

PCR FINGERPRINTING OF DIVERSE GENOMES FROM BACTERIAL STRAINS USING UNIVERSAL RICE PRIMER (URP)

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ABSTRACT

Twenty primers of 20 mer referred to universal rice primer (URP) were developed from a repetitive sequence of rice genome. URP-PCR protocol employed stringent PCR with high annealing temperature throughout the thermo-cycling reaction, giving high reproducibility. Under the PCR condition, each single URP primer produced characteristic fingerprints from diverse genomes of bacterial species. The universal application of URP-PCR was demonstrated by applying it to 24 strains from *Pectobacterium carotovorum* subsp. *carotovorum*, 41 *Agrobacterium vitis* strains, 3 *Xanthomonas* spp. 5 *Pseudomonas* spp, *Rhizobium* sp. plant pathogenic bacteria, human and animal pathogenic bacterial strains including 6 *Escherichia coli*, 4 *Salmonella* spp., 7 *Mycobacterium* spp and 3 *Blucella abortus* strains. In addition, thermophilic bacteria were randomly isolated from high temperature compost and their URP-PCR polymorphisms were characterized with genetic relatedness. PCR approach using URP primers will be useful for studying DNA diversity of diverse prokaryotic genomes, especially at inter- and intra species levels.

Keywords: Diverse bacterial strains, PCR polymorphism, Universal Rice Primers

INTRODUCTION

The classic taxonomical methods of bacteria, relies on fatty acids, nutritional composition, and biochemical properties and so on (Garrity et al., 2002). However, it has been pointed out that microorganisms tend to lead to other consequences due to environmental effects, and that their shorter

generation and higher rates of metamorphosis have been a limiting factor in the bacterial classification system. The sequence of 16S rDNA was adopted as a new classification tool of the bacterial class (Chang et al., 1997), providing an opportunity for the revision of bacterial taxonomy to take place. In particular, inter transcribed spacer (ITS)-16S

rDNA sequence region is widely used as a species-specific identification of bacterial species (Chang et al., 1997, Garrity et al., 2002). However, since these rDNA regions are highly preserved areas, there is a limit to find polymorphism at level of inter-species such as subspecies, pathovar and race strains within species. Various molecular typing methods such as extragenic palindromic (REP) sequence, 124-127 enterobacterial repetitive intergenic consensus (ERIC) sequence 154 bp and BOX that are derived from repetitive sequences located in bacterial genomes have widely been used for discrimination of various bacterial strains at inter species level (De Bruijn, 1992, Hulton et al., 1991) Recently, in silico genomic fingerprints were devised to be produced by virtual hybridization of 191 fully sequenced bacterial genomes using a set of 15,264 13-mer probes specially designed to produce universal whole genome fingerprints (Jaimes-Diaz et al., 2011).

Multiple arbitrary amplicon profile (MAAP) that can universally be used in different organisms including plants, animals and microorganisms was developed for the use of single or multiple arbitrary primers (Caetano-Anolles, 1994; Caetano-Anolles and Gresshoff, 1997), and includes the techniques of RAPD (Williams *et al.*, 1990),

AP-PCR (Welsh *et al.*, 1990), and DNA amplification fingerprinting (DAF) (Caetano-Anolles *et al.*, 1992). Because of its simplicity, the RAPD method has been used widely in studies of genetic diversity and in examining phylogenetic and taxonomic relationship of bacterial strains (Caetano-Anolles and Gresshoff, 1997). However, in PCR techniques using arbitrary and RAPD primers, low reproducibility that causes variable PCR results depending on PCR condition has been recognized as a disadvantage factor (Caetano-Anolles *et al.*, 1992).

A repeated DNA fragment (pKRD) of 1,187 bp was isolated from a genomic library of weedy rice distributed in Korea. A homology search showed the pKRD sequence belonged to a putative transposable element, the CACTA-like *En/Spm* family. Furthermore, pKRD was used as a molecular marker to detect genetic variation of rice germplasm (Kang and Kang, 2008). Twenty primers consisting of 20 oligonucleotides were randomly designed from the repetitive

sequence, pKRD (Kang *et al.*, 2002). Under the high stringent PCR condition, twenty primers of them can be applied in genomic fingerprinting of a variety of organisms including animals, other plants, and microorganisms, as well as rice and named as universal rice primer (URP). URP-PCR technology has been applied for accessing genetic diversity of various fungal and bacterial species. Numerous papers have demonstrated that URP-PCR profiles of fungal species are very useful for identifying microbial species at intra and inter species levels (Aggarwal *et al.*, 2010. Aggarwal *et al.*, 2010; Hong *et al.*, 2008; Jana *et al.*, 2005; Kang, 2012; Kang, 2001, Kang, 2002) and further the specific PCR polymorphic bands could be used for finding SCAR PCR primer for diagnosis of bacterial species or strain (Kang *et al.*, 2003; Lim *et al.*, 2007).

