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The Applicability of Species- and Trichothecene-Specific Primers in Monitoring the *Fusarium graminearum* Species Complex and Its Impact on the Surveillance of Fusarium Head Blight in Winter Wheat in Serbia

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Abstract: Monitoring changes in the prevalence of *Fusarium* species and toxin production is an important tool for the integrated control of Fusarium head blight (FHB). However, methods for the high-throughput screening of *Fusarium* populations have been developed using isolates with limited geographic origins. In this study, we used species- and trichothecene-specific primers to monitor the *F. graminearum* species complex (FGSC) originating from Serbia. We also tested the applicability of the primers to the surveillance of FHB. We analyzed two hundred and ten isolates collected from thirty two locations and five winter wheat varieties over a three-year period. Using multiple correspondence analysis (MCA), we investigated associations between Fusarium-damaged kernels (FDK) and location, variety, members of the FGSC, and their predisposition for mycotoxin production. The results revealed that the species-specific primers were not specific for 11% of the *F. graminearum* population. The primer sets were 98.5%, 95.2%, and 92.4% effective in the multilocus genotyping of *Tri7*, *Tri3*, and *Tri5* genes, respectively. We found that individual wheat varieties were associated with isolates that could not be characterized using species- and trichothecene-specific primers. *Alternaria* spp. had a significant influence ($p < 0.001$) on grain infection with *F. graminearum*, indicating the necessity to further investigate its impact on the pathogenesis of the *F. graminearum* clade.

Keywords: winter wheat; trichothecene genotyping; grain infection; FGSC complex

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1. Introduction

Fusarium head blight (FHB) is one of the most economically important wheat diseases. It is caused by members of the *Fusarium graminearum* species complex (FGSC), the *F. tricinctum* species complex (FTSC), *F. culmorum*, *F. cerealis*, *F. poae*, *F. langsethiae*, *F. sporotrichioides*, and the nontoxigenic species *Microdochium nivale* and *M. majus* [1]. The *Fusarium graminearum* species complex, also called *F. graminearum* sensu lato, includes 16 phylogenetically distinct species [2,3].

Some members of the FGSC were formerly referred to as lineages 1–8, but these groups are now described as separate species [4,5]. *Fusarium* species that were formerly designated as lineages within the *F. graminarum* clade are *Fusarium austroamericanum* (Lineage 1), *Fusarium meridionale* (Lineage 2), *Fusarium boothii* (Lineage 3), *Fusarium mesoamericanum* (Lineage 4), *Fusarium acaciae-mearnsii* (Lineage 5), *Fusarium asiaticum* (Lineage 6), *Fusarium graminearum* sensu stricto (Lineage 7), and *Fusarium cortaderiae* (Lineage 8) [4]. In addition to the above-mentioned species, the FGSC includes *F. lousianense*, *F. brasilicum*, *F. gerlachii*, *F. nepalense*, *F. aethiopicum*, *F. ussurianum*, *F.* sp. NRRL 34,461, and *F. graminearum* (Gulf Coast) [2].

Members of the FGSC produce B-type trichothecene mycotoxins [2,6]. Type B includes chemotypes IA (producing 3-AcDON), chemotype IB (producing 15-AcDON), and chemotype II, which synthesizes nivalenol (NIV) and/or fusarenonX (4-ANIV). Aoki et al. [2] reported that not all members of the FGSC produce all types of B-type trichothecene mycotoxins. Only *F. graminearum* s. stricto and *F. asiaticum* are reported to be 3-AcDON, 15-AcDON, and NIV producers. The rest of the FGSC members are mainly NIV and/or 3-AcDON producers, except for *F. boothii*, *F. aethiopicum*, *F. vorosii*, and *F. nepalense,* which produce only 15-AcDON [2]. B-type trichothecene mycotoxins are phytotoxic and have adverse effects on animal and human health [6]. However, the production and virulence of mycotoxins also depend on the host [7–9].

Identification of *Fusarium* spp. relies mostly on comparing multilocus sequence data using markers, which are usually the protein-coding genes of translation elongation factor 1 and/or the second largest subunit (RPB1 and/or RPB2) [10]. Some species-specific primers are more suitable for routine usage [10], but their validation in different regions is still needed. To date, studies on the diversity of trichothecene genotypes in Serbia have been conducted only with isolates of *F. graminearum* s. stricto from a limited number of regions [11,12]. Consequently, further investigation with a broader range of *F. graminearum* populations is required to determine whether species- and trichothecene-specific primers can be used in the routine surveillance of *Fusarium* infection. In addition, the regulatory network for plant responses to abiotic and biotic stress consists of many components that may function antagonistically [13–15]. As a result, the susceptibility responses of the individual wheat varieties to FHB could differ significantly due to the complexity of their specific responses to a combination of environmental factors. The divergence in the susceptibility response of individual varieties in different regions was reported by Župunski et al. [16], but it is unclear whether individual varieties are associated with specific trichothecene genotypes in the *F. graminearum* population.

The main objectives of this study were to identify (1) the effectiveness of species- and trichothecene-specific primers (GzTri7f1/r1, Tri*3*03F/R, Tri*3*15F/R, Minus Tri7F/R, and Tri5F/R) in characterizing *F. graminearum* populations originating from Serbia, (2) the relationship between trichothecene genotypes and winter wheat grain infection, and (3) the diversity of the susceptibility response of individual wheat varieties to FHB collected from a range of locations with different agroecological conditions. We hypothesized that the application of species- and trichothecene-specific primers would provide a good basis for the characterization of *F. graminearum* populations in Serbia and that relationship between trichothecene genotypes and commercial varieties would be revealed. The results of the study indicated a close association between individual wheat varieties and members of a *F. graminearum* population that could not be identified using species-specific primers. Moreover, nonidentified strains showed specificity in their genetic potential for mycotoxin production, indicating that there are members of the FGSC that cannot be identified in a timely manner and thus pose a threat to the efficient surveillance, prediction, and control of FHB.

2. Materials and Methods

2.1. Field Trials and Conventional Disease Assessment

We evaluated 236 isolates of *Fusarium* spp. originating from 59 seed samples of 5 varieties (Simonida, Ilina, Zvezdana, Futura, and NS40S) that were produced in farmers' fields under conventional agricultural conditions (Table S1). Seed samples were collected in a 3-year period (2017–2019) from 32 locations in 4 Serbian regions (Autonomous Province of Vojvodina; Belgrade; Šumadija and Western Serbia; East and Southern Serbia).

After shelling, all samples were initially examined using the agar plate incubation method for seed health testing, recommended by the International Seed Testing Association [17]. Four replicates of 100 seeds were randomly chosen from each sample. Seeds were surface sterilized (1.0% NaOCl for 1 min) and rinsed twice with sterile distilled water. Fungi were first isolated on 2% water agar medium supplemented with streptomycin sulfate and

were then transferred to potato dextrose agar (PDA), V8 Juice Agar (HiMedia, Einhausen, Germany), and water agar for morphological identification at the species level [18,19].

The infection level of a *Fusarium* sp. was estimated as the percentage of *Fusarium*-damaged kernels (FDK) found in the sample: FDK (%) = (Number of kernels in which the *Fusarium* sp. occurred/Total number of kernels) × 100. The *Alternaria*-contaminated kernels (ACK) per sample were calculated as follows: ACK (%) = (Number of kernels in which *Alternaria* spp. occurred/Total number of kernels) × 100 (Table S1).

The isolates of predominant *Fusarium* spp.—the *F. graminearum* clade (210 isolates) and *F. poae* (26 isolates) (Table S2)—were subjected to single-spore isolation on PDA plates prior to DNA extraction. Single-spore isolates were separated by dilution plating. DNA extraction was performed using the procedure of Möller et al. [20]. Briefly, 10% cetyltrimethylammonium bromide (CTAB), Proteinase K, and SEVAG (chloroform/isoamyl alcohol, 24:1, *v*/*v*) were used for DNA isolation. DNA was precipitated using isopropanol, resuspended in 0.1 × TRIS-EDTA buffer (TE), and stored at −20 °C prior to molecular analysis.

