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Development of a Diagnostic Test Based on the Polymerase Chain Reaction (PCR) to Identify Strains of *R. solanacearum* Exhibiting the Biovar 2 Genotype

M. Fegan, G. Holoway, A.C. Hayward, and J. Timmis

R. solanacearum has been classified into three races based on host range (Buddenhagen et al. 1962) and five biovars based on oxidation of sugars and sugar alcohols (Hayward 1964) the only agreement between the two schemes is that *R. solanacearum* biovar 2 is equivalent to *R. solanacearum* race 3. *R. solanacearum* biovar 2/race 3 isolates have a very narrow host range, being limited almost entirely to potatoes. Bacterial wilt (Brown rot) caused by *R. solanacearum* isolates of biovar 2/race 3 is a significant disease of potatoes world wide. Infection of potato tubers with *R. solanacearum* biovar 2/race 3 may become latent under conducive environmental conditions. Other races of *R. solanacearum* can infect potatoes but it is the biovar 2/race 3 phenotype that is the most persistent and potentially the most destructive phenotype for potatoes. Consequently, there is a need for rapid, sensitive diagnostic tests for identification of plant material infected with the biovar 2/race 3 phenotype. Tests for the detection of *R. solanacearum* in potatoes have traditionally been directed against the entire species and have not concentrated on the biovar 2/race 3 phenotype. Skoglund et al. (1993) report on molecular and immunological techniques applied to the identification of infected tubers in Burundi, all the tests were directed toward the species *R. solanacearum* not the biovar 2/ race 3 phenotype. The only test described to identify the race 3/biovar 2 phenotype is the DNA probe based test of Cook and Sequeira (1991). We describe here the first test for the biovar 2/race 3 phenotype of *R. solanacearum* employing the polymerase chain reaction.

Methods and Materials

Bacterial Strains

All *R. solanacearum* and BDB isolates used in this study were cultured on sucrose-peptone agar (Hayward 1964) incubated at 28°C for 48 to 72h. *P. syzygii* isolates were cultured on Casamino Acids medium (Roberts et al. 1990).

DNA Purification

Genomic DNA was isolated using the method of Chen and Kuo (1993) or the method of Boucher et al. (1987).

Southern Hybridisation and Competitive Hybridisation of Whole Genomic DNA of *R. solanacearum*.

DNA extracted from *R. solanacearum* strains ACH0158 and ACH0171 were restricted with the restriction endonuclease *Pst*I. Restricted DNA was electrophoresed in a 0.8% agarose gel in TAE buffer. To identify DNA bands specific for ACH0158 the gel was Southern blotted onto nylon membrane and hybridised with total genomic DNA from ACH0158 and also hybridised with total genomic DNA previously prehybridised with a large excess of competitor ACH0171 DNA as described by Cook and Sequeira (1991).

Cloning of *R. solanacearum* Strain ACH0158 DNA

DNA bands were excised from the agarose gel with a sterile scalpel and recovered from the agarose-gels using the method of Thuring et al. (1975). Eluted DNA-fragments were further digested with *Sau*3A and cloned into the plasmid pBluescript.

Probe Preparation Southern blotting, Hybridisation and Autoradiography

Plasmid inserts to be radioactively labelled were isolated from an agarose gel and labelled using the oligolabelling method (Feinberg & Vogelstein 1983) using a GIGAprime™ DNA labelling kit (Bresatec, South Australia) and $\alpha[^{32}P]$-dATP (Bresatec). Unincorporated nucleotides were removed by centrifugation through Bio-Gel..The specific activity of the probe was determined by scintillation counting.

DNA was transferred from agarose gels to Hybond™-N^+ membranes (Amersham) (Southern 1975). The nylon membranes were prehybridised at 65°C for at least 2 h in prehybridisation buffer. Membranes were then hybridised with alkali denaturated probe which was neutralised prior to addition to the hybridisation solution at 65°C for at least 16 h. After hybridisation membranes were washed at 65°C in 2 x SSC, 0.1% SDS for 30 min 1 x SSC, 0.1% SDS for 30 min and 0.5 x SSC and 0.1% SDS for 30 min. Membranes were wrapped in plastic film to prevent drying and exposed to X-ray film (Fuji) in an X-ray cassette with an intensifying screen for at least 1 h at -80°C.

Sequencing

Cloned *R. solanacearum* DNA was manual sequenced using the sequenase system. Sequence comparisons were performed by a homology search of the GenBank data base using the "Fasta" algorithm of the Genetics Computer Group Sequence Analysis Software Package Version 7.0 (Devereux et al. 1984)

Primer Design

Primers were designed by visual inspection of the sequence information, checking for the absence of homology between primers. The secondary structure of each primer was examined using the "squiggles" program of the Genetics Computer Group Sequence Analysis Software Package Version 7.0 (Devereux et al. 1984).

