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Molecular Identification and Phylogenetic Analysis of *Fusarium* spp. Associated with *Triticum aestivum* L. Based on DNA Barcoding of *β-tub* Gene

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Abstract

Fusarium spp. are active producers of mycotoxins that enter the food chain and pose risks to human health. Identifying pathogenic agents is a key step in developing disease management strategies. For the first time in Bulgaria, we identified *Fusarium* species in wheat, harvest 2024÷2025, through the application of DNA barcoding. For genetic marker and construction of phylogenetic tree, the protein-coding gene β-tub was chosen. Among 26 identified isolates, *F. sporotrichioides* (42.3%) dominated, followed by F. proliferatum 23.1%), *F. avenaceum* (7,7%), *F. armeniacum* (7.7%), and *F. poae* (7,7%). *F. tricinctum* (3.8%), *F. oxisporum* (3.8%), and *F. equiaseti* (3.9%) were weakly expressed. Phylogenetic analysis classified the isolates into 5 species complexes: FSAMSC, FFSC, FTSC, FIESC, and FOSC and highlighted the genetic distances between them. Molecular genetic analysis showed that 84.6% of the wheat samples contained only one species of *Fusarium*, and in 15.4% the co-presence of two species was established. The largest share was in samples with low infestation 2÷4%, which represented 35% (n=32) of all positives. No statistically significant difference was found between the varieties and the level of contamination, as well as between the origin of the selected varieties and the level of contamination.

Keywords: *Fusarium* spp.; wheat; gene *β-tub;* DNA barcoding; phylogenetic analysis

1. Introduction

Fusarium spp. is a cosmopolitan genus of ascomycete fungi, numbering more than 300 species of important economic and ecological importance [Mirghasempour, et al. (2022).]. Although *species* perform a variety of ecological roles: saprophytes, endophytes, symbionts, they are among the best-known pathogens of wheat and cause serious diseases with huge financial losses in the agricultural sector (Ekwomadu and Mwanza, 2023). These adaptive micromycetes are able to colonize roots, stems, leaves, ears and grain. Soil, plant residues and seeds serve as the main sources of inoculum, and chlamydospores can survive in the soil for years, providing initial outbreaks for subsequent infections. in which two or more species can infect the same crop at the same time. Species *F. graminearum, F. culmorum, F. avenaceum, F. poae, F. sporotrichioides, F. equiseti, F. verticillioides, and F. proliferatum* are the predominant species that infect wheat and cause fusariosis by classes (FHB) и *Fusarium* crown rot (FCR) in Europe and Bulgaria including (Beev, 2013.,Rauwane, 2020; Alisaac, E., & Mahlein, A. K. (2023). In Canada and Italy, the dominant pathogen of durum wheat is *F. avenaceum*, with *F. graminearum* present in a secondary role (Tittlemeier et al., 2013; Beccari et al., 2018). Worldwide, the distribution of *Fusarium* spp. in wheat shows significant regional variations related to climatic conditions, crop rotation and specific agrotechnical practices. For example, the combination "maize-wheat" increases the risk of pathogen transmission between crops, especially *F.*

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verticillioides and *F. graminearum*, which creates favorable conditions for the development of FHB in wheat (Liu et al., 2022). Some Fusarium species are active producers of mycotoxins: trichothecenes, fumonisins, zearalenone, eniatin, etc. According to Perochon A and Doohan FM. (2024), these secondary metabolites facilitate the development of cereal diseases during pathogenesis. On the other hand, *Fusarium* mycotoxins accumulate in harvested produce and can have carcinogenic, neurotoxic, mutagenic, teratogenic, estrogenic, hepatotoxic and immunosuppressive effects on animal and human health (Taheur et al., 2019).

Classical identification methods based on morphological characteristics (colony appearance, color and growth rate of mycelium, observation of macroconidia, microconidia, chlamydospores and spore-producing structures (Burgess et al., 1994; Hyde at al., 2010] are seriously hampered by the limited informativeness of the variability of the characters and have difficulty distinguishing species with related morphological characteristics (Leslie and Summerell, 2008).

With the advancement of molecular techniques, DNA barcoding technology has been developed, where species can be identified through variations in short gene sequences (so-called barcodes) located in a standardized genetic locus (specific gene or region) of the studied sample, highly variable at the species level but conservative at the genus level (Guo et al., 2022).

Although the non-coding region ITS was recognized as a major barcode marker for fungi by the International Consortium of Mycologists in Amsterdam, 2011 (Schoch et al., 2012) due to its critical biological role in rRNA and ribosome maturation (Hausner and Wang, 2005), it is not always a reliable tool for *Fusarium* identification (Nilsson et al., 2009; Blaalid et al., 2013).

According to Stielow et al. (2015), unlike other fungal genera, the use of the ITS genetic locus for identifying representatives of the genus Fusarium is very difficult and can be reliably applied only in about 75% of all species. In the search for suitable genetic loci for the genus, protein-coding genes play an increasingly important role in molecular identification due to their high informativeness. As such genes, the gene encoding β-tubulin (β-tub), tef-1α, RNA polymerase (RPB2), etc. are widely used. (Al-Hatmi et al., 2016).

In filamentous fungi, the number of genes in the β-tubulin locus varies between genera and species. The β-tub gene encodes the β-tubulin subunit and includes several exons separated by introns, the location and length of which can vary between species. The combination of conserved and variable regions determines the potential of the β-tub gene and makes it a suitable and reliable marker for species identification and phylogenetic analyses. Within the genus *Fusarium*, the β-tub gene shows significant species differentiation, allowing its use to precisely distinguish closely related taxa. Many ascomycetes possess only one *β-tub* gene, but in F. graminearum, β-tub1 and β-tub2 stand out with different functions during vegetative growth and sexual reproduction, but with common roles in hyphal growth (Li et al., 2019).

The primers for the *TUB* gene were designed by Annis and Panaccione (1998) and were originally designed to cover flank intron 3 of the *Neotyphodium* spp. (Tsai et al. 1994). Subsequently, Tooley et al. (2001) designated the BT5 and BT3 primers for characterization of pathogenic *Claviceps* spp. on sorghum (*Sorghum bicolor*), which are reliably applied for species identification within the genus *Fusarium* [Liu, J.et al (2011); Stoeva, D., et al. (2024).

The aim of this study is to identify *Fusarium* spp. species distributed in the Bulgarian common wheat population by applying the DNA barcoding method based on the β-tub region, to clarify the phylogenetic relationships between the species, as well as to establish the influence of the wheat variety on the level of contamination with *Fusarium* spp.

