A Multiplex PCR Assay for Detection of *Pseudomonas syringae* pv. *actinidiae* and Differentiation of Populations with Different Geographic Origin

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Abstract

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Pseudomonas syringae pv. *actinidiae* is responsible for severe outbreaks of bacterial canker of kiwifruit currently occurring around the world. Although molecular detection methods have been reported, none provide complete selectivity for this pathovar or discriminate among pathogen haplotypes. Therefore, a new multiplex polymerase chain reaction (PCR) assay was developed and validated. The assay was tested on 32 *P. syringae* pv. *actinidiae* isolates and 15 non-*P. syringae* pv. *actinidiae* strains and correctly assigned *P. syringae* pv. *actinidiae* strains to three different haplotypes: a Japanese/Korean group, a European group, and a Chinese group. Two *P. syringae* pv. *actinidiae* isolates from New Zealand were found to belong to the Chinese group whereas two other isolates from New Zealand, which were isolated from kiwifruit plants but which do not cause bacterial canker, tested negative. The described PCR assays has a limit of detection of approximately 5 to 50 pg of purified DNA or of 5×10^2 bacteria/PCR and were shown to work with both artificially and naturally infected plant tissues. Thus, the described method represents a suitable tool for detection of *P. syringae* pv. *actinidiae* and haplotype attribution, in particular, when testing a high number of samples during surveillance and prevention activities.

Pseudomonas syringae is a bacterial species that includes dozens of pathogenic variants that may be highly aggressive on many plant species. These variants are assigned to intraspecific taxa named "pathovars" according to their host range and the symptoms they cause (39). Pathovar *actinidiae* is the taxon of *P. syringae* that causes bacterial canker of kiwifruit (34). This disease was first observed on kiwi in 1984–85 in Hunan Province, China (13), and the pathogen was first isolated and exhaustively described in Japan in 1989 on the green-fleshed species *Actinidia deliciosa* L. (32). The disease was later found in Korea (18,19) and Italy (29). However, *P. syringae* pv. *actinidiae* did not cause severe damages until 2008, when a dramatic outbreak due to a new, more aggressive haplotype occurred in Italy (1–3,23). This outbreak is still continuing today. In 2010, the same haplotype was also reported in Portugal (4), France (36), and New Zealand (12); and, in 2011, it reached Australia (8), Spain (5), Switzerland (11), and Chile (10). The presence of the pathogen was also reported in Turkey (7). Within a few years, this new canker disease has grown to an international pandemic that is now threatening the sustainability of the kiwi industry in all major kiwi-producing countries.

The worldwide *P. syringae* pv. *actinidiae* population consists of different subpopulations (9,14,16,22–24,30,37) that seem to be characterized by different levels of aggressiveness toward kiwifruit. The strains isolated in Europe since 2008 and some of the strains isolated in New Zealand in 2010–11 are considered the most virulent by far, also defined as haplotype PSA-1 or PSA-V (9), based on their aggressiveness and rapidity of their spread. In New Zealand, another population (labeled as haplotype PSA-2 or PSA-LV) was described. Strains belonging to this population only cause leaf spots but no cankers (9).

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In regard to Asia, the data available today suggest that *P. syringae* pv. *actinidiae* strains from Japan and Korea belong to one population within which two different haplotypes can be distinguished based on presence or absence of gene clusters coding for the toxins coronatine and phaseolotoxin (16,21). The only Chinese strains analyzed so far were closely related both to the European and New Zealand strains: only six mutations (single nucleotide polymorphisms [SNPs]) in 3.5 million bp of genome sequence distinguish Chinese *P. syringae* pv. *actinidiae* strains from the European *P. syringae* pv. *actinidiae* strains and only one difference in gene content within a genomic island similar to PPHGI-1 could be detected (24). Interestingly, when two New Zealand PSA-V strains were compared with the European and the Chinese *P. syringae* pv. *actinidiae* strains, they resembled the European strains in regard to the six SNPs but were identical to the Chinese strains in regard to gene content within the PPHGI-1 genomic island (24). Using parsimony, the most likely explanation of this finding is that importation of *P. syringae* pv. *actinidiae* from China into Europe and into New Zealand were two independent events because this scenario can be explained with one lateral gene transfer event, whereas importation of *P. syringae* pv. *actinidiae* from China into Europe and from there into New Zealand would imply two lateral gene transfer events (24).

Due to the co-existence on kiwifruit plants of *P. syringae* pv. *actinidiae* with other pathogens, such as *P. syringae* pv. *syringae* and *P. viridiflava*, as well as fungi, viruses, and insects, neither classical approaches such as isolation and cultivation on appropriate media nor identification by standard laboratory tests and implementation of Koch's postulates are specific enough to determine unequivocally the identity of the pathogen. Furthermore, these procedures are time consuming and, therefore, unsuitable for a rapid response for disease management, quarantine analysis, and determining whether plants are *P. syringae* pv. *actinidiae*-free before planting them in new orchards.

In recent decades, molecular analyses using polymerase chain reaction (PCR) has proven to be a powerful tool for detecting specific bacteria, often without the need for culturing. Also, for *P. syringae* pv. *actinidiae*, various PCR assays have been developed (15,20,28,31) but, unfortunately, most of these assays lack in speci-

ficity (26) and none of them can simultaneously distinguish between different populations of *P. syringae* pv. *actinidiae*. Here, we describe a new, highly specific multiplex PCR assay based on our recent comparative evolutionary genomics study of *P. syringae* pv. *actinidiae* (24). This assay distinguishes *P. syringae* pv. *actinidiae* from all other pseudomonads and can putatively assign *P. syringae* pv. *actinidiae* strains to populations of different geographic origin.

