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MOLECULAR IDENTIFICATION OF MATING TYPE GENES IN ASEXUALLY REPRODUCING *FUSARIUM OXYSPORUM* AND *F. CULMORUM*

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Abstract: Sexually (homothallic and heterothallic) and asexually reproducing species belong to the *Fusarium* genus. So far, there is no known sexual stage of the *F. oxysporum* Schlechtend.: Fr. and *F. culmorum* (W.G. Smith) Sacc. Knowing the reproduction mode is important for the design of successful control strategies, since they are different for clonally and sexually reproducing organisms. In examined sets of asexual *F. oxysporum* and *F. culmorum* isolates, the DNA sequences of mating type genes (idiomorphs *MAT-1* and *MAT-2*) were identified. *MAT-1* sequence was detected for 33 and 40% of *F. oxysporum* and *F. culmorum* isolates, respectively. For the remaining isolates a sequence specific for *MAT-2* was amplified.

Key words: *Fusarium culmorum*, *F. oxysporum*, mating types, *MAT*, PCR

INTRODUCTION

Sexually (homothallic and heterothallic) and vegetative reproducing species belong to the *Fusarium* genus. Many of them are severe plant pathogens. *Fusarium oxysporum* Schlechtend.: Fr. is known as the toxigenic fungus causing stem and crown rot of asparagus and producing moniliformin, and in some cases fumonisins (Waskiewicz *et al.* 2009; 2010). *Fusarium culmorum* (W.G. Smith) Sacc. is an abundant and aggressive pathogen causing brown foot rot of cereals in temperate regions. Moreover, *F. culmorum* is able to biosynthesise highly toxic trichothecenes (Logrieco *et al.* 2003; Nicolaisen *et al.* 2005).

So far, a sexual stage of the *F. oxysporum* and *F. culmorum* is not known, although their intraspecies variability is common (Irzykowska and Baturo 2008; O'Donnell *et al.* 2009). The probable sources of genetic variation are still discussed (Kistler 1997; O'Donnell *et al.* 2009). Sex plays important roles in fungi evolution, *i.e.* the development of a new pathogenic race, and of a new strain resistant to a fungicide. However, no sexual stage is known for more than 15,000 species of fungi including the strongly phytopathogenic ones (Arie *et al.* 2000).

In many fungi, mating occurs between morphologically identical partners. Molecular analysis of mating type genes is a powerful tool for the evolutionary investigation of reproductive life styles and species relationships (Christiansen *et al.* 1998). The mating type locus conferring mating behaviour, consists of dissimilar DNA sequences (idiomorphs) in the mating partners of most ascomycetous fungi (Pöggeler 2001). Idiomorphs are highly dissimilar regions of DNA, which may contain from one to three open reading frames, in isolates of opposite mating type (Dyer *et al.* 2001). In heterothallic species, mating type is controlled by a single locus with two idiomorphic alleles, designated *MAT-1* and *MAT-2* (Turgeon and Yoder 2000). Each *MAT* idiomorph carries one gene encoding a single *MAT*-specific DNA binding protein (Karényi *et al.* 1999). These proteins probably play a crucial role in pathways of cell speciation and sexual morphogenesis as regulatory transcription factors. *MAT* alleles contain a conserved alpha (ALPHA) box domain or a high-mobility-group (*HMG*) box domain, respectively (Yun *et al.* 2000). Homologues of mating type genes have also been found in the genome of homothallic species, where beside their function in the fertilization process, another role is the establishment of nuclear identity (Pöggeler 2001). Each homothallic species carries both *MAT-1* and *MAT-2* genes, organized in a species-specific manner, usually closely linked. In contrast, the basidiomycetous fungi display a multiple mating system (Kothe 2001).

Knowing the reproduction mode is important for the design of successful control strategies, since these strategies are different for clonally and sexually reproducing organisms (McDonald and McDermott 1993). Comparative molecular evolutionary analyses indicate that evolution of *MAT* genes is dominated by strong purifying selection and show that *MAT* is functionally constrained, even in species for which a sexual state is unknown (O'Donnell *et al.* 2004). The present study is focused on molecular detection of the *MAT* idiomorphs in *F. oxysporum* as well

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F. culmorum genomes. Verification of a hypothesis about the possibility of mating is also a part of our study.

MATERIALS AND METHODS

Fungal isolates

Thirty single-spore cultures each of *F. oxysporum* and *F. culmorum* isolates derived in the west of Poland were used in the experiment. Isolates of *F. oxysporum* originated from white and green asparagus spears with visible disease symptoms. Isolates of *F. culmorum* were derived from roots of wheat and rye displaying brown foot rot symptoms. All fungal isolates were identified on the basis of their morphology (Booth 1971; Gerlach and Nirenberg 1982; Kwasna *et al*. 1991).

