Both mating types in the heterothallic fungus Ophiostoma quercus contain MAT1-1 and MAT1-2 genes

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ABSTRACT
In heterothallic Ascomycota, two opposite but distinct mating types control all sexual processes. Using mating crosses, mating types were assigned to ten isolates of the heterothallic fungal species Ophiostoma quercus. Primers were subsequently designed to target the MAT1-1-1, MAT1-1-3 (of the mating type 1 idiomorph), and MAT1-2-1 (of the mating type 2 idiomorph) genes in these isolates. Results showed that all isolates contained the full gene sequence for the MAT1-2-1 gene. In addition, fragments of the MAT1-1-1 and MAT1-1-3 genes were sequenced from all isolates. These results were unexpected, as each isolate from a heterothallic species would typically contain only one of the two possible MAT idiomorphs.

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Introduction
Ophiostoma represents a diverse genus in the Ascomycota with a worldwide distribution (Wingfield et al. 1993). Most species have a close association with tree-infesting bark beetles and some cause serious tree diseases (Hausner et al. 1993). This is true of species in the Ophiostoma piceae complex (Chung et al. 2006; De Beer et al. 2003; Harrington et al. 2001; Kamgan et al. 2008; Uzunovic et al. 2000). This complex includes two of the three known Dutch elm disease pathogens (Ophiostoma ulmi and Ophiostoma novo-ulmi) which, as invasive species, have been responsible for the death of millions of Elm trees in the Northern Hemisphere (Brasier 1990). Other species in the O. piceae complex result in blue-stain of timber and they degrade wood quality. For example, Ophiostoma quercus is responsible for significant economic losses due to sapstain in hardwoods (De Beer et al. 2003; Harrington et al. 2001).

Species of Ophiostoma exhibit mating behaviours that range from strict homothallism through to strict heterothallism. For example, the Dutch elm disease pathogens and O. quercus are heterothallic (Brasier 1984; Brasier & Kirk 1993; Harrington et al. 2001; Solla et al. 2008) with sexual reproduction requiring the interaction of two individuals of opposite mating type (Coppin et al. 1997). Moreover mating-type recognition is not
only universal among the Dutch elm disease pathogens (e.g. Brasier & Mehrotra 1995) but also between the Dutch elm disease pathogens and O. quercus. Thus interspecific pairings between opposite mating types of O. novo-ulmi and O. quercus result in the formation of normal as well as abnormal perithecia (but no ascospores), indicating a common mechanism of control (Brasier 1993). Individuals of homothallic species (e.g. Ophiostoma arduennense and Ophiostoma minus) are typically self-fertile and capable of completing the sexual cycle in the absence of a second individual (Carlier et al. 2006; Gorton & Webber 2000; Grobbelaar et al. 2009).

Sexual reproduction in the Ascomycota is controlled by the genes found at a single mating-type locus (MAT-1) (Coppin et al. 1997; Turgeon 1998) with two idiomorph alleles (Metzenberg & Glass 1990). In heterothallic species, individual isolates usually have either the MAT1-1 or MAT1-2 idiomorph, but they have never been found to contain both idiomorphs (Glass & Nelson 1994; Nelson 1996). In homothallic species, the genomes of all individuals harbour genes of both idiomorphs, frequently in different arrangements of the MAT locus (Elliott 1994; Nelson 1996).

Three genes are commonly located at the MAT1-1 idiomorph, MAT1-1-1, MAT1-1-2, and MAT1-1-3 (Coppin et al. 1997; Elliott 1994; Glass & Nelson 1994). Of these, the α-box protein encoding gene, MAT-1-1 (Coppin et al. 1997; Debuchy & Turgeon 2006), was first identified in Saccharomyces cerevisiae (Astell et al. 1981) and has subsequently been identified in all fungal MAT1-1 idiomorphs (Glass et al. 1990; Kanematsu et al. 2007; Li et al. 2010). The MAT1-1-2 gene encodes an amphipathic α-helix protein with a conserved Histidine, Proline, Glycine (HPG) domain (Debuchy & Turgeon 2006), while the MAT1-1-3 gene encodes a protein with a High Mobility Group (HMG) domain (Coppin et al. 1997; Debuchy & Turgeon 2006). Another HMG domain protein, encoded by the MAT1-2-1 gene, is characteristic of the MAT1-2 idiomorph (Arie et al. 1997; Coppin et al. 1997; Nelson 1996). MAT1-2-1 is generally the only gene located on the MAT1-2 idiomorph and has been found in all MAT1-2 idiomorphs that have been characterized (Arie et al. 1997; Coppin et al. 1997; Kanematsu et al. 2007), including those of the Dutch elm disease pathogens (Paoletti et al. 2005).

