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Molecular Variation and Phylogeny within *Fusarium avenaceum* and Related Species

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Abstract: Many recent articles feature research on the *Fusarium tricinctum* species complex (FTSC), and their authors present different ideas on how the isolates of this species complex can be identified at the species level. In previous studies, our aim was to investigate the phylogeny of FTSC strains, which researchers have morphologically identified as *Fusarium avenaceum*, *Fusarium arthrosporioides*, and *Fusarium anguioides.* In the current study, our phylogenetic maximum parsimony and likelihood analyses of the DNA sequences of the translation elongation factor 1-alpha (*TEF1*) and combined sequences of *TEF1* and beta-tubulin (*TUB2*) supported the existence of at least four main groups among these strains. Main Group I mainly contains *F. avenaceum* strains, while Main Group II contains two subgroups, one of which primarily includes *F. arthrosporioides* strains, and the other mainly includes European *F. anguioides* strains. Main Group III contains strains from different plants that originated from Asia, including two *F. anguioides* strains. *F. avenaceum* strains, which are mostly isolated from different trees, form Main Group IV. A fifth group (Main Group V) was only supported by *TEF1* sequences. The main groups previously found by us based on *TUB2* sequences could be connected to the new species of the FTSC, which were identified based on *TEF1* sequences. In addition, we found strains that significantly differ from Main Groups I-V, and we grouped some of them as single, intermediate, or sister groups. All of the main groups of the present work, and some single and intermediate strains, may represent different species of the FTSC, while the two subgroups of Main Group II constitute intraspecific variation. Regardless of whether they belonged to the main groups, all the analysed strains were able to form different enniatins and 2-amino-14,16-dimethyloctadecan-3-ol, but did not produce beauvericin.

Keywords: *Fusarium anguioides*; *F. arthrosporioides*; *F. avenaceum*; *Fusarium tricinctum* species complex; beta-tubulin; elongation factor 1-alpha; phylogenetic species; enniatins; 2-amino-14,16-dimethyloctadecan

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1. Introduction

The morphological and molecular descriptions of fungi that are closely related to *Fusarium avenaceum* (Fr.) Sacc. have been controversial, and researchers have presented different ideas on how to identify the isolates of this species group at the species level [1–11]. At present, we include *F. avenaceum* isolates, *F. arthrosporioides* and *F. anguioides* isolates, and the different morphologically intermediate isolates of the *F. avenaceum* species complex in the large *Fusarium tricinctum* species complex (FTSC) [9], which, according to Laraba et al. [11], consists of 36 phylogenetically different species, phylospecies FTSC 1–36, based on multilocus sequence analyses. The morphologically described species (morphospecies) *F. avenaceum*, *F. arthrosporioides* Sherb., and *F. anguioides* Sherb. are highly similar, do not have distinct phenotypic boundaries, and typically produce macroconidia, with five septa and little variation in size [12].

In the classical taxonomy, *F. avenaceum* and *F. arthrosporioides* belong to the section *Roseum*, but *F. anguioides* is a member of the section *Arthrosporiella*, based on the morphological features, such as the ability of the latter to form chlamydospore-like cells and blastic or phialidic conidiogenous cells, rarely with two conidiogenous loci [12]. The authors of the other manuals for *Fusarium* species identification do not even mention *Fusarium anguioides* [13,14].

In many cases, the identification of the isolates of these three species based only on morphology leads to species identities that differ from identities determined by DNA-sequence-based methods and mycotoxicological analyses [5,10,15–18]. *Fusarium avenaceum* is one of the *Fusarium* species that are most frequently isolated from plants cultivated in diverse geographic and climatic regions [19–25]. *Fusarium anguioides* and *F. arthrosporioides* occur frequently on different plant species, including cereals and dicotyledons. Both species are closely related to *F. avenaceum* and are often identified as *F. avenaceum* based on morphology [5,16,26]. In an analysis of the pathogenicity, the *F. avenaceum* and *F. arthrosporioides* strains were more aggressive than the *F. anguioides* strains in that they caused a greater reduction in the lengths and larger necroses in winter wheat seedlings [5]. All three species produce mycotoxins, such as moniliformin and enniatins (ENNs), particularly in cereals [10,11,18,23,27–31]. Furthermore, the presence of the enniatin synthase gene in multiple strains is consistent with ENN production [30–32]. There is a correlation between *F. avenaceum* DNA and the moniliformin and enniatin levels in cereal grains [17]. Enniatins also influence the virulence in potato tubers [33].

In Finland, Norway, and northwestern Russia, *F. avenaceum* is one of the most common *Fusarium* species in small-grain cereals [19,23,26,28,34,35]. A recent study on the occurrence of *Fusarium* species and mycotoxins in small-grain cereals grown in the Urals and western Siberia revealed that the proportions of *F. avenaceum* and *F. anguioides* among all morphologically identified *Fusarium* species reached 20% and 13%, respectively. However, the levels of moniliformin in the grain were only positively correlated with *F. avenaceum* infection in the grain [25]. Nelson et al. [36] neotypified *F. anguioides* using strains of the *F. concolor* species complex from China, which are more distantly related to *F. avenaceum* than to the *F. anguioides* strains that Nirenberg identified (e.g., strain BBA 69055 = CBS 173.12) [3,4,15,37]. According to Nelson et al. [36], BBA 69055 is not any more morphologically identifiable as *F. anguioides*, and, according to Jacobs-Venter et al. [3], the isolate CBS 173.12 belongs to *F. avenaceum*. According to Crous et al. [38], the strain BPI 72044 was designated as a neotype of *F. anguioides*, and it was erroneously stated that no material was available for epi- and lectotypification [36]. However, Sherbakoff provided an illustration with the original protologue of *F. anguioides* and deposited cultures of this species in the Cornell University Plant Pathology Herbarium as sample CUP-007479 [39].

According to phylogenetic analyses based on the DNA sequences of the beta-tubulin (*TUB2*) and transcription elongation factor 1-alpha (*TEF1*) genes, as well as the ribosomal intergenic spacer (IGS) region, *F. avenaceum*, *F. arthrosporioides,* and *F. anguioides* isolates form a clade that is closely related to *F. tricinctum* (Corda) Sacc. [5,10,11,15,16,31,40]. In these molecular analyses, isolates that had previously been identified by morphology were resolved as separate groups based on variations in the DNA sequences. Yli-Mattila et al. [5] named the four main groups based on *TUB2* sequences and inter-simple sequence repeat (ISSR) fingerprints: Main Group I primarily contains morphologically *F. avenaceum* strains; Main Group II mostly contains morphologically *F. arthrosporioides* strains; Main Group III mostly contains morphologically *F. anguioides* strains that originated from Europe; Main Group IV includes strains that originated from Asia, one of which was identified as *F. anguioides* based on morphology.

