Full Length Research Paper

Genetic diversity analysis of rep-PCR genomic fingerprinting of *Lysobacter* spp.

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Lysobacter. spp. are considered as important biocontrol bacteria, due to their antagonistic activity against many pathogenic fungi, bacteria and nematodes. Rep-PCR was used to analyze the genetic diversity of 12 *Lysobacter* strains. These strains included *Lysobacter antibioticus* (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4 and LR9-3), *Lysobacter enzymogenes* (DH3, 1-T-1-4) and *Lysobacter capsici* (LG18) isolate from different regions in Yunnan province of China. Rep-PCR was performed using DNA amplification with primers based on short bacterial repetitive elements (ERIC, BOX, IS1113 and J3). The genetic diversity was analyzed through rep-PCR, molecular fingerprint clustering analysis and UPGMA to construct phylogenetic tree. The results show that when the genetic distance was 0.59, IS1113-PCR could cluster the *Lysobacter* strains as 3 species: *L. antibioticus, L. enzymogenes* and *L. capsici*. The 3 species had obvious differences between each other and the rep-PCR technique could be used to detect genetic variation between different *Lysobacter* strains, identification and strain classification.

Key words: *Lysobacter*, Rep-PCR, genetic diversity.

INTRODUCTION

With the development of molecular biology technology, in recent years, a variety of techniques based on PCR has been widely used in genetic diversity analysis of pathogenic fungi and bacteria, especially the appearance and use of rep-PCR, it made the biodiversity research more rapid, convenience and economy (Xiang et al., 2010; Li et al., 1999). Due to the repetitive DNA sequence only in prokaryotes, but not in the eukaryotic chromosomes, rep-PCR technology can specify amplification prokaryotes DNA, and avoid the influence of eukaryotic gene, so it is especially suitable for related eukaryotes prokaryotic symbiotic bacteria and pathogenic bacteria of genetic diversity research (Li and Zhi, 2006). This technique use the highly conserved short repeat sequences in fungal or bacterial genomic DNA as target sequences of primers to amplify through PCR. Through agarose gel electrophoresis separating DNA fragments with different sizes, each species or strains can produce specific DNA fingerprints. Rep-PCR was used to analyze the genetic diversity of *Xanthomonas* strains and results indicated

rep-PCR was a useful tool for detecting genetic variation among strains of *X.o.pv.oryzicola* and identification of strains as well as classification studies (Ji et al., 2002).. REP-PCR and BOX-PCR were used to study the genetic diversity of *Ralstonia solanacearum* and also indicated that this tool was scientific and effective (Li et al.,2011).

Lysobacter. spp. belong to the *Xanthomonadaceae* family, produce yellow to brownish black pigment, rod-shaped cells, rounded ends, no flagella, the edge of the colony is clear and smooth, have the ability to slip (Ji, 2011). *Lysobacter* are widely distributed in nature and found in soil, rivers, sewers, and other extreme environment (Christensen and Cook, 1978). *Lysobacter* species are recognized as new bacterial predators with the arsenal of bioactive small molecules, the biosynthetic mechanisms and biosynthetic genes for cyclodepsipeptide lysobactin, cyclic lipodepsipeptides, cephem-type β-lactams and polycyclic tetramate macrolactams which make them as biocontrol agents and promising drug producers (Xie et al., 2012). In 1978, Christensen named 4 species of this genus of bacteria: *Lysobacter enzymogenes, Lysobacter antibioticus, Lysobacter brunescens* and *Lysobacter gummosus* (Christensen and Cook, 1978). With the development of bacterial taxonomy technology and a large number of professional database updating, this bacteria was reclassified from *Stenotrophomonas* to *Lysobacter* spp. (Sullivan et al., 2003; Islam et al., 2005). Forepart, the research mainly focused on its activity of various extracellular enzymes, they act on the pathogen cell wall and other targets, causing lysis and inhibition (Reichenbach, 2006). *Lysobacter* spp. are considered as new microbial pesticides, and produce extracellular enzymes and antibiotics that can inhibit the occurrence of several plant diseases. Anovel antifungal compound (HSAF, 1) was isolated from *L. enzymogenes* C3 and can disrupt sphingolipids important to the polarized growth of filamentous fungi, thus, *L. enzymogenes* C3 was used in the biological control of fungal diseases of plants (Lou et al., 2011). *L enzymogenes* OH11 secretes chitinases that hydrolyzed the pathogenic fungal cell wall contributing to suppression of proliferation (Postma et al., 2009); *Lysobacter* sp. strain sbk88 and *L. enzymogenes* 3.1t8 can produce antibiotics that are involved in suppression of *Pythium aphanidermatum* and *Aphanomyces cochlioides* (Kato et al., 1998). *L. enzymogenes* OH11 can control *Pseudomonas solanacearum* (Jiang et al., 2005) and *L. antibioticus* 13-1 was shown to control *Xanthomonas oryzae pv. Oryzae* and *Erwinia carotovora* subsp. *carotovor , Ecc* postulated by niche exclusion by colonizing the crop rhizosphere and competition for nutrients (Wei et al., 2014; Zhang et al., 2011; Wu et al., 2010).

