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Research Article

Identification of *Xanthomonas campestris* pv. *campestris* races 4 and 9 by Molecular Marker-Based Approach

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Abstract Black rot, a disease of significance affecting vegetable *Brassica* crops, is primarily caused by the bacterium *Xanthomonas campestris* pv. *campestris* (*Xcc*). When the disease spreads extensively in the field, it can lead to substantial yield losses, particularly under favorable environmental conditions. Controlling the spread of this disease is challenging, and the primary approach involves utilizing resistant cultivars or disease-free seeds. Among the various methods available for identifying different *Xcc* races, Polymerase Chain Reaction (PCR)-based molecular markers have proven to be highly reliable. To date, the PCR method has successfully identified *Xcc* races 1 to 7. In this study, molecular markers were developed for races 4 and 9 through the sequencing and alignment of the whole genome sequences of *Xcc* races, closely related *Xanthomonas campestris* (*Xc*) pathovars, and two *Xanthomonas* species. These designed markers were subsequently validated by PCR with bacterial genomic DNA samples from *Xcc* races and 7 other bacteria. The results indicated successful amplification only for race 4 and race 9, yielding amplicon sizes of 1080 bp and 830 bp, respectively, while the other strains failed to amplify. Furthermore, the amplicons from races 4 and 9 were cloned and sequenced, confirming that both races exhibited matching sequences after alignment. Consequently, the molecular marker method offers a rapid and efficient means of differentiating between *Xcc* races 4 and 9 within a few hours, presenting itself as a viable alternative to conventional methods that rely on the use of differential cultivars of *Brassicaceae* for identifying *Xcc* races.

Key words Black rot, Cabbage, Molecular marker, *Xanthomonas campestris* pv. *campestris*, PCR

Introduction

Xanthomonas campestris pathovar *campestris* (*Xcc*) is a small, aerobic, rod-shaped, and Gram-negative bacterium, which causes the black rot disease in cabbage and other *Brassica* vegetables. It is considered the most serious disease that infects almost all the important cruciferous crops such as cabbage, cauliflower, broccoli, brussels sprouts, kohlrabi, and kale as well as the model plant *Arabidopsis* (An ShiQi et al. 2011; Soengas et al. 2007; Vicente et al. 2001). *Xcc* enters the plants through the hydathodes and wounded leaves and develops V-shaped yellow lesions with necrotic and blackening veins at the leaf margins (Cook et al. 1952; Williams 1981). In Korea, *Xcc* is one of the most damaging diseases in cabbage-growing fields (Kim 1986).

Conventional approaches for identifying the causal agent of plant diseases have traditionally involved the utilization of visual symptom assessment, microscopy techniques, and culturing methods (Ward et al. 2004). Nevertheless, reliance on these straightforward, cost-effective, and time-consuming methods can sometimes lead

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to erroneous conclusions, particularly when there are striking similarities between disease symptoms and the morphological characteristics of pathogens. This inherent limitation highlights the heavy dependence of these methods on prior knowledge and experimental expertise (Nezhad 2014). Up to now, *Xcc* races have been classified into eleven races based on race determination using the differential cultivars method (Cruz et al. 2017; Fargier et al. 2007; Kamoun et al. 1992; Vicente et al. 2001). Among eleven races, races 1 and 4 are predominant in *Brassica oleracea* whereas race 6 is predominant in *Brassica rapa* (Lema et al. 2012; Vicente et al. 2013). Although race 9 of *Xanthomonas* is not predominant in every part of the world, the recent development in the seed trade between the countries poses a potential threat of spreading the race to other countries. Therefore, it is important to detect the presence of these races. However, the identification of *Xcc* races based on differential cultivars is time-consuming and labor-intensive. Furthermore, the process of culturing pathogens for identification purposes is time-consuming and, in some cases, not feasible due to the inability to culture certain pathogens (Cardenas et al. 2008; Rinke et al. 2013).

The rapid advancements in molecular biotechnology have carried out the way for the application of rapid and dependable detection methods, including PCR (Ward et al. 2004). The development of race-specific molecular markers with PCR-based techniques is very effective, quick, and simple. Molecular identification of pathogens through PCR saves time (within a few hours) and is less laborious for detecting plant pathogenic bacterial races (Song et al. 2014). The emergence of Next-Generation Sequencing (NGS), has introduced a pioneering approach to diagnostics, and methods for detecting and identifying phytopathogens (Chalupowicz et al. 2019). These advancements have paved the way for DNA-based NGS, which encompasses many steps including variant/mutation annotation and interpretation (Qin 2019). Various variations, including single nucleotide polymorphisms (SNPs), insertions and deletions (INDELs), and structural variations can be discovered using the population genomics datasets based on NGS (Potgieter et al. 2020). These variations can be exploited to design molecular markers and can be used for plant pathogen diagnosis (Afrin et al. 2020; Rubel et al. 2019a). Many researchers have used PCR-based molecular markers to identify bacterial and fungal pathogens. Two (Sequence Characterized Amplified Region) SCAR markers were developed to detect the fungus *Fusarium oxysporum* f. sp. *melonis* race 2, caused vascular wilt of cucurbits (Luongo et al. 2012). The new *Xanthomonas oryzae* pv. *oryzae* K3a in rice plants was identified using a race-specific marker-based PCR technique (Song et al. 2014). Race 3 of *Pseudomonas syringae* pv. *phaseolicola* in halo blight of common bean was identified by PCR-based molecular markers (Schaad et al. 1995). This study aimed to develop a specific molecular marker for the identification of *Xcc* races 4 and 9.

Materials and Methods

Bacterial strains and culture conditions

The 16 bacterial strains were used in this study, including nine *Xcc* races (races 1-9); three *Xc* pathovars: *X. campestris* pv. *incanae* (*Xci*), *X. campestris* pv. *raphani* (*Xcr*), and *X. campestris* pv. *zinniae* (*Xcz*); two *Xanthomonas* species: *X. axonopodis* pv. *dieffenbachiae* (*Xad*), and *X. campestris* pv. *vesicatoria* (*Xcv*) and two other plant pathogenic

bacteria: *Pseudomonas syringae* pv. *maculicola* (*Psm*), and *Erwinia carotovora* subsp. *carotovora* (*Ecc*) (Table 1). All the bacterial isolates were grown on King's B medium (KB) for 48 hours at 30℃ (King et al. 1954).

Table 1. List of bacterial strains used in this study.

SL.	Bacterial Strains	Races	Host	Country	Collection Year	References
1	*X. campestris* pv. *campestris* (HRIW-3811)	1	*B. oleracea*	US	2017	Vicente et al. (2001)
2	*X. campestris* pv. *campestris* (HRIW-3849A)	2	*B. oleracea* var. *botrytis*	US	2017	
3	*X. campestris* pv. *campestris* (HRIW-5212)	3	*B. oleracea* var. *gemmifera*	UK	2017	
4	*X. campestris* pv. *campestris* (HRIW-1279A)	4	*B. oleracea* var. *capitata*	UK	2017	
5	*X. campestris* pv. *campestris* (HRIW-3880)	5	*B. oleracea* var. *capitata*	Australia	2017	
6	*X. campestris* pv. *campestris* (HRIW-6181)	6	*B. rapa*	Portugal	2017	
7	*X. campestris* pv. *campestris* (HRIW-8450A)	7	*B. oleracea* var. *capitata*	UK	2017	
8	*X. campestris* pv. *campestris* (MBG-145.3)	8	*B. rapa*	Spain	2017	Lema et al. (2012)
9	*X. campestris* pv. *campestris* (NCPPB-1145)	9	-	UK	2022	NCPPB
10	*X. campestris* pv. *incanae* (WHRI-6377)	-	*Matthiola incana*	UK	2017	Vicente et al. (2001)
11	*X. campestris* pv. *raphani* (WHRI-8305)	2	*B. rapa* var. *perviridis*	UK	2017	
12	*X. campestris* pv. *zinniae* (KACC17126)	-	*Zinnia elegans*	South Korea (Suwon)	2017	KACC
13	*X. axonopodis* pv. *dieffenbachiae* (KACC17821)	-	*Anthurium andreanum*	South Korea (Yongin)	2017	
14	*X. campestris* pv. *vesicatoria* (KACC11153)	-	-	South Korea	2017	
15	*Pseudomonas syringae* pv. *maculicola* (ICMP13051)	-	*B. oleracea* var. *capitata*	New Zealand	2016	ICMP
16	*Erwinia carotovora* subsp. *carotovora* (ICMP12464)	-	*B. oleracea* var. *capitata*	New Zealand	2016	

Note: NCPPB-The National Collection of Plant Pathogenic Bacteria, KACC- Korean Agriculture Culture Collection, Jeollabuk-do, Korea; ICMP-International Collection of Microorganisms from Plants, Auckland, New Zealand; HRI-W-Horticulture Research International, Wellesbourne, UK

Extraction of bacterial genomic DNA

The bacterial DNA was extracted from 16 bacterial strains using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. The concentration and quality of all the DNA after extraction were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Then, the DNA was stored at -20℃ for further experiments.