2. Material and Methods

Sample Collection

A total of 101 bread wheat (*Triticum aestivum* L.) symptomless samples were collected during the period June 2024–August 2025 from different main commercial wheat-growing regions in Bulgaria (Figure 1.) from storagefacilities and according to ISO 24333:2009 (Bulgarian Institute for

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Standardization: Sofia, Bulgaria, 2009). The collection includes 59 samples of imported varieties, 28 samples of Bulgarian selected varieties and 14 samples without information on the varietal affiliation. Briefly, 1 kg spot samples of maize with novisible disease symptoms were taken with a bulk profile sampler from 10 points throughoutthe bulk lot (lots ranged from 5 to 100 tons). Multiple samples from appropriate sites wereaseptically poured into a sterile container and manually mixed to obtain a homogeneouscomposite sample. Following their complete mixing, a bulk of samples weighing 10 kg wasformed and manually reduced to samples of 0.5 kg, which were then labeled. Sampling atstorage facilities was chosen to focus on *Fusarium* spp. that persist post-harvest and presenta direct risk to food and feed safety. This strategy reflected the study's aim of assessing contamination under realistic storage conditions. While field sampling may reveal broaderpre-harvest fungal diversity, post-harvest sampling provides relevant insights into thefungal populations that are most likely to enter the food chain.

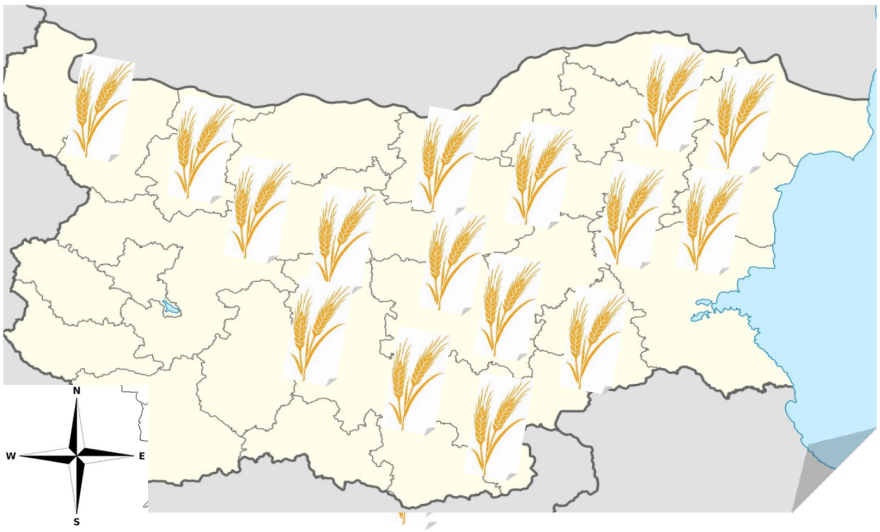

Figure 1. Samples collected from main commercial wheat growing regions in Bulgaria.

Fungal Isolation of Fusarium spp.

One hundred whole seeds from each sample were randomly selected and superficially sterilized with 70% ethanol for 6 min, followed by three rinses with distilled water to eliminate surface microflora. The isolates were dried on a sterile paper towel, and plated onto potato dextrose agar (PDA) (HiMedia©, Maharashtra, India) in 4 Petri dishes (d = 15 cm) (25 seeds in each) for 7 days at a constant temperature of 25 °C and a photoperiod of 16 h/8 h (day/night)[(Bulgarian National Standard (BDS), 1986). After which the number of growing colonies belonging to *Fusarium* spp. was counted. The identification of *Fusarium* spp. was done using keys by Burgess et al. (1992), Gerlach and Nirenberg (1983), and (Leslie and Summerall, 2006). All suspected *Fusarium* spp. colonies were subcultured using the single spore technique (Nirenberg, H. I., 1981; Aboul-Nasr et al., 2014). A spore suspension was prepared in a 10 ml sterile water sample so as to contain 1 to 10 spores. The prepared water agar medium (WA) plates were inoculated with 0.1 ml of suspen-sion and incubated for 18-20 h at 25 °C. For further molecular identification, the resulting single spore cultures were transferred onto synthetic nutrient-poor agar (SNA) and incubated at 25 °C for 7 days to initiate spore formation (Gerlach and Nirenberg, 1983). Each isolate's spores were examined under a micro-scope to identify distinctive characteristics unique to *Fusarium* spp.

Fusarium Species Identification

DNA barcoding stages for *Fusarium* species identification in this study are presented on Figure 2.

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Figure 2. Cultured wheat grains with germinated colonies on PDA medium.

DNA Extraction, PCR Amplification and Sequencing

A 5-day-old mycelium of *Fusarium* spp. strains of SNA plates was used for genomic DNA isolation. Initially, to lysate the fungi's polysaccharide cell wall, the mycelium was frozen for 24 hours at -20 °C and then pulverized with quartz sand. DNA extraction from mycelium of monosporic *Fusarium* cultures was performed by using ,,Animal and Fungi DNA Preparation Kit" (Jena Bioscience GmbH, Jena, Germany) according to the manufacturer's instructions. The concentration of the extracted genomic DNA of each sample was measured using a NanoView Plus spectrophotometer (GE HealthCare Technologies, Inc. Chicago, USA) at a 260–280 nm wavelength. The DNA concentration of all samples was adjusted to 10 ng/µl, in a working volume of 70 µl, and the genomic DNA-extracted samples were stored at -18 °C until further analysis. The optimal annealing temperature was determined based on gradient PCR in the temperature range 51.1-61.2 °C. The following primers were used for amplification: region *β-tub*: F:5'-CGTCTAGAGGTACCCATACCGGCA-3' and R:5'-GCTCTAGACTGCTTTCTGGCAGACC-3' [Tooley et al., 2001][i]. The DNA concentration of all samples was adjusted to 10 ng/µl, in a working volume of 70 µl, and the genomic DNA-extracted samples were stored at -20 °C until further analysis. PCRs were performed in 25 µl volumes containing 2 µl of the extracted DNA, 12.5 µl 2x Master Mix (VWR International BV, Leuven, Belgium), 1 µl of each primer (FOR/REV), and 8.5 µl of nuclease-free water. *β-tub* gene was amplified using the following cycling parameters: 95 °C for 5 min, 30 cycles of 95 °C for 30 s, primers annealing at 51.1 °C for 0,45 min, and 72 °C for 1 min, a final extension at 72.0 °C for 9 min followed by a 4 °C soak in a thermal cycler (QB-96 Thermal Cycler, Quanta Biotech Ltd. Surrey, United Kingdom). Amplicons were size fractioned alongside a 100-bp molecular weight marker (Gene-Ruler™ Ladder Plus, Thermo Fisher Scientific Inc. Waltham, MA, USA) in 1% agarose gels in 1× TAE buffer, stained with 10,000×GelRed™ (Cat. № 41003, Biotium Inc. Flermont, USA), and visualized on a MiniBis photodocumentation system using a transilluminator (ECX-15M Bio Imaging Systems, Bio-Imaging Systems, Inc. Jackson, USA). Sequencing reactions were purified using GeneMATRIX Short DNA Clean-Up Purification Kit (Cat. No. E3515, EURx Ltd. Gdansk, Poland) and sequenced in both directions by a PlateSeq kit (Eurofins Genomics Ebersberg, Gdansk, Germany).

