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ORIGINAL ARTICLE

Genetic diversity study of *Fusarium culmorum*: causal agent of wheat crown rot in Iraq

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Abstract

Fusarium crown rot (FCR), caused by *Fusarium culmorum* (Wm.G.Sm) Sacc., is an important disease of wheat both in Iraq and other regions of wheat production worldwide. Changes in environmental conditions and cultural practices such as crop rotation generate stress on pathogen populations leading to the evolution of new strains that can tolerate more stressful environments. This study aimed to investigate the genetic diversity among isolates of *F. culmorum* in Iraq. Twenty-nine samples were collected from different regions of wheat cultivation in Iraq to investigate the pathogenicity and genetic diversity of *F. culmorum* using the repetitive extragenic palindromic (REP-PCR) technique. Among the 29 isolates of *F. culmorum* examined for pathogenicity, 96% were pathogenic to wheat at the seedling stage. The most aggressive isolate, from Baghdad, was IF 0021 at 0.890 on the FCR severity index. Three primer sets were used to assess the genotypic diversity via REP, ERIC and BOX elements. The amplicon sizes ranged from 200–800 bp for BOX-ERIC2, 110–1100 bp for ERIC-ERIC2 and 200–1300 bp for REP. A total of 410 markers were polymorphic, including 106 for BOX, 175 for ERIC and 129 for the REP. Genetic similarity was calculated by comparing markers according to minimum variance (Squared Euclidean). Clustering analysis generated two major groups, group 1 with two subgroups 1a and 1b with 5 and 12 isolates, respectively, and group 2 with two subgroups 2a and 2b with 3 and 9 isolates, respectively. This is the first study in this field that has been reported in Iraq.

Keywords: genetic diversity, *Fusarium*, pathogenicity, repetitive extragenic palindromic technique (REP-PCR), *Triticum aestivum*

Inroduction

Fusarium culmorum (Wm.G.Sm) Sacc., a fungal plant pathogen with a wide host range, is the causal agent of several diseases on the plants. On wheat, *F. culmorum* causes two important diseases that can cause serious economic losses: head blight and crown rot (Burgess *et al*. 2001; Chakraborty *et al*. 2006; Khalifah and Oadi 2014). Reliable estimates for yield loss due to Fusarium crown rot (FCR) in Iraq are not available, but where data are available, FCR can be devastating. For example, FCR can reduce yields of winter wheat production in the Pacific northwest region of the USA by up to 61% (Smiley *et al*. 2005). FCR also affects grain quality through the production of mycotoxins such as deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEN) and T2-toxin which can be harmful to human, and livestock health (Pestka and Smolinski 2005; Blandino *et al*. 2012; Matny 2015).

Over the past 5 years, FCR has re-emerged as an economically important disease in Iraq, causing significant yield losses to the wheat crop (Matny *et al*. 2012; Matny and Khalifah 2015). A few studies on FCR have been carried out in order to better understand the

genetic diversity present in *F. culmorum* populations and to understand why this disease has re-emerged. Drought conditions in Iraq from 2011 to 2016 very likely contributed to the spread of *F. culmorum* in wheat fields since many studies have shown that dry environments are favorable for *F. culmorum* growth and reproduction (Balmas *et al.* 2006; Scherm *et al.* 2013). FCR has also been reported in other Middle Eastern countries such as Turkey (Tunali *et al.* 2006; Yörük *et al.* 2016), Iran (Hajieghrar 2009; Eslahi 2012) and Syria (El-Khalifeh *et al.* 2009).

Genetic diversity analyses of microorganisms have demonstrated that pathogen diversity depends on global environmental changes and shifts in agro-ecological systems (Saharan and Naef 2008; Gurel *et al.* 2010). *Fusarium culmorum* isolates show high levels of phenotypic and genotypic variability in culture, including colony morphology, pigmentation and sporulation (Puhalla 1981; Miedaner *et al.* 2011; Kollers *et al.* 2013). In addition, variations in aggressiveness and mycotoxin production have been found among various isolates that were collected from different geographic locations (Gang *et al.* 1998; Tunali *et al.* 2012; Winter *et al.* 2013; Ji *et al.* 2015; Matny *et al.* 2017).