PCR Amplification Using Designed Primers

The optimised PCR conditions for primer pair 630/631 were; 25µl (total volume) reaction mixture containing PCR buffer [67mM Tris-HCl (pH 8.8) 16.6mM $(NH_4)_2SO_4$, 0.45% (vol/vol) Triton X-100, 200µg of gelatine per ml] 1.5mM $MgCl_2$, each deoxyribonucleotide triphosphate at a concentration of 200µM, 6 picomoles of each primer(630/631), 25ng of DNA and 0.5 U of *Tth* plus DNA polymerase (Biotech International, Ltd., Perth, Australia). Negative controls that contained all of the ingredients described above except for the template DNA were included in each experiment. PCR amplifications were performed with a MJ Research PTC100 thermocycler programmed as follows: an initial denaturation step at 96°C for 5 min, followed by 30 cycles of 60°C for 30 s, 72°C for 30 min, and 94°C for 15 s, with a final extension step of 72°C for 10 min.

REP-PCR Analysis

The method described by Louws et al. (1994) was employed using the primer BOXA1R (Louws et al. 1994). PCR Amplification was performed on a MJ Research PTC 100 thermocycler in a 25 µl reaction volume containing PCR buffer [67mM Tris-HCl (pH 8.8) 16.6mM $(NH_4)_2SO_4$, 0.45% (vol/vol) Triton X-100, 200µg of gelatine per ml] 1.5mM $MgCl_2$, each deoxyribonucleotide triphosphate at a concentration of 250µM, 30 picomoles of BOX1AR primer, 25ng of DNA and 2.2 U of *Tth* plus DNA polymerase (Biotech International, Ltd., Perth, Australia). Negative controls that contained all of the ingredients described above except for the template DNA were included in each experiment.

The amplification products were resolved on a 3% NuSieve® 3:1 agarose gels (FMC) using TAE buffer electrophoresed at 4V/cm for 3 h. Agarose gels were stained with 0.5 mg/ml ethidium bromide, products were visualised on a UV transilluminator and photographed using an MP4 land camera.

Results

Southern Hybridisation and Competitive Hybridisation of Whole Genomic DNA of *R. solanacearum* and Elution of ACH0158 Specific Genomic DNA Fragments.

Agarose gel resolution of *Pst*I-digested ACH0158 genomic DNA revealed many restriction fragments that were absent from a comparable digest of genomic DNA from *R. solanacearum* strain ACH0171 (Fig. 1; lanes 1 and 2). On standard Southern analysis or using competitive Southern hybridisation analysis (Cook & Sequeira 1991) many DNA fragments were potentially *R. solanacearum* strain ACH0158 specific. The three DNA fragments of highest molecular weight (approx. 27.6, 21.6 and 18.6 kb; indicated in the rectangle superimposed on Fig. 1) were eluted from the gel together.

Cloning of *R. solanacearum* Strain ACH0158 DNA and Testing of Clone Specificity for Biovar 2 Organisms

The three fragments eluted from the agarose gel were further restricted with *Sau*3A and cloned into the *Bam*HI site of pBlueScript. Cloned fragments were tested for specificity for ACH0158 and other *R. solanacearum* biovar 2 isolates by Southern hybridisation. Plasmid pBs2.10 contained an insert that was specific for *R. solanacearum* isolates belonging to biovar 2, hybridising to the 27.6 kb *Pst*I fragment of ACH0158 (results not shown) and an *Eco*RI fragment of approximately 5 kb of biovar 2 isolates ACH0158, SA1S and SA1R (Fig. 2). This cloned fragment did not hybridise to restricted DNA from biovar 3 (ACH0171) or biovar 4 (ACH092) isolates (Fig. 2).

Sequencing

The sequence of the subcloned fragment of *R. solanacearum* DNA from ACH0158 in plasmid pPs2.10 was determined and is shown in Fig. 3

PCR amplification Using Designed Primers

The primer pair 630 - 631 (Fig. 3) were designed and the optimal PCR conditions determined. The primers and PCR protocol were then tested against over 200 *R. solanacearum* isolates. Only isolates of biovar 2 produced the expected amplification product of 357bp. However, a biovar 2 isolate from potato in Indonesia failed to produce an amplification product and three strains of biovar 1 did produce the 357bp product. An example of a typical PCR result is shown in Fig. 4.

