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Molecular Identification of Fusarium spp. Isolated from Wheat Grain Based on Sequencing of Internal Transcribed Spacer (ITS) Region

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Abstract

Molecular identification via Internal Transcribed Spacer region (nrDNA-ITS) sequencing of *Fusarium* spp. isolates from wheat grain originated from Stara Zagora region was performed for the first time in Bulgaria. A total of 60 wheat grain samples (*Triticum aestivum*) were preliminarily morphologically identified at the genus level as *Fusarium* spp. The rDNA-ITS region of all isolates was successfully amplified and the PCR products obtained were directly sequenced. After a comparison of detected sequences with the NCBI database, members of three different fungal genera (*Fusarium*, *Chaetomium*, and *Alternaria*) were identified. Among the *Fusarium* isolates, *F. tricinctum* was prevailing, followed by *F. poae*. A total of three isolates, *F. proliferatum*, *F. graminearum* and *F. equiseti*, were presented with a single probe. The shortest genetic distance (0.004) was detected between *F. tricinctum* isolates. On the basis of genetic distances, fungal isolates were grouped into two main clusters – one comprising *F. tricinctum* isolates and *F. proliferatum*, and the second including *F. equiseti*, *F. graminearum* and *F. poae*. It could be concluded that the rDNA-ITS genome region of the genus *Fusarium* may be used as a suitable marker for early detection, accurate and reliable identification of Fusarium spp. contamination of wheat. The timely and accurate information would assist in the selection of appropriate approaches for control of fusarium infections and possible mycotoxin contamination of agricultural products.

Keywords: *Fusarium* spp., *Triticum aestivum*, Internal Transcribed Spacer region (rDNA-ITS), DNA sequencing

Резюме

За първи път в България е извършена молекулярна идентификация на изолати *Fusarium* spp. от пшеница, произхождаща от региона на Стара Загора, чрез секвениране на ДНК-рибозомен вътрешен транскрибирщ спейсър регион (nrDNA-ITS). Направена е предварителна морфологична идентификация на гъбни изолати от общо 60 проби пшенични зърна (*Triticum aestivum*), до ниво род. Успешно е амплифициран ITS-региона на всички изолати морфологично определени като принадлежащи към род *Fusarium* и получените PCR продукти бяха подложени на директно секвениране. След сравняване на установените нуклеотидни последователности с база дзанни на NCBI, бяха идентифицирани щамове на три различни рода (*Fusarium*, *Chaetomium* и *Alternaria*). Между изследваните изолати на род *Fusarium* преобладава *F. tricinctum*, последван от *F. poae*. Три от видовете - *F. proliferatum*, *F. graminearum* и *F. equiseti* са представени с по един изолат. Най-ниски стойности на генетични дистанции (0.004) има установени между изолатите на *F. tricinctum*. Въз основа на генетичните дистанции, изолатите се групират в два основни клъстера – единият, включващ *F. tricinctum* и *F. proliferatum*, и втори, включващ *F. equiseti*, *F. graminearum* и *F. poae*. В заключение, може да се отбележи, че изследваният rDNA-ITS геномен регион от род *Fusarium* би могъл да се използва в качеството на подходящ маркер за ранно откриване, точна и надеждна идентификация на контаминирана с *Fusarium* spp. пшеница Навременната и точна информация би спомогнала при избора на подходящи подходи за контрол на фузариумните инфекции и възможно замърсяване с микотоксини на селскостопанската продукция.

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Introduction

Microscopic fungi from the genus *Fusarium* are an integral element of soil microflora but are also encountered in the underground and aerial parts of plants, plant debris and other organic substrates (Marasas *et al*., 1984; Burgess and Summerell, 1992). They induce numerous economically important diseases in crops, e.g. head blight in cereals, *Fusarium* root rot, corn cob fusariosis, etc., substantially reducing cereal crop yields and the quality of produce (Parry *et al*., 1995; Draganova *et al*., 2010; Beev *et al*., 2013). The combined effect of several *Fusarium* species, simultaneously infecting more than one crop and producing one or more mycotoxins, is an interesting example of biodiversity. During vegetation and storage, cereals could be attacked by multiple fungal species forming epiphytic microflora. *Fusarium* species, causing infections of cereal crops (corn, wheat, barley, oat, etc.), have a dominant position (Glen, 2007).

