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Original Article

Genetic diversity of *Fusarium semitectum* isolates from rice, using RAPD and REP-PCR markers

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Abstract: In order to determine genetic variability among 33 isolates of *Fusarium semitectum*, morphological and molecular studies were carried out. Isolates from different paddy fields in the north of Iran were separated from each other in four morphotype groups. The isolates showed a high level of variation in aerial mycelium, color of colony, number of septa and growth rate on potato dextrose agar (PDA). Comparison between morphological and molecular methods revealed that in some cases, the groupings of isolates based on morphological method was consistent with molecular groupings with a few exceptions. The results indicate that RAPD-PCR and REP-PCR could be used for monitoring intraspecific genetic variability within *F. semitectum*.

Key words: morphology, DNA fingerprinting, variation, polymorphism

INTRODUCTION

Rice (*Oryza sativa* L.), as a cereal grain, is the most widely consumed staple food for a large part of the world's human population, especially in Asia. It is the grain with the second-highest worldwide production, after maize (FAO 2010). World production of rice has risen steadily from about 200 million tons of paddy rice in 1960 to over 678 million tons in 2009 (FAO 2010).

Species of the genus *Fusarium* Link are among the most important plant pathogenic fungi that commonly infect many economically important crops. *F. semitectum* Berk. & Ravenel [syn: *F. pallidoroseum* (Cooke) Sacc.; *F. incarnatum* (Roberge) Sacc.] is a widespread and common species in tropical, subtropical and Mediterranean regions and regularly associated with a complex of plant diseases (Leslie & Summerell 2006). This cosmopolitan species is a member of *Fusarium* section *Arthrosporiella* that was proposed by Wollenweber & Reinking (1935) and has no known sexual stage (Burgess et al. 1994).

Fusarium has a confusing and unstable taxonomic history. A number of factors, including the lack of clear morphological characters separating species, leading to species concepts that are too broad, together with variation and mutation in culture have conspired to create taxonomic systems that poorly reflect species diversity (Geiser et al. 2004). The morphological characters used for identification of *Fusarium* species are limited in number, sensitive to environmental conditions, subtle and easily misinterpreted. Numerous molecular markers are introduced for the clarification and delineation of fungal taxa. Random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and repetitive extragenic palindromic (REP) (Versalovic et al. 1991) are specific variations of PCR-based molecular markers. RAPD has the advantage of being technically simple and rapidly facilitated. This technique has been used for a long time for fungal genetics and phylogenetic studies (Brown 1996, Miller 1996). The taxonomic status of *F. semitectum* has varied over time. As *F. semitectum* was suggested to be species complex (Leslie & Summerell 2006), DNA markers were applied to assess the extent of intraspecific variation within *Fusarium* species. Feng et al. (2000) used RAPD markers to assess genetic diversity among three races (races 3, 7, 8) of *F. oxysporum* f. sp. *vasinfectum* in China. Twenty-six isolates from 11 provinces in China and three isolates of foreign origin were amplified with 10 decamer primers. From this, 140 RAPD markers were obtained with a polymorphism rate of 87.8 %. In melon, RAPD-PCR was used to differentiate between *F. semitectum* and *F. roseum* isolates (Chen-Shang et al. 1999). Smith et al. (2001) characterized two types of *F. oxysporum* f. sp. *vasinfectum*, a high sporing type (HS) and a low sporing type (LS) by RAPD analysis and pathogenicity tests. Repetitive DNA-based fingerprinting methods are commonly known as Rep-PCR. These methods rely on the amplification of genomic sequences between repetitive elements conserved in prokaryotes including repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX elements. Universal primers theoretically anneal to the intergenic target sites which are randomly dispersed in genomes and provide the amplifications of different lengths of fragments. REP-PCR applications are widespread in plant pathogenic bacteria and are

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only tested in fungal strains of *Fusarium oxysporum* (Edel et al. 1995).

Most strategies for plant disease management based on host resistance require a good knowledge of variability in plant pathogens. That is why the objective of this study was to develop a polymerase chain reaction (PCR) assay to examine genetic variation in a collection of the pathogen. In the present study, genetic diversity among *F. semitectum* isolates obtained from rice paddies was investigated by using RAPD and ERIC/BOX analyses.