In this study, it was demonstrated that URP primers can universally be applied to PCR fingerprinting of diverse genomes from plant, human, animal and industry related bacterial strains at levels of intra and inter

species.

MATERIALS AND METHODS

Bacterial strains and genomic DNA extraction

Fourty one *Agrobacterium vitis* strains were isolated from different grapevine varieties by Hankyong National University. Twenty four strains of *Pectobacterium carotovum* subsp. *carotovorum*, 3 *Xanthomonas* spp. 5 *Pseudomonas* spp, *Rhizobium* sp. of plant pathogenic bacteria were provided by Korea Agricultural Culture Collection (KACC). Thermophilic bacterial strains containing *Thermoactinomyces vulgaris* that are randomly isolated from high temperature fermented compost were obtained from National Institute of Agricultural Sciences (NIAS), Rural Development Administration in Korea. DNA samples of human and animal related bacterial strains included 6 *Escherichia coli* strains (ATCC11105, 111a111b, M15, DH5, and O157:H7), 4 *Salmonella* spp., 7 *Mycobacterium* spp. and 3 *Blucella abortus*

strains were obtained from Medical School in Yeonsei University of Korea. Genomic DNA from gram negative and positive bacteria was extracted following the procedure described by Ausubel et al.(1987).

PCR analysis

URP with 20 mer were provided from JK BioTech, Co. Ltd., Ansong, Korea. PCR reactions were carried out in 50 ul reaction mixtures containing the DNA template (20 to 50 ng of purified DNA), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 uM of each dNTP, 200 ng primer and 2.5 unit *Taq* DNA polymerase (Promega, USA). The reaction mixture was overlaid with a thin layer of sterile mineral oil to prevent evaporation. DNA amplification was performed in a programmable DNA thermal cycler (MJ Research, Inc., USA). The cycling parameters used were initial denaturation at 94°C for 4 min, followed by 35 cycles each consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. After the last cycle, the PCR tubes were incubated at 72°C

for 7 min and were held at 4°C. Amplified products were electrophoresed in a 1.5 % agarose gel in TAE buffer and visualized by staining with ethidium bromide.

PCR polymorphic bands based genetic relationship

PCR polymorphic bands were scored on their presence (value=1) or absence (value=0). The similarity coefficient was calculated by rearranging the scored bands of each rice variety. A dendrogram was constructed with the statistical program NTSYSpc (Rohlf, 2000) using the unweighted pair-group method with arithmetic mean (UPGMA).

RESULTS AND DISCUSSIONS

URP-PCR method was used to study the genetic diversity of 24 *P. carotovorum* subsp *carotovorum* (Pcc) strains causing bacterial soft rot disease on different plant species and 9 additive plant pathogenic bacteria. Fig. 1 shows PCR fingerprinting profile of diverse bacteria including