2.2. Identification of Fusarium Species and Trichothecene Genotyping

2.2.1. Identification of *Fusarium* spp.

Molecular characterization of isolates that were previously identified based on morphology was performed with sequence-characterized amplified region (SCAR) analysis (Table 1). All isolates were first identified with the species-specific primer pair Fg16F/R [21] and classified into the SCAR groups defined by Carter et al. [22] as follows: type 1 (420 bp), type 2 (510 bp), type 3 (540 bp), type 4 (580 bp), type 5 (520 bp), and type 6 (400 bp) (Table S2). The primer pair Fg16F/R yields PCR products of 420 bp (SCAR group 1) and 400 bp (SCAR group 6) for *F. graminearum* s. stricto [3,23–27]. The remaining SCAR groups are associated with other species in the FGSC. PCR reaction mixtures were prepared using the method of Demeke et al. [28]. Briefly, the reaction mixture (25 µL) contained 1 × PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.3)), 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.4 µM of each primer, 0.75 units of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and 25 ng of purified DNA. The cycling parameters are given in Table 1.

Table 1. Primer sets for the identification of *Fusarium* spp.

Primer Set	Nucleotide Sequence (5′-3′)	Size of Amplified PCR Fragment (bp)	PCR Conditions
Fg16F Fg16R	CTCCGGATATGTTGCGTCAA GGTAGGTATCCGACATGGCAA	420 (SCAR 1) 400 (SCAR 6)	Initial Denaturation: 95 °C, 3 min 38 Cycles: 95 °C, 30 s 62 °C, 20 s (Fg16F/R) 60 °C, 20 s (Fp82F/R) 72 °C, 45 s Final Extension: 72 °C, 5 min
Fp82F Fp82R	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGTT	220	
Fgr-F Fgc-R	GTTGATGGGTAAAAGTGTG CTCTCATATACCCTCCG	500	Initial Denaturation: 94 °C, 85 s 25 Cycles: 95 °C, 35 s 53 °C, 30 s 72 °C, 30 s Final Extension: 72 °C, 5 min

If no PCR product was detected with Fg16F/R, another pair of species-specific primers (FgrF/FgcR) [29] was used for further identification (Table 1). The primer pair FgrF/FgcR was reported to be highly specific to all lineages of the *F. graminearum* clade, with the exception of lineage 5 [29]. PCR reaction mixtures (25 µL) were prepared as follows:

1 × PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.3)), 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.5 µM of each primer, 1.25 units of Taq DNA polymerase (Thermo Fisher Scientific), and 25 ng of purified DNA. The cycling parameters are given in Table 1.

Isolates of *F. poae* were identified with the Fp82F/R species-specific primer pair [28] using the same reaction mixture composition as that described for the Fg16F/R primer pair. The cycling parameters are given in Table 1.

The amplification reactions were performed in a Veriti 96-well Thermal Cycler (AB Applied Biosystems). PCR products were separated by electrophoresis in 1.5% agarose gels containing 1 µg mL^{-1} ethidium bromide. Normalization was obtained by using a 100-bp DNA ladder (Bio-Lab, Hong Kong, China).

2.2.2. Trichothecene Genotyping

Trichothecene genotyping of 210 *F. graminearum* isolates was performed using the following primer sets: GzTri7f1/r1; Minus Tri7F/R; and Tri303F/R, Tri315F/R, and Tri5F/R for the sequences of the *Tri7*, *Tri3*, and *Tri5* genes (*TRI* genes) [23,25,30,31] (Table S2). PCR was performed in a 25 µL reaction mixture containing 25 ng of DNA, 1 × PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.3)), 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.5 µM of each primer, and 1.25 units of Taq DNA polymerase. The amplification reactions were performed in a Veriti 96-well Thermal Cycler (AB Applied Biosystems). The cycling parameters for each primer set as well as the sizes of amplified PCR fragments are presented in Table 2. The PCR products of *TRI* genes were separated by electrophoresis in 1.5% agarose gels containing 1 µg mL^{-1} ethidium bromide. Normalization was obtained by using a 100-bp DNA ladder (Bio-Lab).

Table 2. Primer sets for multilocus genotyping of *F. graminearum* populations.

Primer Set	Nucleotide Sequence (5′-3′)	Size of Amplified PCR Fragment (bp)	PCR Conditions
GzTri7f1/ GzTri7r1	GGCTTTACGACTCCTCAACAATGG AGAGCCCTGCGAAAG(C/T)ACTGGTGC	173–250	Initial Denaturation: 94 °C, 2 min 30 Cycles: 94 °C, 30 s 60 °C, 1 min 72 °C, 2 min Final Extension: 72 °C, 10 min
Tri303F/ Tri303R	GATGGCCGCAAGTGGA GCCGGACTGCCCTATTG	586	
Tri315F/ Tri315R	CTCGCTGAAGTTGGACGTAA GTCTATGCTCTCAACGGACAAC	864	
Tri5-F Tri5-R	AGCGACTACAGGCTTCCCTC AAACCATCCAGTTCTCCATCTG	544	Initial Denaturation: 95 °C, 3 min 38 Cycles: 95 °C, 30 s 62 °C, 20 s 72 °C, 45 s Final Extension: 72 °C, 5 min
Minus Tri7F/ Minus Tri7R	TGGATGAATGACTTGAGTTGACA AAAGCCTTCATTCACAGCC	483	Initial Denaturation: 94 °C, 5 min 30 Cycles: 94 °C, 1 min 60 °C, 1 min 72 °C, 1 min Final Extension: 72 °C, 10 min

2.2.3. Qualitative *Tri-5*-Specific PCR Assay and HPLC Analysis

A qualitative *Tri-5*-specific PCR assay was used to assess the genetic potential of 210 *F. graminearum* isolates for mycotoxin production (Table S2). Sanoubar et al. [31] reported a direct relationship between the intensity of the *Tri-5* DNA band and the amount of deoxynivalenol (DON); thus, this feature was used to characterize the *F. graminearum*

clade in addition to trichothecene genotyping with the presence/absence of the *Tri5* gene. Sanoubar et al. [31] did not provide a method for estimating band intensity, and thus, we assumed that it was visually based. In order to standardize the PCR band intensity level used in this study, the *Tri-5* DNA band intensity was estimated relative to the DNA ladder band of 500 bp since the quantity of the PCR products can be estimated relative to the quantity of the DNA bands in the DNA ladder. In this study, estimates of *Tri-5* DNA band intensities were expressed using an ordinal scale. The ordinal scale is the second level of measurement and reports the order of the data without establishing the degree of variation between them. All isolates producing brighter *Tri-5* DNA bands than the 500 bp band in the DNA ladder were designated *Tri5-3*, and all isolates producing *Tri-5* DNA bands with intensities that were equal to or lower than that of 500 bp were designated *Tri5-2* and *Tri5-1*, respectively.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was performed to quantify the mycotoxins of one isolate carrying a *Tri7* deletion. The analysis was performed by the Institute of Public Health, Belgrade, Serbia, using Agilent 1200 and Detector Triple Quad LC/MS 6410 (Agilent Technologies, Santa Clara, CA, USA). LC-MS/MS was performed on *F. graminearum* isolates cultivated on PDA for 4 weeks on corn kernels following the procedure of Logrieco et al. [32].

2.3. Statistical Methods

Multiple linear regression (stepwise regression) was used to analyze the factors that influence grain infection with the most predominant *Fusarium* spp. The year, variety, and location were considered to be categorical variables, while climatic elements (temperature, relative humidity, and total rainfall in May) and grain contamination with competing *Alternaria* spp. were introduced as continuous variables. The climatic factors were obtained for the month of May, which is when anthesis of wheat usually occurs in Serbia (Table S3). The alpha to enter and alpha to remove the influencing factors in stepwise multiple regression were set to 0.15 by default since levels such as 0.05 have reportedly failed to identify important variables [33]. The relationship between grain infection with *F. graminearum* and grain contamination with *Alternaria* spp. was characterized using Spearman's coefficient of correlation.

