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Molecular Variability of *Fusarium verticillioides* (Sacc.) in Maize from Three Agro-Ecological Zones of Southwest Nigeria

Olumayowa Mary Olowe, Adeboyega Christopher Odebode, Odunayo Joseph Olawuyi, Ayodele Adegboyega Sobowale

Department of Botany, University of Ibadan, Ibadan, Nigeria
Email: mayolowe@yahoo.com

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Abstract

This study investigated the characterisation of *Fusarium verticillioides* strains using Amplified Fragments Length Polymorphism (AFLP) EcoR1-G/ Mse1-C primer combination. This was done to determine the amount of genetic variability present in *F. verticillioides* population. The objective of this study is to carry out molecular characterization of *F. verticillioides* strains from infected ear of maize. Six isolates were obtained in maize ear from 10 locations in three agro-ecological zones of Southwest Nigeria. Polymorphic bands detected were 164 from one base extension of EcoR1 and Mse1 primers in the selective amplification. Primer combination EC + MC produced the highest polymorphic bands of 58, while primer combination ET + MC had the highest percentage polymorphism (100%). Genetic similarity (67%) was observed among strains from Ibadan (IBD) (34, 36), Igbooho (IGH) (39), Ado-Ekiti (EKT) (46, 47), Saki (SAK) (52) and Igboora (IGB) (57, 58, 59 and 60). Strains 19 and 20 from Ilesha (ILH) had the highest pairwise similarity coefficient at 97% grouped in cluster II. The dendrogram delineated *F. verticillioides* strains into four major cluster groups with 77% similarity and other sub-groups within. *Fusarium verticillioides* strains could therefore be heterogeneous, and thus amplified fragment length polymorphism is an ideal tool for distinguishing the pathogenic variants of *Fusarium verticillioides*.

Keywords

Fusarium verticillioides, Amplified Fragment Length Polymorphism, Southwest Nigeria, Maize

1. Introduction

Maize is one of the staple foods for most of the population especially in areas adaptable

for their production [1]. It is a major cereal crop for livestock feed and human nutrition [2] [3] [4] [5]. Though, its production in Nigeria is constrained by biotic factors such as pests and diseases [2].

Fusarium verticillioides (Sacc.) is a maize pathogen causing root, stalk and ear rots in Nigeria [6] [7]. It is found in plant residue in almost every maize field at harvest which its symptoms vary widely and range from asymptomatic plants to severe rotting of all plant parts [8]. Symptomless infection exists in maize leaves, stems, roots and grains whereby its presence is ignored in many cases because it does not cause visible damage to the plant [9]. This suggests that some strains of *F. verticillioides* produce disease in maize while others do not [10].

The knowledge of variability in pathogen is required by host resistance to develop effective disease management strategies [11]. Conventional markers used to study the variability in pathogens are based on the use of differential hosts, culture characteristics, morphological markers and biochemical tests [12] [13]. These markers distinguished pathogens on the basis of the physiological characters such as pathogenicity and growth behaviours which are highly influenced by the host age, inoculum quality and environmental condition. The use of molecular markers is therefore necessary.

The genetic characterisation of *Fusarium* species using several molecular techniques has been adopted; they are RAPD (Random Amplified Polymorphic DNA), PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism) [14]. The application of AFLP in DNA fingerprinting of microbes particularly fungi had been reported [15] [16]. This technique provides a high degree of flexibility due to different restriction enzymes, the nature and number of selective nucleotides that can be manipulated to limit amplification fragments generated in order to account for differences in genome size.

This study aimed to assess the genetic relatedness of *F. verticillioides* from maize in three agro-ecological zones of Southwest Nigeria.

2. Materials and Methods

2.1. Collection of Diseased Maize Samples

Six maize ear showing rot symptoms were randomly obtained from Derived Savanna (Ilora, Ijebu-Ode, Igboora and Eruwa), Rainforest (Ado-Ekiti, Akure, Ilesa and Ibadan) and Guinea Savanna (Saki, Igboho) of Southwest Nigeria between May to July, 2013.

