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Molecular and morphological diversity of *Fusarium* species in Finland and northwestern Russia

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Abstract

In 2001 the range of the total *Fusarium* contamination percentage of infected seeds was between 0% and 44%, while in 2002 the contamination level was 2–25% in naturally infected Finnish samples and 5–14.5% in six samples from northwestern Russia. The most common *Fusarium* species in barley were *F. avenaceum*, *F. arthrosporioides*, *F. sporotrichioides* and *F. culmorum*, while in spring wheat the most common *Fusarium* species were *F. avenaceum*, *F. arthrosporioides*, *F. culmorum*, *F. sporotrichioides* and *F. graminearum*. In most cases, molecular identification with species-specific primers corresponded to the morphological analyses and allowed the identification of degenerated and otherwise morphologically difficult cultures. It was even possible to separate most of the *F. arthrosporioides* isolates from Finland from the closely-related *F. avenaceum* isolates. In the phylogenetic analysis of combined β-tubulin, IGS and ITS sequences most European *F. arthrosporioides* formed a separate clade from most isolates of *F. avenaceum* and from all isolates of *F. tricinctum*. Most of the species-specific primers also amplified DNA extracted from grain samples. It was, for instance, possible to detect *F. avenaceum* in all barley samples with contamination levels higher than 1% and in all spring wheat samples with contamination levels higher than 3%. The detection level for *F. graminearum* was at a contamination level of 3–5% and that for *F. culmorum* at a contamination level of 1–5%. In addition, the first Finnish *F. langsethiae* isolate was found by means of species-specific primers.

Introduction

Fusarium avenaceum (teleomorph *Gibberella avenacea*; Booth and Spooner, 1984) causes damping-off, root rot, stalk rot and/or fruit rot under conditions unfavourable to its hosts (Gerlach and Nirenberg, 1982). In Finland, this weak pathogen, together with the closely related *F. arthrosporioides* and *F. tricinctum* (teleomorph *Gibberella tricincta;* El-Gholl et al., 1978), is frequently found on cereal grains (Ylimäki et al., 1979; Ylimäki, 1981; Yli-Mattila et al., 1996, 2002a), where it causes seedling and head blight. *Fusarium culmorum*, *F. poae*, *F. sporotrichioides*, and *F. graminearum* are also common *Fusarium* species in Finnish cereals (Yli-Mattila et al., 2002b).

The purpose of the present study was to study the detection, occurrence and diversity of different *Fusarium* species in grains during the growing seasons 2001–2002 in Finland and northwestern Russia. In addition, the *Fusarium* isolates from the years 1998 (Eskola et al., 2001), 2001 and 2002 were re-identified by different species- and strain-specific primers and the phylogenetic sequence data from *F. avenaceum*/*F. arthrosporioides*/*F.tricinctum* isolates (Yli-Mattila, 2002a) was reanalysed. The different primers (Paavanen-Huhtala, 2000; Konstantinova et al., 2002;

Yli-Mattila et al., 2002a) and DNA extraction methods (Paavanen-Huhtala, 2000; Konstantinova et al., 2002) were applied to different grain samples and pure cultures. We were also interested to find out how common the markers (Paavanen-Huhtala, 2000; Yli-Mattila et al. 2002a) found in *F. avenaceum* isolates of main group II were among *F. avenaceum*, *F. arthrosporioides* and *F. tricinctum*.

Materials and methods

Grain samples and Fusarium *isolates*

During the year 1998, 43 rye samples, 15 barley, 6 oats and 4 wheat were collected in southern Finland (Eskola et al., 2001; Yli-Mattila et al., 2002b). The contamination percentages of each dried sample were studied and the *Fusarium* isolates from the grain samples were identified by morphology (Eskola et al., 2001).

Fresh grain samples (200 seeds per sample) from the years 2001 to 2002 (Tables 1 and 2) were used for the analysis of fusaria, since drying at high temperatures may decrease the amount of living fungi. The isolation and morphological identification of the fungi took place as described in Eskola et al. (2001). The total amount of *Fusarium* isolates identified by morphology in 1998–2002 was ca. 3500, of which ca. 1000 were re-identified by the species-specific primers.

DNA extraction

Mycelia were grown on potato dextrose agar (PDA) for 4–5 days at 25 °C under constant light

Table 1. Detection of *F. avenaceum* (JIA/FA-ITS), *F. poae* (Fp82/PoaeIGS), *F. graminearum* (Fg11), *F. culmorum* (Fc01) and *F. tricinctum* (FA-ITS) by species-specific primers from ground seeds of grain samples of the year 2001