All obtained sequence data were manually edited and aligned using the MUSCLE algorithm [Edgar, 2004][ii] in the MEGA software v. 11.0.13 [Tamura, 2021][iii]. To identify *Fusarium* spp. among the generated sequence data, the Basic Local Alignment Search Tool (BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used.

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3. Results

Based on the mycological analysis of the samples studied, it was found that *Fusarium* spp. was identified in the epiphytic microflora of 82.2% (n=83) of the samples studied, with the degree of contamination in individual samples varying between 0÷46%. The average value for 2024 was 7.2% ± 8.30, and for 2025—5.5% ± 2.80. The largest share, 35% (n=32), was occupied by samples with relatively low levels of contamination between 2 and 4%, followed by samples with 5÷6% content of *Fusarium* spp. (17%) (n=16). In samples of wheat variety Factor from the Dobrich region, the highest levels were found (46%)—2024 and variety Nikolay, harvest 2024, Sadovo region (38%). According to the conditions of the experiment, the influence of the wheat variety on the contamination with *Fusarium* spp. for the two research years 2024 and 2025 (Table 1).

Table 1. Descriptive statistics on the contamination with *Fusarium* spp. of different varieties of common wheat grown in Bulgaria.

Variety	Number of samples	Mean, %	Standard deviation, %	Coefficient of variation,%	Minimum	Maximum
Avenue	21	3,85	3,27	84,9	0,0	11,0
Armstrong	2	5,50	3,53	64,2	3,0	8,0
Annapurna	4	6,00	3,36	56,1	4,0	11,0
Apilko	6	7,00	4,81	68,8	1,0	13,0
Boryana	2	5,50	4,95	89,9	2,0	9,0
Gizda	2	7,00	2,83	40,4	5,0	9,0
Enola	5	5,20	3,70	71,1	0,0	9,0
Pibrak	4	6,50	9,81	150,9	0,0	21,0
Sadovo 1	2	**3,00**	4,24	141,4	0,0	6,0
Sashec	2	4,00	5,66	141,4	0,0	8,0
Silverio KWS	2	**15,00**	11,3	75,42	7,0	23,0
Sobel	4	9,75	7,81	80,0	3,0	21,0
Sofru	11	5,36	7,16	133,4	0,0	25,0
Terres	2	2,50	3,53	141,4	0,0	5,0
Factor	6	9,50	17,93	188,7	0,0	46,0

The influence of the factors "varietal affiliation" and "origin of selected varieties" of wheat (Bulgarian/foreign) grown in Bulgaria on contamination with *Fusarium* spp. Data from the one-factor analysis of variance (ANOVA) showed a lack of statistically significant influence ($p>0.05$).

Results of Molecular Identification of Fusarium spp.

After purification of the amplification products (500 bp), 26 representative isolates were selected for sequencing by the Sanger method. Analysis of the sequences using the *β-tub* DNA barcode revealed nine *Fusarium* species: *F. sporotrichioides*, *F. proliferatum*, *F. poae*, *F. armeniacum*, *F. equiseti*, *F. tricinctum*, *F. avenaceum* and *F. oxisporum* (Figure 3 and 4.).

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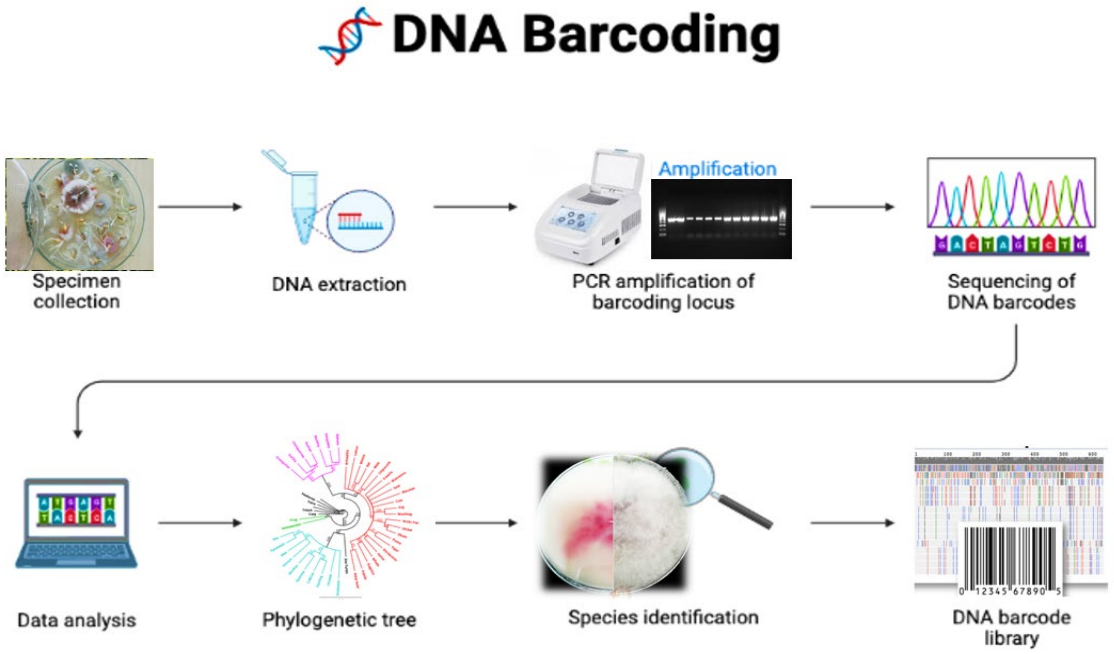

Figure 3. DNA barcoding stages for *Fusarium* species identification in this study.