Pectobacterium carotovorum subsp. *tetraploid* grapevine cultivars of *Vitis vinifera* *carotovorum* (Pcc), *Xanthomonas* (Kyohoand, Daebong), which are relatively *axonopodis* pv. *citri*, *Pseudomonas* spp., sensitive to *A. vitis*, were intensively planted *Pseudomonas solanocearum*, *Rhizobium* in farm fields (80%) of Ansong and Cheonan *leguminosarum*, *Pseudomonas* pv areas of Korea. Forty one strains of *phaseoliala*, *Pseudomonas marginalis*, *Agrobacterium vitis*, the causal agent of *Xanthomonas campestris* pv. *campestris* and crown-gall disease on grapevine (Fig. 2 A), *Pseudomonas syringae* pv *syringae* using originating from different geographical URP 4. The PCR data revealed that the regions and 16 grapevine cultivars including majority of the bacterial strains have multiple 35 Kyoho cultivar of Korea, were banding patterns showing their PCR characterized by PCR fingerprinting using polymorphisms. Furthermore, the results URP 2 and URP 4(Fig. 2 B, C). *A. vitis* distinguished clearly difference among the strains originated from Kyoho cultivar of Pcc strains, showing their high genetic grapevine showed relatively simple genetic diversity. In the case of *Pseudomonas* species, diversity of the four PCR types, while the *A. vitis* *Pseudomonas* pv *phaseoliala* and *vitis* strains originated from other grapevine *Pseudomonas marginalis* were divided as cultivars showed various genetic diversities closely related group showing analogous with 8 types. Comprehensively, UPGMA PCR pattern among the *Pseudomonas* species. analysis based genetic relationship using the URP-PCR polymorphic bands showed 41 *A. vitis* *Agrobacterium vitis*, a soilborn bacterium, is *vitis* strains are genetically clustered into a causal agent of crown gall on grapevines. large seven groups and 17 subgroups in the Crown gall disease of grapevine has rapidly become problematic in Korea because groups (Data not shown).

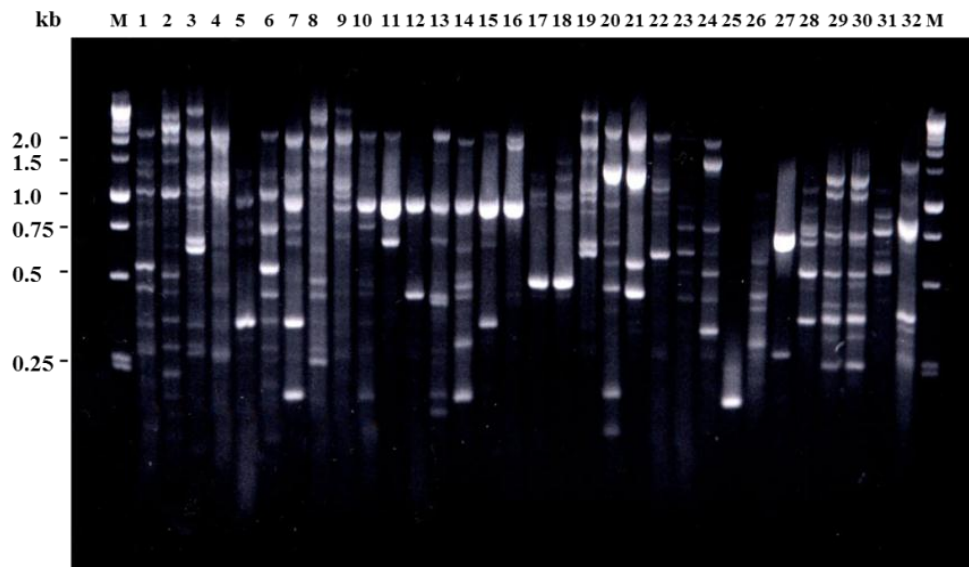
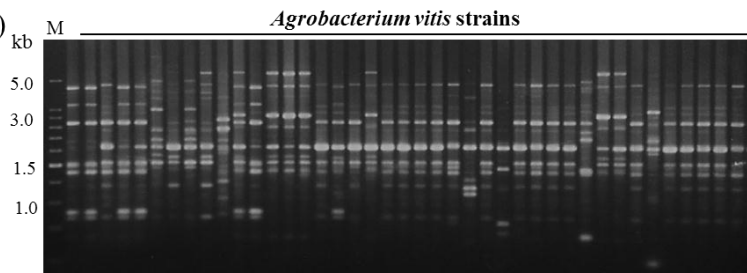


Fig. 1. PCR fingerprinting of different plant pathogenic bacterial strains by URP 4. M: 1kb DNA ladder (Promega), 1-24: *Pectobacterium carotovoum* subsp. *carotovorum*, 25: *Xanthomonas axonopodis* pv *citri*, 26: *Pseudomonas* sp. 27: *Pseudomonas solanocearum*, 28: *Rhizobium leguminosarum*, 29: *Pseudomonase* pv *phaseoliala*, 30: *Pseudomonas marginalis*, 31: *Xanthomonas campestris* pv. *campestris*, 32: *Pseudomonas syringae* pv *syringae*

(A)



(B)



(C)