Recent studies of the MAT genes have revealed their importance in the biology and evolution of fungi (Bennett et al. 2003; Strandberg et al. 2010; Zaffarano et al. 2010). For example, comparisons of MAT DNA sequences in different fungi have improved our understanding of the evolution of homothallic and heterothallic mating strategies (Arie et al. 1997; Bennett et al. 2003; Conde-Ferrazé et al. 2007; Fraser & Heitman 2004; Li et al. 2010; Martin et al. 2011; Steenkamp et al. 2000; Turgeon 1998). Also, the availability of information on the mating idiomorphs allowed for the assessment of the presence of MAT genes in the genome of apparently asexual species (Foster & Fitt 2003; Mandel et al. 2007; Turgeon 1998). At the intra-species level, knowledge regarding the distribution of MAT genes has also shed light on the preferred reproduction mode (i.e. sexual versus asexual) of certain fungal populations (Britz et al. 1998; Linde et al. 2010; Zhan et al. 2002). Such information is particularly important for fungal pathogens, as sexual and asexual reproduction have markedly different effects on the population structures of the pathogens, which in practical situations require different disease management strategies (McDonald & Linde 2002).

Analysis of the distribution of mating types within a population of a heterothallic fungus may be accomplished using either conventional mating studies or DNA-based approaches. Conventional mating tests are laborious and time-consuming as they involve mating all available isolates in every possible combination and subsequent assignment of mating specificities. This traditional approach has been used widely for heterothallic species of Ophiostoma (Brasier & Kirk 1993; De Beer et al. 2003; Grobbelaar et al. 2009; Harrington et al. 2001; Zhou et al. 2004). However, the mating-type designations obtained under laboratory conditions do not always reflect the situation in natural environments (Marra et al. 2004; Marra & Milgroom 2001). Also, not all the individuals examined are necessarily equally fertile under the conditions tested, and this can lead to erroneously assigned mating types. In contrast, DNA-based approaches are relatively inexpensive and have been shown previously to provide reliable mating-type assignments (Cherif et al. 2006; Dyer et al. 2001; Yokoyama et al. 2004). These DNA-based methods are, however, dependent on the availability of sequence information for the MAT locus, because MAT idiomorph-specific PCR assays exploit the inherent differences in the MAT genes (Dyer et al. 2001; Steenkamp et al. 2000).

For Ophiostoma, MAT sequence information is available only for the Dutch elm disease pathogens O. ulmi, O. novo-ulmi, and Ophiostoma himal-ulmi (Jacobi et al. 2010; Paoletti et al. 2005, 2006). Since mating-type recognition occurs between the Dutch elm disease pathogens and also between O. novo-ulmi and O. quercus, the aim of the present study was to attempt to characterise the MAT genes in O. quercus using primers derived from the MAT sequences of O. novo-ulmi.

Materials and methods

Isolates and mating studies

Ten Ophiostoma quercus isolates originating from single spores were used in this study (Table 1). These isolates were obtained from Quercus, Acacia, and Eucalyptus hosts in Africa, Europe and North America. Their mating-type specificities have been determined in previous studies (Brasier & Kirk 1993; De Beer et al. 2003; Kamgan et al. 2008). For routine cultivation of these isolates, malt-extract agar (MEA; 20 g L−1 malt extract [Biolab, Merck], 20 g L−1 agar [Biolab, Merck]) medium and an incubation temperature of 25 °C were used.

