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Analysis of the association between *Fusarium verticillioides* strains isolated from rice and corn in Iran bymolecular methods

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ABSTRACT

Fusarium verticillioides (teleomorph: *Gibberella moniliformis* = *G. fujikuroi* mating population A) is a common fungal pathogen of maize and rice crops worldwide. Fungal phytopathogens are cause of many plants diseases and much loss of crop yields, especially in tropical and subtropical regions [2]. All Isolates from infected corn samples were determined by specific primers but none of the isolates collected from infected rice were determined by specific primers (which were designed for corn isolates) as *F. verticillioides*. They were identified according to mating type (applying standard isolates). Results determined that 6 out of 14 rice isolates were characterized as *MAT1*, while the others were *MAT2*. Additionally, all the isolates were reconfirmed using morphological analysis. Rep-PCR was used to determine genetic diversity of 55 isolates of *F. verticillioides* from infected ears and stems of *Zea maize* and *Oryzae sativa* from different corn and rice producing areas of Iran. Reproducing genomic fingerprints was done by amplifying each strain according to PCRs of entero bacterial repetitive intergenic consensus (ERIC) and BOX sequences. Corresponding conserve repetitive element motifs in the genomes of diverse bacterial species were used to compare *F. verticillioides* isolates in rice and corn. In total, all 55 isolates were evaluated and comparisons were shown as a dendrogram produced by an UPGMA cluster analysis based on the Jaccard's similarity coefficient. Fifty-five isolates were divided into 43 groups determined as 3 groups with 3 individuals, 6 groups with 2 individuals and 34 groups form a single member in 65% similarity. Results suggested that *F. verticillioides* isolates from rice and corn are genetically different. It is possible that they belong to two *forma specials*. Cluster analysis shows that Rep-PCR is a convenient and rapid method for analysis of genetic diversity and strain differentiation in *F. verticillioides*. Universal primers theoretically anneal to intergenic target sites that are randomly dispersed in genomes and provide amplification of different length fragments.

Keyword: Rep-PCR, *Fusarium verticillioides*, genetic diversity

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INTRODUCTION

Fusarium verticillioides (Saccardo) Nirenberg (teleomorph: *Gibberella moniliformis* Wineland = *G. fujikuroi* mating

population A) is a major pathogen affecting of corn crops worldwide [15,16] and is the most frequently reported *Fusarium* species in corn [10]. Fusarium ear rot is the most destructive disease caused by *F. verticillioides*. The pathogen can cause stand reduction and poor kernel quality and poses a potential threat to human and animal health from mycotoxins such as moniliformin, fusarin C, fusaric acid and fumonisin produced by the fungus [3]. Standardized methods for documenting diversity and distribution have been lacking[17]. Repetitive DNA based fingerprinting methods are commonly known as Rep-PCR. This method relies on the amplification of genomic sequences between the repetitive elements conserved in prokaryotes including repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX elements [19]. Universal primers theoretically anneal to the intergenic target sites that are randomly dispersed in genomes and provide the amplification of different lengths of fragments. Rep –PCR amplifications are widespread in plant pathogenic bacteria and only tested in fungal strains of *Aspergillus fumigatus*[1], *Fusarium oxysporum*[4], Tilletia species [12], *Fusarium solani*[8]*and Fusarium culmorum*[6]. Presently, there is no frequent use of Rep-PCR in genotyping of plant pathogenic fungi. The objective of this research was to test Rep-PCR as a rapid and cost-effective method in the production of isolates associated with outbreaks of disease. A research showed that an *enterobacterial* repetitive intergenic consensus PCR (ERIC- PCR) and PCR restriction fragment length polymorphism (PCR-RFLP) were useful methods for genotyping *Fusarium* isolate [5].