Most studies on *Lysobacter* in recent years were all focused on its biological control, gene cloning and protein (Qian, 2009; Nian, 2015; Liu, 2012). Irene de Bruijn et al. (2015) studied genomics and metabolic profiling of *Lysobacter* in 2005 (de Bruijn et al., 2015). rep-PCR was utilized to analysis 12 strains of *Lysobacter* in order to investigate the diversity of this important biocontrol bacterial genome and lay the foundation for application.

MATERIALS AND METHODS

Test materials and instruments

Test strains and culture medium

L. antibioticus (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4, LR9-3), *L. enzymogenes* (DH3, 1-T-1-4) and *L. capsici* (LG18) were isolated from different regions in Yunnan province of China.

NA medium: sucrose 10 g, peptone 5 g, beef extract 3 g, yeast extract 1 g, agar 15 to 18 g, water after dissolving to volume 1000 mL, adjusted to a pH of 7.0, high-pressure sterilized.

R_2A medium: peptone 0.5 g, acid hydrolyzed casein 0.5 g, glucose 0.5 g, amylogen 0.5 g, yeast extract 0.5 g, $C_3H_3NaO_3$ 0.3 g, $K_2HPO_4 \cdot 3H_2O$ 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.005 g, agar 15 g, dissolved in water after constant volume to 1000 mL, adjusted to a pH of 7.0, high-pressure sterilized.

LB liquid medium: peptone 10 g, yeast extract 5 g, 10 g sodium chloride, dissolved in water after constant volume to 1000 mL, adjusted to a pH of 7.0, high-pressure sterilized.

Major instruments and reagents

Super clean bench, Constant temperature shaking table, Small high-speed centrifuge, Thermostatic water bath, PCR instrument (Takara), High-pressure steam sterilization pot, Image master UV imaging system , Bacterial Genomic DNA Extraction Kit (Beijing Taike Biotechnology Co., Ltd. 50 times), PCR primers by Takara Biotechnology (Dalian) Co., Ltd. synthesis; Takara SYBR premix ExTaq (perfect real time kit purchased from Takara Biotechnology (Dalian) Co., Ltd.

Isolation and identification of *Lysobacte*r from soil samples

Dilution coated plate separation method was used (Wang et al., 2007), the separation is mainly based on the color and shape of colonies to screen *Lysobacter* in NA and R_2A plates, then 16S rDNA of bacterial universal PCR amplification primers 27F (AGAGTTTGATCCTGGCTCAG)/1492R (GGTTACCTTGTTACGA CTT) are used. PCR reaction system (25 μL): 2 x EasyTaq PCR Supermix 12 μL, primer 27F 1 μL, primer 1492R (10 μmol / L) 1 μL, DNA template (50 ng/ μL) 1 μL, ddH$_2$O complement 25 μL. The initial denaturing temperature was 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for 10 min. Amplification products was by 1.0% agarose gel electrophoresis to recover. After purification, 16S rDNA fragments sequencing by Shanghai Biological Engineering Co., Ltd. sequencing primers are 27F and 1492R. Sequencing results blast in GenBank were then obtained with high similar sequence. A total of 12 strains of bacteria were isolated, purified and identified from the collected soil samples (Table 1).

Rep-PCR and genetic diversity analysis of *Lysobacter*

Lysobacter genome DNA extraction (referring to Beijing Taike Biotechnology Co., Ltd. bacterial genomic DNA extraction kit), and

Table 1. The ecological distribution of the different species of *Lysobacter.*

Strain Name	Origin Agrotype	Agrotype	Croppattern
L. ant 13-1	Kunming City, Yunnan Province	Rhizosphere soil	Rice
L. ant HY	Fuyuan County of Qujing city in Yunnan Province	Rhizosphere soil	Konjak
L. ant 13-6	Fuyuan County of Qujing city in Kunming Province	Rhizosphere soil	Konjak
L. ant 6-B-1	Fengqing County of Lincang city in Yunnan Province	Rhizosphere soil	Konjak
L. ant 13-B-1	Fengqing County of Lincang city in Yunnan Province	Rhizosphere soil	Konjak
L. ant 6-T'-4	Fengqing County of Lincang city in Yunnan Province	Rhizosphere soil	Konjak
L. ant LJ6-3	Shilin County of Kunming city in Yunnna Province	Rhizosphere soil	Panax notoginseng
L. ant LJ6-4	Shilin County of Kunming city in YunnanProvince	Rhizosphere soil	Panax notoginseng
L. ant LR9-3	Shilin County of Kunming city in Yunnan Province	Rhizosphere soil	Panax notoginseng
L. enz DH3	Fengqing County of Lincang city in Yunnan Province	Rhizosphere soil	Cayenne
L. enz 1-T-1-4	Fengqing County of Lincang city in Yunnan Province	Rhizosphere soil	Konjak
L. cap LG18	Fuyuan County of Qujing city in Yunnan Province	Rhizosphere soil	Potato

L. ant: *Lysobacter antibioticus*; L. enz: *Lysobacter enzymogenes* ; L. cap: *Lysobacter capsici.*

then rep-PCR was done.