Materials and Methods

Bacterial strains and culturing. A set of 32 strains (Table 1) was selected from a collection of about 150 *P. syringae* pv. *actinidiae* strains isolated between 1984 and 2011 from almost all areas of kiwifruit cultivation infested by the disease. The selected strains, which are representative of all *P. syringae* pv. *actinidiae* populations described to date, were used for assay development and validation. All strains available to us from Japan (six strains), Korea (three strains), China (three strains), and New Zealand (four strains) were included. On the other hand, due to the availability of about 120 European strains, a random selection of 16 was made (but acting so that every interested country was represented by at least one strain); this included 7 strains from all Italian regions where the disease has been reported to date, 2 from Spain, 3 from Portugal, 3 from France, and 1 from Switzerland. Additionally, strains belonging to 10 pathovars related to *P. syringae* pv. *actinidiae* were used as negative controls: *P. syringae* pv. *theae* (two strains); *P. syringae* pv. *avellanae,* today reclassified as *P. avellanae* (17) (three strains); *P. syringae* pv. *morsprunorum*; *P. syringae* pv. *tomato*; *P. syringae* pv. *maculicola*; *P. syringae* pv. *papulans*; *P. syringae* pv. *syringae* (two strains); *P. syringae* pv. *lachrymans*; *P. syringae* pv. *aptata*; and *P. syringae* pv. *pisi*. An individual of *P. viridiflava* isolated from *A. chinensis* was also included (Table 1). All bacterial strains are stored in the Bacterial Culture Collection of the Department of Agriculture, Forestry, Nature and Energy of the University of Tuscia at –80°C in nutrient glycerol agar. Strains were routinely grown on nutrient agar (NA) for 48 to 72 h at 27°C.

DNA extraction. Genomic DNA was extracted from single colonies grown for 48 h at 27°C on NA medium. Approximately 2 × 10^9 cells were used for DNA extraction with the PureLink Genomic DNA kit (Invitrogen) according to the manufacturer's in-

Table 1. Bacterial strains used in this study, their source, geographic origin, and host of isolation

Pseudomonas sp. and pathovar	Strain code	Source[a]	Isolation country, (site), year	Host species (cultivar)
Pseudomonas syringae pv. *actinidiae*	KW1	Y. Takikawa	Japan (Shizuoka) 1984	*Actinidia deliciosa* (Hayward)
	KW11[b]	Y. Takikawa	Japan (Shizuoka) 1984	*A. deliciosa* (Hayward)
	4911	CFBP	Japan (Shizuoka) 1984	*A. deliciosa* (Hayward)
	4912	CFBP	Japan (Shizuoka) 1984	*A. deliciosa* (Hayward)
	5095	CFBP	Japan (–) 1988	*A. chinensis* (–)
	5097	CFBP	Japan (–) 1988	*A. chinensis* (–)
	Kn2	Y. J. Koh	Korea (–) 1997	*A. chinensis*(–)
	23663	KCTC	Korea (Jeonnam) 1989	*A. chinensis* (–)
	23664	KCTC	Korea (Jeonnam) 1989	*A. chinensis* (–)
	PSA92	Scortichini	Italy (Latium) 1992	*A. deliciosa* (Hayward)
	7285	CFBP	Italy (Veneto) 2008	*A. chinensis* (Jin Tao)
	7286[b]	CFBP	Italy (Latium) 2008	*A. chinensis*(Hort16A)
	7287	CFBP	Italy (Latium) 2008	*A. deliciosa* (Hayward)
	490	DAFNE Unitus	Italy (Calabria) 2010	*A. chinensis* (Jin Tao)
	1To	DAFNE Unitus	Italy (Piedmont) 2010	*A. deliciosa* (Hayward)
	15ER	A. Calzolari	Italy (Emilia Romagna) 2011	*A. deliciosa* (Hayward)
	829	DAFNE Unitus	Spain (Galicia) 2011	*A. chinensis* (Jin Tao)
	830	DAFNE Unitus	Spain (Galicia) 2011	*A. chinensis* (Jin Tao)
	820	DAFNE Unitus	Portugal (Valença) 2011	*A. deliciosa* (Erica)
	832	DAFNE Unitus	Portugal (S.ta Maria da Feira) 2011	*A. deliciosa* (Hayward)
	835	DAFNE Unitus	Portugal (Vila Boa de Quires) 2011	*A. deliciosa* (Hayward)
	1F	Anses	France (Aquitaine) 2010	*A. chinensis* (Jin Tao)
	5F	Anses	France (Rhone Alpes) 2010	*A. deliciosa* (Hayward)
	14F	Anses	France (Aquitaine) 2010	*A. chinensis* (Hort16A)
	LSV38.17	…	Switzerland (–) 2011	…
	CH2010-5[b]	L. Hwang	China (Shaanxi) 2010	*A. chinensis* (Hongyang)
	CH2010-6	L. Hwang	China (Shaanxi) 2010	*A. chinensis* (Hongyang)
	CH2010-7	L. Hwang	China (Shaanxi) 2010	*A. chinensis* (Hongyang)
	18839	ICMP	New Zealand (Bay of Plenty) 2011	*A. deliciosa* (Hayward)
	18875	ICMP	New Zealand (Bay of Plenty) 2011	*A. deliciosa* (Hayward)
	18804	ICMP	New Zealand (Bay of Plenty) 2010	*A. chinensis* (–)
	18882	ICMP	New Zealand (Nelson) 2010	*A. chinensis* (Gold)
P. syringae pv. *theae*	2598	NCPPB	Japan (–) 1970	*Thea sinensis*
	2599	NCPPB	Japan (–) 1970	*T. sinensis*
P. syringae pv. *avellanae*[c]	4224	NCPPB	Italy (–) 1994	*Corylus avellana*
	4226	NCPPB	Greece (–) 1987	*Corylus avellana*
	Pav 34	DAFNE Unitus	Italy (–)	*C. avellana*
P. syringae pv. *morsprunorum*	1871	NCPPB	Italy (–) 1965	*Prunus avium*
P. syringae pv. *tomato*	1323	CFBP	France (Loire) 1971	*Lycopersicon esculentum*
P. syringae pv. *maculicola*	2038	NCPPB	Unite Kingdom (–) 1967	*Brassica oleracea*
P. syringae pv. *papulans*	1754	CFBP	Canada (–) 1973	*Malus sylvestris*
P. syringae pv. *aptata*	5428	CFBP	Morocco (–) 1996	*Cucumis melo*
P. syringae pv. *lachrymans*	6463	CFBP	Hungary (–) 1958	*C. sativus*
P. syringae pv. *pisi*	2105	CFBP	New Zealand (–) 1969	*Pisum sativum*
P. syringae pv. *syringae*	3909 B,1	A. Calzolari	Italy (Emilia Romagna) 2009	*A. chinensis*
	4250,1	A. Calzolari	Italy (Emilia Romagna) 2009	*A. chinensis*
P. viridiflava	4254 A,1	A. Calzolari	Italy (Emilia Romagna) 2009	*A. chinensis*

