Molecular diversity of the *Fusarium fujikuroi* species complex from maize

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Abstract Random amplified polymorphic DNA (RAPD) markers were used to estimate genetic relationship and diversity of *Fusarium* species associated with pink ear rot of maize. Three *Fusarium* species – *F. verticillioides*, *F. temperatum* and *F. subglutinans* sensu stricto were collected from contaminated corn kernels. A collection of 41 randomly selected *Fusarium* isolates (16 *F. verticillioides*, 16 *F. temperatum* and nine *F. subglutinans* sensu stricto) was screened for polymorphism with thirty decamer primers. Seventeen primers that had generated a high number of polymorphic loci were used for the analysis of genetic diversity. The primers yielded a total of 146 polymorphic loci, an average of 8.59 polymorphic bands per primer. The percentage of polymorphic bands (%P) was 46.1%. The RAPD-based UPGMA analysis demonstrated that although *F. temperatum* and *F. subglutinans* s.s. form two distinct groups they are more similar to each other than to *F. verticillioides*. *Fusarium verticillioides* was the most heterogenous species (Nei's genetic diversity 0.180), while the least heterogenous one was *F. subglutinans* s.s. whose genetic diversity was 0.098. The analysis of the translation elongation factor 1- α and β -tubulin gene sequences as well as the mating type ratio were employed to support the RAPD data.

Keywords *Fusarium fujikuroi* species complex · Genetic diversity · Pink ear rot of maize · Random amplified polymorphic DNA

Introduction

Fusarium ear rot of maize, called also pink ear rot, is an economically important fungal disease commonly occurring in areas where maize is cultivated. The disease results in yield losses and the reduction of grain quality. A major economic threat is associated with this disease as *Fusarium* species possess the ability to synthesize mycotoxins in affected plants. The mycotoxins produced by these fungi are regarded as a potential threat to human and animal health (Voss et al. 2007; Desjardins 2006) and have a phytotoxic effect on plants as well (Lamprecht et al. 1994). The fumonisins are considered to be the most hazardous type due to their general toxicity and possible carcinogenicity to humans (Rheeder et al. 1992; IARC 1993). The occurrence of *Fusarium* ear rot – due to the prevalence, pathogenicity and toxigenic potential of its causative agents – is associated with significant economic losses in the world maize production (Logrieco and Visconti 2004; Wu 2007).

Fusarium ear rot is caused by several *Fusarium* species, including members of *Fusarium fujikuroi* species complex (FFSC). In maize-growing areas of Poland the predominant species are: *F. temperatum* and *F. subglutinans* sensu stricto, which are included in the so-called American clade according to O'Donnell et al.

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(1998b), and *F. verticillioides* – previously described as *F. moniliforme* (Seifert et al. 2003) – representing the African clade of the *Fusarium fujikuroi* species complex.

Fusarium temperatum and *F. subglutinans* sensu stricto are closely related species with a high level of morphological similarity. The number of septa and the shape of macroconidial basal cell are the morphological features that distinguish these two species (Scaufflaire et al. 2011). Both species resemble a polyphyletic and cosmopolitan taxon, *F. subglutinans* sensu lato. A large number of cryptic species were described in the last two decades within *F. subglutinans* sensu lato. Besides *F. temperatum* and *F. subglutinans* s.s., this complex taxon includes species pathogenic to various plants, such as bananas – *F. concentricum* (Nirenberg and O'Donnell 1998), mangoes – *F. mangiferae*, *F. sterilihyphosum* (Britz et al. 2002) and *F. mexicanum* (Otero-Colina et al. 2010), sugarcane – *F. sacchari* (Leslie et al. 2005) or pine trees – *F. circinatum* (Nirenberg and O'Donnell 1998; Britz et al. 1999). Originally isolated from maize, *F. temperatum* was described in 2011 by Scaufflaire et al. as a distinct group within a collection of *F. subglutinans* strains. On the basis of the polyphasic approach proposed by Taylor et al. (2000) for species identification, *F. temperatum* can be distinguished from *F. subglutinans* s.s. mainly by the differences in their mycotoxin activity as well as translation elongation factor 1- α (EF 1- α) and β -tubulin DNA sequences (Scaufflaire et al. 2011).

Whereas *F. temperatum* and *F. subglutinans* sensu stricto have been found to date only on maize (Scaufflaire et al. 2011), *F. verticillioides* is associated with a wide range of plant species. Beyond maize, *F. verticillioides* may infect rice, pineapples, sugarcane, sorghum, peas and fig trees (Nirenberg and O'Donnell 1998; O'Donnell et al. 1998b; Muleńko et al. 2008; Kvas et al. 2009; Hsuan et al. 2011; Stępień et al. 2013). The wide range of *F. verticillioides* host plants may be associated with the difficulty of defining clear species boundaries (Van Hove et al. 2011). With the implementation of new concepts of species definition, it has been shown that some known FFSC species characterized by a high level of genetic diversity consist of very similar twin taxa (Leslie 1995; Steenkamp et al. 2002; Kvas et al. 2009).

The purpose of this study was to assess the genetic diversity in the populations of *Fusarium* species associated with the pink ear rot of maize. For this purpose

RAPD markers were used. This technique has been reported to be suitable for assessing interspecific differences among *Fusarium* spp. strains collected from different hosts (Jiménez et al. 2000) and genetic variation within a population, e.g. in the case of *Fusarium verticillioides* strains (Kini et al. 2002).