In the present work, we aimed to investigate the diversity of the *TEF1* sequences and mycotoxins of geographically and morphologically different strains of *F. avenaceum* and related species to ascertain whether any of the four main groups based on *TUB2* sequences and ISSR fingerprints are supported by *TEF1* sequences [4,5,32,40]. We also investigated how the main groups based on *TUB2* sequences, as well as the *TEF1* haplotypes defined by

Kulik et al. [32] and the *TEF1* sequences published by Stakheev et al. [40], correspond to the resolutions in the FTSC of O'Donnell et al. [4] and Laraba et al. [11]. We first compared the *TEF1* sequences of the four main groups of *F. avenaceum* isolates [5] to the sequences in GenBank, and subsequently to the various groups of *F. avenaceum* isolates used in the different studies [4,11,24,32,40]. Finally, we analysed combined *TUB2* and *TEF1* sequences to confirm the results.

2. Materials and Methods

The list of the studied isolates and their abbreviations can be found in Table 1. Morphological analyses were performed macroscopically and microscopically [12,36,39]. DNA was extracted using the CTAB method, according to the common protocol [41] or by using a GenElute™ Plant 241 Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA), as described by Yli-Mattila et al. [42]. A fragment of *TEF1* was amplified and sequenced from both ends using the oligonucleotide primers EF1 and EF2, as described by O'Donnell et al. [43]. Five DNA sequences were submitted to GenBank, with the accession numbers OK564509, OK564510, OK564511, OK564512, and OK564513. The *TEF1* sequences were aligned using MUSCLE [44]. The maximum-parsimony (MP) and maximum-likelihood (ML) consensus trees were constructed using Mega 11 [45], in which clade support was assessed by 500 ML bootstrap pseudoreplicates of the data.

Table 1. *Fusarium* strains used in the present study and identified based on morphological and molecular characteristics.

Group	FTSC *	Morphological Species	Strain No.	Alternative Strain No. **	Origin	Substrate	Year
Main Group I	4	*F. avenaceum*	MFG 118702	av48 [c]	Russia, Pskov	barley, grain	2009
		F. arthrosporioides	MFG 58655	ar57 [c]	Russia, Leningrad	oat, grain	2013
		F. avenaceum	BBA 64151	a2 [b]	Germany	*Solanum*	1980
		F. arthrosporioides	BBA 71186	ar7 [b]	Germany	*Bellis*	1999
		F. anguioides	MFG 114605	an41 [c]	Russia, Kaliningrad	barley, grain	2008
		F. anguioides	MFG 119913	an42 [c]	Russia, Kirov	oat, grain	2008
		F. anguioides	MFG 108904	an45 [c]	Russia, Pskov	barley, grain	2008
		F. arthrosporioides	BBA 67701	ar5 [b]	France	cereals	1993
		F. avenaceum	DDPP 04401	NA ***	Poland	wheat	NA
		F. avenaceum	KK12	NA	Hungary	wheat	NA
		F. avenaceum	F1	NA	Hungary	wheat	NA
		F. avenaceum	18	NA	Hungary	wheat	NA
		F. avenaceum	DDPP 042836	NA	Poland	wheat	NA
		F. avenaceum	DDPP 04032	NA	Poland	wheat	NA
		F. avenaceum	K-0238	NA	Russia	wheat	NA
		F. avenaceum	FCR R6679	NA	Australia	soil	NA
		F. avenaceum	NRRL 26890	NA	Finland	wheat	NA
		F. avenaceum	FRC R-671	NA	USA, New York	alfalfa	NA
		F. avenaceum	FaLH27	NA [d]	Canada	winter wheat	2001
Main Group II	4	*F. anguioides*	MFG 112804	an37 [c]	Russia, Novgorod	barley, grain	2008
		F. arthrosporioides	BBA 70576	ar1 [b]	Finland	barley	1986
		F. avenaceum	FaLH03	NA [d]	Canada	spring wheat	2001
		F. avenaceum	NRRL 54939	Fa05001 [d]	Finland	barley	2005
		F. avenaceum	F093	NA	Canada	lupin	NA
		F. avenaceum	NRRL 25128	NA	Poland	Ichneumonidae	NA
		F. avenaceum	CBS 15957	NA	Italy	*Fagus sylvatica*	NA
		F. avenaceum	DDPP 09a1	NA	Poland	wheat	NA
		F. anguioides	MFG 74005	NA	Russia	wheat	NA
		F. avenaceum	F-132	NA	Russia	wheat	NA
		F. anguioides	MFG 111502	NA	Russia	barley	NA

Table 1. *Cont.*

Group	FTSC *	Morphological Species	Strain No.	Alternative Strain No. **	Origin	Substrate	Year
Main Group V	4	*F. avenaceum*	NRRL 36069	CBS 101627	UK	carnation	NA
		F. avenaceum	NRRL 53589	CBS 386.62	Netherlands	winter wheat	NA
		F. avenaceum	NRRL 53729	CBS 121289	Switzerland	winter wheat	NA
		F. avenaceum	CBS 409.86	NA	USA	barley	NA
		F. avenaceum	05-003	NA	Switzerland	wheat	NA
		F. avenaceum	376	NA	Switzerland	wheat	NA
Intermediate Group Between Main Groups I and II	4	*F. avenaceum*	IBT 40026	NA	Denmark	wheat	NA
		F. avenaceum	CBS 409.86	NA	USA	barley	NA
		F. avenaceum	DDPP 04401	NA	Poland	wheat	NA
		F. avenaceum	379	NA	Switzerland	wheat	NA
		F. avenaceum	CML 3545	NA	Brazil	wheat	NA
		F. avenaceum	FCR R-9495	NA	USA, California	lisianthus	NA
		F. avenaceum	MFG 80310	NA	Russia	oat	NA
		F. avenaceum	F-2307	NA	Germany	wheat	NA
		F. avenaceum	NRRL 36374	CBS 239.94	Netherlands	carnation	NA
		Gibberella tricincta	NRRL 39591	ICMP 5244	New Zealand	garden pea	NA
		F. avenaceum	NRRL 53733	CBS 121294	Switzerland	winter wheat	NA
Unknown Group I	22	*F. avenaceum*	FRC R-6739	NA	Germany	codling moth	NA
		F. avenaceum	BBA 63201	a4 [b]	Austria	*Ulmus scabra*	1974
Main Group III	11	*F. anguioides*	MFG 58314	an47 [c]	Russia, Vladivostok	rudbeckia, leaves	2010
		Fusarium sp.	CML 3541	NA	Brazil	wheat	NA
		F. avenaceum	FRC-R9369	NA	Canada	lisianthus	NA
		F. avenaceum	JRBK-5	NA	China, Hubei	walnut	NA
		F. avenaceum	SICAUCC 18	NA	China	*Polygonatum cyrtonema*	NA
		F. sp. nov-9	MRC 2532	NA	Japan	soybean	NA
		F. avenaceum	Fv5-9	NA	China	*Fragaria ananassa*	NA
	27	*F. anguioides*	BBA 69055	an3 [b]	Japan	wheat, grain	1994
Main Group IV	5	*F. avenaceum*	BBA 70177	a49 [a], NRRL 26893	Finland	apple	NA
		Fusarium sp.	NRRL 52726	NA	Turkey	NA	NA
		Fusarium sp.	KOD 810	NA	USA, New Hampshire	fir	NA
		F. avenaceum	10992	NA	New Zealand	*Pinus radiata*	NA
		F. avenaceum	E19/2-4	NA	Slovenia	*Acer pseudoplatanus*	NA
Unknown Group II	30	*F. arthrosporioides*	BBA 70561 [b,e]	ar2 [b]	Finland	barley	1986
Unknown Group III	?	*F. avenaceum*	MFG 80212	NA	Russia	oat	NA
Unknown Group IV	34	*F. avenaceum*	BBA 70575 [b,e]	a5 [b]	Germany	*Cytisus*	1997
	9	*F. torulosum*	NRRL 22747	NA	Hungary	barley	NA
	3	*F. tricinctum*	NRRL 25481	NA	NA	NA	NA
		F. tricinctum	BBA 64485	t6 [b]	Germany	wheat	1986
	2	*F. acuminatum*	NRRL 36147	NA	NA	NA	NA
		F. acuminatum	NRRL 62622	CS4907	NA	NA	NA

* FTSC marked according to [11]; ** alternative names of strains are from [5,16,46–48], for a, b, c, d, and e, respectively; *** NA: not available.