Whole genome re-sequencing and alignment of *Xcc* races

The whole genome sequences of *Xcc* races: race 1 (B100 and CFBP1869), race 3 (ATCC33913), race 4 (CFBP5817), race 9 (str.8004), and *Xci* (CFBP1606R), *Xcr* (str.756C) and *Xcv* (str.85-10) were taken from the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). The genomes of races 2, 5, 6, 7, and 8 were sequenced and aligned with the available genome sequences mentioned above using the Integrative Genomics Viewer (IGV) (https://software.broadinstitute.org/software/igv/) to identify the variant regions.

Primer designing and PCR conditions

The flanking DNA sequences near the variant regions were used to design primers using Primer3 (https://primer3.ut.ee/), thereafter twenty primer pairs were designed (Supplementary Table S1). PCR was performed with a 10 μL reaction mixture containing 1 μL (30 ng μL^{-1}) of DNA, 0.5 μL of each 10 pmol forward and reverse primers, 5 μL of 2X Prime Taq Premix (GenetBio, Daejeon, Korea) and 3 μL of sterile distilled water. The PCR conditions for race 4 and race 9-specific markers were adjusted with denaturation at 94℃ for 2 minutes followed by 30 cycles (94℃ for 20 seconds, 70℃ for 30 seconds, and 72℃ for 20 seconds) and terminated by a final elongation at 72℃ for 2 minutes, 25℃ overnight (Supplementary Table S1). The PCR products were analyzed with gel electrophoresis using 1.5% agarose at 100 V for 30 minutes and visualized with a gel documentation system under UV light (320 nm). The size of PCR products was determined using HiQ 100 bp DNA ladder. Additionally, reported primers for specific amplification of *Xcc* races 1-7 (Supplementary Table S1) were used for the validation using *Xcc* race 9 DNA.

Evaluation of the sensitivity and specificity of primers

To evaluate the sensitivity and specificity of 'XccR9-2F2-2R1' primer, 30 ng μL^{-1} DNA of races 4 and 9 was taken and serially diluted up to 10^{-4} dilution (30 ng μL^{-1}, 3 ng μL^{-1}, 0.3 ng μL^{-1}, 0.03 ng μL^{-1}, 0.003 ng μL^{-1}). Thereafter, 1 μL of DNA from each dilution was used for PCR amplification using the PCR conditions described above.

Cloning and sequencing of races 4 and 9

The PCR amplicons of *Xcc* races 4 and 9 using the 'XccR9-2F2-2R1' primer were further cloned and sequenced. The visible bands were excised from the agarose gels (0.8%) and the amplicons were purified in mini-columns using the Wizard SV gel and PCR cleanup system (Promega, Madison, WI, USA). The amplicons were cloned into *E. coli* (DH5α) using the TOPcloner blunt kit (Enzynomics, Daejeon, Korea) following the manufacturer's instructions. Thereafter, the single colonies grown overnight on solid Luria Bertani (LB) media containing ampicillin (1 mg mL^{-1}) were picked. Subsequently, these selected colonies were cultured in liquid LB media to isolate plasmid DNA. The plasmid DNA isolations were purified with QIAprep Spin Miniprep Kit (50). The alignment of cloned sequences was performed with Multiple Sequence Alignment by ClustalW online (https://www.geno

me.jp/tools-bin/clustalw). Additionally, the cloned and sequenced amplicons were subjected to ORF prediction using NCBI tool ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The predicted ORF sequences were searched for protein homology in the NCBI database using the Blastp tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).

Bio-PCR assay

The susceptible cabbage lines were artificially inoculated with strains of *Xcc* races 1 to 9. Three plants for each race were inoculated at 35 days after sowing, and the three youngest leaves were inoculated using the clipping method followed by dipping into a bacterial suspension (10^8-10^9 CFU/mL) of *Xcc* races (Vicente et al. 2001). After 2 weeks, when the symptoms appeared on the leaves, three infected leaves were collected for bio-PCR assay. Afterward, the leaves with black rot symptoms were cut about 1 cm, sliced into small pieces, and soaked in 2 mL of sterile water for 1 hour at room temperature. Following this, 1 mL of bacterial ooze was transferred into a 1.5 mL microcentrifuge tube and incubated at 65℃ for 10 min. Eventually, 5 μL of the exudates were used for the bio-PCR assessment with the PCR condition stated above.

Results

Identification of variant regions and primer designing

The comparison of whole genome sequences, including *Xcc* races (1-9), two *Xc* pathovars (*Xci* and *Xcr*), and one other *Xanthomonas* species (*Xcv*), and the alignment of these available complete genome sequences allowed us to identify the variant regions (Fig. 1). Variant regions specific to *Xcc* race 4 and race 9 were identified using the Integrative Genomics Viewer (IGV) tool. Flanking genomic sequences of these variants were then utilized to initially design twenty pairs of primers to detect the *Xcc* races 4 and 9 (Supplementary Table S1). Out of these 20 pairs of primers, only one primer pair namely 'XccR9-2F2-2R1' (forward primer sequence-CGAACAGCAAAGGCAGATACAG; reverse primer sequence - CATGCGCATAGCGGCCCGCCTTG) able to differentiate *Xcc* races 4 and 9 from *Xcc* races 1-8, *Xc* pathovars, *Xanthomonas* species and other plant pathogenic bacteria. None of the markers developed for *Xcc* race 1-7 gave a specific reported amplicon with *Xcc* race 9 DNA (Figs. 2b-2h).

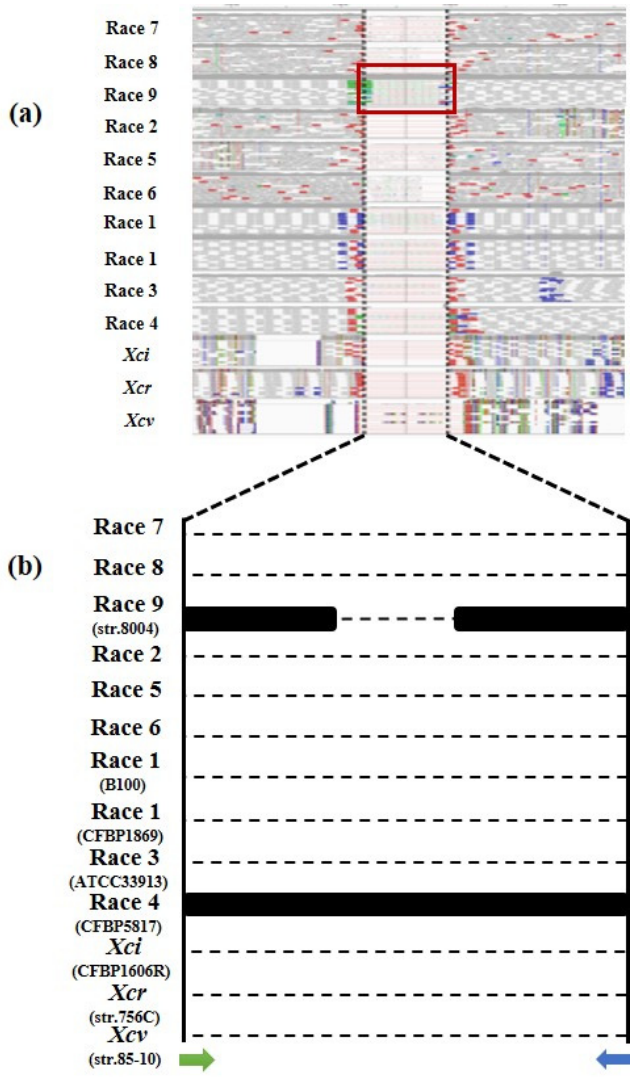

Fig. 1. a) Alignment of the whole genome sequences of *Xcc* races (1-9), two other *Xc.* pathovars (*incanae* and *raphani*), and another *Xanthomonas* species (*X. campestris* pv. *vesicatoria*); b) Line diagram representation of PCR amplification pattern with XccR9-2F2-2R1 primer; Dashed lines represent deletion region; Solid black bar represents similar genomic regions; Green arrow and blue arrows represent forward and reverse primers, respectively.

