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ORIGINAL ARTICLE

PCR-based assay for the detection of *Xanthomonas euvesicatoria* causing pepper and tomato bacterial spot

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Abstract

Aims: To develop a PCR-based assay for *Xanthomonas euvesicatoria* detection in culture and *in planta*.
Methods and Results: A fragment of 1600 bp specific for *X. euvesicatoria* was found by repetitive extragenic palindromic sequence-PCR. Among the primers designed on the basis of the partially sequenced fragment, the primers Xeu2.4 and Xeu2.5 direct amplification of the expected product (208 bp) for all the *X. euvesicatoria* strains and not for other related and unrelated phytopathogenic bacteria or saprophytic bacteria isolated from pepper and tomato phyllosphere. The assay permits the detection of *X. euvesicatoria* in pure culture, with a limit of detection of two bacterial cells and 1 pg of DNA per PCR, and in extracts obtained from asymptomatic inoculated tomato and pepper plants.
Conclusions: Primers Xeu2.4 and Xeu2.5 provide a specific, sensitive and rapid assay for the detection of *X. euvesicatoria* in culture and in pepper and tomato plants.
Significance and Impact of the Study: Because *X. euvesicatoria* is a quarantine organism in the European Union, and it is subjected to stringent international phytosanitary measures, this highly sensitivity PCR-based assay is suitable for its detection in pepper and tomato plant materials to avoid the introduction and spread of the bacterium.

Introduction

Bacterial spot of pepper and tomato plants, incited by bacterial spot-causing xanthomonads (BSX): *Xanthomonas euvesicatoria*, *Xanthomonas vesicatoria*, *Xanthomonas perforans* and *Xanthomonas gardneri* (Jones *et al.* 2004), is one of the most economically important disease; especially, in tropical and subtropical countries. For many years, the disease was attributed to *Xanthomonas campestris* pv. *vesicatoria*, which was considered to be a relatively homogeneous organism (Jones *et al.* 1998). In the 1990s, the bacterium was found to be composed of two genetically and phenotypically distinct groups (A and B), which Vauterin *et al.* (1995) placed into *Xanthomonas axonopodis* pv. *vesicatoria* (A) and *X. vesicatoria* (B). Two other BSX pathogenic on tomato plants, assigned to groups C and D, were successively described (Jones *et al.* 2000). Jones *et al.* (2004) demonstrated that groups A, C and D have <70% DNA relatedness with each other, with the type strain of *Xanthomonas axonopodis* and with the currently classified species within the *Xanthomonas* genus. For these reasons, they renamed A, C and D groups as *X. euvesicatoria*, *X. perforans* and *X. gardneri*, respectively. They also stated that B group can be retained in *X. vesicatoria*.

BSX are quarantine organisms in the European Union (EPPO A2 list) and are subjected to stringent international phytosanitary measures. Because of the economic and legal importance of the disease, the accurate detection of BSX in pepper and tomato seeds where they can survive for a long period, as well as in asymptomatic greenhouse-grown transplants, is critical and is also the best means to prevent further bacterial dissemination (Stall 1993). Although diagnostic protocols based on the use of semi-selective media and serological tests were developed to detect BSX (McGuire *et al.* 1986; Tsuchiya *et al.* 2003), they present many inconvenience (O'Brien *et al.* 1967; Gitaitis *et al.* 1987)

Nucleic acid-based techniques, which have shown more promise, have been developed for BSX detection (Kuflu and Cuppels 1997; Obradovic *et al.* 2004; Cuppels *et al.* 2006). Because none of them has high specificity for *X. euvesicatoria* the most worldwide bacterial species among BSX, we developed a set of primers, designed on the sequence of a DNA fragment from repetitive extragenic palindromic sequence (REP)-PCR fingerprinting, which permit to develop a rapid, sensitive and specific PCR protocol for the detection of this bacterium in culture and *in planta*.

Materials and methods

Bacterial strains and DNA extraction

Bacterial strains used in the present study are listed in Table 1. For DNA extraction, bacteria were cultivated in Luria–Bertani (LB) broth for 16 h at 27°C in an orbital shaker at 200 rev min^{-1}, and the genomic DNA was extracted with the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, St Louis, MO, USA). DNA concentrations were estimated by measuring the absorbance at 260 and 280 nm.