[a] Acronyms for microbial collection: CFBP = Collection Française de Bactéries associées aux Plantes; KCTC = Korean Collection for Type Culture; ICMP = International Collection of Microorganisms from Plants; NCPPB = National Collection of Plant Pathogenic Bacteria.
[b] Strains representative of the three main populations selected to be used in sensitivity tests.
[c] Now only *Pseudomonas avellanae*.

structions for gram-negative bacteria. The concentration of the eluted DNA was estimated by a fluorimetric device (Qubit; Invitrogen, Life Technologies Italia), adjusted to a final concentration of 50 ng/µl with Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and kept at –20°C until use.

Primer design and multiplex-PCR amplification. All primer pairs were designed from sequenced *P. syringae* pv. *actinidiae* genomes available from the National Center for Biotechnology (NCBI) and with primer 3 software (http://frodo.wi.mit.edu. Primers were synthesized by Primm srl. The position of the amplicons in the genome and the relative primer sets used for the detection of *P. syringae* pv. *actinidiae* populations in multiplex-PCR (m-PCR) are specified in Table 2. Reactions were first carried out independently for each primer pair. Each reaction consisted of a 50-µl total volume mixture containing 25 µl of GoTaq Colorless Master Mix 2X (Promega Corporation), 1 µl of template DNA, 0.8 µM each primer, and sterile distilled water (SDW) to the final volume. All four primers were used in each reaction mixture, with primer concentration adjusted to obtain amplifications with comparable intensity. The 50 µl of m-PCR mixture consisted of 25 µl of GoTaq Colorless Master Mix 2X (Promega Corporation), 1 µl of template DNA, 0.30 µM primer pair *P. syringae* pv. *actinidiae*, 0.80 µM primer pair Europe, 0.60 µM primer pairs J/K and China (Table 2), and SDW to the final volume. Amplifications were carried out in a C1000 gradient thermal cycler (Bio-Rad Laboratories srl) and optimized to the following conditions: an initial denaturation step at 95°C for 10 min; followed by 40 serial cycles of a denaturation step of 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 75 s; and a final extension step at 72°C for 5 min. A negative control (no template DNA) was included in each batch of m-PCRs. The amplification products were detected by electrophoresis of 5 µl of each amplification mixture in a 2% agarose gel in 1× Tris-acetate-EDTA buffer. Gels were stained with GelRed (Biotium Inc.). At least three replicate PCR amplifications were carried out on different days for each strain.

Specificity, sensitivity, and reproducibility. The sensitivity of the assay was tested using both pure DNA and bacterial suspensions of one representative strain for each of the investigated populations (Table 1, strains labeled with [b]). Purified genomic DNA (50 ng) of each representative strain was serially diluted in 10-fold increments in SDW down to 5 fg of DNA per PCR. Also, bacterial suspensions of each representative strain were prepared from log-phase cultures on nutrient broth and adjusted to a concentration of 5×10^8 CFU ml^{-1}; then, each suspension was 10-fold diluted seven times to 5×10^1 CFU ml^{-1} in SDW. These dilutions were used to determine both the initial concentration of each bacterium by surface plating of 100 µl onto NA plates, as well as for subsequent crude DNA extraction for PCR. DNA was extracted by heating 100 µl of each dilution at 95°C for 10 min; then, the suspensions were centrifuged for 2 min at 12,500 × *g* and 1 µl of the supernatant was added directly to the PCR mixture. All m-PCRs performed to assess both specificity and limits of detection of the assay were done in triplicate.