Detection of *Xcc* races 4 and 9 using 'XccR9−2F2−2R1' primer

In this study, we developed a marker for early and quick detection of *Xcc* race 4 and race 9. The designed primers were validated with bacterial DNA of *Xcc* races, *Xc* pathovars, *Xanthomonas* species, and other plant pathogenic bacteria by PCR amplifications (Table 1). Among twenty designed primers, only one primer pair 'XccR9-2F2-2R1' was able to differentiate the *Xcc* race 9 with an amplicon size of 830 bp (Fig. 2i and Supplementary Fig. S1). Interestingly this primer gave an amplicon size of 1080 bp with *Xcc* race 4 DNA whereas all the other *Xcc* races (races 1, 2, 3, 5, 6, 7, and 8), *Xc* pathovars (*Xci*, *Xcr*, and *Xcz*), *Xanthomonas* species (*Xad* and *Xcv*), and other plant pathogenic bacteria (*Psm* and *Ecc*) used in this study were unamplified (Fig. 2i). Despite this,

the primer pair 'XccR9-2F2-2R1' amplified an amplicon in *Xcc* race 4 and race 9, but it was able to differentiate between both races (Fig. 2i). Therefore, the primer pair 'XccR9-2F2-2R1' can be used as a diagnostic molecular marker for detecting both races 4 and 9.

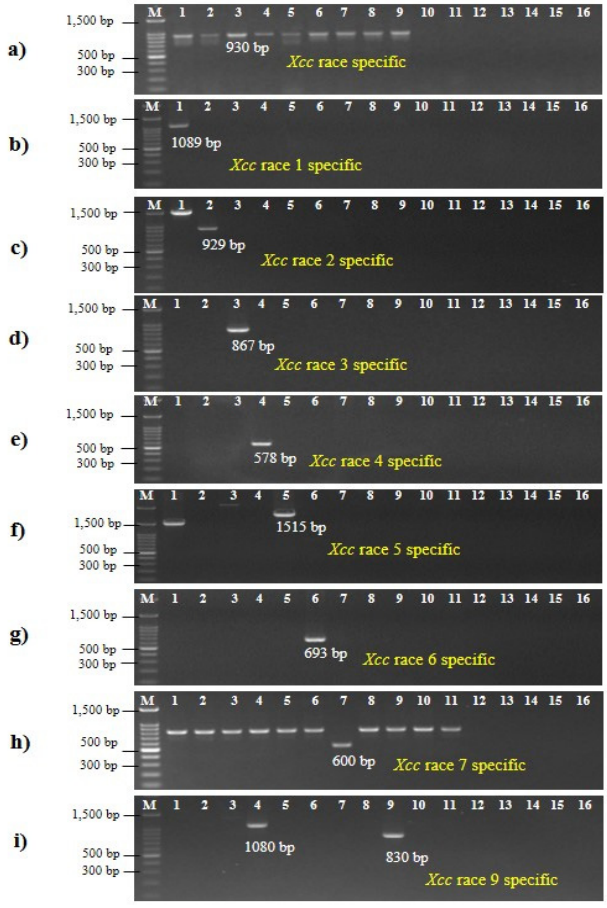

Fig. 2. Agarose gel electrophoresis of PCR products of *Xcc* race 4 and race 9 DNA using race a 9-specific primer, and *Xcc* race specific primer and specific primer sets of *Xcc* races 1-7 reported by previous studies. a) *Xcc* specific (Xcc-53, 930-bp); b) *Xcc* race 1-specific (Xcc-47R1, 1089-bp); c) *Xcc* race 2-specific (Xcc-R2-89-2, 929-bp); d) *Xcc* race 3-specific (XccR3-49, 867-bp); e) *Xcc* race 4-specific (Xcc2-46R4, 578-bp); f) *Xcc* race 5-specific (XccR5-89.2, 1515-bp); g) *Xcc* race 6-specific (XccR6-60, 693-bp); h) *Xcc* race 7-specific (Race 7-1F-1R, 600-bp); i) *Xcc* race 9-specific (XccR9-2F2-2R1, 830-bp) amplified from genomic DNA of *Xcc* races, *Xc.* pathovars and other plant pathogenic bacteria. Lane M: DNA ladder-100 bp; Lanes 1-9: *Xcc* races (1-9); Lane 10: *X. campestris* pv. *incanae* (WHRI-6377); Lane 11: *X. campestris* pv. *raphani* (WHRI-8305); Lane 12: *X. campestris* pv. *zinniae* (KACC17126); Lane 13: *X. axonopodis* pv. *dieffenbachiae* (KACC17821); Lane 14: *X. campestris* pv. *vesicatoria* (KACC11153); Lane 15: *Pseudomonas syringae* pv. *maculicola* (ICMP13051); Lane 16: *Erwinia carotovora* subsp. *carotovora* (ICMP12464).

Marker sensitivity

We further conducted tests to evaluate the efficiency and sensitivity of the newly developed marker 'XccR9-2F2-2R1'. Remarkably, this marker successfully detected DNA from *Xcc* race 4 and race 9 even at a very low concentration of 0.003 ng μL^{-1}(Fig. 3).

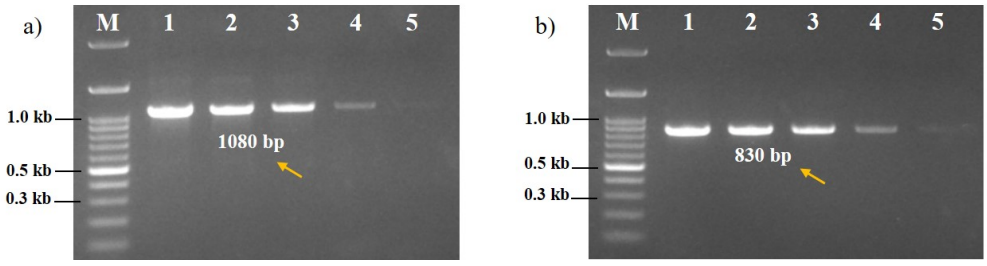

Fig. 3. Efficient detection of different rates of DNA concentrations by PCR amplification using XccR9-2F2-2R1 primer. a) Detection of genomic DNA of race 4; b) Detection of genomic DNA of race 9. Lane M: DNA ladder (100-bp); Lane 1: 30 ng μL^{-1}; Lane 2: 3 ng μL^{-1}; Lane 3: 0.3 ng μL^{-1}; Lane 4: 0.03 ng μL^{-1}; Lane 5: 0.003 ng μL^{-1}.

Cloning and sequencing

Cloning and sequencing of the PCR amplicons produced by the 'XccR9-2F2-2R1' primer in races 4 and 9 DNA revealed a 190 bp deletion in race 9 when compared to race 4. Therefore, this marker can be used proficiently to differentiate race 4 and race 9 with amplicon sizes of 830 bp and 1080 bp, respectively (Supplementary Figs. S2a, S2b).

The cloned and sequenced amplicons were subjected to Open Reading Frame (ORF) prediction using NCBI tool ORF Finder. The number of ORFs found in race 9 and race 4 cloned fragments was six and nine, respectively. The largest ORF found was 843 nucleotides and 630 nucleotides in length and encoded for 280 amino acids and 206 amino acids in race 4 and race 9, respectively (Supplementary Figs. S2c, S2d). This protein has significant homology with the protein-encoding IS5 transposase family, but the protein sequence of the race 9 amplicon has a 71 bp deletion when compared to the protein sequence of the race 4 amplicon (Fig. S2d).