For the analysis of the association between qualitative variables (variety; location; presence of *F. graminearum* s. stricto, *F. graminearum* s. lato, and nonidentified members of *F. graminearum* clade; level of grain infection), multiple correspondence analysis (MCA) was performed. For more convenient data visualization, the level of grain infection was defined using a scale from 1 to 4 as follows: 0%–9%—level of grain infection 1; 10%–19%—level of grain infection 2; 20%–29%—level of grain infection 3; >30%—level of grain infection 4. According to Lenc et al. [34], varieties that exceed 11% kernel infection should be considered susceptible.

The relative frequencies of *Tri7* alleles per location, FGSC groups, and varieties were calculated in a two-way relative frequency table, where the counts of *Tri7* alleles in each row were divided by the total number of isolates per row. Associations between *Tri7* alleles and locations, FGSC groups, and varieties were evaluated using Cramer's V-square and Kappa statistics. The relative frequencies of *Tri-5* DNA band intensity estimates per variety and FGSC groups were calculated in the same manner as that indicated for *Tri7* alleles using two-way relative frequency tables. The effectiveness of primer pairs was expressed as the percentage of isolates that produced PCR products relative to the number of isolates analyzed with the given primer set.

Multiple regression and other statistical analyses, except for MCA, were performed using Minitab 17 Statistical Software (2010) (trial version) [35]. The MCA of qualitative variables was performed using XLSTAT 2019 (trial version) in Microsoft Excel [36].

3. Results

The most prevalent fungal species isolated from the collected wheat seeds were members of the *F. graminearum* clade, *Alternaria* spp., and *F. poae*. However, the average grain infection significantly differed among years (p = 0.023) and varieties (p = 0.008). *Alternaria* spp. predominated with an average ACK of 22% in 2018 and 44% in 2019, while the members of the FGSC were prevalent in 2017 with an average FDK of 38% (Figure 1a). On average, Simonida and Ilina had the lowest FDK (12%) compared with Zvezdana (22%), Futura (41%), and NS40S (70%) (Figure 1b). The average grain infection with *F. poae* did not exceed 10% in the three-year period.

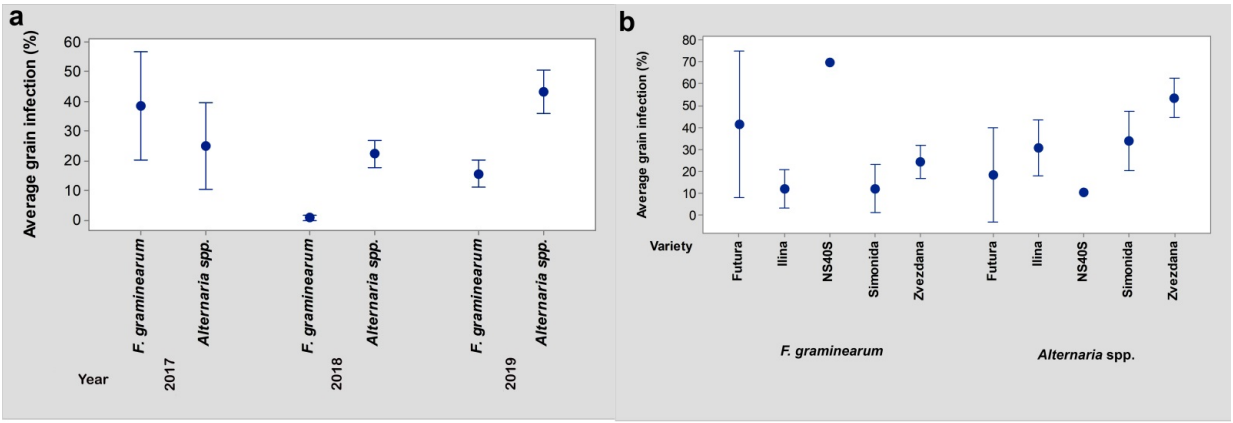

Figure 1. Average grain infection with the *F. graminearum* clade and *Alternaria* spp. in a three-year period (2017–2019) from 32 locations in Serbia. (**a**) The effect of the year on grain infection with the *F. graminearum* clade and *Alternaria* spp.; (**b**) the effect of the variety on grain infection with the *F. graminearum* clade and *Alternaria* spp.

In the three-year period, *Alternaria* spp. were more commonly recovered than members of the FGSC and *F. poae* in all locations, except in Valjevo and Kula, where the average grain infection with the FGSC ranged between 40% and 50% (Figure 2).

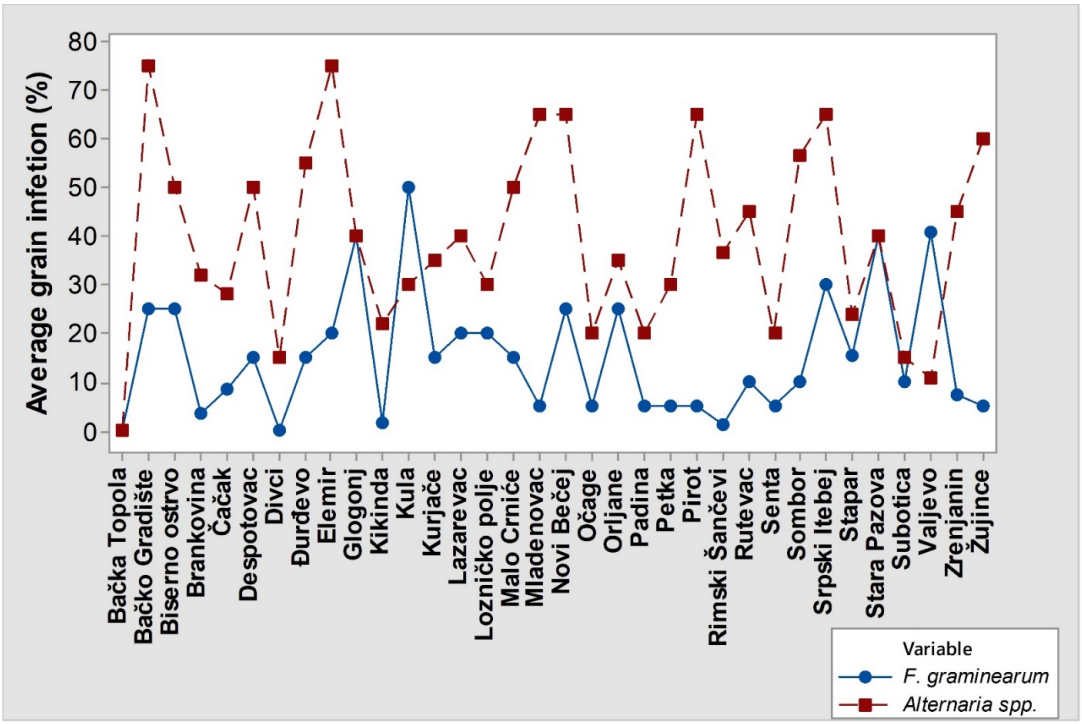

Figure 2. The effect of the location on grain infection with the *F. graminearum* clade and *Alternaria* spp. in a three-year period (2017–2019) in Serbia.

Climatic conditions in the month of May, which is when anthesis occurs, were conducive to FHB infection in 2017 and 2019 (Table S3). The average temperature in May was between 14.4 °C (2019) and 17.7 °C (2019), but in 2018, it exceeded the average monthly temperature of 17.2 °C, which has been reported to be the upper limit for grain infection with *F. graminearum* s. stricto [5]. The average temperature in the month of May in 2018 ranged from 19.9 °C to 20.6 °C in all locations, except in Čačak, where it was 17.5 °C. Average total rainfall in May ranged from 30.1 mm (2018) to 208.6 mm (2019), and average relative humidity ranged from 59% (2018) to 82% (2019).

Multiple stepwise regression applied to the categorical variables (year, variety, and location) and the continuous variables (grain contamination with *Alternaria* spp.) showed that the level of grain infection with *F. graminearum* was determined by the year and variety. It was also affected by grain contamination with *Alternaria* spp. ($p < 0.001$) and the *Alternaria* spp. × year interaction ($p = 0.004$), giving the model an (R^2) of 70% (Table S4). The correlation between the infection level of *F. graminearum* and the contamination level of *Alternaria* spp. was also determined by Spearman's correlation coefficient. A high negative correlation was confirmed in Southern and Eastern Serbia ($r = -0.781$, $p = 0.038$) and Šumadija and Western Serbia ($r = -0.733$, $p = 0.025$). In the Autonomous province of Vojvodina and the region of Belgrade, the correlation between the infection level of *F. graminearum* and the contamination level of *Alternaria* spp. was not significant.

