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Genetic diversity among *Fusarium* species associated with sorghum stalk rot in Southern Ethiopia

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Fusaria are very diverse and destructive pathogens affecting different crops. However, their identity and diversity are unresolved in countries like Ethiopia, where various crop species are grown under differing environmental conditions. The objectives of this paper were to identify *Fusarium* spp. associated with sorghum stalk rot in Southern Ethiopia, and elucidate the genetic diversity within and between the species. For this purpose, Fusaria associated with sorghum from two locations in Southern Ethiopia were isolated. Sequencing of the elongation factor 1-alpha gene (EF-1α) was used for species identification. In addition, AFLP analysis was employed for further diversity studies within and between the *Fusarium* spp. Sequence analyses revealed the presence of two *Fusarium* spp. The first was identified as *Fusarium andiyazi*, while the identity of the second remains to be solved. AFLP analysis clustered the isolates into two major groups. The Dice similarity coefficients ranged from 0.39 to 0.91 for isolates of *F. andiyazi* while isolates within the new *Fusarium* spp. had a Dice similarity coefficient varying between 0.69 and 0.96. Cluster analysis and principal coordinate analysis clearly indicated a genetic separation between the two species. Both groups were pathogenic to mature sorghum plants following a toothpick inoculation test. More researches are required to identify the new species and elucidate the pathogenicity of the isolates.

Key words: EF-1α, *Fusarium andiyazi*, genetic similarity, sequence analysis, *Sorghum bicolor.*

INTRODUCTION

Sorghum (*Sorghum bicolor*, (L.) Moench) is the fifth most important cereal accounting for more than 65 million tons of annual production on over 45 million ha of land worldwide (FAO, 2017). The bulk of sorghum is produced in less developed nations (Berenji and Dahlberg, 2004), however, USA is the leading producer with more than 12 million tons of production (FAO, 2017). In Ethiopia, the crop is grown on more than 2 million ha of land making it one of the three most important crops both in terms of area coverage and total production (5 million tons) (CSA, 2018).

Sorghum is grown for its various purposes in different parts of the world. It serves as a major source of food and is also used as feed source for livestock especially in developed nations. In addition, sorghum is used as raw material for industries and for the production of bio-fuel. Despite its versatile use and ability to withstand adverse environmental conditions including moisture stress and

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high temperatures, sorghum production is hampered by various biotic stresses among which diseases caused by different pathogens are one (Thakur and Mathur, 2000; Chala et al., 2011; 2012; Eshte et al., 2015). *Fusarium* species that cause stalk rot, ear rots and grain mold are among the major pathogens that infect sorghum (Frederiksen and Odvody, 2000; Leslie et al., 2005). The fungus *Fusarium* belongs to the most harmful pathogens of cultivated crops all over the world (Antonia, 1995; Ramdial et al., 2017).

Once they occur in the field or storage, *Fusaium* spp. are known to cause significant qualitative and quantitative yield reduction (Parry et al., 1995; Brandfass and Karlovsky, 2008). In addition, they produce mycotoxins that pose serious health risks to humans and animals that feed on contaminated grains (D'Mello et al., 1999; Pestka and Smolinsky, 2005; Antonissen et al., 2014; Wu et al., 2014 and Duan et al., 2016). Despite continued efforts to manage diseases caused by Fusaria, they still pose serious threats to grain producers across the world (Brandfass and Karlovsky, 2008). Since the early reports by Wollenweber and Reinking (1935), lots of researches have been conducted on the taxonomy/genetic diversity of the genus *Fusarium* (Summerell et al., 2011; O'Donnell et al., 2015; Laurence et al., 2016; Moussa et al., 2017 and Valente et al., 2017). However, research on *Fusarium* spp. from sorghum has been given only peripheral importance (Leslie et al., 2005). The only exceptions, in this regard, are earlier reports by Claflin (2000) and Leslie (2000; 2002), which identified more than 10 *Fusarium* species from sorghum, with many of them known to infect the stalk and grain. On the other hand, the identity and diversity of *Fusarium* species infecting sorghum in Africa, particularly in Ethiopia remains unresolved. In Ethiopia, there are limited reports (Ayalew, 2002; Ayalew et al., 2006; Chala et al., 2014; Taye et al., 2016; 2018) on *Fusarium* spp. and associated mycotoxins from sorghum even though the country is one of the primary centers of origin and diversity for the crop. The objectives of this work were: i) to identify *Fusarium* spp. associated with sorghum stalk rot in Southern Ethiopia; and ii) to elucidate the genetic diversity within and between the species.

