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Research Article

Bipolaris species associated with rice plant: pathogenicity and genetic diversity of *Bipolaris oryzae* using rep-PCR in Mazandaran province of Iran

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Abstract: Ninety one monoconidial *Bipolaris* isolates were obtained from lesions on different parts of rice in different locations of Mazandaran province during the summer of 2009. *Bipolaris* species were identified using morphological features such as color and shape of colony and color and size of conidia and conidiophores. The isolates were separated into two species; 85 (93.4%) isolates belonged to *Bipolaris oryzae* and the remaining 6 (6.6%) isolates to *Bipolaris cynodontis*. Therefore *B. oryzae* is regarded as the major cause of rice brown spot disease in Mazandaran province. In order to analyze genetic diversity among *B. oryzae* isolates, 71 isolates were subjected to fingerprinting analysis by rep-PCR using BOX and REP primers. In cluster analysis, 15 clonal lineages and 54 haplotypes were identified. The largest clonal lineage contained with 36 haplotypes was the most common lineage. These results also indicate a relatively high level of genetic diversity among *B. oryzae* isolates. Also, pathogenicity test of a few *B. oryzae* isolates (12 isolates) was conducted under greenhouse condition and showed that those isolates were pathogenic to rice seedlings of cv. Tarom. All isolates produced some leaf spots 24 h after inoculation.

Keywords: *Bipolaris*, Rice, Genetic diversity, REP and Box Primers, Pathogenicity test, Mazandaran province.

Introduction

Rice (*Oryza sativa* L.) is one of the most important crops in terms of contributing to human diet and value of production (Bockelman and Dilady, 2003) and is the staple food for nearly two-thirds of the World population. However, rice plant is susceptible to several leaf spot diseases including blast and brown spot which adversely affect grain yield and quality, causing significant yield losses. Brown spot, caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker, is one of the most important seed borne diseases of rice and is an economically important foliar disease (Ou, 1985). In 1942, an outbreak of the disease caused yield losses of 90% which resulted in famine in Bengal (Ghoze *et al*., 1960) and was one of the major reasons for the death of 2 million people (Stuthman, 2002). It causes seedling blight and damages the foliage and panicles of rice, particularly when rice is grown in nutritionally deficient or unfavorable soils (Marchetti and Peterson, 1984). Distinctive symptoms of the disease

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include light reddish-brown lesions or lesions with a gray center surrounded by dark to reddish-brown margin with a bright yellow halo (Ou, 1985). Brown spot was reported by Petrak for the first time in Iran (Safari Motlagh and Kaviani, 2008b). The reported causal agents of the disease in Guilan province (north Iran) are *B. oryzae, B. victoriae* and *B. bicolor* (Safari Motlagh and Kaviani, 2008a), while in Fars and Kohgiloyeh Boyerahmad provinces (south Iran), causal agents include *B. oryzae, B. tetramera* and *Exserohilum rostratum* (Razavi, 1992), and *B. oryzae* and *B. sorghicola* have been reported as causal agents of brown spot in Mazandaran province (Khosravi, 1998).

Molecular techniques have been increasingly used to explore genetic variability in fungi (Caligiorne *et al*., 1999). Genetic variability of different species of *Bipolaris* has been studied by different molecular methods including RAPD (Kumar *et al*., 2011) and PCR-RFLP (Weikert-Oliveira *et al.,* 2002). In the present study, the genetic variation among *B. oryzae* isolates was determined by rep-PCR (Repetitive Extragenic Palindromic-PCR genomic fingerprinting) marker. Although rep-PCR primers were designed for repeated elements in prokaryotic genomes, this method has been also applied to differentiate species of the genus *Tilletia* (McDonald, *et al.,* 2000**)**, and to identify genetic variation and characterize variability at inter-and/or intra-specific levels of several fungal genera including *Verticillium*, *Fusarium*, *Stagonospora*, *Septoria*, and *Leptosphaeria* (Arora *et al*., 1996; Healy *et al.,* 2004; Tymon and Pell 2005).