There are many methods and techniques used for studying the genetic diversity of microorganisms, including repetitive extragenic palindromic (REP-PCR), also known as repetitive DNA-based fingerprinting. The amplification of prokaryotic genomic sequences between the repetitive elements include: repetitive extragenic palindromic (REP) sequences, entero-bacterial repetitive intergenic consensus (ERIC) sequences and BOX elements. REP-PCR applications are in widespread use among studies of plant pathogenic bacteria, but among eukaryotic microbes, have only been tested in *F. oxysporum* (Edel *et al.* 1995). The principle aim of this study was to characterize the genetic diversity among *F. culmorum* isolates collected in Iraq through REP-PCR and associate the results with their geographic distribution and pathogenicity towards wheat at the seedling stage.

Materials and Methods

Plant material and fungal isolation

Wheat plants exhibiting FCR symptoms were collected from seven provinces representing different agricultural zones in Iraq. All samples were collected in paper envelopes and necessary data (sample number, place and date of collection and host cultivar name) were recorded. The samples were brought to the laboratory and kept in a well-ventilated area at room temperature (25 ± 3°C and 30% humidity) until the samples could be processed. The crowns of wheat plants were cut into 0.5–1.0 cm segments and treated with a 10% sodium hypochlorite (bleach) solution (diluted from commercially-available concentrated bleach) for 2 min, washed with sterile water and dried with filter paper. All samples (4 pieces/plate) were placed in 9 cm Petri dishes containing potato dextrose agar (PDA). Fifty milligrams of Agrimycin-343 was added to the medium after autoclaving. Plates were incubated at 25 ± 2°C for 5 days and then a single *Fusarium* spp. spore was picked up (under a microscope at 400X) using a needle according to colony and spore characterization methodologies (Booth 1971; Leslie and Summerell 2006) and placed in a new petri dish containing PDA for use in pathogenicity assays.

Pathogenicity assay

For the pathogenicity assays, *F. culmorum* isolates were grown on autoclaved millet seed. First, 1 kg of millet seed was soaked in water for 12 h, then the water was drained, and several 250 ml flasks were filled with 50 g each of this millet seed and autoclaved at 121°C and 1.5 kg · cm^{-1} pressure for 20 min. One disc (0.5 cm) of a 7-day-old *F. culmorum* colony was placed in each flask and incubated at 25 ± 2°C for 14 days. Pathogenicity tests were then performed on wheat seedlings. A 1 : 1 mixture of sterile soil and peat moss was autoclaved at 121°C and 1.5 kg · cm^{-1} pressure for 1 h. This process was repeated after two separate days. Pots (5 × 10 cm) used in the greenhouse experiments were filled with the soil mixture and 5 g of *F. culmorum* inoculum was added to each pot in the top 5 cm surface layer of the soil. All pots were watered and placed in the greenhouse for 2 days at 25 ± 2°C. Three seeds of *Triticum aestivum* L. cv. Abu-Ghreeb1 (a commonly used cultivar in Iraq) were sown in each pot, and each treatment was repeated three times. Plants were irrigated with sterilized water as needed. After 35 days, crown rot symptoms and stem discoloration characteristic of FCR were apparent on the inoculated plants. FCR severity was calculated by measuring the length of discoloration relative to seedling height. The FCR severity index was obtained by multiplying this ratio by the number of leaf-sheath layers with necrosis. The FCR index was calculated according to the following formula: length of stem discoloration/seedling heigh) × × number of leaf sheath layers with necrosis (Mitter *et al.* 2006).

Fungal growth and DNA extraction

Twenty-nine *Fusarium* spp. isolates were grown on PDA media in 9 cm Petri dishes for 7 days at 25 ± 2°C. The REDExtract-N-AMP™ Plant PCR kit (Sigma-Aldrich, St. Louis, MO, USA) was used for DNA extraction

according to manufacturer's instructions. Briefly the hyphal tip of the mycelia was harvested with a sterilized needle and placed in 0.2 ml collection tubes, to which 50 µl of extraction solution was added, followed by incubation at 95°C for 10 min. The DNA concentrations and quality were measured to ensure quality using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Qualitative analyses of DNA were carried out via agarose gel electrophoresis.

Amplification of fungal DNA

Species identification of *Fusarium* spp. isolates was determined by amplifying and sequencing the translation elongation factor 1 alpha (TEF-1α) gene. Forward (EF1) 5'-ATGGGTAAGGA(A/G)GACAAGAC-3' and reverse (EF2) 5'-GGA(G/A)GTACCAGT(G/C)ATCA TG-3' (O'Donnell *et al*. 2000) primers were used to amplify the *TEF-1*α gene. The PCR reaction solution was prepared at a total volume of 20 µl. The PCR conditions were as follows: an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 50 s, annealing at 53°C for 50 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Amplification products were visualized on 1.0% agarose gel stained with SYBR™ safe DNA gel stain in 1X TAE (Invitrogen™, Carlsbad, CA, USA). Amplification of a product approximately 700 bp long was generated by PCR from the DNA template.