No.	Cereal	Cultivar/drying (+/−)	Origin	Fus.%	JIA	Fp82/ PoaeIGS	Fg11	Fc01	FA-ITS
1	W.wheat	Ramiro (−)	SW Finland	1	±	−/±	−	−	−
3	Barley	Rolfi (−)	SW Finland	6.5	+	+/+	−	−	+
5	Barley	Inari (−)	SE Finland	44	+	+/+	−	−	+
7	Barley	Inari (−)	SE Finland	8	+	+/+	−	−	+
9	Barley	Scarlett (−)	SW Finland	15.5	+	+/+	−	−	+
11	Barley	Saana (−)	Southern Finland	8.5	±	−/±	−	−	−
13	S. wheat	Reno (−)	Southern Finland	16	+	+/+	−	−	+
15	Barley	Scarlett (−)	SW Finland	29.5	+	+/+	−	−	+
17	S. wheat	Kruunu (−)	SW Finland	6.5	+	±/+	−	−	−
19	Barley	Saana (−)	SE Finland	7.5	+	−/+	−	−	−
21	S. wheat	Tjalve (−)	SE Finland	18.5	+	−/+	±	+	+
23	Barley	Saana (−)	SW Finland	4.5	−	+/+	−	±	−
25	Barley	Scarlett (−)	SW Finland	8.5	−	+/+	−	+	−
27	Barley	Extract (−)	SW Finland	23.5	+	−/+	−	−	−
29	Barley	Saana (+)	Southern Finland	0.5	+	+/+	−	±	−
30	Barley	Scarlett (+)	Southern Finland	5.5	−	+/+	−	+	+
31	W. wheat	Ramiro (+)	SW Finland	0	−	−/±	−	−	−
32	W. wheat	Ramiro (+)	SW Finland	0	−	−/±	−	−	−
33	Barley	Saana (−)#	Western Finland	30.5	+	+/+	−	−	+
35	S. wheat	Vinjett (−)#	Western Finland	40.5	+	−/−	+	−	±
36	S. wheat (=sample 35 after drying)	Vinjett (+)	Western Finland		+	−/−	+	−	±
37	Barley	Mette (−)	SW Finland	16.5	−	−/±	−	±	−
38	S. wheat	Tjalve (+)	SW Finland	*	−	/−	−	−	*
39	Oats	Roope (+)#	Western Finland	*	±	/−	+	−	*
40	Oats	Leila (+)#	Western Finland	*	±	/−	+	−	*
41	S. wheat	Mahti (+)#	Western Finland	*	±	/−	+	−	*
42	Rye	Amilo (−)	SW Finland	2	−	/+	−	−	*

SW = southwestern, SE = southeastern. Fus. % = *Fusarium* contamination %. + = positive reaction, − = negative reaction, ± = weak reaction, * = not determined, # = harvested after a one-week-rain.

Table 2. Detection of *F. avenaceum* (JIA), *F. poae* (Fp82/PoaeIGS), *F. graminearum* (Fg11, 175/430), *F. sporotrichioides* (Pfusf/Fspor) and *F. culmorum* (Fc01, 175/430) by species-specific primers from the seed surfaces of grain samples from 2002

No.	Cereal	Cultivar/drying (+/−)	Origin	Fus. %	JIA	PoaeIGS	Fg11	Pfusf/ Fspor	Fc01	Pfusf/ Flanr	175/430
1	Barley	Saana (+)	SW Finland	15	±	±	−	−	−	−	+
2	Barley	Saana (−)	Southern Finland	3	−	−	−	−	−	−	±
3	Barley	Inari (−)	SE Finland	23	+	−	−	−	−	+	−
4	Barley	Extract (−)	SW Finland	16	+	−	−	−	−	+	±
5	Barley	Extract (−)	SW Finland	25	±	−	−	−	−	−	−
6	Barley	Cellar (−)	SW Finland	15	+	−	−	−	−	+	+
7	Barley	Saana (−)	Western Finland	12	−	−	±	−	−	+	±
8	Barley	Scarlett (−)	SE Finland	15	(±)	−	−	−	−	+	−
9	Oats	Belinda (−)	SE Finland	5	−	±	−	−	−	+	−
10	S. wheat	Mahti (−)	SW Finland	17	−	±	−	−	−	−	+
11	S. wheat	Reno (−)	Southern Finland	5	−	−	−	−	−	−	−
12	S. wheat	Reno (−)	Southern Finland	3	−	−	−	−	−	−	−
13	S. wheat	Reno (−)	Southern Finland	13	+	±	−	−	−	−	+
14	S. wheat	Vinjett (−)	SW Finland	5	−	−	−	−	−	−	±
15	S. wheat	Vinjett (−)	Western Finland	13	−	−	±	−	−	−	+
16	S. wheat	Tjalve (−)	SE Finland	6	(±)	−	−	−	−	−	+
32	S. wheat	Leningradka 97 (+)	Leningrad region	5	+	−	*	−	−	*	−
33	Barley	Suzdalets (+)	Leningrad region	7	±	−	−	−	−	±	−
34	Barley	Crinichniy (+)	Leningrad region	14.5	(±)	−	−	−	−	−	−
36	Barley	Crinichniy (+)	Leningrad region	7	−	±	−	−	−	−	−
41	Oats	Borus (+)	Leningrad region	3	*	*	*	*	*	*	*
77	S. wheat	Tjalve (+)	SW Finland	*	±	+	−	*	*	−	+
94	Oats	Veli (+)	SW Finland	*	−	+	+	*	*	+	+
102	Oats	Aarre (+)	Southern Finland	*	−	+	−	*	*	+	−
103	Oats	Aarre (+)	Southern Finland	*	−	+	+	*	*	(±)	±
109	Oats	Aarre (+)	Central Finland	*	±	+	±	*	*	−	±
110	Oats	Roope (+)	Western Finland	*	−	+	±	*	*	+	+
112	Oats	Veli (+)	Western Finland	*	−	+	+	*	*	+	±
115	Oats	Veli (+)	Western Finland	*	±	+	±	*	*	+	+

SW = southwestern, SE = southeastern, Fus % = *Fusarium* contamination %, + = positive reaction, − = negative reaction, ± = weak reaction, (±) very weak reaction, * = not determined, S. wheat = spring wheat.

before DNA extraction. DNA was extracted from the mycelia (Paavanen-Huhtala et al., 1999) and from naturally-infected grain samples from the year 2001 (Konstantinova et al., 2002). Another extraction method was modified from Taylor et al. (2001) for the grain samples from 2002. In this method, 10 g of grains were first soaked for 5 min and then vortexed for one min in 20 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The mixture was then transferred to Bioreba bags, in which the grains were treated with a Bioreba hand homogeniser. Filtered seed soak (15 ml) was centrifuged (10,000 rpm at 4 °C) and DNA was extracted with the Dneasy® Plant Mini Kit of Qiagen or with GenElute™ Plant Genomic DNA Kit of Sigma (or in some cases with the chloroform/octanol method used for fungal cultures).