MATERIALS AND METHODS

Isolation and storage of isolates

Fusariumverticillioides strains were obtained from the fungal collection of the laboratory for plant pathology, University of Tehran. These strains had been isolated from infected ears and stems of corn (*Zea mays* L.) from fields located in the main maize growing areas of Iran [18]. The other 14 isolates were isolated from *Oryzae sativa* obtained from provinces of Mazandaran and Gilan [7]; the two most important rice producing areas in Iran. Sampling sites of these accessions are shown in Table 1. Fungal cultures were transferred into the Saboroud broth (peptone – glucose 1:4) and incubated in a dark condition for 6-7 days at 24ºC. When they reached the satisfied growth state, mycelium was filtered through Whatman No.1 filter paper and turned to powder by liquid nitrogen. All of the monoconidial isolates were tested using specific species primers (that had been designed for corn isolates) for *F.verticillioide.* However, rice isolates did not show any interaction with the primers (as it was mentioned the primers were made for corn isolates). So, they were identified according to mating type test (applying standard isolates). The standard sexual crosses were carried out according to the method cited by [9].

Crossing and mating type

In this study, isolates were single-spored before using in sexual crosses. In addition, assay was repeated twice. Tester female strains were inoculated on carrot agar (CA) plates and the *Fusarium* isolates (male strains) were inoculated on complete medium (CA) and incubated at 25ºC for 7 days. After incubation, mycelia from male strains were suspended in 1.5 ml 0.25% Tween 60 solution and spread on the surface of a CA plate that was already colonized by a tester strain of mycelium. The fertilized plates were incubated at 27ºC and checked twice a week for 2 months to observe production of perithecia. *Fusarium* isolates were cultured as single conidia and then transferred to carnation leaf agar (CLA) and potato dextrose agar (PDA) plates. After that, plates were incubated with a 12/12 h photoperiod under cold white and black light fluorescent lamps [11].

Extraction genomic DNAs from fungal mycelium

Genomic DNAs were extracted and purified using the fast DNA kit (Pr881609, Cinna pure, Iran).

Quantification of extracted DNAs

The resulting DNA was quantified with the Nanodrop set.

Polymerase chain reaction (PCR) to identified F.verticillioides isolates

Each strain was identified by species specific primers [14]. VER1 (5- CTTCCTGCGATGTTTCTCC-3) VER2 (5-AAT TGG CCA TTG GTA TTA TAT ATC TA-3).

PCR condition

VER - PCR was performed in a volume of 50 µl containing 20mM Tris - HCL (PH 8), 50mM Kcl, 2mM MgCl2, 200µM concentration of the VER primers VER-1 and VER-2, 10 ng of genomic DNA, and 2.5U of Taq polymerase. The following PCR condition were used with a model 2400 thermo cycler (Perkin-Elmer, Norwalk, conn).The PCR

parameters were: 35 cycles of denaturation at 94ºC for 1min, 1min at 56ºC, annealing at 72ºC for 1 min and a final extension at 72ºC for 7 min.

ERIC-PCR and BOX-PCR

ERIC-1 and ERIC-2 primers have been described [19]. Fingerprints were assigned to each different type in any band that was observed. The protocol recommended by the manufacturer, was followed using the alternative protocol for maximum yield. Rep-PCR primers were ERIC 1[12](5- ATG TAA GCT CCT GGG GAT TCA C -3), ERIC 2(5- AAG TAA GTG ACT GGG GTG AGC G) and BOX-AIR (Mc Donald *et al*., 2000) (5- CTA CGG CAA GGC GAC GCT GAC G -3) for BOX-PCR.

PCR condition

ERIC-PCR was performed in a volume of 50 µl containing 20mM Tris - HCL (PH 8), 50mM Kcl, 2mM $MgCl_2$, 200µM concentration of the ERIC primers ERIC-1 and ERIC-2, 10 ng of genomic DNA, and 2.5U of *Taq* polymerase. The following PCR condition were used with a model 2400 thermo cycler (Perkin-Elmer, Norwalk, conn).The PCR parameters were: 40 cycles of denaturation at 94ºC for 1min, 1min at 36ºC, annealing at 72ºC for 2 min and a final extension at 72ºC for 15 min. BOX- PCR mix was contained 100 ng genomic DNA, 0.2 mm of each dNTP, 50 pmol of each primers, 1U of Taq DNA polymerase (Takara), 2.5 ll of 10 Ex-Taq Buffer free Mg^{2+} and 3 mm $MgCl_2$ in 25 ml of total volume. The following PCR conditions were used with a model 2400 thermocycler (Perkin-Elmer, Norwalk, conn). 30 cycles of denaturation at 94ºC for 1 min, 1min at 52ºC, annealing at 65ºC for 8 min and a final extension at 65ºC for 16 min. Amplified products were resolved by electrophoresis in tris-borate - EDTA buffer with a 10% (wt/vol) polyacrylamide gel electrophoresis (PAGE), stained with ethidium bromide for 30 min and photographed under UV light using the gel documentation system (Bio Rad, USA). An amplisize molecular ruler 50- to 2,000-bp ladder (Bio-Rad, Barcelona, Spain) was loaded twice in each gel. ERIC and BOX analyses were repeated once and only reproducible bands were scored.