The sequence data for all the *TUB2* and several *TEF1* sequences were downloaded from GenBank, and we also referred to those reported in previous studies [4,11,16,32,40,46]. One can find all of the sequences in GenBank based on the strain names specified in Table 1. The accession numbers of the sequences used in Figures 1–5 and Supplementary Figures S1–S5 can be found in Supplementary Table S1.

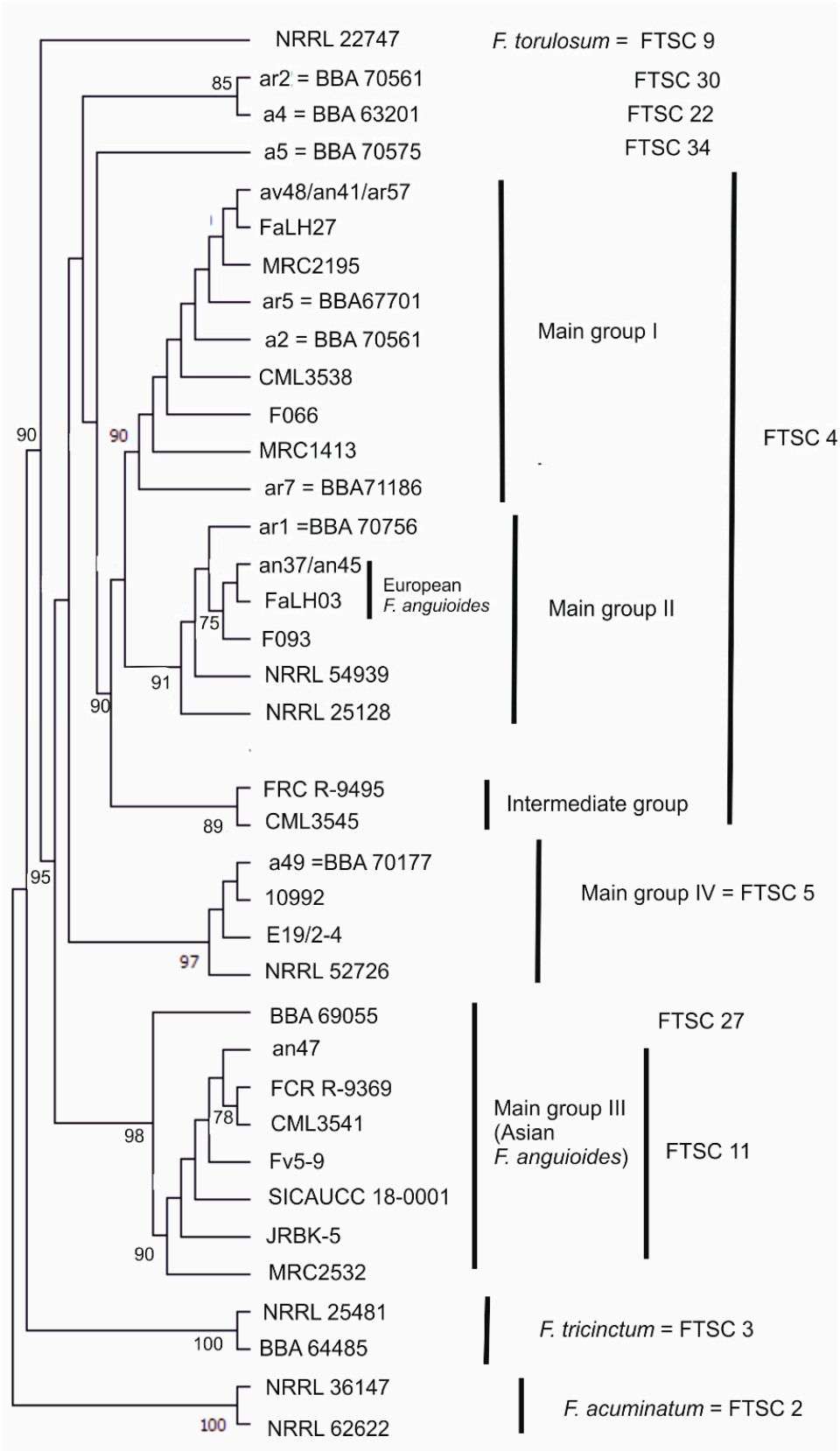

Figure 1. Maximum-parsimony consensus tree of translation elongation factor 1-alpha (*TEF1*) sequences. Bootstrap values are indicated above nodes based on 500 pseudoreplicates of the data. Only branches present in more than 50% of the trees are shown. *Fusarium tricinctum* and *F. acuminatum* strains are outgroups.