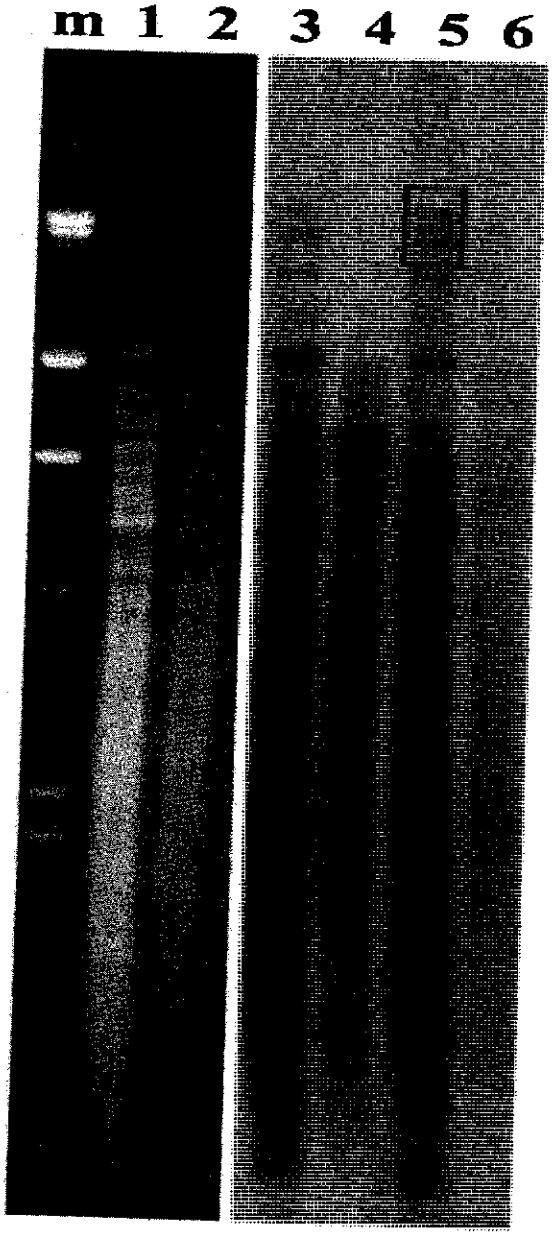

Fig. 1. Southern hybridisation and competitive hybridisation of whole genomic DNA of *R. solanacearum*. Lane 1 contains lambda bacteriophage DNA restricted with *Hin*dIII showing fragments of 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb. Lane 2 and 3 show PstI digested DNA from strains ACH0158 and ACH0171 stained with ethidium bromide. Lanes 4 and 5 show autoradiographs of Southern transfers of lanes 3 and 4 after probing with labelled ACH0158 whole genomic probe. Lanes 6 and 7 are similar Southern hybridisations to lanes 4 and 5 where the probe had been prehybridised with a large excess competitor 0171 DNA (Cook and Sequeira 1991). The bands that were eluted from the gel and cloned are enclosed inside a rectangle.

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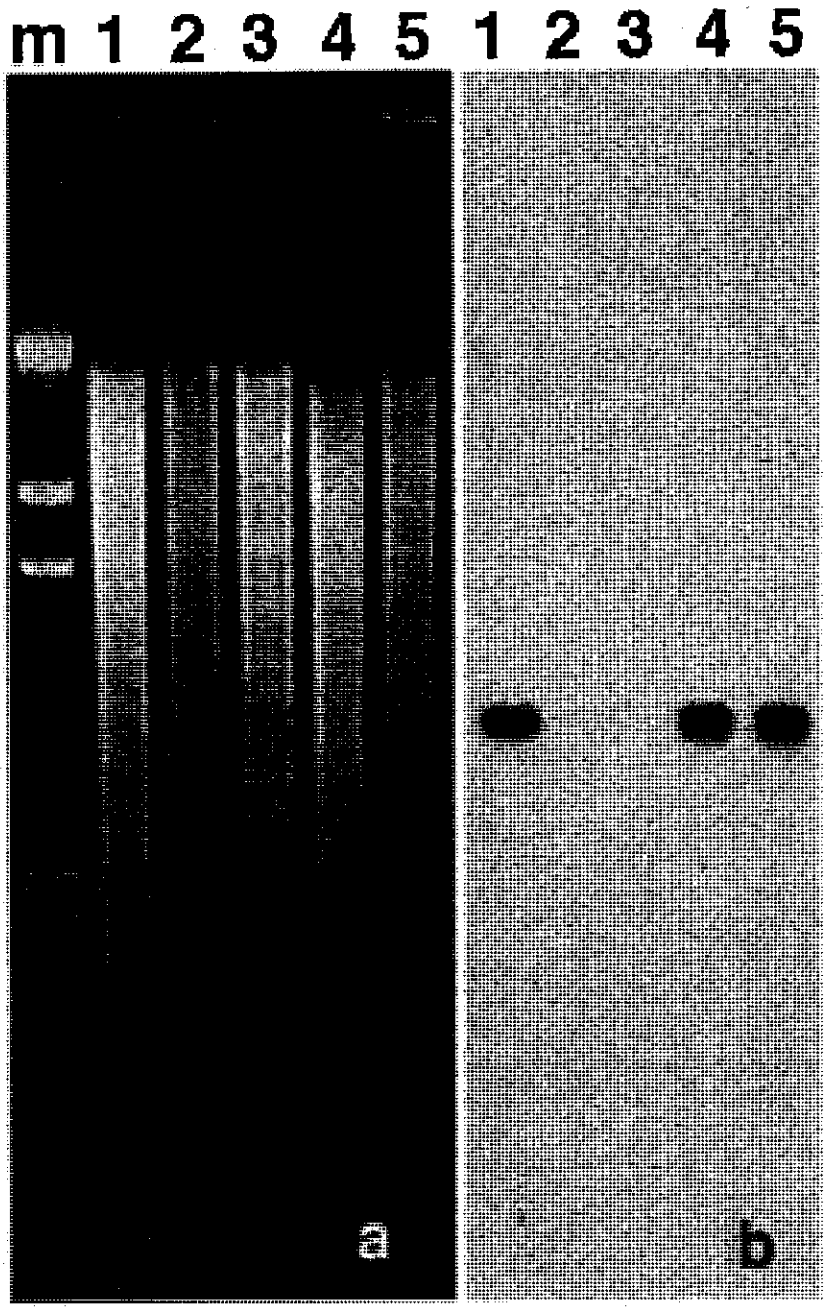

