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

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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 Silvera 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*.

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'-ATGTAAGCTCTGGGGATTCA) and ERIC2: (5'-AAGTGACGTGGGGGGrAgGcG) were tested for *E. pyrifoliae* and a panel of other phytopathogenic bacteria. PCR amplification was carried out in a 25 µl reaction volume 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 × 10⁸ 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 (Qiogene, 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...
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 using primers designed in this study. To confirm the uniformity of the primer set, 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 × 10^6 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 templates: we used a cell suspension of E. pyrifoliae WT3 (1 × 10^6 CFU/ml) mixed with one of E. amylovora ATCC 15580, Pantoaea stewartii subsp. stewartii LMG 2712, Breneria rubrifaciens LMG 5117, Pectobacterium carotovorum subsp. atrosepticum ATCC 15580, or Pectobacterium carotovorum subsp. atrosepticum ATCC 19510 (1 × 10^7 CFU/ml) or plant tissues artificially infected with E. pyrifoliae (1 × 10^7 CFU/ml). Total DNA from infected plant tissues as the templates: we used a cell suspension of E. pyrifoliae WT3 (1 × 10^6 CFU/ml) mixed with one of E. amylovora ATCC 15580, Pantoaea stewartii subsp. stewartii LMG 2712, Breneria rubrifaciens LMG 5117, Pectobacterium carotovorum subsp. atrosepticum ATCC 15580, or Pectobacterium carotovorum subsp. atrosepticum ATCC 19510 (1 × 10^7 CFU/ml) or plant tissues artificially infected with E. pyrifoliae (1 × 10^7 CFU/ml). Total DNA