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GENETIC POLYMORPHISM OF *FUSARIUM CULMORUM* ISOLATES ORIGINATING FROM ROOTS AND STEM BASES OF BARLEY

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Abstract: *Fusarium culmorum* is an etiologic agent of barley foot rot. The identification and variability evaluation of *F. culmorum* isolates, originating from roots and stem bases of spring barley, was carried out using molecular methods. Species-specific SCAR primers were successfully applied to identify *F. culmorum* isolates from northern and south-eastern Poland. To determine DNA polymorphism on intraspecies level RAPD technique was used. Twenty three RAPD markers revealed DNA polymorphism suitable to assess genetic variation among isolates examined. Cluster analysis of RAPD data identified a few groups of isolates. In some cases grouping of isolates was correlated with their geographic origin.

Key words: barley, *Fusarium culmorum*, molecular markers, stem base disease, PCR

INTRODUCTION

Fusarium spp. can cause seedling damage and diseases of roots, stem bases, heads and grain (Baturo 2007). Stem base disease complex of barley comprises three main diseases: eyespot, sharp eyespot and brown foot rot. In temperate regions *Fusarium culmorum* (W.G. Smith) Sacc. is one of the most abundant and aggressive pathogen. In addition to causing the disease, *F. culmorum* is a producer of mycotoxins such as highly toxic trichothecenes (Bottalico and Perrone 2002). Deoxynivalenol (DON) and its acetylated derivatives 3- or 15-acetyldeoxynivalenol (3-ADON or 15-ADON, re-

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spectively), nivalenol (NIV) and and fusarenone X (FUS) are produced by certain isolates of *F. culmorum* (Nicolaisen *et al.* 2005; Logrieco *et al.* 2003).

Visual assessment of infected plants is often insufficient to diagnose the species of causal agent of the disease, particularly when different organisms can induce similar symptoms. Mixed infection of several *Fusarium* species and other root rot pathogens occurs frequently. Considerable expertise is required to differentiate morphologically *F. culmorum* from other *Fusarium* spp. because their traits exhibit variation on a continuous scale that may overlap between species (Schilling *et al.* 1996).

Because *Fusarium* species infecting barley differ in pathogenicity, fungicide sensitivity and their ability to produce mycotoxins, correct identification and detailed characterization of pathogens is very important (Nicholson 2004). As a consequence, many techniques based on PCR have been developed. They are based on anonymous fragments and on rDNA sequence including internal transcribed spacers – ITS (Kulik *et al.* 2004; Möller *et al.* 1999; Nicholson *et al.* 1998; Schilling *et al.* 1996) or on structural genes (Chandler *et al.* 2003; Jurado *et al.* 2005; Kerényi *et al.* 2004; Nicolaisen *et al.* 2005). Molecular analysis is independent from environmental conditions (e.g. light, temperature), medium contents and the developmental stage of organism examined.

Description of the genetic variability level is also important because pathogen populations with high genetic variation are potentially capable of rapidly evolving responses to changing environmental conditions (McDonald *et al*. 1994). One of the common methods for detecting intraspecies polymorphism is random amplified polymorphic DNA technique (Williams *et al.* 1990). This technique has been applied to a wide range of organisms including *Fusarium* spp. (Amoah *et al.* 1995; Chełkowski *et al.* 1999; Khalil *et al.* 2003; Ouellet and Seifert 1993; Schilling *et al.* 1996).

The present study was undertaken to identify and assess the differentiation of *F. culmorum* isolates originating from several varieties of spring barley using molecular methods. The principal aims of this study were to evaluate the impact of geographic origin and host origin on *F. culmorum* intraspecies variability.

MATERIALS AND METHODS

Fungal isolates and preparation of single spore cultures

Isolates were obtained in 2005–2006, in the North and South-East of Poland from 14 spring barley cultivars. *F. culmorum* was isolated from roots and stem bases of barley displaying disease symptoms (Table 1). Small plant tissue fragments were washed for 15 minutes in tap water, surface-sterilised in 1% sodium hypochlorite for 2 minutes, washed three times in sterile water and plated onto PDA medium, pH 5.5. All isolates, after 7 days of incubation in 23°C, were first transferred to test-tubes with PDA medium, next on Petri dishes with PDA or SNA medium and identified according to mycological keys (Leslie and Summerell 2006; Kwaśna *et al.* 1991). To prepare single spore cultures all isolates were transferred to Petri dishes with SNA medium and incubated at 23°C for 10 days. A very small fragment of sporulating mycelium was smeared onto 2% water agar. Sixteen hours later, single, germinated conidia were transferred into test-tubes with PDA medium.

