Specific and Sensitive Detection of *Enterobacter mori* Using Reliable RT-PCR

M. M. Lou, State Key Laboratory of Rice Biology and Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Ministry of Agriculture, Institute of Biotechnology, Zhejiang University, Hangzhou 310058, China; G. L. Jin, Institute of Bioinformatics, Zhejiang University, Hangzhou 310029, China; and W. X. Tian, G. Q. Zhang, X. Y. Fan, F. Wang, B. Zhu, and G. L. Xie, State Key Laboratory of Rice Biology and Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Ministry of Agriculture, Institute of Biotechnology, Zhejiang University

Abstract

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Enterobacter mori, the causal agent of bacterial wilt in mulberry, is becoming a serious disease in mulberry orchards in China. Because no effective control strategy has been devised for this disease, the reliable screening of mulberry material for latent infection became necessary. Hence, a fast polymerase chain reaction (PCR) assay for the detection of *E. mori* was developed in this study. The primers were designed within regions of the RNA polymerase β -subunit (*rpoB*) gene. The method is fast and simple and showed 100% sensitivity (no false negatives) and 100% specificity (no false positives), which was tested with 4 representative *E. mori* strains, 9 *Enterobacter* type strains, 2 strains

of the other major mulberry bacterial pathogens (*Ralstonia solanacearum* and *Pseudomonas syringae* pv. *mori*) in China, 7 strains of other plant-associated pathogens, and 50 unidentified epiphytic bacterial isolates from mulberry plants. The real-time PCR assays reliably detected the DNA at at least 10 fg/ μ l and the bacterial cells at 10² CFU/ml from mulberry shoots and roots suspension. The strong positive reaction in testing of all symptomatic plants (with 100% positive) and parts of asymptomatic latent infected plant samples (with 36.4% positive) provided proof that this method is reliable and sensitive and suitable for screening plant material with latent infections of *E. mori*.

Mulberry (*Morus alba*) is a genus of 10 to 16 species of deciduous trees that are domestic in warm temperate and subtropical regions of Asia, Africa, Europe, and the Americas, with the majority of the species native to Asia (10). Mulberry leaves are ecologically important as the sole nutritional source for the silkworm (*Bombyx mori*), the cocoon of which is used to make silk. China produces about 75% of the world's raw silk, which is valued at nearly \$2 billion (5). Therefore, epidemiology of some mulberry diseases is of great concern in China.

In summer 2006, a severe outbreak of a foliar decline disease was noted in mulberry (*M. alba* L. 'Hushang') in Zhejiang and Guangxi provinces, which are the two major mulberry-growing areas in China (17). The disease was characterized by severe wilt, especially on 1- or 2-year-old mulberry plants, that resulted in premature plant death. Leaf wilt symptoms generally started on older leaves at the bottom of the plant and spread to the younger leaves. The leaves of infected plants became withered and dry and turned dark brown and, eventually, the plants became defoliated. The root xylem of infected plants was moist and discolored with brown stripes. In the past few years, this mulberry wilt disease (MWD) has become increasingly more serious, and the economic losses are more than a million dollars every year (data provided by the Agriculture Department of Zhejiang Province).

Early studies identified the MWD pathogen as *Enterobacter cloacae* (17) whereas, recently, we found that MWD was caused by a new species and has been described as *E. mori* (18,20). The genus *Enterobacter* consists of gram-negative, facultatively anaerobic, rod-shaped bacteria with 21 species in the genus. This genus in-

cludes several plant pathogens and many human pathogens (3,9,18). Thousands of hectares of mulberry orchard have been infected by *E. mori* (data provided by the Agriculture Department of Zhejiang Province); therefore, it is important to establish a fast and specific polymerase chain reaction (PCR) detection system to block the pathogen from transmission. In recent years, several real-time (RT)-PCR approaches in the detection of phyto-bacteria have been designed and validated, with significant enhancements in speed, sensitivity, and specificity (6). Thus, development of a species-specific PCR assay for *E. mori* could facilitate its fast and efficient detection. However, rapid diagnosis methods for *Enterobacter* spp. have been scarcely reported; only a detection system based on RT-PCR for *E. sakazakii*, which is a human opportunistic pathogen, has been established (2,13).