DNA sequencing

The PCR products of *TEF-1*α were prepared for sequencing by cleaning up with the QiAquick® PCR purification kit (Envigado, Colombia). Sequencing was carried out commercially (ACGT, Inc., Chicago, IL, USA). The sequencing chromatograms were read and aligned using MEGA6 software (Tamura *et al*. 2013) and the sequences were compared with those in GenBank (http://www.ncbi.nlm.nih. gov/) for the *TEF-1*α gene using the basic local alignment search tool (BLAST). All sequences of the isolates were sent to GenBank to obtain accession numbers.

Genetic diversity study

Fusarium culmorum isolates collected from different regions of Iraq were genotyped using three primer pairs designed to amplify multiple regions of the genome simultaneously: 1 – REP1R-Dt (5′-III NCGNCATCNGGC-3′) and REP-2G (5′-GCGGCTT ATCGGGCCTAC-3′) for REP; 2 – ERIC 1 (5′-ATGTA AGCTCCTGGGGATTCAC-3′) and ERIC 2 (5′-AA GTAAGTGACTGGGGTGAGCG-3′) for ERIC, and 3 – BOX-A1R (5′-CTACGGCAAGGCGACGCTGA CG-3′) (Versalovic *et al*. 1991) for BOX-ERIC2. The PCR conditions were: an initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, an annealing step at 50°C (BOX and ERIC) or 37°C (REP) for 1 min, and an extension at 72°C for 2 min, followed by a final extension at 72°C for 15 min (Belkum *et al*. 1993). Amplification products were visualized on 1.5% agarose gels with SYBR™ safe DNA gel stain in 1X TAE (Invitrogen™). A total of 410 potential markers were generated by this method for genotyping. REP-PCR markers were evaluated together in pair-wise comparisons. Single and shared fragments were analyzed by using Multivariate Statistical Package (MVSP) 3.22 program and the similarity was calculated according to minimum variance (Squared Euclidean) (Kovach 2001).

Results

Pathogenicity tests for *F. culmorum* on wheat seedlings demonstrated that the collected isolates varied in their pathogenicity toward wheat cultivar Abu-Grheeb1, and ranged from 0.001 to 0.890 on the FCR severity index. However, some isolates (IF 0003, IF 0013, IF 0024) were non-pathogenic, with a score of 0.00 on the FCR severity index. The isolates that had the highest FCR severity index scores were: IF 0021, IF 0028, IF 0045, IF 0046, IF 0015, and IF 0005. Isolates IF 0021, IF 0028, IF 0045 and IF 0046 were isolated from Baghdad while IF 0015 and IF 0005 came from Anbar and Diyala provinces, respectively (Table 1).

The results of species-specific identification using TEF-1α demonstrated that all isolates used in this study were in fact *F. culmorum*.

The genetic diversity study showed monomorphic and polymorphic bands pattern. The annealing temperature for the BOX, ERIC and REP primers used in this study were different than the output provided by Gurel *et al.* (2010). In this study, we found that the optimum annealing temperatures for the primers were 50°C for BOX and ERIC, and 37°C for REP. The PCR bands for the final amplification products were between 200–800 bp for BOX-ERIC2, 110–1100 bp for ERIC-ERIC2, and 200–1300 bp for REP. A total of 410 polymorphic markers were identified in this study for the *F. culmorum* isolates, including 106 for BOX, and 175 for ERIC, and 129 for the REP-PCR. These markers were used to study the minimum variance for the *F. culmorum* strains (Fig. 1).

Minimum variance cluster analysis was used to detect the variance between the *F. culmorum* isolates (Fig. 2). The dendrogram illustrated in this study separated the *F. culmorum* isolates into major groups as

Table 1. *Fusarium culmorum* accessions used in this study including loci information, *TEF-1α* gene test and disease severity index