MATERIALS AND METHODS

Isolation and identification of fungal strains

Diseased rice ears were collected from different paddy fields in Guilan, Mazandaran and Golestan provinces in the North of Iran in summers of 2011-2012. Five rice seeds of each sample were surface-sterilized with a 1% sodium hypochlorite solution for 1 min, rinsed twice in sterile distilled water and dried on sterile Whatman paper in a laminar flow cabinet. All sterilized samples were placed onto the selective medium peptone pentachloronitrobenzene agar (PPA) (Nash & Synder 1962) and incubated at 25°C for 7 days. All *Fusarium* isolates were subcultured on Potato Dextrose Agar (PDA) (Merck, Darmstadt, Germany) and Carnation-leaf Agar (CLA) (Fisher et al. 1982), using a single spore technique (Leslie &Summerell 2006). PDA cultures were incubated at 25°C, and CLA and SNA cultures were incubated at 25°C with on/off black light (20W-220V) cycles of 12 hours each for 2-4 weeks. Cultural characteristics were assessed visually and microscopically. Colony morphology (color, texture, sporodochia and growth rate) was recorded from cultures grown on PDA. The morphology of macroconidia, microconidia, conidio-genous cells and the chlamydospores was assessed from cultures grown on SNA and CLA. Morphological identification of isolates was made according to Nelson et al. (1983) and Leslie and Summerell (2006).

DNA extraction

Fungal DNA was isolated according to method proposed by Zhu et al. (1993). For DNA extraction, each isolate was grown in 25 ml of yeast extract in mechanical shaking (150 rpm) for 5 days at 25°C. Mycelia were harvested by filtration, freeze-dried for 48 h, and then stored at –20°C prior to DNA extraction. Total DNA was extracted by using the CTAB (Cetyl Trimethyl Ammonium Bromide) method. For this, 1 g of frozen mycelium was ground in liquid nitrogen using a mortar and pestle into a very fine powder. The powder was suspended in 10 ml of CTAB solution (50 mM Tris buffer pH 8.0, 100 mM EDTA, 150 mM NaCl) and incubated at 65°C for 45 min. DNA was extracted by adding an equal volume of chloroform/ isoamyl alcohol (24:1 v/v) and mixed thoroughly but gently and then centrifuged at 11000 rpm for 15 min at 10°C. Aqueous viscous supernatant was removed to a fresh tube and precipitated with 600 µl of ice-cold isopropanol and shaking for 5 min at 150 rpm. The mixture was centrifuged at 11000 rpm for 10 min at 10°C. Pellet was washed with 70% ethanol, dried completely, and dissolved in 100 µl amount of TE buffer.

RAPD Amplification

Ten random decamer primers (OPC and OPD, SinaClon, Iran, Table 1) were tested for PCR amplification of *F. semitectum* isolates. Amplifications were performed in a total volume of 25 µl containing 0.5 µl dNTPs mix (10mM), 1 µl primer (12.5 pmol), 20 ng template DNA, 1.2 µl $MgCl_2$, 1.5 µl reaction buffer (10X), 1.5 U *Taq* DNA polymerase and 18.5 µl deionized water. The DNA amplification was done in a thermal cycler (BioRad, USA) using the following PCR cycles. The first denaturation step of 2 minutes at 94 °C, followed by 35 cycles of denaturation for 30 sec at 94 °C, annealing for 1 min at 38 °C, extension at 72°C for 2 min and final extension at 72 °C for 5 min with holding temperature at 4°C for 10 min. Amplifications were performed twice to confirm consistency of the method. Negative control (without template DNA) was maintained for each set of experiment to test for the presence of non-specific banding.

ERIC-BOX Amplification

For ERIC and BOX the two oligonucleotide primer pairs used for PCR amplification had 22 nucleotides in length each (Table 1). Reactions were carried out in a 25 µl volume containing 20 ng template DNA, 2 µl buffer (10X), 0.5 µl dNTPs mix (10mM), 0.8 µl $MgCl_2$ (50mM), 0.3 µl each of primers, 1.5 U *Taq* polymerase and deionized water 18.5 µl. The program included an initial denaturation at 95°C for 2 min, 35 cycles with denaturation at 94°C for 30 sec, annealing at 52°C for 1 min, extension at 65°C for 2 min and final extension at 65°C for 8 min with holding temperature at 4°C for 10 min.