REP-PCR analysis

To identify DNA fragments putatively specific to *X. euvesicatoria*, 64 strains of this bacterium, all the BSX used in the present study and other related xanthomonads were subjected to REP-PCR analysis (Rademaker and de Bruijn 1997). Amplicons were separated by electrophoresis on 2% (w/v) agarose gels in 0·5 × TAE buffer at 50 V and 4°C for 14 h. Cluster analysis was performed on a similarity matrix, which was produced using the Jaccard coefficient and subjected to the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm, using NTSYSpc software (Exeter Software, New York, NY, USA), ver. 2·1.

A DNA fragment of 1600 bp, putatively specific to *X. euvesicatoria* (NCPPB 2968^T strain), was partially sequenced by PRIMM Srl Milano (Italy). The sequence (598 bp) was deposited in the GenBank database under the accession number FJ445513.

PCR assay specificity

On the basis of the REP-amplified 1600 bp fragment sequence, eight primer sets were designed. PCRs were performed in 50-μl reaction mixture containing 1 mmol l^{-1} $MgCl_2$, 10 pmol l^{-1} of each primer, 250 μmol l^{-1} of each deoxynucleotide triphosphate, two U of Taq DNA polymerase (Invitrogen, Merelbeke, Belgium) and 25 ng of bacterial DNA. The PCR temperature profile comprised an initial denaturation step at 94°C for 3 min, 35 cycles at 94°C for 45 s, 64°C for 50 s and 72°C for 50 s, and a final extension at 72°C for 10 min.

The PCR products were separated by 1·3% (w/v) agarose gel electrophoresis in 0·5 × TAE buffer for 2 h at 110 V and visualized under UV light. Because the Xeu2.4 (5′-CTGGGAAACTCATTCGCAGT-3′) and Xeu2.5 (5′-TTGTGGCGCTCTTATTTCCT-3′) primer set was the only set specific for *X. euvesicatoria* detection, it was used in the successive experiments.

PCR assay detection limits

Increasing bacterial cell (2×10^1 to 2×10^9 CFU ml^{-1}) or DNA (0·5 ng ml^{-1} to 25 μg ml^{-1}) concentrations of NCPPB 2968^T and DAPP-PG 34 *X. euvesicatoria* strains were used as templates (10 μl of bacterial cells or one μl of DNA) in the earlier-described PCR protocol, except for an initial denaturation step of 95°C for 5 min. for bacterial cells. Bacterial cells, grown for 24 h in LB broth at 27°C, were suspended in sterile distilled water and spectrophotometrically adjusted to a concentration of about 10^9 bacteria per ml. To determine the concentration of each suspension, 10-fold dilution series were prepared in sterile distilled water to 10^{-8}, and 100 μl of each dilution was spread onto Nutrient Agar plates. After 36–48 h of incubation at 27°C, CFUs for each dilution were counted.

In planta detection of *Xanthomonas euvesicatoria*

The youngest leaves of tomato (*Solanum lycopersicum* L., cv. UC82) and pepper (*Capsicum annuum* L., cv. Seinor) plants, at the third-fourth true leaf stage, were partly infiltrated using a glass atomizer, with 10^6 bacteria per ml suspensions in 10 mmol l^{-1} $MgCl_2$ of a number of BSX strains (see Fig. 2). Three, 10 and 20 days after the inoculations, small leaf pieces were surface-sterilized by immersing them in 70% (v/v) ethanol and homogenized with 500 μl of sterile distilled water. After the addition of 500 μl of 0·5 mol l^{-1} NaOH containing 0·5% (w/v) PVP (Sigma PV-40), the homogenates were vortexed and left for 15 min at room temperature. Homogenate supernatants were 100-fold diluted in sterile distilled water and five μl used as template for Xeu2·4/Xeu2·5-based PCR assay performed as described earlier.