Band scoring and data analysis

ERIC-PCR and BOX-PCR bands were scored as present (1) or absent (0). Data analysis was performed by an UPGMA cluster analysis based on the Jaccard's similarity coefficient. Reactions were carried out three times to confirm reproducibility.

RESULTS

All 55 *F. verticillioides* isolates obtained from corn and rice were reconfirmed with specific species primers (showing a 0.58 kb monomorphic band) and a mating type test. In the present study, 12 isolates of *F. verticillioides* isolated from rice were cross-fertilized with tester strains of the mating population A (*G. moniliformis*). Results showed that 12 out of 14 isolates were cross-fertile with tester strains of the mating population A. Results also determined that 6 out of 12 rice isolates were characterized as *MAT1*, while the others were *MAT2*. Corresponding conserve repetitive element motifs in the genomes of diverse bacterial species were used to compare *F. verticillioides* isolates in rice and corn. In total all 55 isolates were evaluated and compared in a dendrogram produced by an UPGMA cluster analysis based on the Jaccard's similarity coefficient. Cluster analysis showed that the isolates were divided into two groups with 70% similarity based on their host. Both monomorphic and polymorphic banding patterns were obtained in Rep-PCRs. The 55 types comprised 43 different groups. The fingerprints obtained from ERIC-PCR comprised 3 to 12 amplification bands of rice isolates and 3-8 bands of corn isolates ranging in size from 0.2 to 3 kb and 0.2 to 1.5 kb respectively (Fig. 2.b, c). Forty-one groups were obtained with 55 isolates (Table 1). Likewise, the fingerprint obtained with BOX-PCR comprised, 3-8 bands of rice isolates and 3-12 bands of corn isolates ranging in size from 0.2 to 1.2 kb and 0.35 to 3 kb respectively (Fig.1.d, e). These results confirmed the expected genetic differences between *F. verticillioides* obtained from rice and corn isolates. Results showed that rice isolates are totally separate from corn isolates as they remained on top of the dendrogram. The 55 isolates comprising of 43 different profiles had approximately 65% similarity. The rice and corn fungal isolates included 10 and 33 genetic groups respectively. As mentioned above, these 55 isolates were divided into 43 groups with 3 individuals, six groups that included 2 individuals and 34 isolates that formed a single member with 65% similarity. In general, the rice fungal isolates demonstrated genetic diversity compared with the corn isolates. Additionally, the standard isolate obtained from South Africa was totally different from the other isolates.

Table 1. Origin and genetic characteristics of *Fusarium verticilllioides* species from rice and corn in Iran

