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Genetic diversity of *Fusarium* endophytes strains from sorghum (*Sorghum bicolor* L.) tissues in Burkina Faso

Gilles I. Thio¹*, Elisabeth P. Zida¹, James B. Neya¹, Ednar G. Wulff², Ole S. Lund³ and Birte Boelt⁴

¹Institut de l’Environnement et de Recherches Agricoles (INERA), 01 BP 476 Ouagadougou 01, Burkina Faso.
²Division of Plant Diagnostics, Danish Veterinary and Food Administration, Ministry of Food, Agriculture and Fisheries, Soendervang 4, DK-4100 Ringsted, Denmark.
³Department of Plant Biology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.
⁴Department of Agroecology, Science and Technology, Aarhus University, Forsoegetsvej 1, DK-4200 Slagelse, Denmark.

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The diversity and genetic differentiation of populations of *Fusarium* species associated with sorghum fields, both endophytes obtained from sorghum performing and non performing plants and isolates obtained from two sampling periods were investigated. *Fusarium* specific Internal Transcribed Spacer 2 (FITS2) primers set were used to assess genetic variability of 32 isolates from susceptible *Fusarium* spp. endophytes from *Sorghum* tissues. *Fusarium thapsinum* (*Gibberella thapsina*) with 68.75% of the isolates constituted the majority of *Fusarium* spp. isolated in performing plants. *Gibberella thapsina* species identified are described as non-pathogenic and associated to performing plant of sorghum. Previously, some species of *Fusarium thapsinum* have been recognized as pathogenic and responsible for yield losses in several cereal crops including *Sorghum bicolor* produced in Burkina Faso. The other *Fusarium* spp. identified in this study including *Fusarium subglutinans*, *Fusarium chlamydosporum*, *Gibberella intermedia*, *Fusarium dlaminii*, *Fusarium oxysporum*, *Fusarium proliferatum*, and *Fusarium* spp. An additional unknown fungi species were also identified. A diverse population of 10 sequence types was found, although 8 sequence types represented nearly two-thirds of the isolates studied. The sequence types were placed in different phylogenetic clades within *Fusarium* spp., and endophytic isolates were not monophyletic. Phylogenetic analysis from Neighbor-Joining/UnWeighted Neighbor-Joining showed a high genetic relationship among these 32 isolates of *Fusarium* spp. and high variation in FITS sequence of them. The use of specific phylomarker of the genus *Fusarium* allowed to identify the endophytic species of this genus and to establish the phylogenetic relationships between the endophytic species of *Fusarium*. The phylogenetic analysis revealed three groups of the fungi. However, no relationship between these groups and the geographical origins of these fungi has been established.

Key words: *Fusarium thapsinum*, endophyte, FITS2 marker, sorghum.

INTRODUCTION

Sorghum (*Sorghum bicolor*) is the fifth most important grain crop in the world and the main cereal crops grown in sub-Saharan Africa in terms of cultivated area, production and consumption (FAOSTAT, 2015). In Burkina Faso, sorghum is the main staple crop in terms of annual production, which is grown for human food
nutrition. Sorghum production is subject to abiotic stresses including drought, and various biotic agents such as the soilborne and seedborne fungal diseases which frequently lead to significant crop yield and grain density losses (Katilé et al., 2010). One of the major diseases of sorghum is grain mould. The disease is caused by several fungal genera, including Fusarium, Leptosphaeria, Cochliobolus and Cladosporium (Pak et al., 2016). These fungi are capable of producing mycotoxins in grains which are harmful for human and animal consumption (Agriopoulos et al., 2020). Fusarium moniliforme (Fusarium thapsinum) is one of the most important fungal species that colonize sorghum plant tissues and are mostly considered as pathogens. Some species of F. moniliforme isolated from farmer’s fields were associated with sorghum plant performance under drought conditions and may be a potential beneficial endophyte. The term “endophyte,” originally introduced by De Bary (1866), refers to any organisms occurring within plant tissues, distinct from the epiphytes that live on plant surfaces. Carroll (1986) defines endophytes as mutualists, those fungi that colonize aerial parts of living plant tissues and do not cause symptoms of disease. Therefore, latent pathogens known to live symptomlessly inside the host tissues and organisms that have an epiphytic phase in their life cycle are also endophytes (Schulz and Boyle, 2005, 2006). Endophytes are thought to play multiple physiological and ecological roles in the mutualistic association with their host plants (Ilis et al., 2017). These symbiotic associations are characterized by the early formation of particular of organs and new tissues for the signaling and nutrient communications between plants and microorganisms (Hiruma et al., 2016; Zipfel and Oldroyd, 2017). Subsequently, considerable evidence indicated endophytic associations to be important for the plant immune system (Soliman et al., 2015), disease suppression (Terhonen et al., 2016), nutrient acquisition (Hiruma et al., 2016), plant fitness (Khare et al., 2018) and tolerance to abiotic stresses (Chagas et al., 2018; Shahzad et al., 2017; Silva, 2017). Many endophytes are known to be an important source of secondary metabolites and plant hormones (Hardoim et al., 2015; Muria-Gonzalez et al., 2015; Teimoori-Boghsani et al., 2020) and have the potential to synthesize various bioactive metabolites that may be used as therapeutic agents against numerous diseases (Aharwal et al., 2016; Duan et al., 2019).