Materials and methods

Fungal strains

Fusarium isolates were recovered from corn seeds obtained directly from naturally infected maize cobs with *Fusarium* ear rot symptoms, collected during four harvest seasons 2007–2010 in fields of central part of Poland, Radzików. *Fusarium* isolates were recovered from corn seeds with as well as without signs of *Fusarium* infection. After cob trashing, symptomatic corn kernels were directly plated on the synthetic nutrient agar (SNA), while healthy looking i.e. asymptomatic kernels were previously surface disinfected. Plates with 5 kernels on surface of agar medium were incubated in the light for 4–5 days at a room temperature. Identification of *Fusarium* sp. colonies was performed on the basis of morphological characters according to Nelson system of taxonomy (1983) and “The *Fusarium* Laboratory Manual” by Leslie and Summerell (2006). Single-spore *Fusarium* strains were obtained following the method described by Burgess and Liddell (1983). All strains were deposited in the Culture Collection of the Warsaw Agricultural University Herbarium (WAUF).

Most corn ear samples were collected from the single corn field of the Plant Breeding and Acclimatization Institute in Radzików, Poland (Mazowieckie province). Only in the case of *F. subglutinans* s.s. were isolates originating from other maize-growing areas outside Poland also included (Table 1).

Molecular identification

Single-spore strains mycelia grown for 5–7 days on potato dextrose agar (PDA) and then scraped off the surface of the media were used for the DNA extraction. Isolation of genomic DNA was achieved with the use of the modified CTAB method (Doohan et al. 1998; Tomczak et al. 2002).

Table 1 *Fusarium verticillioides*, *F. temperatum* and *F. subglutinans* s.s. strains analysed in the study

Species	Strain No.	Origin	MAT allele	EF 1- α	β -tubulin
<i>F. verticillioides</i>	KFI-633	Poland	MAT1–1	n/a	n/a
	KFI-392	Poland	MAT1–1	n/a	n/a
	KFI-522	Poland	MAT1–1	n/a	n/a
	KFI-388	Poland	MAT1–1	n/a	n/a
	KFI-622	Poland	MAT1–2	n/a	n/a
	KFI-610	Poland	MAT1–1	n/a	n/a
	KFI-680	Poland	MAT1–2	n/a	n/a
	KFI-693	Poland	MAT1–2	n/a	n/a
	KFI-694	Poland	MAT1–2	n/a	n/a
	KFI-523	Poland	MAT1–1	n/a	n/a
	KFI-373	Poland	MAT1–2	n/a	n/a
	KFI-524	Poland	MAT1–1	n/a	n/a
	KFI-525	Poland	MAT1–1	n/a	n/a
	KFI-377	Poland	MAT1–1	n/a	n/a
	KFI-445	Poland	MAT1–2	n/a	n/a
	KFI-527	Poland	MAT1–1	n/a	n/a
<i>F. temperatum</i>	KFI-612	Poland	MAT1–2	MT461303	MT461329
	KFI-615	Poland	MAT1–2	MT461304	MT461335
	KFI-617	Poland	MAT1–1	MT461305	MT461336
	KFI-618	Poland	MAT1–2	MT461309	MT461337
	KFI-620	Poland	MAT1–2	MT461312	MT461338
	KFI-650	Poland	MAT1–2	MT461313	MT461330
	KFI-653	Poland	MAT1–1	MT461311	MT461331
	KFI-654	Poland	MAT1–1	MT461314	MT461332
	KFI-655	Poland	MAT1–1	MT461308	MT461333
	KFI-657	Poland	MAT1–1	MT461315	MT461328
	KFI-662	Poland	MAT1–1	MT461306	MT461339
	KFI-664	Poland	MAT1–1	MT461316	MT461340
	KFI-690	Poland	MAT1–2	MT461318	MT461334
	KFI-669	Poland	MAT1–1	MT461317	MT461341
	KFI-687	Poland	MAT1–1	MT461310	MT461342
	KFI-695	Poland	MAT1–1	MT461307	MT461343
<i>F. subglutinans</i> s.s.	KFI-607	Poland	MAT1–1	MT461327	MT461352
	KFI-717	South Korea	MAT1–1	MT461324	MT461344
	KFI-719	South Korea	MAT1–1	MT461319	MT461350
	KFI-720	USA	MAT1–1	MT461321	MT461346
	KFI-722	China	MAT1–1	MT461326	MT461349
	KFI-723	China	MAT1–1	MT461323	MT461348
	KFI-718	South Korea	MAT1–2	MT461320	MT461347
	KFI-721	USA	MAT1–2	MT461322	MT461351
	KFI-724	USA	MAT1–2	MT461325	MT461345

n/a – not available

Due to the high morphological similarity within FFSC, the final species identification was confirmed using molecular methods. Species-specific SCAR (Sequence Characterized Amplified Regions) markers (Mulè et al. 2004) were used for the initial identification of *Fusarium verticillioides* and *F. subglutinans* sensu lato. More detailed species identification was needed for *F. subglutinans* sensu lato species complex. According to numerous studies (O'Donnell et al. 1998a; Yli-Matila et al. 2002; Harrow et al. 2010) the morphologically similar *Fusarium* species can be distinguished on the basis of the DNA sequence polymorphism in the translation elongation factor 1- α (TEF1- α) and β -tubulin gene. Whereas TEF1- α region is phylogenetically informative for most of the *Fusarium* species (Knutsen et al. 2004), additional sequence data from other genes is needed to differentiate *F. temperatum* from *F. subglutinans* sensu stricto and other closely related FFSC species (Geiser et al. 2004; Scaufaire et al. 2011). Therefore, the analysis was carried out on the basis of the DNA sequence data from both regions as described by O'Donnell et al. (1998b). The DNA sequences were amplified with the use of the primers developed by O'Donnell and Cigelnik (1997) and Geiser et al. (2004) respectively.

The PCR products were sequenced at the Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw, Poland and assembled using DNA Baser Assembler v5.15 (Heracle BioSoft). The identification of each fungal strain was verified by the comparison of the obtained sequences with the data from NCBI GenBank (www.ncbi.nlm.nih.gov). The TEF1- α and β -tubulin partial gene sequences of *F. temperatum* and *F. subglutinans* s.s. were deposited in NCBI GenBank. The accession numbers for the sequences used in this study are provided in Table 1.