Isolate	*Origin*	*Host*	*Response to VER1/2*	*Mating type*
Z1	*Semnan*	*Zea mays*	+	-
Z2	*Semnan*	*Zea mays*	+	-
Z3	*Semnan*	*Zea mays*	+	-
Z4	*Semnan*	*Zea mays*	+	-
Z5	*Semnan*	*Zea mays*	+	-
Z6	*Golestan*	*Zea mays*	+	-
Z7	*Golestan*	*Zea mays*	+	-
Z8	*Golestan*	*Zea mays*	+	-
Z9	*Golestan*	*Zea mays*	+	-
Z10	*Golestan*	*Zea mays*	+	-
Z11	*Golestan*	*Zea mays*	+	-
Z12	*Tehran*	*Zea mays*	+	-
Z13	*Tehran*	*Zea mays*	+	-
Z14	*Tehran*	*Zea mays*	+	-
Z15	*Tehran*	*Zea mays*	+	-
Z16	*Tehran*	*Zea mays*	+	-
Z17	*Tehran*	*Zea mays*	+	-
Z18	*Tehran*	*Zea mays*	+	-
Z19	*Tehran*	*Zea mays*	+	-
Z20	*Khouzestan*	*Zea mays*	+	-
Z21	*Khouzestan*	*Zea mays*	+	-
Z22	*Khouzestan*	*Zea mays*	+	-
Z23	*Khouzestan*	*Zea mays*	+	-
Z24	*Khouzestan*	*Zea mays*	+	-
Z25	*Khouzestan*	*Zea mays*	+	-
Z26	*Khouzestan*	*Zea mays*	+	-
Z27	*Kermanshah*	*Zea mays*	+	-
Z28	*Kermanshah*	*Zea mays*	+	-
Z29	*Kermanshah*	*Zea mays*	+	-
Z30	*Kermanshah*	*Zea mays*	+	-
Z31	*Kermanshah*	*Zea mays*	+	-
Z32	*Isfahan*	*Zea mays*	+	-
Z33	*Isfahan*	*Zea mays*	+	-
Z34	*Isfahan*	*Zea mays*	+	-
Z35	*Isfahan*	*Zea mays*	+	-
Z36	*Isfahan*	*Zea mays*	+	-
Z37	*Isfahan*	*Zea mays*	+	-
Z38	*Mazandaran*	*Zea mays*	+	-
Z39	*Fars*	*Zea mays*	+	-
Z40	*Fars*	*Zea mays*	+	-
Z41	*South Africa*	*Zea mays*	+	-
O1	*Mazandaran*	*Oryzae sativa*	-	*MAT2*
O2	*Mazandaran*	*Oryzae sativa*	-	-
O3	*Mazandaran*	*Oryzae sativa*	-	*MAT2*
O4	*Mazandaran*	*Oryzae sativa*	-	-
O5	*Mazandaran*	*Oryzae sativa*	-	*MAT1*
O6	*Gilan*	*Oryzae sativa*	-	*MAT1*
O7	*Gilan*	*Oryzae sativa*	-	*MAT2*
O8	*Gilan*	*Oryzae sativa*	-	*MAT1*
O9	*Gilan*	*Oryzae sativa*	-	*MAT2*
O10	*Gilan*	*Oryzae sativa*	-	*MAT1*
O11	*Gilan*	*Oryzae sativa*	-	*MAT2*
O12	*Gilan*	*Oryzae sativa*	-	*MAT1*
O13	*Gilan*	*Oryzae sativa*	-	*MAT1*
O14	*Gilan*	*Oryzae sativa*	-	*MAT1*