To confirm the identity of all isolates used in this study, the ribosomal RNA (rRNA) internal transcribed spacer regions (ITS 1 and 2) and the 5.8S gene were amplified and sequenced using the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). Each 25 μl PCR reaction contained 1 U Roche FastStart Taq mixture and reaction buffer (Roche, Mannheim, Germany), 2.5 mM MgCl2, 0.25 mM of each dNTP, 0.2 mM of each primer and 20–50 ng of template DNA. The latter was prepared for each isolate by scraping mycelium from the surface of 4–6-week-old MFA cultures and subjecting the harvested mycelium to a salt-based DNA extraction method (Aljanabi & Martinez 1997). PCRs were performed on an Eppendorf
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host</th>
<th>Country</th>
<th>Collector</th>
<th>Mating group</th>
<th>ITS</th>
<th>Genbank acc. Nr.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMW 2520</td>
<td>Eucalyptus chips</td>
<td>South Africa</td>
<td>ZW de Beer</td>
<td>A</td>
<td>AF493241&lt;sup&gt;a&lt;/sup&gt;</td>
<td>JQ319599&lt;sup&gt;f&lt;/sup&gt;</td>
<td>FJ865421 (De Beer et al. 2003)</td>
</tr>
<tr>
<td>2521</td>
<td>Eucalyptus chips</td>
<td>South Africa</td>
<td>ZW de Beer</td>
<td>B</td>
<td>FJ441283&lt;sup&gt;e&lt;/sup&gt;</td>
<td>JN225450&lt;sup&gt;i&lt;/sup&gt;</td>
<td>FJ8654420 (De Beer et al. 2003)</td>
</tr>
<tr>
<td>14307</td>
<td>Acacia mearnsii</td>
<td>Uganda</td>
<td>J Roux</td>
<td>B</td>
<td>FJ959044</td>
<td>JQ319600&lt;sup&gt;i&lt;/sup&gt;</td>
<td>FJ865425 (Kamgan et al. 2003)</td>
</tr>
<tr>
<td>17256&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A. mearnsii</td>
<td>Uganda</td>
<td>J Roux</td>
<td>A</td>
<td>FJ959042</td>
<td>JQ319601&lt;sup&gt;i&lt;/sup&gt;</td>
<td>FJ865422 (Kamgan et al. 2008)</td>
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<tr>
<td>17257&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A. mearnsii</td>
<td>Uganda</td>
<td>J Roux</td>
<td>A</td>
<td>FJ959045</td>
<td>JQ319602&lt;sup&gt;i&lt;/sup&gt;</td>
<td>FJ865424 (Kamgan et al. 2008)</td>
</tr>
<tr>
<td>27845</td>
<td>H 2190</td>
<td>Quercus sp.</td>
<td>Canada</td>
<td>A</td>
<td>FJ959043</td>
<td>JQ319603&lt;sup&gt;i&lt;/sup&gt;</td>
<td>FJ865423 (Kamgan et al. 2008)</td>
</tr>
<tr>
<td>27846</td>
<td>H 1039</td>
<td>Quercus sp.</td>
<td>UK</td>
<td>A</td>
<td>JQ319591</td>
<td>JQ319604&lt;sup&gt;i&lt;/sup&gt;</td>
<td>FJ865426 (Brasier &amp; Kirk 1993)</td>
</tr>
<tr>
<td>27847</td>
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<td>Quercus sp.</td>
<td>UK</td>
<td>B</td>
<td>JQ319590</td>
<td>JQ319606&lt;sup&gt;i&lt;/sup&gt;</td>
<td>FJ865413&lt;sup&gt;g&lt;/sup&gt;</td>
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<tr>
<td>27848</td>
<td>H 1042</td>
<td>Quercus sp.</td>
<td>UK</td>
<td>B</td>
<td>EF429089&lt;sup&gt;g&lt;/sup&gt;</td>
<td>JQ319607&lt;sup&gt;i&lt;/sup&gt;</td>
<td>FJ865412&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Accession numbers shown in bold represent sequences produced in this study.

- **a** CMW = Culture collection of the Forestry and Biotechnology Institute (FABI), University of Pretoria, South Africa; CBS = Centraal bureau voor Schimmelcultures, Utrecht, The Netherlands; H = From the collection of Brasier & Kirk (1993).
- **b** All three of these single ascospore isolates were obtained from one isolate, CMW 5826, as tester strains for *O. quercus* (Kamgan et al. 2008).
- **c** Sequences already in NCBI database from previous studies.
- **d** Sequence produced using primer pair 11af/11cr.
- **e** Sequence produced using primer pair Mt1af/R.
- **f** Sequence produced using primer pair Mt3cf/R.
- **g** Sequence produced using primer pair OqMt1F/OqMt1R.