Table 1. The origin of *Fusarium culmorum* isolates

Isolate code	Isolates' origin				Year of isolation
	Part of host plants	Barley variety	Field localization	Region	
Fc 0503	stem base	Justina	Chrząstowo	Kujavia-Pomerania	2005
Fc 0505	root	„	Chrząstowo	„	2005
Fc 0611	stem base	„	Chrząstowo	„	2006
Fc 0613	root	„	Chrząstowo	„	2006
Fc 0501	stem base	Orthega	Kończewice	„	2005
Fc 0502	root	„	Kończewice	„	2005
Fc 0508	stem base	Nadek	Kończewice	„	2005
Fc 0509	root	„	Kończewice	„	2005
Fc 0647	stem base	Mauritia	Kończewice	„	2006
Fc 0648	root	„	Kończewice	„	2006
Fc 0524	stem base	Widawa	Kończewice	„	2005
Fc 0526	stem base	Bolina	Kończewice	„	2005
Fc 0528	stem base	Tolar	Kończewice	„	2005
Fc 0519	stem base	Lailla	Kończewice	„	2005
Fc 0605	stem base	Antek	Sobiejuchy	„	2006
Fc 0607	root	„	Sobiejuchy	„	2006
Fc 0602	stem base	Johan	Sobiejuchy	„	2006
Fc 0603	root	„	Sobiejuchy	„	2006
Fc 0609	stem base	Prosa	Sobiejuchy	„	2006
Fc 0610	root	„	Sobiejuchy	„	2006
Fc 0637	stem base	Start	Gruczno	„	2006
Fc 0639	root	„	Gruczno	„	2006
Fc 0642	stem base	Stratus	Lisewo	Pomerania	2006
Fc 0651	stem base	Justina	Bałcyny	Varmia and Mazuria	2006
Fc 0650	stem base	„	Bałcyny	„	2006
Fc 0615	stem base	„	Osiny k/Puław	Lublin Region	2006
Fc 0616	root	„	Osiny k/Puław	„	2006
Fc 0523	stem base	„	Osiny k/Puław	„	2005
Fc 0517	stem base	Refren	Osiny k/Puław	„	2005
Fc 0516	root	„	Osiny k/Puław	„	2005
Fc 0624	stem base	„	Osiny k/Puław	„	2006
Fc 0626	root	„	Osiny k/Puław	„	2006
Fc 0628	stem base	„	Osiny k/Puław	„	2006
Fc 0629	root	„	Osiny k/Puław	„	2006
Fc 0521	stem base	„	Osiny k/Puław	„	2005

DNA preparations

Mycelia from 6-day-old cultures, grown on liquid medium (5 g/l of glucose, 1 g/l of yeast extract), were collected by vacuum filtration using a Büchner funnel. DNA was extracted and purified using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's recommendations with slight modification.

Species-specific PCR and RAPD assays

Two species-specific primers *Fc*_for: 5'- GATGCCAGACCAAGACGAAG-3' and *Fc*_rev: GATGCCAGACGCACTAAGAT-3' (Schilling *et al.* 1996) (Sigma-Genosys, Pampisford, UK) were used for molecular identification of *F. culmorum*. The amplification techniques were carried out using a *Taq* PCR Core Kit (QIAGEN, Inc., Valencia, USA) in a total volume of 5 µl. The reaction mixture was prepared according to Irzykowska *et al.* (2005a). Amplification was carried out in a Biometra T*personal* 48 thermocycler (Whatman Biometra, Goettingen, Germany) using the following programme: initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min and extension at 72°C for 2 min. The amplification was ended with an additional extension at 72°C for 5 minutes.

The RAPD-PCR reactions were carried out using a *Taq* PCR Core Kit (QIAGEN, Inc., Hilden, Germany) as described before (Irzykowska *et al.* 2005b). Twenty random 10-mer primers: OPC 01-10 and OPJ 05-15 (Qiagen Operon, Cologne, Germany) were used to screen the isolates for polymorphism. Amplification was carried out in a Biometra T*personal* 48 thermocycler (Whatman Biometra, Goettingen, Germany) using the following program: initial denaturation for 2 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 sec, primer annealing at 37°C for 1 min and extension at 72°C for 2 min. The amplification was ended with an additional extension at 72°C for 5 min. PCR was repeated twice to check reproducibility.