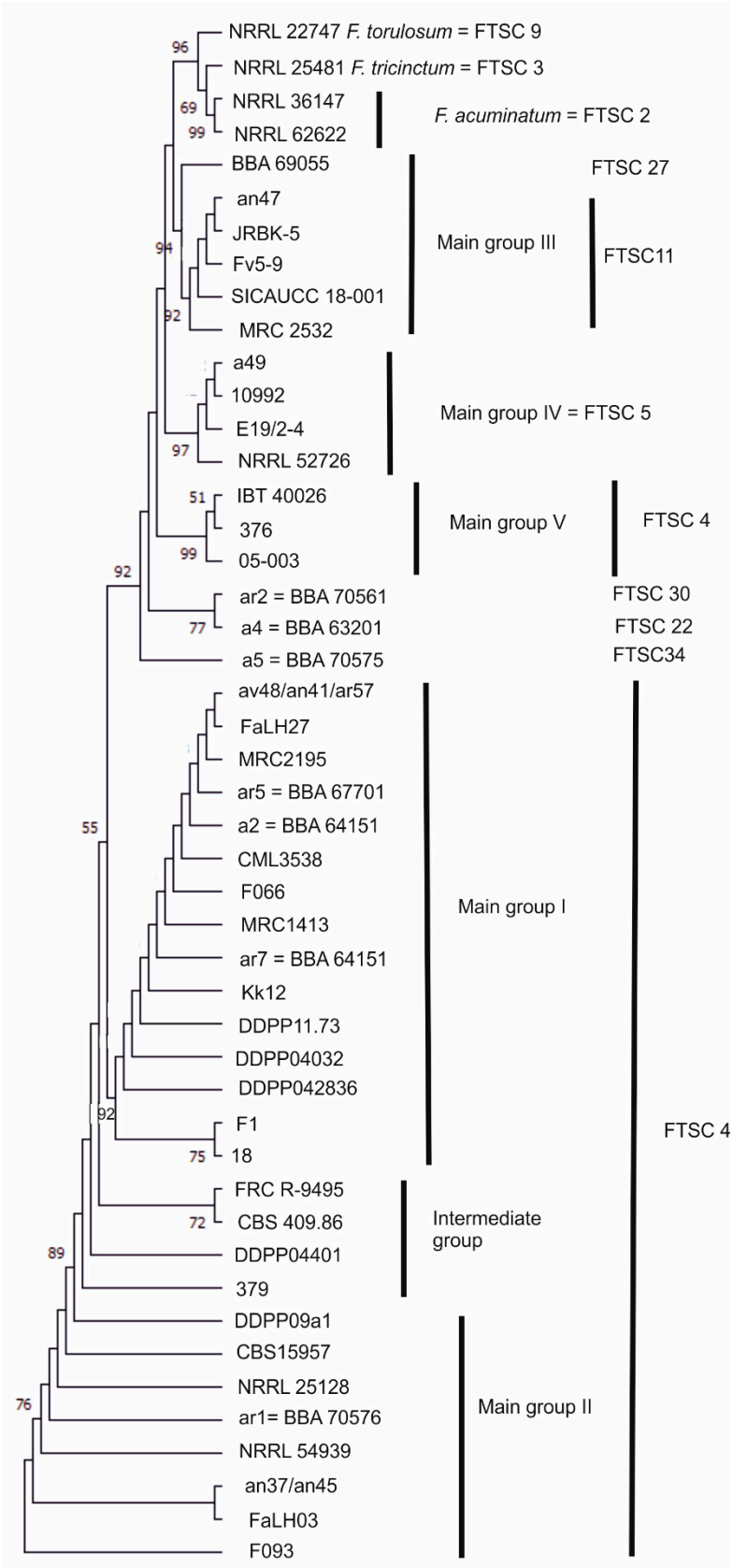

Figure 2. Maximum-parsimony consensus tree of translation elongation factor 1-alpha (*TEF1*) sequences, including strains from the work of Kulik et al. [32] (2011). Bootstrap values are indicated above nodes based on 500 pseudoreplicates of the data. Only branches present in more than 50% of the trees are shown. *Fusarium tricinctum* and *F. acuminatum* strains are outgroups.

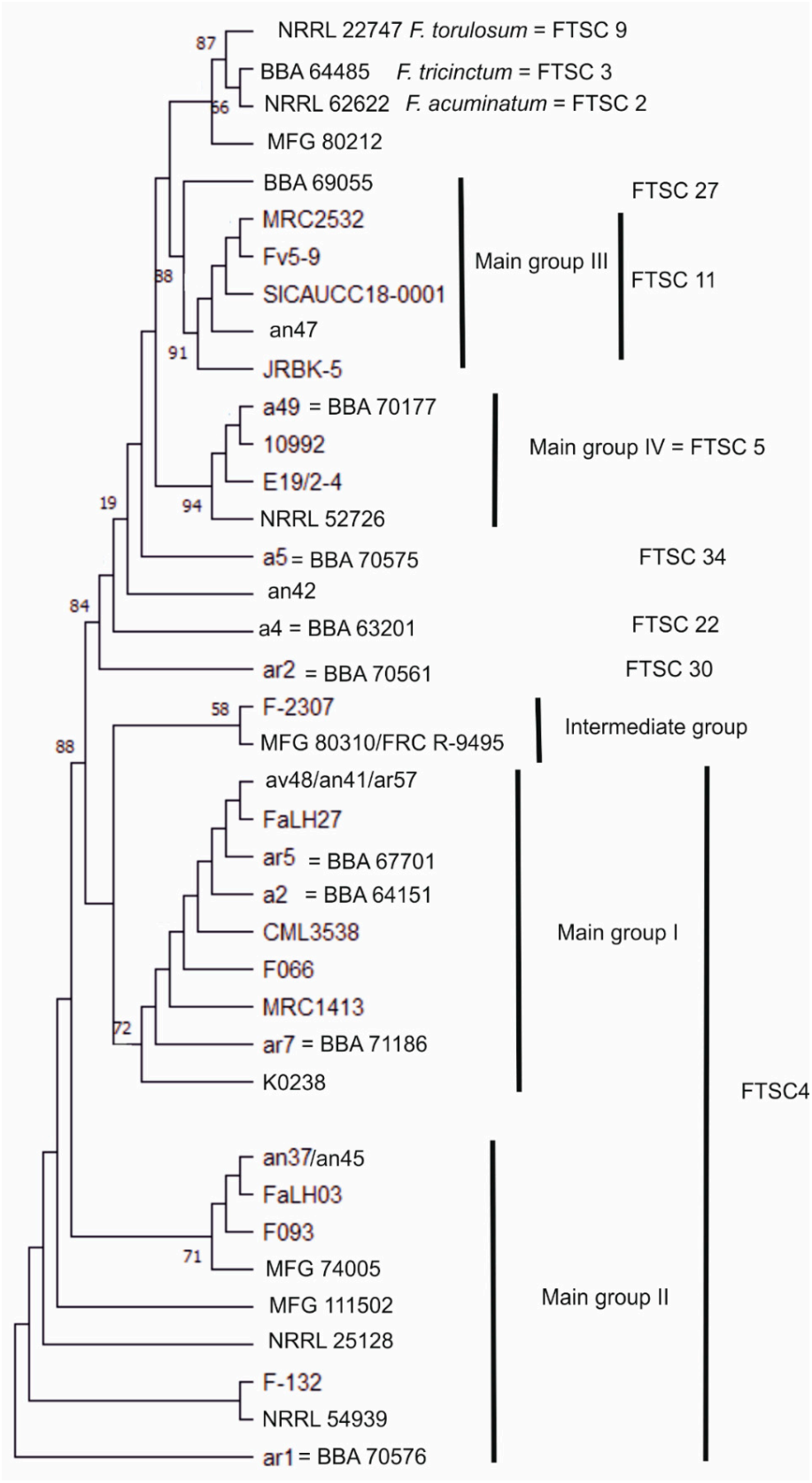

Figure 3. Maximum-parsimony consensus tree of translation elongation factor 1-alpha (*TEF1*) sequences, including strains from the work of Stakheev et al. [40] (2016). Bootstrap values are indicated above nodes based on 500 pseudoreplicates of the data. Only branches present in more than 50% of the trees are shown. *Fusarium tricinctum* and *F. acuminatum* strains are outgroups.