No.	Accession no.	Isolate no.	Species	Location	*TEF-1α* gene	FCR* severity index
1	KY205745	IF 0003	*F. culmorum*	Karbala	+	0.00
2	KY205746	IF 0004	*F. culmorum*	Karbala	+	0.030
3	KY190104	IF 0005	*F. culmorum*	Diyala	+	0.286
4	KY190106	IF 0006	*F. culmorum*	Diyala	+	0.001
5	KY190111	IF 0007	*F. culmorum*	Diyala	+	0.074
6	KY190107	IF 0008	*F. culmorum*	Diyala	+	0.002
7	KY190127	IF 0009	*F. culmorum*	Kirkuk	+	0.098
8	KY190118	IF 0013	*F. culmorum*	Anbar	+	0.00
9	KY190123	IF 0014	*F. culmorum*	Anbar	+	0.011
10	KY190116	IF 0015	*F. culmorum*	Anbar	+	0.309
11	KY190108	IF 0017	*F. culmorum*	Najaf	+	0.022
12	KY205747	IF 0021	*F. culmorum*	Baghdad	+	0.890
13	KY190121	IF 0022	*F. culmorum*	Baghdad	+	0.294
14	KY190126	IF 0024	*F. culmorum*	Diyala	+	0.00
15	KY190122	IF 0026	*F. culmorum*	Anbar	+	0.050
16	KY190112	IF 0028	*F. culmorum*	Baghdad	+	0.543
17	KY205748	IF 0029	*F. culmorum*	Baghdad	+	0.004
18	KY190109	IF 0030	*F. culmorum*	Kirkuk	+	0.001
19	KY190113	IF 0031	*F. culmorum*	Kirkuk	+	0.030
20	KY190114	IF 0032	*F. culmorum*	Kirkuk	+	0.002
21	KY190117	IF 0033	*F. culmorum*	Kirkuk	+	0.004
22	KY190124	IF 0040	*F. culmorum*	Babylon	+	0.076
23	KY190110	IF 0041	*F. culmorum*	Babylon	+	0.005
24	KY190119	IF 0042	*F. culmorum*	Babylon	+	0.001
25	KY205749	IF 0044	*F. culmorum*	Diyala	+	0.019
26	KY190105	IF 0045	*F. culmorum*	Baghdad	+	0.350
27	KY190125	IF 0046	*F. culmorum*	Baghdad	+	0.200
28	KY190120	IF 0047	*F. culmorum*	Baghdad	+	0.008
29	KY190115	IF 0052	*F. culmorum*	Anbar	+	0.010

*Fusarium crown rot

shown in Figure 2. Group 1 includes 17 isolates and may be divided into two sub-groups (1a and 1b) consisting of 5 and 11 isolates, respectively. All isolates in this group were collected from northern and central sites in Iraq. Group 2 consists of 12 isolates and may also be divided into two subgroups (2a and 2b) consisting of 3 and 9 isolates, respectively. Group 2a isolates originated from central Iraq while group 2b isolates came from northern, central, and southern Iraq.

Isolates IF0022 and IF0026 shared the highest similarity values (100% similar). There was no relationship between the geographic origin of the isolate and its genetic relationship to other isolates (Fig. 2). One reason is the use of seed that has not been certified by the Iraqi Ministry of Agriculture and the exchange of seed between farmers across different regions leads to the transfer of the pathogen with the seed from one province to another. Also, some farmers obtain their seed from local markets where the seed source is unknown.

Discussion

Genetic diversity studies of pathogen populations are used to understand the importance of genetic changes in plant pathogens and the relationships between adaptation of pathogens and climate change, and their influence on management of plant pathogens (McDonald and Linde 2002). *Fusarium culmorum* has also been reported to cause seed-borne diseases of pre- and post-emergence seedling death and is one of the causal species of Fusarium head blight (FHB) (Polley