Electrophoresis conditions

The PCR products were separated by electrophoresis (4 V/cm) in 1.5% agarose gels with 1x TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.0) and visualised under UV light following ethidium bromide staining. A Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas GMBH, St. Leon-Rot, Germany) was used as a molecular size standard for PCR products.

Statistical analysis

Polymorphic bands were scored and analyzed by Treecon for Windows version 1.3b software (Van de Peer and de Wachter 1994). The coefficients of genetic similarity (S) of the investigated isolates were calculated using the following formula (Nei and Li 1979):

$$S_{ij} = 2N_{ij}/(N_i+N_j)$$

where N_{ij} is the number of alleles present at *i*-th and *j*-th isolates, N_i – the number of alleles present at the *i*-th isolate, N_j – the number of alleles present at the *j*-th isolate, *i*, *j* = 1, 2, …, 35. Basing on calculated coefficients isolates were grouped hierarchically using the unweighted pair group method of arithmetic means (UPGMA). The relationship among isolates was presented in the form of a dendrogram.

RESULTS

Molecular identification of *F. culmorum* isolates

The mycological identification of *F. culmorum* isolates was verified by qualitative molecular analysis. The *Fc*_for and *Fc*_rev species-specific primers used in polymerase chain reaction were designed after specific RAPD fragment sequencing.

The reaction conditions were slightly modified from original protocol described by Schilling *et al*. (1996) Annealing temperature was enhanced to 56°C to increase reaction specificity and reaction mixture volume was decreased from 50 µl (in original protocol) to 5 µl to reduce analysis cost. The suitability of these primers for identification of *F. culmorum* originating from different varieties of spring barley was confirmed. A 472 bp SCAR marker was amplified in specific PCR for each *F. culmorum* isolate examined, confirming mycological diagnostics (Fig. 1).

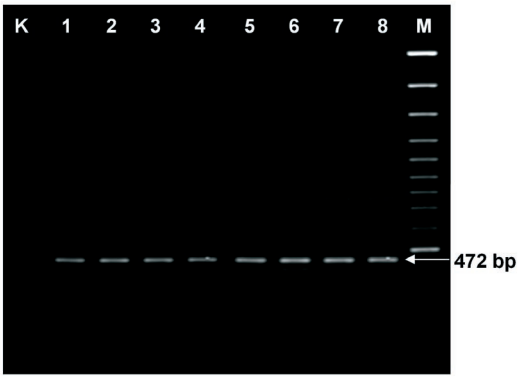

Fig. 1. Species-specific PCR of DNA from *Fusarium culmorum* cultures. Lanes 1–4 isolates from roots, 5–8 isolates from stem bases, Lane K – negative control, Lane M – a Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas)

Intraspecies genetic variability

To assess genetic variation of *F. culmorum* isolates twenty arbitrary primers were tested. Amplification fingerprints of some of them were monomorphic or not reproducible, so they were excluded from further analysis. Finally eight primers generating stable and polymorphic banding patterns were chosen. Twenty-three polymorphic DNA fragments were obtained, from 2 with a few primers to 5 with OPJ-05 primer and on the average, almost 3 polymorphic amplicons per primers (Table 2). The size of PCR products ranged from 0.2 to 3.0 kb (Fig. 2). The comparison of each band profile for each primer was done on the basis of the presence (1) *versus* absence (0) of RAPD products of the same length. Each band was assumed to represent a single genetic locus. The RAPD data were used for grouping isolates by the UPGMA method and to prepare the dendrogram representing genetic variability of *F. culmorum* isolates (Fig. 3). Cluster analysis resolved two groups at the 57% similarity level. The highest genetic similarity was revealed between the isolates Fc 0611 and Fc 0613 (both isolated in Chrząstowo), and also Fc 0528 and Fc 0642. In some cases clusters on the dendrogram corresponded with geographical origin of isolates.