Development and use of primers specific to main group II of F. avenaceum

In order to obtain main group II (Yli-Mattila et al., 1997; 2002a) specific fragments, amplifications for the RAPD analysis were carried out with 40 different RAPD–PCR oligonucleotide primers of series OPA1-20 and OPB1-20 (Operon Technologies, Alameda, CA, USA) alone and with 14 RAPD primer combinations as described by Paavanen-Huhtala et al. (2000). Amplification products including strain-specific fragments were then cloned according to Paavanen-Huhtala (2000) using the TOPO TA cloning kit (K4500-01; Invitrogen, Leek, the Netherlands).

The plasmid DNA was isolated using a boiling method slightly modified from Sambrook et al. (1989). The main difference was in the constituents

of STET buffer (8% sucrose, 0.5% Triton X-100, 50 mM EDTA (pH 8), 10 mM Tris–HCl (pH 7.5)) and in the additional RNase treatment. The plasmid DNA purification was done according to Paavanen-Huhtala (2000).

DNA fragments ligated into plasmids were sequenced with the M13 Forward and Reverse primers and oligonucleotides were designed according to the sequences obtained (Paavanen-Huhtala, 2000). Some DNA fragments amplified with two different RAPD primers were directly sequenced after purification with the same primers as used in the amplification (Burt et al., 1994).

Main group II-specific primers Fa5f/r, Fa8f/r and Fa17f/r (Table 3) were tested in 25 µl standard PCR reactions as described by Paavanen-Huhtala (2000). Primer pairs Fa5f/r (forward/reverse) and Fa8f/r were used together in multiplex PCR. In order to avoid false negative results in the specific PCR, the quality of the DNA was tested with the primers ITS1 and ITS4 (White et al., 1990).

Primers specific for different Fusarium *species*

Primer pair JIAf/r specific for *F. avenaceum* (Turner et al., 1998), FA-ITSf/r specific for *F. avenaceum* and *F. tricinctum*, (Turner et al.,

Table 3. Sequences of the *Fusarium* species-specific primers

Name of the primer (size of the product)	Sequence 5′–3′	Specificity of species-specific primers
Fa5f(1071 bp)[1]	GGGGTCTTGCCACTCAGCTTGT	Mainly in *F. arthrosporioides*
Fa5r	GGGGTCTTGCGGATCATGTGCT	
Fa8f(604)[1]	GTGACGTAGGGAAACTGCCTGG	Mainly in *F. arthrosporioides* and *F. tricinctum*
Fa8r	GTGACGTAGGACCAGAGATGTA	
Fa17f(1550 bp)[1]	ACCGCTTGTACCGTACCGTCAAT	Mainly in *F. arthrosporioides*
Fa17r	GACCGCTTGTTGCATCTAGGTAG	
JIAf(300 bp)[2]	GCTAATTCTTAACTTACTAGGGGCC	*F. avenaceum*/*F. arthrosporioides*
JIAr	CTGTAATAGGTTATTTACATGGGCG	
FA-ITSf(272 bp)[2]	CCAGAGGACCCAAACTCTAA	*F. avenaceum*/*F. tricinctum*/*F. arthrosporioides*
FA-ITSr	ACCGCAGAAGCAGAGCCAAT	
Fc01f(300)[4]	ATGGTGAACTCGTCGTGGC	*F. culmorum*
Fc01r	CCCTTCTTACGCCAATCTCG	
PoaeIGS(306)[5]	CAAGCTCTCCTCGGAGAGTCGAA	*F. poae*
CNL12	CTGAACGCCTCTAAGTCAG	
Fp82f(200 bp)[6]	CAAGCAAACAGGCTCTTCACC	*F. poae*
Fp82r	TGTTCCACCTCAGTGACAGGTT	
PulvIGS(610)[5]	GAACCGTCCGGCACCCATCC	*F. sporotrichioides*/*F. langsethiae*
CNL12	CTGAACGCCTCTAAGTCAG	
Fg11f(400 bp)[3]	CTCCGGATATGTTGCGTCAA	*F. graminearum*
Fg11r	GGTAGGTATCCGACATGGCAA	
175f(245 bp)[7]	TTTTAGTGGAACTTCTGAGTAT	*F. culmorum*/*F. graminearum*
430r	AGTGCAGCAGGACTGCAGC	
Pfusf(300 bp)[8]	CCGCGCCCCGTAAAACG	F. *sporotrichioides*
Fspor	ACTGTGTTTGCACACAGATC	
Pfusf(300 bp)[8]	CCGCGCCCCGTAAAACG	*F. langsethiae* (+ a few *F. sporotrichioides* strains)
Flanr	CTGTCGGTAAGGACAGATC	

[1] Paavanen-Huhtala (2000).
[2] Turner et al. (1998).
[3] Doohan et al. (1998).
[4] Nicholson et al. (1998).
[5] Konstantinova and Yli-Mattila (in press).
[6] Parry and Nicholson (1996).
[7] Mishra et al. (2003).
[8] S. Klemsdal (2004, personal communication).