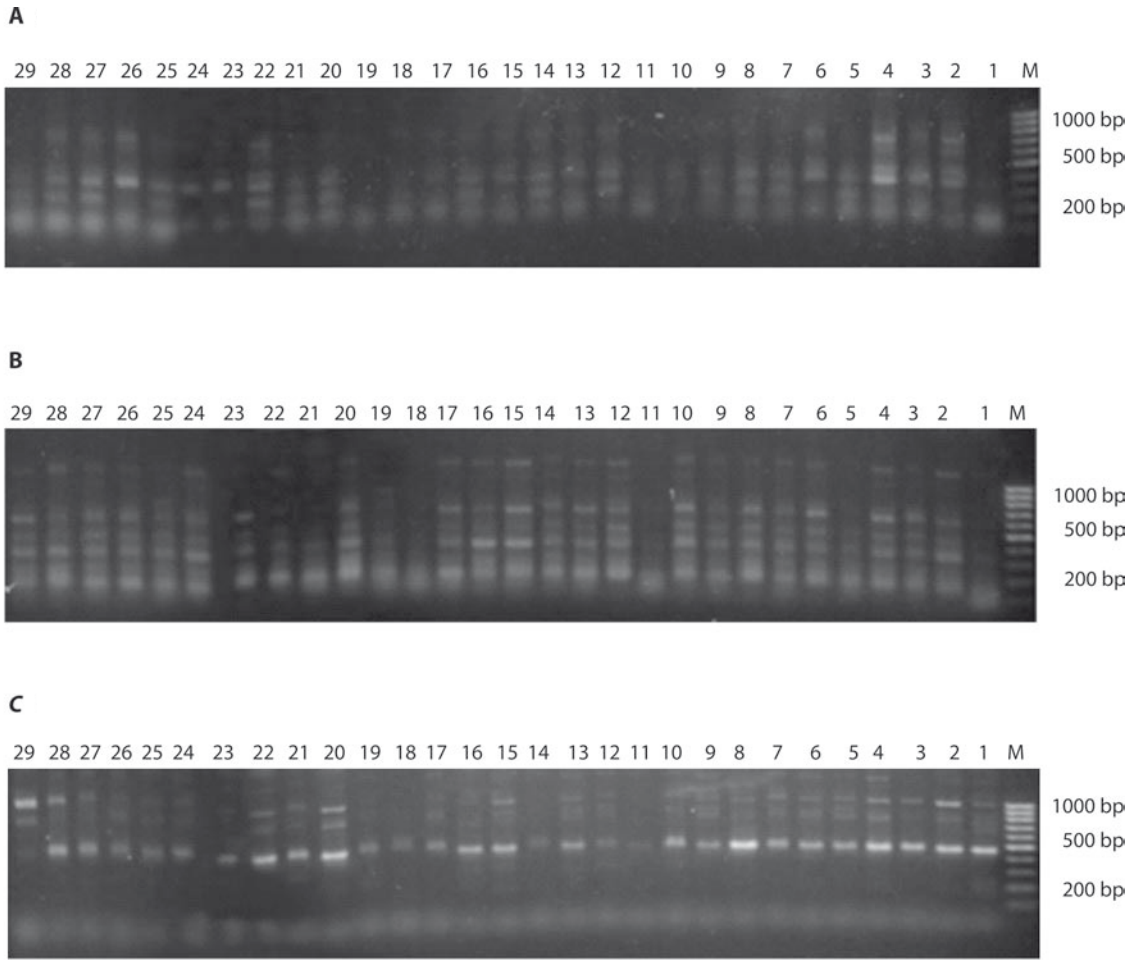

Fig. 1. Fingerprint pattern for 29 *Fusarium culmorum* isolates by using three primer pairs: A – BOX-ERIC2, B – ERIC-ERIC2, C – REP

and Turner 1995). In this study, 89.7% of the *F. culmorum* isolates were pathogenic towards wheat at the seedling stage, while a smaller (10.3%) number of isolates were non-pathogenic.

The results of our study on the genetic diversity among 29 isolates of *F. culmorum* collected from different regions of Iraq showed that there was no relationship between geographic location and genetic similarity of the isolates. We suggest that this means that *F. culmorum* populations have the ability to survive and adapt to different and extreme variations in climate, from the cold area in northern Iraq with temperatures ranging from –5 to 10°C in winter and 35 to 45°C in summer, to central and southern Iraq where temperatures range from around 5 to 15°C during winter to 45 to 55°C in the summer.

Many studies of DNA analysis have investigated genetic variability and population structure of *F. culmorum* using a variety of molecular markers, such as random amplified polymorphic DNA (RAPD) (De Nijs *et al*. 1997; Gargouri *et al*. 2003; Yörük and Albayrak 2013) and restriction fragment length polymorphism (RFLP) (Nicholson *et al.* 1993; Llorensa *et al*. 2006). These studies suggest that there is extensive genetic diversity in *F. culmorum* populations. In addition, Mishra *et al.* (2003) found a high degree of intra-specific polymorphism among *F. culmorum* isolates using inter-simple sequence repeat (ISSR) analysis. Albayrak *et al.* (2016) also studied the relationship between *Fusarium* spp. isolates according to their species and geographic regions by using ISSR markers. In another study, Bayraktar and Fatma (2010) found that ISSR markers have a high degree of intra- and interspecific polymorphisms among *Fusarium* spp. Finally, Gargouri *et al.* (2003) used RAPD markers to study the genetic variability and population structure of *F. culmorum* isolated from wheat stem bases.

