GENETIC DIVERSITY OF ANTAGONISTIC BACTERIA AGAINST SHEATH BLIGHT AND BAKANAE RICE DISEASE BY RAPD

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ABSTRACT

In this research genetic diversity of 19 antagonistic bacterial strains were isolated from rice field contaminated with *Rhizoctonia solani* and *Fusarium moniliforme*. Based on morphological, biochemical, physiological and pathogenicity characteristics, total cellular protein profiles (SDS-PAGE) and PCR with specific primers the antagonistic strains were identified. The antagonistic strains of *R.solani* included B4, B6, B17, B18, B22, B24, B41 and B42 and that of *F.moniliforme* included F1, F6, F12, F15, F16, F18, F21, F24 were identified as *Pseudomonas fluorescens* and F14, F19, F32 and F35 were identified as *Bacillus cereus.* Out of fifteen RAPD primers, five primers were selected based on their ability to amplify all antagonistic strains. Twenteen strains were grouped based on RAPD analysis. Intra population gen diversity and distributed genetic diversity were assessed by POPGENE software. Nei's genetic distance for A, B and C population is 0.22, 0.28 and 0.29 respectively. RAPD analysis revealed that strains B18 and F12 formed distinict group compared to other strains and showed maximum similarity with B24 and F18 strains. Other strains were placed in separated subgroups. Strains B41 and B6 showed maximum genetic distance. Molecular analysis using PCR based RAPD method is thus useful to differentiate such strains at the intra specific level.

Keywords : Rice, antagonistic bacteria, diversity genetic**,** RAPD

INTRODUCTION

Much of the nature of bacterial species and their diversity remains a mystery due to the very large numbers of taxa, the potential difficulty in quantifying them, and the fact that they are relatively cryptic in nature. Of the many bacterial species that live on the leaves of plants, only those associated with agricultural and crop plants tend to be isolated, identified, and characterized (Gosse *et al.,* 2005). Microorganisms are increasingly being considered for the biological control of sheath blight and bakanae rice disease (Niknejad Kazempour, 2004; Niknejad Kazempour and Elahinia 2007). Limited knowledge of antagonistic bacteria for biological control agents has retarded their commercial development and widespread use (Olubukola *et al.,* 2002)**.**
The total genetic diversity of a species is a key factor in its persistence and conservation. Because realistic sample sizes are far smaller than the total population, it is impractical to exhaustively characterize diversity of most populations (Rauch and Bar-Yam, 2005).
Identification of specific microorganism in environmental samples is important for risk analysis of released bacteria. Molecular biological techniques provides highly species methods for the detection and identification of such bacteria (Hansen and Winding, 1997). The application of Random Amplified Polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Wiliames *et al.,* 1990), for production of isolate specific DNA fingerprints is specially promising (Hadrys *et al.,* 1992). This technique has the advantages that no DNA sequence information of the organism is needed, and bacteria do not have to be genetically marked. In addition, polymorphic RAPD fragment have been used for the development of isolate and species specific prob for bacteria (Fani *et al.,* 1993 ; Manulis *et al.,* 1994), fungi (Dobrowolski and O'Brien 1993; Potenza *et al.,* 1994) and plant (Aitken *et al.,* 1994) for distinguishing between closely related organisms.

The aime of the present study was to genetic diversity of *Pseudomonas fluorescens* and *Bacillus cereus* antagonistic isolates against sheath blight and bakanae rice disease by RAPD.

MATERIALS AND METHODS

Bacterial strains and media

Nineteen strains of *Pseudomonas fluorescens* and *Bacillus cereus* isolated from different geographical areas (Guilan province, Iran). *P. fluorescens* and *B. cereus* strains were grown in Luria broth (LB) or King's medium B broth (KB) . Liquid media cultures were generated from –80°C freezer stocks in 15% glycerol by overnight shaking at 220 rpm in LB at 25°C. The cultures were initiated at an OD600 = 0.05. Media were amended with appropriate antibiotic cyclohexamid (50 µg/ml).