Mating types of the fungal strains were determined by a multiplex PCR assay according to thermal conditions described by Mohammadian et al. (2011), with the use of primers developed by Steenkamp et al. (2000).

RAPD assay

For the purposes of the analysis 16 Polish strains of *F. verticillioides* and *F. temperatum* each were selected randomly from the obtained *Fusarium* population and reflected nearly equal frequency of these two species isolated from maize. Additionally nine strains of *F. subglutinans* s.s. were included to the studies. Due

to the rare occurrence of this fungus only one isolate was of domestic origin and the additional eight isolates came from the areas outside Poland (Table 1).

Thirty RAPD primers (Operon Technologies) of recognized usefulness in the assessment of diversity within fungi of the *Fusarium* genus (El-Yazeed et al. 2011; Saharan 2010; Latiffah et al. 2008; Iqbal et al. 2006; Pamphile and Azevedo 2002; Viljoen et al. 1997) were used for this study. In the preliminary study selected primers were screened for reproducibility and polymorphism.

In order to ensure reproducible results, all of the amplifications were performed on only one thermal cycler, Veriti 96-Well Thermal Cycler (Applied Biosystems, USA). The reproducibility of the RAPD bands was found mainly by optimisation of PCR components and reaction conditions for the target species. Varying concentrations of selected RAPD primers, MgCl₂ and template DNA were used in the preliminary analysis. Furthermore, following the results obtained by Skorić et al. (2012), the high concentration of Taq polymerase (3 U in the 12 μ l reaction mixture volume) was used to provide the high intensity of the detectable bands.

As a result of previously performed gradient PCR with the temperature range of 34–40 °C, the annealing temperature of 37 °C was proved to be the most optimal for obtaining clear bands in RAPD patterns. Therefore, the amplification program was as follows: one cycle at 95 °C for 1 min followed by 10 cycles at 94 °C for 5 s, 37 °C for 30 s, 72 °C for 30 s and 35 cycles with extended to 60 s elongation step (94 °C for 5 s, 37 °C for 30 s, 72 °C for 60 s), finally one cycle at 72 °C for 7 min.

At this stage of the research, in order to ensure that only reproducible bands are scored in the course of the analysis, a series of PCR reactions (in two replications) were conducted with selected primers (OPAC 19, OPAA 12) using previously established reagent concentrations and thermal cycling parameters. The obtained amplification profiles for both primers exhibited no differences.

Each 12 μ l reaction mixture consisted of 0.6 μ l of template DNA (50 ng/ μ l), 1.2 μ l of RAPD primer (10 μ M/ μ l), 0.3 μ l of dNTPs, 0.06 μ l of Dream Taq DNA Polymerase (Thermo Fisher Scientific, USA) (5 U/ μ l), 0.9 μ l of 25 mM MgCl₂, 1.2 μ l of 10 $\times$ PCR buffer (Thermo Fisher Scientific, USA) and 7.74 μ l of sterile distilled water.

The result of each amplification was visualized in 1.3% agarose gel (containing 0.1 mg/ml ethidium bromide) electrophoresis in $1\times$ TBE (Tris-Borate Buffer). The agarose gel electrophoresis was performed using an Owl™ D4 Horizontal Electrophoresis System (Thermo Fisher Scientific, USA). The images of the banding patterns were acquired under UV light using a INGenius LHR2 gel imaging system (SynGene, UK).

RAPD data analysis

The amplification profiles were analysed in GeneTools 4.02.01 (Syngene, UK) and CLIQS 1D (TotalLab, UK). The size of RAPD PCR products was referenced to the GeneRuler 1 kb SM0313 DNA size marker (Thermo Fisher Scientific, USA). The number of all amplicons were counted and presented in the form of a binary matrix, which was prepared on the basis of the presence (“1”) or absence (“0”) of specific bands.

The binary matrix combining the data records for all the 41 *Fusarium* isolates was analysed in programs: PopGene v.1.32 (Yeh et al. 1999), Past 3.14 (Hammer et al. 2001) and GeneAEx 6.5 (Peakall and Smouse 2006, 2012).

The relevant parameters to be considered in order to estimate the efficiency of RAPD markers were:

- the total number of bands (n_T) as well as the number of polymorphic (n_P) and monomorphic bands (n_M);
- the percentage of polymorphic bands for all assays (%P) and per single assay unit (n_P/U),
- polymorphism information content (PIC) according to the formula of De Riek et al. (2001) for dominant markers:

$$PIC = 1 - [p^2 + (1-p)^2], \text{ where } p \text{ is the frequency of the marker,}$$

- resolving power (RP) and mean resolving power (MRP) according to Prevost and Wilkinson (1999):

$$RP = \sum BI, \quad MRP = 1/n \sum BI_i,$$

where ‘BI’ is band informativeness calculated as $BI = 1 - [2 \times (0.5 - p)]$.

and where ‘p’ is the proportion of individuals containing the band;

- effective multiplex ratio (EMR) according to Powell et al. (1996) formula:

$$EMR = \%P \times nU;$$

- marker index (MI) according to Powell et al. (1996) formula:

$$MI = PIC \times EMR.$$

In order to determine the diversity within and among the surveyed *Fusarium* species the following diversity indices were calculated with the use of PopGene software version 1.32 (Yeh et al. 1999) and GenAEx 6.5 (Peakall and Smouse 2006, 2012):

- gene diversity (h) according to Nei (1973): $h = 1 - (p^2 + q^2)$, where ‘p’ is the frequency of the RAPD band and $q = 1 - p$;
- coefficient of genetic differentiation (G_{ST}), which was estimated on the basis of the value of total genetic diversity (H_T) and genetic diversity within populations (H_S) according to Nei (1973):

$$G_{ST} = \frac{H_T - H_S}{H_T}$$

- Shannon-Weaver diversity index (I) according to the formula of Shannon and Weaver (1949):

$$I_{sw} = -1 \times [p \times \ln(p) + q \times \ln(q)],$$

where ‘p’ is the frequency of the RAPD band and $q = 1 - p$,

- the number of migrants per generation (N_m) which is regarded as indirect estimation of the amount of gene flow between populations was calculated by the formula of McDermott and McDonald (1993):

$$N_m = 0.5(1 - G_{ST})/G_{ST}.$$

In addition, genetic similarity (GS) was estimated among all the 41 tested *Fusarium* strains. Calculations were based on four similarity coefficients: Jaccard (1908); Kulczyński (1927); Dice (1945) – estimated with the formula of Nei and Li (1979) – and Ochiai (1957). Correlation coefficients between the GS values

obtained with the use of various measures were determined with the significance level of $\alpha = 0.001$.

