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Deletion of the *MAT-2* mating-type gene during uni-directional mating-type switching in *Ceratocystis*

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Abstract *Ceratocystis eucalypti* is strictly heterothallic, with single ascospore strains representing one of two opposite mating types. Most other *Ceratocystis* species, including *C. virescens*, *C. pinicola*, and *C. fimbriata*, are homothallic. In the homothallic species, the MAT-2 strains are self-fertile, while MAT-1 strains are self-sterile and grow more slowly than MAT-2 strains. The current hypothesis is that self-fertility of MAT-2 strains is due to the deletion of the *MAT*-2 mating-type gene, resulting in the expression of the *MAT*-1 mating type. These mutant MAT-1 strains are able to cross with MAT-2 strains. Part of the *MAT*-2 mating-type gene in *C. eucalypti*, *C. pinicola*, and *C. fimbriata* was amplified using degenerate primers designed from the conserved *MAT*-2 HMG DNA-binding motif. The expected approximately 300-bp PCR products were cloned and sequenced. Specific primers were designed that amplified 210-bp fragments only in MAT-2 isolates of *C. eucalypti*, *C. virescens*, *C. pinicola*, and *C. fimbriata*. These fragments were present in self-fertile field isolates and self-fertile progeny but were absent in the self-sterile (MAT-1) progeny from selfings of *C. virescens*, *C. pinicola*, and *C. fimbriata*, thus supporting the hypothesis that the *MAT*-2 mating-type gene is deleted during uni-directional mating-type switching. A Southern-blot analysis was performed to confirm the deletion of *MAT*-2 gene in self-sterile progeny. The DNA sequence data for the *C. eucalypti MAT*-2 mating-type gene was increased to 1371-bp using TAIL-PCR and uneven PCR, representing a portion of the complete *MAT*-2 gene DNA sequence.

Key words Heterothallic · Homothallism · Mating-type switching · Mating-type genes · *MAT*-1 · *MAT*-2 · Gene deletion · *Ceratocystis*

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Introduction

Two different mating-type systems exist amongst species of the ascomycetous genus *Ceratocystis sensu stricto* Ellis & Halsted. A number of species in this genus, such as *Ceratocystis eucalypti* Yuan and Kile (Kile et al. 1996) and *Ceratocystis fagacearum* (Bretz) Hunt (Hepting et al. 1952), are strictly heterothallic, with single-ascospore isolates being one of two opposite mating types (MAT-1 or MAT-2). Most species of *Ceratocystis*, including *Ceratocystis fimbriata* Ellis & Halsted (Andrus and Harter 1933; Olson 1949) and members of the *Ceratocystis coerulescens* (Münch) Bakshi complex, such as *Ceratocystis pinicola* and *Ceratocystis virescens* (Bakshi 1951; Harrington and McNew 1997), are homothallic, with selfings giving rise to self-fertile and self-sterile ascospore progeny. The self-fertile strains behave as MAT-2, and the self-sterile strains as MAT-1, in crosses (Harrington and McNew 1997, 1998). Progeny of a selfing event in these species generally segregate in a 1:1 ratio; half of the progeny are self-fertile (MAT-2), and the other half are slower growing, self-sterile (MAT-1), strains (Harrington and McNew, 1997). This apparent switching of the expression of mating type in *Ceratocystis* is only found in MAT-2 strains and is referred to as uni-directional mating-type switching (Perkins 1987; Harrington and McNew 1997). The

MAT-1 strains remain self-sterile, so the switching event is not reversible (Harrington and McNew 1997), in contrast to bi-directional mating-type switching in *Saccharomyces cerevisiae* (Hicks et al. 1979).

Self-sterile (MAT-1) strains of *Ceratocystis pinicola* Harrington and Wingfield (1998), earlier known as *C. coerulescens*, have slower growth than self-fertile (MAT-2) strains. It has been suggested that all strains carry the *MAT*-1 mating-type gene and that the *MAT*-2 mating-type gene is deleted during mating-type switching (Harrington and McNew 1997). This deletion may result in the deletion or loss of expression of more than a single gene, which might explain the slower growth of the self-sterile (MAT-1) strains.

A detailed understanding of mating-type genes in ascomycetes has been mainly restricted to two yeast species, namely *S. cerevisiae* (Herskowitz 1988, 1989) and *Schizosaccharomyces pombe* (Kelly et al. 1998), and three genera of filamentous asocmycetes, i.e. *Neurospora* (Glass et al. 1990; Staben and Yanofsky 1990), *Podospora* (Debuchy and Coppin 1992; Debuchy et al. 1993) and *Cochliobolus* (Turgeon et al. 1993; Yun et al. 1999). The mating-type loci from the discomycete *Pyrenopeziza brassiciae* has also recently been cloned and sequenced (Singh and Ashby 1998).