These results indicate the specificity of the years, the locations, and the responses of the varieties to mycobiota associated with winter wheat grain. In order to determine the structure of the *F. graminearum* population in Serbia and its possible association with differences in the grain infection of the winter wheat varieties in a three-year period, we conducted a molecular analysis on single-spore isolates using species- and trichothecene-specific primers.

3.1. Effectiveness of Species and Trichothecene-Specific Primers for the Characterization of the F. graminearum Population in Serbia

We used two sets of species-specific primer pairs (Fg16F/R and FgrF/FgcR) for the routine monitoring of the *F. graminearum* population in Serbia. Sequence characterized amplified region (SCAR) analysis with Fg16F/R revealed that *F. graminearum* s. stricto was the most common member of the *F. graminearum* population (79.5%). Almost all of the *F. graminearum* s. stricto isolates (99.5%) were classified into SCAR group 1 with a PCR product of 420 bp. However, 1 out of the 210 isolates produced a type 6 SCAR product of 400 bp (Figure 3a, Table S2).

The members of the FGSC that did not produce a PCR product with Fg16F/R primers were subjected to analysis with the FgrF/FgcR primer pair. Isolates that produced a PCR product accounted for 9.5% of the population and were assigned to *F. graminearum* s. lato (Table S2). Isolates that did not produce a PCR product with any primer pairs represented 11% of the *F. graminearum* population and were B-type trichothecene producers, as indicated by their *Tri7* and/or *Tri3* genotypes (Table S2). Nonidentified isolates had morphological features that could not be distinguished from other members of the *F. graminearum* clade. Among 26 isolates that were identified morphologically as *F. poae*, only one was not confirmed using FP82F/R species-specific primers (Table S2).

Multilocus genotyping of *F. graminearum* isolates was performed using the primer sets GzTri7f1/r1; Minus Tri7F/R; and Tri303F/R, Tri315F/R, and Tri5F/R for sequences of the *Tri7*, *Tri3*, and *Tri5* genes. The GzTri7f1/r1 primer set identified eight *Tri7* alleles in the *F. graminearum* population originating from Serbia. GzTri7 PCR products varied between 173 and 250 bp depending on the number of 11 bp inserts in the *Tri7* gene (Figure 3b). The majority of isolates (22%) contained three 11 bp repeats, followed by isolates with six 11 bp repeats (18%) and one 11 bp repeat (17%). Although one *Tri7* allele dominated at every location, Cramer's V-square (0.23) and Kappa (0.00) statistics revealed no significant association between locations and *Tri7* alleles. *Tri7* alleles were not significantly associated with groups of FGSC members, as indicated by Cramer's V-square (0.08) and Kappa (0.00) statistics. Cramer's V-square (0.08) and Kappa (0.00) statistics also indicated a lack of

association between *Tri7* alleles and the five winter wheat varieties. The relative frequencies of the *Tri7* alleles per location and FGSC groups are provided in Tables S5 and S6.

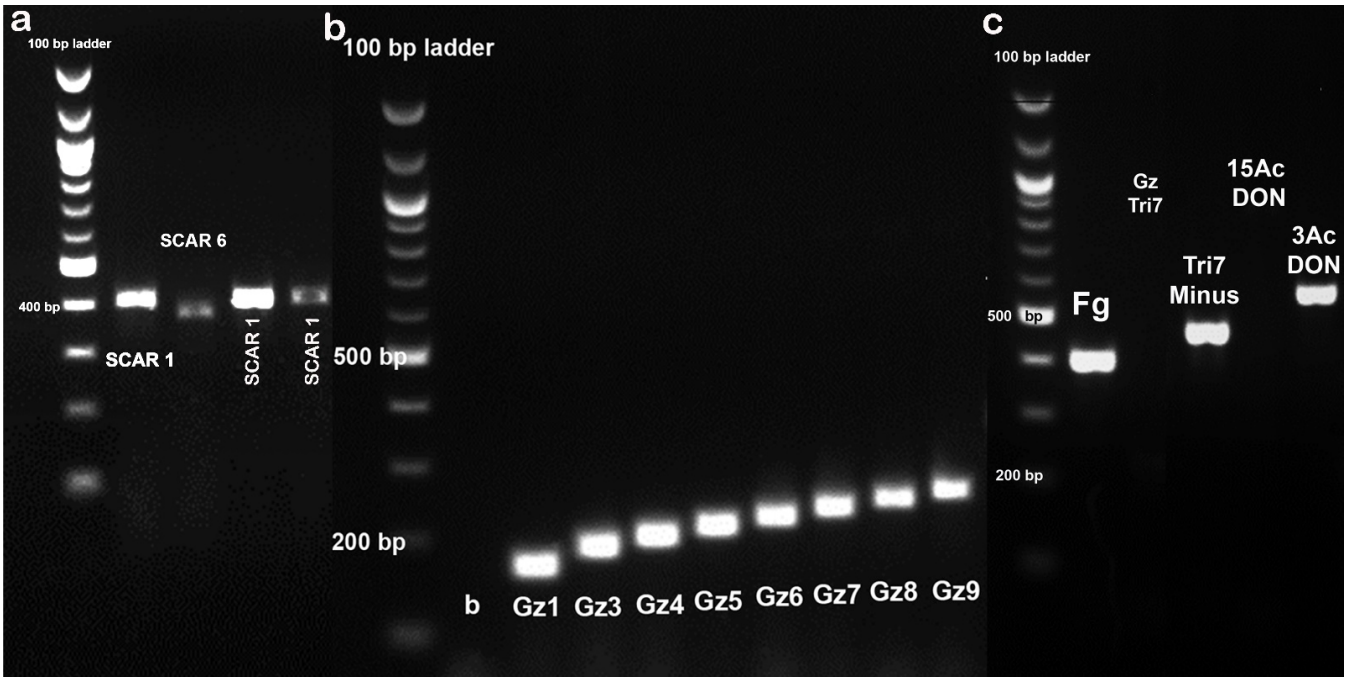

Figure 3. Diversity of Fg16 target and *Tri7* genes. (**a**) SCAR (Sequence characterized amplified region) analysis of the *F. graminearum* clade with the Fg16F/R primer pair identifying SCAR 6 and SCAR 1 PCR products; (**b**) *Tri7* gene diversity in the *F. graminearum* population: b—blank; Gz1—PCR product of the *Tri7* allele with one 11 bp repeat; Gz3—PCR product of the *Tri7* allele with three 11 bp repeats; Gz4, Gz5, Gz6, Gz7, Gz8, and Gz9—PCR products of *Tri7* alleles with four, five, six, seven, eight, and nine 11 bp repeats, respectively; (**c**) trichothecene genotyping of *F. graminearum* s. stricto based on the Tri7 deletion: Fg16—PCR product of the Fg16F/R primer pair; GzTri7—the GzTri7f1/r1 primer pair for the Tri7 gene yielded no PCR product; tri7Minus—PCR product of the Minus Tri7F/R primer pair confirming the deletion of the Tri7 gene; 15-AcDON—the Tri315F/R primer pair for 15-AcDON yielded no PCR product; 3-AcDON—PCR product of the Tri303F/R primer pair for 3-AcDON.

Among the *F. graminearum* s. stricto isolates, only one carried a *Tri7* deletion and produced a PCR product with the MinusTri7f/r primer pair (Figure 3c). Further analysis with Tri303F/R and Tri315F/R primer sets identified this genotype as a 3-AcDON producer (Figure 3c). We performed additional testing with HPLC only on this one isolate in order to confirm the result obtained with the trichothecene-specific primer pair. The HPLC analysis was in agreement with the molecular results, showing that the concentration of 3-AcDON (7110 µg/kg) was higher than that of 15-AcDON (15 µg/kg) and DON (2290 µg/kg).