Table 1 – *Ophiostoma quercus* isolates used in this study. Mating groups were arbitrarily assigned to indicate the mating specificity of the 10 isolates.
Table 2 – Primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
<th>Primer binding region in O. novo-ulmi MAT1-1 and MAT1-2 idiomorphs</th>
<th>Region amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td>OqMt1F</td>
<td>TGCCCAAGAAAGGAAAGACTGG</td>
<td>1653 1672&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-1 idiomorph</td>
</tr>
<tr>
<td>OqMt1R</td>
<td>GGTTATGGGAGACAGGAA</td>
<td>1493 1512&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-2 idiomorph</td>
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<tr>
<td>OqMt2</td>
<td>GCACACACCTTGGCCAGGA</td>
<td>119 138&lt;sup&gt;c&lt;/sup&gt;</td>
<td>MAT1-1-1 gene</td>
</tr>
<tr>
<td>Seq9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GGGGATGTAAAAGGAAC</td>
<td>1188 1204&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-1-3 gene</td>
</tr>
<tr>
<td>M1aF</td>
<td>CCGAGCTCTCAGATAATAA</td>
<td>4622 4641&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-2 gene</td>
</tr>
<tr>
<td>M1aR</td>
<td>GAAACTCCCGAGCGGTA</td>
<td>5324 5341&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-1-2 gene</td>
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<tr>
<td>11aF</td>
<td>TCCCTTCTCGTCCCTCT</td>
<td>4817 4834&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-1-1 gene</td>
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<tr>
<td>11cR</td>
<td>CGATGCTTCTGGATTTTG</td>
<td>5076 5095&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-2 gene</td>
</tr>
<tr>
<td>Mt2aF</td>
<td>GAGTCATCTTACCGAAACA</td>
<td>2961 2980&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-1-3 gene</td>
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<tr>
<td>Mt2aR</td>
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<td>2916 2934&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-1-2 gene</td>
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<tr>
<td>Mt2bF</td>
<td>AATGGGATCTATCTCACC</td>
<td>2911 2928&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-1-3 gene</td>
</tr>
<tr>
<td>Mt2bR</td>
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<td>3597 3614&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-1-3 gene</td>
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<tr>
<td>M3cF</td>
<td>CTCCAGCTCTTCTCTTCT</td>
<td>1650 1667&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>M3cR</td>
<td>GAAATTCATTTGTCATCC</td>
<td>2291 2310&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MAT1-1-3 gene</td>
</tr>
</tbody>
</table>

<sup>a</sup> From Paoletti et al. (2005).
<sup>b</sup> Sequence positions corresponding to sequence from O. novo-ulmi isolate H327 – Accession number FJ858801.
<sup>c</sup> Sequence positions corresponding to sequence from O. novo-ulmi isolate R66 – Accession number AY887028.
kit (Promega, Madison, USA) after which cloned inserts were amplified directly from colonies using the vector-specific primers T7 and SP6 (Butler & Chamberlin 1982; Dunn et al. 1983). The latter PCRs utilized the same PCR reaction and cycling conditions as before, with the only exception that 30 amplification cycles instead of 35 were used. These PCR products were also purified and sequenced as before, except that primers T7 and SP6 were used. PCR products produced from primer sets 11af + 11cR and M3cF + M3cR were not cloned, but sequenced directly as described above using the original primers.

To confirm the identity of sequenced fragments of the MAT idiomorphs, comparisons were made with the available sequences for the Dutch elm disease pathogens (Jacobi et al. 2010; Paoletti et al. 2005, 2006) by making use of the NCBI nucleotide database and BLASTn (Zhang et al. 2000). Predicted protein sequences for O. quercus were obtained by using the online version of the de novo prediction program AUGUSTUS (Stanke et al. 2006) as well as by comparison to the predicted protein sequences for O. novo-ulmi (Jacobi et al. 2010; Paoletti et al. 2005).

For analysis of MAT1-1 fragments, the produced O. quercus sequences were compared with the same region of the previously determined MAT1-1 sequences for O. novo-ulmi. These included two representative sequences for O. novo-ulmi isolate H327 (accession numbers FJ858801 and EU163846). For analyses of the MAT1-2 fragments, we included only the ORF and intron sequences of the MAT1-2-1 gene determined previously for O. novo-ulmi isolate PG402 (accession number AY887024). These comparisons were facilitated by constructing multiple alignments with the online interface of the alignment program MAFFT v. 6 using the G-INS-i strategy (Katoh et al. 2002). All protein and nucleotide sequence analyses, visualisation and conservation calculations were done using the CLC Main Workbench v. 6.1 (CLC Bio, Aarhus, Denmark).

**Results**

**Isolates and mating study**

The ITS sequences of the ten *Ophiostoma quercus* isolates used in this study had a nucleotide sequence similarity ranging from 99.3% to 100% when compared to that reported for the ex-neotype culture of *O. quercus* (isolate CBS 117913, accession number AY466626) (Grobbelaar et al. 2009), confirming their identity as *O. quercus*. The results of the mating tests (Fig 2) on solid medium confirmed the heterothallic behaviour of *O. quercus* previously determined (Brasier & Kirk 1993; De Beer et al. 2003; Kamgan et al. 2008). Of the 55 mating tests performed, none of the ten self-pairings produced perithecia or ascospores (Fig 1B). In contrast, only nine positive matings (Fig 1A) were observed among the 45 remaining mating combinations. In all cases of positive mating, the ascomata produced abundant ascospores that were viable on MEA medium. Similar results were obtained when this trial was repeated. None of the self-pairings produced perithecia, but six positive matings were scored among the remaining 45 combinations. The data