Turgeon et al. (1995) reported that the *Cochliobolus heterostrophus MAT*-2 idiomorph encodes for a mating-specific DNA-binding protein, which includes a high mobility group (HMG) DNA-binding site, similar to HMG regions found in *Neurospora crassa mt a*-1 (Staben and Yanofsky, 1990) and *Podospora anserina* FPR1 (Debuchy and Coppin 1992). These conserved amino-acid sequences have been used in the design of degenerate primers for the amplification of the *MAT*-2 HMG box in pyrenomycetes and in loculoascomycetes (Arie et al. 1997). Using these degenerate primers, we amplified part of the *MAT*-2 mating-type gene from selected species of *Ceratocystis* and tested whether the *MAT*-2 mating-type gene is deleted during uni-directional mating-type switching.

Materials and methods

C. eucalypti strains used in this study included 50 single ascospore progeny resulting from a cross between isolates C639 (MAT-1) and C642 (MAT-2). Twenty progeny each were also analyzed from selfings of *C. virescens* isolate C74 and *C. pinicola* isolate C795. Collection information on these isolates was reported earlier (Harrington et al. 1998). The *C. fimbriata* isolates C856 and C1099 are from *Prunus* in California and from *Ipomoea* in Papua New Guinea, respectively. All fungal isolates are maintained in the culture collection of T.C. Harrington. DNA from the isolates studied was extracted according to the method described by DeScenzo and Harrington (1994). Degenerate primers NcHMG1 (5′-CCYCGYCCYCCYAAYGCNTAYAT-3′) and NcHMG2 (5′-CGNGGRTTRTARCGRTARTNRGG-3′), designed for the amplification of the conserved *MAT*-2 HMG box in *N. crassa* and *P. anserina* (Arie et al. 1997), were used in PCR amplification reactions. The PCR reactions were performed as described by Arie et al. (1997) using *Taq* DNA Polymerase (Promega Corporation, USA) or Expand High-Fidelity *Taq* DNA Polymerase (Boehringer Mannheim, Germany), with primer annealing at 55 °C. The PCR products were separated on 2% agarose gels, stained using ethidium bromide, and visualized under UV light. The expected approximately 300-bp (base pairs) PCR products were extracted from gel slices using the QIAquick Gel Extraction Kit (Qiagen Inc., USA) or Geneclean II (Bio 101, Inc., USA). These products were cloned using the pGEM-T Easy Vector System (Promega Corporation, USA) or the pCR-Script Amp SK(+) Cloning Kit (Stratagene, USA). Both strands of the cloned fragments were sequenced with the M13 reverse and M13 universal primers, or T7I and SP6, using the ABI PRISM 377 DNA Sequencer and Genetic Analyzer (Perkin-Elmer, USA). The *MAT*-2 HMG box sequence data of *C. eucalypti*, *C. pinicola*, and *C. fimbriata* were used to design specific, non-degenerate primers. PCR reactions with the specific primers were performed as described for the degenerate primers (Arie et al. 1997), with primer annealing at 58 °C. The *C. eucalypti MAT*-2 specific primers EUM2-1 (5′-GACATCAAGCCGTCAAGACCG-3′) and EUM2-2 (5′-GTCTTTTTGTATGCTTCGGCC-3′) were tested against field isolates and MAT-1 and MAT-2 single-ascospore progeny of *C. eucalypi* and *C. virescens*. The *C. pinicola MAT*-2 specific primers COER2-1 (5′-GACACCAAGACGTCAAAGCC-3′) and COER2-2 (5′-GCTTTTCTTGTAAGTTTCAGC-3′) were tested against MAT-1 and MAT-2 single-ascospore progeny of *C. pinicola*. The *C. fimbriata* specific primers CFM2-1 (5′-GCTACATTTTGTATCGCAAAGAC-3′) and CFM2-2 (5′-TAGTGGGGATATGTCAACATG-3′) were tested against field isolates and single-ascospore strains of *C. fimbriata*. Genomic DNA from single-ascospore progeny of *C. fimbriata* (C1099) and *C. virescens* (C74) were double-digested with both *Eco*RI and *Pst*I. The resulting restriction products (5 µg) were separated on 1% agarose gels and blotted onto nylon membranes (Hybond-N+, Amersham Pharmacia Biotech, Piscataway, N.J.) per the manufacturer's instructions. The PCR product generated as previously described from the amplification of the *MAT*-2 gene of *C. virescens* or *C. fimbriata* was gel-purified (Geneclean II Kit, Bio 101, Vista, Calif.) and labeled with ^{32}P (RadPrime DNA labeling system, Gibco BRL, Rockville, Md.). Membranes with the digested, genomic DNA were hybridized with the respective probe in modified Church and Gilberts buffer and washed at the highest stringency (0.1 × SSC, 0.1% SDS at 65 °C) per the manufacturer's instructions. The membranes were then exposed to phosphor screens for at least 72 h and scanned using a Storm 840 phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). A lambda *Hin*dIII marker was used to determine the molecular weights of the hybridized fragments (FragmeNT analysis software, Molecular Dynamics, Sunnyvale, Calif.). Thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier 1995) was used for the amplification of the flanking regions of the *MAT*-2 HMG box in *C. eucalypti*. The PCR reaction mixtures and primers for TAIL-PCR were as described by Arie et al. (1996), and the PCR cycling conditions were done as described by Liu and Whittier (1995), using the primers EUM2-1 or EUM2-2 in the primary TAIL-PCR reaction. This PCR reaction was followed by a secondary TAIL-PCR reaction using the primers ETP1 (5′-AATCAGCTAGCATCCGTG-3′) or ETP2 (5′-GAATCTGAAGAGCTATGG-3′). Single PCR products produced by the secondary TAIL-PCR reactions were cloned and sequenced. Further DNA sequencing of the flanking regions of the *MAT*-2 mating-type gene of *C. eucalypti* utilized the uneven PCR technique (Chen and Wu 1997). The primary reactions were performed with the primer ETP1, followed by a secondary reaction with the primer ETP3 (5′-CGATACGACTATCTGGTTGC-3′) and 0.5 µl of the ETP1 primary PCR reaction product as template DNA. The 25 µl primary PCR reaction mixture contained 50 ng of template DNA, 2 units of *Taq* DNA polymerase (Advanced Biotechnologies Ltd., UK), the buffer supplied with the enzyme, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM ETP1 and 2 pg of the OPB12 primer (5′-CCTTGACGCA-3′) (Operon Technologies, Inc., USA). Thermal cycling conditions for the primary and secondary PCR reactions were as described by Chen and Wu (1997). Single bands produced by the secondary uneven-PCR reactions were cloned and sequenced. The DNA sequences from the 3′ conserved flanking region of the *MAT*-1 and