The objectives of this study were: 1-identification of *Bipolaris* species associated with rice leaf spot symptoms in Mazandaran province, 2-characterization of the genetic variation of *B. oryzae* isolates using DNA fingerprinting and 3-evaluation of the aggressiveness of different *B. oryzae* isolates on Tarom cultivar.

Materials and Methods

Sampling

Leaves and panicles of six rice cultivars including Tarom, Tarom hashemi, Fajr, Neda, Khazar and an unknown cultivar with symptoms of brown spot were collected from rice fields of different counties in Mazandaran province, north Iran, during July and September 2009 using Xia *et al*. (1993) method. Samples were dried and stored in a refrigerator at 4 °C.

Fungal isolation and identification

Single lesions of diseased samples were excised and sterilized by immersion in 0.5% sodium hypochlorite for 1-5' and then washed three times with sterile distilled water. The specimens were placed on wet filter paper in sterilized Petri dishes and incubated under (nUV light) at 25-30 °C for 2-5 days to induce sporulation of the fungi. The isolated fungi were purified by single spore method and were maintained on sterilized filter paper discs at 4 °C in the refrigerator. A total of 91 isolates were obtained. Species identification was carried out according to the characteristics described by Ellis (1976) and Sivanesan (1987). The colony morphology of all isolates was studied on PDA by incubating the plates at 26 °C for 5 days. All isolates were grown on PDA medium for 2-3 days. Then, a plug of each isolate was transferred to tap water agar (TWA) + wheat straw medium and incubated 10-20 days at 20-26 °C and 12 h light/darkness photoperiod. Morphological characteristics of conidium and conidiophore, type of conidial germination and structure of hilum were studied microscopically.

DNA extraction and rep-PCR analysis

For DNA extraction the fungi were grown on potato dextrose broth medium for 5 days at 25 °C (Liu *et al*., 2000). Mycelia were harvested by vacuum filtration using Buchner funnel and were freeze-dried, lyophilized and then macerated in liquid nitrogen. DNA was extracted from 71 isolates of *B. oryzae* using a Core-one^{tm} Plant Genomic DNA isolation Kit

(Corebio, Korea) according to the manufacturer's instructions. The extracted genomic DNA was electrophoresed on 1 % agarose gel, and detected by staining with ethidium bromide.

REP 1R (IIIICGICGICATCIGGC) and REP 2I (ICGICTTATCIGGCCTAC) and BOX 1R (CTACGGCAAGGCGACGCTGACG) primers were selected to screen all the isolates (McDonald *et al*., 2000). DNA amplification reaction was carried out in a final volume of 20µl, which consisted of 2mM dNTPs mix (for BOX primer, 1mM), 10pM of each primer, 2.5 unit of Taq DNA polymerase, 30ng DNA template, 25mM of $MgCl_2$ and 2.5µL of 10X reaction buffer. All of the reactions were performed in a model Palm cycler (Corbett Research, CG 1-96 Australia) thermal cycler under the following conditions: The initial denaturation at 94 °C for 7 min, followed by 35 cycles of denaturation (3 min at 94 °C and 30 sec at 92 °C), annealing (1 min at 49 °C), extension (7 min at 72 °C) and an extra extension step for 10 min at 72 °C. The PCR cycle for rep-PCR was similar to those of BOX PCR. However, the annealing temperatures were 39 °C for REP primer. Amplified DNA fragments were separated by electrophoresis on 1.2% agarose gel in 1X TBE buffer, stained with ethidium bromide and DNA bands were visualized and photographed under UV light utilizing the gel documentation system, Eagle Eye II (Stratagene).

Data analysis

DNA fingerprints were scored visually and recorded according to the position of bands for each of the 71 isolates as 1, for the presence and 0, for the absence of a band. Cluster analysis was used to examine genotypic relationships among the isolates. The phenograms of the isolates were constructed based on the similarity matrix using the Unweighted Pair Groups Method with Arithmetic mean (UPGMA) algorithm with NTSYS-pc version 2.02e and similarity matrix based on Jaccard's similarity coefficient.

