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Notes

Species-specific Detection of *Erwinia pyrifoliae* by PCR Assay Using Enterobacterial Repetitive Intergenic Consensus (ERIC) Primers

Duck Hwan Park[1], Shree Prasad Thapa[2], Won-Sik Kim[3], Jang Hyun Hur[2] and Chun Keun Lim[1]*

[1]*Department of Applied Biology and* [2]*Division of Biological Environment, Kangwon National University, Chuncheon 200-701, Korea*

[3]*Norgen Biotek Corporation 3430 Schmon Parkway, Thorold, ON L2V 4Y6, Canada*

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We designed a sensitive and specific PCR-based method with enterobacterial repetitive intergenic consensus (ERIC) primer to detect *Erwinia pyrifoliae*, which cause shoot blight in Asian pear, from a mixed culture and infected plant materials. The primers specifically detected only *E. pyrifoliae* and showed no cross-reactivity with other bacterial phytopathogens.

Keywords **:** detection, *Erwinia pyrifoliae*, ERIC, *Pyrus pyrifolia*

Erwinia pyrifoliae, a member of the family enterobacteriaceae, is the etiological agent of shoot blight disease in Asian pears (*Pyrus pyrifolia* cv. Singo) and is endemic to South Korea (Rhim et al., 1999; Shrestha et al., 2003). The disease symptoms are similar to those of fire blight caused by *Erwinia amylovora* and bacterial shoot blight of pear (BSBP) caused by Japanese *Erwinia* spp. (Matsuura et al., 2007; Van der Zwet and Keil, 1979). Therefore, it is necessary to devise a non-symptom-based method to distinguish this disease from one another.

Enterobacterial repetitive intergenic consensus (ERIC) sequences are highly conserved DNA sequences of size 124-127 bp. They are located in the intergenic chromosomal regions in members of the family enterobacteriaceae (Sharples and Lloyd, 1990). These sequences are of interest to researchers because they have been used to fingerprint bacterial genomes and for genetic analysis and epidemiological investigation of pathogens, including *Vibrio parahaemolyticus*, *Campylobacter* spp., *Escherichia coli*, *Salmonella enterica*, and *Aeromonas* spp. (Da Silverra et al., 2002; Houf et al., 2005; Mouwen et al., 2005; Szczuka and Kaznowski, 2004; Versalovic et al., 1991; Wong and Lin, 2001). Thus, it may be possible to use ERIC sequences as target regions for the development of specific and sensitive genetic markers for *E. pyrifoliae*.

*Corresponding author.
Phone) +82-33-250-6437, FAX) +82-33-256-8254
E-mail) chunkeun@kangwon.ac.kr

Previously, Shrestha et al. (2007) attempted to develop primers specific to the *E. pyrifoliae* ERIC sequences, but the specificity of these primers was limited to *E. amylovora* and chromosomal DNA. Further, the specificity of these primers to *E. pyrifoliae* from mixed cultures including other phytopathogenic bacteria and infected plant materials was not tested. Although PCR is rapid and sensitive with purified DNA, it yields rather poor results with crude plant extracts. In this study, we designed sensitive primers corresponding to positions 20-39 (forward) and 638-657 (reverse) of the deduced ERIC sequences of *E. pyrifoliae*. We also confirmed the feasibility of using these primers to detect *E. pyrifoliae* from a mixed culture of bacteria and artificially infected plant materials such as twigs, leaves, and immature pears.

To amplify the conserved ERIC regions and specific regions derived from the ERIC sequences of *E. pyrifoliae*, the primers ERIC1R: (5'-ATGTAAGCTCCTGGGGATTCAC), ERIC2: (5'-AAGTAAGTGACTGGGGTGAGCG) (Sharples and Lloyd, 1990) and EpERF: (5'-GCGGTCATAGTGGCAATGAT), EpERR: (5'-GCACCTGCGATGCAAAGATG) were tested for *E. pyrifoliae* and a panel of other phytopathogenic bacteria. PCR amplification was carried out in a 25 µl reaction volumes with 20 pM of primer, 20 µM of dNTPs (Promega, USA), 1 U of *Taq* DNA polymerase (Biotools, Madrid, Spain), and 10 ng of DNA. As an alternative template, we used 5 µl of bacterial suspension (boiled for 10 min with 0.1 M NaOH) prepared in distilled water with an optical density value of 1.0 (ca. 1×10^8 CFU/ml). PCR amplification was carried out using a DNA thermal cycler (Bio-Rad, USA) with the following steps: initial denaturation at 94°C for 2 min, annealing at 58°C for 30 sec, extension for at 72°C for 1 min (30 cycles), and final extension at 72°C for 7 min. Next, 5 µl of each amplified PCR product was electrophoresed on a 0.7% agarose gel (Qbiogene, CA, USA), stained with ethidium bromide, and visualized on a UV transilluminator and photographed under UV light.