Assay evaluation on kiwifruit samples artificially inoculated with *P. syringae* pv. *actinidiae*. For direct detection of *P. syringae* pv. *actinidiae* in kiwifruit tissues, artificially inoculated plants were used. One-year-old plantlets of *A. deliciosa* 'Hayward' and *A. chinensis* 'Hort16A' were maintained in a growth chamber with photoperiod of 16 h of day and 8 h of night at 25°C; for artificial inoculation, plants were enclosed in clear sterile plastic bags overnight to promote stomatal opening, then were sprayed with 5 ml of a *P. syringae* pv. *actinidiae* (strain 7286) bacterial suspension (10^8 CFU ml^{-1}) in SDW. Control plants were inoculated with the same amount of SDW. Symptoms appeared after 10 to 15 days; then, approximately 100 mg of leaf tissue surrounding necrotic spots was aseptically removed, crushed in a sterile mortar, and used directly for DNA extraction with the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The same procedure was carried out for control plants. Aliquots of extracted DNA (1 µl each) were directly used for m-PCR amplification as described above.

Assay evaluation on kiwifruit samples naturally infected with *P. syringae* pv. *actinidiae*. Leaves of kiwifruit plants with symptoms referable to *P. syringae* pv. *actinidiae* were collected in two orchards of both *A. deliciosa* Hayward (6-year-old plants) and *A. chinensis* 'JinTao' (3-year-old plants) (30 samples per orchard in total). The protocol described for artificially inoculated plants was also used for DNA extraction and for m-PCR amplification. Moreover, for each sample, a portion of symptomatic tissue was used for classical bacterial isolation. About 100 mg of leaf tissue were homogenized and plated on NA. Plates were incubated at 27°C for 24 to 48 h and observed under the microscope. In all cases, a single morphotype was recovered. Single colonies were then restreaked on the same medium and incubated for another 24 h. Approximately 2×10^9 cells were used for DNA extraction using the PureLink Genomic DNA kit (Invitrogen) according to the manufacturer's instructions for gram-negative bacteria and analyzed by m-PCR as described above.

Results

Primer design. A first primer pair was designed to specifically detect *P. syringae* pv. *actinidiae* but not any related *P. syringae* pathovar. For this, a primer pair was designed to amplify a portion of the *hopZ3* gene sequence that is conserved among the nine completely sequenced *P. syringae* pv. *actinidiae* strains but different from the *hopZ3* sequences of pv. *theae* and all other pathovars retrievable from the NCBI database with the BLAST algorithm. The predicted amplicon, referred to as the "*P. syringae* pv. *actinidiae*" amplicon, is 311 bp long and the primers sequences are *P. syringae* pv. *actinidiae* F (CAGAGGCGCTAACGAGGAAA) and *P. syringae* pv. *actinidiae* R (CGAGCATACATCAACAGGTCA). The corresponding sequence is located in the published genome sequence of *P. syringae* pv. *actinidiae* strain CFBP7286 between base pairs 7,677 and 7,987 of scaffold 12383_336, contig 1 (accession number: AGNO01000134).

A region within the PPHGI-1-like genomic island (24) was chosen to design three additional primer pairs to distinguish European, Chinese, and Japanese/Korean *P. syringae* pv. *actinidiae* strains from each other. The first primer pair, which was first described by Mazzaglia et al. (24), was designed to amplify a region present only in the sequenced genomes of *P. syringae* pv. *actinidiae* strains isolated during the current bacterial canker of kiwifruit outbreak in

Table 2. Primers used in this study: amplicon name, primer sequence, name of the *Pseudomonas syringae* pv. *actinidiae* strain based on which the primer pair was designed, position of the amplicon in the strain's genome, and accession number of the genome used for primer design

Amplicon	Primer sequence (5′–3′)	Strain	Amplicon position in genome	Accession number
P. syringae pv. *actinidiae*	CAGAGGCGCTAACGAGGAAA CGAGCATACATCAACAGGTCA	CFBP7286	7,677–7,987, scaffold 12383_336, contig_1	AGNO01000134
Europe	TGGTGATCGTCTGGATGTGT ATTATGCTCCTGGCTCATGG	CFBP7286	54,930–55,662, scaffold 12383_2218, contig_5	AGNO01000423
China	GGAGTTCCAGCAACTGACG CGCTCAAGATCCTTTTCCAT	CH2010-6	28,169–28,777, scaffold 12384_102, contig_1	AGUH01000096
Japan/Korea	AGCAACGGTGGTTTGTTTTC AAATGTTTGCCAGCCAAGTC	M302091	8,118–8,371, contig_26.3	AEAL01000253

Europe. The PCR product size of this "European" amplicon is 733 bp and the primer sequences are Europe F (TGGTGATCGTCT GGATGTGT) and Europe R (ATTATGCTCCTGGCTCATGG). This region is located in the published genome sequence of *P. syringae* pv. *actinidiae* strain CFBP7286 between base pairs 54,930 and 55,662 of scaffold 12383_2218 contig 5 (accession number: AGNO01000423). The second population-specific primer pair was designed to amplify a 254-bp region present only in the sequenced genomes of *P. syringae* pv. *actinidiae* strains isolated in Japan and Korea; the primers are J/K F (AGCAACGGT GGTTTGTTTTC) and J/K R (AAATGTTTGCCAGCCAAGTC). The amplified region is located in the published genome sequence of *P. syringae* pv. *actinidiae* strain M302091 between base pairs 8,118 and 8,371 on contig 26.3 (accession number: AEAL01000253). The last primer pair was designed to amplify a 609-by fragment of the PPHGI-1 island present only in the sequenced genomes of *P. syringae* pv. *actinidiae* strains isolated in China; the primer sequences are China F (GGAGTTCCAGCA ACTGACG) and China R (CGCTCAAGATCCTTTTCCAT). The PCR product is located in the published genome sequence of *P. syringae* pv. *actinidiae* strain CH2010-6 between base pairs 28,169 and 28,777 on scaffold 12384_102 contig_1 (accession number: AGUH01000096).