Pathogenicity test

Based on three items including molecular differences in gel pictures, site of collection and the part of rice plant from which the isolates were collected, 12 isolates of *B. oryzae* were selected and cultured on TWA + wheat straw at 25 °C under 12 h light and 12 h darkness conditions for 14 days, then conidial suspensions were prepared by scraping the surface of culture plates and washing in sterile water containing one drop of Tween 20. Then the fungal suspension was filtered through 2 layers of cheese cloth. Concentration of conidia was adjusted to 5×10^4 using a haemocytometer. Pathogenicity was tested on rice plants in greenhouse. Thirty days old plants were sprayed with conidial suspension of selected isolates. Control seedlings were sprayed with distilled water containing a drop of Tween 20. The plants were covered with transparent plastic sheets to provide adequate humidity and kept at room temperature. The inoculated plants were observed after 7 days and infected leaves were scored using a rating (r) of 0-9, denoting proportions of Brown spot disease over the whole leaf area (IRRI, 2002). Koch's Postulates were completed to prove the pathogenicity of 12 isolates of *B. oryzae* in greenhouse conditions. Virulence of isolates were evaluated and compared based on the Disease Index formula (Gao *et al*., 2011). Disease index was calculated according to the following equation:

$$\text{Disease Index (\%)} = [\sum (r \times n_r)/(9 \times N_r)] \times 100$$

where r = rating value, n_r = number of infected leaves with a rating of r and N_r = total number of leaves tested.

This test was conducted in a completely randomized design where isolates and pots were considered as treatments and replicates respectively. Three pots were used for each isolate. Data analysis was done using SAS software version 9.1.

Results

Species identification

According to identification keys of Sivanesan (1987) and Ellis (1971), a total of 91 isolates

belonging to two species of *Bipolaris* genus were identified of which 85 isolates belonged to *B. oryzae* (Ito and Kurib) Drechsler ex Dastur and 6 isolates belonged to *Bipolaris cynodontis* (Marignoni) Shoem. Average diameter of colony growth of *B. oryzae* and *B. cynodontis* on PDA medium was 7cm and 3.25 cm respectively after 5 days at 25 °C (Figs. 1-A, B). Microscopic characteristics of *B. oryzae* were as follows: (Figs. 1-C, E). Conidiophores single or in small groups, mostly straight, sometimes flexuous and geniculate, light to dark brown and paler toward the apex, septate, 300-630 µm long and 5-7.5 µm in width. Conidia pale to light brown, straight, smooth, often curved, navicular, obclavate and occasionally cylindrical, 40-154.5 × 9.5-24.5 µm with maximum of 14 distosepta, Hilum often protruding.

Microscopic characteristics of *B. cynodontis* were as follows: (Figs. 1-D, F). Mycelium gray to dark gray, 2.5-3.75 µm diameter. Conidiophores short and thick, single or in small groups, pale to light brown, septate, smooth, simple, straight and rarely curved and geniculate, simple and rarely branching, 55-167.5 µm long, 5-7µm in width, conidiogenous nodes are verruculose. Conidia cylindrical, slightly curved and usually broadest in the middle and tapering towards the rounded ends with 3-9 distosepta, yellow to dark brown, 20-70 × 8.75-16.25 µm.

DNA fingerprinting and rep-PCR analysis

The rep-PCR analysis of *B. oryzae* resulted in complex fingerprint patterns. Thirty one and 42, totally 73, appreciable DNA bands were reproduced by BOX and REP primers respectively. Total amplified polymorphic DNA bands were 55 of which 28 bands belonged to REP primer and the rest belonged to BOX primer. Using the BOX primer, the percentage of polymorphic loci was 64.28% whereas with REP primer it was 90.32%. In other words, 75.34% polymorphism was observed among isolates. The amplified appreciable bands in BOX ranged in length from 300 to 2500 bp whereas for the REP they ranged from 500 to 3000 bp (Figs. 2-A, B).