To confirm the specificity of the PCR assay, we used 30 bacterial isolate: 10 *E. pyrifoliae* isolates and 20 isolates of

Table 1. List of bacterial strains used in this study

Bacterial species	Biological origin	Geographic origin	Source
1. *Erwinia pyrifoliae* WT3	*Pyrus pyrifolia*	Korea	KCCM10283 (Shrestha et al., 2003)
2. *E. pyrifoliae* WT15	*P. pyrifolia*	Korea	KACC13945 (Shrestha et al., 2003)
3. *E. pyrifoliae* WT18	*P. pyrifolia*	Korea	KACC13946 (Shrestha et al., 2003)
4. *E. pyrifoliae* WT19	*P. pyrifolia*	Korea	KACC13947 (Shrestha et al., 2003)
5. *E. pyrifoliae* WT20	*P. pyrifolia*	Korea	KACC13948 (Shrestha et al., 2003)
6. *E. pyrifoliae* WT30	*P. pyrifolia*	Korea	KNUCPB953 (Shrestha et al., 2003)
7. *E. pyrifoliae* Ep1	*P. pyrifolia*	Korea	DSM 12162
8. *E. pyrifoliae* Ep4	*P. pyrifolia*	Korea	DSM 12394
9. *E. pyrifoliae* Ep8	*P. pyrifolia*	Korea	DSM 12393
10. *E. pyrifoliae* Ep16^T	*P. pyrifolia*	Korea	DSM 12163
11. *E. amylovora* ATCC 15580^T	*Pyrus cummunis*	UK	Dye D.W.
12. *E. amylovora* LMG 1877	*Cydonia oblonga*	Denmark	Hockenhull, J.
13. *E. amylovora* LMG 1946	*Pyrus cummunis* cv. *durondeau*	Belgium	Vantomme R.
14. *E. amylovora* LMG 2068	*Rubus idea*	USA	Hayward A.
15. *Pectobacterium carotovorum* subsp. *atrosepticum* ATCC 33260^T	*Solanum tuberosum*	UK	Graham, D.
16. *P. carotovorum* subsp. *carotovorum* ATCC 15713^T	*S. tuberosum*	Denmark	Hellmers, E.
17. *P. carotovorum* subsp. *wasabiae* ATCC 43316^T	*Eutrema wasabi*	Japan	Goto, M.
18. *P. chrysanthemi* ATCC 11663^T	*Chrysanthemum*	USA	Burkholder, W.H.
19. *E. rhapontici* ATCC 29283^T	*Rheum rhaponticum*	UK	Starr, M.P.
20. *Xanthomonas axonopodis* pv. *glycines* 8ra	–		E.J. Braun
21. *X. campestris* pv. *vesicatoria* KNUCPB 07	–	Korea	Lim, C.K
22. *Pantoea stewartii* subsp. *stewartii* ATCC 8199^T	*Zea mays*	USA	Margraert et al.
23. *P. stewartii* subsp. *stewartii* LMG2712	*Z. mays*	USA	Williams, L.
24. *P. stewartii* subsp. *indologenes* ATCC 51785^T	*Setaria italica*	India	LMG
25. *P. annanatis* LMG 2665^T	*Ananas comosus*	Brazil	Robbs, C.
26. *P. dispersa* LMG2603^T	*Soil*	Japan	Gavini, F. CUETM
27. *Brenneria rubrifaciens* LMG 5117	*Juglans regia*	USA	PDDCC
28. *B. rubrifaciens* ATCC 29291	*J. regia*	USA	Starr, M. P.
29. *Pseudomonas fluorescens* Gpf01	*Panax ginseng*	Korea	Lim, C. K.
30. *P. syringae* ATCC 53543	–	USA	Eastman Kodak Co

^{T}Type strain, ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganism; KACC, Korean Agricultural Culture Collection; KCCM, Korean Culture Centre of Mircoorganisms; KNUCPB, Kangwon National University Collection of Phytopaghogenic Bacteria; LMG, Laboratorium voor Microbiologie.