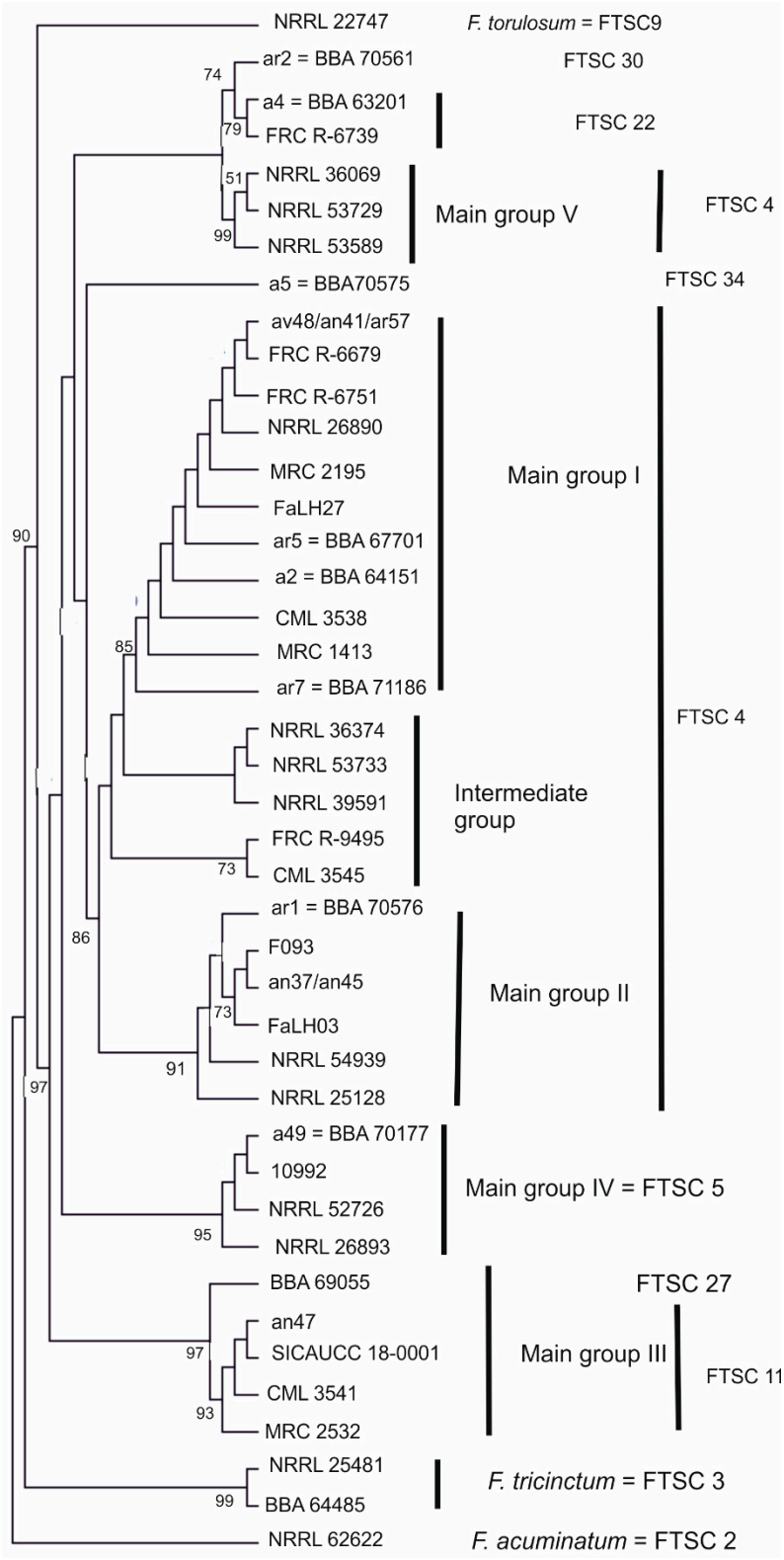

Figure 4. Maximum-parsimony consensus tree of translation elongation factor 1-alpha (*TEF1*) sequences, including strains from the work of Laraba et al. [11] (2022). Bootstrap values are indicated above nodes based on 500 pseudoreplicates of the data. Only branches present in more than 50% of the trees are shown. *F. tricinctum* and *F. acuminatum* strains are outgroups.

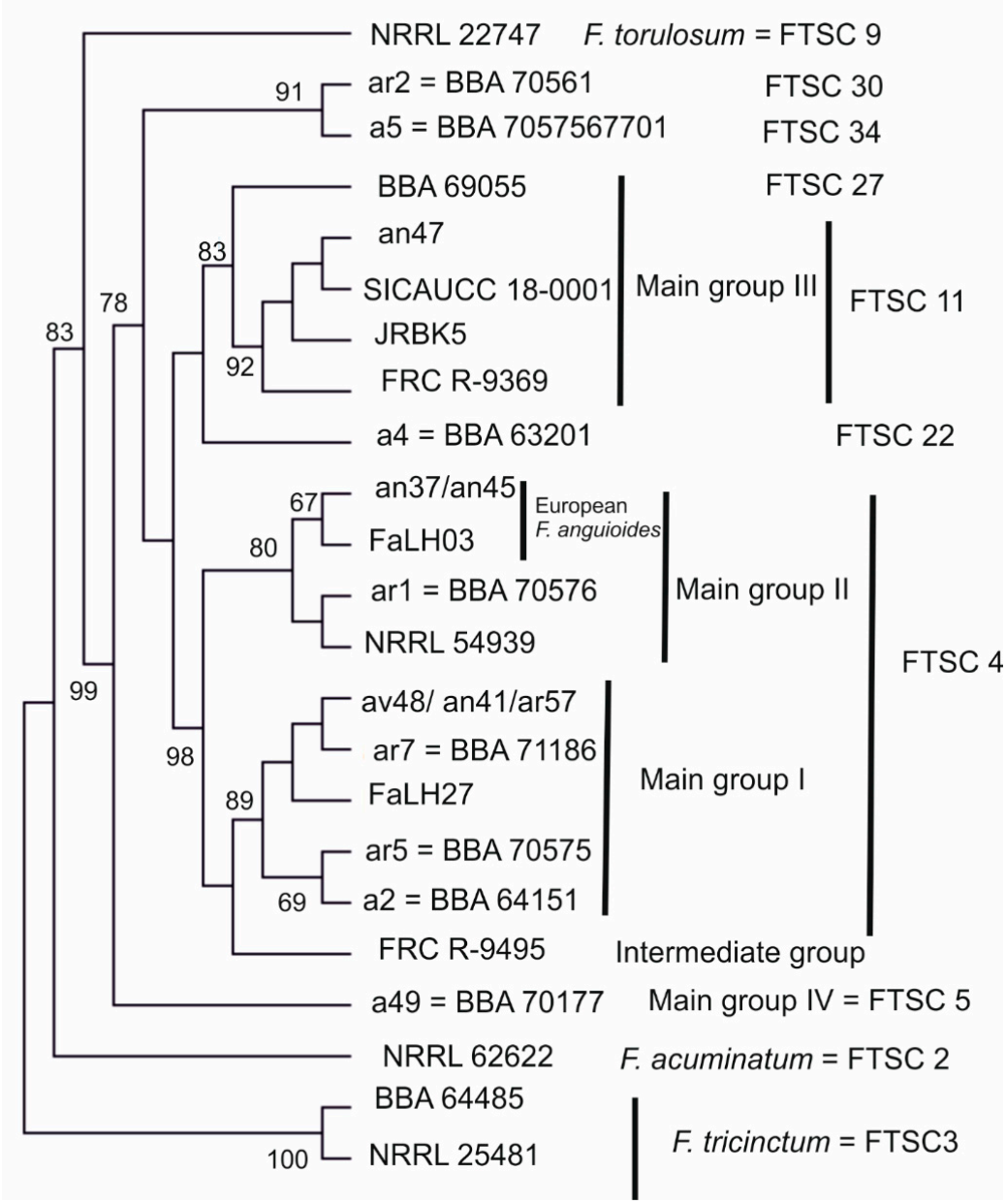

Figure 5. Maximum-parsimony consensus tree for combined translation elongation factor 1-alpha (*TEF1*) and *TUB2* sequences. Bootstrap values are indicated above nodes based on 500 pseudoreplicates of the data. Only branches present in more than 50% of the trees are shown. *Fusarium tricinctum* and *F. acuminatum* strains are outgroups.