In addition to the genetic diversity and variation in pathogenicity towards wheat presented in this study, populations of *F. culmorum* are also characterized by high levels of phenotypic variability in culture, such as sporulation, pigmentation, mycotoxin production, and colony morphology (Puhalla 1981). Gang *et al.* (1998) found a large variation among *F. culmorum* isolates

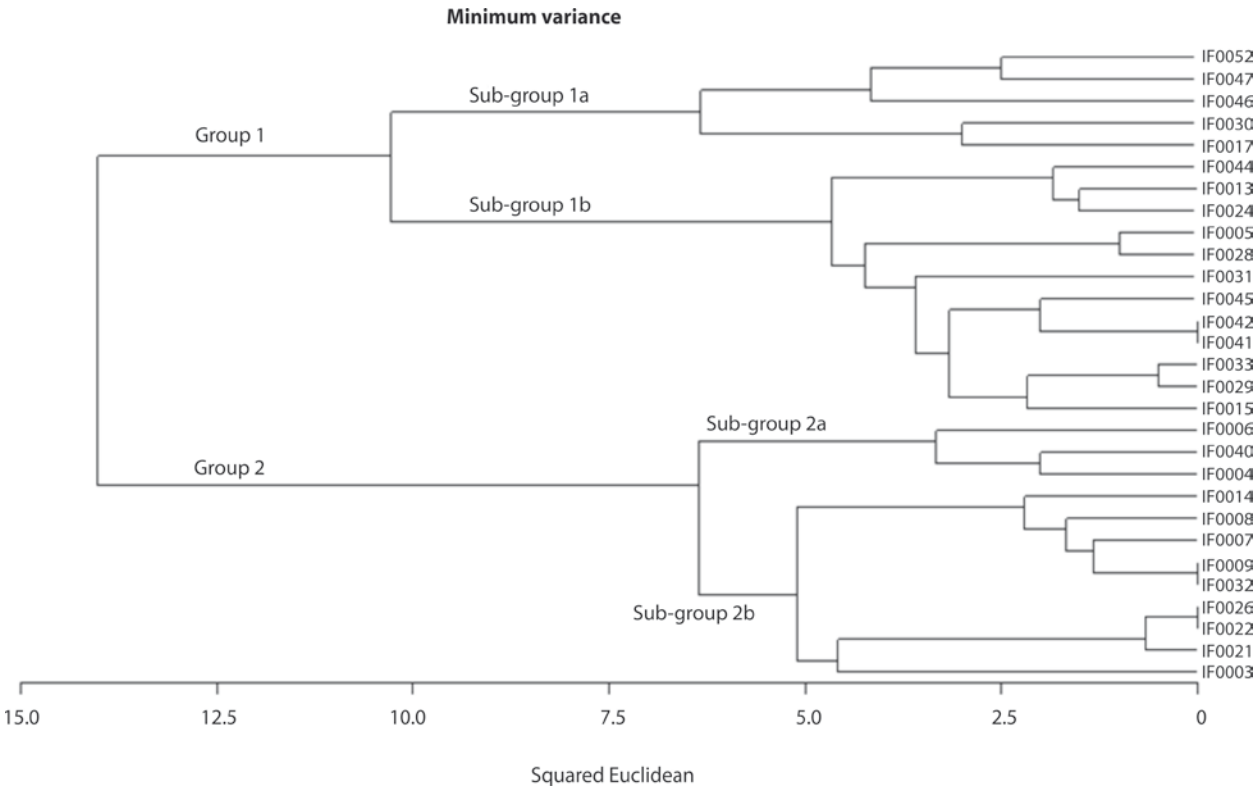

Fig. 2. Dendrogram of 29 *Fusarium culmorum* isolates generated based on the number of bands and position of appearance for three primers BOX-ERIC2, ERIC-ERIC2 and REP by using Multivariate Statistical Package (MVSP) 3.22 program to show minimum variance (Squared Euclidean)

collected from the various geographic areas for aggressiveness, race designation and mycotoxin production.

This is the first report of the genetic diversity of *F. culmorum* populations present in Iraq using the REP-PCR method. Two groups of *F. culmorum* were identified in this study according to minimum variance (Squared Euclidean). This is the first study which reported on the genetic diversity of FCR in Iraq. Although the study was limited to 29 isolates due to difficulties in completing more extensive sampling, it provides an initial glimpse into the genetic diversity and variation in pathogenicity present in *F. culmorum* populations in Iraq.

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