Fig. 2. Hybridisation of pPs2.10 to *Eco*RI digested DNA from *R. solanacearum* strains ACH092, ACH0158, ACH0171, SA1S and SA1R. (a) Lane 1 contains lambda bacteriophage DNA restricted with *Hin*dIII showing fragments of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kb. Lanes 1-5 show *Eco*RI digested genomic DNA from strains ACH0158, ACH092, ACH0171, SA1S and SA1R stained with ethidium bromide. (b) Lanes 1-5 show the corresponding lanes from (a) after transfer and Southern hybridisation with pPs2.10. SA1S is a Biovar 2 strain isolated from potato in South Australia. SA1R is a phenotype-converted derivative of SA1S.

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REP-PCR analysis

All biovar 2 isolates produced a similar banding pattern. An example of the results of REP-PCR analysis can be seen in Fig. 5. The same pattern was also produced by three isolates of biovar 1 (CIP03, CIP430 and CIP433; marked in a rectangle on Fig. 5).

Discussion

The object of this study was to detect DNA sequences that are specific for *R. solanacearum* strains of the biovar 2/race 3 phenotype which are potentially useful for the development of diagnostic tests. The approach of Southern and competitive hybridisation (Fig .1) followed by cloning and screening of cloned fragments for specificity to *R. solanacearum* phenotypes was successful in identifying *R. solanacearum* biovar 2 specific DNA fragments (Fig. 2). The biovar 2-specific 570bp fragment cloned into plasmid pBs2.10 was sequenced and primers designed for specific amplification of *R. solanacearum* biovar 2. The primers, tested against greater than 200 *R. solanacearum* strains of all biovars amplified all isolates of biovar 2 tested with two exceptions, strain R784 isolated from potato in Indonesia and strain ACH0732 isolated from tomato in the Northern Territory in Australia. These two isolates are not typical biovar 2 isolates, strain R784 belongs to the Indonesian subgroup of isolates (Fegan unpublished data) that are phylogenetically distinct from typical biovar 2 isolates (Taghavi et al. 1996). Strain ACH0732 is an aberrant biovar 2 isolate that is also phylogenetically distinct from the typical biovar 2 isolates (Taghavi et al. 1996). Both isolates produce rep-PCR patterns that are atypical of biovar 2 organisms (Fegan and Brunori unpublished data).