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**Fig 1 – Mating in *Ophiostoma quercus***

(A) Cross between two *O. quercus* isolates of opposite mating types [CMW 2520 (MAT A) × CMW 2521 (MAT B)] inoculated onto agar with wood pieces. Inocula indicated with squares. Abundant sexual ascomata (B) are produced all along the interaction zone (dashed line) between the two isolates. Some asexual conidiophores (C) were also produced. (D) Control cross of two identical isolates [CMW 2520 (A) × CMW 2520 (A)] forming no ascomata, but only some asexual conidiophores (E). Scale bars a, d = 5 mm; b, c, e = 100 μm.
for the two trials are given in Fig 2, showing a total of nine unique positive mating combinations. Based on these results, the isolates were separated into two groups of five isolates, and respectively assigned ‘A’ and ‘B’ mating types (Table 1).

The second mating test using liquid cultures of the isolates followed the technique of Brasier & Gibbs (1975). Similar to the tests only using solid media, none of the self-pairings produced perithecia. Seven positive mating reactions were observed, five of these between isolates of opposite mating types. The two remaining mating interactions were between the MAT B isolate CMW 2521 and the MAT B isolates CMW 17258 and CMW 27847 (Fig 2), while no positive matings were observed for CMW 2521 mated with any of the MAT A isolates. Again all of the positive matings produced fully developed perithecia with abundant ascospores, which were viable when transferred to MEA.

**PCR, cloning, and sequencing of the mating-type genes**

Using the MAT1-1 idiomorph-specific primer pair OqMt1F + OqMt1R (Table 2), it was possible to amplify and sequence a 180 bp fragment from the genome of the ten Ophiostoma quercus isolates (Table 1). The BLASTn results confirmed that the sequence of this fragment was similar to those previously determined for MAT-1 isolates of Ophiostoma novo-ulmi (Paoletti et al. 2006). None of these amplicons showed any sequence similarity to fragments amplified from the MAT1-1 idiomorph of Ophiostoma spp. examined in the present or previous studies (Paoletti et al. 2005). The sequence of this 180 bp fragment overlapped with the last 37 nucleotides of the MAT1-1-3 gene of O. novo-ulmi (Jacobi et al. 2010), while the remainder of the fragment shared similarity with 143 nucleotides of the 3’ non-coding region immediately following the MAT1-1-3 gene (Jacobi et al. 2010; Paoletti et al. 2006). The nucleotide sequence of the MAT1-1 fragments for the ten O. quercus isolates were identical. In two of the isolates (CMW 1034 and CMW 2521), a second fragment of approximately 600 bp was co-amplified, but its sequence showed no similarity to any MAT gene or to any other sequence in the NCBI database and was thus excluded from subsequent analyses.

In order to extend the MAT1-1-3 sequence, the O. quercus-based primer Mt3cF was used with Mt3cR (Table 2) in PCRs with DNA from all isolates used in this study (Table 1). This primer pair allowed amplification and sequencing of a 602 bp portion (598 bp for isolate CMW 27845) of the MAT1-1-3 gene only in MAT A isolates as well as the MAT B isolate CMW 2521 (Table 1, Fig 3). Combination of these fragments with those obtained using primers OqMt1F + 1R resulted in sequence fragments of 766 bp (762 bp for isolate CMW 27845) in length. Sequence comparisons showed that this fragment is homologous to the 3’ end of the 728 bp MAT1-1-3 ORF and a region downstream to it in O. novo-ulmi isolate H327 (accession number FJ858801). Here, 623 bp of the O. quercus sequence overlapped with the ORF and the remainder corresponded with the downstream region. Five of the O. quercus isolates (CMW 2520, CMW 2521, CMW 27846, CMW 17257, and CMW 17258) shared 100 % sequence similarity with each other, and 79 % similarity with O. novo-ulmi isolate H327 in this 623 bp portion of the MAT1-1-3 ORF. Ophiostoma quercus isolate CMW 27845 shared 84 % sequence identity with the other O. quercus isolates and 75 % identity with O. novo-ulmi isolate H327 for this 623 bp region. For all but one (CMW 27845) of the isolates of O. quercus, three introns (50 bp, 54 bp, and 64 bp in length, respectively) were predicted. In isolate CMW 27845, only two introns were predicted and these included intron 1 of 56 bp and intron 2 68 bp in length. The latter intron spans the end of the sequence and could be incomplete. All intron boundaries are based on de novo predictions made by AUGUSTUS, and the true boundary positions can only be confirmed by RNA sequencing. AUGUSTUS predictions of the O. quercus sequences yielded peptides containing an HMG-box conserved domain similar to that predicted for the