2.2. Isolation of *Fusarium verticillioides*

Some infected and healthy grains obtained from the sampled maize ear were washed under running water, surface sterilized for one minute in 10% sodium hypochlorite solution, and then rinsed in three changes of sterile water, followed by drying with sterile paper towel. These were then placed on solidified potato dextrose agar in petri-dishes and incubated at room temperature (28˚C ± 2˚C) for 7 days. Observation was made daily on mycelial growth. Pure cultures were obtained by subculturing onto fresh plates.

2.3. Morphological Characteristic of *Fusarium verticillioides*

Morphological identification according to [17] and [18] was carried out at the pathology unit of International Institute of Tropical Agriculture (IITA) Ibadan. Morphological identification of *F. verticillioides* involved observable characters on the cultured plates (rate of growth, pigment production, presence or absence of mycelium and mycelium colour), while the microscopic identification involved distinctive structures and characters (phialides, chlamydospores, microconidia and macroconidia).

2.4. Extraction of Genomic DNA of *Fusarium verticillioides*

Genomic DNA was extracted using a DNeasy tissue kit according to the manufacturer's instruction. DNA was eluted in 200 µl of elution buffer (buffer AE of the kit) and stored at −20˚C. DNA yield were measured using Nanodrop spectrophotometer, while the quality was assessed by agarose gel electrophoresis, restriction enzyme digestion and AFLP analyses using the procedure described by [19].

2.5. Restriction and Ligation of Adapters

Two restriction endonucleases, EcoRI and Mse1 were used to digest 100 ng of genomic DNA in 10 µL reaction, which was incubated for 37˚C for 3 hours and inactivated at 70˚C for 15 minutes. Digested and undigested DNA were separated on 1% agarose gels in TBE buffer. The ligation reaction volume of 10 µl was prepared to contain of 5 U of T4 DNA ligase, ligase buffer 100× BSA, 5 µM EcoRI (Forward and reverse) and 50 µM MseI (forward and reverse). After combining 10 µL of ligation mix with the digested DNA fragment, ligation was allowed to proceed for 5 hours at 37˚C. The adapter-ligated DNA (20 µL) was diluted 1:5 with autoclaved water to serve as template in the pre-selective PCR and was stored at −20˚C.

2.6. Pre Selective and Selective PCRs

The description of the adapters for pre-amplification and selective amplification primer sequence used in this study is shown in **Table 1**. Pre-selective amplification of the dilute template DNA was carried out with AFLP primer combinations (5 µM Pre EcoRI and 50 µM Pre MseI) without selective nucleotides and twenty microliter PCR reactions containing 8.0 µl of dilute template DNA, 2.0 µl of PreEcoRI (5 UM), 0.2 µl of PreMseI (5 UM) primer, 5 µ/Ul of Taq DNA polymerase, 2 µl of 10× Taq DNA polymerase buffer, 0.6 µl of 10 mM DNTPs and 0.6 µl of 50 mM of Mg_2 were performed. Pre-amplification reactions were performed for 20 cycles of: 30 s at 94˚C, 30 s at 94˚C, 56˚C at 30 s and 1 min at 72˚C. Following pre-amplification, the reactions were diluted 10 folds with autoclaved water and used as template for selective amplification. Selective amplification were performed using three (3) selected combination of primers with one selective nucleotides (EcoRI + T, EcoRI + G, EcoRI + C) and one MseI primers (MseI + C). The reactions were carried out in volumes of 20 µl of PCR buffer containing 6 µl of dilute preamplified template DNA, 0.2 µl of 10 ng EcoRI, 0.7 µl of 10 ng MseI, 0.3 µl of 10 mM dntps, 2 µl of reaction buffer and 0.3 µl of 50 nM. The program ran for 11 cycles at 94˚C for 30 s, 94˚C for 30 s, 72˚C for 60 s, 94˚C for 30 s, 56˚C for 30 s and 72˚C for 60 s. 7 µl of formaldehyde dye was added to 20 µl of each sample and the samples were

Table 1. Description of the primers used for amplified fragment length polymorphism.