In this study, we designed a pair of primers (Em-rpoBF and Em-rpoBR) which amplified a 307-bp portion of the RNA polymerase β -subunit gene, *rpoB*. This primer pair proved solely specific for *E. mori*. The main objective of this research was to develop a PCR-based technique to detect *E. mori* in field samples without bacteria purification and DNA extraction procedures. We believe this technique can be used for both in planta screening of latent infections and reliable identification of isolated bacteria.

Materials and Methods

Bacteria isolates. Ninety-six *E. mori* strains, which originated from Zhejiang and Guangxi provinces (two of the most important provinces in mulberry planting in China), were isolated from diseased mulberry plants. Four representative strains were selected for further analysis, having been proven as the model strains for the 96 *E. mori* strains (18). *Ralstonia solanacearum*, *Pseudomonas syringae* pv. *mori*, nine type strains of the *Enterobacter* complex, and seven other phyto-bacteria were used as controls in evaluating this PCR technique for the detection of *E. mori*. Fifty mulberry epiphytic strains (MES) from roots, shoots, and leaves were also isolated and selected as negative controls in PCR detections. All of the isolates used in this study are presented in Table 1.

Plant material. Fifty-five mulberry samples were collected from Zhejiang province, including the site where MWD was first observed (Tonglu town; Table 1). Of these samples, 11 originated from mulberry showing typical MWD symptoms while 44 were

Corresponding authors:

B. Zhu, E-mail: bzhu@zju.edu.cn

G.-L. Xie, E-mail: glxie@zju.edu.cn

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Table 1. Bacterial strain and mulberry plant material samples used in this study, their biological origin, geographic origin, year of isolation, and *Enterobacter mori*-specific polymerase chain reaction results (PCR)