Table 2. Description of RAPD primers

Primer code	Sequence 5′ to 3′	Number of polymorphic bands
OPC-05	GATGACCGCC	2
OPC-06	GAACGGACTC	2
OPC-07	GTCCCGACGA	4
OPC-08	TGGACCGGTG	3
OPC-09	CTCACCGTCC	2
OPJ-05	CTCCATGGGG	5
OPJ-13	CCACACTACC	3
OPJ-14	CACCCGGATG	2
	Total	23

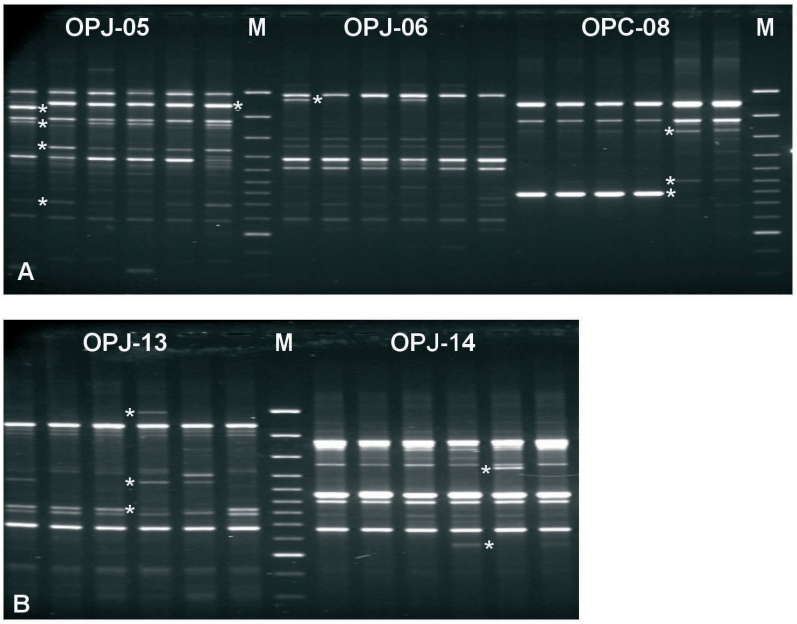

Fig. 2. Examples of RAPD-PCR patterns of *Fusarium culmorum* isolates amplified with primers: (A) OPJ-05, OPJ-06, OPC-08 and (B) OPJ-13, OPJ-14. Polymorphic bands are marked with asterisks. Lane M – a Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas)

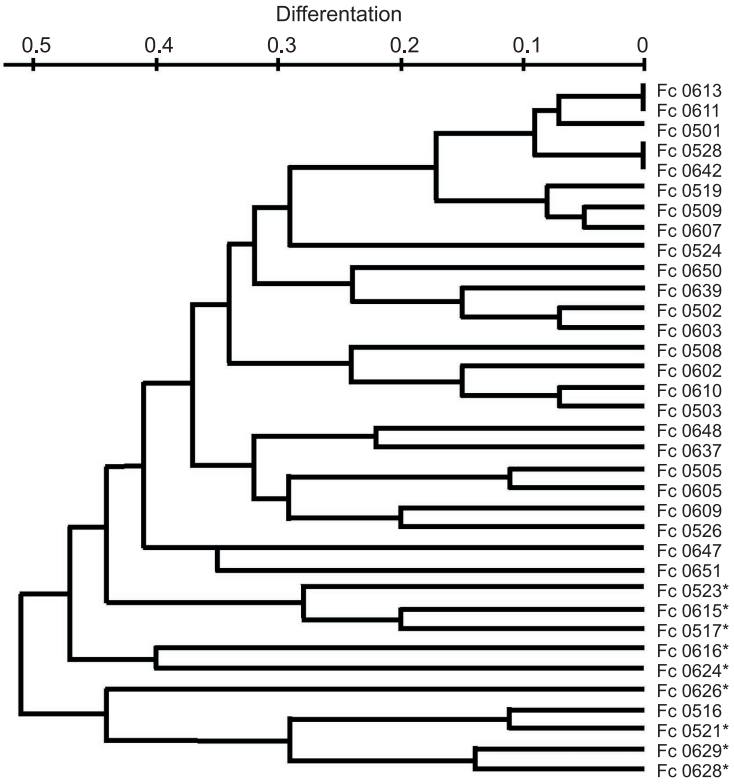

Fig. 3. Dendrogram of *Fusarium culmorum* isolates based on random amplified polymorphic DNA data analysis. The isolates were grouped hierarchically using the unweighted pair group method of arithmetic means (UPGMA). Isolates with asterisks originated from Lublin Region