Apart from the two atypical biovar 2 isolates that did not produce the expected PCR product the only other unusual result was the production of a PCR product by three strains of *R. solanacearum* of biovar 1. These three strains (CIP03, CIP430 and CIP433) were isolated from potato in Peru and all produce the same rep-PCR profile as produced by biovar 2 isolates (Fig. 5). One of these isolates CIP03 has previously been recognised as being atypical (Marín and El-Nashaar 1993). Marín and El-Nashaar (1993) report that CIP03 differed from other biovar 1 strains tested in their study in biochemical activities, colony morphology and pathogenicity. CIP03, with the exception of the oxidation of the three disaccharides that differentiate biovars 1 and 2, had similar biochemical properties to biovar 2 organisms (Marín and El-Nashaar 1993). In terms of pathogenicity CIP03 exhibited a pathogenicity profile similar to biovar 2/race 3 strains, including a high pathogenicity index on *Solanum nigrum.*

While this primer pair has yet to be completely validated we believe that the primers 630/631 will detect all *R. solanacearum* strains that are of the same genotype as the typical biovar 2/race 3 strains isolated from potato and could be used for the detection of these organisms in infected potato tubers.

1 GATCTTGTAA GCCTTGGTAC CCAGGTGGTG CCACGCTTCC TTCCCATCGC 50

51 TGAAGCCAAG GGCGCAGTTC CACACCCGTG ACCTGATAGT TGAAACTGCC 100

101 CAGCAGGTCG CCATTCCCAT **ACAGAATTCG ACCGGCACGC** CGAGCCTGAA 150

630---------->

151 CCTTGCGCGC GGTGGCCAAA CTCATCTGGG CCATTCTTGC GAAACGACTT 200

201 GCCTTGCTGC TGCCAAATCG CCGTGCCGAT GGTCAATGGT GACAACGGTT 250

251 TCCACTTCGT ACCATCCGGC GCCAGCCCTT TGTCATGGCG CTCCTGATTC 300

301 ACCGCAAACA GCGATTCGCC GATGCTTCCC AGCATCTGCT GGGGCGTAAT 350

351 CACTTCCTGG CGCACTGCAC TCAACGCTTG CAGCAGGTGT TCGGCTTGAA 400

401 ATT**CGTAGGC GAATTGCATG TGATTGCCCC** GTGGTGATGG AGATGCGCCA 450

<----------631

451 GCGAGGCCGC CCCACCTATT TCTTGTAGAC CAACCGCCCG ATACGCTGTT 500

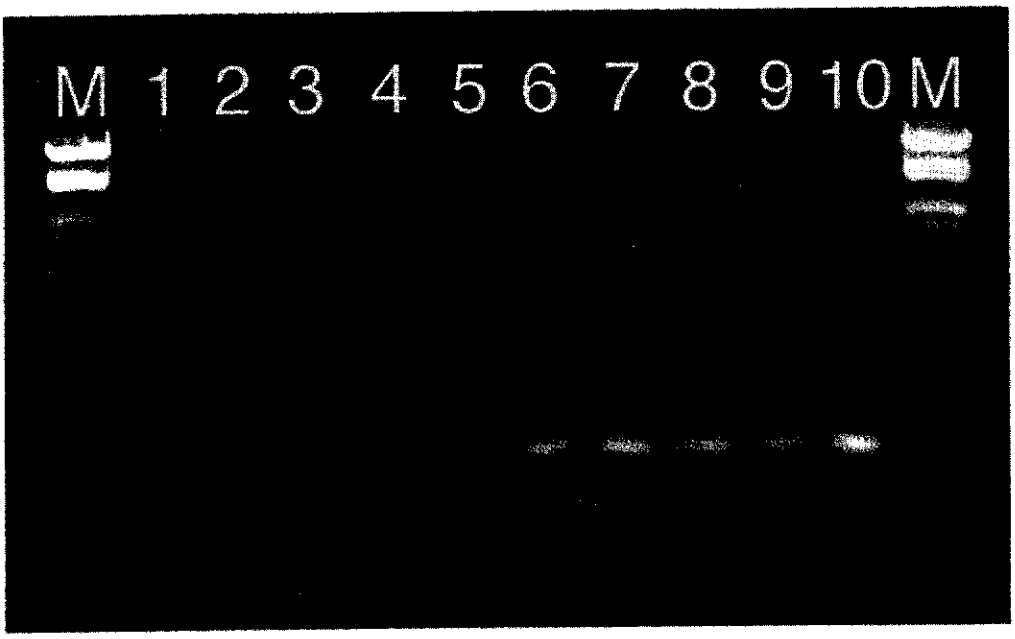
501 TATCGAGGGG CCGCGCGGTC TTCCGGCGCT TCGGTTCCCA TGAACGTGAC 550

551 ACGCCTGTCC TAGAGCGACC 570

Fig. 3. Nucleotide sequence of the cloned DNA fragment of *R. solanacearum* strain ACH0158in plasmid pPs2.10. Primers 630 (forward) and 631 (reverse) are shown in bold

Acknowledgments

The authors wish to acknowledge the financial support received from ACIAR projects PN9015 and PN9452.