Strains, samples	IDs ^a	Symptoms	Biological origin	Origin, year of isolation	PCR	Isolation ^b
<i>Enterobacter mori</i>	R2-2	...	<i>Morus alba</i>	Tonglu, China, 2005	+	...
<i>E. mori</i>	R6-2	...	<i>M. alba</i>	Chun'an, China, 2005	+	...
<i>E. mori</i>	R11-2	...	<i>M. alba</i>	Lin'an, China, 2006	+	...
<i>E. mori</i>	LMG25706 ^T	...	<i>M. alba</i>	Guangxi, China, 2006	+	...
<i>E. cloacae</i> subsp. <i>dissolvens</i>	LMG2683 ^T	...	<i>Zea mays</i>	1922	–	...
<i>E. cloacae</i> subsp. <i>cloacae</i>	ATCC13047 ^T	...	Spinal fluid	1960	–	...
<i>E. cancerogenus</i>	LMG2693 ^T	...	<i>Populus canadensis</i>	Czech Republic, 1964	–	...
<i>E. pyrinus</i>	LMG22970 ^T	...	<i>Pyrus pyrifolia</i>	Yusung, Korea, 1988	–	...
<i>E. amnigenus</i>	LMG2784 ^T	...	Soil	France, 1943	–	...
<i>E. turicensis</i>	LMG23730 ^T	...	Fruit powder	Zurich, Switzerland, 2005	–	...
<i>E. helveticus</i>	LMG23732 ^T	...	Fruit powder	Zurich, Switzerland, 2005	–	...
<i>E. nimipressuralis</i>	LMG10245 ^T	...	<i>Ulmus</i> sp.	United States, 1968	–	...
<i>E. agglomerans</i>	LMG2557	...	<i>Pyrus communis</i>	United Kingdom, 1958	–	...
<i>Ralstonia solanacearum</i>	M5	...	<i>M. alba</i>	China, 1975	–	...
<i>Pseudomonas syringae</i> pv. <i>mori</i>	LMG5074	...	<i>M. alba</i>	Hungary, 1983	–	...
<i>Pantoea ananatis</i>	LMG2665 ^T	...	<i>Ananas comosus</i>	Brazil, 1965	–	...
<i>Burkholderia cepacia</i>	LMG1222 ^T	...	<i>Allium cepa</i>	United States, 1949	–	...
<i>Dickeya chrysanthemi</i>	LMG2468	...	<i>Saccharum officinarum</i>	Australia, 1953	–	...
<i>Pectobacterium atrosepticum</i>	CGMCC1.1000	...	<i>Solanum tuberosum</i>	China	–	...
<i>Xanthomonas axonopodis</i> pv. <i>poinsetticola</i>	LMG849	...	<i>Euphorbia pulcherrima</i>	India, 1950	–	...
<i>X. arboricola</i> pv. <i>poinsetticola</i>	LMG5403	...	<i>Euphorbia pulcherrima</i>	New Zealand, 1972	–	...
<i>Acidovorax avenae</i> subsp. <i>citrulli</i>	LMG2255	...	<i>Citrullus lanatus</i>	United States, 1978	–	...
Mulberry epiphytic strains	MES1-50	...	<i>M. alba</i>	Zhejiang, China, 2008	–	...
Samples ^c						
PT-1	...	+	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-2	...	–	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-3	...	–	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-4	...	–	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-5	...	–	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-6	...	–	Fengtian, <i>M. alba</i>	Tonglu, China, 2009	–	–
PT-7	...	–	Fengtian, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-8	...	–	Fengtian, <i>M. alba</i>	Tonglu, China, 2009	–	–
PT-9	...	–	Fengtian, <i>M. alba</i>	Tonglu, China, 2009	–	–
PT-10	...	–	Fengtian, <i>M. alba</i>	Tonglu, China, 2009	–	–
PT-11	...	+	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-12	...	–	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-13	...	+	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-14	...	–	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-15	...	+	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-16	...	–	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-17	...	–	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-18	...	–	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-19	...	+	Qiangsang, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-20	...	+	Jinshi, <i>M. alba</i>	Tonglu, China, 2009	+	+
PT-21	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	–	–
PT-22	...	+	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	+	+
PT-23	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	+	+
PT-24	...	–	Yu711, <i>M. alba</i>	Chun'an, China, 2009	–	–
PT-25	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	–	–
PT-26	...	+	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	+	+
PT-27	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	+	+
PT-28	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	–	–
PT-29	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	–	–
PT-30	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	–	–
PT-31	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	–	–
PT-32	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	–	–
PT-33	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	–	–
PT-34	...	+	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	+	+
PT-35	...	–	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	+	+
PT-36	...	+	Nongsang, <i>M. alba</i>	Chun'an, China, 2009	+	+
PT-37	...	–	Nongsang, <i>M. alba</i>	Lin'an, China, 2009	+	+
PT-38	...	–	Nongsang, <i>M. alba</i>	Lin'an, China, 2009	+	+
PT-39	...	+	Nongsang, <i>M. alba</i>	Lin'an, China, 2009	+	+
PT-40	...	–	Nongsang, <i>M. alba</i>	Lin'an, China, 2009	–	–
PT-41	...	–	Nongsang, <i>M. alba</i>	Lin'an, China, 2009	–	–
PT-42	...	–	Nongsang, <i>M. alba</i>	Lin'an, China, 2009	–	–
PT-43	...	–	Nongsang, <i>M. alba</i>	Lin'an, China, 2009	–	–
PT-44	...	–	Nongsang, <i>M. alba</i>	Lin'an, China, 2009	–	–
PT-45	...	–	Nongsang, <i>M. alba</i>	Lin'an, China, 2009	+	+
PT-46 to PT-55	...	–	Nongsang, <i>M. alba</i>	Lin'an, China, 2009	–	–

^a T = type strain; LMG = BCCM-LMG Bacteria Collection, Gent, Belgium; M5 = acquired from Institute of Plant Protection, Chinese Academy of Agricultural Science.

^b Isolation of *Enterobacter mori* from 55 samples.

^c Samples collected from different provinces of China; PT = plant template.

symptomless. The tested samples consisted of shoots and roots (when they were available).