Morphological identification of Fusarium endophytic species was previously performed and several Fusarium spp. Including F. moniliforme (Gibberella thapsina), Fusarium subglutinans, Fusarium chlamydosporum, Fusarium proliferatum, Fusarium oxysporum, and Fusarium solani were identified (Bacon et al., 2001; Demers et al., 2015). Both morphological and molecular identifications are essential for elucidating the fungal species of fungus and establishing genetic relationships within species (Laura et al., 2010). Internal transcribed spacer (ITS) markers are successfully used for characterization of molecular or genetic diversity of many organisms including plants, fungi, and bacteria (Cros et al., 1993). Some ITS markers notably ITS2 are used as phylomarkers for detection of intra and interspecific relationships within populations (Banerjee et al., 2007; Lei et al., 2012) and for validation of species status (Dabert, 2006). The focus of the study is highly relevant as a follow up on our previous finding that many Fusarium spp. was significantly associated with well growing young plants of sorghum in Burkina Faso (Zida et al., 2014). This study actually identifies the benefit of Fusarium endophyte species associated in performing plant used as specific PCR primer set of Fusarium spp. by amplification of the ITS2 region. The research also established phylogenetic relationship of the 32 endophytic Fusarium spp. identified.

MATERIALS AND METHODS

Site of sorghum tissues collection in Burkina fields

Sorghum plant tissues were collected in farmer’s fields in Burkina Faso. A total of 9 sites under two agro-ecological zones; the sahelian zone with an average annual precipitation ranging from 300 to 600 mm and the north sudanian zone (Soudano-Sahelian) with 600 to 900 mm precipitation were considered (Figure 1). In each site, 5 fields arbitrary chosen were investigated for sorghum plant tissue (leaves, stems and roots) sampling. Sorghum tissues were collected during two sampling periods, first sampling (S1) and the three leaves stage) and harvested sampled collected at maturity (S2). Two types of plants divided into performing plant (PP) and non-performing plants (NP) were collected according to their vigor and behavior to drought in farmers’ fields. For each field, 10 plants, 5 performing plants and 5 non-performing plants were considered for tissues sampling.

Fungal endophyte isolation and morphological identification

Sorghum endophytic fungi were isolated according to the protocols described by Petrini (1992). Sorghum leaf, stem and root tissues were cut into 12 to 15 mm pieces. The fragments were surface sterilized in 70% ethanol (v/v) for 1 min, immersed in sodium hypochlorite (NaOCl) 3% for 4 min and, then in 70% ethanol for 30 s and finally washed three times successively in sterilized distilled water. The growth media, potato dextrose agar (PDA) was used for fungal isolation. After drying under the laminar flow hood, pieces were transferred to Petri dishes containing autoclaved PDA previously aseptically supplemented with streptomycin in order to suppress bacterial growth. A total of 450 sorghum plants were investigated. Sorghum fragments (leaf, stem, and root) were plated in Petri dishes, 12 from each of the 450 investigated plants. Plates were incubated in darkness for 9 days at 28°C. Each

*Corresponding author. E-mail: glithiolpr@gmail.com.