Results

REP-PCR analysis

To identify DNA fragments putatively specific to *X. euvesicatoria*, the strains of this bacterium and related xanthomonads described in Table 1 were subjected to

Table 1 Bacterial strains used in this study and Xeu2·4/Xeu2·5-based PCR assay specificity

Bacterial species	Strain*	Biological origin	Geographic origin	PCR
Xanthomonas euvesicatoria	CFBP 6811	*Capsicum annuum*	Brazil	+
	LMG 668	*C. annuum*	Cook Island, New Zealand	+
	82-8	*C. annuum*	Florida, USA	+
	CFBP 1604	*C. annuum*	Guadeloupe	+
	DAPP-PG 29, 30, 31, 32, 33, 34, 35, 37, 38, 39, 40, 41, 42, 43, 44, 45, 75, 77, 78, 79, 80, 82, 86, 87, 88, 89, 93, 94, 95, 96, 99, 119, 122, 128, 129, 137, 142, 145, 148, 231, 232, 233, 234, 236, 237, 240, 243	*C. annuum*	Italy	+
	LMG 926	*Capsicum frutescens*	Hungary	+
	NCPPB 2968^T	*C. frutescens*	USA	+
	LMG 909	*Capsicum* sp.	Côte d'Ivoire	+
	LMG 913	*Capsicum* sp.	Senegal	+
	LMG 910	*Capsicum* sp.	South Morocco	+
	CFBP 5594, 5601	*Solanum lycopersicum*	Guadeloupe	+
	75-3	*S. lycopersicum*	Florida, USA	+
	CFBP 5597	*S. lycopersicum*	Martinique	+
	LMG 914	*S. lycopersicum*	Senegal	+
	LMG 929	*S. lycopersicum*	USA	+
	LMG 667	*S. lycopersicum*	–	+
	LMG 905	–	–	+
Xanthomonas vesicatoria	DAPP-PG 114	*C. annuum*	Italy	–
	LMG 934	*S. lycopersicum*	Brazil	–
	LMG 925	*S. lycopersicum*	Hungary	–
	LMG 920, DAPP-PG 223, 466, 468	*S. lycopersicum*	Italy	–
	LMG 911^T, 916	*S. lycopersicum*	New Zealand	–
	LMG 919	*S. lycopersicum*	Zimbabwe	–
Xanthomonas gardneri	NCPPB 881	*S. lycopersicum*	ex Yugoslavia	–
Xanthomonas perforans	NCPPB 4321	*S. lycopersicum*	USA	–
Agrobacterium tumefaciens	DAPP-PG 252	–	Spain	–
Clavibacter michiganensis subsp. *michiganensis*	NCPPB 1064	*S. lycopersicum*	Italy	–
Dickeya chrysanthemi	NCPPB 402	*Chrysanthemum morifolium*	–	–
Escherichia coli	DAPP-PG 669	–	–	–
Pantoea agglomerans	CFBP 3845	–	–	–
Pectobacterium carotovorum subsp. *carotovorum*	LMG 2408	*Zantedeschia aethiopica*	Denmark	–
Pseudomonas corrugata	DAPP-PG 58	*S. lycopersicum*	Crete, Greece	–
	NCPPB 2445^T	*S. lycopersicum*	UK	–
Pseudomonas savastanoi pv. *savastanoi*	DAPP-PG 507	*Olea europaea*	Italy	–
	LMG 2209^T	*O. europaea*	ex Yugoslavia	–
Pseudomonas syringae pv. *aptata*	LMG 5059^T	*Beta vulgaris*	USA	–
P. syringae pv. *maculicola*	DAPP-PG 671	–	–	–
P. syringae pv. *tabaci*	GSPB 1209	*Nicotiana tabacum*	Göttingen, Germany	–
P. syringae pv. *tomato*	NCPPB 1106^T	*S. lycopersicum*	UK	–
P. syringae pv. *syringae*	DAPP-PG 53	*Capsicum annum*	Italy	–
Pseudomonas viridiflava	LMG 2352^T	*Phaseolus* sp.	Switzerland	–
Ralstonia solanacearum	DAPP-PG 650	*Solanum tuberosum*	Egypt	–
	DAPP-PG 653	Soil	Egypt	–
	DAPP-PG 648	Water	Egypt	–
	DAPP-PG 652	Weeds	Egypt	–
Xanthomonas arboricola pv. *juglandis*	LMG 747^T	*Juglans regia*	New Zealand	–
Xanthomonas axonopodis pv. *phaseoli*	GSPB 330	*Phaseolus vulgaris*	Germany	–
	LMG 7455^T	*P. vulgaris*	USA	–
X. axonopodis pv. *vignicola*	DAPP-PG 588	*Vigna unguiculata*	Mozambique	–