DNA extraction. All the bacteria used in this study were grown on BD Difco 0001-17 nutrient agar (NA; Difco Laboratories, Detroit) at 30°C. DNA was extracted from bacterial cultures by a bacterial genomic DNA extraction kit (TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit Ver. 2.0; TaKaRa, Dalian, China). The concentration of DNA was measured using a spectrophotometer (NanoDrop2000C; Thermo Fisher Scientific Inc.) and its density adjusted to 100 ng/μl with an optical density at 260 or 280 nm of approximately 1.70 to 1.90.

Plant template preparation. To eliminate the DNA extraction procedure, a fast plant template (PT) preparation method was used in this study. Pieces (2 to 3 cm long) of a mulberry sample's roots or shoots were cut and the surface of the sample was disinfected by dipping with 75% ethanol for 30 s. After the ethanol was removed, smaller pieces were cut and covered with sufficient volume (approximately 1 ml) of sterile distilled water (SDW) in 1.5-ml tubes. Samples were incubated with shaking (120 rpm) for 30 min at room temperature (about 20°C). Supernatants were centrifuged for 10 min at 2,000 rpm, transferred to a new 1.5-ml tube, and centrifuged for 20 min at 8,000 rpm. Pellets were resuspended in 300 μl of SDW. PT was directly used as a PCR template in further analysis.

Bacterial suspension preparation. A single colony of the four representative strains was grown in Luria-Bertani medium and used to prepare a 1×10^9 CFU/ml suspension in SDW. A series of 10-fold dilutions was made from each suspension (10^9 to 10^0 CFU/ml). The optical density of the suspension was adjusted and counted as in a previous assay development (4).

Sequencing and PCR primers design. PCR primers for *E. mori* detection were designed from the RNA polymerase β-subunit gene, *rpoB*. Before designing the specific primers, the fragments of partial *rpoB* of *E. mori* and closely related species were sequenced and the specific regions which are unique to *E. mori* identified (Table 2). PCR reactions comprised 1 μl of DNA template, 5 μl of 10× PCR buffer and 0.25 μl of Taq DNA polymerase (TaKaRa), 0.25 μl of 20 mM dNTP, and 0.2 μM CM₇ and CM_{31b} primers (7) (Table 2). Amplification was initiated by denaturation at 95°C for 5 min, followed by 35 cycles at 95, 56, and 72°C for 30 s at each temperature. A final extension at 72°C for 5 min was included. The products were sequenced by BGI Company in Shanghai, China. The products and other sequences which were downloaded from GenBank (Table 3) were aligned

by ClustalW (15). In these alignments, two specific regions that are unique to *E. mori* were identified and used as specific primers (Table 2).

PCR and RT-PCR assay conditions in *E. mori* detection. PCR reactions comprised 2 μl of template, 2.5 μl of 10× PCR buffer and 0.15 μl of Taq DNA polymerase (TaKaRa), 0.15 μl of 20 mM dNTP, and 0.2 μM each primer. SYBR Green I RT-PCR was performed using SYBR Premix Reagent Trial Pack (TaKaRa). The standard mixture contained 2 μl of template, 12.5 μl of 2× SYBR Premix, 0.15 μl of 20 mM dNTP, and 0.2 μM each primer. The amplification was initiated by denaturation at 95°C for 5 min; followed by 40 cycles at 95, 65, and 72°C for 30 s at each temperature; with a final extension at 72°C for 5 min.

Specificity in *E. mori* detection. The specificity of PCR assays was checked by amplification of: (i) 96 strains of *E. mori* of different origins; (ii) 9 type strains of *Enterobacter* spp. that were closely related to *E. mori*; (iii) 2 strains of *R. solanacearum* and *P. syringae* pv. *mori*; (iv) 7 strains of other plant-associated pathogens; and (v) 50 unidentified MES, using standard methods in this study.

Sensitivity in *E. mori* detection. The sensitivity of PCR assays was determined with a serial dilution of *E. mori* DNA from 10 ng/μl to 1 pg/μl and bacteria-plant tissue mixture with *E. mori* cells from 10^9 to 10^0 CFU/ml. The mixture was prepared by adding 1 ml of bacterial suspensions containing 10^9 to 10^0 CFU to freshly harvested, disinfected mulberry tissues (pieces 2 to 3 cm long) and then incubated with shaking at 120 rpm for 30 min at room temperature (about 20°C). These suspensions (1 μl) were used as template in PCR reactions. In addition, 2 μl of PT was directly used in PCR assays.