1998), Fc01f/r (Nicholson et al., 1998) and 175f/430r (Mishra et al., 2003) specific for *F. culmorum*, PoaeIGS/CNL12 (Konstantinova and Yli-Mattila, in press) and Fp82f/r (Parry and Nicholson, 1996) specific for *F. poae*, PulvIGS/CNL12 (Konstantinova et al., 2002; Konstantinova and Yli-Mattila, in press) specific for *F. sporotrichioides*/*F. langsethiae*, Pfusf/Fspor specific for *F. sporotrichioides* (S. Klemsdal, personal communication), Pfusf/Flanr specific for *F. langsethiae* (+ a few *F. sporotrichioides* isolates, S. Klemsdal, personal communication) and Fg11f/r (Doohan et al., 1998; Waalwijk et al., 2003) specific for *F. graminearum* were tested and used to confirm the morphological identification of *Fusarium* isolates (Table 3).

Phylogenetic analyses

The POY 2.7 program (Wheeler, 1996; Gladstein and Wheeler, 2001) in the computers of CSC (Scientific Computing, Espoo, Finland) was used to analyse the same combined IGS, β-tubulin and ITS sequences as in the NJ analysis of Yli-Mattila et al., (2002a), without previous alignment and excluding of gaps. The command line used for the parallel analyses of POY was poy-parallel-solo-spawn 7-noleading – norandomizeoutgroup-gap 1 – maxtrees 20-appoxbuild-multibuild 5 – random 5 – fitchtrees-seed 1-slop 1-checkslop 2 names of the sequence files > name of the results file. The list of isolates and accession numbers for the POY analysis can be found in Yli-Mattila et al., (2002a).

Results

ITS amplification

In some cases there was a positive ITS-PCR amplification, even when the concentration or quality of target DNA was too low for specific primers, especially if the amplified fragment was long. Two amplification products were often obtained with ITS1 and ITS4 primers from DNA extracted from ground barley and wheat grains. The longer fragment was probably from the plant genome, since according to Hsiao et al., (1995), the ITS regions of barley are about 600 bp long.

Main group II-specific primers

Ninety-one percent of the *F. arthrosporioides* isolates collected in Finland in 1998 had a marker produced by the Fa17f/r primer pair (Figure 1), while only 2% of *F. avenaceum* isolates (main group II) and none of the *F. tricinctum* isolates from the

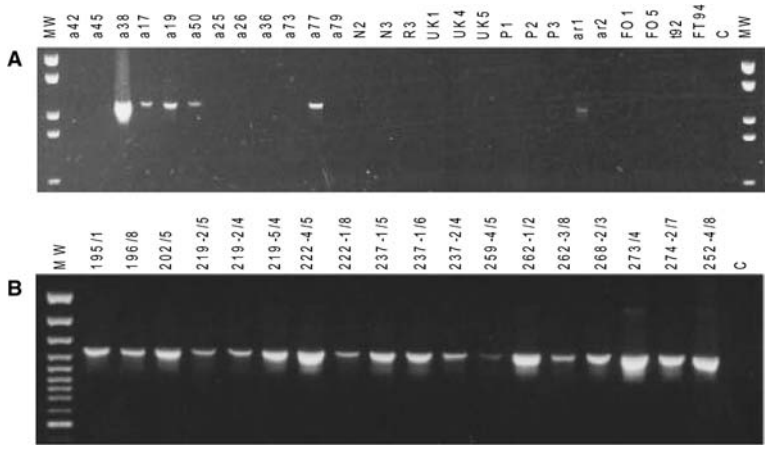

Figure 1. PCR products of ca. 1550 bp obtained with specific primer pair Fa17f/r. (A) Five *F. avenaceum* isolates (main group II) and one *F. arthrosporioides* (ar1) isolate had a positive result. 16 *F. avenaceum* (main group I), two *F. oxysporum* (FO1, FO5), *F. tricinctum* (t92, FT94) isolates and *F. avenaceum* isolates N2 and N3 from Norway, R3 from Russia, UK1, UK2 and UK3 from the United Kingdom and P1 and P2 from Poland gave a negative result. The list of isolate code numbers can be found in Figure 3 or in Paavanen-Huhtala (2000). MW is the molecular marker VI (Boehringer/Mannheim), whose markers are 2176, 1766, 1230, 1033, 653, 517, 473, 394, 298, 234, 220 and 154 bp. (B) PCR products obtained from one *F. avenaceum* (252-4/8) and 15 *F. arthrosporioides* isolates from Finland from 1998. C is the negative control and MW the molecular weight marker (1 kb ladder).

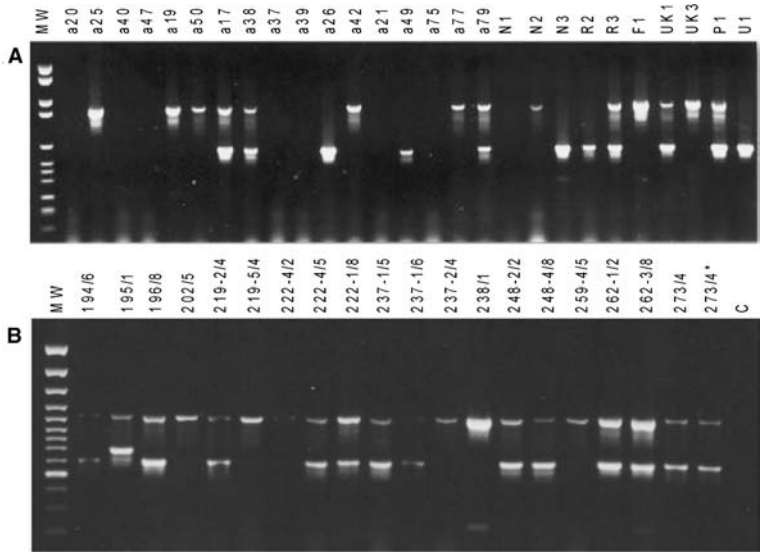

Figure 2. PCR products obtained with specific primer pairs Fa5f/r (upper band, 1071 bp) and Fa8f/r (lower band, 604 bp) from isolates of *F. avenaceum*. (A) The list of the isolate code numbers can be found in Figure 3 or in Paavanen-Huhtala (2000). *F. avenaceum* isolates N1, N2 and N2 are from Norway, R2 and R3 from Russia, F1 from France, UK1 and UK3 from the United Kingdom, P1 from Poland and U1 from the USA. C is the negative control and MW the molecular weight marker as in Figure 1A. (B) PCR products obtained with specific primer pairs Fa5f/r and Fa8f/r from *F. arthrosporioides* isolates collected in Finland in 1998. MW is the molecular weight marker (1 kb ladder).