A plant may become infected with *Fusarium* spp. at any stage of its development, depending on both the plant species and the infecting species. Therefore, having in mind that most *Fusarium* species possess specific mycotoxigenic profiles, the early and accurate identification of *Fusarium* spp. is essential for predicting the potential risk of infection and prevention of mycotoxin occurrence along the food chain. The unambiguous identification of mycotoxigenic *Fusarium* species is still an exceptionally critical problem because the number of species in the genus is constantly changing according to various taxonomic systems. A considerable number of *Fusarium* spp. classifications have demonstrated a lack of uniform criteria for identifying species affiliation. Thus, various names are used for the same species, or different species are grouped under the same name. The issue is further complicated by the fact that *Fusarium* species are exceptionally variable and often mutate. Also, many toxigenic strains degenerate and lose toxin-producing ability when stored on artificial nutrient media in laboratory conditions. The information from species recognition criteria used so far – morphological, biological, and phylogenetic species recognition specific for a given region, rarely matches (Edel *et al*., 2001; Desjardins, 2006; Raja *et al*., 2017). The main approach to classification of *Fusarium* spp., which is still independently used, is the morphological approach although, in many instances, the information it provides could be misleading. The reason is the occurrence of some processes in fungi, such as hybridization, cryptic speciation and convergent evolution (Brun and Silar, 2010; Hughes *et al*., 2013). The independent application of the morphological recognition of microfungi is often limited to a number of morphological features. For example, cultures of some endosymbiotic fungi that routinely produce secondary metabolites do not exhibit spores. In other spore-forming species, a considerable variation in the shape and size of some asexual structures (conidia, spores) exists within the same strain (Ko *et al*., 2011). In these cases, the exact identification of species affiliation of the respective strain is very challenging. To avoid such limitations, it is advised to combine the morphological approach for identification of microscopic fungi with other contemporary methods of analysis, which would deliver accurate and consistent results. To this end, a number of mycologists recommend using methods based on DNA sequencing for identification of fungi with high genetic diversity level (Hibbett *et al*., 2011; Taylor and Hibbett, 2013).

The application of molecular techniques for species recognition of mold fungi dates back to more than two decades with nuclear ribosomal operon primers described by White *et al.* (1990) including three nuclear ribosomal genes encoding a large ribosomal unit (nrLSU-26S or 28S), a small subunit (nrSSU-18S) and an internal transcribed region functioning as a spacer (ITS1, 5.8, ITS2). The identification of molds on the basis of sequencing, also known as DNA barcoding, is based on the utilization of the nrDNA-ITS region. The meeting of the international consortium of mycologists held in 2011 in Amsterdam proposed 6 DNA regions from the fungal genome and one of them, namely the ITS region, was officially approved for a DNA barcoding marker (Schoch *et al*., 2012). Additional fungal studies have provided support for the ITS region as a suitable fungal barcode (Seena *et al*., 2010; Kelly *et al*., 2011). Moreover, most metagenomic studies of fungi use the ITS region in their studies to identify fungi using modern sequencing technology which can aid in the rapid identification of fungi (Nilsson *et al*., 2011; Wang *et al*., 2011; Ihrmark *et al*., 2012). This method for molecular genetic identification requires comparing the studied sequence to the respective reference sequence from a database, e.g. GenBank at the National Center for Biotechnology Information, the European Nucleotide Sequence Archive of the European Molecular Biology Laboratory, etc. (Cardinali *et al*., 2016; Yahr *et al*., 2016).