Bio-PCR assessment

A bio-PCR assay was conducted to validate the competency of the developed markers in detecting the presence of *Xcc* races 4 and 9 from infected leaves. Cabbage leaves were artificially infected with various strains of *Xcc* races (1-9) strain. None of the samples infected with *Xcc* races 1, 2, 3, 5, 6, 7, and 8 showed amplification, whereas races 4 and 9 yielded amplicon sizes of 1080 bp and 830 bp, respectively. Therefore, the results demonstrated amplification with a base-pare size equivalent to positive control DNA (gDNA of *Xcc* race 4 and race 9) (Fig. 4). This finding affirmed the potential of the novel developed marker that can directly detect the bacterial ooze from soaking leaves of cabbage without the genomic DNA extraction of bacteria within a few hours with an accurate and reliable identification.

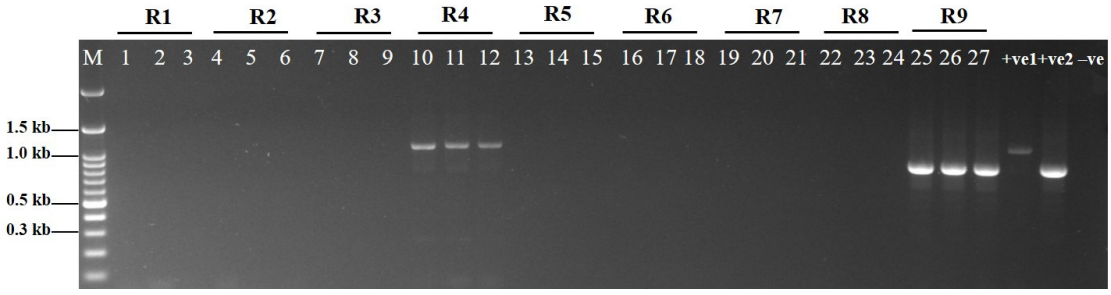

Fig. 4. Bio-PCR assessment using race 9-specific primer (XccR9-2F2-2R1) to detect 27 samples from infected cabbage leaves after inoculation with strains of *Xcc* races 1-9. Lane M: DNA ladder (100-bp); Lane 1-3: race 1; Lane 4-6: race 2; Lane 7-9: race 3; Lane 10-12: race 4; Lane 13-15: race 5; Lane 16-18: race 6; Lane 19-21: race 7; Lane 22-24: race 8; Lane 25-27: race 9; +ve1: positive control of gDNA of race 4, +ve2: positive control of gDNA of race 9; -ve: negative control of distilled water.

Discussion

The establishment of races and differentiation of plant pathogens is associated with the presence of *Avr* genes in pathogens and its interaction with the corresponding R gene in the plant system, which became the basis of race differentiation using the differential cultivars. However, the system of race differentiation using differential cultivars requires rigorous efforts in growing plants, microbial culturing, optimization of culturing conditions, and skilled personnel. On the other hand, the use of molecular diagnostics of plant pathogens is less tedious. Identifying the race of a plant pathogen is an important criterion for the management of the disease. There have been various reports on the molecular diagnosis of plant-pathogenic bacteria and fungi (Hariharan et al. 2021; Tewari et al. 2019). The utilization of race-specific molecular markers in this study facilitated rapid, reliable, and precise identification of *Xcc* race 4 and race 9. The availability of genomic sequences for *Xcc* races (races 1- 9) and other *Xc* pathovars (*Xci*, *Xcr*, and *Xcv*) simplified the identification of highly variable genomic regions and the development of specific markers for race 4 and race 9 (Fig. 1). Alignment of genome sequences provides useful data about the variants. However, these variants need to be analyzed and interpreted with the help of certain tools. IGV is one of the tools for the visualization and interpretation of variants (Robinson et al. 2017). This study also used the IGV software for variant calling to design the specific primers. The previously developed marker by Rubel et al. (2017) specifically detected *Xcc* race 4 (Fig. 2e, Supplementary Table S1), whereas our marker provides the advantage of simultaneous detection of *Xcc* races 4 and 9. The race-specific markers developed in this study provided rapid and effective tools, requiring minimal labor and offering high reliability, for specifically detecting and differentiating races 4 and 9 from other *Xcc* races. In contrast, the cultivar-based race determination method requires extensive fieldwork and additional labor. Our 'XccR9-2F2-2R1' primer displayed the ability to identify *Xcc* races 4 and 9 through PCR amplification even at very low concentrations of genomic DNA of races 4 and 9, highlighting the high efficiency and sensitivity of the marker quality (Fig. 3).

Moreover, PCR-based molecular marker techniques have been successfully employed for the detection of bacterial and fungal pathogens. *Xanthomonas* pathovars were detected in *Brassica* seeds and plants, and *X. campestris*

was specifically distinguished from other *Xanthomonas* using PCR (Berg et al. 2005). In Korea, Rubel et al. (2017) developed specific markers using SCAR for the detection of *Xcc* races 1 and 4. Wang et al. (2010), in China, reported highly reliable PCR-based SCAR markers for detecting wheat stripe rust races CYR32 and CYR33 caused by *Puccinia striiformis* f. sp. *tritici*. Pasquali et al. (2007), successfully employed an inter-retrotransposon sequence-characterized amplified region (IR-SCAR) marker to identify race 1 of *F. oxysporum* f. sp. *lactucae* in lettuce.

PCR-based methods have also proven effective in identifying other pathogens, including race 3 of *Pseudomonas syringae* pv. *phaseolicola* in halo blight of common bean (Schaad et al. 1995), *Xcc* in black rot disease (Singh et al. 2014), *Xoo* in rice bacterial blight (Song et al. 2014), and *Colletotrichum* spp. causing anthracnose disease in sweet persimmon (Iee et al. 2002). Therefore, this PCR-based molecular marker holds promise for the rapid identification of *Xcc* race 4 and race 9 in cabbage fields affected by black rot disease caused by *Xcc* worldwide.

Insertion sequences (IS) and transposons play a significant role in bacterial pathogenicity and evolution, including *Xanthomonas*, by facilitating genome rearrangement (Ferreira et al. 2015). In the case of *Xanthomonas*, it has been reported that IS elements might potentially introduce inversions and rearrangements, thereby contributing to race diversity (Ochiai et al. 2005). In this study, we conducted sequence analysis of the amplicons from race 4 and race 9 using the 'XccR9-2F2-2R1' marker, which indicated the presence of transposases belonging to the IS5 family. However, the presence of a complete gene in race 4 likely aided its adaptation through genome rearrangement, and further studies are required to confirm the role of this gene.

In conclusion, this study developed a molecular marker-based PCR amplification for the specific identification of races 4 and 9 through the realignment of whole genome sequences of *Xcc* races (1-9), two *Xanthomonas* species (*Xci* and *Xcr*), and one other *Xc* pathovar (*Xcv*). The newly developed marker (XccR9-2F2-2R1) illustrated a quick and reliable detection of race 4 and race 9 among various bacterial strains such as *Xcc* races, *Xanthomonas* species, and other plant pathogenic bacteria. Therefore, to our knowledge, this novel marker proved as a useful and rapid diagnostic tool for accurate and reliable detection of *Xcc* races 4 and 9. Additionally, this marker can be used as an alternative method compared to a traditional one using differential cultivars of *Brassicas* for race identification, which is time-consuming and labor-intensive.