MAT-2 mating-type idiomorphs of *C. eucalypti* were amplified using the primers ETP3 and EUM2-6I (5′-GGAACCAACCATATGGACTG-3′). PCR reaction mixtures were as described by Arie et al. (1997), with the PCR primer annealing performed at 55 °C. The expected 130-bp single PCR fragments were purified and sequenced.

Results and discussion

Degenerate primers designed for the amplification of the *MAT*-2 HMG box in all pyrenomycetes (Arie et al. 1995) were successfully used in the amplification of the expected approximately 300-bp PCR fragment of the *MAT*-2 HMG box in *C. eucalypti* (GenBank accession numbers: AF164170–AF164172), *C. pinicola* (GenBank accession number: AF164193–AF164194), and *C. fimbriata* (GenBank accession number: AF164169). The sequence data were used for the design of specific *MAT*-2 HMG box primers. The *C. eucalypti* primers only produced amplification products in the MAT-2 field isolates of *C. eucalypti*, *C. virescens*, *Chalara neocaledoniae* and *Chalara australis*; and the *C. pinicola*-specific primers amplified the *MAT*-2 region in only *C. pinicola* and *C. douglasii* MAT-2 isolates (Witthuhn et al. 2000). The *C. fimbriata* primers amplified the *MAT*-2 region in only *C. fimbriata* MAT-2 isolates. No *MAT*-2 HMG box amplification products were produced in MAT-1 strains of *Ceratocystis* species.

The specific primers EUM2-1 and EUM2-2, designed for the amplification of the *MAT*-2 HMG box in the strictly heterothallic *C. eucalypti*, amplified the expected 210-bp PCR fragment only in the 25 MAT-2 progeny and not in the 25 MAT-1 progeny of *C. eucalypti*. No silent copies of the *MAT*-2 mating-type gene were detected in *C. eucalypti* MAT-1 strains.