Specificity. The identification of a *P. syringae* pv. *actinidiae* strain as a member of the European population requires the amplification of both the "*P. syringae* pv. *actinidiae*" and the "European" amplicon; identification as a member of the Japanese/Korean group requires the amplification of both the "*P. syringae* pv. *actinidiae*" and the "J/K" amplicon; and identification as a member of the Chinese population requires the amplification of both the "*P. syringae* pv. *actinidiae*" and the "Chinese" amplicons. Results employing all four primers in a single m-PCR assay with 32 *P. syringae* pv. *actinidiae* strains and 15 isolates of different *P. syringae* pathovars and *Pseudomonas* spp. are shown in Figure 1. The expected *P. syringae* pv. *actinidiae* fragment of 311 bp and the expected J/K fragment of 254 bp were amplified from the six Japanese and three Korean strains (lanes 2 to 10). Of the 16 European strains assayed (lanes 12 to 25), 15 produced a PCR amplification of the expected *P. syringae* pv. *actinidiae* fragment (311 bp) and the expected European amplicon (733 bp). The single exception was strain PSA92, which gave the amplification profile typical of Japanese/Korean strains (lane 11). The expected *P. syringae* pv. *actinidiae* fragment of 311 bp and Chinese amplicon of 609 bp were amplified from the three Chinese strains assayed (lanes 26 to 28). Four strains were assayed from New Zealand, only two of which cause cankers on kiwi (9). Amplicons indicative of the Chinese strain group were amplified from the two canker-causing strains (Fig. 1, lanes 29 and 30). DNA was not amplified from the other two strains, suggesting that these two strains are not in pv. *actinidiae* (Fig. 1, lanes 31 and 32). Finally, all strains belonging to different *P. syringae* pathovars or *Pseudomonas* spp. did not produce any amplicons (lanes 33 to 47).

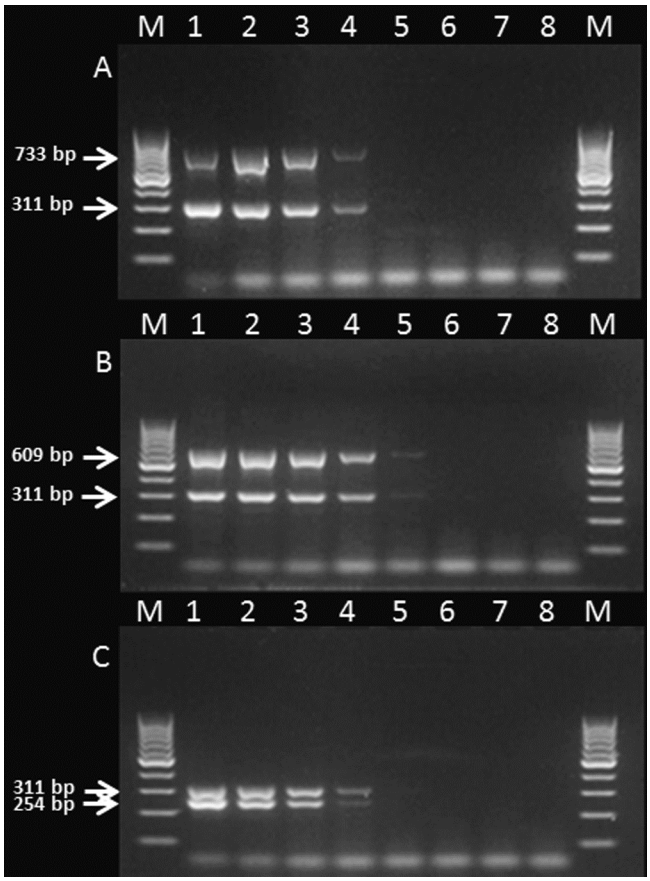

Fig. 2. Sensitivity of detection of purified DNA with all four primer pairs. Polymerase chain reaction amplification from a 10-fold dilution series of purified DNA from *Pseudomonas syringae* pv. *actinidiae* strains **A,** KW11; **B,** 7286; and **C,** CH2010-5. Lane 1, 50 ng; lane 2, 5 ng; lane 3, 0.5 ng; lane 4, 50 pg; lane 5, 5 pg; lane 6, 0.5 pg; lane 7, 50 fg; lane 8, control, no template DNA. M, molecular marker (GeneRuler 100-bp DNA ladder; Genenco).