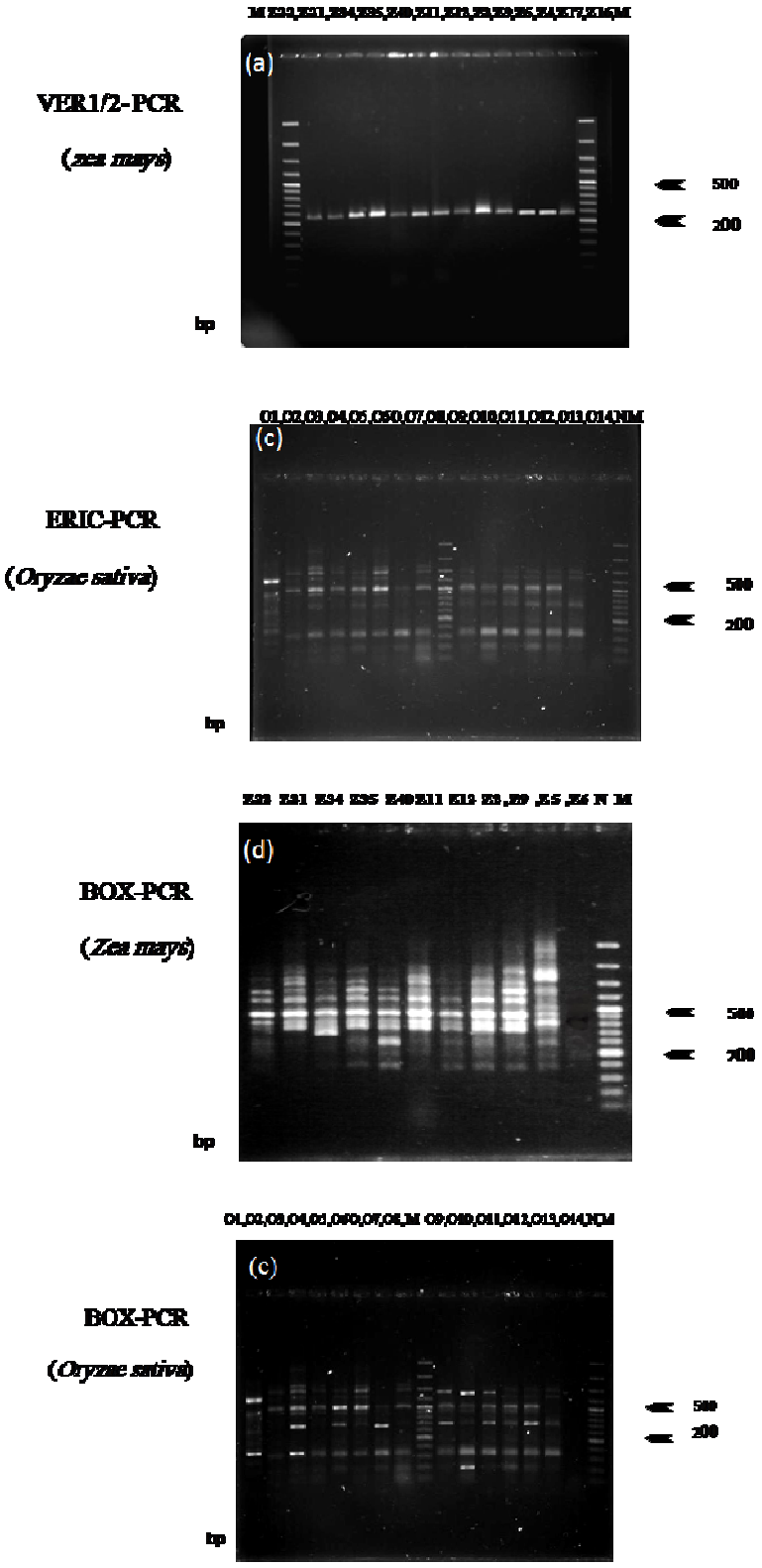


Fig. 1. (a) Specific species – PCR (ver1/2).(b,c) enterobacterial repetitive intergenic consensus (ERIC).(d,e) box- PCR fingerprinting pattern of *F. verticillioides*. M: 1Kb DNA ladder (fermentase), N:(negative control).

Figure 2. Dendrogram was generated using weighted pair group method with arithmetical averages(UPGMA) and jaccard`s coefficient of *F. verticillioides* isolates.