Cluster Analysis of rep-PCR

Bands were assigned a number with respect to their migration distance within the gel. For each individual, the presence or absence of each band was determined and designated 1 if present or 0 if absent in order to obtain binary banding data. Therefore monomorphic and polymorphic DNA bands were used and the combined BOX and rep-PCR patterns were analyzed to generate a dendrogram. Similarity matrices from binary banding data of each of the two primer combinations were derived with the Similarity for Qualitative Data program (SIMQUAL) in the Numerical Taxonomy and Multivariate analysis System for personal computer (NTSYS-pc) version 2.0 (Rohlf, 1993) and estimates for similarity were based on Jaccard's similarity coefficient. Matrices of similarity were calculated using UPGMA (Unweighted Pair Group Method with Arithmetic mean) and eventually a dendrogram was constructed by the NTSYS-pc program for all of the selected isolates (Fig. 3). Dendrogram indicates the genetic relationship between *B. oryzae* isolates based on the total number of amplified fragments. Fifteen clonal lineages or fingerprinting groups that are nominated A to O were obtained with a similarity coefficient of 0.8. Clonal lineage A allocated maximum number of isolates and haplotypes and included isolates from all counties of province except Behshahr (Table 1). Three isolates belonged to clonal lineage B; clonal lineages C and D had 2 isolates each (Table 2). Clonal lineage A included isolates from all six rice cultivars as shown in Table 3; also these 53 isolates were obtained from different parts of rice plants including seed, leaf, panicle axis, panicle neck and flag leaf sheath (Table 4). A fairly wide range in the value (0.54 to 1) of Jaccard similarity coefficient was observed among the isolates.

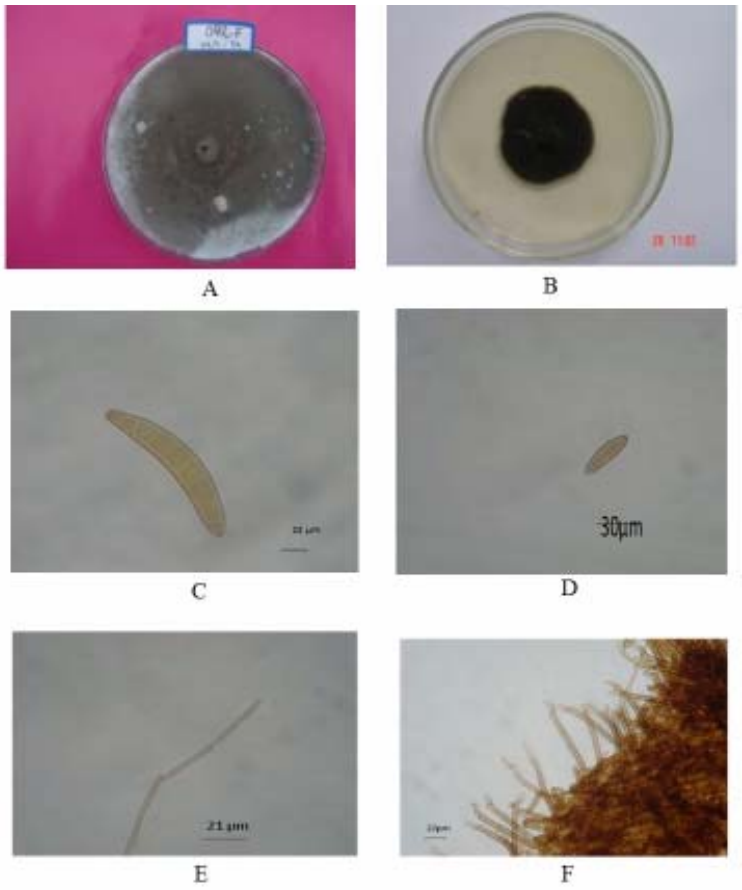

Figure 1 *Bipolaris oryzae* on PDA medium after five days (A), *Bipolaris cynodontis* on PDA medium after five days (B), *Bipolaris oryzae* conidium (C), *Bipolaris cynodontis* conidium (D), *Bipolaris oryzae* conidiophore (E) and *Bipolaris cynodontis* conidiophore (F).