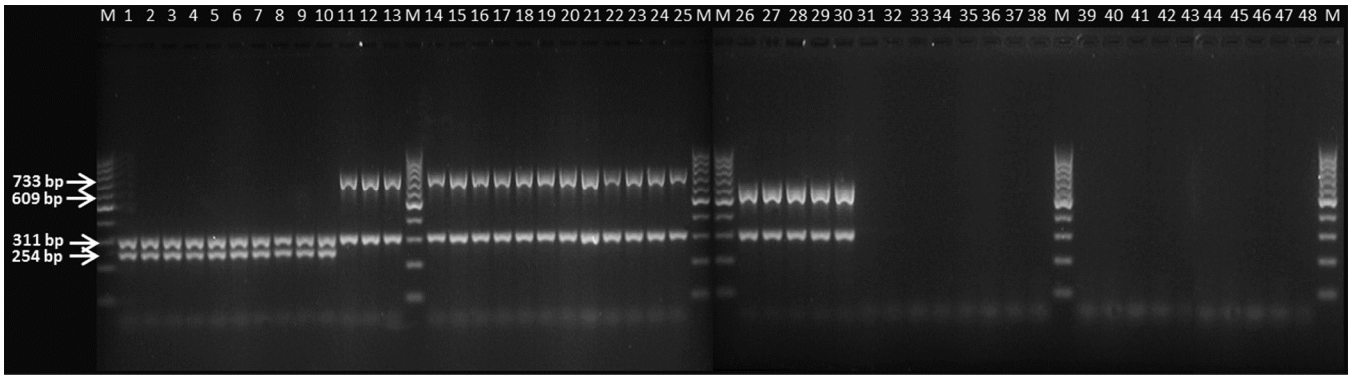

Fig. 1. Multiplex polymerase chain reaction amplification with all four primer pairs. Lanes 1–6: Japanese *Pseudomonas syringae* pv. *actinidiae* strains (KW1, KW11, 4911, 4912, 5095, and 5097); lanes 7–9: Korean *P. syringae* pv. *actinidiae* strains (Kn2, 23663, and 23664); lanes 10–25: European *P. syringae* pv. *actinidiae* strains (PSA92, 7285, 7286, 7287, 490, 1To, 15ER, 829, 830, 820, 832, 835, 1F, 5F, 14F, and LSV38.17); lanes 26–28 Chinese *P. syringae* pv. *actinidiae* strains (CH2010-5, CH2010-6, and CH2010-7); lanes 29–30: New Zealand *P. syringae* pv. *actinidiae* strains (18839 and 18875); lanes 31–32: New Zealand strains isolated from kiwifruit plants but that do not cause canker (18804 and 18882); lanes 33–34: *P. syringae* pv. *theae* (2598 and 2599); lane 35: *P. syringae* pv. *tomato*; lane 36: *P. syringae* pv. *papulans*; lane 37: *P. syringae* pv. *aptata*; lane 38: *P. syringae* pv. *lachrymans*; lane 39: *P. syringae* pv. *pisi*; lanes 40–41: *P. syringae* pv. *syringae* (3909 b,1 and 4250,1); lanes 42–44: *P. avellanae* (NCPPB 4224, NCPPB4226, and Pav34); lane 45: *P. syringae* pv. *morsprunorum*; lane 46: *P. syringae* pv. *maculicola*; lane 47: *P. viridiflava*; lane 48: control, no template DNA. M: molecular marker (GeneRuler 100-bp DNA ladder; Genenco). Arrows indicate the *P. syringae* pv. *actinidiae* amplicon (311 bp), European amplicon (733 bp), Japanese/Korean amplicon (609 bp), and Chinese amplicon (254 bp), respectively.

Sensitivity. The sensitivity of the m-PCR assay was in the range from 5 to 50 pg for all tested strains when 1-µl aliquots of serial 10-fold dilutions of purified DNA of the selected representative strains were used (Fig. 2). The detection limit for m-PCR assay was also investigated using 10-fold serially diluted bacterial suspensions. Amplification of the expected fragments was clearly visible on the gels down to a concentration corresponding to 5×10^2 CFU/PCR for all tested strains (Fig. 3). However, the fragment of 311 bp was often visible even using only 5×10^1 CFU/PCR.

***P. syringae* pv. *actinidiae* detection in artificially inoculated and naturally infected kiwifruit samples.** The pathogen was successfully detected in all the symptomatic samples obtained from artificially inoculated plantlets of both *A. deliciosa* and *A. chinensis* after amplification from 1 µl of extracted DNA. No amplification products were obtained from water-inoculated plants (Fig. 4).

The pathogen was also detected without any failure in all 60 symptomatic samples obtained from naturally infected plants of both *A. deliciosa* and *A. chinensis* after amplification from 1 µl of extracted DNA (Fig. 5). All samples produced the two amplicons referable to the European type. The molecular analysis of bacteria isolated from the same samples fully confirmed these results.