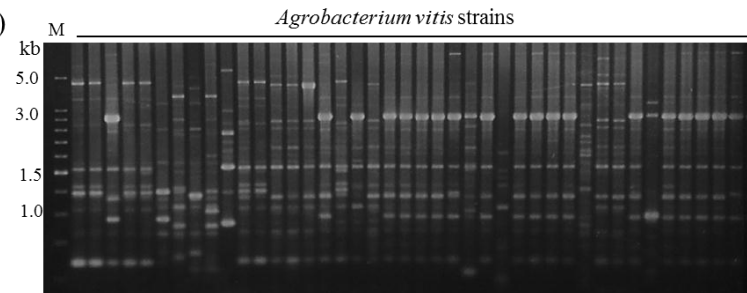


Fig. 2. URP-PCR polymorphism of *Agrobacterium vitis* strains, causal agent of crown gall on grapevine (A) detected by URP 2 (B) and URP 4 (C)

PCR-based methods are cheaper, easier and provide faster results; these methods include Random Amplified Polymorphic DNA PCR (RAPD-PCR), Repetitive extragenic palindromic PCR (REP-PCR) and Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR). These methods have been successfully applied for typing many bacteria (Anita et al. 2005; Johnson and Bryan, 2000; Williams et al. 1990). RAPD. Nevertheless, AP-PCR have been pointed out as low reproducibility problems as they are applied with annealing temperatures below 40°C. On the other hand, the URP-PCR can achieve stable PCR results because it has a high annealing temperature of 55°C. REP-PCR and ERIC-PCR fingerprinting methods using repeated sequence DNA of bacteria are limited in bacterial species retaining the sequence, whereas URP-PCR can universally apply in genomes from diverse bacteria strains without the reasonable sequence information on bacteria in advance. In particular, species or strain specific PCR fragment generated from URP-PCR products could be used in developing SCAR primers, which useful for specifically detecting *Agrobacterium vitis* and *Pectobacterium carotovorum* subsp. *carotovorum* (Kang et al., 2003; Lim et al., 2007).

Table 1. Oligonucleotide sequences of URP primers and their PCR applicability
on bacterial strains

Primers	Sequences (5'-3')	GC Conte nt	Tm (°C)	Applicability on bacterial strains
URP1	ATCCAAGGTCCGAGACAACC	50	65	Yes
URP2	CCCAGCAACTGATCGCACAC	50	65	Yes
URP3	AGGACTCGATAACAGGCTCC	50	66	Yes
URP4	GGCAAGCTGGTGGGAGGTAC	50	65	Yes
URP5	ATGTGTGCGATCAGTTGCTG	50	67	Yes
URP6	TACATCGCAAGTGACACAGG	50	68	No
URP7	AATGTGGGCAAGCTGGTGGT	55	74	Yes
URP8	GATGTGTTCTTGGAGCCTGT	50	65	Yes
URP8	GGACAAGAAGAGGATGTGGA	50	65	ND
URP9	TACACGTCTCGATCTACAGG	50	65	No
URP10	AAGAGGCATTCTACCACCAC	50	65	No

Genomic DNA of 225 strains in 45 bacterial species were used as templates for URP-PCR fingerprinting. The applicability of URP-PCR represents Yes, No and ND (Not determined)

In the following experiment, URP-PCR was applied to identification between or within human and animal -related bacterial species. As representative bacterial materials, genomic DNAs extracted from *Mycobacterium* spp. including 4 *Mycobacterium intracellulae*, strains, 4 *M. avium* strains, 3 *M. scroflaceum* strains, *M. fortuitum*, *M. gallinarum*, *M. canetti*, and *M. smegmatis*. *M. intracellulae*, *M. avium* and *M. scroflaceum* strains that are the most common cause of cervical lymphadenitis in children, *M. fortuitum* is one of the many species of nontuberculosis mycobacteria (NTM) that are commonly found in the environment. *M. canettii*, a novel pathogenic taxon of the *Mycobacterium tuberculosis* complex (MTBC) (Hirsh et al., 2004), *M. smegmatis* is commonly used in work on the *Mycobacterium* genus due to its being a "fast

grower" and non-pathogenic (Simner et al., 2013).