MATERIALS AND METHODS

Isolate collection

Twenty sorghum stalks with visible rotting were randomly collected from sorghum fields in Southern Ethiopia during a routine field survey. The stalk samples were stored in paper bags at room temperature until isolation. Geographic description of the locations is given in Table 1.

Isolation, identification and storage of the isolates

Infected stalks were cut into pieces, surface sterilized using 0.5% sodium hypochlorite (NaOCl) solution for 90 s, and rinsed three times in sterile, distilled water. The cut and surface-sterilized stalks were placed on potato dextrose agar (PDA) and incubated at 25°C under continuous fluorescent light for 10 days. After 10 days of incubation, sporulation was observed in the PDA plates. Isolates were identified as Fusaria based on pigmentation and conidial morphology. On these bases, a total of 37 single spore isolates were transferred to new PDA plates. The isolates were grouped into two groups based on pigmentation and form of conidia. Pure cultures were maintained on potato dextrose agar (PDA) amended with 50 ppm of streptomycin, and stored at 4°C as stock cultures.

Molecular characterization

DNA extraction

Approximately 100 mg of fresh mycelium per isolate was crushed in liquid nitrogen using mortar and pestle. Fine powdered mycelium was transferred to a 2 ml microcentrifuge tube and genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. The quality of the extracted DNA was controlled on 0.8% agarose gels and the DNA was stored at -20°C.

Species identification

Elongation factor 1-alpha genes of seven isolates, randomly selected from the two morphological groups, were partially sequenced using the EF-1α primers that is, EF-728F: 5´-CATCGAGAAGTTCGAGAAGG-3´ and EF-986R: 5´-TACTTGAAGGAACCCTTACC-3´ (Carbone and Kohn, 1999). The resulting sequences were BLAST searched with the NCBI nucleotide database for molecular species identification.

AFLP analysis

AFLP analysis was conducted following the method developed by Vos et al. (1995) with modifications that include the use of fluorescent labeled primers instead of radioactive labeled isotopes. Six combinations of *Mse*I and *Eco*RI primers were used for selective amplification (Table 2). The primers differ by two selective nucleotides at their 3´ ends and the *Eco*RI primers were labeled with the fluorescent dye FAM (6-carboxyfluorescein). The selective amplification reaction mix contained 1.6 µl dNTP (2.5 mM), 2 µl of 10× polymerase chain reaction (PCR) buffer, 0.08 µl of Taq DNA polymerase (5 U/µl), and 5 µl *Mse*I (6 ng/µl) and 1 µl *Eco*RI (1 pmol) primers to which 5 µl of 10 fold diluted preamplification PCR product was added as a template. The PCR amplification conditions were as follows: 1 cycle of 94°C for 30 s, 65°C for 30 s and 72°C for 60 s; 12 cycles where the annealing temperature was lowered by 0.7°C for each cycle; 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s; finally 72°C for 7 min. The accuracy of the analysis was checked by running a randomly selected sample in duplicates.

Data scoring and analysis

Amplification products were separated in an ABI3730 DNA analyzer (Applied Biosystems Inc., Foster City, California) following the manufacturer's protocol and using GeneScan-1200 LIZ size standard (Applied Biosystems). The presence (1) and absence (0) of peaks were scored using Gene-Mapper software version 4.0 (Applied Biosystems Inc., Foster City, California), checked manually, and only clear and unambiguous peaks with fluorescence greater than or equal to 100 arbitrary units were entered into a binary data matrix for further analysis. The binary matrices were

Table 1. Geographic origin of *Fusarium* isolates.

Isolates	Origin	Altitude (m)	Latitude	Longitude
Fa_{1-16}	Welayita	1947-1952	6°58‘-6°60‘	37°51‘-37°53‘
Fsp_{1-22}	Gidole	1297-1590	5°40’-5°42’	37°22’-37°24’

then used to calculate genetic similarities between the isolates based on the Dice similarity coefficient (Dice, 1945), and the unweighted pair-group method with arithmetic average (UPGMA) was used to construct a genetic similarity tree with the help of the NTSYS-pc software, version 2.0 (Exeter Biological Software, Setauket, NY). To further elucidate the genetic relationship among the tested isolates, principal coordinate analysis was conducted using the software GenAlEx6 (Peakall and Smouse, 2006).