The ribosomal spacer has a number of advan-

tages which make it a convenient target region for molecular genetic identification of fungi, namely: 1) easy amplification with universal primers, complementary to gene sequences encoding ribosomal DNA molecules; 2) the ITS region is represented as multiple copies (over 200) in the genome as a mosaic of permanent (conserved) and diverse nucleotide sequences allowing for easy amplification even from small amounts of diluted DNA or degraded samples; 3) numerous studies demonstrated that the ITS region had a high degree of fungal interspecies variability (Nilsson *et al*., 2009; Blaalid *et al*., 2013). A report in the Journal of Natural Products, containing summarized data for the use of different methods for fungal analysis and the distribution of published research in 2010-2016 showed an increasing tendency for utilization of nrDNA-ITS as a marker of early fungal species recognition (42%), as compared to independently applied morphological (8%) and other methods (Raja *et al*., 2017).

The aim of the present study was to perform molecular identification of *Fusarium* spp. strains isolated from wheat grain originated from Stara Zagora region, Bulgaria, by mycological examination and sequencing of the nrDNA-ITS region.

Materials and Methods

Material for analysis

A total of 60 wheat grain samples were collected from 21 different locations in Stara Zagora region (lands of villages: Zagore, Badeshte, Pastren, Mogila, Sarnevo, Hrishteni, Dalboki, Oriacohvitca, Sabrano, Khan Asparuch, Malka Vereia, Rakitnica, Yavorovo, Vinarovo, Svoboda, Zetyovo, Spasovo, Sredetc, Znamenosetc, Merichlery, Galabovo) in the period from July to August 2018 at harvest-time, from storage facilities according to ISO 24333:2009. Briefly, 1 kg of incremental grain samples without any visible disease symptoms was taken with bulk profile sampler from 10 positions throughout the bulk consignment (amount of consignments varied from 5 to 500 tonnes). They were collected in a mixing vessel and after they had been thoroughly mixed a 10 kg bulk sample was formed. The amount of the final sample was 0.5 kg.

Isolation and identification of fungi by cultural methods

One hundred whole grains were collected from each sample after being superficially sterilized with 70% ethanol for 5 min, followed by three rinses with distilled water to eliminate surface microflora. Grains were placed in 4 Petri dishes (d=15 cm) (25 grains in each) on Czapek Dox medium (MERCK®). Inoculated plates were incubated at 22-25°C for 7 days (BDS 11374/86). After incubation, the number of grown colonies belonging to the genus *Fusarium* was calculated.

The affiliation of colonies to the genus level was done according to Pitt & Hocking (2009). All suspected *Fusarium* spp. colonies were subcultured, using the single spore technique (Pitt & Hocking,2009). A suspension of spores was prepared in a 10 ml sterile water blank so that it contained 1–10 spores per low-power (10X) microscope. The water agar medium plates were inoculated with 0.1 ml of suspension and incubated for 18-20 h at 25°C. For further molecular identification, the resulting single spore cultures were transferred onto a selective medium (SNA) (Nirenberg, 1976) and incubated at 25°C for 7 days.

Molecular methods

DNA isolation. Genomic DNA from Fusarium spp. strains was isolated from ingrown on SNA plates mycelium with diameter 1±2 cm, initially ground with quartz sand to degrade the polysaccharide cellular wall. For isolation of DNA from samples, the Plant DNA Preparation Kit (Jena Bioscience, Germany) was used according to manufacturer's instructions. The concentration of isolated genomic DNA in each sample was measured on a NanoView Plus (GE Healthcare) spectrophotometer at a wavelength of 260-280 nm.

For PCR amplification of the nrDNA-ITS region, the primer pair (Bioneer, Korea) proposed by White *et al.* (1990) was used. PCR reactions were run with genomic DNA amount of 200 ng. To this end, 1.5mM Red Taq Master mix reaction mixture (VWR, Denmark) with a total volume of 50 µl was prepared. Amplification was performed on a thermocycler VWR Doppio (General Electric, UK). The optimum annealing temperature of primers (52.5°C) was preliminarily determined by repeated PCR amplification of the same sample within the temperature range 49.3-60.7°C.

