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Full Length Research Paper

Genetic variation in a population of *Bipolaris oryzae* based on RAPD-PCR in north of Iran

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Isolates of *Bipolaris oryzae* were analysed by RAPD techniques to determine the amount of intraspecific genetic variability. In order to do RAPD-PCR, seven primers were applied. At first, DNA of all isolates was isolated, and then DNA was amplified in thermocycler by using seven primers at a thermal program. As the result, segments of DNA by the size of 200 - 300 bp were produced. Cluster analysis using UPGMA method gave three groups. Levels of polymorphism were observed between DNA of different isolates. The pattern of RAPD bands could not show the direct correlation between polymorphism and climates or geographical areas.

Key words: *Bipolaris oryzae*, rice, genetic variation, RAPD.

INTRODUCTION

Brown spot is one of the most important seedborne diseases of rice in Guilan province of Iran (Safari Motlagh et al., 2005). It affects the grain yield, both in wet and dry seasons, with heavy natural infection (Bakonyi et al., 1995). It was first associated with necrotic spots on the leaves of cultivated rice (Ou, 1985). At first the causal agent of brown spot disease by Breda de Haan was named *Helminthosporium oryzae* (Gangopadhyay and Padmanabhan, 1987). Nowadays, the *graminicolous Helminthosporium* species were divided into three genus based on colony, conidiophore and conidial morphology, type of condial germination and the type of hilum structure: *Bipolaris, Drechslera, Exserohilum* (Sivanesan, 1987).

The telemorphs were from Ascomycetes and consist of: *Cochliobolus, Pyrenophora* and *Setosphaeria,* respectively (Sivanesan, 1987*).*

Some authors have used the molecular markers to study of the relationship among these species (Bakonyi et al., 1995; Abadi et al., 1996). Increasingly, molecular biology techniques have been used to explore genetic variability in fungi (Caligiorne et al., 1999) including: PCR and RAPD-PCR. This method was applied for diagnosis of *Rhizoctonia solani* (Nicholson and Parry, 1996), *Microdochium nivale* and some plant pathogenic fungi (Parry and Nicholson, 1996). Isolates of *Bipolaris sorokiniana* were analyzed by RAPD to determine the amount of intraspecific genetic variability and to study host pathogen interactions (Oliveira et al., 2002). Genetic variation were analysed by PCR and PCR-RFLP among pathogens causing "*Helminthosporium*" diseases in rice, maize and wheat (Weikert-Oliveira et al., 2002). The aim of this investigation was to study the genetic variation of *Bipolaris oryzae* population as the causal agent of rice brown spot disease prevalent in paddy fields of Iran.

MATERIALS AND METHODS

Samples of *B. oryzae* were collected from rice fields of Guilan province of Iran, by using Xia et al. (1993) method.

Morphological study was applied in TWA+ wheat straw media (Sivanesan, 1987). Total DNA was extracted from mycelium according to the procedure of Liu et al. (2000). According to the protocol, samples for DNA extraction were grown on potato-dextrose broth for seven days at 22 - 25°C. 200 mg of myceli were harvested, frozen in liquid nitrogen. Then 500 µL lysis buffer was added. The tubes were then left at room temperature for 10 min. After adding 150 µl of 5 M potassium acetate, the tubes were vortexed briefly

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Abbreviations: RAPD-PCR, Random amplified polymorphic DNA-polymerase chain reaction; **RFLP,** restriction fragment length polymorphism; **UPGMA,** unweighted pair group method with arithmetic mean.

Table 1. RAPD primers used in this study.

Code	Sequence $5' \rightarrow 3'$	OD (µg/ml)
A01	CCCAAGGTCC	15
A02	GGTGCGGGAA	13
A06	GAGTCTCAGG	9
A08	ACGCACAACC	14
B06	GTGACATGCC	11
B07	AGATGCAGCC	10.5
OPC13	AAGCCTCGTC	13

OD, Optical density.

and spun at 8,000 rpm for 1 min. The supernatant was transferred to another 1.5 ml Eppendorf tube; an equal volume of isopropyl alcohol was added. The tubes were mixed by inversion briefly. The tube was spun at 10000 rpm for 2 min and the supernatant discarded. The resultant DNA pellet was washed in 300 µL of 70% ethanol. After the pellet was spun at 10,000 rpm for 1 min, the supernatant was discarded. The DNA pellet was airdried and dissolved in 50 µL of 1X Tris-EDTA and 1 µL of the purified DNA was used in 25 to 50 µL of PCR mixture.

Then for determining DNA quantity as empirical, the genomic DNA extracted was electrophoresed in 1.4% agarose gel and detected by staining with ethidium bromide.

The primers used in this study were A01, A02, A06, A08, B06, B08 and OPC13 (Oliveira et al., 2002). For standardization of the amplification conditions and selection of the primers, the genomic DNA extracted from original samples of *B. oryzae* was used as a template for the amplifications (Table 1). All reactions were carried out in a volume of 25 µL with 10 ng DNA, 12 mmol/L of each dNTP, 1.25 µmol of primer, 2.5 µL of 10X reaction buffer (50 mmol KCl + 20 mmol Tris-HCl, pH 8.4, 5 mmol $MgCl_2$): 50 µg/ml bovin serum albumin and 1 unit Taq DNA polymerase.