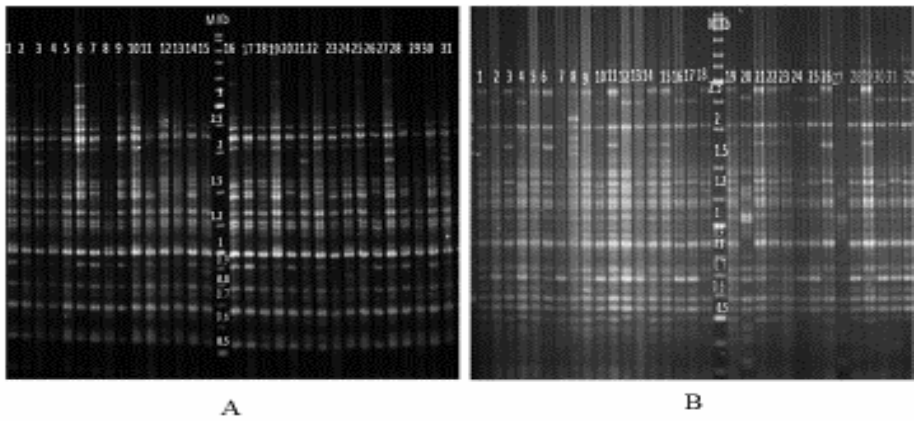

Figure 2 DNA replication patterns of *Bipolaris oryzae* isolates using Box (A) and REP primer (B) in 1.25% agarose gel. (100 bp GenerulerTM DNA Ladder Mix).