same year had this marker. All *F. arthrosporioides* isolates also produced a PCR product with primer pair Fa5f/r, while only 17 of 84 *F. avenaceum* isolates and none of the *F. tricinctum* isolates formed this product. The product of primer pair Fa8f/r was more frequent in *F. arthrosporioides* (14/23) and *F. tricinctum* (12/20) than in *F. avenaceum* (10/84) isolates. The band produced by primer pair Fa5f/r was always of the same size, but there was variation (isolate 195/1) in the size of the band produced by primer pair Fa8f/r (Figure 2).

In 2001–2002 the proportion of *F. arthrosporioides* isolates with the markers of primer pairs Fa17f/r, Fa5f/r and Fa8f/r was lower than in 1998. In the combined results of the years 1998–2002 (Table 4), 67% of the *F. arthrosporioides* isolates had the group II specific markers of primer pairs Fa17f/r or Fa5f/r and Fa8f/r, while less than 4% of *F. avenaceum* isolates (group II) had them (Table 4). 62% of *F. tricinctum* isolates had the marker of primer pair Fa8f/r without the marker of Fa5f/r, while less than 1% of *F. avenaceum* and *F. arthrosporioides* isolates had the same combination of markers. Most of the *Fusarium* spp. isolates, which were not identified by morphological characters, could be identified as *F. avenaceum*/*F. arthrosporioides* or *F. tricinctum* using strain-specific primers together with primer pairs JIAf/r (specific for *F. avenaceum*/*F. arthrosporioides)* and FA-ITSf/r (specific for *F. avenaceum*/*F. tricinctum*/*F. arthrosporioides*, Tables 4 and 5).

PCR with species-specific primers in pure cultures

The *F. avenaceum*-specific primer pair JIAf/r amplified a fragment (ca. 300 bp) from all the 84 *F. avenaceum* isolates tested and from 23 *F. arthrosporioides* isolates, but not from the 20 *F. tricinctum* isolates of the year 1998. The morphological identification of *F. arthrosporioides* isolates was verified by H. Nirenberg, according to whom five isolates (195/1, 248-2/2, 262-1/2, 262-3/8, 273/4) were degenerated; all of them except 248-2/2, however, could still be identified by their brownish colour and pyriform conidia. Three isolates (196/8, 222-4/5, 237-1/6) produced brownish colour but not pyriform conidia and could not be identified with certainty, but in all likelihood they are *F. arthrosporioides*. All these degenerated isolates could be identified with primer pair Fa17f/r. All *F. avenaceum*, *F. arthrosporioides* and *F. tricinctum* isolates tested from the year 1998 yielded a

Table 4. Amplification results of *F. avenaceum*, *F. arthrosporioides* and *F. tricinctum* isolates collected in 1998, 2001 and 2002 using primer pairs Fa5f/r, Fa8f/r, Fa17f/r and JIAf/r

Primers				Morphological identification				Molecular identification
Fa5	Fa8	Fa17	JIA	*F. arthrosp.* (%) n = 107	F. *tricinctum* (%) n = 73	*F. avenaceum* (%) n = 160	*F.* sp. (%) n = 11	
–	–	–	–	4	34	1	27	*F. tric.*
–	+	–	–	2	62	1	36	*F. tric.*
–	–	–	+	7	1	71	36	*F. aven.*
+	–	–	+	16	0	16	0	*F. aven.*
–	+	–	+	4	3	8	0	*F. aven.*
+	‘–	–	–	12	0	0	0	*F.* sp.
+	–	+	+	18	0	1	0	*F. arthr.*
+	+	+	+	24	0	0	0	*F. arthr.*
+	+	–	+	14	0	2	0	*F. arthr.*
–	–	+	+	7	0	0	0	*F .arthr.*
–	+	+	+	4	0	0	0	*F. arthr.*
% of isolates, which could be confirmed by using primers				67%	96%	95%	100%	

The morphological and molecular identification results are marked. + = PCR product obtained, – = no PCR product obtained, n = number of isolates studied.

PCR product of the expected size (270 bp) with primer pair Fa-ITSf/r. The product was stronger in *F. avenaceum* and *F. arthrosporioides* isolates than in *F. tricinctum* isolates. A weak product was also obtained in *F. sambucinum* isolates (results not shown).