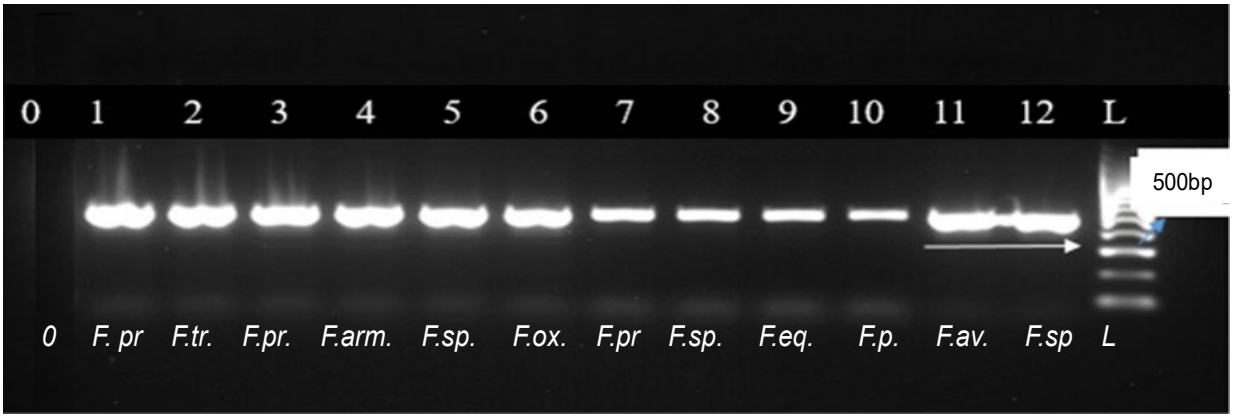

Figure 3. Agarose gel electrophoresis (1%) used for separation of the different PCR products. Lanes labeled "0" correspond to blank control; 1-12: *F. pr.- F. proliferatum, F.tr. -F. tricinctum, F.arm.—F. armeniacum F.sp.—F. sporotrichioides, F. oxysporum, F.eq.- F equiseti, F.p.- F. poae, F.av.—F. avenaceum.*

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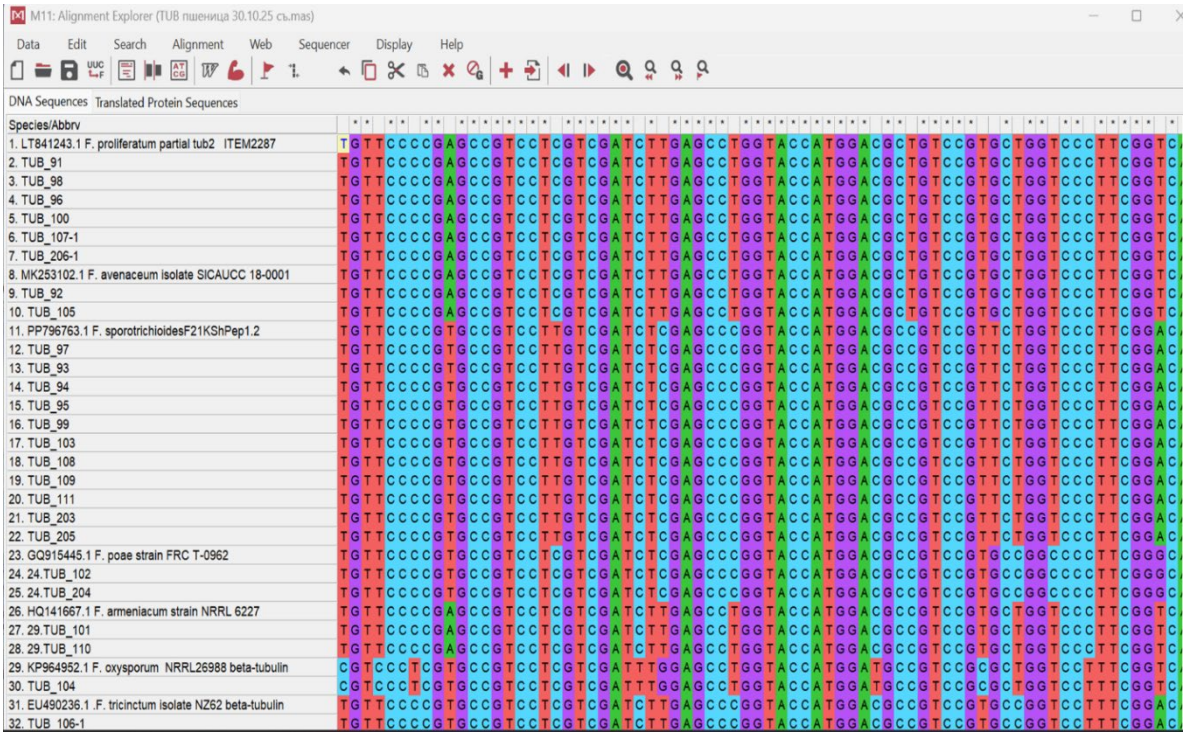

Figure 4. Fragment of aligned and compared sequence data of region *β-tub* from *Fusarium* spp. isolates, with reference. *C, G, T, A-nucleotides.

Sequence analysis by the β-tub barcode revealed eight *Fusarium* species: *F. sporotrichioides*, *F. proliferatum*, *F. avenaceum*, *F. armeniacum*, *F. poae*, *F. equiseti*, *F. tricinctum* and *F. oxysporum* with varying degrees of presence (Table 2, Figure 5).

Table 2. Species identification of *Fusarium* isolates according to the DNA barcode *β-tub*.

№	Growing region	Variety	Harvest	ID	Identified species
1	Svilengrad	Sobel	2024	91	*F. proliferatum*
2	Ivaylovgrad Avenue	Avenue	2024	92	*F. avenaceum*
3	Ivaylovgrad Avenue	Avenue	2024	93	*F. sporotrichioides*
4	Ruse	Sobel	2024	94	*F. sporotrichioides*
5	Sadovo	Gizda	2024	95	*F. sporotrichioides*
6	Sadovo	Gizda	2024	96	*F. proliferatum*
7	Sadovo	Nikolay	2024	97	*F. sporotrichioides*
8	Nova Zagora Avenue	Avenue	2024	98	*F. proliferatum*
9	Kyustendil Annapurna	Annapurnaa	2024	99	*F. sporotrichioides*
10	Karlovo	Apilko	2024	100	*F. proliferatum*
11	Dobrich	Factor	2024	101	*F. armeniacum*
12	Dobrich	Pibrak	2024	102	*F. poae*
13	Dobrich	Apilko	2024	103	*F. sporotrichioides*
14	Sliven	Sofru	2025	104	*F. oxysporum*
15	Dobrich	Pibrak	2025	105	*F. avenaceum*
16	Sliven	Avenue	2025	106	*F. tricinctum*
17	Chirpan	Gizda	2025	107	*F. proliferatum*
18	Nova Zagora Armstrong	Armstrong	2025	108	*F. sporotrichioides*
19	Plovdiv	Pibrak	2025	109	*F. sporotrichioides*
20	Vidin	Avenue	2025	110	*F. armeniacum*

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21	Silistra	Diamond	2025	111	*F. sporotrichioides*
22	Stara Zagora Avenue	Avenue	2025	112	*F equiseti*
23	Vratsa	Avenue	2025	203	*F. sporotrichioides*
24	Plovdiv Armstrong	Armstrong	2025	204	*F. poae*
25	Silistra Annapurna	Annapurna	2025	205	*F. sporotrichioides*
26	Silistra	Sofru	2025	206	*F. proliferatum*