<table>
<thead>
<tr>
<th></th>
<th>2520</th>
<th>17256</th>
<th>17257</th>
<th>27845</th>
<th>27846</th>
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<tr>
<td>A</td>
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</table>

**Fig 2 – Condensed results of the mating studies.**

CMW numbers and assigned mating type are shown for all isolates. Self-matings and matings between isolates of the same mating type that produced no perithecia are excluded. The two columns for each mating interaction indicate the results of the two repeats for the agar block (two blue columns) and liquid broth (yellow column) mating test. Mating interactions between opposite mating types were expected to be positive for a strict heterothallic fungus, and 15 positive matings were observed. Matings between isolates of the same mating type (e.g. CMW2521 versus CMW17258) were expected to be negative, but two positive reactions were seen in the liquid broth mating test (shown in blocks). A + indicates a positive mating reaction with the formation of perithecia, while a – indicates the absence of perithecia and was scored as a negative result.
Fig 3 – Diagrammatic representation of the gene information currently available for Ophiostoma.

For *O. novo-ulmi*, three MAT genes are predicted for a MAT1-1 isolate (NCBI accession number FJ858801) (A), while only a single gene is present in a MAT1-2 isolate (B). In this study, the MAT1-2-1 gene was amplified from both MAT A and MAT B isolates of *O. quercus* (C, D). In addition, all five MAT A isolates contain a partial MAT1-1-1 (266 bp) and a large fragment of the MAT1-1-3 (766 bp) gene (C), while four of the MAT B isolates also encode fragments of the MAT1-1-3 (180 bp) and MAT1-1-1 (266 bp) genes (D). For the MAT B isolate CMW2521, a large fragment of the MAT1-1-3 (766 bp) and MAT1-1-1 (712 bp) genes was amplified in addition to the MAT1-2-1 gene (E). The structure and gene order of the *O. quercus* idiomorphs are implied from that of *O. novo-ulmi*. Dark bars indicate the presence of an intron. Stars indicate the $\alpha$-box conserved domain. Diamond shapes represent HMG-boxes for the MAT1-1-3 (filled) and MAT1-2-1 (clear) genes. Dashed lines and boxes indicate sections of the idiomorph and coding regions for which sequence is not available. The diagrams are not drawn to scale.

*O. novo-ulmi* MAT1-1-3 gene and other fungal MAT1-1-3 genes (Figs 3 and 4).

For the primers based on the *O. novo-ulmi* MAT1-1-1 and MAT1-1-2 sequences (Table 2), those that target MAT1-1-2 did not yield amplicons in any of the isolates used in the study. However, with the MAT1-1-1 primer pair Mt1aF + Mt1aR (Table 2), a 712 bp fragment was amplified and sequenced from the MAT B isolate CMW 2521, but not from any other isolate (Table 1, Fig 3). But when the primer pair Mt1aF + Mt1cR was used, we were able to amplify a 266 bp fragment from the remaining nine *O. quercus* isolates (Table 1, Fig 3). Comparison to the *O. novo-ulmi* MAT1-1-1 sequence (accession number FJ858801) revealed that the sequence from isolate CMW 2521 spanned 47 bp of the single intron and 665 bp of the coding region. The *O. quercus* MAT1-1-1 sequences produced using primer pair Mt1aF + Mt1cR mapped to a 266 bp region coding for the MAT1-1-1 $\alpha$-box in *O. novo-ulmi*. Peptide prediction of the CMW 2521 MAT1-1-1 sequence with AUGUSTUS and subsequent BLASTp analysis showed that this sequence harbours a large fragment of the expected conserved $\alpha$-box motif predicted for *O. novo-ulmi* (accession number ACZ53927), with a 97 % similarity in the region between these two isolates. In addition, direct translation of the *O. quercus* MAT1-1-1 fragments produced from primer pair Mt1aF + Mt1cR also showed high amino acid homology to the *O. novo-ulmi* $\alpha$-box protein.