DNA was used to perform rep-PCR with ERIC primers using the manufacturer's protocol. The primer pair Eric1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′, Eric2: 5'-AAGTAAGTGACTGGGGTGAGCG-3′ was used and composed by the Shanghai born industry and Biological Engineering Co., Ltd. synthetic. PCR reaction system: 10 x PCR buffer, 50 ng of DNA template, 400 µM dNTP, primer F 10 pmol, primer R 10 pmol, 2 U Taq enzyme, ddH$_2$O complement 25 µL. The initial denaturing temperature was 95°C for 7 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 65°C for 8 min, with a final extension at 65°C for 15 min.

The primer BOX: 5′-CTACGGCAAGGCGACGCTGACG-3′ was used and composed by the Shanghai born industry and Biological Engineering Co., Ltd. synthetic. PCR reaction system: 10 × PCR buffer, 50 ng of DNA template, 400 µM dNTP, primer F 10 pmol, primer R 10 pmol, 2 U Taq enzyme, ddH$_2$O complement 25 µL. The initial denaturing temperature was 95°C for 7 min, followed by 35 cycles of 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min, with a final extension at 65°C for 15 min.

The primer J3: 5′-GCTCAGGTCAGGTCGCCTGG-3′ was used and composed by the Shanghai born industry and Biological Engineering Co., Ltd. synthetic. PCR reaction system: 10 × PCR buffer, 50 ng of DNA template, 400 µM dNTP, primer F 10 pmol, primer R 10 pmol, 2 U Taq enzyme, ddH$_2$O complement 25 µL. The initial denaturing temperature was 95°C for 7 min, followed by 35 cycles of 94°C for 1 min, 68°C for 1 min, and 65°C for 8 min, with a final extension at 65°C for 15 min.

The primer pair IS1113: TX1: 5′-TGTAGTGGACCTTCGAA-3′, TX2: 5′-ACGAGCGATTGATCAGG-3′ was used and composed by the Shanghai born industry and Biological Engineering Co., Ltd. synthetic. PCR reaction system: 10 x PCR buffer, 50 ng of DNA template, 400 µM dNTP, primer F 10 pmol, primer R 10 pmol, 2 U Taq enzyme and ddH$_2$O complement 25 µL. The initial denaturing temperature was 95°C for 7 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 65°C for 8 min, with a final extension at 65°C for 15 min.

The end of amplification, 8 µL PCR product was taken in 0.5 * TAE buffer through the concentration of 1.5% agarosegel electrophoresis, the voltage drop to 100 V, electrophoresis was done for 5-6 h, after the completion of the electrophoresis, using ethidium bromide staining, decolorization, in gel imaging system amd the collection and preservation of image was done.

Approximately 25 mL of the post amplification reactions were separated by electrophoresis in a 1.5% agarose gel in TAE buffer,

stained with an ethidium bromide solution (10 mg/mL), decolorization and photo documented under UV light. An optimized comparative study of the diversity of the obtained banding patterns showed the presence or absence of repetitive elements using photographic images.

Rep-PCR amplified products of the gel map to read the band, have band (mark "1"), no band (mark "0"), using software NTSYS for cluster analysis, and using unweighted pair group method with arithmetic mean (UPGMA) for molecular fingerprint clustering analysis and constructing system tree pattern.

RESULTS

Analysis of rep-PCR fingerprint banding patterns

The Box PCR showed 11 different molecular fingerprints with bands ranging from 250 to 7000 bp for the 12 isolates of *L. antibioticus* (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4, LR9-3), *L. enzymogenes* (DH3, 1-T-1-4) and *L.capsici* (LG18) (Figure 1).

The Eric PCR showed 17 different molecular fingerprints with bands ranging from 250 to 7000 bp for the 12 isolates of *L. antibioticus* (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4 and LR9-3), *L. enzymogenes* (DH3, 1-T-1-4) and *L.capsici* (LG18) (Figure 2).

The IS1113 PCR showed 5-15 different molecular fingerprints with bands ranging from 250 to 7000 bp for the 12 isolates *L. antibioticus* (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4, LR9-3), *L. enzymogenes* (DH3, 1-T-1-4) and *L.capsici* (LG18) (Figure 3).

The J3 PCR showed 8-16 different molecular fingerprints with bands ranging from 250 to 7000 bp for the 12 isolates of *L. antibioticus* (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4 and LR9-3), *L. enzymogenes* (DH3, 1-T-1-4) and *L. capsici* (LG18) (Figure 4)