Table 1. Primers used for RAPD-PCR amplification of *Fusarium semitectum* isolates.

Primer name	Primer sequence *5'-3'*	Primer name	Primer sequence *5'-3'*
OPC- 5	GATGACCGCC	OPD- 2	GGACCCAACC
OPC- 7	GTCCCGACGA	OPD- 3	GTCGCCGTCA
OPC- 9	CTCACCGTCC	OPD- 5	TGAGCGGACA
OPC- 10	TGTCTGGGTG	OPD- 8	GTGTGCCCCA
OPC- 18	TGAGTGGGTG	OPD- 9	CTCTGGAGAC
ERIC	AAGTAAGTGACTGGGGTGAGCG		
BOX 1AR	CTACGGCAAGGCGACGCTGACG		

PCR products were electrophoresed on 1.5% agarose gel by using 1X TAE buffer (Fermentas Life Sciences, Canada), stained with Red safe, visualized in a UV-transilluminator and the gels were photographed using GelDoc system.

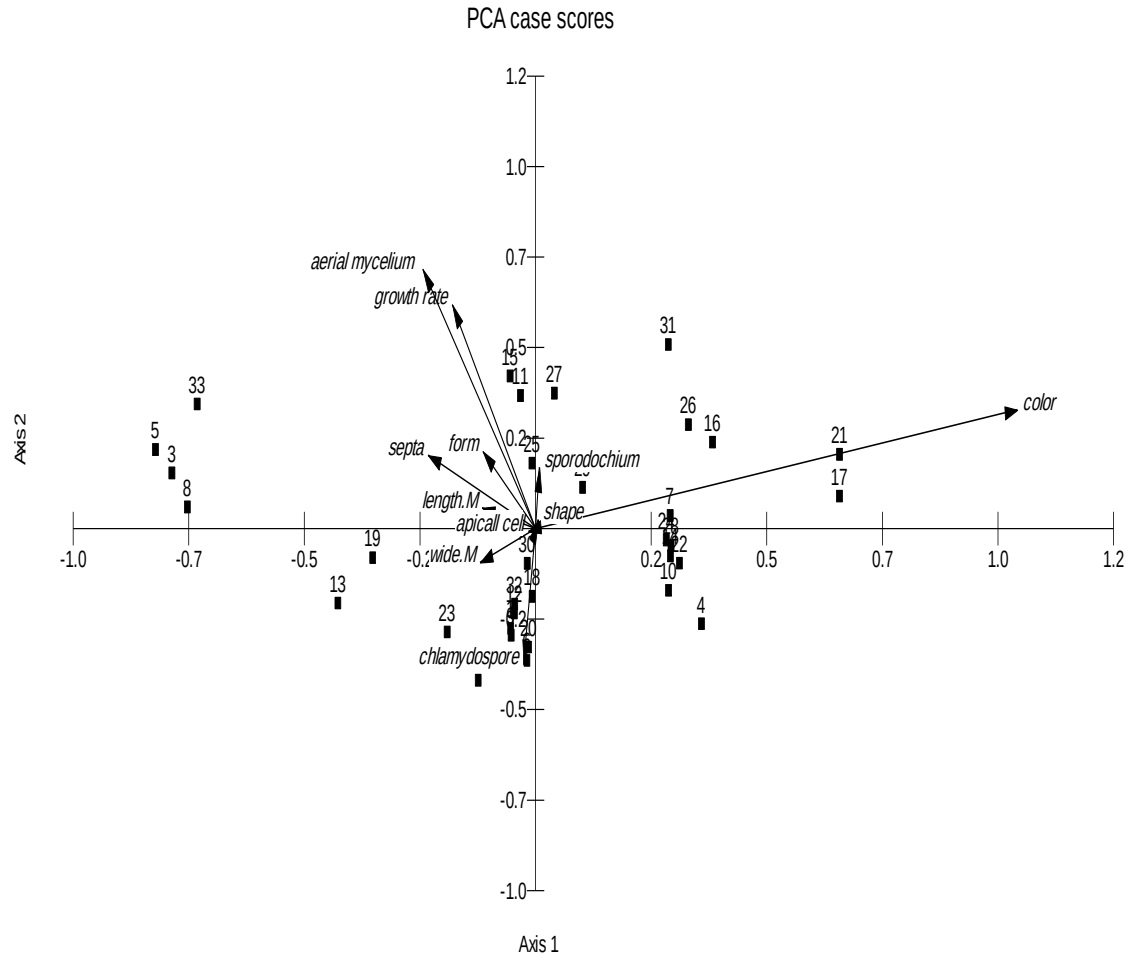
Data analysis

Polymorphic RAPD markers were manually scored as binary data with presence as "1" and absence as "0". Monomorphic markers were not scored. The similarity matrix was then subjected to the unweighted pair-group method with arithmetical mean (UPGMA) cluster analysis based on Nei & Li (1979).