DNA preparations and species-specific polymeraze chain reaction (PCR) assays

PCRs with species-specific primers were used to confirm the identification of *Fusarium* spp. based on morphological features. Mycelia from 6-day-old single spore cultures of *F. oxysporum* and *F. culmorum* grown on liquid medium (5 g/l of glucose, 1 g/l of yeast extract), were collected by vacuum filtration with a Büchner funnel. DNA was extracted and purified using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's recommendations. In the polymerase chain reaction, a forward primer: 5′-CAGCAAAGCATCAGACCACTATAACTC-3′ and a reverse primer: 5′-CTTGTCAGTAACTGGACGTTGGTACT-3′ (Mulè *et al.* 2004a, 2004b) were used to detect *F. oxysporum*, while *Fc*_forward: 5-GATGCCAGACCAAGACGAAG-3′ and *Fc*_reverse: 5′-GATGCCAGACGCACTAAGAT-3′ were used for *F. culmorum* (Schilling *et al.* 1996) (Sigma-Genosys, Pampisford, UK). The amplification reactions were carried out using a *Taq* PCR Core Kit (QIAGEN, Inc., Hilden, Germany). The reaction mixtures and amplification conditions were described earlier (Irzykowska and Bocianowski 2008; Waskiewicz *et al.* 2010). Amplification was carried out in a Biometra T*personal* 48 thermocycler (Whatman Biometra, Goettingen, Germany).

Diagnostic PCR for mating type

To identify the mating type genes in the genomes of *F. oxysporum* and *F. culmorum* isolates, conserved portions of the ALPHA or HMG box of the *MAT-1* and *MAT-2* idiomorph were amplified with degenerate oligonucleotide primer pairs: a forward (ALPHA): 5-CGCCCTCTKAAYGSCTTCATG-3′ and a reverse (ALPHA): 5-GGARTARACYTTAGCAATYAGGGC-3′ for *MAT-1*, and a forward (HMG): 5-CGACCTCCCAAYGCYTACAT-3′ and a reverse (HMG): 5-TGGGCGGTACTGGTARTCRGG-3′ for *MAT-2* (Kerényi *et al.* 2004). The PCRs were carried out using a *Taq* PCR Core Kit (QIAGEN, Inc., Hilden, Germany) with reaction mixture containing: 5 ng of fungal DNA, 0.2 mM of each dNTP, 0.5 μM of each primer and 0.5 U of *Taq* DNA polymerase in 1′reaction buffer with 2.5 mM magnesium chloride. For amplification, an initial denaturation was done at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C (*MAT-1*) or 58°C (*MAT-2*) for 45 s and extension at 72°C for 2 min. The amplification ended with an additional extension at 72°C for 5 min.

The electrophoresis conditions

The PCR products were separated by electrophoresis (4 V/cm) in 1.5% agarose gels with 1 x TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.0) and visualised under ultraviolet 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.

RESULTS

Molecular identification of *F. oxysporum* and *F. culmorum* isolates

Among sixty examined isolates, one half of the set displayed morphology typical for *F. oxysporum*, while the remaining 30 isolates were identified as *F. culmorum*. Mycological identification of fungal isolates was verified by qualitative molecular analysis. PCR products obtained for *F. oxysporum* and *F. culmorum* were the 534 and 472 base pair, respectively (Fig. 1A, B). Identification based on PCR with species-specific primers was 100% consistent with morphological identification of isolates. No one isolate failed to show a specific band. Such results confirm that examined isolates from Poland did not possess genomic alterations in the primer annealing sites, and that length of genomic regions tagged by earlier described primers has not changed in the way detectable on agarose gel.