DNA Extraction

For bacterial DNA extraction, the isolates were grown overnight, in nutrient broth (Merck, Darmstadt, Germany), at 26 °C and the DNA was extracted as described by Martins et al*.,* 2005. One tube of 1.5 ml was used to centrifuged the cells at 13,000 × g for 5 min and the pellet was suspended in 200 µl Tris 0.1 mol L-1 and added with 200 µl of lysis solution (NaOH 0.2 N and 1% SDS), mixed and deproteinazed with 700 µl of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v), homogenized and centrifuged 10 min at 13,000 × g .To precipitate DNA, 700 µl of cold absalute isopropanol was added and spinned, washed in 70% ethanol and centrifuged. Precipitated DNA is dried at room temperature and suspended in 100 µl of water. The method described by Ausubel *et al.,* (1996) was performed comparing 24 strains. The samples from the both methods were electrophoresed on 1.5% agarose gels, stained with ethidium bromide and photographed under UV light. The RAPD was performed according to Ortiz-Herrera *et al.,* (2004).

PCR conditions for amplification and electrophoresis

Amplification was carried out in a 25 µl volume in 0.5 ml microtube using a Hybaid programmable thermal controller. Each 25 µl PCR reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 µM of each nucleotide (dATP,dCTP, dGTP, and dTTP), 0.25 µM of each primer, 100 ng DNA and 1 U of *Taq* DNA polymerase (Promega Corp., Madison, WI). A 25 µl sterile, mineral-oil overlay was added to reduce evaporation. DNA amplification was carried out in a PTC-100 programmable DNA thermal cycler (MJ Research, Watertown MA). The amplification was performed as follows: initial 1 min 94°C denaturation; 45 cycles of 1 min 94°C, 1 min 36°C, 2 min 72°C; and 5 min 72°C extension. Amplified fragments were separated in 1.5 % agarose gel using TBE buffer and were visualized and photographed using a Gel Documentation System, GDS 8000 (BioRad., California, USA), after staining with ethidium bromide.

Estimating population genetic structure with RAPD data

The estimation of population genetic structure in an analysis of this data was analyzed by assuming that the populations are in Hardy-Weinberg equilibrium. The estimates were calculated using the following methods:
1. The frequency of the 67 RAPD bands detected with the five primer were calculated and estimates of genetic diversity (Ĥ) of each population were obtained using Shannon's information measure (Lewontin, 1972); modified for RAPD analysis by Chakraborty and Rao (1991) which is defined as Ĥ = ΣPi. loge Pi, where Pi is the frequency of the ith RAPD bands in each population.
2. The genetic diversity obtained from Shannon's index of the 3 populations, detected by the five primers.
3. Nei's original measures of genetic identity and genetic distance (Nei, 1972).
 were calculated.
4. A dendrogram was constructed from the genetic distance matrix using UPGMA method. The variation in RAPD patterns was analyzed by analysis of molecular variance, AMOVA, (Excoffier *et al.,* 1992).

Table 1. Sequences of random primers listed in the RAPD analysis of *Pseudomonas fluorescence* populations.

Code	Sequence (5' to 3')
P4	**5'-GCG-GGA-GAC-<C>-3'**
P5	**5'-CCC-ACT-GAC-<G>-3'**
P6	**5'-CGG-AGA-GCG-<A>-3'**
P8	**5'-CCT-CAC-CTG-<T>-3'**
P9	**5'-TGG-GCT-CGC-<T>-3'**

Table 2. Information of produced ragment by different primer.

Primer	No. of produced fragment	No. of polymorphic Fragment	No. of monomorphic Fragment	Percentage of polymorphic for each primer
P4	8	8	-	100
P5	14	13	1	92.80
P6	18	16	2	88.88
P8	17	17	-	100
P9	10	10	-	100

16 Based on the number of shared amplification products, Nei M and Li , 1979, a matrix of genetic distance between individuals was obtained using a RAPD distance program v. 1.04 calculated from presence / absence data. Components of variance attributable to differences between regions, between populations within regions, and between individuals within populations were estimated from this matrix using AMOVA. AMOVA variance components were used as estimates of the genetic diversity within and between populations and regions. The number of permutations for significance testing was set at 100 for all analyses. From *phi* statistics (F statistics) gene flow (Nem) can be approximated as Nem = 1/4 (1/FST – 1), (Wright, 1951; Fischer and Matthies, 1998).

RESULTS AND DISCUSSION

A total of 50 RAPD primers, 5 primers were chosen based on the quantity and quality of their amplified fragments and were then used to survey all strains of antagonistic bacteria (table 1). The presence or absence of amplified DNA bands was scored for 67 band positions (table 2). All bands scored were between 0.3 and 0.5 kb (Figure 1). The final presence absence matrix contained scores for 67 individuals each at 67 band positions (primer P4: 8 bands, P5: 14, P6: 18, P8 17: P9 10).

Table 3. Information of three populations ragment by different parameters.