The data analysis was performed by using the MVSP (Multi Variate Statistical Package) 3.1 program (Kovach Computing Services, Wales, UK) to analyze the relationship among all isolates of *F. semitectum*. To determine the statistical significance of the dendrogram branches, the data were bootstrapped with 1000 replicates using the program Past (Hammer et al. 2001). Genetic diversity and population differentiation parameters were calculated using PopGene software v. 1.32 (Yeh et al. 1999).

RESULTS

All 33 isolates obtained from diseased rice paddy fields in the north of Iran were identified as *F. semitectum* based on morphological characters such as the presence of monoblastic and polyblastic conidiogenous cells, sickle- and spindle-shaped macroconidia and mesoconidia appeared as rabbit-ears *in situ*. Some isolates of *F. semitectum* produced chlamydospores singly, in chains or in clusters. Formation of orange and light sporodochia was also observed. In general, isolates of *F. semitectum* could be recognized by tan to brown and orange colony appearances. *F. semitectum* isolates form abundant uniform to floccose mycelium and growth rate between 2-7 cm in 5 days after incubation at 24˚C. Principal component analysis (PCA) shows that the color of colony, aerial mycelium and number of septa are chief characters in grouping the isolates (Fig 1).



Vector scaling: 1.09

Fig. 1. Principal Component Analysis (PCA) among 33 isolates of *Fusarium semitectum* based on morphological characters.

Morphological characters indicated that all isolates of *F. semitectum* can be divided into four different morphotypes (Table 2). Among the 33 isolates, 9 isolates were grouped as morphotype A, 13 isolates as morphotype B, 9 isolates morphotype C and 2 isolates as morphotype D. Of the 10 RAPD primers surveyed, eight primers (except OPC-9 and OPD-9) produced reproducible and scorable polymorphic bands and were used in molecular analysis of all isolates. Primers OPC-5 (with 11 polymorphic bands) and OPD-8 (with 2 polymorphic bands) produced the highest and lowest number of fragments being the most and least informative primers used in the analysis. The size of RAPD fragments ranged from 500 to 2500 bp. RAPD analysis of isolates revealed the presence of 11 lineages at the arbitrary level of 60% similarity (Fig. 2). The isolates 1, 6 and 16 showed a relative genetic similarity of 100% by RAPD-PCR. Also, a relative genetic similarity of 90% was obtained for five pairs of isolates.

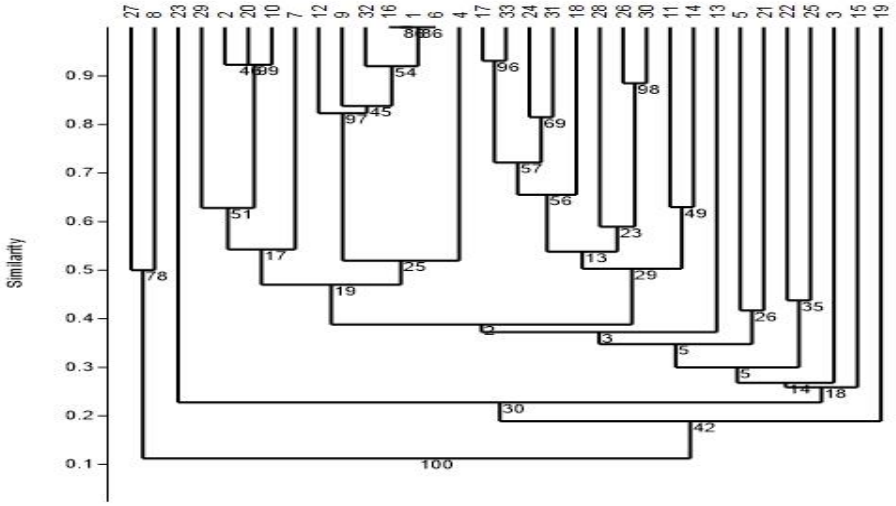


Fig. 2. UPGMA dendrogram showing relationship among 33 isolates of *Fusarium semitectum* based on analysis of 8 random amplified polymorphic DNA (RAPD) primers.