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References

Boucher C.A, Van Gijsegem F, Barberis P, Arlat AM, Zischek C (1987) Pseudomonas solanacearum genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. J Bacteriol 169:5626 - 5632

Buddenhagen I, Sequeira L, Kelman A (1962) Designation of races in Pseudomonas solanacearum. Phytopathology 52:726

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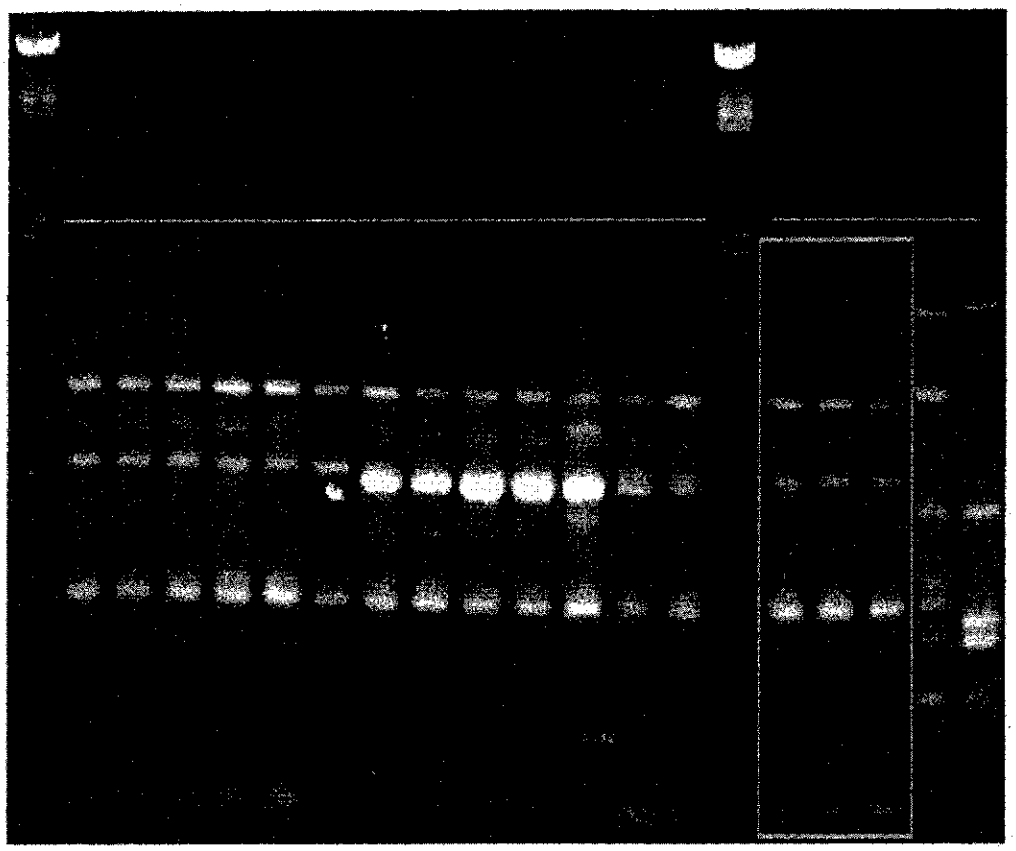

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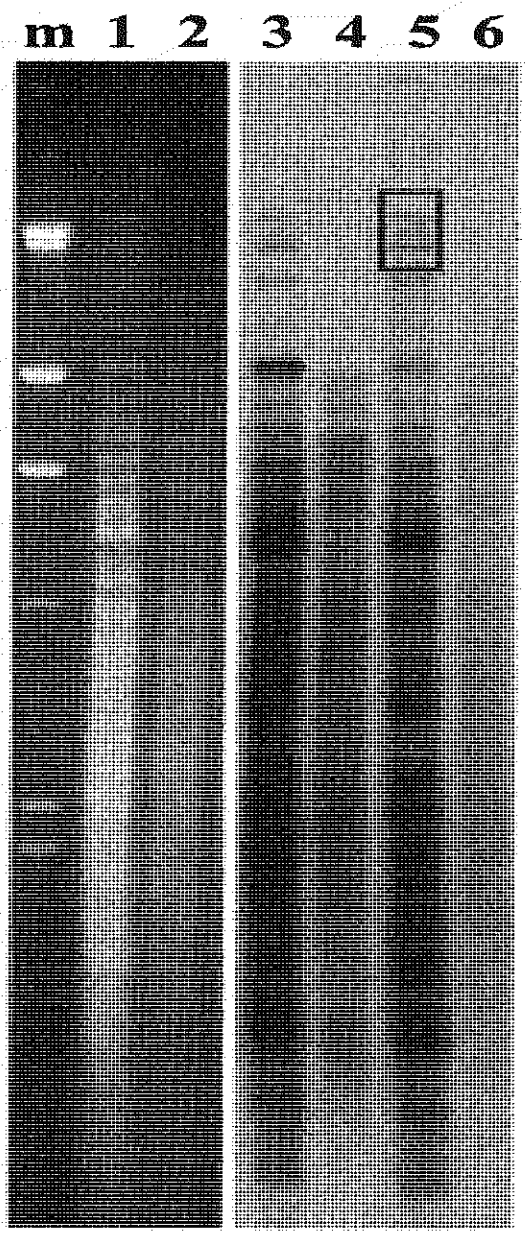