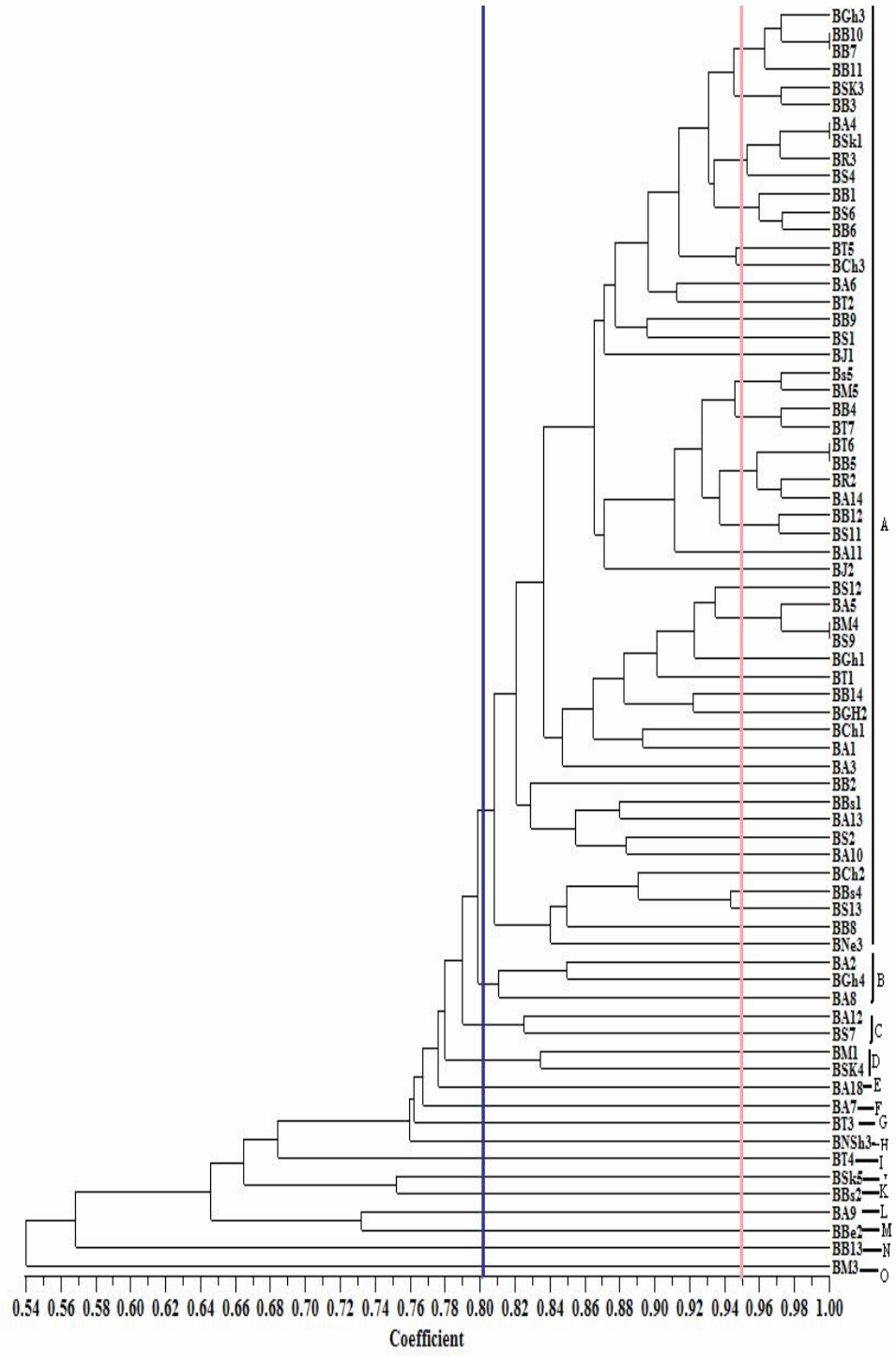

Figure 3 Dendrogram for the 71 isolates of *B. oryzae* obtained from rice plants using UPGMA method by NTSYS-pc-2.02 software that has been established with analysis of binary matrix and rep-PCR method. English letters on the vertical axis represent lineage names.

Table 1 Abundance and distribution of clonal lineages and their haplotypes in geographic areas of Mazandaran province.

Sampling locations	Number of isolates	Number of haplotypes	Number of isolates in each clone													
			A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Amol	15	15	9	2	1		1	1						1			
Babol	14	14	13													1	
Sari	9	9	9														
Qaemshahr	4	4	3	1													
Savadkooh	5	5	2		1	1						1					
Jooibar	2	2	2														
Neka	1	1	1														
Behshahr	1	1													1		
Mahmood abad	4	4	2														1
Noshahr	1	1															
Chaloos	3	3	3							1							
Tonekabon	7	7	5						1		1						
Ramsar	2	2	2														
Babolsar	3	3	2										1				

Table 2 The frequency of *Bipolaris oryzae* isolates from rice in clonal lineages, identified among 71 isolates using two primers, BOX and REP.

Clonal lineage	Number of isolates	Abundance of isolates (%)	Number of identified haplotypes
A	53	74.65	36
B	3	4.22	3
C	2	2.81	2
D	2	2.81	2
E	1	1.41	1
F	1	1.41	1
G	1	1.41	1
H	1	1.41	1
I	1	1.41	1
J	1	1.41	1
K	1	1.41	1
L	1	1.41	1
M	1	1.41	1
N	1	1.41	1
O	1	1.41	1

Table 3 The number of isolates within each clonal lineage obtained from different cultivars of rice.

Colonal lineage	Number of isolates in each clonal lineage					
	Tarom	Tarom hashemi	Fajr	Neda	Khazar	High yield
A	25	19	2	2	2	2
B	1	3				
C	1	2	1			
D	1	1				
E	1	1				
F	1				1	
G	1	1				
H	1	1				
I	1	1				
J	1			1		
K			1			
L	1	1				
M	1					
N	1	1				
O	1	1				

Table 4 The number of isolates within each clonal lineage, obtained from different parts of rice.