Results of PCR fingerprint analysis

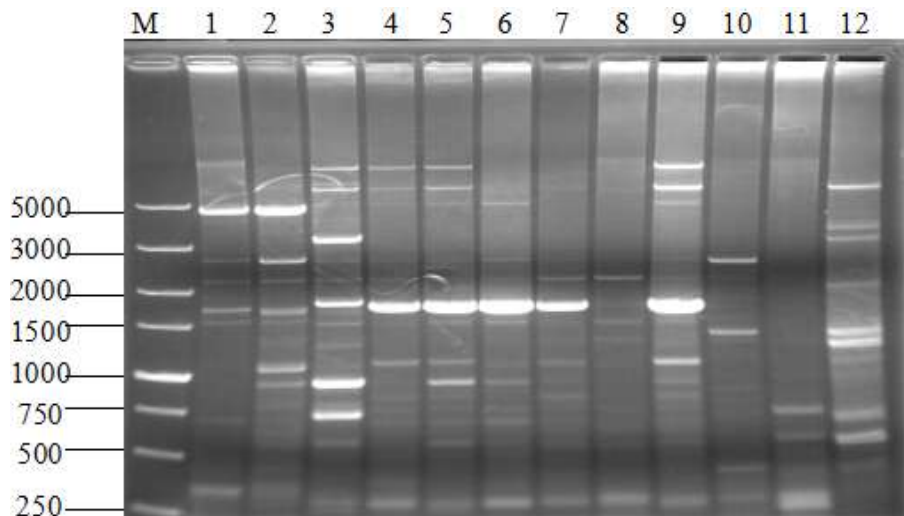UPGMA cluster analysis was performed on the DNA
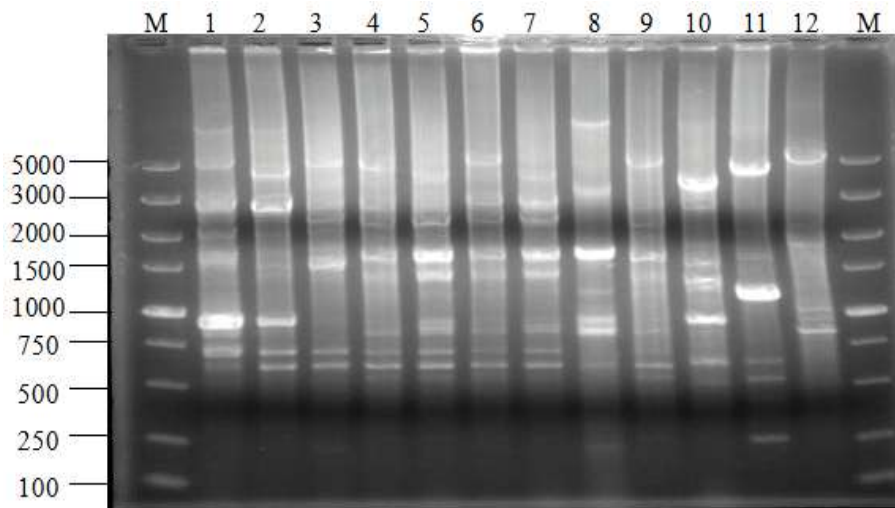
Figure 1. Box-PCR fingerpringing patterns from genomic DNA of *Lysobacter* strains. M: marker, 1: *L. antibioticus* HY, 2: *L. antibioticus* 13-1, 3: *L. antibioticus* 13-6, 4: *L. antibioticus* 6-B-1, 5: *L. antibioticus* 13-B-1, 6: *L. antibioticus* 6-T'-4, 7: *L. antibioticus* LJ6-3, 8: *L. antibioticus* LJ6-4, 9: *L. antibioticus* LR9-3, 10: *L. enzymogenes* DH3, 11: *L. enzymogenes* 1-T-1-4, 12: *L. caisici* LG18.
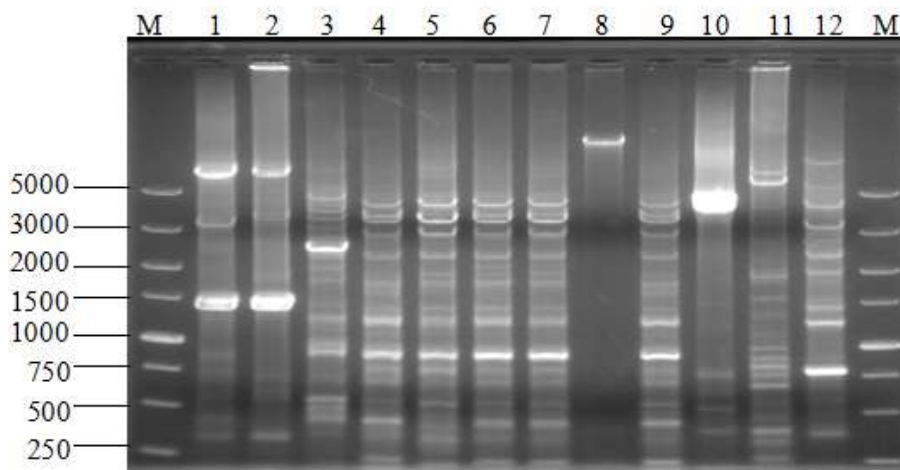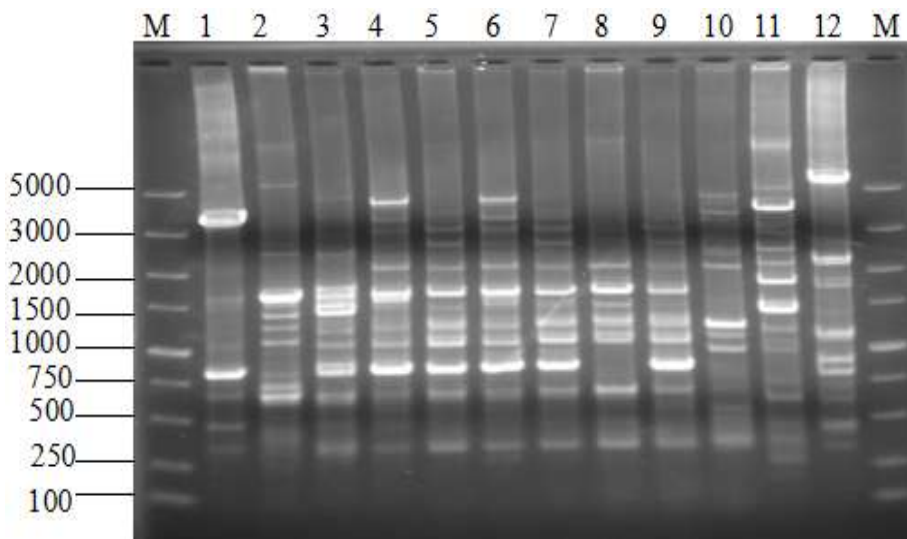