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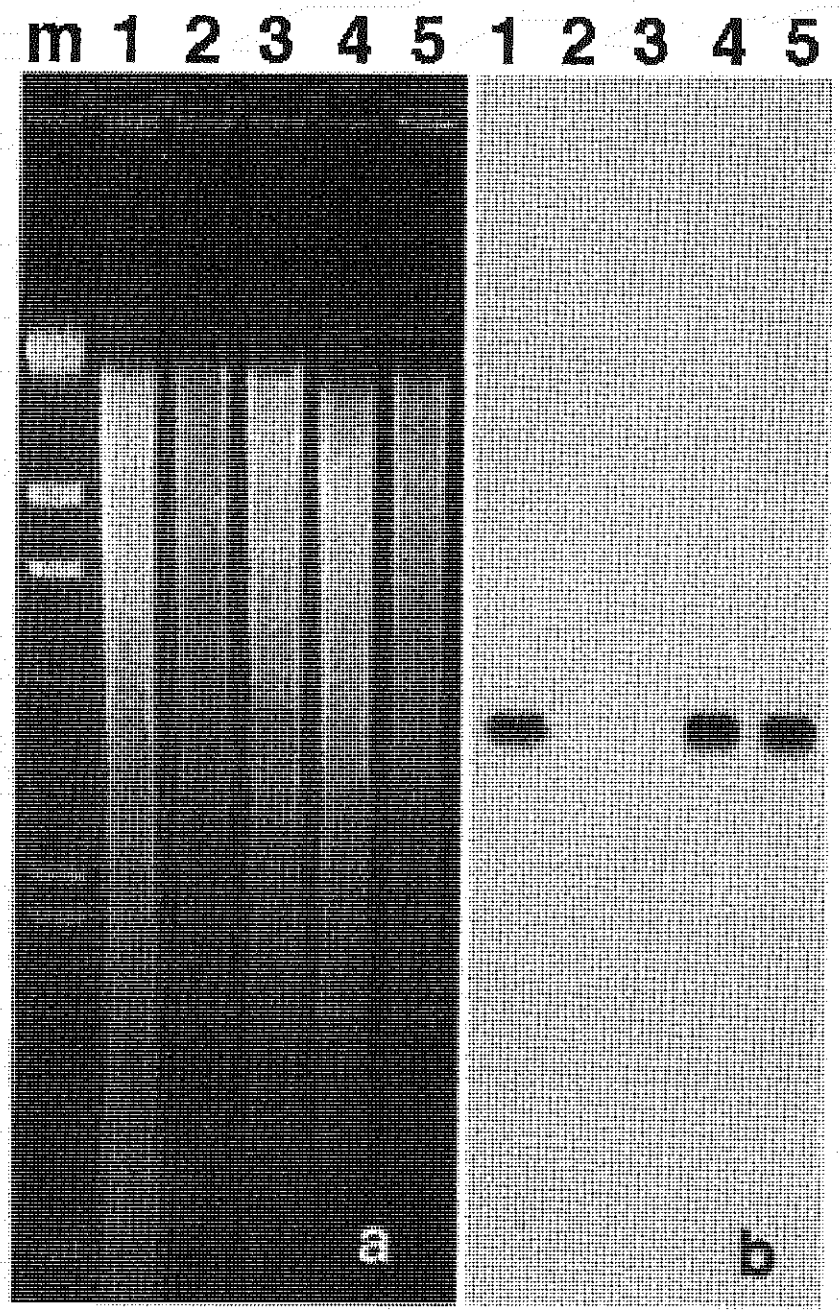