The PCR products obtained were identified by horizontal electrophoresis on 2% agarose gel diluted in 1×TBE buffer, at 80V/60 min. and were observed on MiniBis photo documentation system under UV light with GelCapture image acquisition software. The measurement of fragment length was verified with DNA marker (GeneRuler™, 50 bp., Fermentas).

Direct sequencing. A total of 12 samples (PCR products) were sequenced for the studied nrDNA-ITS region in the Bioneer research lab, South Korea. Sequences for each of the samples

provided by Bioneer were used for subsequent software processing. We also used Bioneer's oligo synthesis supports for the ITS custom primer.

Bioinformatic analysis. Identification of isolates was performed by comparing each sequence to a respective reference sequence in the NCBI database using Nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). DNA Sequence Assembler v4. (2013) was used for conversion of sequences from TXT to FASTA format. Sequences were edited, assembled and aligned by the CLUSTALW tool, using the MEGA7 v.0.26. software. The same software was used to determine genetic distances between the studied fungal isolates, and a phylogenetic tree was reconstructed by the Neighbour-joining method (Saitou and Nei, 1984; Tamura *et al*., 2004).

Results

Mycological examination analysis

Mycological examinations of 60 wheat grain samples harvested in 2018 yielded 12 strains belonging to *Fusarium* spp. The main mycoflora of wheat samples determined by morphological examination of the fungal isolates comprised 9 genera of microscopic fungi: *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., *Alternaria* spp., *Mucor* spp., *Rhizopus* spp., *Cladosporium* spp., *Rhizoctonia* spp. and *Nigrospora* spp. The contamination rate of individual samples with *Fusarium* spp. ranged from 0 to 4%, and rates for other genera (*Alternaria* and *Mucor*) varied within broader ranges. For the rest of genera, contamination rates varied insignificantly. It should be noted that in 2017-2018, a period characterized by low soil and air humidity during the vegetation of wheat, contamination with different members of the genus *Fusarium* was expectedly low. Out of the 60 studied wheat samples, 12 (20%) were positive for *Fusarium* spp., whereas the other 48 (80%) were negative. This was probably due to the agricultural activities performed during wheat vegetation, and host resistance to fungal diseases.

Data analysis from PCR amplification of fungal isolates

The rDNA-ITS region of 12 fungal strains isolated from wheat was successfully amplified by gradient PCR and fragments with the expected length were obtained (Fig. 1). The amplicon lengths for *Fusarium* isolates varied from 515 to 557 bp. The highest amplified product length was that of *Alternaria infectoria* – 578 bp, while that the length of *Chaetomium* spp. was 551 bp.

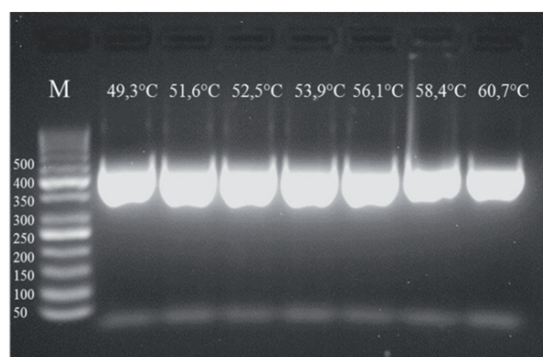

Fig. 1. M – Marker (DNA Ladder, 50 bp), Lanes 2-8 – PCR samples of nrDNA-ITS region in studied fungal isolates obtained at different annealing temperatures.

Data analysis from nrDNA-ITS region sequencing in Fusarium spp. samples

The analysis comprised 12 nucleotide sequences. The sequence alignment was done according to the CLUSTALW ***algorithm*** allowing for the elimination of all missing nucleotides (gaps). The fragment with a total of 12 aligned sequences (Fig. 2) clearly shows the nucleotide identity of 10 of the studied fungal isolates (samples 1, 2, 4, 5, 6, 7, 9, 10, 11 and 12) and nucleotide variation for samples 3 and 8. The nucleotide sequences were submitted in the GenBank under the following accession numbers: MN982764 for *Fusarium* spp., MN982876 for *Chaetomium* spp. and MN982879 for *A. infectoria*.