The Fc01f/r primer pair amplified a fragment of 570 bp from all *F. culmorum* isolates from 1998 except for two. The PCR product of 400 bp with primer pair Fg11f/r showed that one of these isolates was *F. graminearum*; the other could not be identified with any of the primers tested. One isolate identified as *F. graminearum* gave negative amplification results with *F. graminearum* and *F. culmorum*-specific primers but positive results with primer pairs Fa5f/r and Fa8f/r; accordingly, it might be *F. avenaceum* of main group II or *F. arthrosporioides*. Four isolates identified as *F. poae* gave negative results by the *F. poae*-specific primer pairs PoaeIGS/CNL12 and Fp82f/r, and three of these isolates were shown to be *F. sporotrichioides*/*F. langsethiae* by primer pair PulvIGS/CNL12; the fourth was identified as *F. tricinctum* by Fa5f/r, Fa8f/r and Fa-ITSf/r primers (Table 5). They were later confirmed by M. Torp and H. Nirenberg as *F. sporotrichioides* and *F. tricinctum*, respectively. All four *F. sporotrichioides* isolates gave negative results with primer pair PulvIGS/CNL12 but positive results with primer pair Fa-ITSf/r, and were later identified as *F. tricinctum* by M. Torp and H. Nirenberg. Similar results were obtained later using these primers, but the primer pair PulvIGSr/CNL12, originally designed to separate subgroup II of the new species *F. langsethiae* from other *F. langsethiae* isolates (Konstantinova et al., 2002; Konstantinova and Yli-Mattila, in press), sometimes gave cross-reactions with *F. poae* isolates (results not shown).

In 2001 and 2002 over 90% of the morphological identifications were also confirmed by species-specific primers; though in some cases mixtures of two species (Table 5) were found. The first isolate of *F. langsethiae* from Finland was found among the isolates from barley in 2001 (grain sample 19, Table 1) using species-specific primers Pfusf/Flanr (S. Klemsdal 2004, manuscript). It was later morphologically confirmed by M. Torp. The primer pair 175f/140r, designed for the identification of *F. culmorum* (Mishra et al., 2003), was found to give a PCR product also with *F. graminearum* isolates, which is in accordance with known ITS sequences (e.g. accession number AF006344) in GenBank. A few *F. avenaceum*, *F. arthrosporioides* and *F. tricinctum* isolates of the years 2001 and 2002 could be re-identified using species- and main group II-specific primers (Tables 4 and 5). In several cases it was not possible to confirm the morphological identification of *F. tricinctum* iso-

Table 5. Examples of *Fusarium* isolates collected in 1998, 2001 (T-isolates) and 2002 (P-isolates) in Finland and re-identified by *F. avenaceum*/*F. arthrosporioides*-specific JIA; *F. avenaceum*/*F. arthrosporioides*/*F. tricinctum*-specific Fa17f/r, Fa5f/r, Fa8f/r and FA-ITSf/r; *F. culmorum*-specific Fc01f/r and 175f/430r; *F. graminearum*-specific Fg11f/r; *F. sporotrichioides*/*F. langsethiae*-specific Pfusf/Fspor, PulvIGS/CNL12 and Pfusf/Flanr and *F. poae*-specific PoaeIGS/CNL12

Species	Code	Source	Origin	JIA	Fa17	Fa5	Fa8	FA-ITS	Fc01	175f/430r	Fg11	Pfusf/ Fspor	Pulv-IGS	Poae-IGS	Pfusf/ Flanr
(*FC*) → *F. graminearum*	237-3/7	Ilmajoki	Barley			−	−		−		+				
(*FC*) → unknown species	194/4	S Finland	Rye			−	−	−	−		−				
(*FG*) → *F. arthrosporioides*/*F. avenaceum*	259-3/3	Lahti region	Org. rye			+	+		−		−				
(*FP*) → *F. sporotrichioides*	192/7	Artjärvi	Rye			−	−						+	−	
(*FP*) → *F. sporotrichioides*	219-5/3	Lahti region	Oats/ wheat			−	−						+	−	
(*FP*) → *F. sporotrichioides*	248-1/4	Kotka region	Rye			−	−						+	−	
(*FP*) → *F. tricinctum*	262-2/3	Rymättylä	Oats			−	−	+					−	−	
(*FS*) → *F. tricinctum*	222-3/3	Kotka region	Rye			−	−	+					−	−	
(*FS*) → *F. tricinctum*	237-1/1	Ilmajoki	Barley			−	−	+					−	−	
(*FS*) → *F. tricinctum*	248-4/3	Kotka region	Rye			−	−	+					−	−	
(*FS*) → *F. tricinctum*	262-3/2	Nousiainen/ Mellilä	Oats			−	−	+						−	
F. sp. → *F. avenaceum*	T377	W Finland	Barley	−	−	−	+	+				−			
(*FA*) → *F. tricinctum*	T122	W Finland	Barley	−	−	−	±	+							
(*FT*) → *F. avenaceum*	T110	SE Finland	Barley	±	−	−	+	+							
(*FAr*) → *F. avenaceum*	T88	S Finland	Wheat	±	−	−	−	+							
(*FAr*) → *F. avenaceum*	T40	SE Finland	Barley	+	−	+	−								
(*FAr*) → *F. avenaceum*	T25	S Finland	Wheat	±	−	±	−	+							
(*FS*) → *F. langsethiae*	T113	SE Finland	Barley									−			+
(*FA*) → *F. avenaceum*/*F. culmorum*	T31	S Finland	Barley	+	−	−	+		+						
(*FC*) → *F. graminearum*	P244	SW Finland	Barley						−	+	±				
(*FS*) → *F. poae*	P36	SW Finland	Wheat										−	±	−
(*FT*) → *F. avenaceum*	P111	SW Finland	Oats	+	−	−	−	+							

Species names in parentheses indicate the morphological identification of isolates before the study. Species names after the sign (→) were obtained after re-identification. F. sp. = *Fusarium* species, FC = *F. culmorum*, FG = *F. graminearum*, FP = *F. poae*, FS = *F. sporotrichioides*, FA = *F. avenaceum*, FT = *F. tricinctum*, FAr = *F. arthrosporioides*, S = Southern, W = Western, SE = Southeastern, SW = Southwestern, + = PCR product obtained was of the expected size, − = no PCR product obtained. A blank means that amplification was not performed.

lates using the FA-ITSf/r primer pair, since all *F. tricinctum* isolates did not give a PCR product. In most of these cases, however, the negative result with the JIAf/r primer pair and the positive result with the Fa8f/r primer pair supported the identification of *F. tricinctum* isolates (Table 5).