The obtained similarity index matrices were subsequently used for the cluster analysis in Past 3.14 (Hammer et al. 2001). With the use of principal coordinate analysis (PCoA) and the unweighted pair group method with arithmetic mean (UPGMA), the genetic relatedness of all the 41 tested *Fusarium* strains was shown on graphs and dendrograms, respectively.

For separate species, the phylogenetic network analyses was performed with SplitsTree 4.16.0 using the Neighbor-Net algorithm (Huson and Bryant 2006). To verify the presence of recombination, the PHI (Pairwise Homoplasy Index) test (Bruen et al. 2006) was performed.

Nucleotide sequence analysis

For the purpose of analysis, the obtained sequences of the translation elongation factor 1- α and β -tubulin gene were visually inspected using Chromas 2.6.6 (Technelysium, Australia) and then aligned with the ClustalW algorithm implemented in MEGA 7.0.6 (Kumar et al. 2016). The low-quality read and alignment-ambiguous regions were excluded from subsequent analyses.

The genetic diversity analysis was conducted on all of the obtained sequences as well as on their concatenations. The nucleotide diversity, the GC content, the number of segregating sites and estimates of Theta (Θ) per site were determined using the DnaSP 5.10.01 (Rozas et al. 2009). The same software was used to perform Tajima's Neutrality Test (Tajima 1989).

The phylogenetic analysis was performed with the use of maximum likelihood (ML) method with 1000 bootstrap replicates and visualised with MEGA 7.0.6. *F. verticillioides* strain 7600 (Sequence ID: NC_031676.1) was used as outgroup.

Results

The effectiveness of the selected RAPD primers

Out of the 30 pre-analysed oligonucleotide RAPD primers (Operon Technologies, USA), the 17 primers (Table 2) generating clear, stable amplification profiles with a large number of bands were finally selected for this study. Thirteen remaining primers were discarded due to the insufficient number of generated amplicons (OPAC 03, OPAC 12, OPAD 04, OPAD 05, OPC 02)

or the total lack of amplification products (OPAA 16, OPAA 17, OPAD 15, OPP 03, OPX 02, OPX 12, OPX 14, OPX 18).

A total of 317 amplification products were generated with the use of the 17 selected RAPD primers with 41 *Fusarium* spp. strains. The band sizes ranged from 105 to 3134 bp. The number of products generated by a single decamer RAPD primer was found to range from 9 – with primer OPAC 17 – up to 27 bands with primers OPX 07 and OPX 09 (Table 2).

The RAPD markers selected for the analysis proved to be effective in discriminating the 41 *Fusarium* strains. Nearly half of the generated loci (46.1%) were polymorphic. The frequencies of polymorphic bands ranged from 34.0% (primer OPAD 10) to 68.3% (OPAD 12). The highest frequency of polymorphic bands that exceeded 60% was noted when OPAA 09, OPAA 12 (Fig. 1), OPAD 12 and OPX 15 primers were used (Table 2).

On average, 18.65 products were obtained in a single PCR reaction with one primer. The effectiveness of the RAPD technique, expressed by the average number of polymorphic bands per reaction, was 8.59 (Table 2).

While according to De Riek et al. (2001) the maximum value of polymorphism information content (PIC) for dominant markers such as RAPD is 0.5, the average PIC in this study was 0.261. The PIC value ranged from 0.215 (OPAA 13) to 0.315 (OPX 15). Apart from few markers indicating PIC values below 0.25 (OPAA 13, OPAC 17, OPAC 10, OPAD 10, OPAC 19, OPX 01), most of the markers used in the study were polymorphic and informative, out of which three markers (OPAC 11, OPL 15 and OPX 15) could be regarded as – according to Botstein et al. (1980) – highly informative ones with PIC value of greater than 0.3.

The average resolving power of primers (RP) in this study was 5.69 ranging from 2.63 (OPAC 17) to 8.35 (OPL 15), while mean resolving power (MRP) varied from 0.29 (OPAC 17) to 0.45 (OPL 15, OPX 15) with mean value of 0.37. Although primer OPL 15 showed the highest noted RP, MRP and PIC values, the percentage of polymorphic bands generated with this primer was one of the lowest among all tested markers (39.5%).

As indicated by the marker index (MI) combining the values of PIC and EMR, the two primers that were proven to be the most effective in differentiation of analysed *Fusarium* species were OPAA 09 and OPAD 12. Both primers were characterized by the highest MI value of 2.75, while the average MI value in this study