The reactions were performed on a thermalcycler programmed for one initial cycle with 1 min at 94°C, 5 min at 35°C, 2 min at 72°C and 45 cycles of 1 min at 94°C, 1 min at 35°C and 2 min at 72°C.

The assays were repeated at least twice with each primer in different experiments.

Amplification products were electrophoresed in 1.4% agarose gel and detected by staining with ethidium bromide. The RAPD bands were scored as 0 (fragment absent) and 1 (fragment present) in a data matrix and then, distance values were subject to phenetical and cluster analysis using the softwares package NTSYS-pc-2.02, Popgene, Arlequin 2.00 (Sneath and Sokal, 1973; Rohlf , 1998; Yeh et al., 1999; Schneider et al., 2000).

RESULTS AND DISCUSSION

In this investigation, the genetic variation existing among isolates of *B. oryzae* was studied by analyzing the DNA polymorphisms in isolates recovered from different rice cultivars. Out of the seven primers, four primers A01, A08, B06 and B07 revealed the best profiled of strains.

The RAPD profiled showed a high level of genetic variability among the *B. oryzae* isolates. The most gene-rated and polymorphic bands were amplified at the time of application of primer A01 and the least generated and polymorphic bands were amplified with application of primers A02 and B06. 70 polymorphic fragments were detected in the original samples, ranging from 200 – 300 bp. The phenogram from cluster analysis can be divided *B. oryzae* into 3 groups with a similarity coefficient of 0.75 (Figure 1). The softwares package Popgene and Arlequine indicated that average gene diversity of population of *B. oryzae* were 0.617934 ± 0.364925. The number and percentage of polymorphic loci in this population were 37 and 42.05%, respectively (Table 2).

Standard diversity indices were as follows: Number of gene copies: 19; number of haplotypes: 19; number of original haplotypes in sample: 19; number of loci: 6; number of usable loci: 6; loci with less than 5% missing data; number of polymorphic sites: 6 and gene diversity: 1.0000 ± 0.0171.

In this investigation, RAPD-PCR technique was applied to DNA fingerprinting *B. oryzae* isolates. RAPD profiles did not reveal polymorphism that directly correlated climatic factors or host origin with geographic source of the isolates of *B. oryzae.*

Haplotypes of *B. oryzae* were variated. Host specific pressure did not effect on the genetic variation of this species, because no significant correlation was found between cultivar type and fungus species, but some of the isolates of fungus with high genetic similarity, have had the same origin (Figure 1).

Weikert et al. (2002) reported that species of *Drechslera, Bipolaris* and *Exserohilum* are genetically distinct and demonstrated that the telemorphs of the analyzed species presented high genetic similarity in their electrophoretic profiles when compared to the respective anamorph of each species. According to Burdon and Silk (1997), plant pathogenic fungi most commonly rely on mutation and recombination as the main source of genetically based variation. Within a species, the gene flow between popu-lations supplement these processes as propagules spread from one epidemiological area to another and from one deme to the next.

Gene flow, along with other evolutionary forces, can result in the spread of single genes (or DNA sequences), genotypes and even in the establishment of whole population different regions (McDermott and McDonald, 1993).