Population	No. of antagonistic bacteria	No. of alells (Na)	Effective (Ne)	Genetic diversity based on Nei (H)	No. of polymorphic fragment in the population (Pj)	Percentage of polymorphic (P)
A	8	1.725	1.5237	0.2914	29	72.5
B	6	1.725	1.4769	0.28	29	72.5
C	5	1.60	1.375	0.2208	24	60

Table 4. Genetic distance of three population on based RAPD data.

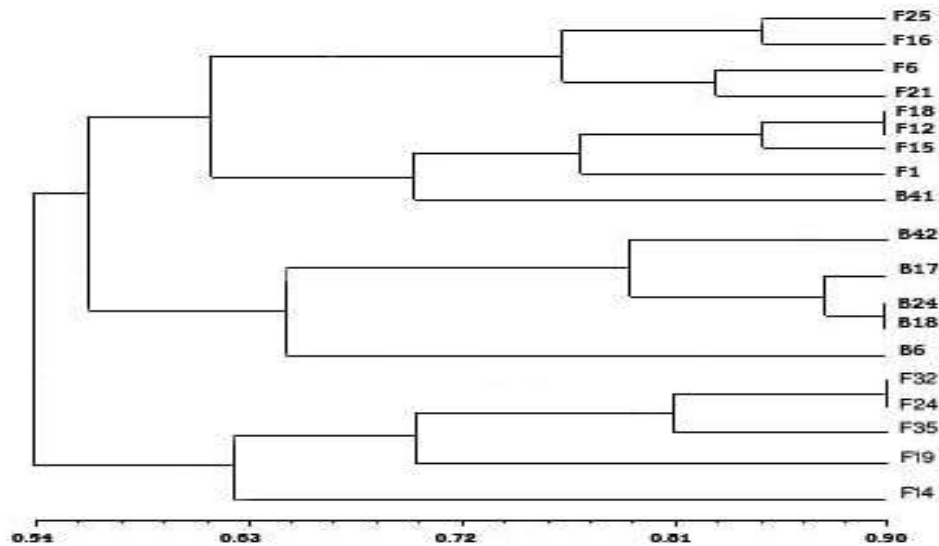| Population	A	B	C
A	***	0.7710	0.7394
B	0.26	***	0.7566
C	0.3019	0.2775	***

Fig.1. Dendogram showing relationship between three populations *Pseudomonas fluorescens* and *Bacillus cereus* antagonistic bacteria strains of *Fusarium moniliforme* and *Rhizoctonia solani* using UPGM based on Jaccard similarity index from RAPD-PCR data.

The results of PCR – RAPD examination with 5 primer for 3 population of antagonistic bacteria are shown in table 2. Among the 67 bands generated by the 5 selected primers, 3 were present among all the samples analysed and correspond to monomorphic bands (table 3). The remaining 64 variable bands were selected as RAPD markers (represent 88.88 % total bands). Primer P4, P8 and P9 detected the most and genetic diversity (table 4). The population of A exhibited the most RAPD variability (0.2914) and population of B exhibited the least RAPD variable (0.2208).

According to genetic distance, dendrogram showed that three population divided into three groups. Population A (*Pseudomonas fluorescens*, antagonistic strains of *Fusarium moniliforme*) are in first group, population of B (*Pseudomonas fluorescens*, antagonistic strains of *Rhizoctonia solani*) is second group and population C (*Bacillus cereus*, antagonistic strains of *Fusarium moniliforme*).

The genetic distance with based and unbased are 0.3019 and 0.26 respectively (table 4). It is indicated low distance between three groups but clustering analysis of individual shows that the individual of each population scattered among the population. This situation indicated low genetic differences among three populations.

RAPD analysis has been proved to be a high resolution method for detection of genetic variation between and within population. Fischer and Matthies (1998) pointed out an important suggestion that because of the high resolution of the method significant genetic variation could be detected within and between population with relatively small sample size. Thus RAPD is likely to be appropriate method for analysis of genetic variation in this study. Also the possibility to obtain an unique genotype when RAPD markers are used in genetic identification will depend of three parameters ; number of bands analysed, number of variants per band and the frequency of variant in the sample (Abuin et al., 2002). This study is the first repotted provides an essential basis on genetic information of antagonistic bacteria strains in Iran. The level of RAPD variation and distribution in population of antagonistic bacteria against sheath blight and bakanae rice disease have been investigated. In conclusion, this investigation demonstrates that fragments obtainedby RAPD can be used for population genetic diversity of antagonistic bacteria in the rice field.

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