Primers and their functions	Sequences (5'-3')
EcoRI-Adapters 1 and 2	
E A 1.1	CTCGTAGACTGCGTACC
E A 1.2	ATTGGTACGCAGTCTAC
MseI-Adapters 1 and 2	
M A 1.1	GACGATGAGTCCTGAG
M A 1.2	TACTCAGGACTCAT
Preamplification	
Pre EcoRI (+0)	GTAGACTGCGTACCAATTC
Pre MseI (+C)	ACGATGAGTCCTGAGTAAC
Selective amplification	
EcoRI +C	AGACTGCGTACCAATTCC
EcoRI +T	AGACTGCGTACCAATTCT
EcoRI +G	AGACTGCGTACCAATTCG
MseI +C	GATGAGTCCTGAGTAAC

Source: [16].

denatured for 5 minutes at 95˚C. Each sample was loaded on a 6% polyacrylamide gel containing 400 µl ammonium persulphate (APS). Gels were cast using long and short plates with 0.25 mm thick spacers and comb. The long plates were treated with sigma-cote, while the short plates were treated with a binding solution. Electrophoresis was performed at a constant power 60 W and a constant temperature of 47.5˚C for 3 h. Amplicons were revealed with silver nitrate stain.

2.7. Data Analysis

For each primer combination, all bands were manually recorded for presence (1) or absence (0) of each band in AFLP analysis. Bands of different electrophoretic mobility were assumed to be non-allelic, while bands of the same mobility were assumed to be allelic. A dendrogram was constructed from the similarity matrix using unweighted pair group method with arithmetic average (UPGMA) described by Sneath and Sokal (1973). Cluster analysis was performed using NTSYSpc (NTSYS-For Numerical Taxonomy System) version 2.1 [20].

3. Results

A total number of 164 bands were produced from three primer combinations out of which 135 were polymorphic, and correspond to an average of 45 polymorphic bands per primer combination (**Table 2**). Most of the bands were polymorphic, while few were monomorphic. The maximum number of bands generated by EC + MC combination was 61 bands while the minimum obtained with EG + MC primer combination was 50 bands (**Figure 1**). The polymorphic information content (PIC) for each marker varied between 0.7734 and 0.8249, EG + MC and ET + MC primer combinations had the maximum with the same PIC values, while ET + MC primer combination had the lowest PIC value (**Table 2**).

The *Fusarium verticillioides* strains were grouped into five (5) clusters from three (3) Agro-ecological zones. The cluster three (3) had the highest number of *F. verticillioides* strains from Ilora (2, 3, 4, 5, 6), Ilesha (21), Akure (26, 27, 29), Ibadan (31, 32), Igbooho

Table 2. Characterisation of the Amplified Fragment Length Polymorphism (AFLP) primers based on Polymorphic Information Content (PIC).

Primer combination	NPB	TNB	PPB	PIC	AN
EG + MC	40	50	80.0	0.8249	59
ET + MC	45	53	84.9	0.7734	58
EC+MC	50	61	82.0	0.8249	59
Total	135	164	246.9	2.4232	176
Mean	45	54.7	82.3	0.8077	58.7

NPB: Number of Polymorphic Bands, TNB: Total Number of Bands, PPB: Percentage Polymorphic Bands, AN: Allele Number.