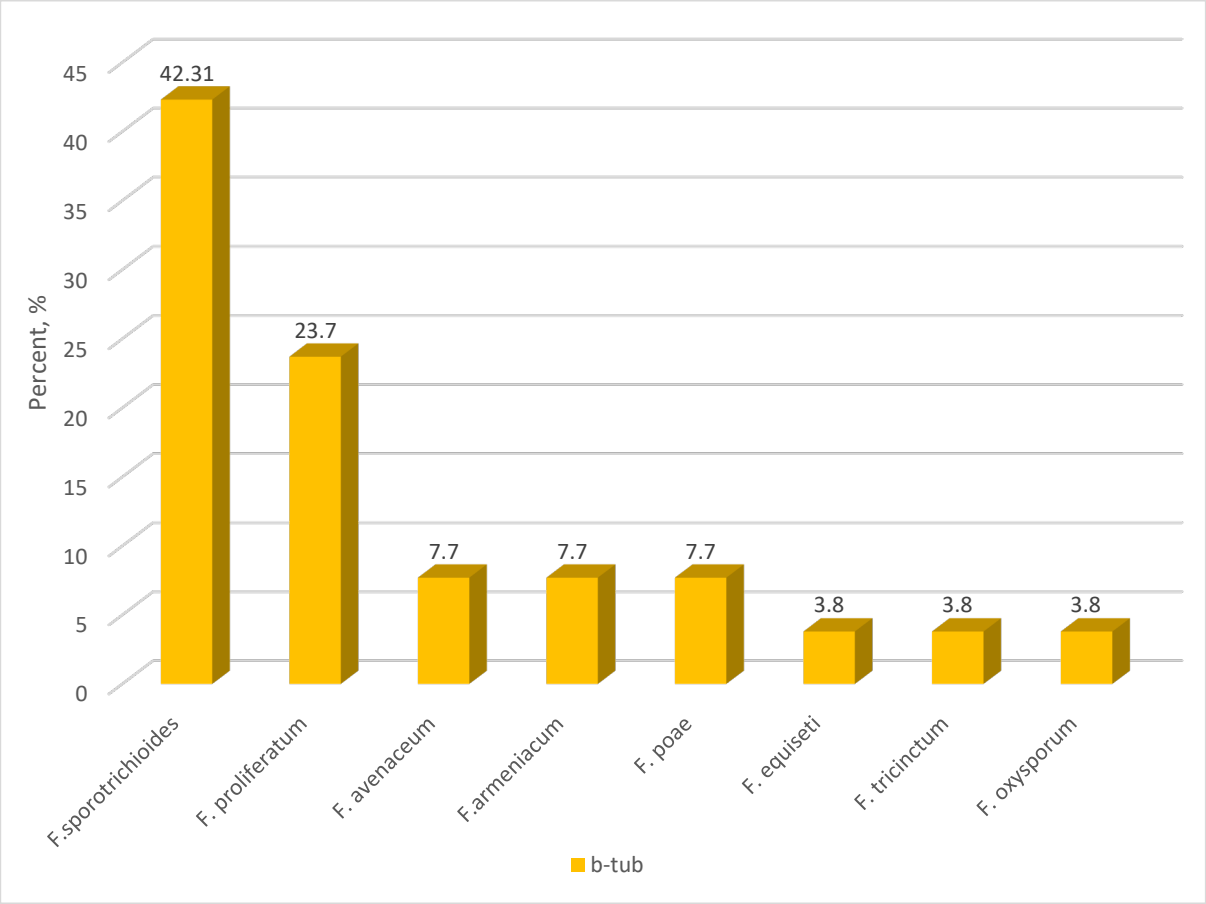

Figure 5. Percentage distribution of molecularly identified *Fusarium* species based on b-tub barcoding.

Molecular genetic analysis showed that 92.2% of the wheat samples contained only one Fusarium species, and in 7.8% (n=4) the co-presence of two species was established. In the samples from the Ivaylovgrad region, Avenue variety and the Sadovo region, Gizda variety, F. avenaceum/F.sporotrichioides; F. sporotrichioides/F. proliferatum were isolated and identified. In the samples from the Ivaylovgrad region (No. 2 and 3), Avenue variety and the Sadovo region (No. 5,6), Gizda variety, F.avenaceum/F.sporotrichioides; F. sporotrichioides/F. proliferatum were isolated and identified (Table 2).

The results of the analysis show that in Southern Bulgaria the species diversity is wider (7 species), compared to Northern Bulgaria (five species). *F. equiseti*, *F. oxysporum* and *F. tricinctum* are found only in Southern Bulgaria, while in Northern Bulgaria the species *F. armeniacum* is found (Figure 6).

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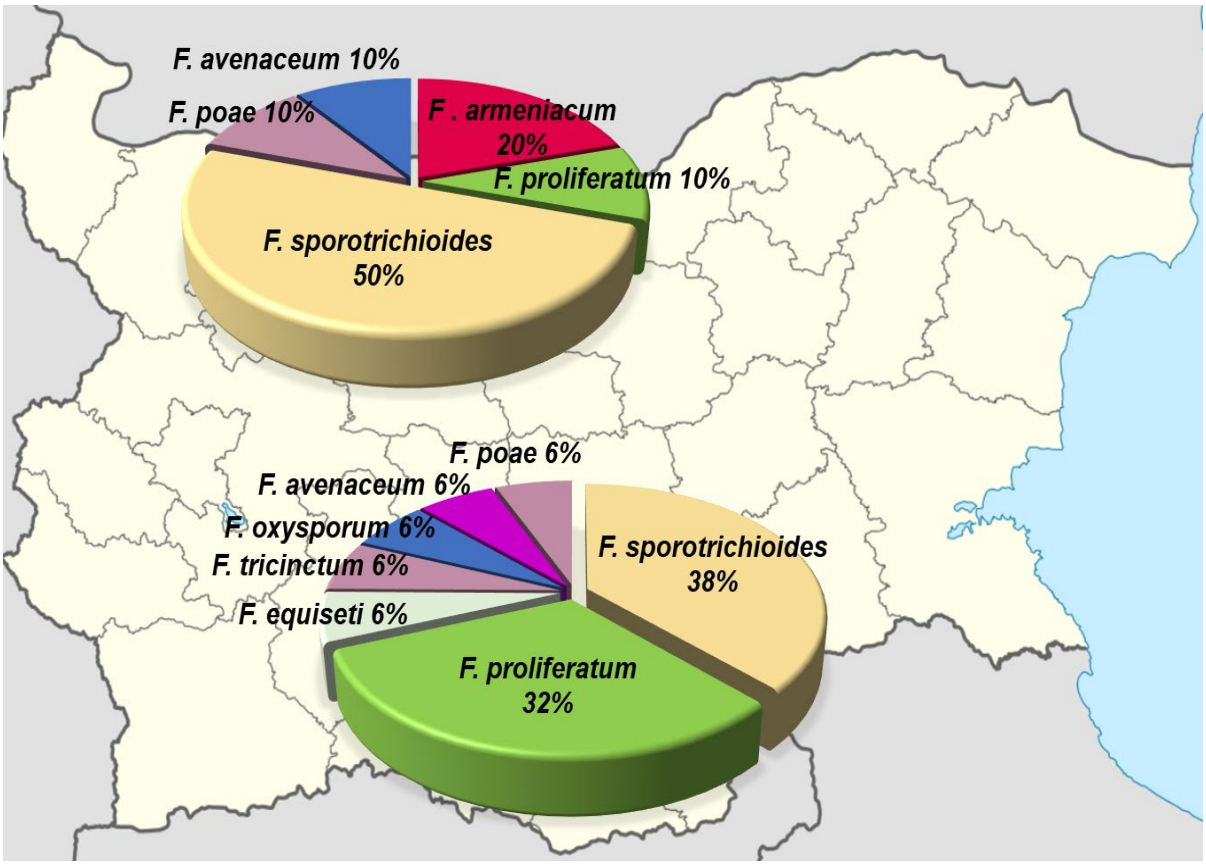