Figure 2. ERIC-PCR fingerpringing patterns from genomic DNA of *Lysobacter* strains. M: marker, 1: *L. antibioticus* HY, 2: *L. antibioticus* 13-1, 3: *L. antibioticus* 13-6, 4: *L. antibioticus* 6-B-1, 5: *L. antibioticus* 13-B-1, 6: *L. antibioticus* 6-T'-4, 7: *L. antibioticus* LJ6-3, 8: *L. antibioticus* LJ6-4, 9: *L. antibioticus* LR9-3, 10: *L. enzymogenes* DH3, 11: *L. enzymogenes* 1-T-1-4, 12: *L. caisici* LG18.

fingerprints and showed that only primer IS1113 can distinguish *L. antibioticus* (r1-9), *L. enzymogenes*(r10-11) and *L. capsici L.capsici* (r12) as three groups (Figure 5). According to different geographical locations, we can reclassified *L. antibioticus* HY and *L. antibioticus* 13-6 both from Fuyuan County of Yunnan Province as the first group, *L. antibioticus* 13-1 from Kunming City of Yunnan Province as the second group, *L. antibioticus* 6-B-1, 13-B-1 and 6-T'-4 from Fengqing County of Lincang City in Yunnan Province as the third group, *L. antibioticus* LJ6-3

and LJ6-4 from Shilin County of Yunnan Province as the fourth group, *L. antibioticus* LR9-3 from Shilin County of Kunming city in Yunnan Province as the fifth group, *L. enzymogenes* 1-T-1-4 and DH3 both from Fengqing County of Lincang city in Yunnan Province as the sixth group and *L. capsici* LG18 from Fuyuan County of Qujing city in Yunnan Province as the seventh group (Table 1). The results confirmed that as compared to the similarity between species, the similarity of the isolates within in the species was higher (Table 2).

Figure 3. IS1113 -PCR fingerpringing patterns from genomic DNA of *Lysobacter* strains. M:marker, 1: *L. antibioticus* HY, 2: *L. antibioticus* 13-1, 3: *L. antibioticus* 13-6, 4: *L. antibioticus* 6-B-1, 5: *L. antibioticus* 13-B-1, 6: *L. antibioticus* 6-T'-4, 7: *L. antibioticus* LJ6-3, 8: *L. antibioticus* LJ6-4, 9: *L. antibioticus* LR9-3, 10: *L. enzymogenes* DH3, 11: *L. enzymogenes* 1-T-1-4, 12: *L. caisici* LG18.


Figure 4. J3 -PCR fingerpringing patterns from genomic DNA of *Lysobacter* strains. M:marker, 1: *L. antibioticus* HY, 2: *L. antibioticus* 13-1, 3: *L. antibioticus* 13-6, 4: *L. antibioticus* 6-B-1, 5: *L. antibioticus* 13-B-1, 6: *L. antibioticus* 6-T'-4, 7: *L. antibioticus* LJ6-3, 8: *L. antibioticus* LJ6-4, 9: *L. antibioticus* LR9-3, 10: *L. enzymogenes* DH3, 11: *L. enzymogenes* 1-T-1-4, 12: *L. caisici* LG18.

DISCUSSION

The BOX, ERIC, IS1113 and J3 repeat sequences are distributed widely in three species of *Lysobacter* genome DNA, and confirmed rep-PCR technology can distinguish different species and used for the determination of *Lysobacter* group genetic diversity. Primers of BOX,