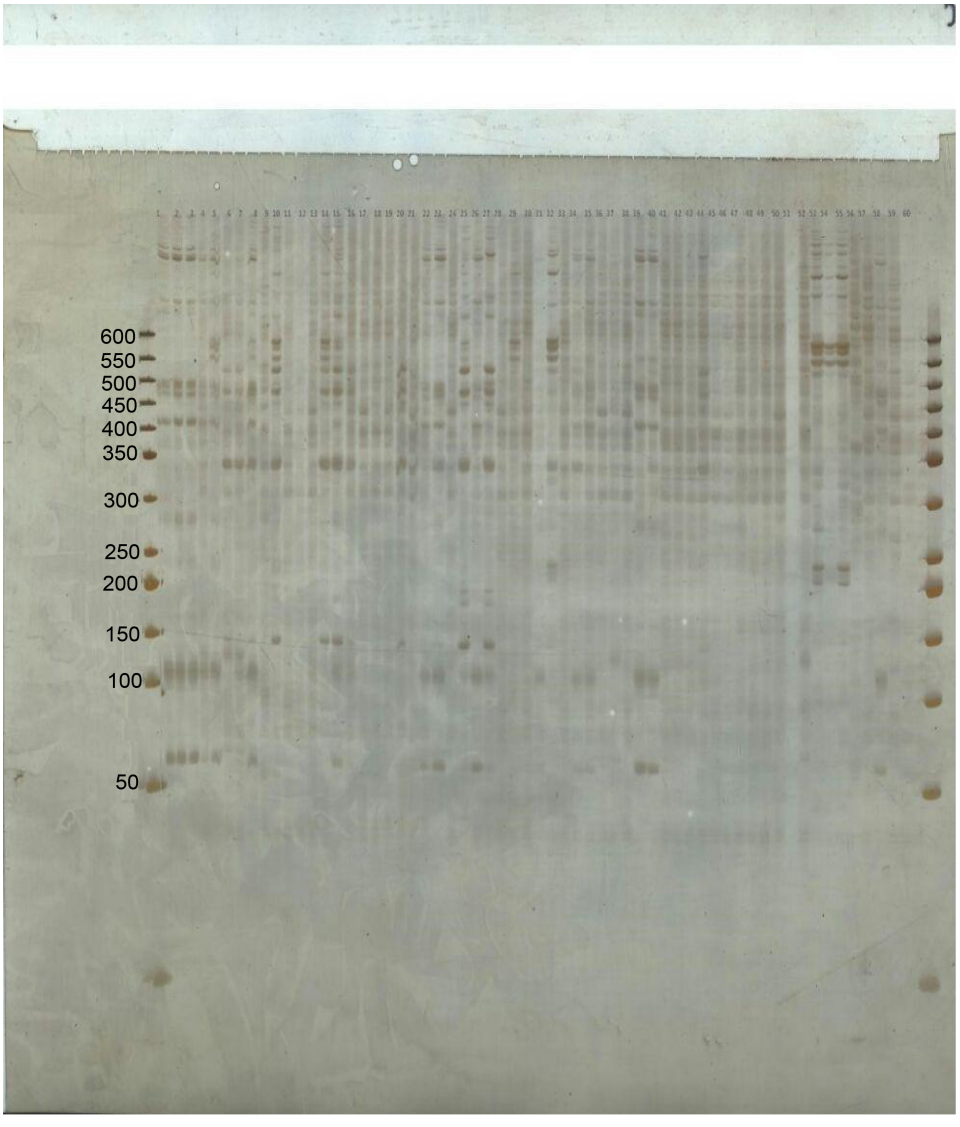

Figure 1. Amplified Fragment Length Polymorphism (AFLP) Products from *Fusarium verticillioides* strains using EcoRI-G/MseI-C Primer Combination.

(38, 41, 42), Ado-Ekiti (43, 44, 45) and Saki (53), while cluster I recorded the least number of *F. verticillioides* strains (**Table 3**).

The genetic relationships of *F. verticillioides* strains were also represented in the dendrogram (Figure 2). The coefficient of similarity ranged from 0.67 to 0.97. There were five main clusters constituting all samples with similarity coefficient of 0.72. Group 1 had two subgroups consisting of six strains; 1 (ILR), 10 (ERW), 49 (SAK), 25 (AKR), 33 (IBD) and 24 (ILH). 24 (ILH) had the highest genetic distance within the group. The group II in the dendrogram had 2 sub-groups of 10 *F. verticillioides* strains of which the highest occurrence were from Eruwa (ERW), though, strains 17 (IJB) was the most distanced within the group. Group I and II had coefficient similarity at 0.87, while Group III with 5 sub-groups had the highest number of strains. Strains 2 and 3 (ERW) formed coefficient similarity with strains 4 and 5 (ERW) at 0.876, while strains 6 (ERW) and 29 (AKR) were the most distanced within the group. The fourth group had 5 subgroups containing 16 isolates in which strain 54 from SAK was the most distanced within the group. Strains from IBD (34, 36), IGH (39), EKT (46, 47), SAK (52) and IGB (57, 58, 59, and 60) were distinctly separated from other strains at 0.67 similarities. The closest strains are 19 and 20 in cluster II from ILH, with the highest pairwise similarity coefficient of 0.97 (Figure 2).