In a qualitative *Tri5*-specific PCR assay, 85% of the isolates produced high-intensity DNA bands on agarose gels (designated *Tri5-3*) (Figure 4a). In 7.6% of the isolates, no *Tri5* DNA PCR product was obtained. Isolates producing DNA bands that were fainter than *Tri5-3* to varying degrees, designated *Tri5-2* and *Tri5-1* (Figure 4a), accounting for an additional 7.6% of the isolates (Table S2). The genetic potential for mycotoxin production was the highest in the group of isolates belonging to *F. graminearum* s. stricto, with 93% of isolates possessing high mycotoxin production potential (*Tri5-3*) (Figure 4b).

A *Tri5-3* PCR product brighter than the 500 bp band of the DNA ladder indicates FGSC isolates with a high predisposition for DON production; *Tri5-2* and *Tri5-1* PCR products with a band intensity that is equal to or fainter than the 500 bp band of the DNA ladder indicate FGSC isolates with a lower predisposition for DON production than *Tri5-3*.

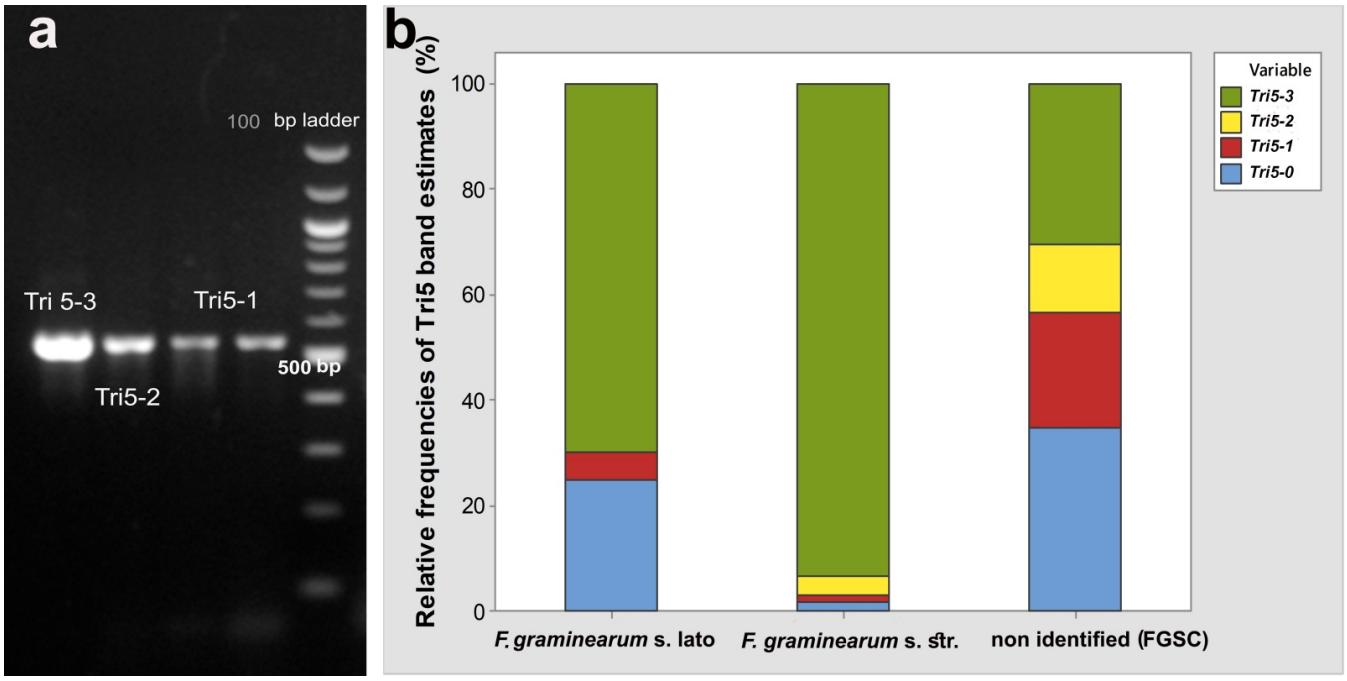

Figure 4. *Tri-5* genotyping and potential for mycotoxin production of 210 FGSC (*F. graminearum* species complex) isolates from Serbia. (**a**) Qualitative *Tri-5*-specific PCR assay resulting in three levels of *Tri-5* DNA band intensities; (**b**) relative frequencies of *Tri5* band intensity estimates among *F. graminearum* s. stricto, *F. graminearum* s. lato, and nonidentified (FGSC).

Trichothecene Genotypes of the Population and the Effectiveness of Primer Sets for Multilocus Genotyping

In the *F. graminearum* population, there were seven different genotypes with respect to the presence/absence of PCR products of the *Tri7*, *Tri5*, and *Tri3* genes (Table 3 and Table S2. The dominant genotype, type 1 (185 out of 210 isolates), was DON with respect to *Tri7*, 15-AcDON with Tri315F/R, and produced a PCR product for the *Tri5* gene. The lowest genotypic diversity was detected in *F. graminearum* s. stricto, which had only three genotype types (types 1, 2, and 3). *F. graminearum* s. lato and nonidentified isolates had more genotype diversity, with six types (1, 3, 4, 5, 6, and 7) and five types (1, 3, 4, 5, and 6), respectively. Genotypes 3, 5, and 6 (18 out of 210) carried the *Tri7* gene but did not produce a PCR product for 15-AcDON and/or the *Tri5* gene (Table 3). These genotype types were not identified as 3-AcDON or NIV chemotypes. These results indicate specificity in the structure of the respective genes, and this reduced the effectiveness of the GzTri7f1/r1, Tri315F/R, and Tri5F/R primers in trichothecene genotyping of FGSC members originating from Serbia.

Three genotype types (types 2, 4, and 7) did not produce a PCR product with GzTri7f1/r1 and accounted for 1.9% of the *F. graminearum* population. One genotype (type 2) carried a *Tri7* deletion, which was confirmed by its PCR product with the Minus-Tri7f/r primer pair, and was proven to be 3-AcDON (Figure 3c). The remaining isolate lacking GzTri7f1/r1 PCR product (types 4) was 15-AcDON producer, as evidenced by the results with the Tri315F/R primer pair (Table 3).

The results obtained in the molecular characterization of the *F. graminearum* population were further subjected to MCA in order to investigate their association with winter wheat grain infection in different varieties cultivated in diverse environmental conditions.

Table 3. Molecular genotypes in the *F. graminearum* population and the effectiveness of primer pairs.

			Tri7		*Tri5*	*Tri3*	
FGSC	**Number of Isolates**	**Genotype Type**	**GzTri7f1/r1**	**MinusTri7f/r**	**Tri5F/R**	**Tri303F/R (3-AcDON)**	**Tri315F/R (15-AcDON)**
F. graminearum s. stricto	163	1	+	-	+	-	+
	1	2	-	+	+	+	-
	3	3	+	-	-	-	+
F. graminearum s. lato	10	1	+	-	+	-	+
	3	3	+	-	-	-	+
	1	4	-	-	-	-	+
	4	5	+	-	+	-	-
	1	6	+	-	-	-	-
	1	7	-	-	+	-	-
Nonidentified	12	1	+	-	+	-	+
	5	3	+	-	-	-	+
	1	4	-	-	-	-	+
	3	5	+	-	+	-	-
	2	6	+	-	-	-	-
Effectiveness of primer pairs			98.5%	100%	92.4%	100%	95.2%

FGSC (*F. graminearum* species complex); (+) indicates that the PCR product is present; (-) indicates that the PCR product missing.

3.2. Geographic Distribution of FGSC Members and Their Association with Winter Wheat Grain Infection in Serbia

The geographic distribution of FGSC members and the levels of grain infection of five winter wheat varieties are presented in Figure 5. MCA was performed in order to discern a global pattern within the data and to identify associations between grain infection (FDK) and location, variety, the ability of isolates to produce mycotoxins, and the presence of *F. graminearum* s. stricto, *F. graminearum* s. lato, and nonidentified (FGSC) isolates.

MCA indicated that the first two dimensions contributed to 30% of the overall variability, and not all points were equally well explained by the two dimensions (Figure 6, Table S7). For some items, more than two dimensions were required to adequately represent the data. These results indicate that multiple factors influence the association between the predisposition for mycotoxin production and grain infection.