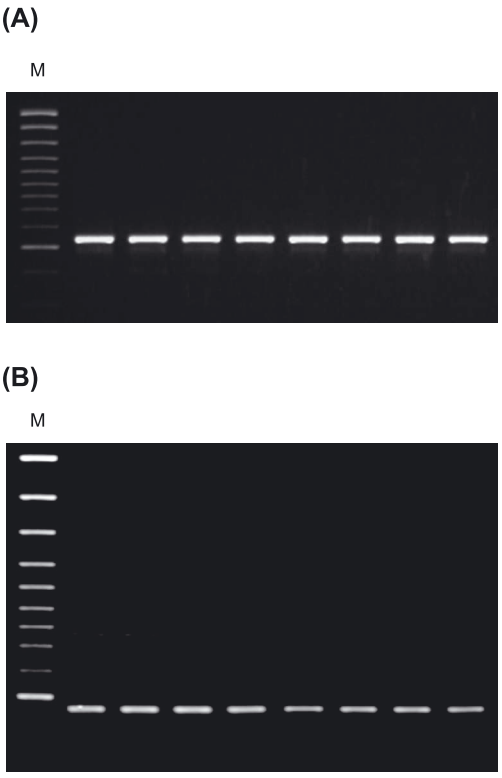
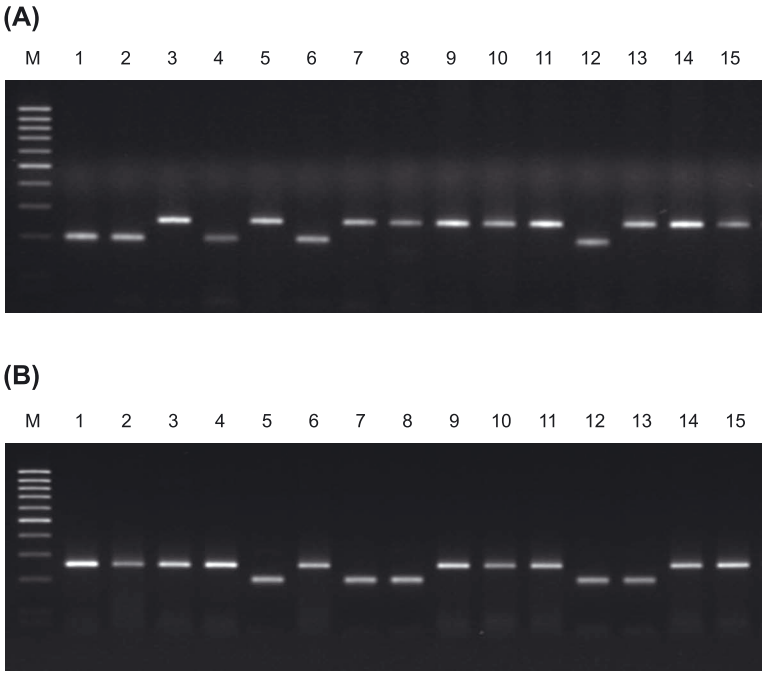


Fig. 1. Examples of species-specific PCR products for (A) *F. oxysporum* (534 bp) and (B) *F. culmorum* (472 bp). Lane M – Gene Ruler ™ 100 bp DNA Ladder Plus (Fermentas)

Mating type assessment by PCR

In examined sets of asexual *F. oxysporum* and *F. culmorum* isolates, DNA sequences encoding mating type genes were detected. Isolates for which a 200 base pair fragment was amplified with ALPHA primers, were designated as the *MAT-1* type. The remaining isolates, for which a 260 base pair PCR product was amplified with HMG primer pairs, were designated *MAT-2* (Fig. 2 A, B).



(A)
Lanes 1, 2, 4, 6, 12 – amplicon of *MAT-1* specific ALPHA box (200 bp)
Lanes 3, 5, 7–11, 13–15 – amplicon of *MAT-2* specific HMG box (260 bp)
Lane M – a Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas)

(B)
Lanes 5, 7, 8, 12, 13 – amplicon of *MAT-1* specific ALPHA box (200 bp)
Lanes 1–4, 6, 9–11, 14, 15 – amplicon of *MAT-2* specific HMG box (260 bp)
Lane M – a Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas)

Fig. 2. Examples of PCR amplification of mating-type specific sequences from *F. oxysporum* (A) and *F. culmorum* (B)

Table 1. Origin and mating type of *F. oxysporum* and *F. culmorum* isolates

F. culmorum			*F. oxysporum*		
isolate	host (cereals)	mating type	isolate	host (asparagus)	mating type
1	2	3	4	5	6
Fc-1	wheat	*MAT-2*	Fox-1	white spear	*MAT-1*
Fc-2	wheat	*MAT-2*	Fox-2	green spear	*MAT-1*
Fc-3	rye	*MAT-2*	Fox-3	white spear	*MAT-2*
Fc-4	rye	*MAT-2*	Fox-4	white spear	*MAT-1*
Fc-5	wheat	*MAT-1*	Fox-5	green spear	*MAT-2*
Fc-6	wheat	*MAT-2*	Fox-6	white spear	*MAT-1*
Fc-7	rye	*MAT-1*	Fox-7	green spear	*MAT-2*
Fc-8	rye	*MAT-1*	Fox-8	green spear	*MAT-2*
Fc-9	wheat	*MAT-2*	Fox-9	white spear	*MAT-2*
Fc-10	rye	*MAT-2*	Fox-10	green spear	*MAT-2*
Fc-11	rye	*MAT-2*	Fox-11	white spear	*MAT-2*
Fc-12	rye	*MAT-1*	Fox-12	white spear	*MAT-1*
Fc-13	rye	*MAT-1*	Fox-13	white spear	*MAT-2*
Fc-14	wheat	*MAT-2*	Fox-14	white spear	*MAT-2*

