i>A</i>	<i>G</i>	<i>G</i>	<i>G</i>
QV3 (2014)	KU984446	A	-	-	-	-	-	-
PSA1 (2014)	KU984445	-	T	-	-	-	-	-
RC6 (2104)	KU984442	-	-	-	C	T	-	-
RL5 (2014)	KU984443	-	-	-	-	-	C	C
Reference 313 (2010)		<i>AAG (K)</i>	<i>ATC (I)</i>	KU984449	<i>CCA (P)</i>	<i>GGG (G)</i>	<i>S (AGC)</i>	<i>TGC (S)</i>
QV3 (2014)	-	AAA(K)	-	KU984447	-	-	-	-
PSA1 (2014)	-	-	TTC (F)	KU984448	-	-	-	-
RC6 (2104)	-	-	-	KU984451	CCC (P)	GGT (G)	-	-
RL5 (2014)	-	-	-	KU984450	-	-	ACC (T)	TCC (S)

580

581 **Figures captions**

582

583 **Fig.1**– Disease severity indices to evaluate the pathogenicity of the *P. s. pv. actinidiae* strains inoculated on plant and
584 leaf discs of *Actinidia chinensis* var. *deliciosa* ‘Hayward’.

585

586 **Fig. 2** – ERIC PCR fingerprinting of representative strains of *P.s. pv. actinidiae* from different geographic area (a).
587 Lane M – 1 kp plus ladder (Qiagen). Lanes 1-6: strains isolated in 2014. Lanes 7 and 9: strains K2 and KW11. Lanes 8
588 and 11: strain VT439 and RM310. Lanes 12-14: strains isolated in 2010. Dendrogram (b) generated after cluster
589 analysis of genetic similarity based on ERIC PCR fingerprinting of all the strains of *P.s. pv. actinidiae*. The dendrogram
590 was constructed using UPGMA method and Dice similarity index.

591

592 **Fig. 3** – RAPD PCR fingerprinting obtained with OPB-13 primer for genomic DNAs of *P.s. pv. actinidiae* strains.
593 Lanes 1-12: Piedmont strains 2010, L1 - 38/10; L2 - 36/10 c1; L3 - 36/10 c2; L4 - 309; L5 - 314; L6 - 74/10; L7 - 310;
594 L8 - 41/11; L9 - 229; L10 - 313; L11 - 39/10; L12 - 34/10; Lanes 13-19: Japanese, Korean and Latium strains, L13 -
595 K2; L14 - KW11; L15 - vt511; L16 - vt439; L17 - lt23; L18 - CFBP7286; L19 - rm310; Lanes 20-40: Piedmont strains
596 2014, L20 - rc1; L21 - rc2; L22 - rc4; L23 - rc6; L24 - rl1; L25 - rl2; L26 - rl4; L27 - rl5; L28 - cc1; L29 - cc2; L30 -
597 cc5; L31 - cc6; L32 - psa1; L33 - psa3 ; L34 - psa8; L35 - psa9; L36 - qv2; L37 - qv3; L38 - qv4; L39 - qv5; L40 - ba3;
598 Lane 41 - Positive control ; Lane C: Negative control ; M: 1 kb Plus ladder (Qiagen).

599

600 **Fig. 4** - Dendrogram of similarity generated by rep and RAPD-PCR fingerprinting of *P. s. pv. actinidiae* strains. Cluster
601 analysis was performed on combined dataset matrix of concatenated fingerprinting markers using Dice similarity index
602 and UPGMA clustering algorithm. The scale indicates the degree of similarities between strains. Number at nodes
603 represent bootstrap percentage estimated from 1000 replications of the dataset.

604

605 **Fig. 5** - Principal Coordinates analysis (PCoA) obtained from the dataset matrix of fingerprinting generated by rep and
606 RAPD-PCR of *P.s. pv. actinidiae* strains analysed in this study. The figure displays the diagram of Coordinate 1 versus
607 Coordinate 2.