In-vitro growth rate of Fusarium isolates

To study the phenotypic characters (growth rate and colony morphology) of the *Fusarium* isolates, 5 mm portions of the 38 single spore isolates were transferred from the stock cultures and cultivated on PDA at 25°C in the dark. After five days of incubation, 3 mm mycelia plugs were taken from the actively growing edges of each isolate, transferred to the centre of four replicate PDA plates, and incubated in the dark at 25±2 and 30±2°C. For each isolate, radial growth was recorded at 24 h intervals for 7 days.

RESULTS

Species identification and characterization

Sequence analysis

Based on sequencing of the elongation factor 1-alpha gene (EF-1α) of isolates, the Fusaria were categorized into two groups. The first group of isolates were identified as *F. andiyazi*. The sequence of the second group of isolates did not match with the sequence of *Fusarium* species deposited in NCBI, and hence their identity remains to be resolved.

Morphological and cultural characterization

F. andiyazi isolates produced both macro- and micro-conidia and had a white mycelium that become some what pale through time. Isolates belonging to the unidentified *Fusarium* species also produced both micro- and macro- conidia on PDA while their mycelium consistently appeared white with a mass of orange colored spores. Isolates of similar morphological appearance were also obtained from sorghum and finger millet grains collected from different locations in Ethiopia (data not shown).

Isolates belonging to the two species also varied in terms of radial growth rate. The growth rate of isolates belonging to *F. andiyazi* ranged from 8 to 14 mm/day and 8 to 12 mm/day at 25±2 and 30±2°C, respectively. On the other hand, isolates belonging to the new species grew considerably faster (10-17 mm/day) at 25°C than at 30°C (7-12 mm/day). When five isolates representing *F. andiyazi* and 10 isolates from the new *Fusarium* species were used to inoculate the stalks of mature sorghum plants using the toothpick inoculation method (Cumagun et al., 2009), all of them produced typical lesions that were absent in the control plants. This suggested the pathogenicity of both species to sorghum and proved that they were responsible for the stalk rot of sorghum.

AFLP analysis

AFLP analysis of 38 isolates clustered them into two major groups (Figure 1). The first major group consists of 16 isolates identified as *F. andiyazi* by sequence analyses. Dice similarity coefficient for isolates belonging to this major group varied from 0.39 to 0.91 (Table 3). Results differentiated isolates of *F. andiyazi* into two sub-groups. The first sub-group consists of 7 isolates while the second sub-group is made of the remaining 9 isolates. The second major group consists of 22 isolates belonging to the unidentified *Fusarium* species and hence named as *Fusarium* spp. Isolates within this group had a Dice similarity coefficient ranging between 0.69 and 0.96, and hence they were considered as genetically more similar with one another than those within *F. andiyazi*, and likely represent a single species. One isolate within this group had 69% similarity while the rest had at least 70% similarty between each other. The six primer combinations used in this study generated a total of 200 clearly scorable bands. Of these, 71 (35.5%) were unique to the new *Fusarium* species while 70 bands (35%) were unique to *F. andiyazi* isolates. The remaining 59 bands (29.5%) were shared across the species. Of the 71 bands unique to *Fusarium* spp., 31 (56%) were polymorphic while 60 (86%) bands unique to *F. andiyazi* were also polymorphic.

Principal coordinates analysis (PCO) also revealed the population subdivision within and between the two *Fusarium* species. Accordingly, the isolates were categorized into three groups (Figure 2). The first three principal coordinates accounted for 89.4, 4.5 and 2.3% of the total variation, respectively. PCO grouped 16 of the *F. andiyazi* isolates that formed the first two clusters of the UPGMA tree into two groups. The first group was made of 9 isolates while the second PCO group consisted of 7

Table 2. Nucleotide sequences of adapters and primers used in the AFLP analysis.