The mycotoxin production on cracked corn kernels in eight strains that were included in the phylogenetic analyses of the present work was evaluated, as previously described by [11]. In addition, mycotoxins were analysed in the strains MFG 151200, MFG 103100, MFG 108904, MFG 112804, MFG 48314, BBA 69055 [5], BBA 64134, BBA 64215, BBA 70561, BBA 67800 [16], the NRRL 13826 strain in *F. avenaceum*, the MFG 58549 strain in *F. reticulatum* Mont., the MFG 60500 strain in *F. graminum* Corda, and the strains MFG 42305, MFG 58674, and MFG 60360 in *F. acuminatum* (Table 2). After 15 days, cultures were extracted with 50 mL acetonitrile/water (86:14 *v*/*v*) by shaking at 30 rpm for 2 h. High-performance liquid chromatography–mass spectrometry (HPLC–MS) analyses of the extracts were performed using a Dionex Ultimate U3000 liquid chromatography system connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific). The detection and quantification of the

metabolites were based on comparisons of the retention times, masses, and mass spectra to purified standards, with a detection level of 1 ng toxin/µL.

Table 2. Mycotoxins produced by strains of the *Fusarium tricinctum* species complex compared with *F. graminum* and *F. reticulatum* strains on cracked corn substrate.

Main Group/FTSC *	Morphological Species	Strain No. **	Mycotoxin Amount (ng/mL)					
			BEA ***	ENN B	ENN B1	ENN A	ENN A1	AOD
−/4	*F. avenaceum*	NRRL 13826	1.0	20.0	5.2	0	LOQ	LOQ
I/4	*F. arthrosporioides*	BBA 71186 [a]	0	22.7	5.8	0	LOQ	41.5
I/4	*F. anguioides*	BBA 63598 [a]	0	0	0	0	0	LOQ
I/4	*F. avenaceum*	MFG 151200 [b]	0	10.5	1.5	0	LOQ	20.1
II/4	*F. arthrosporioides*	BBA 64134 [a]	0	5.2	LOQ	0	LOQ	19.6
II/4	*F. arthrosporioides*	BBA 64215 [a]	0	0	0	0	0	LOQ
II/4	*F. anguioides*	MFG 103100 [b]	0	8.4	2.2	0	LOQ	31.5
II/4	*F. anguioides*	MFG 108904 [b]	0	LOQ	LOQ	0	LOQ	13.9
II/4	*F. anguioides*	MFG 112804 [b]	0	2.0	LOQ	0	LOQ	11.2
III/11	*F. anguioides*	MFG 58314 [b]	0	1.6	LOQ	0	0	125.8
III/27	*F. anguioides*	BBA 69055 [a,b]	0	41.2	21.2	0	3.9	LOQ
–/22	*F. avenaceum*	BBA 63201 [a]	0	4.8	LOQ	0	LOQ	LOQ
−/30	*F. arthrosporioides*	BBA 70561 [a]	0	LOQ	LOQ	0	1.7	1.5
−/3	*F. tricinctum*	BBA 64485 [b]	0	LOQ	LOQ	0	LOQ	4.3
−/2	*F. acuminatum*	MFG 42305	0	LOQ	LOQ	0	0	1.2
−/2	*F. acuminatum*	BBA 67800 [a]	0	36.0	38.5	LOQ	19.8	27.6
−/2	*F. acuminatum*	MFG 58674	0	25.1	22.5	LOQ	10.3	21.8
−/2	*F. acuminatum*	MFG 60360	0	22.1	20.4	LOQ	8.1	12.3
−/−	*F. graminum*	BBA 65242 [a]	0	0	0	0	0	LOQ
−/−	*F. graminum*	MFG 60500	0	LOQ	LOQ	0	LOQ	LOQ
−/−	*F. reticulatum*	MFG 58549	0	4	2.8	0	1.1	170.9

* FTSC marked according to [11]; ** strain numbers are from [16] and [5] for a and b, respectively. *** BEA: beauvericin; ENN: enniatin; AOD: 2-amino-14,16-dimethyloctadecan-3-ol. LOQ values are below the limit of mycotoxin quantitation (<1 ng/mL). "0" means that no toxin was detected.

3. Results

3.1. Molecular Characterisation of Strains

According to the *TEF1* sequences of 570 nucleotides, the strains can be divided into four main groups, which are supported by bootstrap values of >90% in the MP tree (Figure 1) and >83% in the ML tree (Supplementary Figure S1). Main Group I was represented by the *F. avenaceum* strains av48 and a2, the *F. arthrosporioides* strains ar57, ar7, and ar5, and the *F. anguioides* strain an41, which all originated from Europe [5], as well as the isolates MRC 1413 of unknown origin, F066 from Canada [48], CML 3538 from Brazil, and MRC 2195 from South Africa. Main Group II was represented by the *F. arthrosporioides* strain ar1 and the *F. avenaceum* strain NRRL 54939 from Finland [48], the NRRL 25128 strain from Poland, the *F. anguioides* strains an37 and an45 from the European part of Russia, and strain F093 from Canada [48]. Two subgroups within Main Group II were supported by bootstrap values of 75% and 61% in the MP and ML trees, respectively. The strains FRC R-09495 (USA) and CML 3545 (Brazil) were in an intermediate group that was between Main Groups I and II in the phylogenetic tree (Figure 1, Supplementary Figure S1).

The third main group included the *F. anguioides* strain an47, as well as other Asian strains, including MRC 2532 (FTSC 11), SICAUCC 18, and JRBK-5, the strain FRC R-9369 from Canada, and the strain CML 3541 from Brazil. BBA 69055 was separated from the other strains of Main Group III. The fourth main group was represented by strain a49 from Finland, strain 10992 from New Zealand, strain E19/2-4 from Slovenia, and strain NRRL

52726 (FTSC5) from Turkey. Most of the strains from Main Group IV were isolated from trees. The *Fusarium arthrosporioides* strain ar2 from Finland, the *F. avenaceum* strain a4 from Austria, and the *F. avenaceum* strain a5 from Germany were separated from all the other *F. avenaceum* strains, as well as from the *F. tricinctum*, *F. acuminatum* (Ellis and Everh), and *F. torulosum* (Berk. and M.A. Curtis) Nirenberg strains (Figure 1, Supplementary Figure S1).