Acknowledgments

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References

1. Afrin KS, Rahim MA, Rubel MH, Natarajan S, Song J-Y, Kim H-T, et al. 2018. Development of race-specific molecular marker for Xanthomonas campestris pv. campestris race 3, the causal agent of black rot of crucifers. Can J Plant Sci. 98: 1119-1125.
2. Afrin KS, Rahim MA, Jung H-J, Park J-I, Kim H-T and Nou I-S. 2019. Development of molecular marker through genome realignment for specific detection of Xanthomonas campestris pv. campestris race 5, a pathogen of black rot disease. J Microbiol Biotechnol. 29: 785-793.
3. Afrin KS, Rahim MA, Rubel MH, Park J-I, Jung H-J, Kim H-T, et al. 2020. Development of PCR-based molecular marker for detection of Xanthomonas campestris pv. campestris Race 6, the causative agent of black rot of Brassicas. The Plant Pathol J. 36: 418-427.
4. An ShiQi AS, Lu GuangTao LG, Su HuiZhao SH, Li RuiFang LR, He YongQiang HY, Jiang BoLe JB, et al. 2011. Systematic mutagenesis of all predicted gntR genes in Xanthomonas campestris pv. campestris reveals a GntR family transcriptional regulator controlling hypersensitive response and virulence. Mol Plant-Microbe Interact. 24: 1027-1039.
5. Berg T, Tesoriero L, Hailstones D. 2005. PCR-based detection of Xanthomonas campestris pathovars in Brassica seed. Plant Pathol. 54: 416-427.
6. Cardenas E, Tiedje JM. 2008. New tools for discovering and characterizing microbial diversity. Curr Opin Biotechnol. 19: 544-549.
7. Chalupowicz L, Dombrovsky A, Gaba V, Luria N, Reuven M, Beerman A, et al. 2019. Diagnosis of plant diseases using the Nanopore sequencing platform. Plant Pathol. 68: 229-238.
8. Cook A, Walker J, Larson R. 1952. Studies on the disease cycle of black rot of crucifers. Phytopathology. 42: 162-167.
9. Cruz J, Tenreiro R, Cruz L. 2017. Assessment of diversity of Xanthomonas campestris pathovars affecting cruciferous plants in Portugal and disclosure of two novel X. campestris pv. campestris races. J Plant Pathol. 99: 403-414.
10. Fargier E, Manceau C. 2007. Pathogenicity assays restrict the species Xanthomonas campestris into three pathovars and reveal nine races within X. campestris pv. campestris. Plant Pathol. 56: 805-818.
11. Ferreira RM, de Oliveira ACP, Moreira LM, Belasque Jr J, Gourbeyre E, Siguier P, et al. 2015. A TALE of transposition: Tn 3-like transposons play a major role in the spread of pathogenicity determinants of Xanthomonas citri and other xanthomonads. MBio. 6: 10-1128.
12. Hariharan G, Prasannath K. 2021. Recent advances in molecular diagnostics of fungal plant pathogens: a mini review. Front Cell Infect Microbiol. 10: 600234.
11. Iee S-P, Lee Y-S. 2002. Development of rapid molecular detection marker for Colletotrichum spp. in leaf and fruit tissues of sweet persimmon. J Microbiol Biotechnol. 12: 989-992.
13. Kamoun S, Kamdar HV, Tola E and Kado CI. 1992. A vascular hypersensitive response: role of the hrpK locus. Mol Plant Microbe Interact. 5: 22-33.
14. Kim BS. 1986. Testing for Detection of Xanthomonas campestris pv. campestris in crucifer seeds and seed disinfection. Korean J Plant Pathol. 2: 96-101.
15. Kim Y-H, Mao S, Sahu N, Somaddar U, Kim H-T, Watanabe M, et al. 2023. Molecular marker development for the rapid differentiation of black rot causing Xanthomonas campestris pv. campestris race 7. Plant Pathol J. 39: 494.
16. King EO, Ward MK, Raney DE. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J Lab Clin Med. 44: 301-307.
17. Lema M, Cartea ME, Sotelo T, Velasco P, Soengas P. 2012. Discrimination of Xanthomonas campestris pv. campestris races among strains from northwestern Spain by Brassica spp. genotypes and rep-PCR. Eur J Plant Pathol. 133: 159-169.
18. Luongo L, Vitale S, Haegi A, Belisario A. 2012. Development of SCAR markers and PCR assay for Fusarium oxysporum f. sp. melonis race 2-specific detection. J Plant Pathol. 94: 193-199.
19. Nezhad AS. 2014. Future of portable devices for plant pathogen diagnosis. Lab Chip. 14: 2887-2904.
20. Ochiai H, Inoue Y, Takeya M, Sasaki A, Kaku H. 2005. Genome sequence of Xanthomonas oryzae pv. oryzae suggests contribution of large numbers of effector genes and insertion sequences to its race diversity. JARQ. 39: 275-287.
21. Pasquali M, Dematheis F, Gullino ML, Garibaldi A. 2007. Identification of race 1 of Fusarium oxysporum f. sp.

lactucae on lettuce by inter-retrotransposon sequence-characterized amplified region technique. Phytopathology. 97: 987-996.
22. Potgieter L, Feurtey A, Dutheil JY, Stukenbrock EH. 2020. On variant discovery in genomes of fungal plant pathogens. Front Microbiol. 11: 626.
23. Qin D. 2019. Next-generation sequencing and its clinical application. Cancer Biol Med. 16: 4-10.
24. Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng J-F, et al. 2013. Insights into the phylogeny and coding potential of microbial dark matter. Nature. 499: 431-437.
25. Robinson JT, Thorvaldsdóttir H, Wenger AM, Zehir A, Mesirov JP. 2017. Variant review with the integrative genomics viewer. Cancer Res. 77: 31-34.
26. Rubel MH, Natarajan S, Hossain MR, Nath UK, Afrin KS, Lee J-H, et al. 2019a. Pathovar specific molecular detection of Xanthomonas campestris pv. campestris, the causal agent of black rot disease in cabbage. Can J of Plant Pathol. 41: 318-328.
27. Rubel MH, Natarajan S, Nath UK, Denison MIJ, Hee-Jeong J, Hoy-Taek K, et al. 2019b. Development of a marker for detection of Xanthomonas campestris pv. campestris races 1 and 2 in Brassica oleracea. Hortic Environ Biotechnol. 60: 511-517.
28. Rubel MH, Robin AHK, Natarajan S, Vicente JG, Kim H-T, Park J-I, et al. 2017. Whole-genome re-alignment facilitates development of specific molecular markers for races 1 and 4 of Xanthomonas campestris pv. campestris, the cause of black rot disease in Brassica oleracea. Int J Mol Sci. 18: 2523.
29. Schaad NW, Cheong S, Tamaki S, Hatziloukas E, Panopoulos N. 1995. A combined biological and enzymatic amplification (BIO-PCR) technique to detect Pseudomonas syringae pv. phaseolicola in bean seed extracts. Phytopathology. 85: 243-246.
30. Singh D, Raghavendra B, Rathaur PS, Singh H, Raghuwanshi R, Singh R. 2014. Detection of black rot disease causing pathogen Xanthomonas campestris pv. campestris by bio-PCR from seeds and plant parts of cole crops. Seed Sci. Technol. 42: 36-46.
31. Soengas P, Hand P, Vicente J, Pole J, Pink D. 2007. Identification of quantitative trait loci for resistance to Xanthomonas campestris pv. campestris in Brassica rapa. Theor Appl Genet. 114: 637-645.
32. Song E-S, Kim S-Y, Noh T-H, Cho H, Chae S-C, Lee B-M. 2014. PCR-based assay for rapid and specific detection of the new Xanthomonas oryzae pv. oryzae K3a race using an AFLP-derived marker. J Microbiol Biotechnol. 24: 732-739.
33. Tewari S and Sharma S. 2019. Molecular techniques for diagnosis of bacterial plant pathogens. In *Microbial diversity in the genomic era*, Pp. 481-497. Elsevier.
34. Vicente JG, Conway J, Roberts S, Taylor J. 2001. Identification and origin of Xanthomonas campestris pv. campestris races and related pathovars. Phytopathology. 91: 492-499.
35. Vicente JG, Holub EB. 2013. X anthomonas campestris pv. campestris (cause of black rot of crucifers) in the genomic era is still a worldwide threat to brassica crops. Mol Plant Pathol. 14: 2-18.
36. Wang B, Hu X, Li Q, Hao B, Zhang B, Li G, et al. 2010. Development of race-specific SCAR markers for detection of Chinese races CYR32 and CYR33 of Puccinia striiformis f. sp. tritici. Plant Dis. 94: 221-228.
37. Ward E, Foster SJ, Fraaije BA, McCartney HA. 2004. Plant pathogen diagnostics: immunological and nucleic acid-based approaches. Ann Appl Biol. 145: 1-16.
38. Williams PH. 1981. Black rot: A continuing threat to world crucifers. Plant Dis. 64: 736-742.