Table 1 (Continued)

Bacterial species	Strain*	Biological origin	Geographic origin	PCR
	LMG 828	*Vigna unguiculata*	Sudan	–
	LMG 8752^T	*V. unguiculata*	Texas, USA	–
Xanthomonas campestris pv. *campestris*	DAPP-PG 303, 305	*Brassica oleracea*	Italy	–
	LMG 568^T	*B. oleracea*	UK	–
Xanthomonas citri pv. *malvacearum*	DAPP-PG 71, 81	*Gossypium* sp.	Greece	–
	DAPP-PG 222	*Gossypium* sp.	Oklahoma, USA	–
Xanthomonas fuscans subsp. *fuscans*	LMG 837	*Phaseolus vulgaris*	South Africa	–
Xanthomonas hortorum pv. *pelargonii*	DAPP-PG 491	*Pelargonium peltatum*	Italy	–
	LMG 7314^T	*P. peltatum*	New Zealand	–
Xanthomonas translucens	DAPP-PG 264	*Hordeum vulgare*	Germany	–
X. translucens pv. *arrhenatheri*	DAPP-PG 265	*Arrhenatherum elatius*	Germany	–
X. translucens pv. *phlei*	DAPP-PG 281	*Pleum pratense*	Germany	–
X. translucens pv. *poae*	DAPP-PG 263	*Poa pratensis*	Germany	–
Unknown, Gram-negative	Sa4, Sa5, Sa6, Sa7, Sa8	*C. annuum*	Italy	–
Unknown, Gram-positive	Sa3, Sa9, Sa10, Sa11	*C. annuum*	Italy	–
Unknown, Gram-negative	Sa1, Sa2	*S. lycopersicum*	Italy	–

–, unknown biological and/or geographic origins.

*CFBP (Collection Française de Bactéries Phytopathogènes, France), DAPP-PG (Phytopathogenic bacteria collection of the Arboriculture and Plant Protecion Section, Department of Agricultural and Environmental Sciences, University of Perugia, Italy), GSPB (Göttinger Sammlung Phytopathogener Bakterien, University of Göttingen, Germany), LMG (Culture Collection Laboratorium voor Microbiologie, University of Gent, Gent, Belgium) and NCPPB (National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, UK).

REP-PCR analysis. From the similarity matrix, a dendrogram was obtained, which was a good representation of the matrix (cophenetic value = 0·97; see Fig. S1 in Supporting Information). At an arbitrary 70% similarity cut-off value, all the *X. euvesicatoria* strains are present in a cluster, while *X. vesicatoria* in two clusters, with a similarity of 42·8%. Neither *X. perforans* nor *X. gardneri* grouped with other clusters. The same results were obtained when the analysis was repeated. REP-PCR analysis also revealed that the *X. euvesicatoria* strains exhibited a specific 1600 bp fragment, which is not present in the other bacteria tested. When the partial nucleotide sequence of this fragment was compared by BLASTx with the protein sequences present in the GenBank, it showed high identity with the sequence (YP_364868.1) of a hypothetical protein XCV3137 of *X. campestris* pv. *vesicatoria* strain 85-10, which now belongs to *X. euvesicatoria*.

PCR assay specificity and sensitivity

Xeu2.4 and Xeu2.5 primers, used at low $MgCl_2$ concentration (1 mmol l^{-1}) in the PCR, are specific for *X. euvesicatoria* detection. In fact, the expected DNA band of 208 bp was obtained only in all the *X. euvesicatoria* strains tested (Table 1). The assay sensitivities were 2×10^2 CFU ml^{-1} (Fig. 1a) and 1 ng ml^{-1} of DNA (Fig. 1b), corresponding to two bacterial cells and 1 pg of DNA of *X. euvesicatoria* NCPPB 2968^T per PCR. Same results were obtained when the *X. euvesicatoria* DAPP-PG 34 was used (data not shown).