Table 2. Characteristics of isolates *F. semitectum* sampled from different rice paddies in the North of Iran during 2011-12

Isolate number	Geographical origin	Colony color	Arial mycelium	No of macroconidial septa (*)	Morphotype
1	Rasht	Brown	Yes	3(5)	A
2	Bandar Anzali	Orange	Yes	3(4)	B
3	Astaneh	Orange	No	5(3)	C
4	Langrood	Orange	Yes	3(5)	B
5	Roodsar	Beige	Yes	5	D
6	Roodsar	Brown	Yes	3(5)	A
7	Roodsar	Orange	Yes	3(5)	B
8	Kelachay	Orange	No	5(3)	C
9	Ramsar	Brown	Yes	3(5)	A
10	Ramsar	Orange	Yes	3(5)	B
11	Ramsar	Orange	No	5(3)	C
12	Ramsar	Brown	Yes	3(5)	A
13	Ramsar	Orange	Yes	3(5)	B
14	Chalus	Orange	Yes	3(5)	B
15	Noshahr	Brown	Yes	3(5)	A
16	Mahmudabad	Orange	No	5(3)	C
17	Mahmudabad	Orange	Yes	3(5)	B
18	Ferydunkenar	Orange	Yes	3(5)	B
19	Ferydunkenar	Orange	Yes	5(3)	B
20	Behnemir	Brown	Yes	3(5)	A
21	Joybar	Orange	No	3	C
22	Joybar	Orange	Yes	5(3)	B
23	Joybar	Orange	Yes	3(5)	B
24	Sari	Orange	Yes	5(3)	B
25	Sari	Orange	No	5(3)	C
26	Neka	Orange	No	5(3)	C
27	Behshahr	Brown	Yes	3(5)	A
28	Behshahr	Orange	Yes	3(5)	B
29	Behshahr	Orange	No	3(4)	C
30	Behshahr	Brown	Yes	3(4)	A
31	Bandar Gaz	Orange	No	3(5)	C
32	Bandar Gaz	Brown	Yes	3(5)	A
33	Bandar Gaz	Beige	yes	3(4)	D

(*): sometimes

The polymorphic loci in each morphotype ranged from 52.27% to 97.73% (Table. 3). Morphotype C showed the highest percentage of polymorphic loci among all morphotypes while morphotype D exhibited the lowest amount of polymorphism. The estimates of gene diversity (h) for all loci in each of the morphotypes ranged from 0.261 to 0.391. Also, the values of Shannon's information index (I) were similar to Nei's gene diversity index for all morphotypes, ranging from 0.362 to 0.559 among all morphotypes.

Polymorphic banding patterns were obtained in ERIC/BOX-PCR. PCR products were in the range of 300-3000 bp. Totally 156 molecular markers were evaluated to calculate the similarity among isolates. Sixteen groups were obtained with the 33 isolates with 60% genetic similarity (Fig. 3). Two groups of isolates showed a relative genetic similarity of 100% by ERIC/BOX-PCR, were placed in the same groups by RAPD-PCR. No correlation was found between the origin and clustering patterns of isolates. Most isolates from the same site showed different culture characteristics and were clustered in different groups.For example, isolates 1, 18 and 30 were grouped in the same cluster, but were collected from different locations.

DISCUSSION

Thirty-three *Fusarium semitectum* isolates were obtained from different regions and showed considerable morphological variation. Study of five *Fusarium* species with regard to macroscopic and microscopic characteristics showed that there is a large morphological variation of isolates within one species (Onyike 1991). In case of *F. equiseti*, considerable overlapping in the morphology of strains was observed. The isolates varied in color, aerial mycelium, shape of macroconidia and number of septa in macroconidia. This morphological variation meant that some morphological characteristics were unreliable for identification of the species. Burgess et al. (1989) observed morphological changes due to the effects of different environmental conditions and considered constant environmental conditions necessary for taxonomic studies in *Fusarium*.