Supplementary

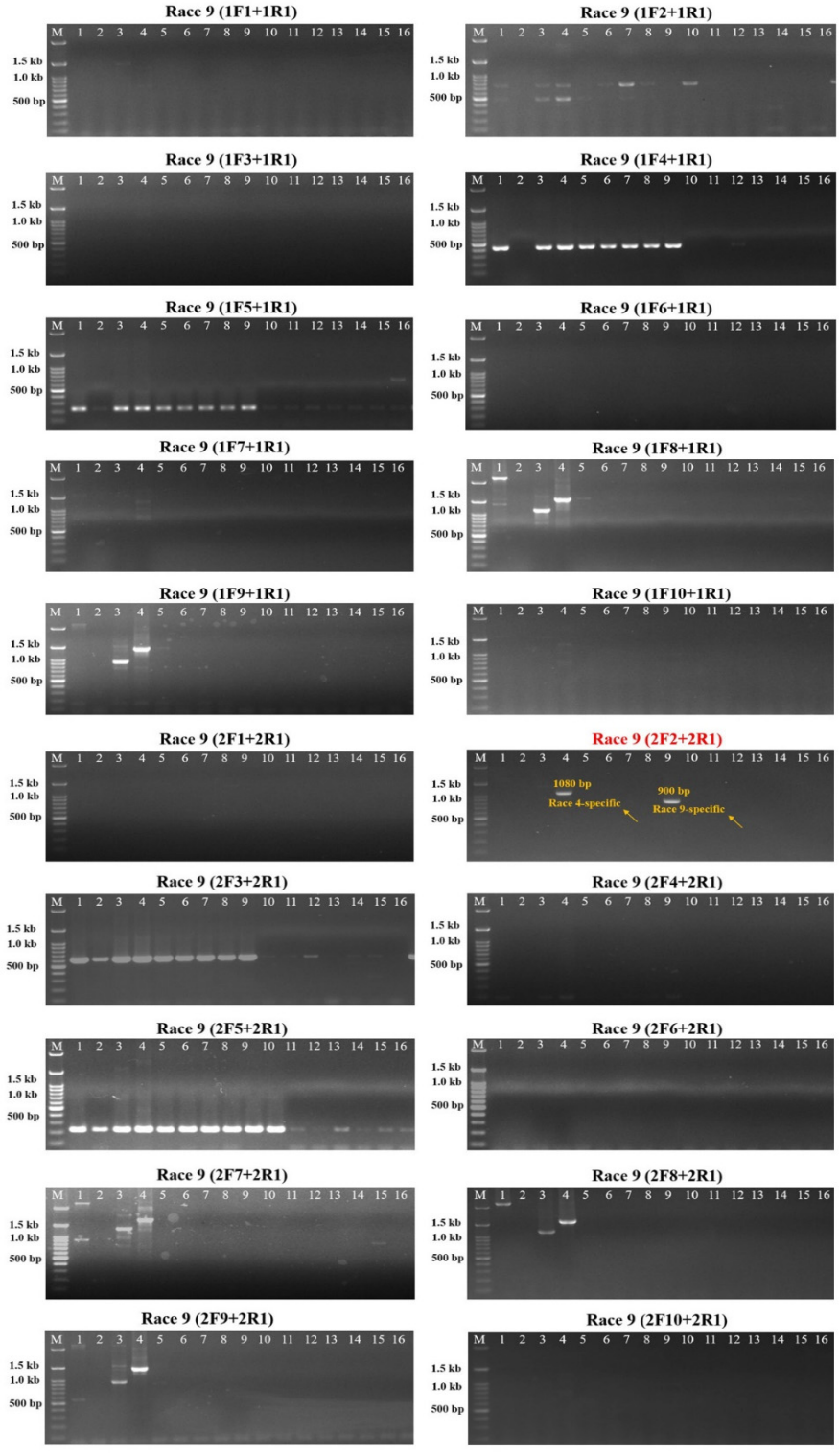

Fig. S1. PCR products on agarose gel using 20 primer pairs to differentiate races 4 and 9 to test among *Xcc* races and other bacterial strains.

a) Cloned and reference sequences of *Xcc* races 9 and 4

>*Xcc* race 9 (830 bp)

CGAACAGCAAAGGCAGATACAGCAAGAGCAGAGCCAACCACACCCTGCAAGGGCCTTGACCTGAAACTTGCAGTTCGACTTAGTCG
CCCCTGAAAAACCCCCAATCACCCAACGCCCGCAAGGCCTTGATGTCTGCAGTGGCGTGCCAGAACTACCAGTTTTCGCTACGCTG
AAGGCTGGTTTCTGGCAATTTCCACTCATGCATACACGCCGTCCTGCTGCCGAGCACATGCCTGCCGAGGAGTTGTTTCGTTCGCG
CCTGGAGAACCAGATCGATCTGCGGCATCCGCTGGCGCAGCTGAGCCAACGGATGCCGTGGACGGCGTTGGAGCAAGCACTTTCAT
CGCGCTTGCCGGCCACCCAGGCTGGTGGCGGTCGGCCGGCATTGCCGGTGCGGCTGATCGCCGGTTTGCTCTACCTCAAACACGCC
TACGACCTGTCCGATGAGGCGGTGTGCGAGCGTTGGCTGGAAAATCCGTATTGGCAGTTTTTCACTGGCGAGGTCGTGTTCCAGAC
GCGCTTGCCGTGCGATGCCAGCTCGCTGACGCGCTGGCGTCAGCGGCTTGACGAAGCGGGGATGGAAGAGTTGCTGGCACACACCA
TCAACGCTGCACATGCCATGCAGGCGGTGGACGCACGCGAGTTGTCGCGGGTGATCGTGGACACCACGGTGCAGGAAAAGGCGATT
GCCTATCCGACCGATAGTCGGTTGCTGGAGGTGGCACGCAAGAAACTGGTGCTGCTGGCCAAGCGTTACGGCATCGGGTTGCGGCA
GAGCTACGCACGGCAAGGTCCGGCCCTGAGCCG**CAAGGCGGGCCGCTATGCGCATG**

>*Xcc* race 4 (1080 bp)

CGAACAGCAAAGGCAGATACAGCAAGAGCAGAGCCAACCACACCCTGCAAGGGCCTTGACCTGAAACTTGCAGTTCGACTACATGC
CCCCTGCGTCCTACGCCTTCGATGGAGCAGACGGATGCCTATCAGATCACTGTCTGACAGTGGTTTGGTCTCACCCGCAACGGATG
TAATGCGGGTGTAGCCACCATGGTTCACGAAGCAATGTTTCGAGATTGCTGCACGTTGGCTGCATGCCAATCTCGCCAGCTGAAAG
CAAGGCAGTCTTGAAAATCGTGGGCAACGGTCAAGCCGATGCCCACAAGGCCCCGCAAGAACTACGTTATTTAGTCGCCCCTGAAA
AACCCCCAATCACCCAACGCCCGCAAGGCCTTGATGTCTGCAGCGGCGTGCCAGAACTACCAGTTTTCGCTACGCTGAAGGCTGGT
TTCTGGCAATTTCCACTCATGCATACACGCCGTCCTGCTGCCGAGCACATGCCTGCCGAGGAGTTGTTTCGTTCGCGCCTGGAGAA
CCAGATCGATCTGCGGCATCCGCTGGCGCAGCTGAGCCAACGGATGCCGTGGACGGCGTTGGAGCAAGCACTTTCATCGCGCTTGC
CGGCCACCCAGGCTGGTGGCGGTCGGCCGGCATTGCCGGTGCGGCTGATCGCCGGTTTGCTCTACCTCAAACACGCCTACGACCTG
TCCGATGAGGCGGTGTGCGAGCGTTGGCTGGAAAATCCGTATTGGCAGTTTTTCACTGGCGAGGTCGTGTTCCAGACGCGCTTGCC
GTGCGATGCCAGCTCGCTGACGCGCTGGCGTCAGCGGCTTGACGAAGCGGGGATGGAAGAGTTGCTGGCACACACCATCAACGCTG
CACATGCCATGCAGGCGGTGGACGCACGCGAGTTGTCGCGGGTGATCGTGGACACCACGGTGCAGGAAAAGGCGATTGCCTATCCG
ACCGATAGTCGGTTGCTGGAGGTGGCACGCAAGAAACTGGTGCTGCTGGCCAAGCGTTACGGCATCGGGTTGCGGCAGAGCTACGC
ACGGCAAGGTCCGGCCCTGAGCCG**CAAGGCGGGCCGCTATGCGCATG**