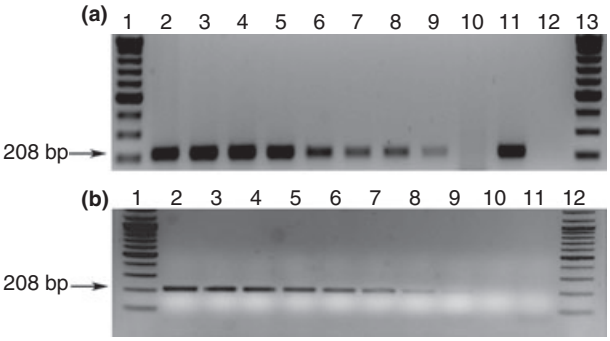

Figure 1 Limits of detection of Xeu2.4/Xeu2.5-based PCR assay for *Xanthomonas euvesicatoria* NCPPB 2968^T cells (a) and purified DNA (b). (a) Lanes 2 through 10, cells at the concentrations of 2×10^9, 2×10^8, 2×10^7, 2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2 and 2×10^1 CFU ml^{-1}. Lane 11, *X. euvesicatoria* NCPPB 2968^T DNA equivalent to 25 ng. (b) Lanes 2 through 10, DNA concentrations equivalent to 25, 12·5, 6·25, 1, 0·5, 0·05, 0·005, 0·001 and 0·0005 μg ml^{-1}, respectively. Lanes 12 (a) and 11 (b), negative control. Lanes 1 and 13 (a) and lanes 1 and 12 (b), molecular weight marker DNA Ladder Mix (MBI Fermentas, Burlington ON, Canada).

Detection of *Xanthomonas euvesicatoria* in plant extracts

Xeu2.4/Xeu2.5-based PCR assay and the extraction procedures used permitted us to specifically detect the presence of all *X. euvesicatoria* strains used in asymptomatic inoculated pepper (Fig. 2) and tomato (see Fig. S2 in Supporting Information) plants, 3 days after the inoculation. Similar results were obtained in symptomatic plants, 10 and 20 days after the inoculation (data not shown).

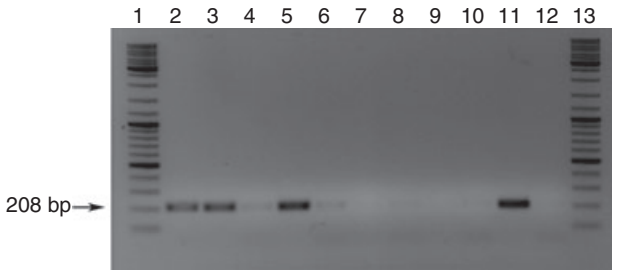

Figure 2 Specificity of Xeu2.4/Xeu2.5-based PCR assay for the detection of *Xanthomonas euvesicatoria* in asymptomatic inoculated pepper plants, 3 days after the inoculation. Extracts obtained from plants inoculated with: *X. euvesicatoria* NCPPB 2968^T, 82-8, DAPP-PG 34 and 75, LMG 668 (lanes 2 through 6), *Xanthomonas vesicatoria* LMG 911^T (lane 7), *Xanthomonas gardneri* NCPPB 881 (lane 8), *Xanthomonas perforans* NCPPB 4321 (lane 9); extracts obtained from control plants (lane 10); lane 11, 25 ng of DNA from *X. euvesicatoria* NCPPB 2968^T. Lane 12, negative control. Lanes 1, 13 molecular weight marker DNA Ladder Mix.

Discussion

Detection methodologies for phytopathogenic bacteria have benefited greatly from modern molecular technology involving specific amplification of target DNA fragments; especially, for quarantine bacteria (De Boer *et al.* 2007). A number of molecular methods, based on PCR, have been developed for the detection of BSX. Obradovic *et al.* (2004) described a method based on the amplification of a region of the *X. campestris* pv. *vesicatoria hrp* gene cluster, which detects all the BSX and each species only after restriction enzyme analysis of the amplicon produced. Based on the probe of Kuflu and Cuppels (1997), a PCR-based protocol was developed to detect all BSX, which has a moderate level of specificity for *X. euvesicatoria* (Cuppels *et al.* 2006). The method developed by Park *et al.* (2009), based on the amplification of a *rhs* family gene, has been validated on very few BSX strains not including all four different bacterial species.