Adapters	Primer sequences	Function
E*co*RI	5´CTCGTAGACTGCGTACC3´ CATCTGACGCATGGTTAA5´	Adapter
M*se*I	5´GACGATGAGTCCTGAG3´ TACTCAGGACTCAT5´	Adapter
E*co*RI	5´GACTGCGTACCAATTC3´	Nonselective primer
M*se*I	5´GATGAGTCCTGAGTAA3´	Nonselective primer

	Primer sequences	
Selective Primer combination	**E*co*RI (5´→3´)**	**MseI (5´→3´)**
E12 × M16	GAC-TGC-GTA-CCA-ATT-CAC	GAT-GAG-TCC-TGA-GTA-ACC
E19 × M15	GAC-TGC-GTA-CCA-ATT-CGA	GAT-GAG-TCC-TGA-GTA-ACA
E19 × M16	GAC-TGC-GTA-CCA-ATT-CGA	GAT-GAG-TCC-TGA-GTA-ACC
E20 × M17	GAC-TGC-GTA-CCA-ATT-CGC	GAT-GAG-TCC-TGA-GTA-ACG
E21 × M16	GAC-TGC-GTA-CCA-ATT-CGG	GAT-GAG-TCC-TGA-GTA-ACC
E21 × M17	GAC-TGC-GTA-CCA-ATT-CGG	GAT-GAG-TCC-TGA-GTA-ACG