Letters in red - forward primer
Letters in green - reverse primer in reverse complimentary
Letters highlighted in blue - deletion part in race 9

b) Alignment of *Xcc* races 9 specific DNA fragments with reference race 4

```
RACE 9              CGAACAGCAAAGGCAGATACAGCAAGAGCAGAGCCAACCACACCCTGCAAGGGCCTTGAC
RACE 4              CGAACAGCAAAGGCAGATACAGCAAGAGCAGAGCCAACCACACCCTGCAAGGGCCTTGAC
                    ************************************************************

RACE 9              CTGAAACTTGCAGTTCGACT----------------------------------------
RACE 4              CTGAAACTTGCAGTTCGACTTACATGCCCCCTGCGTCCTACGCCTTCGATGGAGCAGACG
                    ********************

RACE 9              ------------------------------------------------------------
RACE 4              GATGCCTATCAGATCACTGTCTGACAGTGGTTTGGTCTCACCCGCAACGGATGTAATGCG
```

Fig. S2. Sequences analysis of Race9-2F2-2R1 fragment specific to *Xcc* race 9 with reference race 4; a) Cloned and reference sequences of race 9 and race 4; b) Alignments of race 9 specific DNA fragments with reference race 4; c) ORF prediction of race 4 and race 9 used primer 'Race9-2F2-2R1'; d) Alignment of ORFs from *Xcc* races 9 and 4 specific amplicons. Letters in red represent the forward primer; letters in green represent the reverse primer (reverse complement); ' - ' represents the deleted region; ' * ' means similar sequences.

```
RACE 9          ------------------------------------------------------------
RACE 4          GGTGTAGCCACCATGGTTCACGAAGCAATGTTTCGAGATTGCTGCACGTTGGCTGCATGC

RACE 9          ------------------------------------------------------------
RACE 4          CAATCTCGCCAGCTGAAAGCAAGGCAGTCTTGAAAATCGTGGGCAACGGTCAAGCCGATG

RACE 9          ------------------------------TAGTCGCCCCTGAAAAACCCCCAATCACCC
RACE 4          CCCACAAGGCCCCGCAAGAACTACGTTATTTAGTCGCCCCTGAAAAACCCCCAATCACCC
                                               ******************************

RACE 9          AACGCCCGCAAGGCCTTGATGTCTGCAGTGGCGTGCCAGAACTACCAGTTTTCGCTACGC
RACE 4          AACGCCCGCAAGGCCTTGATGTCTGCAGCGGCGTGCCAGAACTACCAGTTTTCGCTACGC
                ****************************.*******************************

RACE 9          TGAAGGCTGGTTTCTGGCAATTTCCACTCATGCATACACGCCGTCCTGCTGCCGAGCACA
RACE 4          TGAAGGCTGGTTTCTGGCAATTTCCACTCATGCATACACGCCGTCCTGCTGCCGAGCACA
                ************************************************************

RACE 9          TGCCTGCCGAGGAGTTGTTTCGTTCGCGCCTGGAGAACCAGATCGATCTGCGGCATCCGC
RACE 4          TGCCTGCCGAGGAGTTGTTTCGTTCGCGCCTGGAGAACCAGATCGATCTGCGGCATCCGC
                ************************************************************

RACE 9          TGGCGCAGCTGAGCCAACGGATGCCGTGGACGGCGTTGGAGCAAGCACTTTCATCGCGCT
RACE 4          TGGCGCAGCTGAGCCAACGGATGCCGTGGACGGCGTTGGAGCAAGCACTTTCATCGCGCT
                ************************************************************

RACE 9          TGCCGGCCACCCAGGCTGGTGGCGGTCGGCCGGCATTGCCGGTGCGGCTGATCGCCGGTT
RACE 4          TGCCGGCCACCCAGGCTGGTGGCGGTCGGCCGGCATTGCCGGTGCGGCTGATCGCCGGTT
                ************************************************************

RACE 9          TGCTCTACCTCAAACACGCCTACGACCTGTCCGATGAGGCGGTGTGCGAGCGTTGGCTGG
RACE 4          TGCTCTACCTCAAACACGCCTACGACCTGTCCGATGAGGCGGTGTGCGAGCGTTGGCTGG
                ************************************************************

RACE 9          AAAATCCGTATTGGCAGTTTTTCACTGGCGAGGTCGTGTTCCAGACGCGCTTGCCGTGCG
RACE 4          AAAATCCGTATTGGCAGTTTTTCACTGGCGAGGTCGTGTTCCAGACGCGCTTGCCGTGCG
                ************************************************************

RACE 9          ATGCCAGCTCGCTGACGCGCTGGCGTCAGCGGCTTGACGAAGCGGGGATGGAAGAGTTGC
RACE 4          ATGCCAGCTCGCTGACGCGCTGGCGTCAGCGGCTTGACGAAGCGGGGATGGAAGAGTTGC
                ************************************************************

RACE 9          TGGCACACACCATCAACGCTGCACATGCCATGCAGGCGGTGGACGCACGCGAGTTGTCGC
RACE 4          TGGCACACACCATCAACGCTGCACATGCCATGCAGGCGGTGGACGCACGCGAGTTGTCGC
                ************************************************************

RACE 9          GGGTGATCGTGGACACCACGGTGCAGGAAAAGGCGATTGCCTATCCGACCGATAGTCGGT
RACE 4          GGGTGATCGTGGACACCACGGTGCAGGAAAAGGCGATTGCCTATCCGACCGATAGTCGGT
                ************************************************************

RACE 9          TGCTGGAGGTGGCACGCAAGAAACTGGTGCTGCTGGCCAAGCGTTACGGCATCGGGTTGC
RACE 4          TGCTGGAGGTGGCACGCAAGAAACTGGTGCTGCTGGCCAAGCGTTACGGCATCGGGTTGC
                ************************************************************

RACE 9          GGCAGAGCTACGCACGGCAAGGTCCGGCCCTGAGCCGCAAGGCGGGCCGCTATGCGCATG
RACE 4          GGCAGAGCTACGCACGGCAAGGTCCGGCCCTGAGCCGCAAGGCGGGCCGCTATGCGCATG

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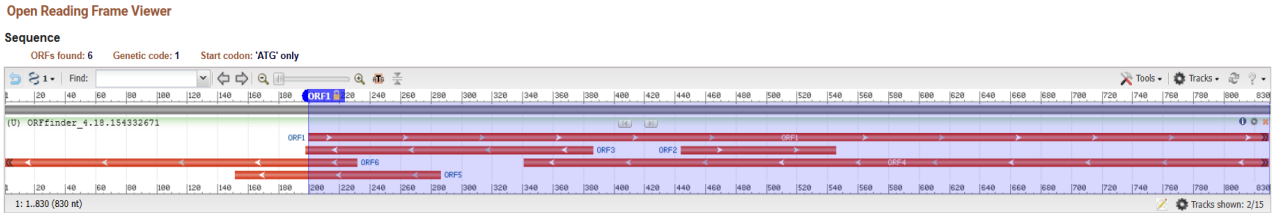
Fig. S2. Continued.

c) ORF prediction of race 4 and race 9 amplicons used primer 'Race 9-2F2-2R1'

> Race 9 used primer race 9 'Race 9-2F2-2R1'

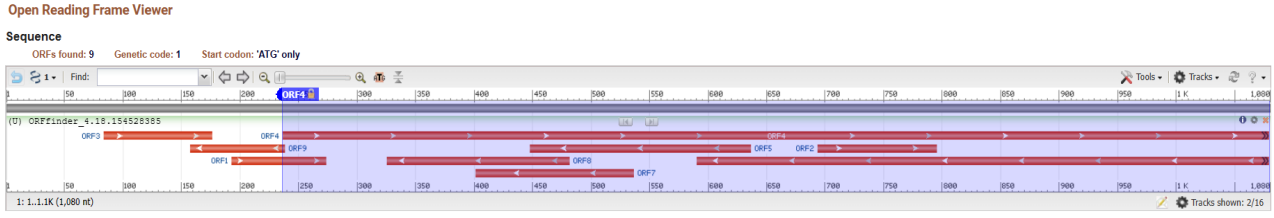


6 ORFs
ORF1

MRIAARLAAQGRTLPCVALPQPDAVTLGQQHQFLACHLQQPTIGRIGNRLFLHRGVHDHPRQLACVHRLHGMCSVDG
VCQQLFHPRFVKPLTPARQRAGIARQARLEHDLASEKLPIRIFQPTLAHRLIGQVVGVFEVEQTGDQPHRQCRPTATSLG
GRQAR

Protein - Partial IS5 transposase

>Race 4 used primer race 9 'Race 9-2F2-2R1'



9
ORFs
ORF4

MPISPAESKAVLKIVGNGQADAHKAPQELRYLVAPEKPPITQRPQGLDVCSGVPELPVFATLKAGFWQFPLMHTRRPAA
EHMPAEELFRSRLENQIDLRHPLAQLSQRMPWTALEQALSSRLPATQAGGGRPALPVRLIAGLLYLKHAYDLSDEAVCE
RWLENPYWQFFTGEVVFQTRLPCDASSLTRWRQRLDEAGMEELLAHTINAAHAMQAVDARELSRVIVDTTVQEKAIA
YPTDSRLLEVARKKLVLLAKRYGIGLRQSYARQGPALSRKAGRYAH

Protein - IS5 family transposase

d) Alignment of ORFs from *Xcc* races 9 and 4 specific amplicons