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isolated fungus was placed into a new PDA culture without streptomycin and incubated at 24°C for 7 days under UV light for 12 h and darkness for 12 h. The identification of fungi was based on macroscopic and microscopic structures observed under the stereomicroscope and compared to compound and/or fungi identification manual published descriptions (Marthur and Kongsdal, 2003). Isolates of each fungal species identified were transferred to Eppendorf tubes containing 2 ml of sterile distilled water and stored at -20°C. The fungal isolates were brought to the Danish Seed Health Centre (DSHC, Denmark) for molecular identification, PCR and sequencing.

Isolates (32) of susceptible *F. moniliforme* were used for molecular characterization (Table 1). 200 µl of each isolate were retransferred to new PDA medium aseptically supplemented with streptomycin antibiotic and incubated at 24°C on a 12-h light/dark cycle for 5 to 7 days. One 5 mm diameter disk of each isolate were sampled and transferred into 50 mL potato dextrose broth (PDB) liquid medium. After 3 to 5 days of growth on orbital shaker, mycelia from each isolate were harvested by vacuum filtration and lyophilized until dry.

**Molecular identification: DNA extraction, amplification, sequencing and data analysis**

Mycelia from each isolate were ground in nitrogen liquid using mortar and pestle. DNA of each susceptible *F. moniliforme* was extracted with the Qiagen DNeasy Plant Mini Kit. To characterize *Fusarium* strains, *Fusarium*-ITS (FITS) primers, FITS-F2 (5'-ACCAGCGGAGGGATCATTAC-3') and FITS-R2 (5'-CTGGGGCAATCCCTGTTGGTT-3') provided by DSHC were used.

PCR was performed using a Master Cycler Gradient thermocycler. The PCR mixture total volume of 21.3 µl contained 1 µl DNA sample (10-100 ng DNA), 18 µl Buffer mix (860 µl MilliQ water, 100 µl Buffer 10X, 20 µl MgCl2 100 mM, 20 µl DNTP 10 mM), 1 µl of each FITS primer (10 pmol/µl), 0.3 µl Taq DNA polymerase (2.5 U/µl, Fermentas, EU). The PCR condition include 94°C for 5 min for initial denaturation, followed by 34 cycle of denaturation at 94°C for 1 min, primer annealing at 61°C for 1 min and extension at 72°C for 1 min. The final extension was set at 72°C for 10 min.

**Gel electrophoresis and bands analysis**

PCR products (5 µl) were analyzed on 0.7% agarose gel in Tris/Borate/EDTA electrophoresis buffer and stained with ethidium bromide solution (14 µl for 1 L of buffer). DNA ladder was used as molecular weight markers to determine the size of bands. After approximately 45 min at 100 mV, the gel was visualized and documented using the UTP-Bio Doc system. Data were analyzed by comparing FITS-2 profiles in terms of presence or absence of each reproducible DNA fragment.

Positive PCR products amplified by the FITS-F2 primers were purified by and desalted using QiAquick PCR purification kit (Qiagen). PCR products were cloned and sequenced using the Eurofins MWG Operon’s sequencing service (Eurofins Genomics LLC). The sequences corresponding to the 32 *Fusarium* spp. isolates were processed by the BLAST program integrated into the BioEdit Alignment software for the molecular identification.

**Phylogenetic reconstruction**

Sequence alignment was carried out using the ClustalW Multiple alignment and a phylogenetic tree was constructed using DARwin6.0.4 software (Thompson et al., 1994).

**RESULTS**

**PCR products analysis**

Electrophoresis and analysis of amplified PCR products

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**Figure 1.** Map of Burkina Faso showing sampling sites and agro-ecological zones.
Table 1. Identity and origin of susceptible *Fusarium* spp. strains used in this study.