Clonal lineage	Number of isolates in each clonal lineage				
	Flag leaf sheath	Panicle neck	Panicle axis	Leaf	Seed
A	31	9	10	1	2
B	3				
C	2				
D	1	1			
E	1				
F				1	
G	1				
H	1				
I	1				
J			1		
K		1			
L	1				
M					
N	1				
O	1				

Pathogenicity test

For pathogenicity tests the local Tarom cultivar, which is the dominant rice cultivar grown, was used. First symptoms appeared 24 hours post inoculation as pinhead spots. Two days later, these points gradually expanded and became oval necrotic spots. In some cases a number of spots joined together and parts of the leaves were blighted.

Discussion

In this investigation, two species of *Bipolaris*, *B. oryzae* and *B. cynodontis*, agents of rice brown spot disease, were identified from rice fields of Mazandaran province of Iran and *B. oryzae* was the dominant species. *B. sorghicola* has been reported from rice fields of this province in previous years. At the same time, *B. victoriae* was reported as the most dominant agent of rice brown spot disease in Guilan province.

Brown spot is one of the important diseases of rice in Iran that causes considerable losses to many cultivars; little research has been done on genetic diversity of its causal agents, *Bipolaris* spp.

Analysis of variance showed that there were differences between virulence of isolates ($P < 0.05$). BCh1 isolate belonging to colonal lineage A, showed the highest disease index. BGh1, BR3, BB1 belonging to colonal lineage A and BBs2 belonging to clonal lineage K showed similar virulence and were placed in the same group. Other isolates belonging to A, J, L and N clonal lineages, were placed in another group (Table 5). The ab group isolates do not show significant difference with a and b groups of isolates in terms of virulence.

Table 5 Relationship between genetic diversity and disease index of 12 isolates of *Bipolaris oryzae*.

Name of isolates	Clinal lineages	Pathogenicity test	D.I. (%)[1]
BB1	A	ab	33
BR3	A	ab	33
BT2	A	b	12
BB9	A	b	16
BS5	A	b	23
BM5	A	b	17
BGh1	A	ab	34
BCh1	A	ab	53
BSk5	J	b	22
BBs2	K	ab	30
BA9	L	b	15
BB13	N	b	12

[1]Disease Index (%).

PCR methodologies employing REP, BOX and ERIC sequences as PCR primer binding sites can be used to study the distribution of repetitive sequences in different genomes. rep-PCR involves the amplification of repetitive sequences such as BOX, enterobacterial repetitive intergenic consensus (ERIC), and repetitive extragenic palindromic (REP) elements and generates DNA fingerprints (Versalovic *et al*., 1991; George *et al*., 1998). Although the rep-PCR method was designed for species or strain differentiation within prokaryotes (Versalovic *et al*., 1991) recently, rep-PCR fingerprinting has been used as between species to characterize *Aspergillus* species and as within species to characterize *Aspergillus fumigatus* (Van Belkum *et al.,* 1993), *Fusarium oxysporum* isolates (Edel *et*

al., 1995) and *Verticillium chlamydosporium* isolates (Arora *et al.,* 1996). In *Leptospheria maculans*, REP, ERIC and BOX primers generated reproducible and specific fingerprints for all members of the species complex, confirming that ERIC-, REP- and BOX- like sequence represent in fungal genomes and represent useful targets for isolate characterization. As opposed to other DNA fingerprinting techniques, the inter-repeat PCR procedures such as ERIC and REP are easier to handle as they involve only rapid minipreparation of DNA, PCR amplification and agarose gel electrophoresis. PCR based procedures that are well adapted for large scale characterizations of pathogenic strains are required for diversity studies (Edel *et al*., 1995); The inter-repeat PCR procedures give results that compare well to other molecular methods (Muiru *et al*., 2010).

rep-PCR is fast, accurate and low cost technique. Seventy one isolates were subjected to rep-PCR and were found to generate high bands and isolates amplified properly and high level of polymorphism was seen. On the other hand, Muiru *et al*. (2010) found few bands in *Exserohilum turcicum* isolates yet over 80% of the isolates amplified properly; this shows that this technique has potential to be used to distinguish strains of fungal pathogens.