Based on the high degree of genetic variation found

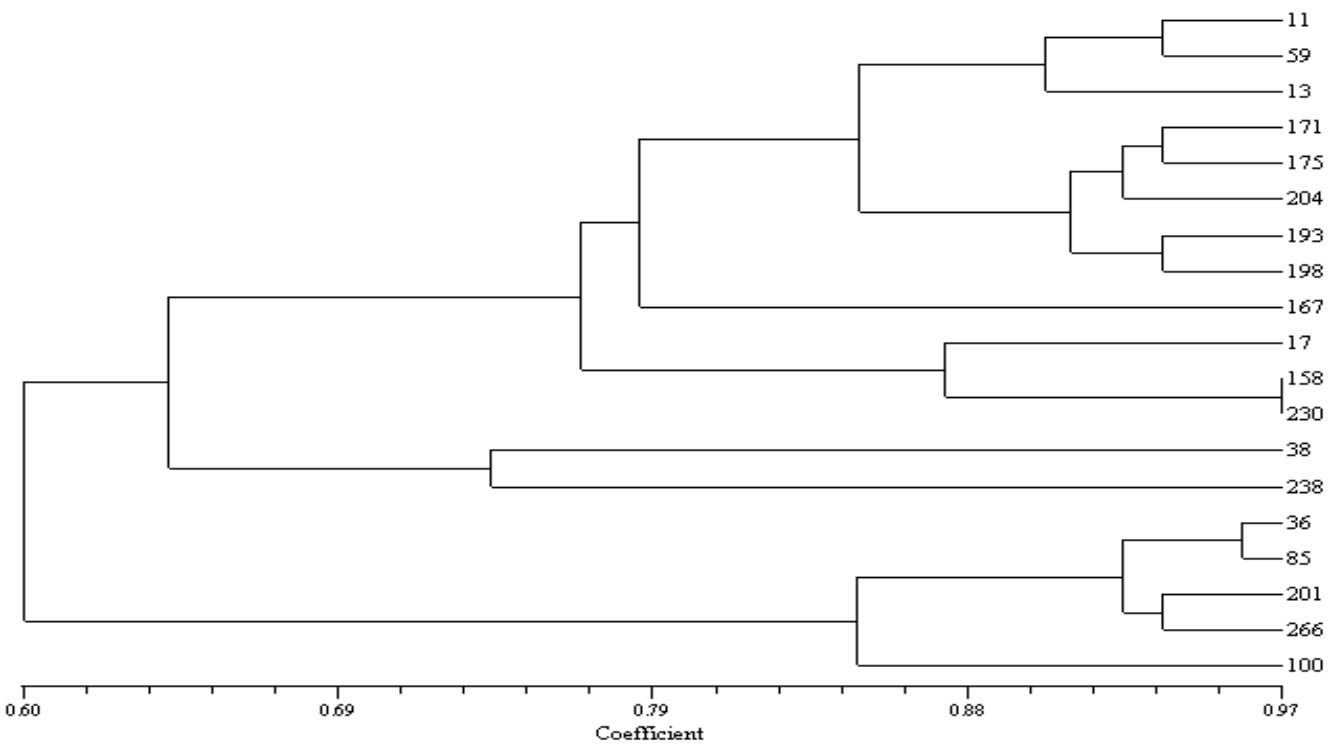

Figure 1. UPGMA-phenogram derived from banding patterns of RAPD analysis.

Table 2. Summary of genic variation statistics for all loci (Nei, 1978).

Locus	Sample size	na*	ne*	h*	I*
A011	19	1	1	0.0000	0.0000
A012	19	1	1	0.0000	0.0000
A013	19	1	1	0.0000	0.0000
A014	19	1	1	0.0000	0.0000
A015	19	1	1	0.0000	0.0000
A016	19	1	1	0.0000	0.0000
A017	19	1	1	0.0000	0.0000
A018	19	1	1	0.0000	0.0000
A019	19	1	1	0.0000	0.0000
B061	8	1	1	0.0000	0.0000
B062	8	1	1	0.0000	0.0000
B063	8	1	1	0.0000	0.0000
B064	8	1	1	0.0000	0.0000
B065	8	1	1	0.0000	0.0000
B066	8	1	1	0.0000	0.0000
B067	8	1	1	0.0000	0.0000
B068	8	2	1.6	0.3750	0.5623
B069	8	2	1.28	0.2188	0.3768
B0610	8	1	1	0.0000	0.0000
B0611	8	1	1	0.0000	0.0000
A081	18	1	1	0.0000	0.0000
A082	18	2	1.2462	0.1975	0.3488
A083	18	1	1	0.0000	0.0000
A084	18	2	1.2462	0.1975	0.3488

Table 2. Continued.

A085	18	2	1.3846	0.2778	0.4506
A086	18	1	1	0.0000	0.0000
A087	18	1	1	0.0000	0.0000
A088	18	2	1.1172	0.1049	0.2146
A089	18	1	1	0.0000	0.0000
A0810	18	2	1.1172	0.1049	0.2146
A0811	18	1	1	0.0000	0.0000
B071	19	1	1	0.0000	0.0000
B072	19	1	1	0.0000	0.0000
B073	19	1	1	0.0000	0.0000
B074	19	1	1	0.0000	0.0000
B075	19	1	1	0.0000	0.0000
B076	19	1	1	0.0000	0.0000
B077	19	2	1.2321	0.1884	0.3365
B078	19	1	1	0.0000	0.0000
B079	19	2	1.1108	0.0997	0.2062
B0710	19	2	1.1108	0.0997	0.2062
B0711	19	2	1.4979	0.3324	0.5147
B0712	19	2	1.1108	0.0997	0.2062
B0713	19	1	1	0.0000	0.0000
B0714	19	1	1	0.0000	0.0000
B0715	19	1	1	0.0000	0.0000
B0716	19	1	1	0.0000	0.0000
B0717	19	1	1	0.0000	0.0000
B0718	19	1	1	0.0000	0.0000
B0719	19	2	1.1108	0.0997	0.2062
Average	16	1.4205	1.1726	0.1101	0.2355

na = Observed number of alleles; ne = effective number of alleles; h = Nei's [1978] gene diversity; I = Shanon's information index.

among *B. oryzae* isolates from Guilan province, it is sug gested that there is considerable gene flow among this population.

In conclusion, application of RAPD-PCR is useful for identification of the fungi at the species level and can provide good results for further studies including intro-duction of resistant varieties.

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