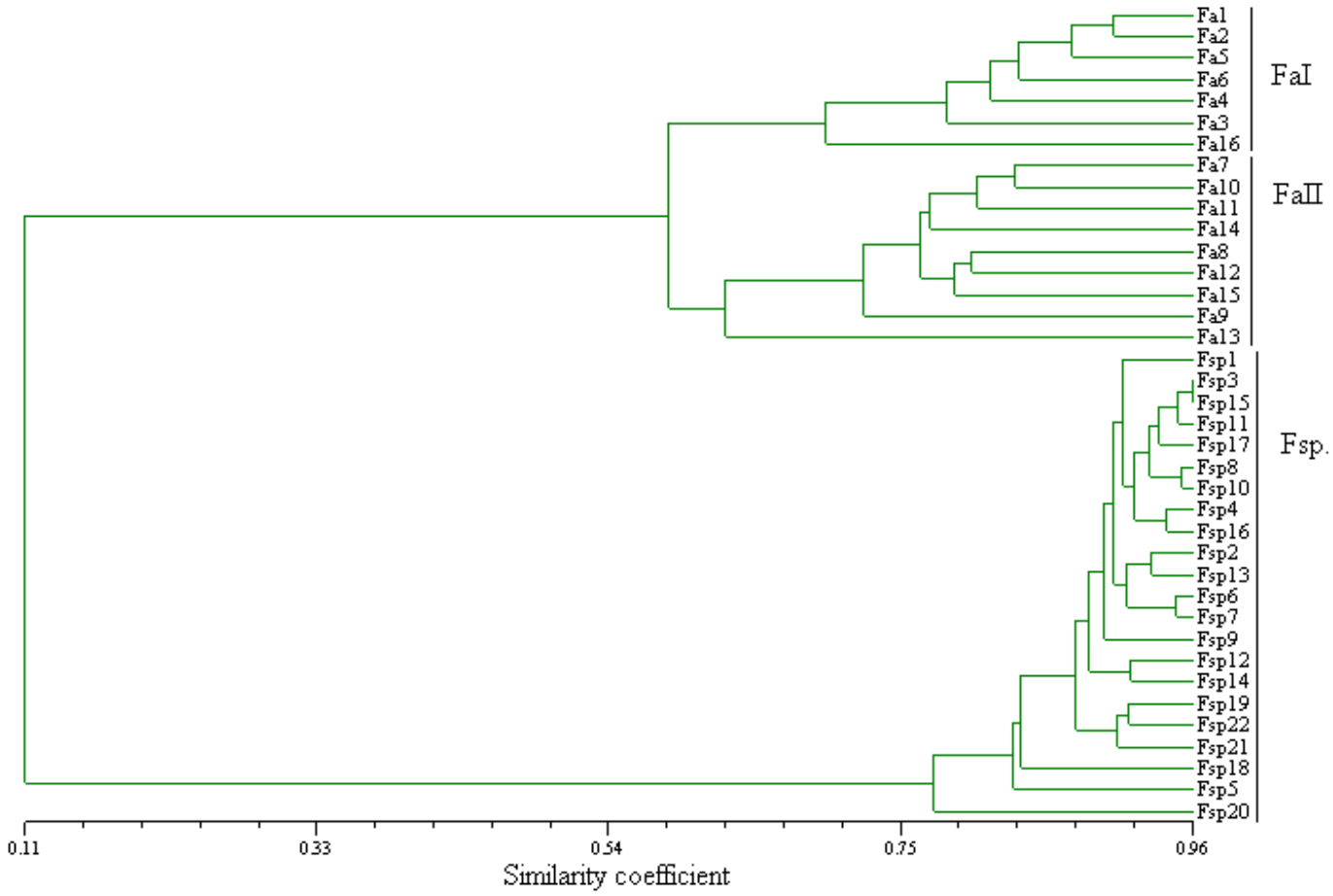

Figure 1. Dendogram showing the genetic diversity of 38 isolates of two *Fusarium* species based on Dice similarity matrix of AFLP bands.

Table 3. Dice similarity index within and between *Fusarium* species.

Species	*F. andyazi*	*Fusarium* spp.
Fusarium andyazi	0.391 - 0.907	0.044 - 0.227
Fusarium spp.	0.044 - 0.227	0.693 - 0.957

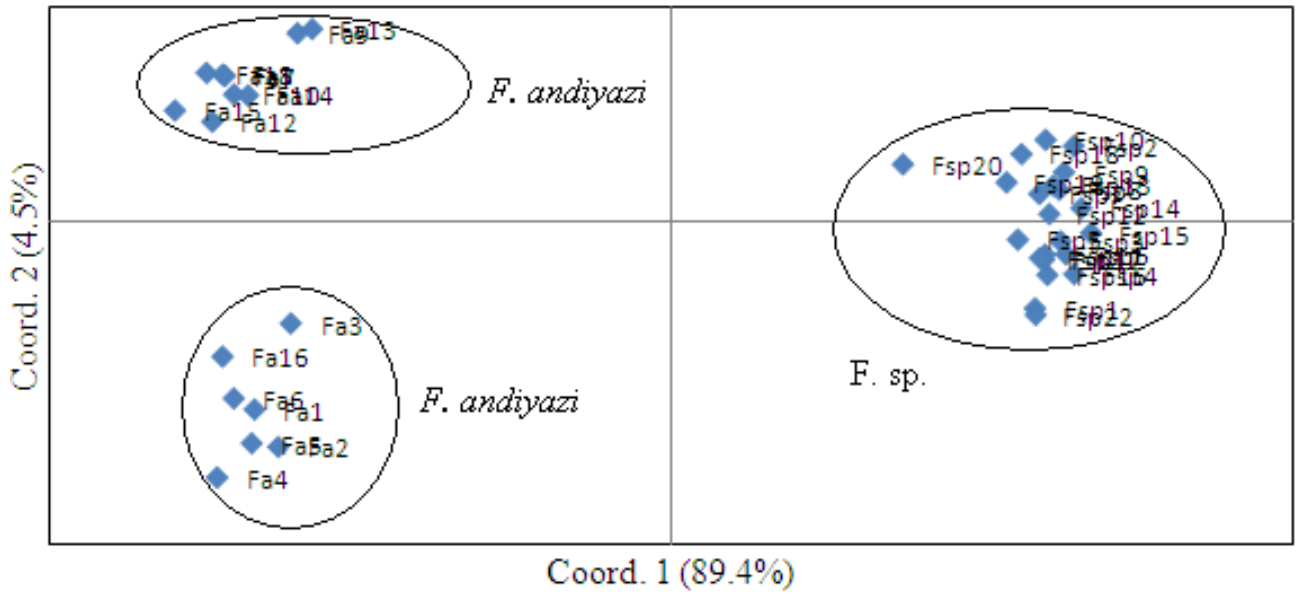

Figure 2. Principal coordinates analysis of 38 *Fusarium* isolates based on AFLP fingerprints.

isolates. The 22 remaining isolates belonging to the new *Fusarium* species were aggregated within a single PCO group with only 1 isolate barely separated from the rest. These results are in line with the cluster analysis of UPGMA.