The isolates of F. *graminearum* s. stricto were closely associated with high levels of grain infection, i.e., exceeding 30% (grain infection-4), and with isolates having a high predisposition for mycotoxin production (*Tri5-3*). *F. graminearum* s. stricto was also positioned in the opposite quadrant from *F. graminearum* s. lato and nonidentified FGSC isolates, indicating the dissimilar profiles of these three groups of FGSC members. Indeed, *F. graminearum* s. lato and nonidentified FGSC members were more associated with locations that had lower grain infection levels (grain infection-2) and with isolates with a low genetic potential for mycotoxin production (*Tri5-0, Tri5-1*). These isolates usually infected the variety found in Simonida. Interestingly, the varieties found in Simonida and Ilina had the lowest average FDK (12%), even though Ilina was more associated with isolates of *F. graminearum* s. stricto with a high potential for mycotoxin production (Figure 7).

The locations where FDK did not exceed 20% (Brankovina, Senta, Despotovac, Pirot, and Kikinda) were closely associated with locations with high levels of grain infection (Kula, Stara Pazova, and Srpski Itebej) due to the predominance of *F. graminearum* s. stricto with a high mycotoxin production potential in both groups of locations. This result indicates that the pathogenicity of *F. graminearum* s. stricto also depends on other conditions and that isolates with a high genetic potential for mycotoxin production do not necessarily cause high levels of disease. The variety found in Zvezdana was associated with isolates with a high potential for mycotoxin production and with locations with all levels of grain infection. This result indicates that the susceptibility response of individual wheat varieties to FHB

could differ significantly due to the complexity of a variety of responses to combinatorial environmental factors.

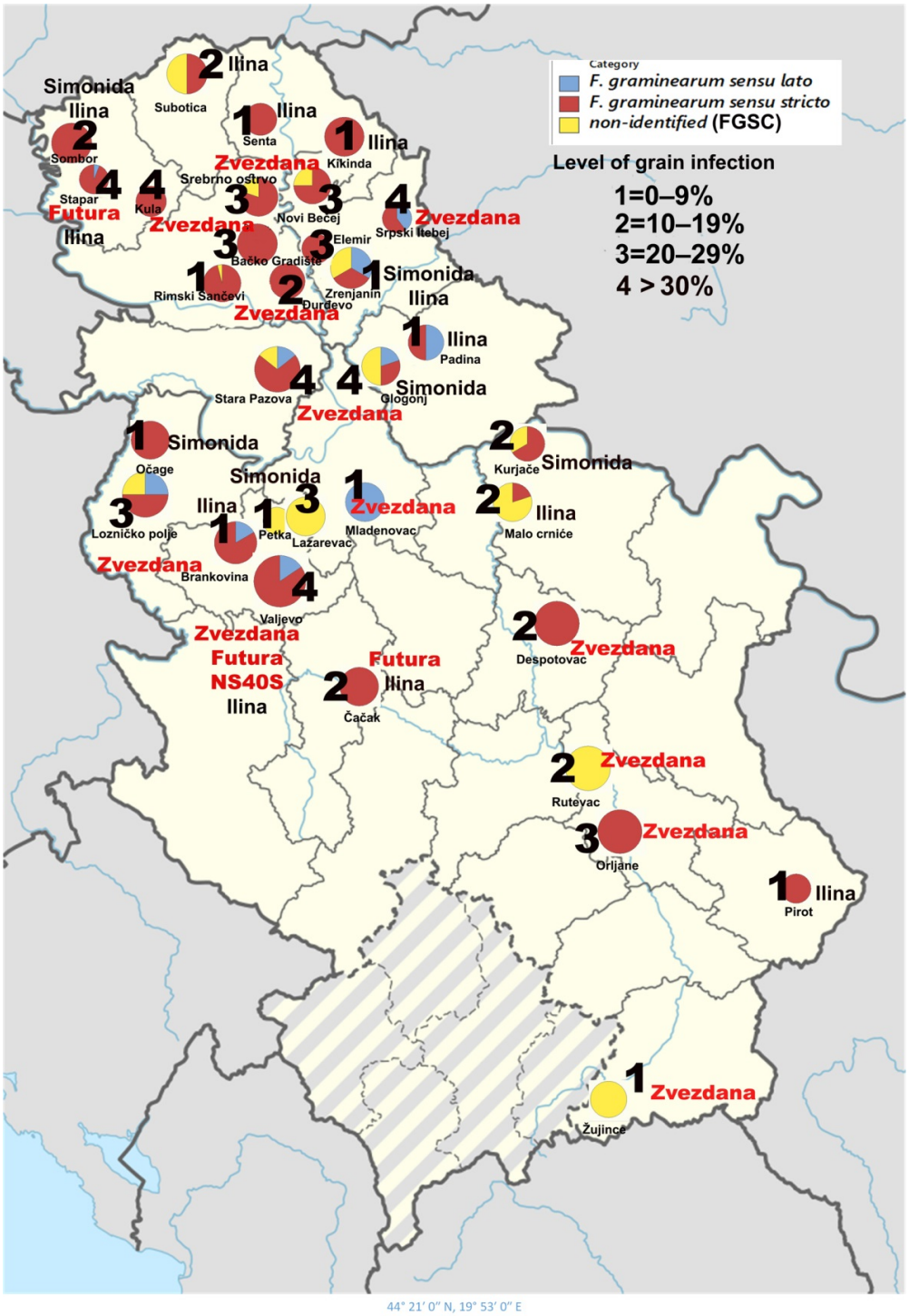

Figure 5. Geographic distribution of FGSC members and the levels of grain infection of five winter wheat varieties in a three-year period (2017–2019) in Serbia.