Table 2 Parameters of RAPD primers used in the study

Primer	Sequence (5'-3')	n _T	n _p	n _M	n _p /U (%)	Bands size range (bp)	PIC	RP	MRP	EMR	MI
OPAA 09	AGATGGGCAG	20	12	8	62.1	116–2271	0.257	5.98	0.36	10.70	2.75
OPAA 12	GGACCTCTTG	14	9	5	64.7	221–2620	0.253	4.08	0.34	8.12	2.06
OPAA 13	GAGCGTCGCT	17	8	9	49.3	205–2217	0.215	5.00	0.31	8.07	1.73
OPAC 10	AGCAGCGAGG	22	8	14	34.9	105–1764	0.225	5.91	0.31	7.18	1.61
OPAC 11	CCTGGGTCAG	17	7	10	38.5	177–2505	0.303	6.04	0.44	5.81	1.76
OPAC 17	CCTGGAGCTT	9	5	4	56.4	345–2562	0.224	2.63	0.29	5.08	1.14
OPAC 19	AGTCCGCCTG	18	9	9	50.0	194–2166	0.231	4.50	0.32	7.41	1.71
OPAD 10	AAGAGGCCAG	26	9	17	34.0	181–2406	0.226	6.03	0.30	7.41	1.68
OPAD 12	AAGAGGGCGT	16	11	5	68.3	107–2731	0.277	5.55	0.39	9.92	2.75
OPF 01	ACGATCCTG	18	7	11	36.9	199–3134	0.281	6.54	0.42	6.16	1.73
OPL 15	AAGAGAGGGG	23	9	14	39.5	187–2603	0.306	8.35	0.45	7.93	2.43
OPX 01	CTGGGCACGA	10	6	4	57.6	350–2202	0.243	3.20	0.35	5.33	1.29
OPX 06	ACGCCAGAGG	21	9	12	42.1	199–2352	0.287	7.20	0.41	7.82	2.25
OPX 07	GAGCGAGGCT	27	12	15	42.8	163–2294	0.257	8.01	0.36	10.27	2.64
OPX 09	GGTCTGGTTG	27	10	17	37.1	130–2659	0.279	7.84	0.42	7.92	2.21
OPX 15	CAGACAAGCC	14	9	5	66.7	211–1908	0.315	4.46	0.45	7.02	2.21
OPX 17	GACACGGACC	18	6	12	35.2	137–2409	0.262	5.39	0.36	5.50	1.44
Total		317	146	171							
Average per primer		18.65	8.59	10.06	46.1		0.261	5.69	0.37	7.51	1.96

nT, total number of generated bands; *nP*, number of polymorphic bands; *nM*, number of monomorphic bands; *nP/U*: percentage of polymorphic band per reaction; *PIC*: polymorphism information content; *RP*: resolving power; *MRP*: mean resolving power; *EMR*: effective multiplex ratio; *MI*: marker index

Fig. 1 RAPD profile of *Fusarium* spp. produced with primer OPAA 12. Lane M is 1 kb DNA ladder, lanes 1–16 represent strains of *F. verticillioides*, 17–32: *F. temperatum*, 33–41: *F. subglutinans* s.s

was 1.96 with the minimum 1.14 noted for OPAC 17. The highest efficiency of OPAA 09 and OPAD 12 – as diversity markers in this study – was confirmed by other coefficients such as effective multiplex ratio (10.70; 9.92) and the high percentage of polymorphic loci (62.1%; 68.3%) (Table 2).

Genetic diversity within populations

Despite the high proportion of polymorphic bands (46.1%) observed in the amplification profiles (Table 2), the genetic diversity within the analysed population of *Fusarium* spp. was rather moderate, as evidenced by the value of Shannon-Weaver index (0.467) (Table 3).

A comparison of this index among the three analysed species revealed that the Shannon-Weaver index was lowest in the *F. subglutinans* sensu stricto (0.148) and highest in the *F. verticillioides* (0.275). The same trend was observed for the Nei's genetic diversity which varied from 0.098 (*F. subglutinans* s.s.) to 0.180 (*F. verticillioides*), with an average of 0.303 (Table 3). The linear relationship between the observed genetic diversity of the analysed species and their polymorphism is presented graphically in Fig. 2.

The G_{ST} value of 0.552 indicated a relatively high level of population differentiation (Table 3). The lowest interpopulation diversity was noted between *F. temperatum* and *F. subglutinans* sensu stricto (0.472). The populations of these two closely related, almost morphologically identical species were characterized by the highest gene flow recorded in the study (0.515). Within the entire sample, the gene flow reached 0.406 (Table 3) and varied from 0.448 to 0.515 (Table 4). Such low level of gene flow seems to be consistent with the observed large genetic diversity noted between the analysed species of *F. verticillioides*, *F. temperatum* and *F. subglutinans* sensu stricto. These results are also confirmed by the conducted analysis of molecular variance (AMOVA), which revealed that a very high variation occurred between species (61%), whilst only 39% occurred among species.

Phylogenetic relationship between *Fusarium* strains

The mean genetic similarity (GS) among the analysed *Fusarium* strains varied from 0.312 (according to Jaccard's similarity coefficient) to 0.441 (Kulczyński's coefficient) (Table 5). In general, the correlation

between similarity coefficients was very high, exceeding more than 0.99, whilst the lowest correlation with other measures was shown by the Jaccard coefficient (0.99661–0.99667; Table 6). The genetic similarity matrix identified according to this coefficient varied from 0.103 to 0.896 (Table 5).

The most consistent classifications of isolates were obtained with the Dice, Kulczyński and Ochiai similarity coefficients. The genetic similarity calculated with these coefficients ranged from 0.186 (according to Dice and Ochiai) and 0.187 (Kulczyński) to 0.945 (for all three coefficients) (Table 5). These three coefficients were very strongly positively correlated ($r > 0.99998$) at the significance level of 0.001 (Table 6).

At the species level, the highest genetic similarity was noted within *F. subglutinans* s.s. (0.762) with only 7.79% variability. The genetic similarities within strains of this species ranged from 0.616 to 0.876. On the contrary, *F. verticillioides* proved to be the most diverse one with the highest coefficient of similarity variation (31.25%). The genetic similarities among *F. verticillioides* isolates ranged from 0.302 to 0.862 (according to the mean value of the four similarity coefficients used) (Table 7).