1	2	3	4	5	6
Fc-15	wheat	*MAT-2*	Fox-15	white spear	*MAT-2*
Fc-16	wheat	*MAT-2*	Fox-16	white spear	*MAT-2*
Fc-17	wheat	*MAT-2*	Fox-17	white spear	*MAT-2*
Fc-18	wheat	*MAT-2*	Fox-18	white spear	*MAT-2*
Fc-19	wheat	*MAT-1*	Fox-19	white spear	*MAT-2*
Fc-20	rye	*MAT-2*	Fox-20	white spear	*MAT-1*
Fc-21	rye	*MAT-1*	Fox-21	white spear	*MAT-2*
Fc-22	wheat	*MAT-1*	Fox-22	white spear	*MAT-1*
Fc-23	rye	*MAT-2*	Fox-23	white spear	*MAT-2*
Fc-24	wheat	*MAT-2*	Fox-24	white spear	*MAT-1*
Fc-25	rye	*MAT-1*	Fox-25	white spear	*MAT-1*
Fc-26	rye	*MAT-2*	Fox-26	white spear	*MAT-2*
Fc-27	wheat	*MAT-1*	Fox-27	white spear	*MAT-2*
Fc-28	wheat	*MAT-1*	Fox-28	white spear	*MAT-1*
Fc-29	rye	*MAT-1*	Fox-29	white spear	*MAT-2*
Fc-30	rye	*MAT-2*	Fox-30	white spear	*MAT-2*

MAT-1 sequence was detected for 33 and 40% of *F. oxysporum* and *F. culmorum* isolates, respectively. There was no correlation between source of isolate and displaying mating type (Table 1). No one isolate failed to show a band with each of the primer pairs (HMG and ALPHA), and no one isolate showed both *MAT-1* and *MAT-2* specific PCR products.

DISCUSSION

Examined isolates of *F. oxysporum* and *F. culmorum* displayed differentiation in the genetic background of mating type. In the genomes of both asexually reproducing species, sequences responsible for mating type inheritance were detected. No one isolate showed both amplicons, which would be distinctive of homothallic species. Similar results were reported for many species from *Fusarium* genus and for *Alternaria alternata* (Fr.) Keissl. (Arie *et al.* 2000; Karényi *et al.* 2004).

The obtained results may suggest that *F. oxysporum* as well as *F. culmorum* have a potentially heterothallic origin. Such a conclusion is in agreement with the hypothesis proposed by Turgeon (1998), that some *Fusarium* species may exhibit a sporadic and cryptic sexual cycle. Taylor *et al.* (1999) also suggested that all fungi with no known sexual stage are originally heterothallic, and most of them should display a sexual reproduction. However, the absence of teleomorphs on the one hand and the source of high intraspecies variability on the other, are still poorly understood.

Previously, it was difficult to determine whether asexual fungi had *MAT* genes because they could not be crossed with each other. Now, the molecular approach makes it possible to detect *MAT* genes also in mitosporic species what is a first step towards learning what causes asexuality. Sequencing of RT-PCR products proved that the *F. oxysporum MAT* genes are expressed (Yun *et al.* 2000). Thus, lack of sexual reproduction must be caused by something other than *MAT* idiomorphs. This may be a result of the functional disorder of the yet unidentified genes that are involved in successful sexual reproduction (Arie *et al.* 2000).

The possibility of mating fungi is a valuable tool for genetic analysis. Molecular discrimination of *MAT-1* and *MAT-2* among *F. oxysporum* as well *F. culmorum* isolates makes recognition of compatible isolates that could be used in future experiments possible, revealing whether they are truly asexual or not. More extensive studies are necessary to assess a hypothetic genetic linkage between particular *MAT* allele and genes controlling physiological features such as pathogenicity, toxicity or resistance to fungicides. Additionally, fast detection of *MAT* alleles will be useful in the future to determine the distribution of *F. culmorum* and *F. oxysporum* isolates displaying different genetic bases of mating.

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