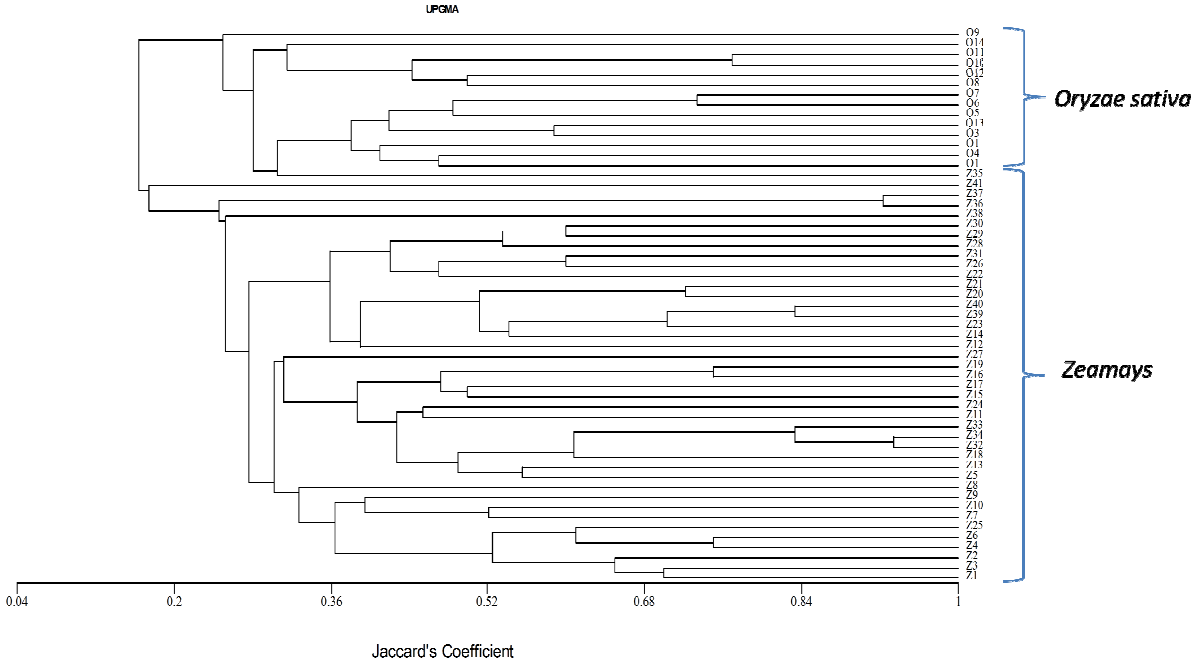
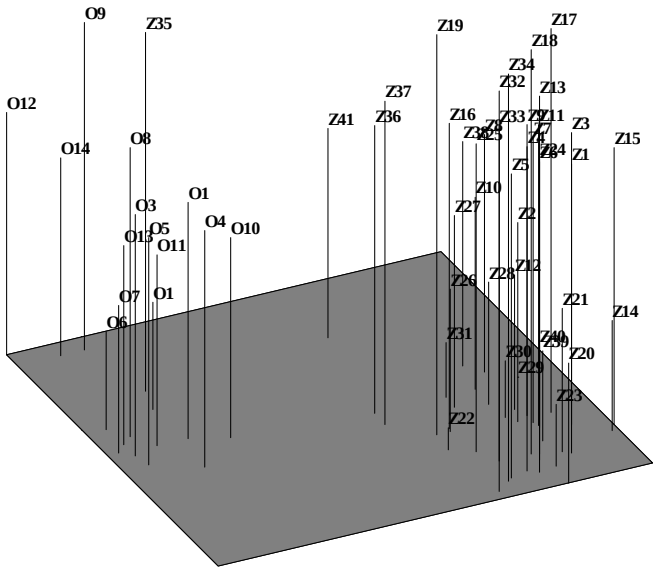


Fig. 3.three dimentional plots, showing the genetic similarity derived from enterobacteria repetitive intergenic consensus ERIC) and BOX-PCR profiles of *F. verticillioides* isolates.



DISCUSSION

This study shows that the host is an important factor in terms of dividing isolates into two different groups. This study evaluated two populations of *F. verticillioides* from two different hosts for genetic variation as assessed with Rep-PCR. Genetic diversity was found to be high (65 %) which could be maintained if sexual recombination is occurring within these populations. Such results as these are key to determining the epidemiology of this pathogen. No difference was observed between environmental and corneal isolate of *F. Verticillioides*. This study demonstrated a high diversity of *F. verticillioides* that caused Fusarium ear rot with a few exceptions; only those isolates collected from the same locations shared the same genotype. ERIC-PCR and BOX-PCR techniques are commonly used for determining types of bacteria and are occasionally used for fungi, they were found to be more discriminatory than other methods. This method was also fast and easy to perform. Combining the results of these two techniques shows that the isolates of infected rice had a different genotype and results also confirmed that all of the isolates from same location yielded from a specific genotype. These results show that Rep-PCR is also a very convenient method for the identification of isolate-specific fingerprints and suitable for phylogenetic analysis in *F. verticillioides*. The origin of intraspecific variation in Rep-PCR is a result of different copy numbers repetitive DNA in bacterial genomes. However, a research was reported that universal primers may not bind to intergenic conserved elements in bacteria [20] , this implies the indefinite of amplification variety size of a fungal fingerprint could be obtained in the case of using only 52ºC annealing temperature. In this work, it is indicated that the random process of priming in terms of fingerprints was reproducible in terms of molecular markers. It is necessary to characterize intergenic repetitive sequences in Fusarium genomes to explain the nature of fungal Rep-PCR. The high diversity between strains could be associated with mutation in primering sites, re-arrangement of chromosal segments or of a recombination process in fungal genomes [13]. This method is convenient and rapid for strain identification and genetic diversity analyses in *F. verticillioides* populations that are widespread in corn and rice breeding areas.

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