Rep-PCR has been useful for identifying genetic variation within phytopathogenic bacteria (Rademaker and de Bruijn 1997). Characterization by REP-PCR of our BSX strains is consistent with the results obtained by Louws *et al.* (1995) who documented that strains of group A (= *X. euvesicatoria*) were relatively homogeneous, whereas those of the group B (= *X. vesicatoria*) were heterogeneous (see Fig. S1 in Supplementary material). Rep-PCR also permits the identification of specific sequences useful for developing PCR-based assays for phytopathogenic bacterial detections (Tegli *et al.* 2002; Moretti and Buonaurio 2007).

By REP-PCR fingerprinting of several BSX strains, we developed a PCR-based assay for the specific detection of *X. euvesicatoria*, which has been validated using 64 strains of the bacterium from different hosts and geographical origins (Table 1). The PCR-based assay developed, which was improved by lowering the Mg^{2+} concentration and enhancing the annealing temperature, is specific for *X. euvesicatoria* as it does not detect all the other bacteria tested (Table 1). The assay has a very high sensitivity, similar to the levels obtained in other studies (Berg *et al.* 2005; Robène-Soustrade *et al.* 2006).

Our PCR protocol permits to detect *X. euvesicatoria* in infected pepper and tomato plants only when the polyphenolic-binding compound PVP is included in the extraction mixture; plant polyphenols form complexes with nucleic acids inhibiting PCR (Koonjul *et al.* 1999). The high sensitivity of our protocol renders it suitable for detecting the target bacterium in symptomless plants. After validation by a ring test, in which a number of different laboratories apply the protocol to the same samples and the results obtained were compared, our protocol could be used in detecting *X. euvesicatoria* in tomato and pepper transplants and seeds, which is the best means to prevent further bacterial dissemination (Stall 1993).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. UPGMA dendrogram (Jaccard coefficient) and genetic relatedness of 81 isolates of *Xanthomonas* strains obtained by repetitive extragenic palindromic sequence (REP)-PCR analysis and REP primer sets. The arrow indicates the cut-off value (70%) for cluster analysis. *Xanthomonas euvesicatoria*: NCPPB 2968^T, 75-3, LMG 667 and 913, CFBP 5597 and 5601; group A = DAPP-PG 39, 233, 234, 236, 237, 240 and 243, CFBP 5594 and 6811, LMG 905, 910, 914; group B = DAPP-PG 77, 79, 89, 93, 94, 128, 137, 145, 231 and 232; group C = 82-8, DAPP-PG 29, 30, 40, 41, 42, 44, 78, 86, 87, 95, 96, 99, 119, 122 and 142; group D = CFBP 1604, LMG 668, 909, 926, 929; group E = DAPP-PG 31, 32, 33, 34, 35, 37, 38, 43, 45, 75, 80, 82, 88, 129 and 148. *Xanthomonas vesicatoria*: LMG 911^T, 916, 919, 920, 925, 934; group F = DAPP-PG 114, 223, 466 and 468. *Xanthomonas gardneri* NCPPB 881. *Xanthomonas perforans* NCPPB 4321. *Xanthomonas axonopodis* pv. *phaseoli* LMG 7455^T. *Xanthomonas fuscans* subsp. *fuscans* LMG 837. *X. axonopodis* pv. *vignicola* LMG 8752^T. *Xanthomonas citri* pv. *malvacearum* DAPP-PG 222.

Figure S2. Specificity of Xeu2.4/Xeu2.5-based PCR assay for the detection of *Xanthomonas euvesicatoria* in asymptomatic inoculated tomato plants, 3 days after the inoculation. Extracts obtained from plants inoculated with: *X. euvesicatoria* NCPPB 2968^T, 82-8, DAPP-PG 34 and 75, LMG 668 (lanes 2 through 6), *Xanthomonas vesicatoria* LMG 911^T (lane 7), *Xanthomonas gardneri* NCPPB 881 (lane 8), *Xanthomonas perforans* NCPPB 4321 (lane 9); extracts obtained from control plants (lane 10). Lanes 1 and 11, molecular weight marker DNA Ladder Mix (MBI Fermentas, Burlington, ON, Canada).

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