The known DNA sequence of the *MAT*-2 mating-type gene of *C. eucalypti* (Witthuhn et al. 2000) was increased from 280-bp to 1371-bp (GenBank accession number: AF182425) with the use of TAIL-PCR (Liu and Whittier 1995) and uneven-PCR (Chen and Wu 1997) techniques. This part of the *MAT*-2 mating-type gene does not appear to include the 5′ portion of the complete mating specific protein (MAT2-1). The putative translation is 122 amino acids (Fig. 1), from base number 289 to base 725, with a single intron (70-bp in size) between bases 449 to 518. A search of homologous amino acids using the BLAST program (version 2.0, National Centre for Biotechnology Information, United States National Institute of Health, Bethesda, Md.) resulted in the highest homology to the conserved HMG boxes of the *Fusarium oxysporum* MAT-2 protein (47 of 89 amino acids, 52%), the *N. crassa* MTa-1 protein (46 of 101, 44%) and the *P. anserina* FPR1 protein (45 of 90, 50%) (Fig. 1).

The primers (ETP3 and EUM2-6I) designed from the 3′ region of the 1371-bp fragment successfully amplified the expected 130-bp single PCR fragment (base 1143 to base 1250) in both MAT-1 and MAT-2 strains of *C. eucalypti*. These results indicate that the conserved region of the mating-type gene that flanks the 3′ end of *MAT*-2 and *MAT*-1 mating-type genes had been sequenced.

The MAT-2 primers EUM2-1 and EUM2-2 were tested against progeny of a selfing event in the MAT-2 isolate (C74) of *C. virescens*. The expected fragment was only amplified in the parent strain and the ten self-fertile, MAT-2 strains. No amplification product was observed in the ten self-sterile, MAT-1 strains (Fig. 2). Similarly, self-fertile and self-sterile single ascospore progeny were recovered from a selfing of the field isolate C795 of *C. pinicola*. The specific primers (COER2-1 and COER2-2), designed for the amplification of the *MAT*-2 HMG box in *C. pinicola*, only amplified the expected 210-bp products in the ten self-fertile, MAT-2 progeny, with no amplification product observed in the ten self-sterile, MAT-1 progeny. The *C. fimbriata* MAT-2 primers amplified a 210-bp fragment from seven self-fertile progeny, but not from five self-sterile progeny, recovered from a selfing of C1099, and from five self-fertile, but not three self-sterile, progeny of a selfing of isolate C856.

The deletion of the HMG box portion of the *MAT*-2 mating-type gene was confirmed through Southern-blot analyses of progeny of selfings of isolates C1099 and C74. The labeled *MAT*-2 HMG box PCR product from *C. fimbriata* only hybridized to the five self-fertile (MAT-2) progeny of *C. fimbriata* (Fig. 3). The same result was found in the hybridization of five self-fertile progeny of *C. virescens* (data not shown). The band size in the case of the *C. fimbriata* MAT-2 strains was 5.0 kb and the band size was 8.3 kb in *C. virescens* MAT-2 strains. No hybridization product was observed in the MAT-1 progeny from the selfing of either *C. fimbriata* and *C. virescens*.

Only MAT-2 strains of *Ceratocystis* species are capable of undergoing uni-directional mating-type switching (Harrington and McNew 1997; Harrington

Fig. 1 The aligned amino acid sequences of the mating specific proteins containing the HMG DNA-binding domain in *C. eucalypti* (GenBank Accession number: AF182425), *Gibberella fujikuroi* (GenBank Accession number: AF100926), *P. anserina* (GenBank Accession number: S22449) and *N. crassa* (GenBank Accession number: P36981)

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Ceratocystis eucalypti MAT-2 MMVLGDDMFDTLQSKEDEIQPKIPRPPNAYILYRKDRHQAVKTDFPNISNNEISKIL
Gibberella fujikuroi MAT-2   NIPGAPEVIFIHRPEKPAFPAKIPRPPNAYILYRKERHHSIKAQRPDITNNEISQVL
Podospora anserina FPR1      NTMNTTVWDPKKRHVQATAEAKIPRPPNAYILYRKDQQAALKAANPGIPNNDISVMT
Neurospora crassa MTA-1      NLFWDPKGIHASAPKEQQKKAKIPRPPNAYILYRKDHHREIREQNPGLHNNEISVIV