Table 3. Genetic diversity parameters evaluated within 3 populations of *Fusarium semitectum.*

Morphotypes	Sample size	Polymorphic loci, %	h	I	ne
A	9	88.64	0.316±0.150	0.475±0.205	1.527±0.304
B	13	95.45	0.384±0.137	0.559±0.175	1.689±0.306
C	9	97.73	0.391±0.116	0.572±0.143	1.694±0.274
D	2	52.27	0.261±0.252	0.362±0.35	1.522±0.50

N: number of isolates, h: Nei's (1973) gene diversity, Shannon's Information index (Lewontin 1972), ne: effective number of alleles

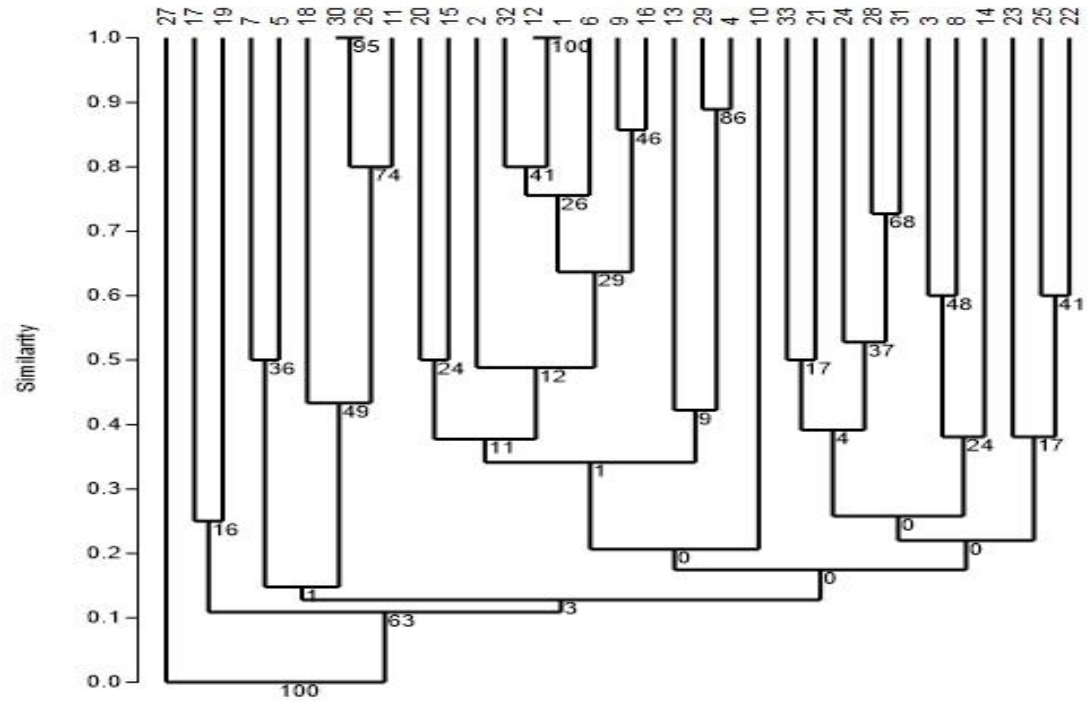


Fig. 3. UPGMA dendrogram showing relationship among 33 isolates of *Fusarium semitectum* based on analysis of primer pairs ERIC/BOX.

Two varieties of *F. semitectum*, *F. semitectum* var. *semitectum* and *F. semitectum* var. *majus* are illustrated by Gerlach & Nirenberg (1982). *F. semitectum* var. *semitectum* produced 1-5 septate while var. *majus* produced 1-7 septate macroconidia (Booth 1971, Gerlach & Nirenberg 1982). The study compared the classification generated from morphological and molecular data. Comparison of the two datasets indicated that in some cases, the grouping of isolates based on morphological method were consistent with molecular groupings with a few exceptions. In this study some isolates of morphotype A (1, 6, 9, 12, 15, 20 and 32) were placed in the same cluster of RAPD and ERIC/BOX analysis. Isolates of this group have brown aerial mycelium and 3(-5) septate macroconidia. This group could belong to *F. semitectum* var. *semitectum*. Molecular marker analysis can be useful in determination of relationships between genotypes and other population data such as morphology, pathogenicity and geographical locations (Mayek-Perez et al. 2001). Molecular analysis has shown that two isolates that were initially grouped in different morphotypes based on morphological characterization, appeared to be identical at the genetic levels when characterized with RAPD analysis. Isolates 1 (morphotype A) and 16 (morphotype C), showed differences in their morphological characteristics but were identical at the genetic level. The results indicated that high levels of intraspecific variability existed within *F. semitectum* isolates obtained from a single host (rice). The high level of intraspecific variation could be due to point mutations (Mishra et al. 2002). Given the fact that sexual reproduction in *F. semitectum* has not been reported either in nature or laboratory conditions so far, the large amount of genetic diversity observed in many studies, demands more attention. The known mechanism in *F. oxysporum* as parasexual recombination can clarify the issue to some extent (Molnar et al. 1990).