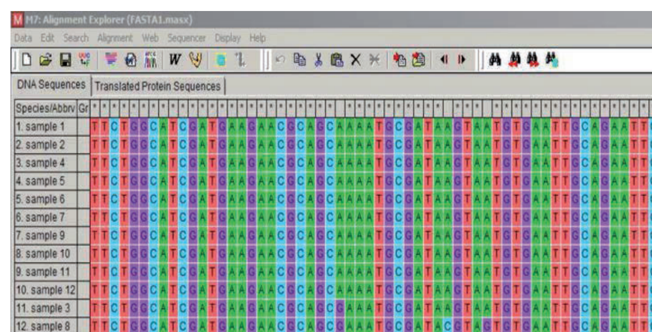

Fig. 2. Fragment with rDNA-ITS region sequences from 12 *Fusarium* spp. isolates in MEGA 7, aligned by CLUSTALW; *A, G, C, T – nucleotides.

Data analysis from the identification of fungal isolates

The identification of fungal isolates was performed by Nucleotide Blast in NCBI by comparing the studied sequences against reference sequences in the database. The species affiliation of the fungal isolates is presented in Table 1.

Table 1. Affiliation of fungal isolates on the basis of nrDNA-ITS region sequencing

Sample No	Species affiliation	Strain / isolate	Fragment size, bp	ID number in GenBank, NCBI
1	*F. tricinctum*	WS11790	535	JX406512.1
2	*F. poae*	5331	515	MG736211.1
3	*Chaetomium sp.*	C7-3	551	MG818934.1
4	*F. poae*	5331	515	MG736211.1
5	*F. tricinctum*	GMG105	534	KJ598871.1
6	*F. tricinctum*	WS11791	538	JX406511.1
7	*F. proliferatum*	JUF0013	557	MH368119.1
8	*A. infectoria*	A-1-6	578	KT223326.1
9	*F. graminearum*	FSHG	552	KU939070.1
10	*F. tricinctum*	WS11791	538	JX406511.1
11	*F. tricinctum*	WBS019	533	KU350729.1
12	*F. equiseti*	s079	523	HQ649909.1

Among the *Fusarium* spp. isolates, *F. tricinctum* was the prevailing species (n=5), followed by *F. poae* (n=2). *F. proliferatum, F. graminearum* and *F. equiseti* were represented by a single isolate, which agrees with previous information provided by Beev *et al*. (2011) about the presence of *F. equiseti* in Bulgarian wheat. Isolate No 3 was recognized as *Chaetomium* sp., and isolate No 8 – as *A. infectoria*. The identity achieved by the analysis is 99%, indicating high reliability of the applied method.

Comparative analysis of Fusarium isolates from wheat grain samples against their reference sequences obtained from NCBI

Genetic distances among studied fungal isolates were calculated by MEGA7 v.0.26 software on the basis of the number of nucleotide substitutions in the analyzed sequences. The shortest genetic distance (0.004) was demonstrated between the *F. tricinctum* isolates, followed by that between the *F. poae* isolates (0.007). The *F. proliferatum* strain, which was differentiated as an independent branch of the phylogenetic tree, was more genetically similar to *F. tricinctum* than to *F. poae,* while *F. equiseti* and *F. graminearum* exhibited higher genetic identity with *F. poae*. The cited values are depicted in Fig. 3. On the basis of genetic distance values, the dendrogram (Fig. 3) was reconstructed using the Neighbour-joining method.

The length of dendrogram branches is given with a figure corresponding to the genetic distances between the studied strains. Neighbour-joining – dendrogram was built on the basis of sequences of the rDNA-ITS region obtained for 10 Fusarium isolates from wheat samples against their reference sequences in the NCBI database. The dendrogram (Fig. 3) showed that fungal isolates were grouped into two main clusters – one comprising 5 *F. tricinctum* isolates and *F. proliferatum*, and the other including 3 isolates of *F. equiseti*, *F. graminearum* and *F. poae* strains.

