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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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Abstract

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

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

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Introduction

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

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

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

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

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

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

Materials and methods

Microorganisms

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

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 $MgCl_2$, 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.
130
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 $MgCl_2$, 0.75
139 µl dNTPs (10 mM), 1 µl each primer (10 mM), 0.2 µl of Taq DNA polymerase (Qiagen) and 20 ng

template DNA. PCR was performed with initial denaturation at 94°C for 1 min, followed by 40 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 extension at 72°C. Rep-PCR with primer GTG_5 was performed following protocols by Versalovic *et al.* (1994).

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

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

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

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

Forward and reverse sequences for each gene were used to create a consensus sequence, using DNA Baser programme (Heracle Biosoft S.R.L., Romania), for multi-alignment using CLUSTALW through MEGA version 6. After cutting the trimmed regions and manual correction, dataset of 380, 887, 550, 518, 681 and 571 bp were obtained, respectively, for the genes *hopA1*, *hrpK1 avrD1*, *cts*, *gapA* and *pgi*.

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

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

Pathogenicity tests

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

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

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

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

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

A disease index (D.I.), ranging from 0 to 5, was assigned, by observing 6 leaves per plant,12 days after inoculation: 0= no symptoms; 1= 1-4% infected area; 2= 5-10% infected; 3= 11-30% infected area; 4= ≥50% infected area; 5= completely necrotic. The classes of infection, used to describe the severity of symptoms, were four: not pathogenic strain (NP; D.I. 0.0-0.4), low virulent strain (LV; 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 virulent strain (HV; D.I. 4.0-5.0). The experiment was performed with three replicates for each strain (18 leaves per strain). The pathogenicity assay was carried out twice.

Results

Pathogen identification

Thirty-three bacterial isolates from symptomatic leaves of kiwifruit cultivated in Northern Italy confirmed to belong to *P.s.* pv. *actinidiae* by amplification of two expected bands of 280 and175 bp, after PCR reaction with two primer pairs of Rees-George *et al.* (2010) and sequencing of 16S region (two sequences were deposited in GenBank with accession numbers KP794939 and KP794940). The pathovar assignation was validated through duplex PCR with the amplification of two PCR product for all strains and, typical fingerprinting profile using rep-PCR with ERIC primer (Fig. 2). By considering the host range test, the populations did not induce any disease symptom on the inoculated lemon fruits tested. Control fruits did not show any disease symptoms.

Molecular characterization

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

Korean strain –K2 (Psa 2) and the Japanese KW11 (Psa 1). UPGMA dendrogram divided the two biovars from biovar 3 (Fig. 2). Rep-PCR through GTG_5 primer revealed also variability within biovar 3, as shown in supplementary Figure S1.

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

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

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

Genetic diversity and principal coordinates analysis

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

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

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

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

Multilocus sequence analysis (MLSA)

A DNA sequence dataset, with partial sequences of *cts, avrD1, hopA1* and *hrpK1* was built. Phylogenetic analysis was performed for each gene sequence dataset. The partial gene sequences of *pgi* and *gapA* were 100% identical for all the isolates considered (data not shown).

Figures S2a and S2b show two major clades for *cts* and *avrD1* genes. For *cts* gene, two clades are shown (Figure S2a; bootstrap value: 88%): clade I, including all Italian isolates, with two cytosines in position 251 and 431, and a clade II, including the Japanese and Korean isolates, with a thymine in position 251 and an adenine in position 431. The same clustering, supported by 64% bootstrap value, is shown for *avrD1* gene (Figure S2b), with all Italian isolates with two adenines in position 43 and 528, and the Japanese and Korean isolates, with a cytosine in position 43 and a guanine in position 528.

Figures S3a and S3b show the Neighbour-Joining analysis of *hrpK1* and *hopA1* genes. Sequences alignment showed very low sequence diversity, but six single nucleotide polymorphisms (SNPs) occurred in four strains isolated in Northern Italy in 2014: two in *hopA1* and four in *hrpK1*. Among the SNPs in *hopA1* and *hrpK1*, two resulted in amino acid variation, while four were silent (Table 3). Amino acid variation occurred from isoleucine (ATC) to phenylalanine (TTC) in strain PSA1 at position 107 and from serine (AGC) to threonine (ACC) at position 82 for RL5 strain. For the Japanese and Korean strains, *hopA1* gene was not amplified.

Pathogenicity tests

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

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

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

Discussion

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

The molecular fingerprinting, obtained by the concatenated matrix of rep-PCR through GTG_5 primer and RAPD PCR analysis, revealed a high level of variability in the population of strains of *P.s.* pv. *actinidiae* from Northern Italy, according to Mazzaglia et al. (2011) for the analysis of Psa

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

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

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

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

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

A MLSA of some effector genes belonging to the T3SS, a conserved secretion system involved in pathogenicity (Lindeberg *et al.*, 2012), was performed to evaluate if the variability in virulence observed could be associated with mutations in these genes. The analysis revealed only four SNPs 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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Compliance with Ethical Standards

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

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Tables

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.

				Pathogenicity assay						
Strain	**Cultivar**	**Geographical location***	**Year**	***in vitro* D.I.**			***in vivo* 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.

Population	***N. strains***	***Na***	***Ne***	***H***	***I***	***PL (%)***
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.

	HopA1			***HrpK1***				
Strain	**Accession Number**	**320**	**321**	**Accession Number**	**31**	**121**	**246**	**625**
Reference 313 (2010)	KU984444	*G*	*A*		*A*	*G*	*G*	*G*
QV3 (2014)	KU984446	A	-	-	-	-	-	-
PSA1 (2014)	KU984445	-	T	-	-	-	-	-
RC6 (2104)	KU984442	-	-	-	C	T	-	-
RL5 (2014)	KU984443	-	-	-	-	-	C	C
Reference 313 (2010)		*AAG (K)*	*ATC (I)*	KU984449	*CCA (P)*	*GGG (G)*	*S (AGC)*	*TGC (S)*
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

Figures captions

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

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

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

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

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