other phytopathogenic bacteria (Table 1). We used serially diluted pure bacterial suspensions and chromosomal DNA (diluted from 50 ng to 0.5 fg) as the PCR templates to determine the sensitivity and limit of detection of the primers. As expected, a 750 bp fragment was amplified from all the *E. pyrifoliae* isolates in the PCR with the ERIC1R and ERIC2 primers (Fig. 1A). To confirm the uniformity of the *E. pyrifoliae*, we then conducted PCR amplification with the new specific primer set (EpERF and EpERR) that specifically amplified ERIC regions only from *E. pyrifoliae* and not those from other phytopathogenic bacteria (Fig. 1B). The assay thresholds were lower than 1×10^4 CFU/ml for the bacterial suspensions (equivalent to 50 bacterial cells per reaction) and 50 pg for DNA (Fig. 2). This low detection limit in terms of the number of cells indicates the high degree of specificity and sensitivity of the primers designed in this study.

To assess the robustness of this assay, we also analyzed the primers in PCR reaction with mixed bacteria and infected plant tissues as the templates: we used a cell suspension of *E. pyrifoliae* WT3 (1×10^4 CFU/ml) mixed with one of *E. amylovora* ATCC 15580, *Pantoea stewartii* subsp. *stewartii* LMG 2712, *Brenneria rubrifaciens* LMG 5110 or *Pectobacterium carotovorum* subsp. *carotovorum* ATCC 15713 (1×10^7 CFU/ml) or plant tissues artificially infected with *E. pyrifoliae* (1×10^6 CFU/ml). Total DNA

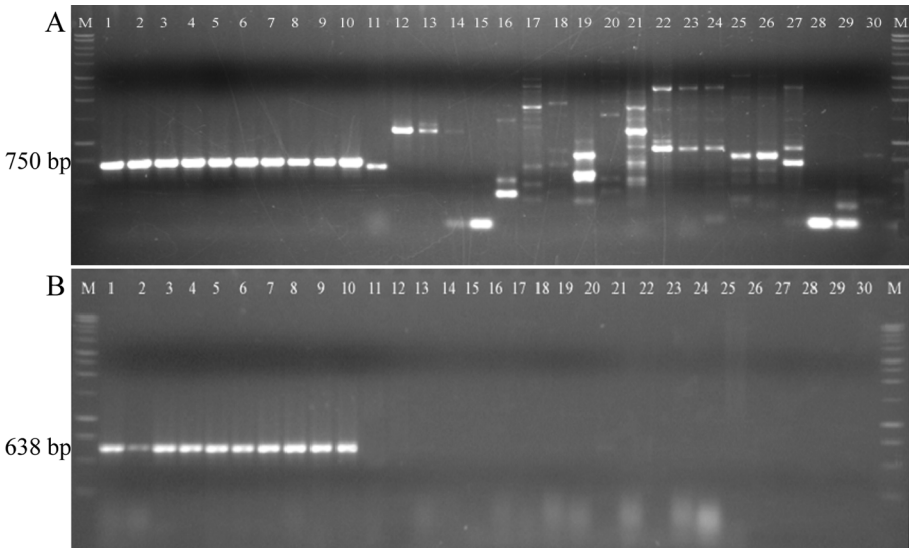

Fig. 1. PCR amplification of *E. pyrifoliae* DNA using (A) random ERIC primers (ERIC1R and ERIC2) and (B) specific ERIC primers (EpERF/EpERR). Lanes 1-10, *E. pyrifoliae* strains (WT3, WT15, WT18, WT19, WT20, WT30, Ep1, Ep4, Ep8 and Ep16). Lanes 11-30, other bacterial strains (listed in Table 1). Lane M, size marker (1kb DNA ladder, Promega™).