4. Discussion

Understanding the diversity of pathogens is necessary to predict the control measures, which include; the use of resistant cultivar, fungicides application or the use of biological agents [21]-[26]. The detected 164 AFLP bands in which 135 were polymorphic (82% polymorphic rate) indicates high genetic diversity in accordance with the findings of [27] and [28] who reported genetic similarity between and among the isolates. Genetic similarity between the isolates which ranged from 0.67 to 0.97 for similar coefficient index is an indication of genetic variation as similarly reported by [29].

It was shown that polymorphism was present in the PCR products from the *F. verticillioides* strains with the AFLP primers used in characterization. There was no association between AFLP patterns and agro-ecological origin of the tested strains, again, the

Table 3. Cluster groups of *Fusarium verticillioides* strains from the three agro-ecological zones.

Cluster group	Total number of strains	Strain codes
I	6	1, 10, 49, 25, 33, 24
II	10	11, 12, 13, 14, 15, 16, 18, 19, 20, 17
III	18	2, 3, 4, 5, 21, 26, 27, 31, 32, 41, 42, 43, 44, 45, 38, 53, 6, 29
IV	16	37, 40, 48, 22, 23, 28, 30, 35, 50, 51, 55, 56, 7, 8, 9, 54
V	10	34 36, 39 46, 47, 52 57, 58, 59, 60