DISCUSSION

The *Fusarium* isolates included in the current study fullfilled the morphological characteristics of *Fusarium* as described in Leslie and Summerell (2006). However, sequence analysis revealed the presence of two *Fusarium* species associated with stalk rot in Southern Ethiopia. The current results are in line with previous works that reported the co-occurence of different *Fusarium* spp. on the same plant (Summerell et al., 2011; Ramdial et al., 2017; Minnaar-Ontong et al., 2017). Results from sequencing confirmed the first species as *F. andiyazi*, a species which was first described by Marasas et al. (2001). This species was subsequently reported to be present in different parts of the world including Australia, Ethiopia, Nigeria, South Africa and United States (Marasas et al., 2001; Marley et al., 2004; Leslie et al., 2005; Leslie and Summerell, 2006 and Summerell et al., 2011). Nevertheless, except for initial reports, no further work has been done on this particular species in Ethiopia to the best of the author's knowledge. As a result, the diversity of this pathogen remains largely unknown to date.

Isolates belonging to the two *Fusarium* species varied not only in terms of their morphology and sequence but they also differed in growth rate, when incubated at 25 and 30°C. Isolates of *F. andiyazi* grew slower than those of the newly recovered *Fusarium* species at both temperatures. Besides, the growth rate of *F. andiyazi* isolates was also consistent across temperatures. Isolates of the same species showed similar growth rate at both of these temperatures in a previous study (Leslie et al., 2005). Isolates of *Fusarium* species on the other hand did not grow consistently across temperatures.

Although cultural/morphological characterizations provide a basis for both inter- and intra-species diversity studies; as suggested in other pathosystems, they may be unstable, highly influenced by the growth environments and rather change with the age of the colonies (Browning et al., 1999; Crouch et al., 2006 and Rivera-Vargas et al., 2006). As a result, such taxonomic features need to be supplemented with other characters like molecular markers that differentiate biological entities at the genetic level. Currently there is a growing

interest to assess the genetic diversity of fungi including Fusaria based on sequence analysis (McDonald et al., 2012; Leavitt et al., 2013; Maphosa et al., 2016; Laraba et al., 2017).

In accordance with sequence analysis and morpho-cultural characterization, AFLP analysis also showed the presence of at least two genetically distinct *Fusarium* populations associated with sorghum stalk rot in Southern Ethiopia. The two *Fusarium* species were not only genetically but also geographically separated as there is more than 50 km distance between the districts from where they were obtained. Backhouse et al. (2001) and Saremi et al., (1999) have reported climatic preferences among *Fusarium* species from both natural and agricultural ecosystems. This report was also supported by Vigier et al., (1997); De Wolf et al., (2003) and Moschini et al. (2004) that ascertained the influence of climate and local weather variations on the recovery of *Fusarium* species. Both UPGMA and PCO analyses of AFLP bands suggested greater variation within *F. andiyazi* than within *Fusarium* species. Leslie et al. (2005) proposed a 40% similarity cut-off to identify strains into a single species. As the most distantly related isolates within this species had a 39% Dice similarity, which is just close to the 40% boundary, it is better not to reach a conclusion that *F. andiyazi* isolates belong to different species.

In the current study, all isolates belonging to *F. andiyazi* were isolated from sorghum stalks collected from the district of Welayita, with elevation ranging from 1947 to 1952 m above sea level (masl), while those belonging to the new *Fusarium* species were isolated from the Gidole district, with elevation of 1297-1590 masl. Based on eleven years weather data from the National Meteorological Agency, Welayita district has a total annual rainfall of 1262 mm, and temperature of 13.6 - 23.4°C (18.5°C average). There is no reliable weather data for Gidole district. However, this relatively low lying district is known to have a more warm and humid weather than Welayita. Preparations are now underway to work on the speciation of isolates belonging to the new species, and to further characterize them on the basis of mycotoxin profiling, mating types and other characteristic features. *F. andyiazi* is not a known mycotoxin producer (Leslie et al., 2005). However, the toxin production potential of isolates belonging to the new *Fusarium* species need to be ascertained especially in light of their isolation from cereal grains. This is of paramount importance as several *Fusarium* spp. are known producers of mycotoxins that pose health risks to consumers of contaminated plant products (Antonissen et al., 2014; Wu et al., 2014; Van der Lee et al., 2015; Duan et al., 2016).

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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