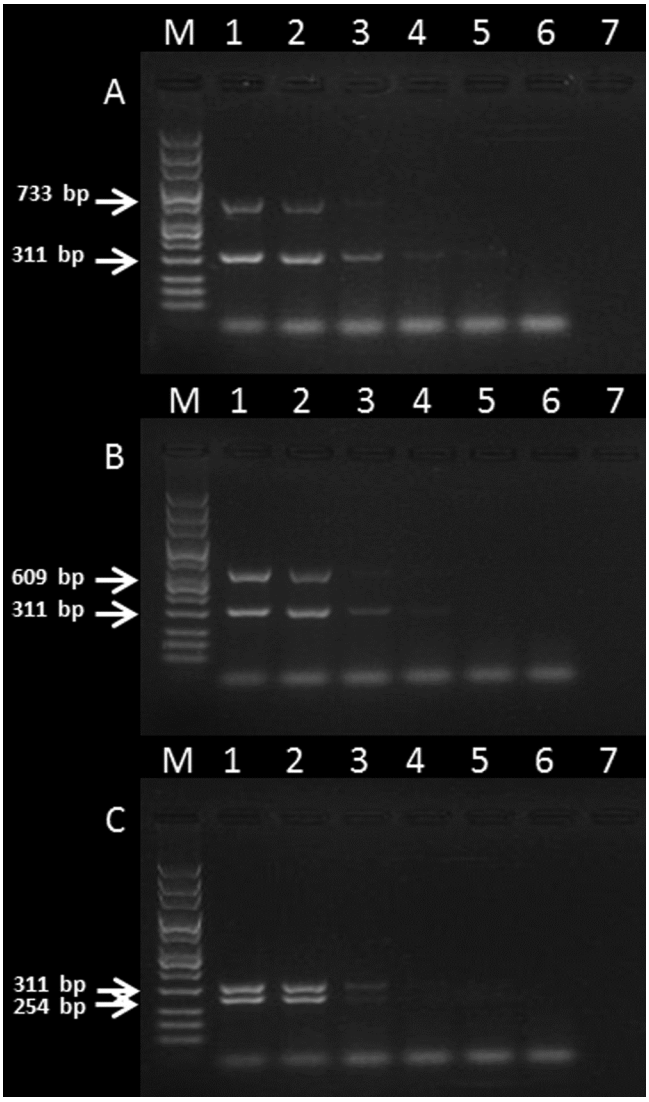

Fig. 3. Sensitivity of detection of bacterial suspensions with all four primer pairs. Polymerase chain reaction (PCR) amplification from a 10-fold dilution series of bacterial suspension of *Pseudomonas syringae* pv. *actinidiae* strains **A,** KW11; **B,** 7286; and **C,** CH2010-5. Lane 1, 5×10^4 CFU/PCR (undiluted sample); lane 2, 5×10^3 CFU/PCR; lane 3, 5×10^2 CFU/PCR; lane 4, 5×10^1 CFU/PCR; lane 5, 5×10^0 CFU/PCR; lane 6, 5×10^{-1} CFU/PCR; lane 7, control, no template DNA. M, molecular marker (SharpMass 100 plus; EuroClone).

Discussion

P. syringae pv. *actinidiae* is difficult to identify by morphological observation, biochemical tests, or pathogenicity assays due to many features shared with other *P. syringae* pathovars and other pseudomonads. However, an accurate identification of *P. syringae* pv. *actinidiae* is important because it is essential to obtain a reliable picture of the phytosanitary situation in an orchard before intervening with any control measures. In fact, the earliest foliar symptoms during the vegetative season can be easily confused with those induced by other more innocuous bacterial pathogens of kiwifruit such as *P. syringae* pv. *syringae* and *P. viridiflava*, which do not require the same drastic interventions required for *P. syringae* pv. *actinidiae*, including the eradication of diseased plants (27). Moreover, an assay able to distinguish *P. syringae* pv. *actinidiae* from closely related bacteria is essential when screening stocks of vegetative material for propagation, planting of new orchards, and phytosanitary controls before importation.

To address this challenge, we described here a novel and rapid method for detection of *P. syringae* pv. *actinidiae* and contemporaneous identification of three *P. syringae* pv. *actinidiae* populations with different geographic origins.

This molecular test is discriminatory: all non-*P. syringae* pv. *actinidiae P. syringae* pathovars and *P. viridiflava* assayed gave a clear negative result while all *P. syringae* pv. *actinidiae* strains

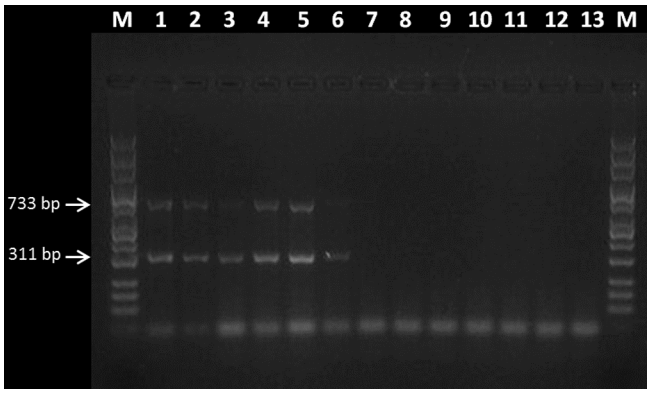

Fig. 4. Multiplex polymerase chain reaction amplification of DNA from symptomatic portions of leaves artificially inoculated with a suspension of *Pseudomonas syringae* pv. *actinidiae* strain 7286 (lanes 1–6) or with sterile distilled water (lanes 7–12); lane 13; negative control, no template DNA; M: molecular marker (SharpMass 100 plus; EuroClone).

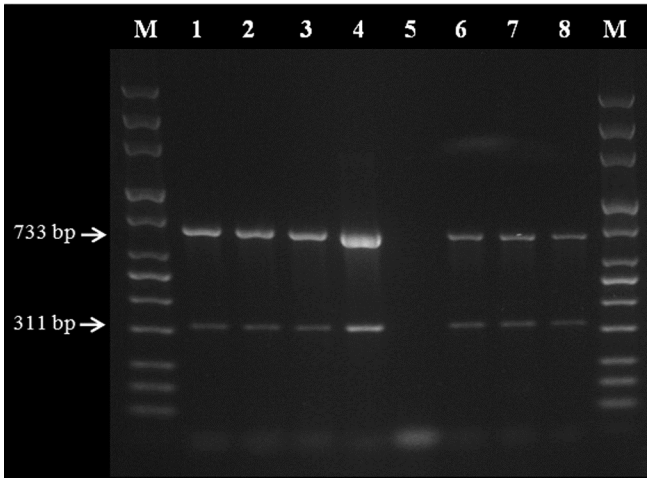

Fig. 5. Multiplex polymerase chain reaction amplification of DNA from symptomatic portions of naturally infected tissues of *Actinidia deliciosa* 'Hayward' (lanes 1–3) and *A. chinensis* 'JinTao' (lane 6–8); lane 4: purified DNA of *Pseudomonas syringae* pv. *actinidiae* strain 7286 as positive control; lane 5; negative control, no template DNA; M: molecular marker (SharpMass 100 plus; EuroClone).

gave the expected amplification pattern, giving additional information about their geographic origin.

