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

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(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  
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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

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## 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 MgCl<sub>2</sub>, 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 MgCl<sub>2</sub>, 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 GTG<sub>5</sub> 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  $\mu$ 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 GTG<sub>5</sub> 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 GTG<sub>5</sub>  
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

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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

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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
<b>Mean (± SE)</b>	-	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
<b>Reference 313 (2010)</b>	KU984444	<i>G</i>	<i>A</i>		<i>A</i>	<i>G</i>	<i>G</i>	<i>G</i>
<b>QV3 (2014)</b>	KU984446	A	-	-	-	-	-	-
<b>PSA1 (2014)</b>	KU984445	-	T	-	-	-	-	-
<b>RC6 (2104)</b>	KU984442	-	-	-	C	T	-	-
<b>RL5 (2014)</b>	KU984443	-	-	-	-	-	C	C
<b>Reference 313 (2010)</b>		<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>
<b>QV3 (2014)</b>	-	AAA(K)	-	KU984447	-	-	-	-
<b>PSA1 (2014)</b>	-	-	TTC (F)	KU984448	-	-	-	-
<b>RC6 (2104)</b>	-	-	-	KU984451	CCC (P)	GGT (G)	-	-
<b>RL5 (2014)</b>	-	-	-	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.