ERIC, IS1113 and J3 are used for PCR, fingerprint and clustering analysis, results show that when genetic distance was 0.59 only IS1113 primer can successfully distinguish *L. antibioticus, L. enzymogenes* and *L. capsici*. IS1113 can be used for analysis of hereditary changes in *Lysobacter* groups, except a few strains, which can be clearly divided into three main genetic
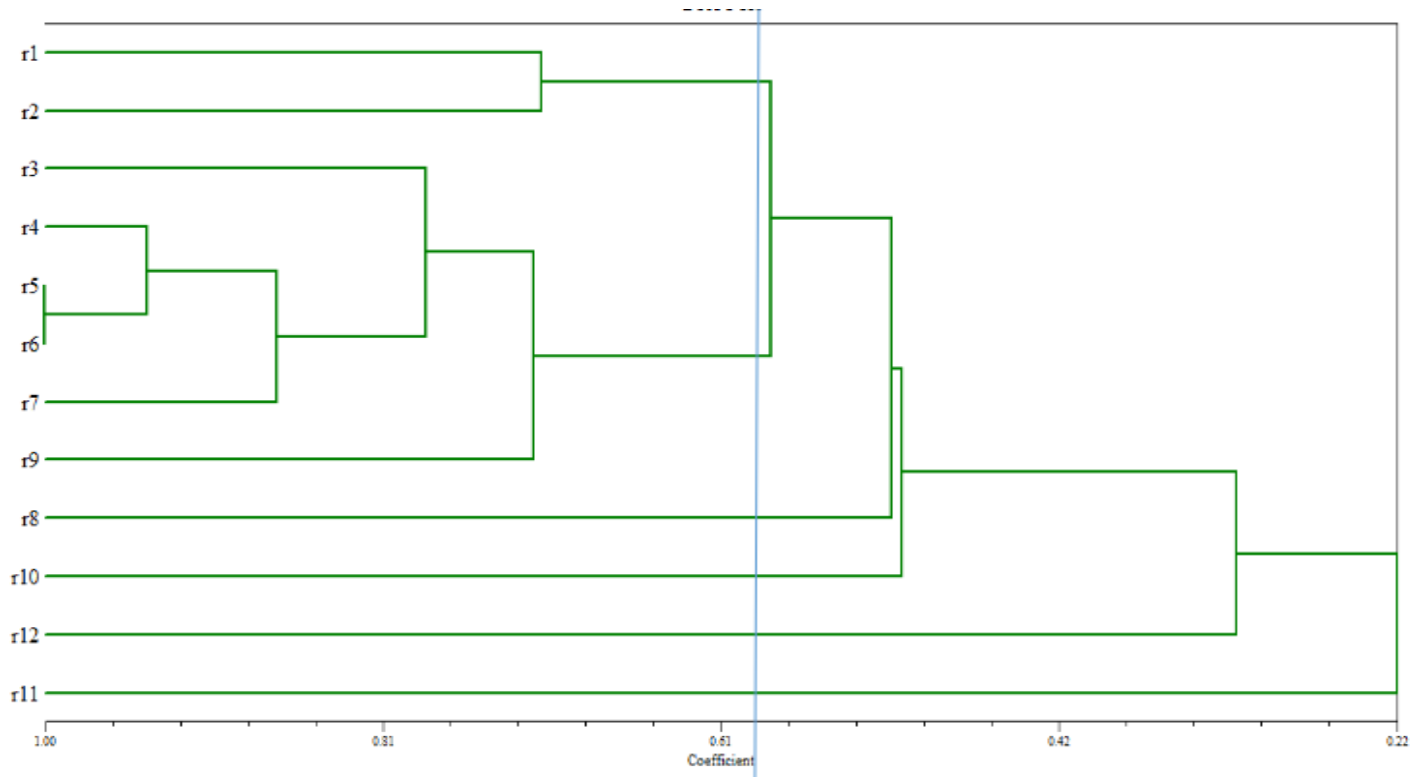
A
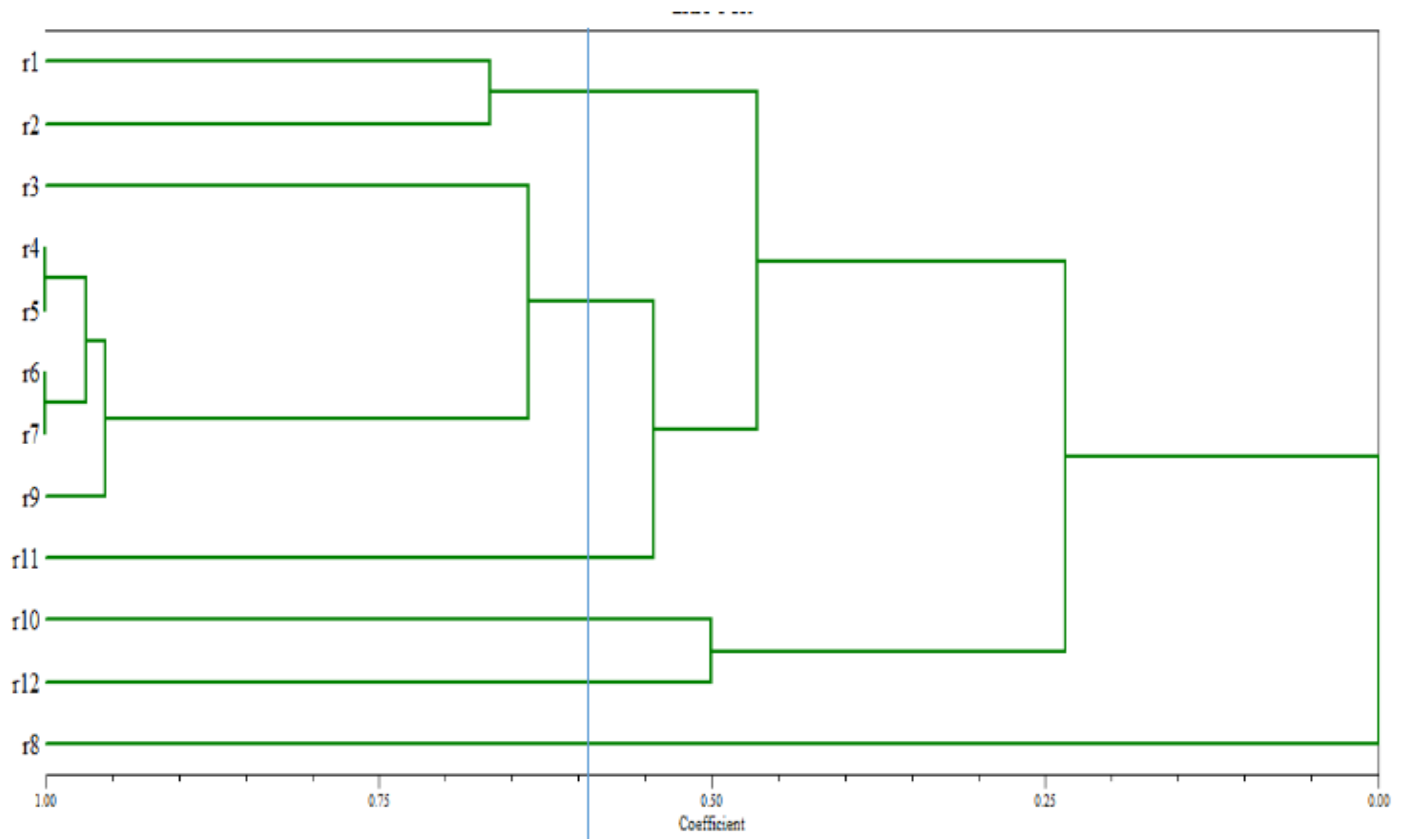
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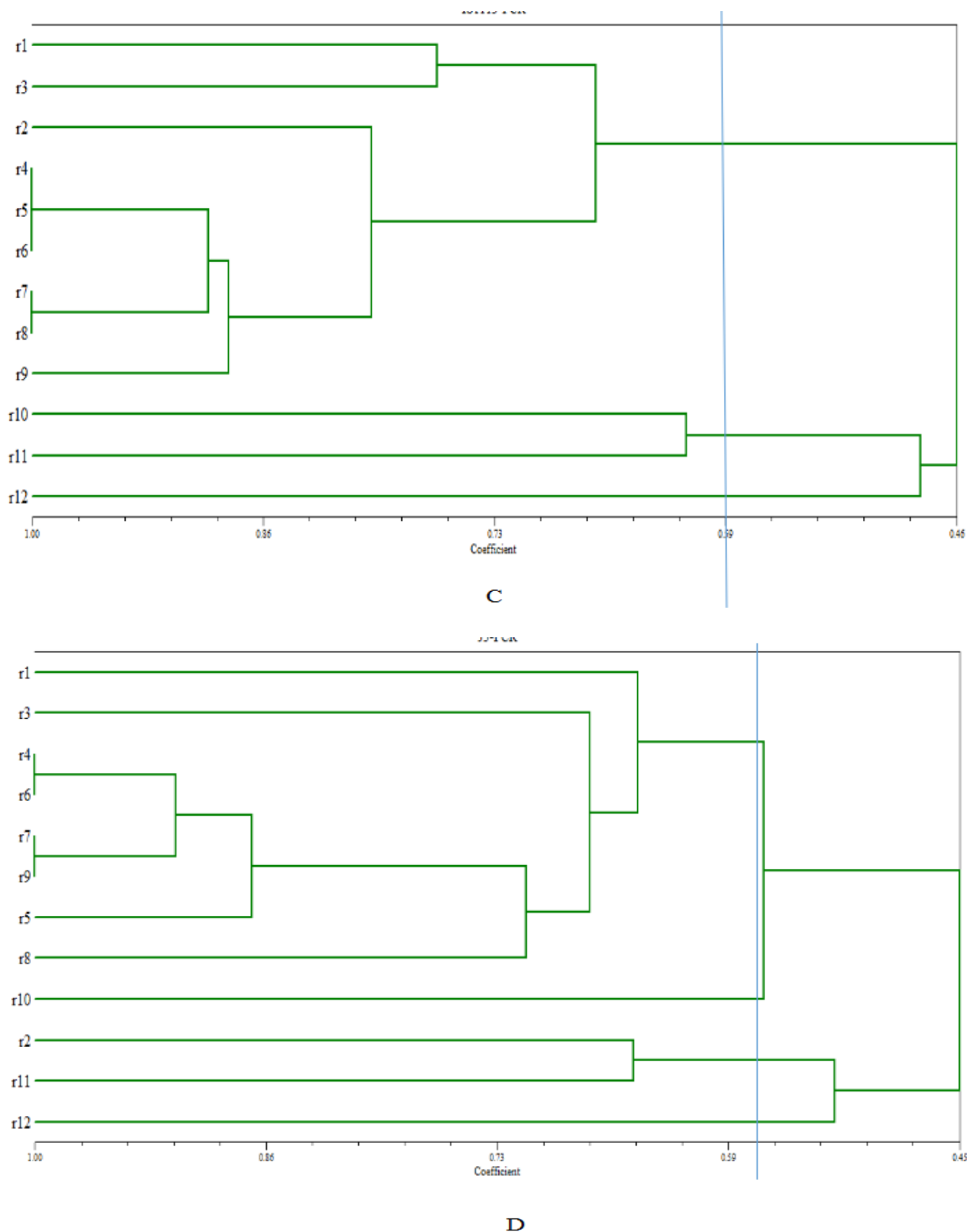
Figure 5. Dendrograms constructed with UPGMA using DNA fingerprints patterns from *Lysobacter* strains. A: BOX-PCR of dendrograms constructed with UPGMA using DNA fingerprints patterns from *Lysobacter* strains; B: ERIC-PCR of dendrograms constructed with UPGMA using DNA fingerprints patterns from *Lysobacter* strains. C: IS1113-PCR of dendrograms constructed with UPGMA using DNA fingerprints patterns from *Lysobacter* strains. D: J3-PCR of dendrograms constructed with UPGMA using DNA fingerprints patterns from *Lysobacter* strains. Strains: r1: *L. antibioticus* HY, r2: *L. antibioticus* 13-1, r3: *L. antibioticus* 13-6, r4: *L. antibioticus* 6-B-1, r5: *L. antibioticus* 13-B-1, r6: *L. antibioticus* 6-T'-4, r7: *L. antibioticus* LJ6-3, r8: *L. antibioticus* LJ6-4, r9: *L. antibioticus* LR9-3, r10: *L. enzymogenes* DH3, r11: *L. enzymogenes* 1-T-1-4, r12: *L. caisici* LG18