The sequence for the full MAT1-2-1 gene (666 bp) was obtained for all 10 *O. quercus* isolates. The AUGUSTUS software predicted that it encodes a protein with 202 amino acid residues and that the gene is interrupted by a single intron of 57 bp. The intron was predicted at a conserved serine position, which is similar to what has been reported for the *O. novo-ulmi* MAT1-2-1 gene (Paolletti et al. 2005). All the *O. quercus* MAT1-2-1 sequences were identical to each other, but 21 polymorphic sites were observed when compared to the *O. novo-ulmi* isolate PG402 (accession number AY887024), resulting in 97 % similarity. Of these polymorphisms, only two occurred in the intron. Seven of the remaining 19 polymorphic sites represented synonymous substitutions, while 12 represented non-synonymous substitutions in the exons of this ORF. Nevertheless, BLASTp analysis with the inferred amino acid sequence against the NCBI database showed similarity to the predicted MAT1-2-1 protein from *Ophiostoma* species and other fungal MAT1-2-1 proteins (Fig 5).

**Discussion**

Results of this study showed that the MAT locus of *Ophiostoma quercus* has a unique structure that has not previously been encountered in any other Ascomycota. Previous work has shown that the MAT1-2 idiophor of *Ophiostoma* species such as *Ophiostoma ulmi*, *Ophiostoma novo-ulmi*, and *Ophiostoma hinal-ulmi* encodes the MAT1-1-2 gene (Paolletti et al. 2005). For the MAT1-1 idiophor, only the MAT1-1-3 gene was comprehensively analysed (Jacobi et al. 2010). However unpublished yet publicly available nucleotide data indicate the *Ophiostoma* MAT1-1-1 idiophor also contains the MAT1-1-2 and MAT1-1-1 genes (Fig 3). This gene organisation is quite common among the Ascomycota (Coppin et al. 1997; Debuchy & Turgeon 2006; Glass & Nelson 1994; Nelson 1996), but elements thought to be exclusively associated with either the MAT1-1 or MAT1-2 idiophors were found in all the *O. quercus* isolates examined. Although the typical heterothallic mating system was
An alignment of the HMG-box domain of the MAT1-1-3 protein.

An alignment of the HMG-box conserved domain characteristic of the MAT1-1-3 protein was done. A plot showing the conservation across the protein fragment is presented at the bottom of the alignment. The O. quercus sequence is representative of the MAT1-1-3 protein sequences produced for all but isolate CMW 27845. Accession numbers: O. quercus sequences — Table 1, O. novo-ulmi subs. novo-ulmi — ACZ53925; Chaetomium globosum — EAQ89965; Cryptophenecia parasitica — AF380365.1; Gibberella fujikuroi — AAG71053; G. zeae — AAG42812; Magnaporthe grisea — BAC65085; Neurospora crassa — AAC37476; Podospora anserina — CAA52051.

observed to function in crosses, isolates shown to represent the two mating types harboured both MAT1-1-3 and MAT1-2-1 sequences. This study highlights the need to perform both behavioural mating tests in culture as well as to determine the mating-type structure using molecular genetic tools in order to fully understand the functional situation.

Previously published mating assignments (Brasier & Kirk 1993; De Beer et al. 2003; Kamgan et al. 2008) as well as those in the current study, made it as possible to assign with confidence ‘A’ or ‘B’ mating designations for all isolates of O. quercus. Traditional mating tests using agar blocks with mycelium produced comparable results, with seven positive matings. Interestingly, two of these were between isolates of the same mating type (Fig 2). Both these matings involved the MAT B isolate CMW 2521, which also mated with the MAT A isolate CMW 2520 and CMW 17256 in the agar based test. The presence of two almost complete copies of the MAT1-1 specific genes MAT1-1-1 and MAT1-1-3 in addition to the MAT1-2-1 gene makes this isolate unique in terms of its genotype. This could thus explain the ability of the isolate to act as both a MAT A and MAT B strain.

In 1975, a mating study was performed with O. novo-ulmi where Elm twigs were dipped into liquid cultures of the same species and differences in the geographical origin have been given as possible reasons for the varied mating success observed (De Beer et al. 2003). An alternative mating test using liquid cultures (Brasier & Gibbs 1975) rather than agar blocks produced comparable results, with seven positive matings. Interestingly, two of these were between isolates of the same mating type (Fig 2). Both these matings involved the MAT B isolate CMW 2521, which also mated with the MAT A isolate CMW 2520 and CMW 17256 in the agar based test. The presence of two almost complete copies of the MAT1-1 specific genes MAT1-1-1 and MAT1-1-3 in addition to the MAT1-2-1 gene makes this isolate unique in terms of its geno-type. This could thus explain the ability of the isolate to act as both a MAT A and MAT B strain.