DISCUSSION

Molecular diagnostics offer a sensitive means of pathogen identification and characterization by analysis of many subtle differences in DNA sequence. Molecular markers application allows avoiding many doubts, which appear during research based on classical methods. Markers are very useful especially in case of fungi such as *F. culmorum,* which never undergo sexual reproduction what narrows down the set of traits available for study (Irzykowska 2006). Two broad approaches were adapted for assays to detect individual fungal species. One of them was based upon polymorphisms within unknown DNA regions. *Fc*_for and *Fc*_rev primers used in this study were obtained after cloning and sequencing RAPD fragment characteristic for a particular species. However, in Schilling *et al.* (1996) study PCR product was not amplified with these primers in case of four *F. culmorum* isolates. In our study specific 472 bp fragment was obtained for all analyzed isolates. Such result demonstrates that analyzed isolates from Poland did not possess genomic alterations in the primer annealing sites and the length of genomic region tagged by the SCAR primers has not been changed by insertion or deletion events.

Genetic variability between 35 isolates examined was also determined using RAPD technique. RAPD is an effective and powerful technique for determining intraspecies genetic variation. This technique has the capacity to generate markers that span the genome without prior knowledge of their sequence. RAPD has been used to analyse genetic variation of several *Fusarium* species, including *F. graminearum* (Ouellet and Seifert 1993), *F. oxysporum*, *F. solani*, *F. moniliforme*, *F. avenaceum* and *F. chlamydosporum* (Khalil *et al.* 2003). Moreover, RAPD technique was applied to determine mating types of several members of the *Fusarium* section *Liseola* (Amoah *et al.* 1995).

In the present study, results obtained from using RAPD technique suggest a medium level of genetic variation among the 35 *F. culmorum* isolates. It is well known that a very high level of intraspecific genetic diversity is typical for species undergoing sexual cycle (Kerényi *et al.* 2004). Meiotic recombination can generate and maintain genotypic variation and results in the reassortment of genes. Cluster analysis was based on UPGMA, which has been used previously with different fungal species (Irzykowska 2007; Peltonen *et al.* 1996; Sun *et al.* 2003; Weber *et al.* 2005). DNA sequences of the most unlike isolates differ at the level of 50–60% in analyzed genome parts. Isolates originating from roots were slightly less polymorphic than those from stem bases. Grouping of isolates did not correlate with a variety of host.

Moreover, the majority of isolates originating from regions of south-eastern part of Poland (Lublin Region) with drier climate were genetically different than those from areas with high relative humidity (Varmia and Mazuria, Kujavia-Pomerania and Pomerania). Possible explanation of the genetic variation is different selection pressure in a region with different climate. The higher incidence of *F. culmorum* when weather conditions were dry was described by Logrieco *et al.* (2003). Assigbetse *et al.* (1994) also reported a correlation between genetic variation and geographic origin of *Fusarium* isolates. According to Edwards *et al.* (2002) *F. culmorum* is more dominant in cooler regions and *F. graminearum* tends to be more dominant in warmer regions. Brennan *et al.* (2005) claimed that climate change can potentially alter the physiology and morphology of both the host and pathogen and is therefore recognized as a serious global environmental problem. At present, likely losses from brown foot rot as a result of change in climate cannot be predicted due to the lack of deep understanding of the influence of temperature and humidity on pathogen biology. Presented results indicate that more extensive studies are necessary to clarify the impact of climate on genetic variability of *Fusarium culmorum*.

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POLISH SUMMARY

GENETYCZNE ZRÓŻNICOWANIE IZOLATÓW *FUSARIUM CULMORUM* POCHODZĄCYCH Z KORZENI ORAZ PODSTAWY ŹDŹBŁA JĘCZMIENIA

Fusarium culmorum jest czynnikiem etiologicznym fuzaryjnej zgorzeli podstawy źdźbła i korzeni jęczmienia. Posługując się metodami molekularnymi przeprowadzono identyfikację i ocenę zróżnicowania izolatów *F. culmorum* pochodzących z korzeni i podstawy źdźbła jęczmienia jarego. Gatunkowo-specyficzne startery SCAR z powodzeniem zastosowano do identyfikacji izolatów *F. culmorum* z północnej i południowo-wschodniej Polski. W celu określenia polimorfizmu DNA na poziomie wewnątrzgatunkowym zastosowano technikę RAPD. Dwadzieścia trzy markery RAPD ujawniły polimorfizm wystarczający do oceny genetycznego zróżnicowania badanych izolatów. Za pomocą analizy klasterów danych RAPD wyznaczono kilka grup izolatów. W niektórych przypadkach grupowanie izolatów było związane z ich geograficznym pochodzeniem.