Table 2. Similarity of IS1113-PCR fingerprints among different strains.

Strain numbers	HY	13-1	13-6	6-B-1	13-B-1	6-T'-4	LJ6-3	LJ6-4	LR9-3	DH3	1-T-1-4	LG18
HY	100											
13-1	0.667	100										
13-6	0.762	0.769	100									
6-B-1	0.636	0.667	0.741	100								
13-B-1	0.727	0.814	0.740	1.000	100							
6-T'-4	0.636	0.815	0.741	1.000	1.000	100						
LJ6-3	0.522	0.786	0.714	0.897	0.897	0.897	100					
LJ6-4	0.522	0.786	0.714	0.897	0.897	0.897	1.000	100				
LR9-3	0.609	0.786	0.714	0.897	0.897	0.897	0.867	0.867	100			
DH3	0.500	0.571	0.571	0.546	0.546	0.546	0.609	0.609	0.522	100		
1-T-1-4	0.308	0.222	0.333	0.421	0.421	0.421	0.400	0.400	0.300	0.615	100	
LG18	0.111	0.435	0.435	0.500	0.500	0.500	0.560	0.560	0.480	0.556	0.400	100

Lysobacter antibioticus: HY, 13-1, 13-6, 6-B-1, 13-B-6-1, 6-T'-4, LJ6-3, LJ6-4, LR9-3. *Lysobacter enzymogenes:* DH3, 1-T-1-4. *Lysobacter capsici:* LG18.

clusters. *Lysobcater* have different structure, activity and biosynthetic pathways, thus the control of pathogenic bacteria is different. So we can classify species quickly through rep-PCR and then use specific biocontrol bacteria to control plant diseases.

It is the first time to apply rep-PCR to study the diversity of *Lysobacter* genome. The method of identifying *Lysobacter* usually uses 16S rDNA of bacterial universal primers to PCR and then amplified products are sequenced, with sequencing results blast in GenBank and then high similar sequence was obtained. This method is time consuming, inconvenient and the results are not accurate because the sequencing results may be inaccurate. Thus, rep-PCR was adopted, this technology has advantages of fast, handy and economic, and dispense with specific probe and southern hybridization. Due to the characteristics of electrophoresis, rep-PCR with strain level spectrum can be used for strain identification and can reflect the differences of genome of close genetic relationship between strains, but the disadvantage is that it does not reflect the differences in the plasmid DNA, and rep-PCR is affected by many factors, such as: primers from different sources or different batches, DNA polymerase or PCR instrument of different types, to an extent, these factors restrict the technology application (Laguerre et al., 1996). In spite of this, under certain experimental conditions, rep-PCR is an important and effective technology for strains identification and clustering. In the later work, Multilocus Sequence Analysis (MLSA) and Restriction Fragment Length Polymorphism Analysis (RFLP) can be used to further research on the origin of their diversity, these will be helpful to further uncover the origin, evolution and phylogeny, and provide important information and scientific basis in the production or use of *Lysobacter* for disease prevention and economic crops treatment.

Conflict of interests

The authors declare that there is no conflict of interest.

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