For all the experiments in this study, the PCR was performed in quintuplicate and the limit of detection was defined by linear regression model as described by Pennacchia et al. (11).

Results

Specificity of the PCR assay. The specificity of the PCR assay was assessed on DNA from a collection of a panel of bacterial strains summarized in Table 1. The Em-rpoBF and Em-rpoBR primers could amplify all 96 *E. mori* strains (in this report, only 4 representative strains are shown) with a positive 307-bp fragment while other strains gave a consistent negative result (Supplementary Figure S1).

Sensitivity of the PCR assay. The sensitivity was determined by traditional PCR and RT-PCR assay methods. The limitation of

Table 2. Nucleotide sequences of polymerase chain reaction primers used and designed in this study

Primer name	Sequence (5'-3')	Length (nucleotides)	Amplicon length (bp)
CM ₇	AACCAGTTCGCGTTGGCCTGG	22	1,000
CM _{31b}	CCTGAACAACACGCTCGGA	19	...
Em-rpoBF	GCCAAGCCGATTCTGGAGCA	21	307
Em-rpoBR	CGTTTCGATTGGACATACG	19	...

Table 3. Sequences from GenBank used for comparison purpose and in primers design

Strains	IDs ^a	Amplicon gene	Accession number	Reference
<i>Enterobacter mori</i>	R2-2	<i>rpoB</i>	EU579855	Wang et al. 2009
<i>E. mori</i>	R6-2	<i>rpoB</i>	EU579856	Wang et al. 2009
<i>E. mori</i>	R11-2	<i>rpoB</i>	EU579857	Wang et al. 2009
<i>E. mori</i>	LMG25706 ^T	<i>rpoB</i>	EU579858	Wang et al. 2009
<i>E. cloacae</i> subsp. <i>dissolvens</i>	LMG2683 ^T	<i>rpoB</i>	AJ543725	Hoffmann et al. 2003
<i>E. cloacae</i> subsp. <i>cloacae</i>	ATCC13047 ^T	<i>rpoB</i>	AJ543726	Hoffmann et al. 2003
<i>E. cancerogenus</i>	LMG2693 ^T	<i>rpoB</i>	AJ566943	Hoffmann et al. 2003
<i>E. pyrinus</i>	LMG22970 ^T	<i>rpoB</i>	DQ836223	Stephan et al. 2007
<i>E. amnigenus</i>	LMG2784 ^T	<i>rpoB</i>	AJ566942	Hoffmann et al. 2003
<i>E. turicensis</i>	LMG23730 ^T	<i>rpoB</i>	DQ779998	Stephan et al. 2007
<i>E. helveticus</i>	LMG23732 ^T	<i>rpoB</i>	DQ779997	Stephan et al. 2007
<i>E. nimipressuralis</i>	LMG10245 ^T	<i>rpoB</i>	AJ566948	Hoffmann et al. 2003
<i>E. agglomerans</i>	LMG1286 ^T	<i>rpoB</i>	EF988970	Brady et al. 2008

^a T = type strain.

DNA or bacterial cell detection by traditional PCR assay in this study was 10 pg/μl and 10⁵ CFU/ml, respectively (Supplementary Figure S2). Mean threshold cycle (Ct) values for each RT-PCR assay against each DNA and bacterial suspension dilution series are listed in Table 4. The equation of the log CFU/ml versus the Ct values obtained was $y = -3.321x + 35.88$, with an R^2 of 0.992 while the equation of the log fg/μl versus the Ct values obtained was $y = -6.672x + 41.99$, with an R^2 of 0.997 (Fig. 1). The detection results of 55 PTs showed that 27 PTs had positive reactions while 28 samples were negative (Fig. 2).