Molecular detection of Fusarium *species in grain samples*

To test whether the species-specific primer approach would be feasible for the molecular detection of *Fusarium* species in Finnish grain samples, DNA was extracted from grains. PCR was most successful by the extraction method of Konstantinova et al., (2002) with whole seeds (Table 1) and by the modified method of Taylor et al., (2001) with the seed surfaces (Table 2). Primer pairs JIAf/r, Fp82f/r, PoaeIGS/CNL12, Fg11f/r, 175f/430r and Pfusf/Flanr gave clear results also with DNA extracted from grains, while the sensitivity of Fc01f/r and Pfusf/Fspor primer pairs was low (Tables 1 and 2). The detection level was 1% in barley and 3% in wheat for *F. avenaceum* with JIAf/r primers, 3–5% for *F. graminearum* with Fg11f/r primers and 1–5% for *F. culmorum* with

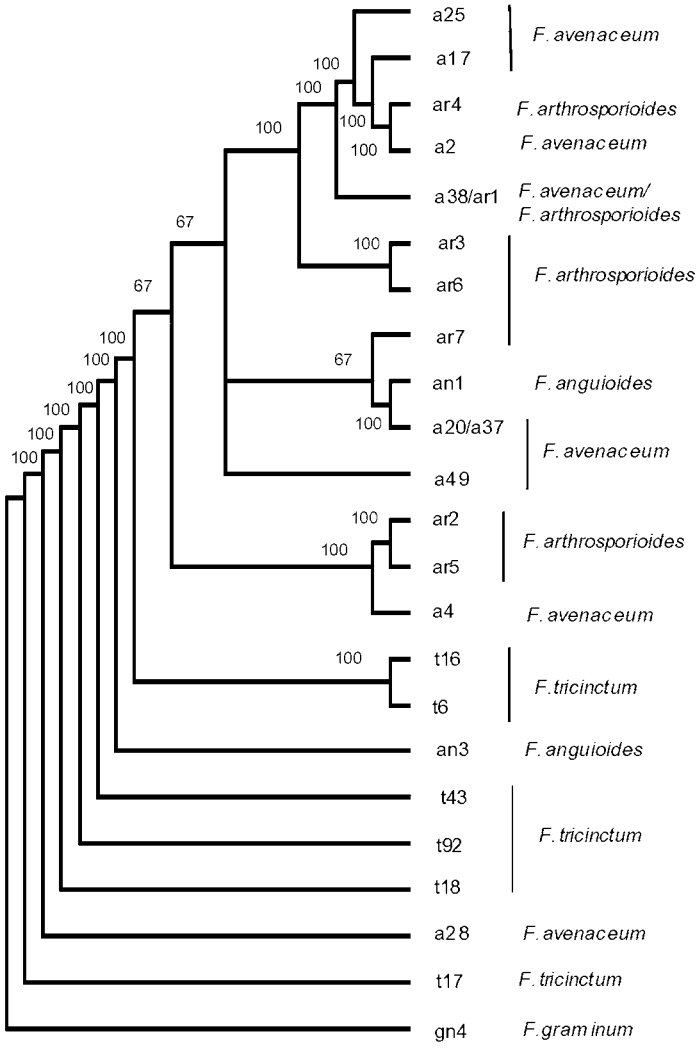

Figure 3. POY consensus tree of three shortest trees with a length of 911 steps for combined β-tubulin, IGS and ITS sequences. Only branches present in more than 50% of the trees are shown. Outgroup *F. graminum* strain gn4 (Yli-Mattila et al., 2002a)

primers 175f/430r (results not shown). The chloroform/octanol and CTAB methods alone with extraction from whole seeds were not successful, probably due to inhibiting compounds.

Phylogenetic analysis

POY analysis of combined β-tubulin/IGS/ITS sequences in *Fusarium avenaceum*, *F. arthrosporioides*, *F. tricinctum* and *F. anguioides* isolates (Yli-Mattila et al., 2002a) gave three trees with a length of 911 steps. In the consensus tree (Figure 3) most of the European *F. arthrosporioides* isolates (ar1, ar4, ar3 and ar6) were in the same clade with *F. avenaceum* isolates of main type II (a17 and a38), while the rest of the *F. arthrosporioides* isolates (ar2, ar5 and ar7) were in two other clades. The morphological separation between *F. tricinctum* and *F. avenaceum*/*F. arthrosporioides* isolates was well supported, except for *F. avenaceum* isolate a28 (with a long gap in the IGS region), which was outside the large *F. avenaceum*/*F. arthrosporioides* clade. *F. avenaceum* isolate a49 was not in either of the three *F. avenaceum*/*F. arthrosporioides* clades, but formed its own group.

Within the *F. tricinctum* isolates, the differences were even greater, and only one clade was well supported. One of the two *F. anguoides* isolates was within the large *F. avenaceum*/*F. arthrosporioides* clade, while the other was outside it.

Discussion

F. avenaceum and *F. arthrosporioides* are very difficult to separate by their morphological characteristics and are often confused with each other. The close relationship between these species is supported by the IGS, ITS and β-tubulin sequence and metabolite profile results (Yli-Mattila et al. 2002a; T. Yli-Mattila, U. Thrane and K. Seifert, unpublished results), according to which several *F. arthrosporioides* isolates from different parts of Europe are closely related to the main group II of *F. avenaceum*. Main group II has been clearly separated from other *F. avenaceum* isolates based on RAPD-PCR (Yli-Mattila et al., 1996) and UP-PCR (Yli-Mattila et al., 1997a).