| Accession number | Isolates No. | Tissues | Plant type | Localities | Agroecological zone |
|------------------|--------------|---------|------------|------------|---------------------|
| 1082/48.801      | 1            | Leaf.2  | PP*        | You        | Sahelian            |
| 1137/48.792      | 2            | Root    | PP         | Bani       | Sahelian            |
| 1136/48.791      | 3            | Leaf.2  | PP         | Bani       | Sahelian            |
| 1104/48.808      | 4            | Leaf.2  | PP         | Ouahigouya | Sahelian            |
| 1170/48.828      | 5            | Leaf.1  | PP         | Ouanda     | North soudanian     |
| 1090/48.805      | 6            | Leaf.2  | PP         | You        | Sahelian            |
| 1153/48.823      | 8            | Leaf.1  | PP         | Ipendo     | North soudanian     |
| 1087/48.804      | 9            | Root    | PP         | You        | Sahelian            |
| 1092/48.796      | 10           | Root    | PP         | Pobe Mengao| Sahelian            |
| 1143/48.795      | 11           | Stem    | PP         | Bani       | Sahelian            |
| 1066/48.827      | 12           | Leaf.2  | PP         | Ouanda     | North soudanian     |
| 1096/48.798      | 13           | Root    | PP         | Pobe Mengao| Sahelian            |
| 1139/48.793      | 16           | Stem    | PP         | Bani       | Sahelian            |
| 1090/48.805      | 18           | Stem    | PP         | You        | Sahelian            |
| 1122/48.813      | 20           | Stem    | PP         | Kouria     | North soudanian     |
| 1171/48.829      | 23           | Leaf.1  | PP         | Zorgho     | North soudanian     |
| 1176/48.833      | 25           | Leaf.1  | PP         | Zorgho     | North soudanian     |
| 1153/48.823      | 27           | Leaf.1  | PP         | Ipendo     | North soudanian     |
| 1069/48.825      | 28           | Leaf.1  | PP         | Dapelgo    | North soudanian     |
| 1117/48.812      | 30           | Leaf.1  | PP         | Kouria     | North soudanian     |
| 1149/48.821      | 32           | Leaf.1  | PP         | Ipendo     | North soudanian     |
| 1176/48.833      | 33           | Leaf.1  | PP         | Zorgho     | North soudanian     |
| 1153/48.823      | 34           | Leaf.1  | NP**       | Ipendo     | North soudanian     |
| 1082/48.801      | 35           | Leaf.2  | NP         | You        | Sahelian            |
| 1086/48.803      | 36           | Leaf.2  | NP         | You        | Sahelian            |
| 1082/48.801      | 37           | Leaf.2  | NP         | You        | Sahelian            |
| 1122/48.813      | 38           | Root    | NP         | Kouria     | North soudanian     |
| 1148/48.819      | 39           | Root    | NP         | Ipendo     | North soudanian     |
| 1139/48.793      | 40           | Stem    | NP         | Bani       | Sahelian            |
| 1122/48.813      | 41           | Root    | PP         | Kouria     | North soudanian     |
| 1103/48.807      | 43           | Stem    | PP         | Ouahigouya | Sahelian            |
| 1092/48.796      | 092          | Leaf.1  | PP         | Pobe Mengao| Sahelian            |

*Performing plant; **Non-performing plant.

revealed the presence of a polymorphic band corresponding to the 28S rDNA gene. The different sizes indicated the presence of 2 groups of fungi. The first group corresponds to the *G. thapsina*, *F. chlamydosporum*, *F. oxysporum*, *Fusarium proliferatum*, *Fusarium dlamini* and *Gibberella* species located at 400 bp. The second group with band size of approximately 380 bp corresponds to the *F. subglutinans* species (Figures 2 and 3).

**The species-specific sequence analysis and clone’s identification**

The present study aims to characterize thirty two isolates of susceptible *Fusarium* spp. species by using molecular approaches, identify the specific sequence of FITS-2 regions from isolates as markers and to establish the relationship between these fungal strains. Majority of the isolates (68.75%) were identified as *G. thapsina*. Only, 12.5% of the isolates were identified as *F. subglutinans*. The six other species identified have each one isolate. Eight specific sequences corresponding to the 8 *Fusarium* spp. identified have been reported in this work. These *Fusarium* spp. were benefit or pathogens to sorghum plant. FITS2 sequences length varies from 138 to 319 bp in *Fusarium* spp. and maximum length being 138 bp and minimum of 319 bp for *Fusarium intermedia* and *G. thapsina*, respectively. Table 2 shows different clone’s specific sequences of *Fusarium* species. Twenty five (25) clones of *Fusarium* have been associated to sorghum performing plants and considered as potential endophytes.

All of the 22 *G. thapsina* species have a common
specific sequence Seq1 in the genome region 1 (Gr1) as described in Table 2. This DNA region Gr1 is a promise for species specific primer designation in Fusarium spp. A second genome region (Gr2) distinguished two groups among the 22 G. thapsina isolates. The first group with 11 G. thapsina isolates has the sequence set GGGGTAC (Seq9) and the others 11 isolates of the second group do not show this sequence (Table 2). Therefore, the results also indicate that the second group of G. thapsina (none Seq9) from the first sampling period in leaf tissue (leaf1).