On the basis of the similarity matrices generated with the use of Dice's, Jaccard's, Kulczyński's and Ochiai's coefficients, the UPGMA cluster analysis was carried out. The resulting dendrograms are shown in Fig. 3. The analysis divided the analysed *Fusarium* strains into two distinct clusters. Interestingly, *F. temperatum* and *F. subglutinans* s.s. strains were grouped together in one cluster. Strains representing these two species were the most similar (0.732) (Table 8). The second, most separated cluster was a group of 16 *F. verticillioides* strains. The dendrograms reflect the large genetic distance separating strains of *F. verticillioides* from the *F. temperatum* and *F. subglutinans* sensu stricto strains which proved to be consistent with their taxonomic classification (Fig. 3). The largest genetic distance was recorded between *F. verticillioides* and *F. subglutinans* s.s. strains (0.359) (Table 8). The results of the UPGMA cluster analysis prepared on the basis of the similarity matrices by Dice (Fig. 3a), Kulczyński (Fig. 3c) and Ochiai (Fig. 3d) show almost identical topology. The only slight difference in topology was noted within the *F. verticillioides*. On the dendrograms generated according to Dice, Jaccard and Ochiai similarity coefficients the isolates KFI-523 and KFI-392 showed a high degree of similarity and made a clear subgroup, while on

Table 3 Genetic diversity indices for *F. verticillioides*, *F. temperatum* and *F. subglutinans* sensu stricto estimated on the basis of RAPD binary matrix

Diversity indices	Species			Mean for species
	<i>F. verticillioides</i>	<i>F. temperatum</i>	<i>F. subglutinans</i> sstrs.s.	
The percentage of polymorphic loci (%P)	59.0%	51.6%	33.4%	46.1%
Nei's genetic diversity (h)	0.180	0.127	0.098	0.303
Shannon-Weaver index (I)	0.275	0.202	0.148	0.463
Coefficient of genetic differentiation (G_{ST})	0.552			
Gene flow (N_m)	0.406			

Kulczyński's dendrogram they were grouped in separate clusters (Fig. 3d).

The relationships of the analysed *Fusarium* species illustrated by the principal coordinate analysis (PCoA) (Fig. 4) were largely congruent with the UPGMA clustering. On the contrary to the UPGMA-based dendrograms however, groups designated as F.t. (containing 16 isolates) and as F.s. (nine isolates) were clearly separated from each other in the two-dimensional PCoA plots of 41 *Fusarium* strains.

The Neighbor-Net phylogenetic networks (Fig. 5) were generated for all *Fusarium* strains as well as for the separate species. The highest degree of genetic similarity was observed within *F. subglutinans* s.s. The results of the analysis show a remarkable diversity within *F. verticillioides* strains. Despite the same geographical origin, *F. verticillioides* strains are clustered into two distinct groups. While most of the *F. verticillioides* strains are grouped together, three MAT1–2 strains – KFI-522, KFI-

527, KFI-522 – form a distinct group. Combined with the mating type data the phylogenetic networks suggest the presence of a closer relationship between strains representing the same mating type (Fig. 5).

Although the phylogenetic analysis based on the translation elongation factor 1- α (EF 1- α) and β -tubulin DNA sequences of *F. temperatum* and *F. subglutinans* sensu stricto does not support this conclusion, it indicates a high level of consistency with RAPD-based results in terms of the interspecies diversity (Fig. 6). Within species, however, the level of nucleotide similarity of the aforementioned regions was high and in the EF 1- α DNA sequences ranged from 98.1–100% and 99.1–100% for *F. subglutinans* s.s. and *F. temperatum* respectively, whereas the β -tubulin DNA sequences showed even higher similarity among tested strains (99.9–100%).

The PHI test performed on the basis of the RAPD phylogenetic network (Fig. 5) detected 552 informative sites and found statistically significant evidence for recombination ($p = 9.653E-4$) among the analysed *Fusarium* strains. This conclusion, however, was not supported by the DNA sequence data. Furthermore, the neutrality tests showed no evidence of selection in both EF 1- α and β -tubulin DNA sequences. Tajima's test values (D) for the sequences of analysed *F. subglutinans* sensu lato strains exceeded 1.5, but were not significant (Table 9).

Fig. 2 The correlation between the level of genetic diversity and polymorphism of the *F. verticillioides*, *F. temperatum* and *F. subglutinans* sensu stricto populations expressed using the Shannon-Weaver index (y axis) and the percentage of polymorphic loci (x axis)**Table 4** The level of gene flow (N_m) between *F. verticillioides*, *F. temperatum* and *F. subglutinans* sensu stricto

	<i>F. verticillioides</i>	<i>F. temperatum</i>
<i>F. verticillioides</i>		
<i>F. temperatum</i>	0.483	
<i>F. subglutinans</i> s.s.	0.448	0.515

Table 5 The genetic similarity of the analysed *Fusarium* spp. population estimated using four similarity coefficients

Similarity coefficient	Min	Mean	Max	Coefficient of variation [%]
Dice	0.186	0.440	0.945	50.13%
Jaccard	0.103	0.312	0.896	68.94%
Kulczyński	0.187	0.441	0.945	50.09%
Ochiai	0.186	0.440	0.945	50.11%

Discussion

The present study on *Fusarium* species responsible for the pink ear rot of maize reveals a moderate level of genetic variation with 46.1% of loci being polymorphic and even higher polymorphism at the species level.

The largest number of polymorphic loci (59%) as well as the highest diversity was recorded within the population of *F. verticillioides* isolates. The Nei's genetic diversity was estimated at 0.180 while the Shannon-Weaver index reached 0.275. Similar results for this species were reported by Reynoso et al. (2009) with the gene diversity value of 0.128 and the percentage of polymorphic loci ranging from 48 to 52%.

The analysed *F. verticillioides* population reveals one of the lowest so far values of genetic similarity of 0.610. Other studies showed much higher genetic similarity of *F. verticillioides* populations: 0.73 (Leslie et al. 2005), 0.76 (Leslie et al. 2004) and 0.91 (Reynoso et al. 2009).