When we compared the *TEF1* sequences presented in Figure 1 to the 515 bp long sequences published by Kulik et al. [32], five main groups were supported by bootstrap values of >89% in the MP tree (Figure 2) and >82% in the ML tree (Supplementary Figure S2). Several strains of Main Groups I and II (strain CBS 15967, and six other European strains) belonged to the same *TEF1* haplotype (Figure 2). Main Group II, including the strains that Kulik et al. analysed [32], belonged to the subgroup that the strain ar1 created.

The strain CBS 409.86 from the United States was in the intermediate group with the strain FRC R-9495. Strain DDPP 04401, which represents a European haplotype [32], and strains 379 and DDPP 09a1 from Europe, were also between Main Groups I and II in the ML tree (Supplementary Figure S2), while, in the MP tree, DDPP09a1 was in Main Group II (Figure 2). We found a new main group (Main Group V) of 14 strains of 2 *TEF1* haplotypes [32] represented by strains 05-003, 376, and IBT 40026, which is related to Main Groups III and IV of the *F. avenaceum* species complex, based on the MP consensus tree. Strains ar2 and a4 formed their own main group in the parsimonious consensus tree, and they, together with strain a5, were again separated from all the other *F. avenaceum* strains (Figure 2, Supplementary Figure S2).

When we compared the *TEF1* sequences used for the construction of Figure 1 to the 525 bp long *TEF1* sequences of the strains published by Stakheev et al. [40], which represent the five main clusters of the phosphate permease gene (PHO) phylogenetic tree, four main groups were supported by bootstrap values of >72% in the MP tree (Figure 3) and >65% in the ML tree (Supplementary Figure S3). We found more strains related to the European *F. anguioides* strains of Main Groups I and II among the strains collected in Russia. Three of these strains (an42, MFG 74005, and MFG 111502) were morphologically identified as *F. anguioides*. The American strain F093 was again in the same subgroup with an37, an45, and MFG 74005, but MFG 111502 was closer to another subgroup of Main Group II. Strain K-2308 was in Main Group I, while strain F-2307 was between Main Groups I and II. Strain MFG 80310 from Russia and strain FRC R-09495 from the United States belonged to the intermediate group between Main Groups I and II. Strains a5, an42, a4, ar2, and MFG 80212 from the Russian Far East were located between Main Groups I and IV and the *F. tricinctum* strain BBA 64485. No strains of Main Groups III, IV, and V were found among the strains, which had been previously analysed by Stakheev et al. [40].

In the fourth comparison, in which we compared the *TEF1* sequences of Figure 1 to the *TEF1* sequences of Laraba et al. [11], five main groups were supported by bootstrap values higher than 86% in the MP tree (Figure 4) and 83% in the ML tree (Supplementary Figure S4). The isolates NRRL 53589 and NRRL 53729 had identical *TEF1* sequences to the isolate IBT 40026 (Figure 2), while strain FRC R-6739 (FTSC 22) was in the same group as the isolate BBA 63201. We found a new subgroup formed by three strains in the intermediate group. BBA 69055 was separated from the other strains of Main Group III, and, according to the information from [11], it belongs to FTSC 27.

Finally, we added 443-nucleotide-long *TUB2* sequences [5] to the *TEF1* sequences. In the combined *TEF1–TUB2* consensus tree of the selected strains, Main Groups I, II, III, and IV were supported by bootstrap values of >80% in the MP tree (Figure 5) and >81% in the ML tree (Supplementary Figure S5). Strains ar5 and a2 formed a subgroup in Main Group I. The American strain FRC R-9495 was again between Main Groups I and II, and strain BBA 69055 separated from Main Group III, while strain ar2 was in the same clade with strain a5, which was supported by bootstrap values of >90% in the MP and ML trees. The shorter *TUB2* sequence of strain 10992 from New Zealand (accession number JX398943) is identical to that of strain a49 of Main Group IV, and it differs from the *TUB2* sequences of all the other strains of the present work.

3.2. Mycotoxin Production of Strains

Different enniatins (ENNs) were produced in most of the isolates on cracked corn substrate, except in *F. anguioides* (BBA 63598), *F. arthrosporioides* (BBA 64215), and *F. graminum* (BBA 65242). The highest ENN levels were produced by the *F. anguioides* strain BBA 69055 from Main Group III (or FTSC 27) and by three *F. acuminatum* strains. The same three *F. acuminatum* strains were the only strains in which we detected ENN A. We only detected beauvericin in one *F. avenaceum* strain from the United States, which was not a part of the phylogenetic investigation. We found AOD levels >100 ng/mL in the *F. anguioides* strain MFG 58314 and the *F. reticulatum* strain MFG 58549.

4. Discussion

Researchers have repeatedly demonstrated the high genetic variability of the strains within the *Fusarium tricinctum* species complex, and they have attempted to classify individual species by highlighting main groups [5,32] or phylospecies [10,11]. However, until now, we have not had clear definitions or Latin binomial names for many species. In a recent study by Cowger et al. [8], more than 20% of the analysed strains closely related to *F. avenaceum* were represented by unnamed species. Moreover, in Mexico, researchers have found many unnamed strains related to *F. avenaceum* [48], which form four distinct evolutionary lineages, which may represent species-level diversity (FTSC species 26, 29, 31, and 32) [11]. In our work, 26% of the analysed strains significantly differed from the five named main groups (I–V), and we grouped them as single, intermediate, or sister groups (Unknown Groups I–IV).

The main groups in the clades that we obtained in the phylogenetic study by using *TEF1* sequences were very similar to those obtained earlier by using *TUB2* sequences [5]. Only the strains BBA 69055 and an42 were moved to another group based on the *TEF1* sequences, and Main Groups II and III of the *TUB2* phylogenetic trees were subgroups in Main Group II of the present work. The phylogenetic tree that we obtained using *TEF1* sequences was in accordance with the phylogenetic tree of the combined *TEF1* and *TUB2* sequences. Based on the results of the present work, three *F. avenaceum* strains, for which the whole genomes were sequenced [49], belong to Main Groups I and II.

Kulik et al. [32] also found five main groups based on their *TEF1* sequences. Their Main Groups I, IV, and V correspond to Main Groups I, II, and V in the present work. The phylogenetic tree of Stakheev et al. [40], based on *TEF1* sequences, also supports Main Groups I and II, while the phylogenetic trees constructed by Kulik et al. [35] and Laraba et al. [11], based on *TEF1* sequences, support Main Group V, which contains morphologically *F. avenaceum* strains like Main Group I.

However, Main Groups I and II could not be clearly found in the phylogenetic trees when other DNA sequences were combined with *TEF1* sequences without *TUB2* sequences [11]. Laraba et al. [11] had only one strain (NRRL 54939) of Main Group II, which was included in the FTSC 4 clade, with strains from Main Group I and the intermediate group in the *TEF1* tree. However, when we added more strains of Main Group II to their phylogenetic *TEF1* tree, Main Groups I and II were well-supported.