The sequence set Seq9 has also been identified in F. dlamini isolate. All of the F. subglutinans isolates and F. chlamydosporum species are characterized by the sequence set GGGACT (Seq10).

**Phylogenetic analysis using DNA sequencing of FITS**

Cluster analysis with FITS-2 profiles formed 3 groups at root 63% (Figure 4). Groups 1 and 2 are homogenous
group and consisting only of the isolates *G. thapsina* species. Group 3 is heterogenous and includes isolates from *G. thapsina*, *F. subglutinans*, *F. intermedia*, *F. oxysporum*, *F. chlamydosporum*, *F. proliferatum*, *F. dlaminii* and *Gibberella* spp. isolate. All of the *Fusarium* spp. analysed are used in this study as closely related and from the common ancestor. Phylogenic analysis and relationship indicate that the four isolates of *F. subglutinans* and *F. chlamydosporum* isolate formed a sub-group and belong to clade 2 (bootstrap value at 59%). Intraspecific diversity was observed among the species *G. thapsina* showed the highest level of intraspecific diversity by forming 3 groups (G1-G3).

Analysis of sequence identity matrix reveals a high penalty for closely related sequences. The sequence similarity analysis within *Fusarium* endophytes isolates indicates values ranging from 0.976 to 0.302. Thus, the highest degree of sequence identity was observed, respectively between the strains 12 Gt and 16 Gt with 0.976, 12 and 20 Gt (0.971), and 12 and 43 Gt (0.971). However, the lowest degree of most diversity was observed between strains 27 F.int. and 39 Gt (0.302). The analysis of sequence identity between *Fusarium sub.* strains ranged from 0.888 to 0.932 (Table 3). The results show the high relationship of 13 strains of *Fusarium* spp. among the 32 isolates.

### DISCUSSION

The current study provides strong evidence of existence of non pathogenic fungal endophytes in sorghum plant.
Figure 4. Molecular diversity of 32 isolates of Fusarium spp. strains obtained by using Neighbor-joining/UnWeighted Neighbor-Joining. DARwin6.0.4 software. 0.1 distance.

Table 3. Sequence Identity Matrix of 13 strains of Fusarium (among a total of 32 strains).

| Seq> | 1.Gt | 2.Gt | 3.Fs | 4.Fs | 5.Gt | 6.Gt | 8.Fc | 9.Gt | 10.Gt | 11.Gt | 12.Gt | 13.Fs | 92.Fs |
|------|------|------|------|------|------|------|------|------|-------|-------|-------|-------|-------|
| 1.Gt | ID   |      |      |      |      |      |      |      |       |       |       |       |       |
| 2.Gt | 0.948| ID   |      |      |      |      |      |      |       |       |       |       |       |
| 3.Fs | 0.685| 0.689| ID   |      |      |      |      |      |       |       |       |       |       |
| 4.Fs | 0.683| 0.721| 0.931| ID   |      |      |      |      |       |       |       |       |       |
| 5.Gt | 0.905| 0.947| 0.674| 0.705| ID   |      |      |      |       |       |       |       |       |
| 6.Gt | 0.911| 0.953| 0.663| 0.693| 0.937| ID   |      |      |       |       |       |       |       |
| 8.Fc | 0.644| 0.651| 0.846| 0.838| 0.633| 0.627| ID   |      |       |       |       |       |       |
| 9.Gt | 0.906| 0.937| 0.654| 0.683| 0.948| 0.96 | 0.614| ID   |       |       |       |       |       |
| 10.Gt| 0.9  | 0.92 | 0.663| 0.675| 0.904| 0.932| 0.623| 0.926| ID   |       |       |       |       |
| 11.Gt| 0.942| 0.959| 0.691| 0.695| 0.92 | 0.932| 0.648| 0.91 | 0.915| ID   |       |       |       |
| 12.Gt| 0.965| 0.959| 0.696| 0.693| 0.921| 0.927| 0.67 | 0.911| 0.911| 0.959| ID   |       |       |
| 13.Fs| 0.685| 0.679| 0.921| 0.932| 0.664| 0.654| 0.819| 0.655| 0.641| 0.668| 0.674| ID   |       |
| 92.Fs| 0.672| 0.668| 0.925| 0.888| 0.653| 0.643| 0.795| 0.645| 0.634| 0.661| 0.666| 0.92 | ID   |

Gt= G. thapsina, Fs= F. subglutinans, Fc= F. Chlamydosporum.