As indicated by the values of Nei's genetic diversity (0.127), the Shannon-Weaver index (0.202) and the percentage of polymorphic bands (51.6%), the analysed population of *F. temperatum* strains exhibited slightly lower level of diversity than *F. verticillioides*. Genetic similarity coefficients (GS) between the *F. temperatum* strains ranged from 0.453 to 0.933, with a mean of 0.731. Similar results were obtained by examination of

F. temperatum isolated from maize in Belgium (Scauflaire et al. 2011), where the genetic similarity ranged from 0.74 to 1.00.

The result of the present study using RAPD reveals very low level of genetic diversity within the population of *F. subglutinans* sensu stricto with an average Nei's genetic diversity estimated at 0.098, the Shannon-Weaver index – 0.148 and only 33.4% polymorphic amplification products observed. Regardless of the diverse origin of isolates, *F. subglutinans* s.s. shows a fairly high level of genetic similarity ranging from 0.616 to 0.876 with mean value of 0.762.

As evidenced by the values of gene diversity (0.303) and the Shannon-Weaver index (0.463), the genetic diversity within the analysed *Fusarium* spp. strains is

Table 7 The genetic similarity of *F. verticillioides*, *F. temperatum* and *F. subglutinans* sensu stricto estimated using four similarity coefficients: Dice, Jaccard, Kulczyński and Ochiai

Species	Similarity coefficient	Min	Mean	Max	Variability [%]
<i>F. verticillioides</i>	Dice	0.336	0.645	0.885	28.96%
	Jaccard	0.202	0.502	0.794	38.16%
	Kulczyński	0.336	0.646	0.885	28.95%
	Ochiai	0.336	0.646	0.885	28.95%
	Mean	0.302	0.610	0.862	31.25%
<i>F. temperatum</i>	Dice	0.494	0.764	0.945	14.46%
	Jaccard	0.328	0.630	0.896	21.07%
	Kulczyński	0.495	0.765	0.945	14.46%
	Ochiai	0.494	0.764	0.945	14.46%
	Mean	0.453	0.731	0.933	16.11%
<i>F. subglutinans</i> s.s.	Dice	0.656	0.794	0.897	6.77%
	Jaccard	0.488	0.662	0.813	11.09%
	Kulczyński	0.663	0.797	0.897	6.61%
	Ochiai	0.659	0.795	0.897	6.69%
	Mean	0.616	0.762	0.876	7.79%

Table 6 Correlation coefficients between measures of genetic similarity

Similarity coefficient	Dice	Jaccard	Kulczyński
Jaccard	0.99667		
Kulczyński	0.99998	0.99661	
Ochiai	0.99999	0.99664	0.99999

Fig. 3 UPGMA dendrogram illustrating the genetic relationships among analysed *Fusarium* spp. strains, generated from RAPD data on the basis of four similarity coefficients: Dice (a), Jaccard

(b), Kulczyński (c) and Ochiai (d). The groups of *F. temperatum* isolates are labelled F.t., *F. subglutinans* s.s. – F.s. and *F. verticillioides* – F.v

Table 8 The genetic similarity (values above diagonal) and diversity (below diagonal) between three *Fusarium* species: *F. verticillioides*, *F. temperatum* and *F. subglutinans* sensu stricto

	<i>F. verticillioides</i>	<i>F. temperatum</i>	<i>F. subglutinans</i> s.s.
<i>F. verticillioides</i>		0.706	0.698
<i>F. temperatum</i>	0.348		0.732
<i>F. subglutinans</i> s.s.	0.359	0.313	

at a fairly high level. The genetic diversity within the species of *Fusarium fujikuroi* species complex seems to be one of the supporting arguments for the regular occurrence of sexual reproduction process. The results of the current study prove that gene recombination occurring during sexual reproduction may be the source of the observed genetic diversity in these anamorphic fungi. What is more, the presence of numerous unique genotypes identified by the molecular markers such as RAPD indicates a small percentage of clones in the population. It is worth noting that all of the *F. verticillioides* and *F. temperatum* isolates originated from one location.

The G_{ST} value of 0.552 indicates that 61% of variation is found within analysed populations of *F. verticillioides*, *F. temperatum* and *F. subglutinans* sensu stricto and only 39% is observed within a species. Such result is in agreement with the very low level of the gene flow between species ($N_m = 0.406$). It is commonly considered that a G_{ST} value higher than “0.25” indicates a very high level of population differentiation (Berg and Hamrick 1997), while the N_m value below “1” allows to conclude that there is no gene exchange between species (McDonald 1997).

While the principal coordinate analysis (PCoA) based on 17 RAPD marker profiles has grouped the *Fusarium* strains into the three clusters according to their taxonomic classification, the UPGMA cluster analysis has grouped all the 41 *Fusarium* strains into two clusters only. The pattern of clustering visible on the dendrograms clearly reflects the level of genetic differentiation between the analysed species: *F. verticillioides*, *F. temperatum* and *F. subglutinans* s.s. The *F. temperatum* and *F. subglutinans* s.s. strains are clustered into one because of their close phylogenetic relationship and high level of morphological similarity. The clustering of strains remains more or less the same on all the

dendrograms regardless of the similarity coefficient used for the analysis.

Surprisingly, despite the fact that the similarity coefficients selected for the analysis are based on different interpretations of the shared absences of specific bands in the amplification profile – while Ochiai's coefficient includes negative co-occurrences, Jaccard's, Dice's and Kulczynski's coefficients include only positive matches – the patterns of diversity revealed by all of the similarity measures are statistically the same. The results of this study indicate that the Jaccard's coefficient – most commonly used next to the Dice index – may strengthen the observed diversity. This index is also considered to be the most appropriate measure of similarity for haploid organisms with dominant markers such as RAPD, AFLP and ISSR, especially for individuals from different taxa (Kosman and Leonard 2005), while Dice's measure is recommended for the comparison analysis of individuals with low levels of similarity as it places more weight on the shared bands (Weising et al. 2005).