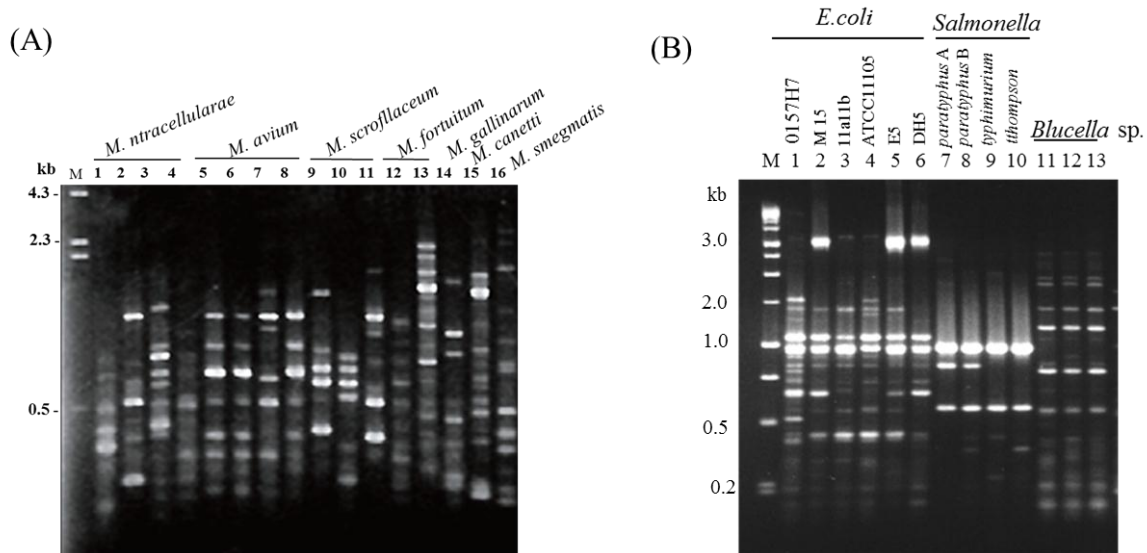


Fig. 3. PCR fingerprinting of human and animal related different bacterial species *Mycobacterium* spp. (A), *Echerichia coli* strains, *Salmonella* spp. and *Blucella* (B) by URP2R.

Mycobacterium avium-intracellulare infection (MAI) is an atypical mycobacterial infection, i.e. one with nontuberculous mycobacteria or NTM, caused by *Mycobacterium avium* complex ("MAC"), which is made of three mycobacteria species, *M. avium*, *M. intracellulare*, and *M. chimaera* (Horsburgh et al., 1985) This infection causes respiratory illness in birds, pigs, and humans, especially in immunocompromised people. As shown in Fig.3 (A), PCR polymorphism dependent on the strains was observed on PCR amplicons

of *Mycobacterium* spp., suggesting URP-PCR is useful for strain typing within the bacterial species. In addition, several human animal pathogenic bacteria were tested for confirming usefulness of URP-PCR. Non pathogenic *Escherichia coli* including strains ATCC11105, 111a111b, M15 and pathogenic *E. coli* O157:H7 that is one of the Shiga toxin-producing type and is a cause of disease, typically foodborne illness of the "colonic escherichiosis" type (Furrer et al., 1990). *E. coli* strains, *Salmonella* spp. and *Blucella abortus* strains were used as

template DNA and were amplified by primer URP-3(Fig. 3B). The *E. coli* O157:H7 specific PCR polymorphic bands were observed on size marker of 0.5 kb and other polymorphic bands were showed in reliance on *E. coli* strains. As a result, six *E. coli* strains were divided into four types. *Salmonella thompson*, *S. typhimurium*, *S. paratyphus* A, and *S. paratyphus* B that are causal agents of Salmonellosis, food poisoning (McClelland et al., 2001; Bej et al., 1994), and animal and human pathogenic bacteria species such as *Brucella* strains were

also examined. A polymorphic band of 0.8 kb was observed on only *S. paratyphus* A, and *S. paratyphus* B, showing its specific PCR fragment in the bacterial species. Nevertheless, URP-PCR polymorphism of *Brucella abortus* strains was identical in the strains. Consequently, URP-PCR yielded distinct PCR profiles that permitted differentiation among them at interspecies level, and furthermore, ubiquitous strain types were observed within *E. coli* strains and *Salmonella* species.