```
ORF_race9       ------------------------------------------------------------
ORF_race4       MPISPAESKAVLKIVGNGQADAHKAPQELRYLVAPEKPPITQRPQGLDVCSGVPELPVFA


ORF_race9       -----------MHTRRPAAEHMPAEELFRSRLENQIDLRHPLAQLSQRMPWTALEQALSS
ORF_race4       TLKAGFWQFPLMHTRRPAAEHMPAEELFRSRLENQIDLRHPLAQLSQRMPWTALEQALSS
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Fig. S2. Continued.

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ORF_race9       RLPATQAGGGRPALPVRLIAGLLYLKHAYDLSDEAVCERWLENPYWQFFTGEVVFQTRLP
ORF_race4       RLPATQAGGGRPALPVRLIAGLLYLKHAYDLSDEAVCERWLENPYWQFFTGEVVFQTRLP
                ************************************************************

ORF_race9       CDASSLTRWRQRLDEAGMEELLAHTINAAHAMQAVDARELSRVIVDTTVQEKAIAYPTDS
ORF_race4       CDASSLTRWRQRLDEAGMEELLAHTINAAHAMQAVDARELSRVIVDTTVQEKAIAYPTDS
                ************************************************************

ORF_race9       RLLEVARKKLVLLAKRYGIGLRQSYARQGPALSRKAGRYAH
ORF_race4       RLLEVARKKLVLLAKRYGIGLRQSYARQGPALSRKAGRYAH
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Fig. S2. Continued.

Table S1. List of primer pairs previously developed including 20 primer pairs that were designed to detect DNA fragments of *Xcc* races 4 and 9 in this study.

Marker	Primer name	Sequences (5′ … 3′)	Annealing conditions	References
Xcc specific	Xcc-53-F-R	F- CGGTGCCAGCGACTCGCCACG R- TCCACGGCGGCGGGCCGATCTG	70℃, 20 sec, 25 cycles	Rubel et al. 2019a
Xcc Race 1 specific	Xcc-47R1-F-R	F- CCTCCTGAGTCATGGCAATGGC R- TAGCAGGGGAGTGCTGCTTGC	65℃, 40 sec, 20 cycles	Rubel et al. 2019b
Xcc Race 2 specific	Xcc-R2-89.2-F-R	F- CGCCTTGATGTAGCCCAGTA R- GGTGGCTTGGACAACGTTC	58℃, 40 sec, 18 cycles	Rubel et al. 2019b
Xcc Race 3 specific	XccR3-49-F-R	F- AAAGAGCCAATGAAGGGCGAACA R- TATGTCAGGCGCATAATCCGCAAT	63℃, 40 sec, 25 cycles	Afrin et al. 2018
Xcc Race 4 specific	Xcc2-46R4-F-R	F- GCGTAGCGAAAACTGGTAGTTC R- GCACAGGCGCACCAGCATATGGC	66℃, 40 sec, 20 cycles	Rubel et al. 2017
Xcc Race 5 specific	XccR5-89.2-F-R	F- GCGAGCGATTTCACCTTCAT R- AATTCGGACAACAGCGCAAT	63℃, 40 sec, 22 cycles	Afrin et al. 2019
Xcc Race 6 specific	XccR6-60-F-R	F- TGTAGAATCTTTAGCTGGCGC R- TTCAATGCATTCCCGACACG	63℃, 40 sec, 22 cycles	Afrin et al. 2020
Xcc Race 7 specific	XccR7-1F-1R	F- TCGAGGCAAAAATGCAGTGC R- AGCAAGGACATCATCGAGGC	70℃, 15 sec, 30 cycles	Kim et al. 2023
Xcc Race 9 specific	Race 9-1F1-1R1	F- CACATGGCACACTTCAAGCTCA R- ATGCGCATAGCGGCCCGCCTTG	70℃, 30 sec, 30 cycles	This work
	Race 9-1F2-1R1	F- CATTACTGCGTCACCGTGGCGGT R- ATGCGCATAGCGGCCCGCCTTG		
	Race 9-1F3-1R1	F- CAATTTCCACTCATGCATACA R- ATGCGCATAGCGGCCCGCCTTG		
	Race 9-1F4-1R1	F- GTTTGCTCTACCTCAAACACGC R- ATGCGCATAGCGGCCCGCCTTG		
	Race 9-1F5-1R1	F- CATCAACGCTGCACATGCCAT R- ATGCGCATAGCGGCCCGCCTTG		
	Race 9-1F6-1R1	F- CAAGGCGGGCCGCTATGCGCAT R- ATGCGCATAGCGGCCCGCCTTG		
	Race 9-1F7-1R1	F- GAAGGACAAACAAAAACTGTAC R- ATGCGCATAGCGGCCCGCCTTG		
	Race 9-1F8-1R1	F- GATCTGAGCGTAGAACCGAC R- ATGCGCATAGCGGCCCGCCTTG		
	Race 9-1F9-1R1	F- CTGAAAGGTGCACAAGGCGAT R- ATGCGCATAGCGGCCCGCCTTG		
	Race 9-1F10-1R1	F- CATGCATTTCGCCATCGACGTA R- ATGCGCATAGCGGCCCGCCTTG		

Table S1. Continued.

Marker	Primer name	Sequences (5′ ⋯ 3′)	Annealing conditions	References
	Race 9-2F1-2R1	F- CTGCAAAATGGGCATGAACCG R- CATGCGCATAGCGGCCCGCCTTG		
	Race 9-2F2-2R1	F- CGAACAGCAAAGGCAGATACAG R- CATGCGCATAGCGGCCCGCCTTG		
	Race 9-2F3-2R1	F- GTCCTGCTGCCGAGCACATGC R- CATGCGCATAGCGGCCCGCCTTG		
	Race 9-2F4-2R1	F- GTTTGCTCTACCTCAAACAC R- CATGCGCATAGCGGCCCGCCTTG		
	Race 9-2F5-2R1	F- CATCAACGCTGCACATGCCATG R- CATGCGCATAGCGGCCCGCCTTG		
	Race 9-2F6-2R1	F- CAAGGCGGGCCGCTATGCGCATG R- CATGCGCATAGCGGCCCGCCTTG		
	Race 9-2F7-2R1	F- GAAGTGGAATGCATCGGTAAG R- CATGCGCATAGCGGCCCGCCTTG		
	Race 9-2F8-2R1	F- GCTGCAGGATCTGAGCGTAGAAC R- CATGCGCATAGCGGCCCGCCTTG		
	Race 9-2F9-2R1	F- CTGAAAGGTGCACAAGGCGATG R- CATGCGCATAGCGGCCCGCCTTG		
	Race 9-2F10-2R1	F- CTACGCCTTCGATGGAGCAGACG R- CATGCGCATAGCGGCCCGCCTTG		