To provide a broader level of resolution, the translation elongation factor 1- α and β -tubulin gene sequence analysis was employed to support the RAPD data obtained in this study. The phylogenetic analysis of the aforementioned regions was in agreement with the RAPD results and supported the separation of species into distinct clades. In terms of the interspecies diversity, both analyses showed similar results indicating higher variation occurring between species than among species. Although phylogenetic network analysis of the RAPD data together with the PHI test suggests the presence of recombination events in the analysed population, no substantial evidence of recombination was detected within the analysed nucleotide sequences.

Although the RAPD based analysis is considered to be a low reproducibility technique (He et al. 1994;

Fig. 4 The phylogenetic relationships of the analysed *Fusarium* species: *F. verticillioides* (*F.v.*), *F. temperatum* (*F.t.*) and *F. subglutinans* sensu stricto (*F.s.*) illustrated by two-

dimensional plots obtained using principal coordinates analysis (PCoA) generated from RAPD data on the basis of four similarity coefficients: Dice (a), Jaccard (b), Kulczyński (c) and Ochiai (d)

Fig. 5 Neighbor-Net phylogenetic network explaining the genetic diversity within the analysed *Fusarium* species: *F. verticillioides*, *F. temperatum* and *F. subglutinans* sensu stricto on the basis of the

RAPD data. For each species analysis, the most distant strain representing other taxon was used as an outgroup

MacPherson et al. 1993; Meunier and Grimont 1993), the result of the analysis shows that the reliability and significance of RAPD in characterization of populations' diversity is high. Some authors even indicate a higher RAPD sensitivity in the diversity determination when compared to other molecular techniques such as RFLP (King et al. 1995; Lin et al. 1995) or SSR (Zargar et al. 2016). As dominant markers, RAPD can be used for an efficient detection of DNA-sequence polymorphisms of many loci throughout the genome. The inability of RAPD markers to identify heterozygotic loci, which is the most frequently listed drawback of binary markers, is of marginal importance for plant pathogenic fungi. As the majority of fungi are haploid organisms, there is a strong and direct relationship of phenotype to their genotype, which is indirectly identified by dominant amplified fragments in a RAPD assay (Taylor et al. 1999). Moreover, some studies tend to show that the discriminatory power of dominant markers is directly related to the number of primers used in the study and thus the number of detected and analysed loci

(Reynoso et al. 2009; Brake et al. 2014; Zargar et al. 2016). Therefore, RAPD has been widely used in the assessment of genetic similarity (Singh et al. 2006), in the analysis of genetic diversity within a species (Collignon et al. 2002) or between closely related species (Jiménez et al. 2000). Within *Fusarium* genus, RAPD has been applicable e.g. in the distinction between non-pathogenic and pathogenic isolates of *F. oxysporum* f. sp. *dianthi* (Manulis et al. 1994), differentiation of special forms of *F. oxysporum* pathogenic to cucumber (Vakalounakis and Fragkiadakis 1999) or in *F. verticillioides* strains differentiation depending on the host plant (Kini et al. 2002).

In this study, the use of RAPD markers proved to be useful and efficient tool to detect the amount of polymorphism in *Fusarium* species associated with the pink ear rot of maize. As expressed by the percentage of polymorphic bands per single assay unit, the effectiveness of the RAPD analysis reached 8.56. Similar result of 10 polymorphic band per assay were obtained by Pamphile

Fig. 6 Maximum likelihood trees based on the concatenated β -tubulin and translation elongation factor 1- α gene DNA sequences of the analysed *F. temperatum* and *F. subglutinans* sensu stricto strains. Both trees were constructed using Kimura's two-parameter model – as determined by the model test algorithm implemented in MEGA 7.0.6 – and tested by bootstrapping (1000 replicates). **a**

Phylogenetic tree showing the relationships between studied *F. temperatum* and *F. subglutinans* sensu stricto strains. **b** Genetic divergence between *F. subglutinans* sensu stricto and *F. verticillioides*. *F. verticillioides* strain 7600 (Sequence ID: NC_031676.1) was used as an outgroup. The scale bar represents the number of substitutions per site

and Azevedo (2002) in the analysis of *F. verticillioides* population obtained from corn in Brazil.

The discriminatory power of RAPD markers used in this study was determined using various parameters. The highest efficiency was demonstrated by the primers

OPAA 09 and OPAD 12, which were characterized by the highest parameters' values: the marker index of 2.75 for both primers, the effective multiplex ratio (10.70; 9.92) and the percentage of polymorphic loci of 62.1% and 68.3%, respectively.

Table 9 Sequence diversity of the translation elongation factor 1- α and β -tubulin gene among *F. temperatum* and *F. subglutinans* sensu stricto strains

Locus	Species	bp	n	GC%	h	s	π	Θ	D
β -tubulin		721	25	55.3	4	6	0.00291	0.00222	0.92422
	<i>F.subglutinans</i>		9	55.8	2	1	0.00031	0.00051	-1.08823
	<i>F.temperatum</i>		16	55.2	2	1	0.00017	0.00041	-1.16221
EF 1- α		582	25	51.6	7	20	0.01474	0.01017	1.62650
	<i>F.subglutinans</i>		9	51.4	3	10	0.00713	0.00705	0.05422
	<i>F.temperatum</i>		16	51.8	4	6	0.00383	0.00317	0.71015
Concatenated sequences		1331	25	53.7	9	26	0.00790	0.00557	1.54449
	<i>F.subglutinans</i>		9	54.0	4	11	0.00317	0.00326	-0.11945
	<i>F.temperatum</i>		16	53.7	5	7	0.00176	0.00161	0.33201

bp - sequence length in base pairs; n - number of sequences; GC% - GC content; s - number of segregating sites; h - number of haplotypes; π - nucleotide diversity; Θ - estimate of Theta per site; D - Tajima's test statistic

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human participants and/or animals This work does not contain any studies with human participants or animals performed by any of the authors.

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