At first, strain PSA92 may appear to be an exception because this strain was isolated in Italy in 1992 but gave the Japanese/Korean amplicon profile. However, this strain was isolated during a very limited outbreak in regard to both time and geography. Using a variety of different molecular methods, including whole-genome sequencing, this strain was previously found to be very similar to Japanese *P. syringae* pv. *actinidiae* strains (9,14,23,24,31,38) and, thus, was probably imported from Japan. After 1992, *P. syringae* pv. *actinidiae* was not reported anymore in Europe until the severe *P. syringae* pv. *actinidiae* outbreak in 2008.

The first primer pair was designed based on *hopZ3*, a gene which encodes a type III secreted effector involved in the pathogenesis of *P. syringae* (38). This gene is present and 100% identical in DNA sequence in all sequenced *P. syringae* pv. *actinidiae* genomes (6,22,24). *P. syringae* pv. *actinidiae hopZ3* allele sequence comparisons of all available *hopZ3* alleles allowed the design of a primer pair specific to *P. syringae* pv. *actinidiae*. That this primer pair amplifies a fragment of the expected size from all *P. syringae* pv. *actinidiae* strains isolated from multiple locations around the world from 1984 to today confirms that this gene is conserved in all currently known *P. syringae* pv. *actinidiae* populations. On the other hand, no amplification was detected with the two *P. syringae* pv. *actinidiae* isolates (*P. syringae* pv. *actinidiae*-LV) from New Zealand that only cause mild foliar symptoms similar to *P. syringae* pv. *syringae* but do not cause any cankers (9). Other *Pseudomonas* taxa also tested negative, confirming the specificity of the developed assay. This suggests that the *hopZ3* primers could be used alone in a PCR assay to detect *P. syringae* pv. *actinidiae* or distinguish *P. syringae* pv. *actinidiae* strains from other pseudomonads.

The other primers were designed from a genomic island that has a gene content and gene order similar to the PPHGI-1 island of *P. syringae* pv. *phaseolicola* (25) and which varies among European, Chinese, and New Zealand *P. syringae* pv. *actinidiae* strains (24). Although these populations are closely related, enough variation is present in the PPHGI-1-like islands among the sequenced strains to develop a PCR assay capable of distinguishing among them.

The results confirm and extend our previous finding that the *P. syringae* pv. *actinidiae* population in Europe is highly homogeneous and distinct from the ones in New Zealand and China (24). Furthermore, several independent m-PCR experiments on additional European isolates obtained since 2008 to date have constantly produced the correct European amplicons (*data not shown*). Not enough isolates are currently available to us to determine whether the *P. syringae* pv. *actinidiae* populations in China and in New Zealand are also homogeneous.

The described assay does not distinguish between the different genotypes known to exist in the Japanese and Korean *P. syringae* pv. *actinidiae* population. If necessary, one could use additional primers targeted to the genes that encode the toxins phaseolotoxin and coronatine, which distinguish the different genotypes in this population from each other (16,28). However, because Japanese and Korean *P. syringae* pv. *actinidiae* strains currently appear limited to these two countries and are not involved in the current worldwide canker of kiwifruit outbreaks, distinguishing among genotypes within the Japanese/Korean population is currently not a priority.

The limit of detection of *P. syringae* pv. *actinidiae* using the m-PCR assay was approximately 5×10^2 CFU ml^{-1} or 5 to 50 pg of purified DNA. This detection limit was independent of the genotype that was used as template. This could become very important in the hypothetical case of simultaneous infection by strains belonging to different populations.

The assay developed here has already proven to be reliable in detecting the pathogen and assigning it to a specific population directly from symptomatic kiwifruit tissues, making the test useful for testing field samples suspected of *P. syringae* pv. *actinidiae* infection directly without the need of going through time-consuming isolation steps. Having a routine analysis protocol for plant samples will permit technicians to precisely evaluate each single plant with suspect symptoms and prompt the farmers to enact immediately any measures to limit the spread of the pathogen or eradicate it. Possibly, the sensitivity of the method is even at a level sufficient to detect latent infections of the pathogen in vegetative tissue or at low levels in pollen, one of the suggested vehicles of spread of *P. syringae* pv. *actinidiae* (33,35), but this remains to be determined.

In conclusion, the newly developed m-PCR assay described here provides a means for rapid and highly discriminatory population-level identification of *P. syringae* pv. *actinidiae* from either isolated bacterial DNA, cultured bacteria, or DNA extracted from symptomatic kiwifruit leaves. The assay can be expected to benefit plant health and quarantine operations, facilitate epidemiological studies, and help development and application of intervention strategies to slow the spread of this recently emerged plant disease.

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