Figure 6. The distribution of the species diversity of *Fusarium* in Southern and Northern Bulgaria.

Phylogenetic Analysis of the Identified Fusarium spp.

Phylogenetic analyses based on *β-tub* gene sequences further separated the 17 isolates into four Fusarium species complexes FSAMSC, FFSC, FIESC and FOSC. The isolates *F. sporotrichioides* and *F. Poae*—members of the FSAMSC complex are grouped in a separate cluster. The isolates of *F. proliferatum*, showing close relationship and clearly distinguished as members of FFSC, are in a separate group, and *F. oxysporum* are separated in a separate cluster. The isolates of the species *F. armeniacum* are separated in a separate subcluster, while *F. equiseti*, *F. tricinctum F. avenaceum* are scattered throughout the tree.

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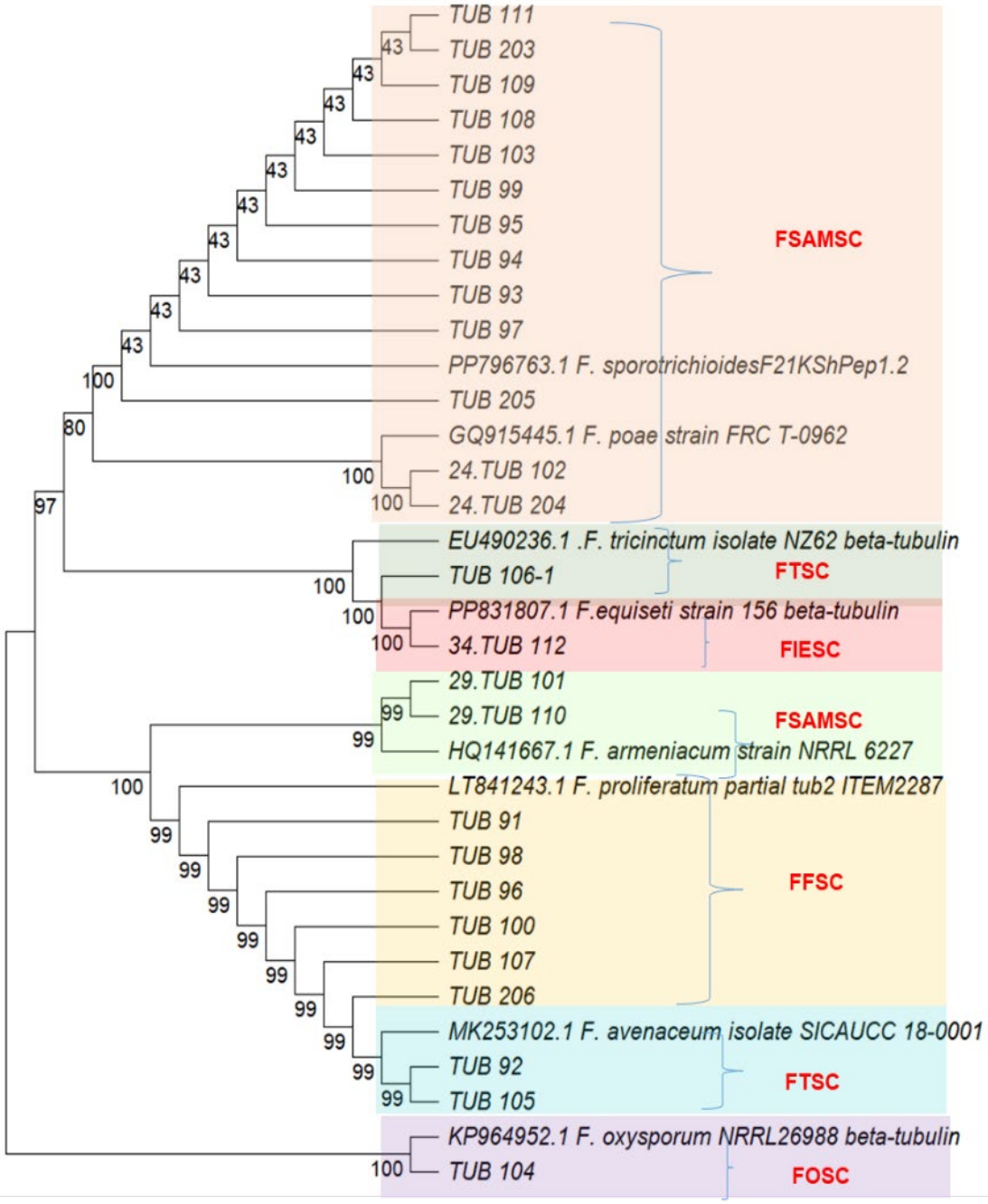

Figure 7. Phylogenetic analysis of *Fusarium* spp. based on the sequence analysis of the protein-coding gene β-tub in the MEGA 12.0 software [Kumar, 2024]. A phylogenetic tree was inferred through the maximum likelihood approach, applying the Tamura (1992) three-parameter substitution model combined with a discrete gamma distribution (TN93 + G) [32]. Support for branches was evaluated using 1000 bootstrap replicates, with bootstrap values of 50 or higher shown at the respective nodes. Positions with gaps or missing data were removed entirely (complete deletion method). Each sequence is labeled with the organism's name followed by its GenBank accession number. The following color scheme was used: *F.equiseti* /FIESC—red; *F. avenaceum* /FTSC—blue; *F. tricinctum* /FTSC– dark green; *F. armeniacum* /FTSC– light green *F. sporotrichioides* and *F.poae* FSAMSC—orange; *F.proliferatum* /FFFC- yellow; F.oxysporum/FOSC—purple.