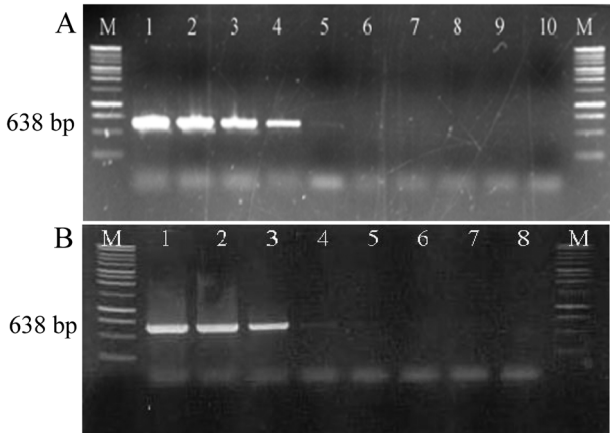

Fig. 2. PCR sensitivity assay of the specific primer. (A) Known concentrations of *E. pyrifoliae* DNA template. Lane 1, 50 ng; Lane 2, 5 ng; Lane 3, 0.5 ng; Lane 4, 50 pg; Lane 5, 5 pg; Lane 6, 0.5 pg; Lane 7, 50 fg; Lane 8, 5 fg; Lane 9, 0.5 fg; Lane 10, water control. (B) Detection limit for *E. pyrifoliae* cell suspension. Lane 1, 1×10^6 CFU/ml; Lane 2, 1×10^5 CFU/ml; Lane 3, 1×10^4 CFU/ml; Lane 4, 1×10^3 CFU/ml; Lane 5, 1×10^2 CFU/ml; Lane 6, 1×10^1 CFU/ml; Lane 7, 1×10^0 CFU/ml; Lane 8, water control. Lane M, size marker (1 kb DNA ladder, Promega™).

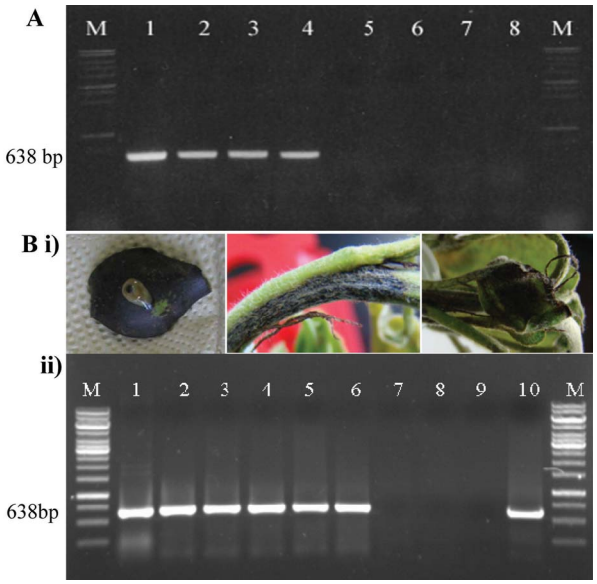

Fig. 3. Specificity of the primers (EpERF and EpERR) to *E. pyrifoliae* from a mixed culture (A) and infected plant tissues (B). (A) Lane 1, *E. pyrifoliae* WT3 and *E. amylovora* ATCC 15580; Lane 2, *E. pyrifoliae* WT15 and *P. stewartii* subsp. *stewartii* LMG 2712; Lane 3, *E. pyrifoliae* Ep1 and *B.rubrifaciens* LMG 5110; Lane 4, *E. pyrifoliae* Ep16 and *P. carotovorum* subsp. *carotovorum* ATCC 15713; Lane 5, *E. amylovora* ATCC 15580; Lane 6, *P. stewartii* subsp. *stewartii* LMG 2712; Lane 7, *B. rubrifaciens* LMG 5110; Lane 8, *P. carotovorum* subsp. *carotovorum* ATCC 15713. (B) (i) Symptoms of *E. pyrifoliae* infection on plant materials (ii) Lanes 1-3, *E. pyrifoliae* strains (WT3, Ep1 and Ep16) from infected immature pears; Lane 4-6, *E. pyrifoliae* WT3 from infected immature pears, twigs and leaves; Lane 7, *E. amylovora* ATCC 15580; Lane 8, symptomless non-inoculated plant materials; Lane 9, water control; Lane 10, *E. pyrifoliae* WT3 chromosomal DNA. Lane M, size marker (1 kb DNA ladder, Promega™).

was extracted using a CTAB-based protocol (Wilson, 1989) from the pellets of artificially infected plant materials (immature pears, twigs and leaves) 4 days post inoculation.

A 638 bp amplicon was obtained from the mixed bacterial cultures and artificially inoculated plant materials 4 days after inoculation (Fig. 3). The results demonstrate the possibility of identifying the shoot blight pathogen under field conditions and the usefulness of our methodology for

field screening.

In conclusion, a rapid PCR assay with advanced ERIC primer sets was developed for the detection of *E. pyrifoliae*. It was found to be highly specific and sensitive for the detection of this organism from plant materials and could be use for routine laboratory diagnosis.

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