High degree of genetic variation was found among *B. oryzae* isolates from Mazandaran province. Also, Safari Motlagh and Anvari (2010) found genetic variation among *B. oryzae* isolates from Guilan province. *Bipoloris* species like *B. sorokiniana* are members of Deuteromycetes and reproduce asexually (Raemaker, 1987). This allows parasexual recombination (Tinline, 1962) to set the fate of variability among the isolates of *Bipolaris* (Jaiswall *et al*., 2007).

It is concluded that application of rep-PCR is useful for identification of the fungi within species level (Gurel *et al*., 2010) and can provide good results for further studies including introduction of resistant varieties. The present investigation establishes molecular variability among the isolates of *B. oryzae.* Clonal lineage A, the largest clonal lineage, included isolates from all counties of the province except Behshahr. No correlation was found between genetic similarity and geographical origin in *B. oryzae* isolates. Suzuki *et al*. (2010) found no such relationship between the fingerprinting and geographical distribution of *Colletotrichum gloeosporioides* isolates either.

Research by Weikert-Oliveira *et al.* (2002) on the isolates of *B. maydis* and *B. sorokiniana* using PCR-RFLP and RAPD markers did not show direct correlation between the polymorphism and climatic conditions and geographic areas either. Results of this study confirm findings by Safari Motlagh and Kaviani (2008b). Also, this marker could very well separate isolates of *B. oryzae* and *B. cynodontis* at 46% similarity. Thus it is a good marker at the species level and is in accordance with the results of McDonald *et al.* (2000).

This is first report on rep-PCR marker used for *Bipolaris* species.

Also, the pathogenicity of all isolates belonging to *B. oryzae* species was proved in greenhouse conditions. Safari Motlagh and Kaviani (2008a) reported pathogenesis of *B. oryzae* isolates on Khazar cultivar. Except BT2 isolate, four isolates that were obtained from the leaves were more virulent than those from other parts of rice plant.

In this study different species of *Bipolaris* were identified and dominant species was determined and high genetic diversity was found among the different isolates of *B. oryzae*.

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گونه‌های *Bipolaris* مرتبط با گیاه برنج: بیماریزایی و تنوع ژنتیکی گونه *B. oryzae* با استفاده از rep-PCR در استان مازندران

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چکیده: در تابستان ۸۷، ۹۱ جدایه از قارچ *Bipolaris* از لکه‌های موجود در برگ‌ها و خوشه‌های گیاه برنج از مناطق برنج‌کاری استان مازندران به‌دست آمد. شناسایی گونه‌ها با استفاده از صفاتی از قبیل رنگ و شکل کلنی، رنگ و اندازه کنیدی و کنیدیوفور انجام گرفت. دو گونه‌ی *Bipolaris oryzae* و *B. cynodontis* شناسایی گردید که ۸۵ جدایه متعلق به گونه *B. oryzae* و ۶ جدایه متعلق به گونه *B. cynodontis* بود. بنابراین *B. oryzae* به‌عنوان عامل اصلی بیماری لکه قهوه‌ای برنج در مازندران شناخته شد. به‌منظور بررسی تنوع ژنتیکی جدایه‌های *B. oryzae*، ۷۱ جدایه با استفاده از نشانگر rep-PCR و دو آغازگر BOX و REP انگشت‌نگاری شدند. با تجزیه خوشه‌ای، ۱۵ دودمان کلنی و ۵۴ هاپلوتیپ شناسایی شد. دودمان کلنی A با ۳۶ هاپلوتیپ به‌عنوان دودمان غالب شناسایی گردید. نتایج، سطح نسبتاً بالایی از تنوع ژنتیکی را در بین جدایه‌های *B. oryzae* نشان داد. علاوه بر این، آزمون بیماریزایی تعداد کمی از جدایه‌های *B. oryzae* در شرایط گلخانه روی برنج انجام پذیرفت و جدایه ها از نظر ویرولانس، تفاوت نشان دادند.

واژگان کلیدی: *Bipolaris*، برنج، تنوع ژنتیکی، rep-PCR، بیماریزایی، مازندران