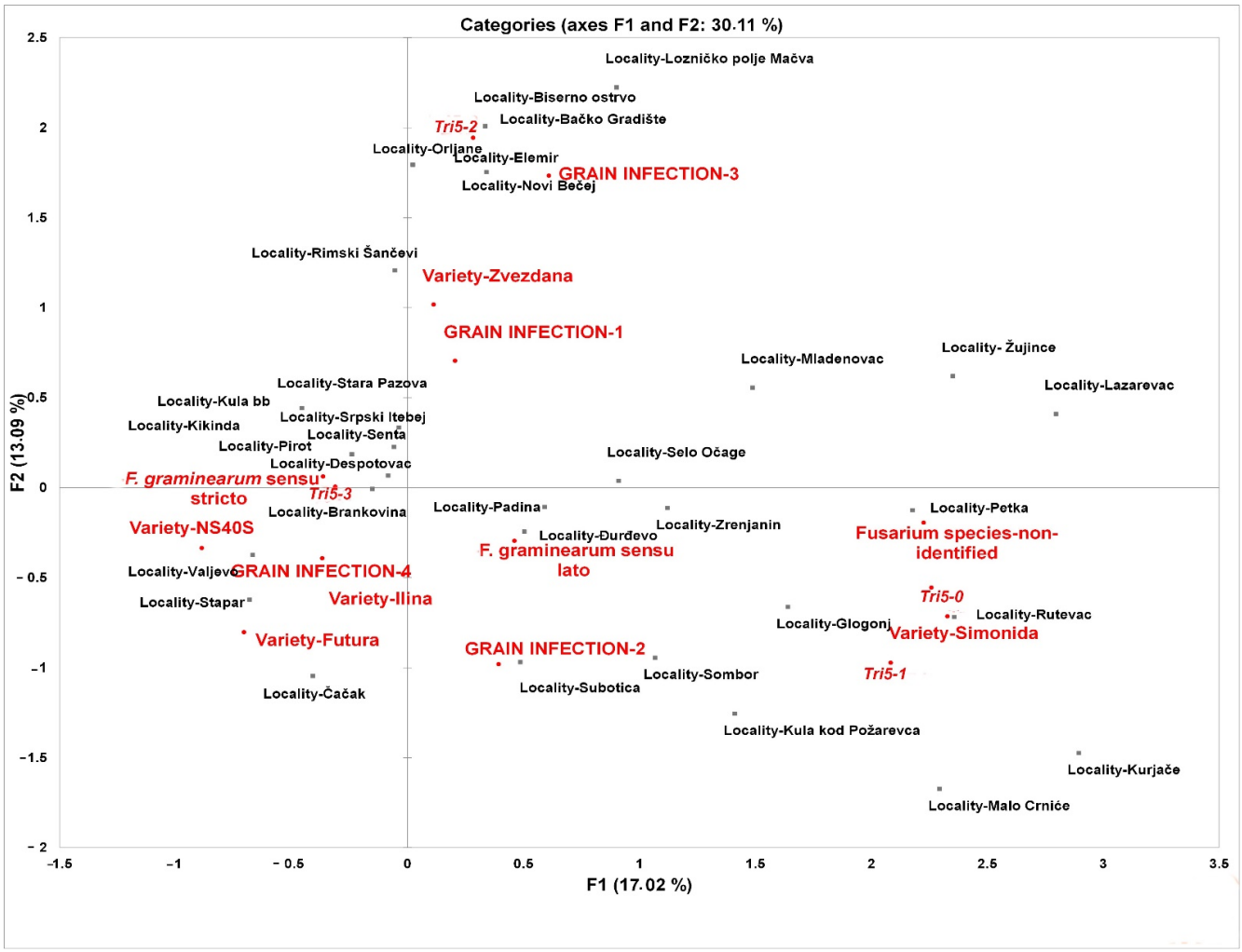

Figure 6. Graphic representation of multiple correspondence analysis (MCA) between grain infection, location, variety, mycotoxin production predisposition of the FGSC, and the presence of *F. graminearum* s. stricto, *F. graminearum* s. lato, and nonidentified (FGSC) isolates.

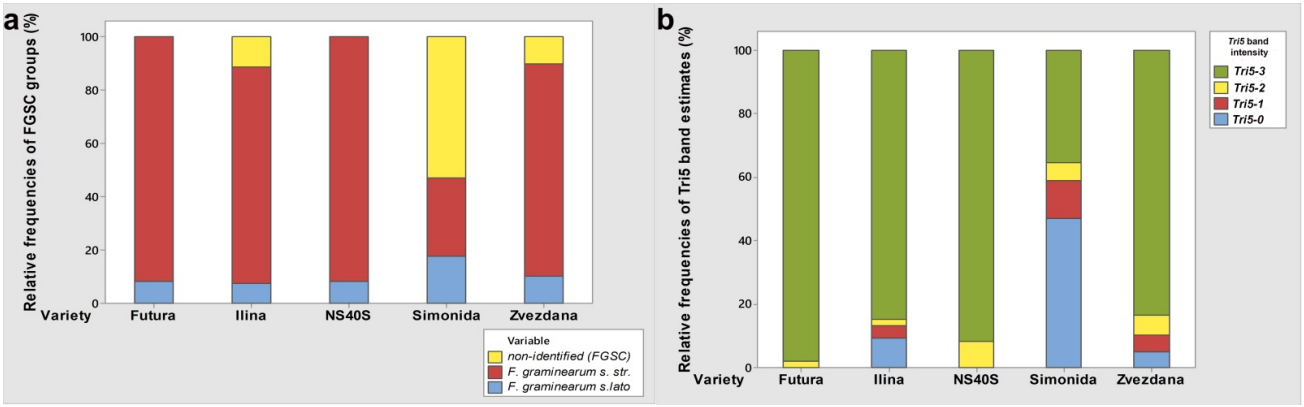

Figure 7. Association between five winter wheat varieties and FGSC groups and isolates differing in mycotoxin production potential. (**a**) Relative frequencies of *F. graminearum* s. stricto, *F. graminearum* s. lato, and nonidentified (FGSC) isolates in five winter wheat varieties; (**b**) relative frequencies of *Tri5* DNA PCR products differing in band intensities and their associations with five winter wheat varieties.

4. Discussion

According to previous reports, *Fusarium* populations should be monitored at two levels: (1) the determination of changes in the diversity of *Fusarium* species in a given environment and (2) the detection of alterations in toxin production [37–39]. Since the relationship between the *TRI* genotype and chemotype can be incongruent, there are inconsistencies in the reports of their relationship and the applicability of methods for trichothecene chemotyping and genotyping [10]. Chemotype data were considered inappropriate for predicting the real risk of toxin production in field conditions due to their dependency on the in vitro induction of trichothecene production [10]. Trichothecene genotyping is the only approach that provides baseline data for the evaluation of mycotoxin risk and the confirmation of the presence of *TRI* genes in a given genome [40]. However, despite the importance of trichothecene genotyping, the efficacy of particular species- and trichothecene-specific primer pairs is still unknown. Consequently, this study tested the applicability and universality of species-specific primers for trichothecene genotyping in *F. graminearum* populations originating from Serbia. We also investigated whether there was an association between molecular data and the grain infection levels of winter wheat varieties in different locations.

4.1. Effectiveness of Species- and Trichothecene-Specific Primers for the Characterization of the F. graminearum Population in Serbia

SCAR analysis with Fg16F/R primers showed that the dominant species in the *F. graminearum* clade was *F. graminearum* s. stricto (79.5%). Among the isolates of *F. graminearum* s. stricto, 166 were classified into SCAR group 1 (420 bp), and one isolate was attributed to SCAR group 6 (400 bp). Previous studies indicated that SCAR type 1 was fully congruent with *F. graminearum* s. stricto and that it can be used to separate *F. graminearum* s. stricto from other phylogenetic species of the FGSC [3,23,24]. The isolates belonging to SCAR group 1 belong to lineage 7/RAPD C [25], and the isolates of SCAR group 1 and lineage 7 represent *F. graminearum* s. stricto [26,27]. Thus, we confirmed the prevalence of *F. graminearum* lineage 7/RAPD C/SCAR group 1 in Serbia, and that there were no changes in the previously reported predominance of *F. graminearum* s. stricto [11,12].

Among 210 isolates of the *F. graminearum* clade, 20.5% could not be identified with the Fg16F/R primer pair. Nearly half of the isolates that could not be identified with the Fg16F/R primer pair (46%) produced a PCR product with FgrF/FgcR, which are reported to be highly specific for all lineages of the *F. graminearum* clade, except for lineage 5 (*Fusarium acaciae-mearnsii*) [29]. Since Fg16F/R is specific for lineages 1, 2, 6, and 7 but not for lineages 3, 4, and 5 [28,41], our results are congruent with the hypothesis that FgrF/FgcR primers have higher specificity in distinguishing members of the *F. graminearum* clade from other causal agents of FHB. Nevertheless, 23/210 isolates were B-type trichothecene DON producers, as indicated by their *Tri3* and/or *Tri7* alleles, but were not identified with either pair of species-specific primers. These nonidentified isolates were morphologically distinct from other B-type trichothecene-producing *Fusarium* species (*F. culmorum*, *F. cerealis*, *F. poae*, and *F. equiseti*). Given that species-specific primers were developed from RAPD amplification products (Fg16F/R), and on the basis of sequences of the IGS region (FgrF/FgcR), the target sequences should be analyzed in more detail in *Fusarium* isolates with divergent origins in order to develop a method with greater discriminating power for routine use worldwide.

Trichothecene genotyping of *Tri7*, *Tri3*, and *Tri5* genes indicated that the most common genotype represented 88% of the *F. graminearum* population originating from Serbia. This genotype was DON with respect to *Tri7*, a 15-AcDON producer, and produced a PCR product for *Tri5*. Interestingly, isolates that were not identified with any species-specific primers, and those identified only with FgrF/FgcR had higher frequencies of trichothecene genotypes that lacked PCR products for *Tri3* and/or *Tri5*. Four of the two hundred and ten isolates had no PCR product for *Tri7*, and one of them was proven to carry a deletion of the gene. This isolate was identified as the only 3-AcDON producer among the 210 strains.