Main Groups I and II of the present work are well-supported in the phylogenetic trees based on the combined *TEF1* and *TUB2* sequences, as well as in those based only on *TEF1* sequences. Thus, it may be necessary to divide FTSC4 into two species. In addition, the intermediate group between Main Groups I and II, based on the *TEF1* sequences of the present work and those of Kulik et al. [32] and Laraba et al. [11], is well-supported. In our previous work, based on only *TUB2* sequences, we could separate the two subgroups of Main Group II of the present work into two main groups [5]. Based on the results of the present work, the subgroup of Main Group II that mainly contains European *F. anguioides* strains is close to the other subgroup of Main Group II that mainly contains *F. arthrosporioides* strains. Thus, we can use morphological identification to indicate to which main group the isolates belong, but we cannot possibly identify the isolates of the *F. avenaceum* species complex by only using morphological characteristics.

According to the results of the *TUB2* analysis, the *F. anguioides* strain BBA 69055 from Japan belongs to the group that is mostly formed by *F. anguioides* strains from Europe [5], while, according to the results of the *TEF1* and combined *TUB2–TEF1* analyses of the present work, the strain is close to, or belongs to, Main Group III of the FTSC strains from Asia, which is similar to the new *Fusarium* sp. nov.-9 (FTSC 11), for which O'Donnell et al. [4] presented only one strain: MRC 2532. According to Laraba et al. [11], BBA 69055 belongs to FTSC 27, which is a sister group of FTSC 11. Main Group III also includes another *F. anguioides* strain, an47, from the Russian Far East. Moreira et al. [24] found more strains that have *TEF1* sequences that are similar to BBA 69055 and MRC 2532 from Brazil, and they identified their strains as FTSC 11. We suggest that it might be better to classify Main Group III (including FTSC 11 and its sister group, FTSC 27) as *F. anguioides*, rather than as the isolates neotypified by Nelson et al. [36], and a comparison of the isolates from Asia and Europe that we analysed in the present work to the original *F. anguioides* CUP-007479 sample of Sherbakoff [39] by using morphological, biochemical, and molecular characteristics would be informative.

In the current study, we could finally discover more strains (Main Group IV), isolated from trees (pine, fir, and acer) of different geographic origins and related to strain a49 (= NRRL 26893), isolated from apple from Finland, which is very different from other isolates based on the isozyme, the RAPD-PCR results, and the IGS and *TUB2* sequences [16,17,47]. These isolates of Main Group IV include strain 10992 from New Zealand, strain E19/2-4 from Slovenia, and strain NRRL 52726 from Turkey [4,37]. According to Laraba et al. [11], strain NRRL 52726 and strain NRRL 26893 belong to FTSC5.

Main Group V is supported based on the *TEF1* sequences obtained in the investigations in [11,32], but it is not supported by the other sequences used by Laraba et al. [11]. This is why Main Group V was included in the FTSC 4 species, although, in the *TEF 1* tree, it is closer to other FTSC species. Thus, the isolates of Main Group V should be further investigated. Based on the *TEF1* and *TUB2* sequences, it seems that Main Groups I–IV of the present work may represent different phylogenetic species of the FTSC, while the two subgroups of Main Group II are more representative of intraspecific variation.

A few isolates could not be grouped with any known isolates, while strain BBA 63201 (= a4) could be grouped with strain FRC R-6739 of FTSC 22 [11]. We found more isolates between the main groups among the isolates based on the *TEF1* sequences that have been analysed in different studies [11,32,40]. The phylogenetic trees based on *TEF1* and *TUB2* sequences support the group of intermediate isolates, which contains two subgroups. Thus, the intermediate group of isolates may also represent a phylogenetic species, as well as other isolates, such as a4, ar2, and a5, and MFG 80212. According to Laraba et al. [11], ar2 is the only strain that represents FTSC 30, and a5 is the only strain that represents FTSC 34, while, according to our results, a4 belongs to FTSC 22. We can use the nucleotides, which are different between the main groups, as markers for identifying isolates.

We found strains producing different ENNs in all the main groups and species that we investigated. This result is in line with the published information on the high amounts of ENNs and the absence of any detected BEA amounts produced by *F. avenaceum* and FTSC strains of different origins [10,21,29–31]. These strains produced different types of ENNs in vitro on rice, but all of them were nonproducers of BEA. BEA production is very rare in FTSC isolates, and researchers have detected it in only two studies [50,51]. Thus, the only strain producing a small amount of BEA in the present work should be further investigated. Our results also correspond to previous studies in which, within a group of ENNs, researchers detected ENN B at the highest level, followed by ENN B1, ENN A1, and ENN A [29–31]. ENN A was specific to the *F. acuminatum* strains. Only two strains, BBA 63598 (isolated from pea in 1928) and the unknown BBA 64215, which belong to Main Groups I and II, respectively, were not able to produce ENNs. More recently, researchers showed that most of the strains of *F. avenaceum* and closely related species produced AOD in amounts of 5.9–2139.3 ng/mL, which indicates the broadly distributed and variable ability to produce this secondary metabolite across the FTSC [10]. Among the strains that we

analysed in our study, the maximum amount of AOD was 125.8 ng/mL, and we detected it in the MFG 58314 strain from Main Group III, which was isolated from rudbeckia from the Far East. We found no clear differentiation in the mycotoxin production between the FTSC strains from the different main groups.

We confirmed a significant intraspecific structure of *F. avenaceum* and related species, as well as the complexity of splitting them into phylogenetic species with the assignment of binominal names that reflect their properties; the FTSC4 of Laraba et al. [11] could be divided into three main groups and one intermediate group. In the future, whole-genome sequencing should be used to confirm the results of the present work, which deals with *F. avenaceum* and related species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14070574/s1, Table S1: Accession numbers of *Fusarium* strains used in the present study. Figure S1: Maximum-likelihood consensus tree of *TEF1* sequences. Bootstrap values are indicated above nodes based on 500 pseudoreplicates of the data. Only branches present in more than 50% of the trees are shown. *F. tricinctum* and *F. acuminatum* strains are outgroups. Figure S2: Maximum likelihood consensus tree of *TEF1* sequences, including strains from the work of Kulik et al. (2011). Bootstrap values are indicated above nodes based on 500 pseudoreplicates of the data. Only branches present in more than 50% of the trees are shown. *Fusarium tricinctum* and *F. acuminatum* strains are outgroups. Figure S3: Maximum-likelihood consensus tree of *TEF1* sequences, including strains from the work of Stakheev et al. (2016). Bootstrap values are indicated above nodes based on 500 pseudoreplicates of the data. Only branches present in more than 50% of the trees are shown. *Fusarium tricinctum* and *F. acuminatum* strains are outgroups. Figure S4: Maximum-likelihood consensus tree of *TEF1* sequences, including strains from the work of Laraba et al. (2022). Bootstrap values are indicated above nodes based on 500 pseudoreplicates of the data. Only branches present in more than 50% of the trees are shown. *F. tricinctum* and *F. acuminatum* strains are outgroups. Figure S5: Maximum-likelihood consensus tree for combined *TEF1* and *TUB2* sequences. Bootstrap values are indicated above nodes based on 500 pseudoreplicates of the data. Only branches present in more than 50% of the trees are shown. *F. tricinctum* and *F. acuminatum* strains are outgroups.

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