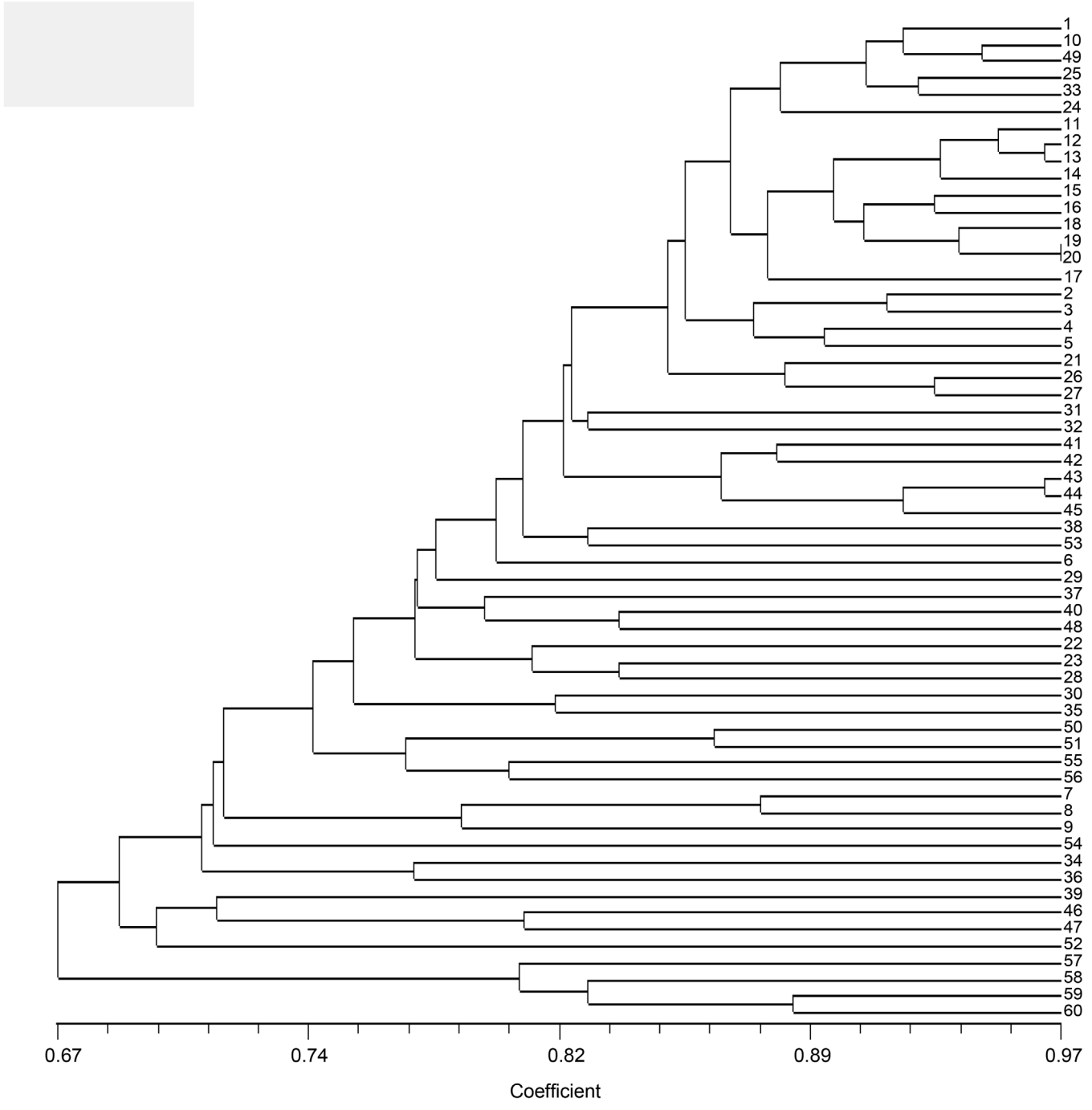

Figure 2. Dendrogram showing *Fusarium verticillioides* strains using three (3) AFLP Primer Combination. Samples: 1-6 (Eruwa), 7-12 (Akure), 13-18 (Ibadan), 19-24 (Ilesa), 25-30 (Igbooho), 31-36 (Saki), 37-42 (Ekiti), 43-48 (Igboora), 49-54 (Ijebu), 55-60 (Ilora).

biology and epidemiology of this pathogen may provide some insight as to during favourable condition. *F. verticillioides* causes many diseases such as root, stalk and ear rots, which is responsible for the decrease in nutritive value, loss in germination, discoloration, increase in fatty acid and secretion of mycotoxins which results in losses of maize production [30] [31] [32] [33] [34].

The dendrogram based on the genetic similarity coefficient established the interrelationship among the isolates from different locations in the three agroecological zones.

The clustering pattern of the isolates, irrespective of the source implies that the genetic distance is not correlated with the agro-ecological distance as similarly reported by [35]. The genetic similarity obtained through AFLP analysis showed level of genetic variation among the strains. The strains do not depicts close relatedness with each other, and strains from different location showed a common clad which supports that migration event are common in the population of *F. verticillioides* as similarly observation was reported by [36].

Random mating population displays a high degree of genotypic diversity due to difficulties in the management of disease as a result of constant appearance of new strains which increase the variability features like fungicides resistance, higher aggressiveness and better fitness in the population [28]. Other factors that could be responsible for increase in genetic diversity are mutation, translocation, chromosomal deletion are common in fungi as well as difference in climatic condition where the isolates were collected [28] [37].

5. Conclusion

In conclusion, molecular characterisation of *Fusarium verticillioides* was investigated to give the genetic variability. Amplified fragment length polymorphism markers should be encouraged in studying the genetic variation in natural population of *F. verticillioides* which can help pathologists to detect the pathogenic potential of this pathogen as well as helping to develop an effective control strategy.

Conflict of Interests

There is no any kind of conflict of interests with any trademark mentioned in this paper, competitive interest, or secondary interest that could have influenced the research. This declaration is carried out by all the authors of the work presented.

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