Discussion

Different *Fusarium* species causing wilt diseases in plants have been also characterized by comparing the ITS region of nonpathogenic and pathogenic species via RFLP assay. For example, the ITS region of 12 *Fusarium* isolates belonging to the section *Elegans, Laseola, Mortiella, Discolor, Gibbosum, Lateritium* and *Sporotrichiella* have been amplified by Data *et al.* (2011) based on universal ITS primers (ITS-1 and ITS-4). The amplified products (ranging from 522 to 565 bp) were digested with seven restriction enzymes. A dendrogram derived from PCR-RFLP analysis of the rDNA region divided the *Fusarium* isolates into three major groups. In conclusion, the authors

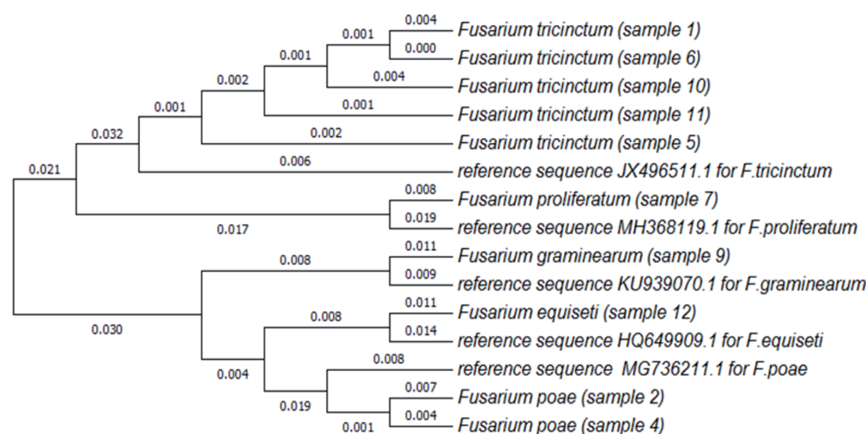

Fig. 3. Neighbour-joining – dendrogram built on the basis of sequences of the rDNA-ITS region of 10 *Fusarium* isolates from wheat samples compared against their reference sequences obtained from NCBI.

pointed out that the assessment of molecular variability based on rDNA RFLP clearly indicated that *Fusarium* species are heterogeneous and most of the *formae speciales* have close evolutionary relationships. In order to use the ITS molecular data to identify plant pathogenic form among *Fusarium* spp, several restriction enzymes specific for each special form were selected by Suda *et al.* (2000) from the mapping data. Schoch *et al.* (2012) pointed out that among the regions of the ribosomal cistron, the ITS region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intra-specific variation.

Conclusions

The results obtained in the present study affirm that the primary microflora of the examined samples comprise a total of 9 genera of microscopic fungi: *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., *Alternaria* spp., *Mucor* spp., *Rhizopus* spp., *Cladosporium* spp., *Rhizoctonia* spp. and *Nigrospora* spp. Among the Fusarium isolates, *F. tricinctum* was prevailing (n=5), followed by *F. poae* (n=2). *F. proliferatum*, *F. graminearum* and *F. equiseti* were represented by a single isolate. The trend for the occurrence of *F. equiseti* in wheat was confirmed. The shortest genetic distance (0.004) was detected between the *F. tricinctum* isolates. The *F. proliferatum* strain was separated as an independent branch of the phylogenetic tree, showing higher genetic similarity to *F. tricinctum* than to *F. poae*. The sequencing analysis allowed determination of phylogenetic relationships between the studied isolates, which formed 2 clearly distinct phylogenetic tree branches. The results clearly indicate that using the genomic rDNA-ITS region was suitable for early detection of wheat seedling contamination with a view to making future decisions and taking the appropriate action needed to prevent Fusarium infections.

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