The estimates of population structure revealed significant level of genetic variability in each morphotype. The similar levels of gene diversity were detected in A, B and C morphotypes. The values of polymorphic loci and Shannon's information index indicated similar levels of genetic variability in A, B and C morphotypes. In contrast, low degree of gene and genotype diversity was detected in D morphotype. RAPD and ERIC/BOX produced different dissimilarity index in the present study. However, grouping of different isolates was consistent in these two methods of analysis. Genetic diversity in *F. semitectum* has been mainly investigated by using molecular markers. Assigbetse et al. (1994) differentiated races of *F. oxysporum* f. sp. *vasinfectum* on cotton by using RAPD as molecular tool. They reported a significant genetic variation in these isolates of *F. oxysporum*. Hawa et al. (2010) reported the genetic variation among different isolates of *F. semitectum* using different molecular markers such as RAPD-PCR and AFLPs. They observed that both the methods were convenient to determine the intra-species genetic variation in the isolates of the same species. The work carried out by Leslie et al. (2007) supports the findings of the present study. They observed inter- and intra-specific genetic variation in different *Fusarium* species. Our results showed that ERIC/BOX is also very convenient for production of isolate-specific fingerprinting and is suitable for phylogenetic analyses in *F. semitectum*. Origin of intra-specific variation in Rep-PCR is a result of different copy numbers of repetitive DNA in bacterial genomes. However, Wilson & Sharp (2006) reported that universal primers may not bind to intergenic conserved elements in bacteria which imply the indefinite nature of amplifications.

There was no correlation between molecular markers used and geographic origin of the isolates. This fact was also reported by Abd-Elsalam et al. (2003) and Ingel et al. (2011).

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تنوع ژنتیکی جدایه های *Fusarium semitectum* به دست آمده از برنج با استفاده از نشانگرهای RAPD و REP-PCR

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چکیده: به منظور تعیین تنوع ژنتیکی ۳۳ جدایه از گونه *Fusarium semitectum*، مطالعات مورفولوژیکی و مولکولی انجام گردید. در این مطالعه جدایه های جمع آوری شده از شالیزارهای مختلف شمال ایران (گیلان، مازندران و گلستان) در چهار گروه مجزا مورفولوژیکی قرار گرفتند. تنوع بالایی در میسلیوم هوایی، رنگ پرگنه، تعداد دیواره عرضی ماکروکونیدیوم و نرخ رشد جدایه ها روی محیط سیب زمینی آگار (PDA) مشاهده شد. برای بررسی میزان تنوع ژنتیکی بین جدایه ها از نشانگرهای مولکولی RAPD و REP-PCR استفاده شد. در دندروگرام ترسیم شده بر اساس تجزیه و تحلیل خوشه ای و روش UPGMA، قطعات DNA تکثیرشده ژنوتیپ ها در حد تشابه ۶۰ درصد به ترتیب در ۱۱ و ۱۶ گروه قرار گرفتند. مقایسه روش مورفولوژیکی با مولکولی نشان داد که در بعضی موارد، گروه بندی جدایه ها بر اساس صفات مورفولوژیکی با گروه بندی مولکولی (با اندکی استثناء) تقریبا یکسان می باشد. نتایج نشان داد که نشانگرهای مولکولی RAPD و REP-PCR می توانند جهت نشان دادن تنوع ژنتیکی درون گونه ای *F. semitectum* مورد استفاده قرار گیرند.

واژه های کلیدی: ریخت شناسی، انگشت نگاری DNA، تغییر پذیری، چند شکلی

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