Evolutionary Analysis by the Maximum Likelihood Method

The phylogeny was inferred using the Maximum Likelihood method and Tamura (1992) model [1] of nucleotide substitutions and the tree with the highest log likelihood (-1 534.17) is shown. The

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percentage of replicate trees in which the associated taxa clustered together (500 replicates) is shown next to the branches [2]. The initial tree for the heuristic search was selected by choosing the tree with the superior log-likelihood between a Neighbor-Joining (NJ) tree [3] and a Maximum Parsimony (MP) tree. The NJ tree was generated using a matrix of pairwise distances computed using the p-distance [4]. The MP tree had the shortest length among 10 MP tree searches, each performed with a randomly generated starting tree. The evolutionary rate differences among sites were modeled using a discrete Gamma distribution across 5 categories (+G, parameter = 0.3006), with 0.00% of sites deemed evolutionarily invariant (+I). The analytical procedure encompassed 59 nucleotide sequences. The complete deletion option was applied to eliminate positions containing gaps and missing data resulting in a final data set comprising 433 positions. Evolutionary analyses were conducted in MEGA12 [5] utilizing up to 7 parallel computing threads.

4. Discussion

In the analysis of 101 samples of common wheat, harvest 2024-2025, an average value for contamination with microscopic fungi of the genus *Fusarium* was found in 2024 7.2% ± 8.30, and respectively for 2025—5.5% ± 2.80. For comparison, most data from other countries report higher values for insemination with mold fungi. For example, Alkadri et al. (2013) reported an average of 2.4% presence of the genus in the surface microbiome of wheat grown in Syria, harvest 2009-2010, with Fusarium spp. being found in 62.5% of the 48 analyzed samples. In wheat samples from Italy, Saudi Arabia, and Ukraine, the genus was present on average with 16÷26%, 32.3%, and 71%, respectively (Covarelli et al.,2015), Mahmud and Shehata (2017), Gritsev et al. (2018) Our results show a relatively low average infection rate with *Fusarium* spp. in wheat, compared to data from other countries.

In a descriptive analysis of the influence of wheat variety on contamination with *Fusarium* spp., no statistically significant difference was found ($p > 0.05$) (Table 11). A similar lack of significant effect of genotype was reported by El Chami et al. (2023) and Spanic et al. (2021), who emphasized the main role of climatic factors in terms of infection dynamics. In Bulgarian conditions, studies by Desheva and Chavdarov (2016) and Dimitrov (2017) also reported that although some varieties exhibit higher resistance, varieties completely resistant to Fusarium infections have not been established. All this emphasizes the need for in-depth studies on the population composition of mycotoxin-producing *Fusarium* species, based on accurate species identification, in order to develop strategies to limit colonization in wheat crops.

In the present study, the results of the molecular genetic analysis conducted based on the β-tub barcode for the purpose of species identification of *Fusarium* spp. in common wheat show that the species composition of this genus in our country is diverse. A total of four key species were identified—*F. sporotrichioides*, *F. proliferatum*, *F. poae* and *F. avenaceum*, which constitute about 80% of the population in the country. Of all the identified species, *F. sporotrichioides* stands out as dominant (42.31%). Although the information about its ecological plasticity is still limited and contradictory, some data indicate that the species develops in a relatively wide temperature range, with the optimum being between 25 and 30 °C (Maragos et al., 2022).

According to other data, the species develops at low temperatures, with minimums around 0 to 35 °C, as a maximum for growth in some sources; the optimum remains 22.5÷30 °C (https://www.fao.org/4/y1390e/y1390e04.htm?utm). This is probably one of the reasons for its leading distribution among *Fusarium* spp. isolates in Bulgaria. In the present study, the frequency of detection of *F. sporotrichioides* was higher in Northern Bulgaria (50%) compared to Southern Bulgaria (38%). (Figure 6), which is in agreement with the observation that the species is highly tolerant to temperature differences. A similar trend was also reported by Gagkaeva et al. (2019), who detected *F. sporotrichioides* in 84.1% of grain samples in the cold regions of the Volga, Urals and Western Siberia, where the species dominates over the known pathogen *F.graminearum* (19.2%). However, in a study from Ukraine, the species *F. sporotrichioides* (18.5%) was the 2nd most common after *F. graminearum* (39.3%), followed by *F. avenaceum* (13.9%), *F. poae* (10.6%) and *F. tricinctum* (8.6%) (Gritsev et al. (2018).

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Since *F. sporotrichioides* is a known producer of more toxic type A trichothecenes (T-2 and HT-2) (Duffeck et al., 2022), its spread in Bulgaria may pose a serious risk to the health of domestic animals and humans. Overall, *F. graminearum* was reported as a "traditional" species on cereal crops in Bulgaria before the 1970s (Karadzhova, 1979; Vrabcheva et al., 1996), but was displaced by *F. verticillioides* (24.6%), *F. poae* (15.4%), and *F. sporotrichioides* (11%) in the period 2001–2005 [Beev, 2009]. More recent data by Gencheva & Beev (2020) for the 2018 harvest, from the Stara Zagora region, the leading species is *F. tricinctum* (41.7%), followed by *F. poae* (16.7%), *F. graminearum* (8.3%), *F.* proliferatum (8.3%), and *F.* equiseti (8.3%). In our study, the species was not identified. The species *F. proliferatum* (23.7%, n=6) is the second most identified species in Bulgaria. The pathogen synthesizes fumonisins, which are particularly dangerous for grain crops and animal health. Its co-presence with *F. sporotrichioides* in sample (No. 6), Sadovo region, 2024, increases the risk of co-accumulation of various mycotoxins, which has economic, nutritional and health importance (Ferrigo et al., 2016; Ma et al., 2024). The *F. poae* identified in this study (7.7%) is widely documented in Europe: in Ukraine, in Italy, in Italy with a frequency of 10.6%, 17.8%, respectively (Gritsev et al., 2018; Pancaldi et al., 2010; Beccari et al. (2018). In our country, it has been documented by Beev et al. (2011) with 16.7% and Gencheva and Beev, (2020) with 16.7%. The species is considered to be sensitive to climatic conditions, with warmer and drier periods favoring its development (Infantino et al., 2023). Among the most common representatives of *Fusarium* spp. in temperate climate zones is F. avenaceum Beccari et al. (2018). In our study it was present with 7.7% (n=2). It is the leading pathogen within the F. tricinctum complex (FTSC) and demonstrates high genetic variability (Beccari et al., 2018). The species is known for the production of "emerging mycotoxins"—moniliformin, bovericin and eniatins (Duffeck et al., 2022). Atypical species for wheat include *F. equiseti*, 3.8% (n=1) whose presence in Bulgaria is probably due to changing climatic conditions globally and in the region. Until recently, this species was not associated with the wheat mycoflora, but data on its presence in our country are now available (Yanashkov and Vatchev, 2016; Gencheva and Beev, 2020). Of interest is the newly identified species *F. armeniacum*, which in our study was isolated for the first time in Bulgaria with 7.7% (n=2). Although it is rarer and occurs mainly in North America and temperate climate zones (Yli-Mattila et al., 2011; O'Donnell et al., 2018), this species deserves attention due to its proven ability to produce trichothecene T-2 toxin (Munkvold, 2017). On the other hand, the species identified by us molecularly, such as *F. sporotrichioides*, *F. poae*, *F. avenaceum*, *F. tricinctum* and *F. equiseti*, often coexist with the most virulent *Fusarium* pathogens—*F. graminearum* and *F. culmorum* (Piacentini et al., 2015; Karlsson et al., 2021; Inbaia et al., 2023). Even species previously considered to be weakly aggressive, such as *F. poae* and *F. sporotrichioides*, turn out to be significant for the development of infection in cereals and are key producers of trichothecenes in wheat (Hietaniemi et al., 2016; Gurikar et al., 2023). According to Stępień's (2011) studies on the optimal temperature range of F. proliferatum, there is a large genetic and phenotypic variability between isolates, which requires differences in temperature optima and water activity of the substrates. The species F. armeniacum, identified only in Northern Bulgaria, seems to develop better at lower temperatures (~25 °C) and higher humidity during the growing season (https://bulletins.cfd.meteo.bg/bull/BuletinNIMH202202.pdf.)