Table 4. *Enterobacter mori* DNA concentration, bacterial dilution series, and mean threshold cycle (Ct) values generated during real-time polymerase chain reaction (PCR) assays

<i>E. mori</i> cell and DNA concentration	Ct value ^a
10 ⁵ CFU/ml	+ (19.17 ± 0.08)
10 ⁴ CFU/ml	+ (24.72 ± 0.10)
10 ³ CFU/ml	+ (37.56 ± 0.11)
10 ² CFU/ml	+ (35.79 ± 0.14)
10 ¹ CFU/ml	– (No Ct)
10 pg/μl	+ (23.74 ± 0.11)
1p g/μl	+ (26.62 ± 0.13)
100 fg/μl	+ (30.39 ± 0.16)
10 fg/μl	+ (33.56 ± 0.12)
1 fg/μl	– (No Ct)
ddH ₂ O control	– (No Ct)

^a Ct: PCR cycle number (in parentheses) at which fluorescence is first detected during a 40-cycle PCR; + and – = positive and negative reactions, respectively.

Discussion

This study presents the development of an effective technique for reliable detection of *E. mori*, the causal agent of mulberry bacterial wilt. The fast PT preparation developed in this study enables these assays to be employed in the detection of the pathogen directly from mulberry materials.

The *E. mori rpoB* assay was designed within the *rpoB* sequences, a gene encoding RNA polymerase β-subunit, which is universal in bacteria (8). This gene has been widely used in *Enterobacteriaceae* classification (1). Although all the bacteria possess this gene, the presence of conserved and variable regions allowed us to design conserved primers flanking the variable region. After sequencing the variable regions, specific primers could be designed in these sequences. In this study, we have evaluated the specificity of the test with 9 *Enterobacter* type strains, 2 strains of the other major mulberry bacterial pathogens in China, 7 strains of other plant-associated pathogens, and 50 MES, because *Enterobacter* spp. were considered the most prevalent epiphytes which had widely been isolated from several species of plants and insects (19). The results suggested that none of the existing *Enterobacter* type strains, the other major mulberry pathogens, and MES produce a positive reaction. All the *E. mori* strains produced strong positive results, which indicated the practicability of this technique in *E. mori* detection.

In this study, 55 PTs were selected as direct PCR template, in which 11 were symptomatic and 44 were asymptomatic. The PCR reaction revealed that the 11 symptomatic and 16 asymptomatic PTs had positive reactions. We further used a streak plate method to obtain every single colony in different shapes and checked whether these colonies included *E. mori* strains by traditional

Fig. 1. **A**, Determination of real-time polymerase chain reaction (RT-PCR) amplification efficiency and detection limits of *Enterobacter mori* cells. **B**, Determination of RT-PCR amplification efficiency and detection limits of *Enterobacter mori* DNA. R^2 are reported on the graph. Cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct values are the average of five replicates.

Fig. 2. **a–c**, DNA binding patterns after specific amplification of *rpoB* regions of 55 plant templates (PTs). M = 100-bp DNA ladder; ck = double-distilled H₂O control; P = *Enterobacter mori* as positive control; 1 to 55 = PT-1 to PT-55.

methods described before (17,18) and the *E. mori*-specific primers. The result revealed that *E. mori* could be isolated from the 11 symptomatic and 16 asymptomatic PTs, whereas this pathogen could not be isolated from other 31 PTs (*data not shown*). The result indicated that our direct detection method was reliable.

The RT-PCR assay was shown to be sensitive enough to detect 10^2 CFU/ml in bacterial suspension. That means that this assay allows the detection of one cell in an RT-PCR reaction (20- μ l system). The $R^2 > 0.99$ in standard curve strongly supported the reliability of the results. Thus, the assay is as sensitive as other reported plant pathogen detection methods (14).

In this study, we used a fast and simple method for in planta detection. Previous reports mainly focused on DNA extraction from plant tissues using several kinds of chemical solutions whereas, in our method, only SDW was required (12,16). It is possible that a more elaborate DNA extraction protocol which would lead to more extracted DNA might increase the sensitivity in PCR detection. However, in this study, we found that the DNA concentration of asymptomatic PT which is associated with latent infections was high enough to be detected. No effective control strategy has been devised to prevent mulberry from being infected by this pathogen. However, as described above, the latently infected mulberry could be detected and certification of *E. mori*-free mulberry saplings by our methods can be used in a preventive disease control program.

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