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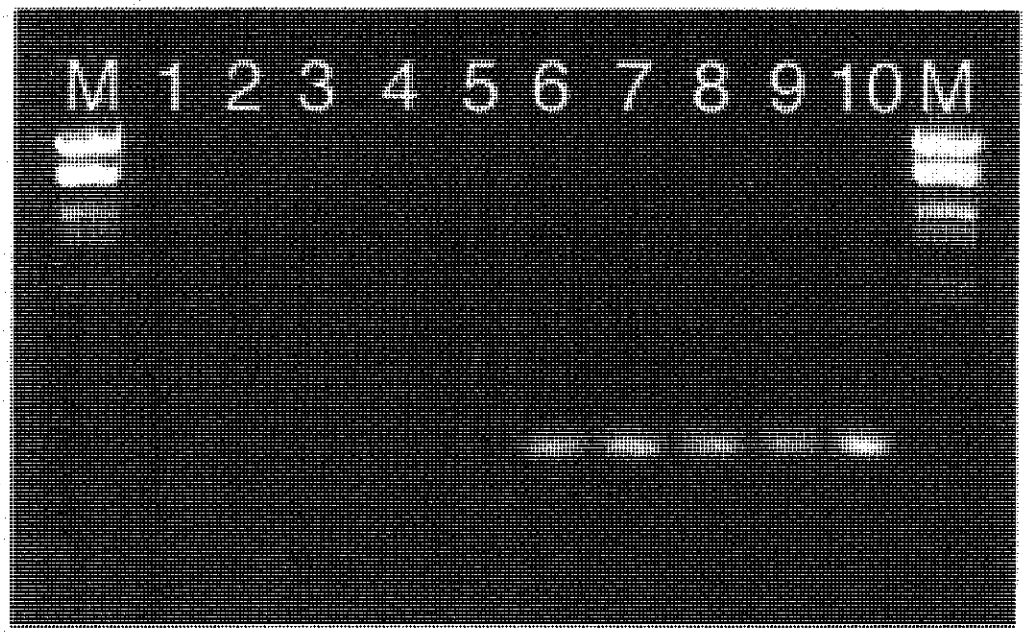

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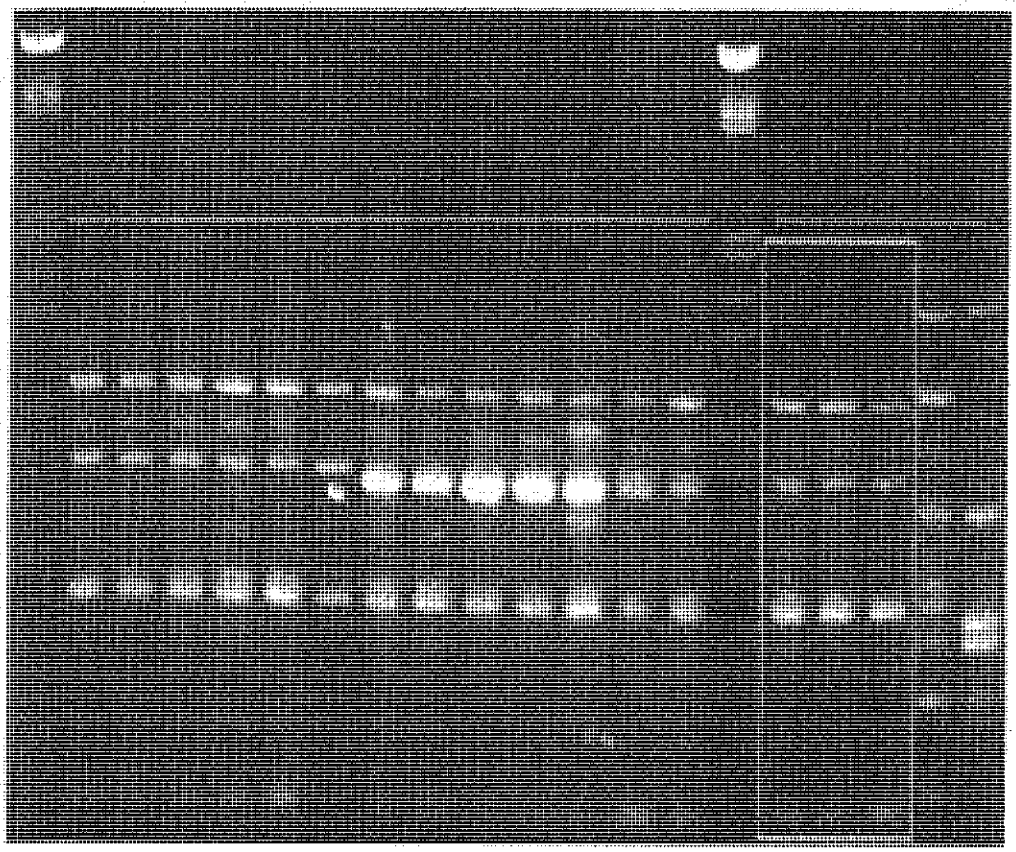

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