Use of fungal endophytes as beneficial bioresource to protect against plant-parasitic has previously been demonstrated (Terhomen et al., 2016; Pavithra et al., 2020). This study reveals differences between Fusarium spp. endophytes associated to sequence variability and plant type (performing and non performing plant). G. thapsina is known to be a seedling pathogen and cause of stalk rot and grain mold of sorghum (Kelly et al., 2017; Nor et al., 2019). The sequence set seq1 identified is a conserved region in G. thapsina rDNA independently to sorghum plant growth period. The sequence set seq9 identified in some G. thapsina isolate from sorghum leaves tissue in early plant growth may indicate a vertical transmission of the endophyte within the plant from leaf to grain in farmer’s field. Molecular phylogenetic relationships among plant pathogenic and nonpathogenic
Fusarium strains have been studied (Fourie et al., 2011; Imazaki and Kadota, 2015). F. thapsinum (F. moniliforme) is known to exist as an endophyte and a facultative pathogen transmitting both vertically as laterally (Bacon et al., 2001). G. thapsina is also known to produce gibberellin mycotoxin in sorghum (Klittich et al., 1997). In this study, G. thapsina (F. thapsinum) strains were identified as a major endophyte fungus associated to sorghum performing plants in field condition. The pathogenicity of G. thapsina to sorghum has not been tested under field conditions, but some strains can cause lesions in sorghum stalks under greenhouse conditions (Stokholm et al., 2016). In this study, four F. subglutinans endophyte with a specific band at 380 bp approximately in electrophoresis gel have been identified. These F. subglutinans endophyte formed a sub-group with F. chlamydosporum isolate and characterized by the sequence set seq10. This specific sequence were associated to performing plants of sorghum in field condition. The role of F. subglutinans as benefit endophyte has been demonstrated (Lee et al., 1995).

Many Fusarium endophytes possess antifungal properties that are useful against a number of plant pathogens in different plant system (Shah et al., 2019). Molecular characterization of the endophytic and biological control mechanisms of Fusarium has been reported (Imazaki and Kadota, 2015; Zhao et al., 2019). The role of Fusarium endophytes in many plant have been described (Ilic et al., 2017). For example, F. proliferatum, has been employed to control grapevine downy mildew caused by Plasmopara viticola (Bakshi et al., 2001; Mondello et al., 2019). F. proliferatum is considered a mycoparasitic, cold-tolerant fungus, capable of controlling the development of P. viticola via secretion of extracellular glucanolytic enzymes (Bakshi et al., 2001; Pancher et al., 2012). Endophytic colonization by the fungus F. oxysporum can result in increased host resistance to pests and diseases, and greater biomass production (Waweru et al., 2011).

In this study, eight species of Fusarium endophytes have been described based on rDNA sequence analyzing and phylogenetic relationship. However, there are few studies that have assessed their effect in the field. Further studies will be necessary to prove the ability of these Fusarium endophyte species and the environmental conditions required to actively infect and colonize sorghum and separate them from the saprophytes. Subsequent investigations also have to determine whether mycotoxins are produced in sorghum tissue by the different species because pathogenicity and mycotoxin production of sorghum derived Fusarium isolates was already proven (Zida et al., 2014).

Conclusion
The Fusarium ITS2 marker provides a powerful tool for studies of intraspecific variation and phylogenies of closely related species of G. thapsina endophytes. In this study, thirty two endophytic Fusarium spp. isolates were molecular identified. Thus, FITS sequences successfully differentiate the species and the different sizes of the amplified products confirm the presence of a codominant and specific FITS marker. This study reveals different between F. thapsina endophytes associated to sequence variability and also the necessity to characterize by molecular approach clones of Fusarium spp.

The Fusarium specific ITS markers (FITS) can be used for Fusarium pathogenic and beneficial endophytic species and associated disease detection. These markers can be used to support traditional identification of fungi or as an alternative approach and to facilitate pathogenicity tests which can be influenced by several biotic and abiotic factors. All of the specific sequences identified could be used for primers design in Fusarium endophyte identification.

CONFLICT OF INTERESTS
The authors have not declared any conflict of interests.

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