The primer pair Fa17f/r can be used as a marker for main group II of *F. avenaceum* (Paavanen-Huhtala, 2000; Yli-Mattila et al., 2002a). Isolates a17 and a38, which are particularly pathogenic and belong to main group II, could be distinguished from all Finnish *F. avenaceum* isolates tested for pathogenicity (Yli-Mattila et al., 1997) since they were the only ones which gave a double PCR product with primers Fa5f/r and Fa8f/r. The PCR products obtained by primer pairs Fa17f/r, Fa5f/r and Fa8f/r have been sequenced (Paavanen-Huhtala, 2000), but very similar sequences were not found. The DNA fragment amplified with Fa17f/r had similarities (60–68% for 90–177 nt) with many NADH hydrogenase sequences.

These markers are clearly more common in *F. arthrosporioides* (all three markers) and *F. tricinctum* (Fa8f/r) than in *F. avenaceum*. With primer pairs, Fa5f/r and Fa8f/r together with JIAf/r >90% of *F. arthrosporioides* and *F. avenaceum* isolates could be distinguished from *F. tricinctum* isolates without the use of primer pair Fa17f/r, while Fa17f/r was useful in identification of *F. arthrosporioides* isolates. Thus, Fa17f/r amplification product and the double product of primer pairs Fa5f/r and Fa8f/r can be used as markers for *F. arthrosporioides*. These are the first molecular markers found for identification of *F. arthrosporioides* and *F. tricinctum* isolates.

The FA-ITSf/r primer pair, which was originally designed for *F. avenaceum* (Turner et al., 1998) was found to be specific for most *F. avenaceum*, *F. arthrosporioides* and *F. tricinctum* isolates. The primer pair JIAf/r designed for *F. avenaceum* (Turner et al., 1998) amplified a fragment from *F. avenaceum* and *F. arthrosporioides* isolates, but not from the *F. tricinctum* isolates, leading to clear discrimination between them. Thus, the primer pair FA-ITSf/r could be used for the identification of most *F. tricinctum* isolates, when used together with JIA and Fa5/Fa8 primer pairs.

In the POY consensus tree of the present work two Finnish (ar1 and ar3) and two European (ar4 and ar6) *F. arthrosporioides* isolates are in the same clade with the Finnish *F. avenaceum* isolates a17 and a38 of main group II and isolate a25 of main group I. All *F. avenaceum* and *F. arthrosporioides* isolates except for a28 belong to a large clade, which is separate from *F. tricinctum* isolates. The grouping of *F. avenaceum* isolate a28 within the *F. tricinctum* isolates in the POY consensus tree may be due to the long overlapping deletion in the IGS region with most *F. tricinctum* isolates. In the NJ and POY consensus trees of β-tubulin se-

quences both a28 and a25 were in the same clade with other *F. avenaceum* isolates of main group I. *F. tricinctum* isolates t16 and t6 formed their own clade in the POY consensus tree, as in the previous NJ consensus tree (Yli-Mattila et al., 2002a). The monophyletic groups within the *F. avenaceum*/*F. arthrosporioides*/*F. tricinctum* species complex are probably new evolving phylogenetic species (Taylor et al., 2000), which are not yet completely separated from each other. This is in accordance with the unpublished results of K. Seifert and U. Thrane (personal communication), and may explain why it is so difficult to find strictly species-specific markers for *F. avenaceum*, *F. arthrosporioides* and *F. tricinctum* isolates. The use of several molecular markers with clearly different frequencies between the two species, as in the present work, may solve this problem. The toxin profiles of the three species also seem to be similar (Langseth et al., 1999; M. Jestoi and T. Yli-Mattila, unpublished data).

The results obtained with species-specific primers confirmed in most cases the identification results based on morphology. In a few cases contradictory results were obtained by molecular methods as compared to morphological methods, especially between *F. culmorum* and *F. graminearum* and between *F. tricinctum*, *F. poae* and *F. sporotrichioides* isolates from 1998. The molecular identification of most isolates was later confirmed by M. Torp and H. Nirenberg. This shows that molecular identification with species-specific primers is a good way of confirming the identification of degenerated and otherwise morphologically difficult cultures.

Most of the species-specific primers worked with DNA extracted from grain samples, but in some cases cross-reactions with plant DNA took place. The results from grain samples by species-specific primers are in accordance with morphological and PCR analyses of the *Fusarium* isolates from the same grain samples (Yli-Mattila et al., 2002b; T. Yli-Mattila, unpublished). In addition, it was possible to detect *F. langsethiae* (Torp et al., 2000; Torp and Nirenberg, in press; Yli-Mattila et al., in press), which is difficult to isolate from grain samples, from several barley and oats samples by species-specific primers.

In conclusion, this study reports the first molecular markers for *F. arthrosporioides* and *F. tricinctum*, which according to their molecular and morphological characters (Yli-Mattila et al., 2002a) are closely related to *F. avenaceum*. The ultimate goal of our future studies is to develop routine DNA extraction and quantitative PCR methods (Cullen et al., 2001; Dan et al., 2001, Schnerr et al., 2001) to enable molecular detection and identification of pathogenic and toxigenic *Fusarium* species or isolates (Bakan et al., 2002; Waalwijk et al., 2003) directly from different types of plant material, without the need for isolation of pure cultures and morphological identification. This will also make it possible in future to prevent the use of mycotoxin-contaminated grain as food and feed. The work dealing with SYBR Green and TaqMan realtime PCR detection of different *Fusarium* species in Finnish grain samples is in progress in our research group based on the species-specific molecular markers.

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