We suspected that the lack of effectiveness of GzTri7F/R, Tri315F/R, and Tri5F/R in the trichothecene genotyping of FGSC originating from Serbia could be due to specificity in the structure of the respective genes. The primers for trichothecene genotyping (GzTri7F/R and Tri315F/R) were developed on the basis of 305 isolates that belong to lineages 2, 6, 7, and 9 and originate from 9 regions (China, France, Germany, Italy, Nepal, USA, Sweden, Poland, and Hungary) [23]. Consequently, further testing of the universality of primers for trichothecene genotyping on FGSC isolates with more divergent origins is highly recommended. The effect of interspecific crossing among FGSC members should also be considered when analyzing the efficacy of molecular methods for identifying *Fusarium* spp. and trichothecene genotyping. Interspecific crossing can occur in the FGSC in the laboratory [42] and between *F. boothii* and *F. graminearum* under field conditions [43]. Interspecific crosses yield hybrids with 100% sequence identity in different genes between multiple *Fusarium* spp. [43]. Thus, interspecific crossing should not be overlooked when evaluating factors that hamper the simultaneous determination of species identity and trichothecene genotyping in high-throughput screening of *F. graminearum* populations. As variation in the sequences of the *TRI* genes can lead to the production of different end-products that cannot be detected using chemical analysis, improving trichothecene genotyping methods and monitoring changes in the amino acid sequences of *TRI* genes would enable the more effective prediction of changes in toxin production.

A *Tri5*-specific PCR assay was used in this study for trichothecene genotyping and testing the mycotoxin production potential of FGSC isolates originating from Serbia. A *Tri5*-specific PCR assay, initially developed to detect trichothecene-producing *Fusarium* species, is also highly informative of the DON-producing potential of *Fusarium* spp. [31]. Sanoubar et al. [31] found a correlation between the visually estimated brightness of *Tri-5* DNA PCR products and the amount of DON produced. Although this method is not quantitatively precise, it is a useful tool for the discrimination of isolates with a high or low predisposition for mycotoxin production. The isolates lacking a *Tri-5* PCR product are expected to be DON-free. However, Sanoubar et al. [31] found that 7% of isolates were negative in the *Tri-5* PCR assay but could produce 11–226 μg kg $^{-1}$ DON. This indicates the possible involvement of other genes in mycotoxin production beyond the *TRI* gene cluster, or the possibility that phytotoxins other than DON are produced and affect *F. graminearum* pathogenesis [31]. Harris et al. [7] reported that the contribution of trichothecene production to virulence depends on the host; thus, specificity in the association between the variety Simonida and isolates with a low potential for mycotoxin production should be examined in more detail in the future.

In this study, the DON-producing populations of *F. graminearum* were divergent in relation to the *Tri7* gene structure, but there was no statistically significant association between *Tri7* alleles and locations, varieties, or members of the FGSC. This result is consistent with previous reports that there is no pattern in the regional distribution of *Tri7* alleles [25]. However, Lee et al. [44] reported that the association between selection pressure and genotype diversity has yet to be resolved and that the aggressiveness of isolates is a complex trait that depends on the host and the environment. Indeed, Ward et al. [45] found high levels of genetic variation within the trichothecene mycotoxin gene cluster (virulence-associated genes) and hypothesized that balancing selection maintained *TRI*-cluster polymorphism, which was also reported to be transpecific. Consequently, further investigation of factors affecting the impact of differences in trichothecene genotypes on the host range and fitness in different environments is needed.

4.2. Geographic Distribution of FGSC Members and Their Association with Winter Wheat Grain Infection in Serbia

We found that three out of five winter wheat varieties (Simonida, Ilina, and Zvezdana) cultivated in all four regions of Serbia (Autonomous province of Vojvodina, Belgrade, Šumadija and Western Serbia, and Southern and Eastern Serbia) were associated with *F. graminearum* s. stricto, *F. graminearum* s. lato, and nonidentified (FGSC) isolates. However, the incidence of three groups of FGSC members was not equal between the three varieties.

The nonidentified and *F. graminearum* s. lato isolates predominated on the variety found in Simonida, while *F. graminearum* s. stricto predominated on the in varieties found in Illina and Zvezdana. The specificity in the association between susceptible varieties and isolates with different genetic potentials for mycotoxin production is also indicated in this study. Isolates showing faint *Tri5* PCR products and isolates lacking the PCR product of the *Tri5* gene were more associated with Simonida than with the rest of the winter wheat varieties. Different lineages of the FGSC are typically reported to vary in their preferences for different crops [43], but we found that FGSC members have preferences for varieties as well.

The genetic potential for mycotoxin production was not the main feature for the discrimination of the location and the variety with respect to high and low levels of grain infection. For example, grain infections in Simonida never exceeded 30%. FDK was also low (12%) for Ilina, which was predominantly infected with *F. gramnearum* s. stricto and isolates with a high potential for mycotoxin production. This result is consistent with other studies that found no correlation between the aggressiveness of *F. graminearum* isolates, lineage, toxin production, and chemotype [46–49]. Since climatic conditions at the time of anthesis were favorable for FHB in all maturing groups in 2017 and 2019, climatic conditions did not hamper the grain infection of the late-maturing variety found in Ilina. Consequently, the specificity of the interaction between the variety and agroecological conditions at a single location could be the main factor that promotes or prevents the occurrence of FHB. Indeed, in Valjevo (Šumadija and Western Serbia), grain infection of the variety found in Ilina in 2017 reached 58%, although in other locations, it never exceeded 20%, even in years that were conducive to FHB.

To date, the management of FHB has relied primarily on combined control strategies that depend on the weather conditions, the timing of fungicide application, variety resistance, tillage practice, and crop rotation [50]. However, interactions between the species that colonize wheat grain and their effect on wheat grain infection/contamination have usually been neglected [51]. We found that the level of grain infection with *Fusarium* spp. depends on the interactions among species that colonize wheat grain and their interactions with agroecological conditions that are specific to each wheat-producing area. Grain contamination with *Alternaria* spp. and its interaction with the year had significant effects on grain infection by *F. graminearum*. These results complement the findings of Jevtic et al. [52], who found antagonistic interactions between *Fusarium* and *Alternaria* spp. on moderately susceptible and susceptible varieties, emphasizing the effect of the variety itself. These findings also support the results of Müller et al. [51], who found that different strains of the same species could act differently in the presence of the same competitor strains. Since mycotoxin production can affect the relationship between competing organisms colonizing wheat grain [51], more studies on the contribution of mycotoxin production to the relationship between *Alternaria* spp and *F. graminearum* are needed to ensure the more reliable prediction and control of FHB.

5. Conclusions

In this study, we showed that species- and trichothecene-specific primers lack universality in the characterization of the *F. graminearum* population and thus affect the efficacy of the FHB surveillance. The results also indicated the diversity of susceptibility response of individual varieties to FHB in different agroecological conditions, with *Alternaria* spp. contamination having a pronounced effect on grain infection with *F. graminearum*. The analyses showed a close association between individual wheat varieties and members of the *F. graminearum* population that could not be identified using species-specific primers. The genetic predisposition of these strains for mycotoxin production also differed when compared with the more common *F. graminearum* s. stricto. These results show that there are members of the FGSC that cannot be identified in a timely manner and thus pose a threat to the efficient surveillance, prediction, and control of FHB.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11040778/s1. Table S1: Averages of grain infection with FGSC, *Alternaria* spp., and *F. poae* in a three-year period in 32 locations in Serbia. Table S2: Diversity of trichothecene genotypes of FGSC from winter wheat in Serbia. Table S3: Climatic conditions in a three-year period (2017–2019) at the time of the anthesis of the winter wheat in agroecological conditions in Serbia. Table S4: Regression analysis of the most influencing factors on grain infection with FGSC in the period 2017–2019. Table S5: Relative frequencies of *Tri7* alleles in DON producers originating from Serbia. Table S6: Relative frequencies of *Tri7* alleles in *F. graminearum* s. stricto, *F. graminearum* s. lato, and nonidentified (FGSC). Table S7: Amount of variance explained by each principal component in the MCA analysis.

Author Contributions: Conceptualization, V.Ž. and R.J.; data curation, M.L. and B.O.; formal analysis, V.Ž.; funding acquisition, R.J.; investigation, V.Ž., M.L., S.M. and B.O.; methodology, V.Ž.; project administration, R.J.; resources, R.J.; software, V.Ž.; supervision, R.J.; validation, R.J.; visualization, V.Ž.; writing—original draft, V.Ž.; writing—review and editing, R.J. and S.M. All authors have read and agreed to the published version of the manuscript.

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