C. eucalypti MAT-2   GKRWREESASIREFYREQAEAYKKTFMEMYPDYRYKPRKASEKKRRRRNITSIALDNEGRSPSTVT
G. fujikuroi MAT-2   GRLWNSETREVRALYKQMEDQKKAEHRRQYPDYQYRPRRPSE-RRRRNNASSDRSTATIAVTQQMT
P. anserina FPR1     GGMWKKESPEVRAEYQRRASEIKAKLMSAHPHYRYVPRRSSE-IRRRAPRRNRAQEVANASPIGEN
N. crassa MTA-1      GNMWRDEQPHIREKYFNMSNEIKTRLLLENPDYRYNPRRSQD-IRRRVSPYLKIKLLNYDVNGNLL
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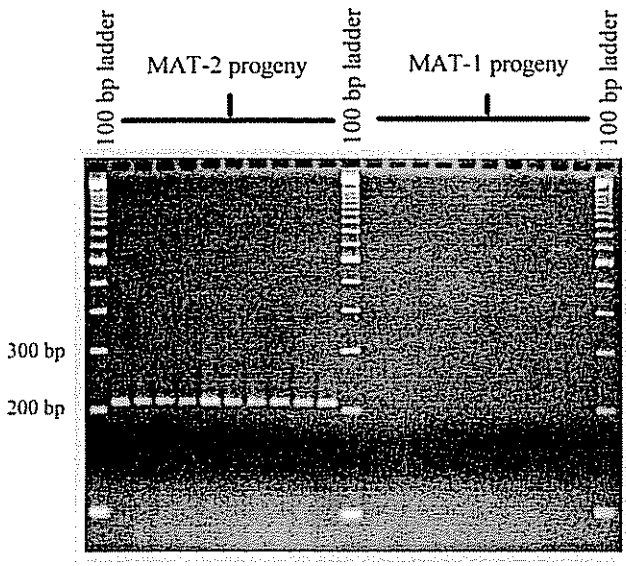

Fig. 2 The PCR amplification products, using the primers EUM2-1 and EUM2-2, of the *MAT*-2 HMG box of progeny of a selfing in *C. virescens*, separated on a 2% agarose gel. *Lanes 1, 12 and 23* 100-bp DNA ladder (Promega Corporation, USA). *Lanes 2–11 C. virescens* self-fertile, MAT-2 progeny. *Lanes 13–22 C. virescens* self-sterile, MAT-1 progeny

et al. 1998), and the *MAT*-2 HMG box was only amplified from self-fertile (MAT-2) isolates of *C. pinicola*, *C. virescens* and *C. fimbriata*. The *MAT*-2 HMG box was amplified from half of the progeny of selfing events in *C. virescens*, *C. pinicola* and *C. fimbriata*, suggesting a Mendelian segregation of the *MAT*-2 HMG box during uni-directional mating-type switching. All the progeny from a selfing should be genetically identical to the MAT-2 parent isolate, but the *MAT*-2 mating-type gene was only amplified in the self-fertile progeny and not in the self-sterile progeny. Absence of the *MAT*-2 HMG box in self-sterile progeny was confirmed by Southern blot analyses. These data suggest that at least a portion of the *MAT*-2 HMG mating-type gene has been deleted from the genome of self-sterile progeny during the selfing event, and the deletion co-segregates with self-sterility and the MAT-1 phenotype. The *MAT*-1 mating-type gene is believed to be present in all isolates of these species but is not expressed in the presence of the *MAT*-2 mating-type gene (Harrington and McNew 1997). However, we do not have *MAT*-1 genetic markers at this time to test this hypothesis.

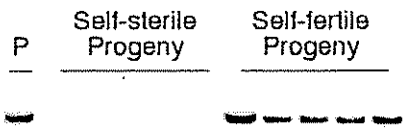

Fig. 3 Southern blot analysis of the self-fertile and self-sterile progeny of a selfing in *C. fimbriata* (C1099). The *MAT*-2 HMG-box PCR product was hybridized against genomic DNA of the parent strain (*P*) and progeny of a selfing event (*self-sterile* and *self-fertile*)

Results from this study provide molecular evidence for the apparent deletion of the *MAT*-2 mating-type gene during uni-directional mating-type switching in species of *Ceratocystis*. It should be noted that ascospores and hyphae of these *Ceratocystis* species are apparently haploid, and the deletion does not involve a transition from the diploid to a haploid state (Harrington and McNew 1997). This is the first report of mating-type switching through gene deletion, and this uni-directional mechanism contrasts sharply, with the bi-directional mating-type system in *Saccharomyces* (Herskowitz 1988, 1989; Hicks et al. 1979) and homothallism without mating-type switching in other ascomycetes (Yun et al. 1999).

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