Although the ITS region is a major barcode marker in fungi, its drawback is insufficient hypervariability to distinguish different species within *Fusarium* species complexes. On the other hand, the combination of conserved and variable regions in the *β-tub* gene establishes it as a reliable locus for species identification in the genus. The use of *β-tub* (619 bp) and its complete sequencing in *F. solani* isolates from India proved that this species contains five exons and four introns, which retain their positions, but are shorter compared to other species of the genus and can serve as species distinction [Tarafder and Datta, 2022]. This structure confirms the evolutionary conservatism of the location of the intron regions and simultaneously reveals taxonomically significant differences in their length. According to the author, in many cases *β-tub* shows better resolution between closely related species than the ITS region and confirms its importance as an effective marker in the molecular identification and barcoding of *Fusarium* spp. According to O'Donnell et al. (2015), however, the

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application of *β-tubulin* (the first protein-coding gene) for molecular identification and phylogeny in the genus has "limited utility in some species complexes due to the presence of different duplicated genes within the same species" (paralogs). The multiple versions of the *β-tubulin* gene in the genome of some Fusarium species from the FSSC, FIESC and FCSC complexes also make identification difficult. Experiments have shown that it does not always provide good resolution between closely related species, e.g., *F.acuminatum/F.armeniacum* or between *F.langsethiae/F. sporotrichioides* (O'Donnell et al., 2015). This discrepancy illustrates some limitations of the β-tubulin region in several representatives of the complex genus *Fusarium*, although in other isolates the marker gives reliable results. This illustrates the importance of using multilocus genotyping to improve the accuracy and reliability of species identification. Due to the high degree of similarity between species at each single locus, the current approach for identification of *Fusarium* spp. is recommended to be multilocus—combining ITS, tef-1*α*, RPB2, *β-tub* and CaM sequences, which enhances the discriminatory potential of the analysis (Island et al., 2014; Han et al., 2023; O'Donnell et al., 2022). This achieves a stable and reliable classification of species that would be indistinguishable with only one marker.

In order to clarify the evolutionary relationships between the studied isolates and their affiliation to the species complexes of the genus *Fusarium*, phylogenetic trees were constructed. The phylogenetic analysis established that the Bulgarian wheat isolates belong mainly to five large evolutionarily related groups-species complexes with a characteristic mycotoxic profile. The representatives of the *F. sambucinum* species complex (FSAMSC) F. *sporotrichioides*, *F. armeniacum*, *F. poae*—species associated with the production of trichothecenes, are the most common with 57.7% (n=15). It is followed by the *F. fujikuroj* species complex (FFSC) with 23.1% (n=6), known for the synthesis of fumonisins and other secondary metabolites. Members of the F. tricinctum species complex FTSC (*F. tricinctum*, *F. avenaceum*, are distinguished by a more moderate presence—11.5%, (n=3), but are proven producers of enyatins, moniliformin and bovericin [Inbaia et al., 2023]. The two complexes FIESC and FOSC are the least widespread (*F.equiseti*, 3.8%, n=1; *F.oxysporum*, 3.8%, n=1). The predominance of representatives from FSAMSC and FTSC suggests a significant risk of contamination of the wheat crop with mycotoxins, while the presence of FIESC and FOSC completes the picture of the pathogenic pressure on the crop. Therefore, molecular analysis not only reveals the species diversity of Fusarium spp. on a regional scale, but also demonstrates which complexes are of greatest importance for crop health and potential safety of production.

The phylogeny distinguished members of FSAMSC, FOSC, FFSC in separate clusters with genetically similar species. However, the phylogenetic analysis based on the *β-tub* gene encountered some limitations. These are the cases with isolates F. equiseti, F. tricinctum and F. avenaceum, which are scattered throughout the tree. The barcode failed to reliably unite F. armeniacum with the other members of FSAMSC, as well as *F. tricinctum* and *F. avenaceum* in the FTSC complex, which put its discriminatory power for all species in doubt and thus reinforced the recommendations of O'Donnell et al., 2022) for multilocus genotyping.

5. Conclusions

The DNA barcoding technique is very useful for the correct identification of species in the complex genus *Fusarium*. The results of this study based on the *β-tub* marker significantly expand our knowledge of the diversity of these pathogens in wheat in Bulgaria. The method can also be used to track the introduction of new species in our country, as a result of climate change or increased international trade in planting material, cereals and feed. The DNA barcoding approach can be applied in geographical areas with a known presence of *Fusarium* spp. or with endemic presence. To build more stable molecular phylogenies, due to the high degree of similarity between species at each single locus, it can be used in combination with other marker genes from the *Fusarium* genome, such as *tef-1α*, ITS, RPB2, and *CaM* sequences, which would enhance the discriminatory potential of the analysis. This achieves a stable and reliable classification of species that would be indistinguishable with only one marker. The summary review of the identified species indicates the potential for contamination of wheat production with mycotoxins. The results are important for epidemiological

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surveillance and for the development of targeted strategies for the management of diseases caused by Fusarium and control of the risk of mycotoxins entering the food chain.

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