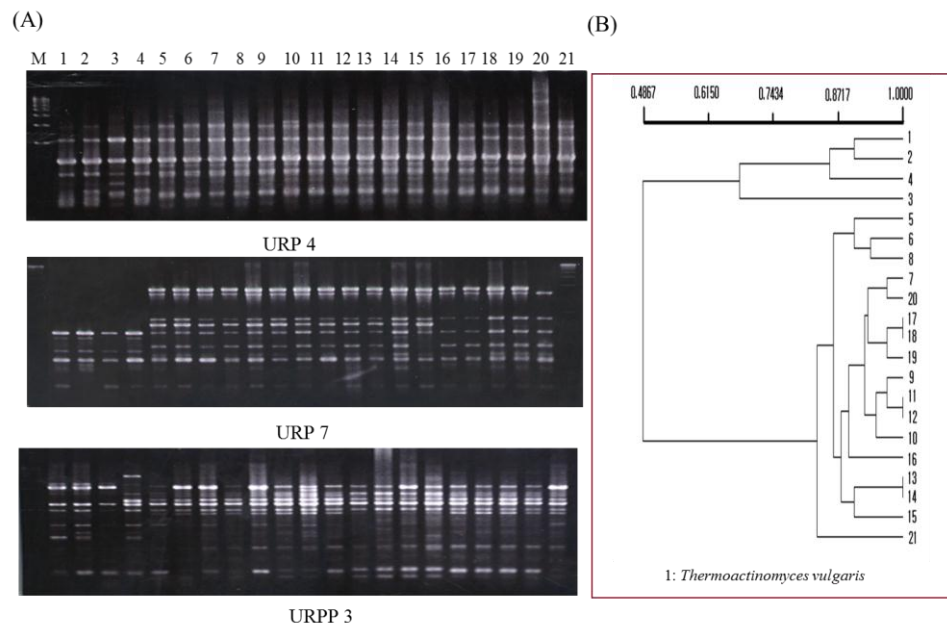


Fig. 4. URP-PCR profiles (A) and the PCR bands based UPGMA dendrogram (B) of hemophilic bacterial strains isolated from compost fermented with high temperature

Finally, thermophilic bacteria that was used for URP-PCR. Genomic DNAs were randomly isolated from high temperature (45-55°C) fermented compost and URP 7 (Fig. 4 A) and resultant

polymorphic bands were scored to construct dendrogram. As showed in dendrogram data of Fig. 4 B, the bacteria were divided into two large groups containing culture collection, *Thermoactinomyces vulgaris* and further five subgroups were formed within the bacterial strains, showing different genetic relationships.

The data obtained from studies of various bacteria suggests that URP-PCR is potentially a valuable tool in the characterization and grouping at inter- and intraspecific levels of various bacterial species associated with medical, agricultural, industrial, and environmental fields. In many experiments using additional bacterial species, it was found URP-PCR allows generating discrete DNA fragments that are species or strain -specific. The species-specific URP-PCR products are easily excised from gels and cloned or re-amplified with the same primer to be used as a probe, providing an alternative strategy in developing specific Sequence-characterized amplified region (SCAR) markers that detect target microbial species or strain. Previously, such strategy was successfully used to develop specific DNA probe that detects *Pectobacterium carotovorum* subsp. *carotovorum* and *A. vitis* among other bacteria (Kang *et al.*, 2003; Lim *et al.*, 2006).

CONCLUSIONS

PCR approach using URP primers offers a powerful tool for studying DNA diversity of prokaryotic genomes, with potential use in taxonomic and phylogenetic analysis, as well as in genotypic screening of strains in species, especially at inter- and intra-species levels. The URP-PCR is clearly different to RAPD (Williams *et al.*, 1990) and AP-PCR method (Welsh *et al.*, 1990) using arbitrary selected-primers because of long primer and high annealing temperature. It was believed that such URP-PCR conditions result in highly reproducible amplification products from organisms. This result suggested universal applicability of URP-PCR to human, animal, plant and industry related bacteria strains. Because URP primers can be applied to a wide range of bacterial species, mass screen of bacterial resources and database construction of DNA profile can be very useful for various purposes such as identifying genetic characteristics of specific strains. However, it remains to elucidate how URP primers designed from

repetitive sequence of rice are able to produce DNA polymorphism on diverse bacteria genomes with high specificity at inter- and intra-species. Nevertheless, it is reasonably assumed that complementary sequences to URP primers are widely dispersed in the diverse bacterial genomes that may play a critical evolutionary role.

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