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In 1975, a mating study was performed with O. novo-ulmi where Elm twigs were dipped into liquid cultures of the
fungus (Brasier & Gibbs 1975). In that study, several isolates were identified as having the ability to self, i.e. self-fertile, and the phenomenon was termed ‘pseudoselfing’. The authors attributed this to a mutation in the MAT locus allowing a switch in the mating specificity. Expression of the new mating type was thought to be suppressed in the mycelial state, but that it could be expressed in the yeast-like liquid culture (Brasier & Gibbs 1975; Brasier 1993). In addition, these ‘pseudoselfing’ isolates had the ability to mate with both A and B testers. The behaviour of isolate CMW 2521 of O. quercus in the current study might also represent a form of pseudoselfing in this fungus. Although selfing was not observed for this isolate even though this was also tested using the technique of Brasier & Gibbs (1975), positive mating reactions were found with isolates of both MAT A and MAT B mating type.

The full HMG-box containing MAT1-2-1 gene was amplified and sequenced for the ten O. quercus isolates included in this study (Fig 3). Although the MAT1-1-3 gene associated with the MAT1-1 idiomorph also encodes an HMG-box motif, a detailed analysis of the MAT1-2-1 and MAT1-1-3 HMG-box domains from O. novo-ulmi indicated that the MAT1-2-1 domain is specific to the MAT1-2-1 idiomorph (Jacobi et al. 2010). Also, the MAT1-2-1 ORF and intron encoded by the 666 bp fragment characterized in this study, shows very high similarity to the MAT1-2-1 sequences reported for the Dutch elm disease pathogens (Paoletti et al. 2005) and other Ascomycota such as Neurospora crassa and Cryptonectria parasitica (Fig 5). This provides confidence that the MAT1-2-1 gene characterized in the present study corresponds to the typical HMG domain encoding gene associated with the typical Ascomycota MAT1-2-1 idiomorph.

In this study, the sequences for two genes usually associated with the typical Ascomycota MAT1-1 idiomorph were determined. From the MAT B isolate CMW 2521 (Table 1), a large portion of the MAT1-1-1 gene was amplified, which encodes the typical MAT1-1-1 α-domain known from other Ascomycota (Coppin et al. 1997; Debuchy & Turgeon 2006; Glass & Nelson 1994; Nelson 1996). In addition, a smaller fragment representing a part of the MAT1-1-1 ORF was amplified from all other isolates in the study using O. quercus specific primers (Table 2). All these fragments spanned the part of the ORF that encodes the α-box domain, characteristic of the MAT1-1-1 protein.

A 180 bp portion of the MAT1-1-3 gene was also found in all ten O. quercus isolates. It was possible to obtain the near-complete sequence for this gene in all MAT A isolates, as well as in the MAT B isolate CMW 2521 (Table 1) but not in any other MAT B isolates. The predicted protein sequence for this region showed high similarity to the MAT1-1-3 sequences for other species, e.g. O. novo-ulmi, Cryptonectria parasitica and Gibberella fujikuroi (Fig 4), providing confidence that this represents the MAT1-1-3 gene.

In the typical heterothallic MAT locus arrangement, the MAT1-1 idiomorph contains at least the α-domain MAT1-1-1 gene in addition to the MAT1-1-3 and MAT1-1-2 genes (Coppin et al. 1997; Nelson 1996; Turgeon & Yoder 2000), while the MAT1-2 idiomorph always contains the MAT1-2-1 gene (Arie et al. 1997; Coppin et al. 1997). Nothing is known regarding the mating-type loci of homothallic Ophiostoma species, but previous research has shown that homothallic mating idiomorphs share similarity with that of heterothallic species. For example, the single MAT locus of the homothallic Gibberella zeae, harbour all four MAT genes, MAT1-1-1, MAT1-1-2, MAT1-1-3, and MAT1-2-1 (Yun et al. 2000). In another example, the Cochliobolus MAT locus is characterized by different organisations ranging from fused single genes to only two MAT genes located in opposite orientation within a single MAT idiomorph (Yun et al. 1999). In this respect, the MAT locus of O. quercus might seem more similar to those of homothallic species because MAT1-1 and MAT1-2 idiomorph-specific sequences were present in single isolates originating from single spores. However, the possibility that the MAT1-1-1 gene identified in this study represents a non-functional pseudogene cannot be excluded because only fragments of this gene could be amplified. The same is true for MAT1-1-3 but less likely in the case of the MAT A isolates (and CMW 2521) as the majority of the gene was sequenced in this study and the amino acid sequence was very similar to that of O. novo-ulmi. Pseudogenes are not subject to functional constraint and are thus likely to diverge relatively rapidly from the original sequence, but we did not find evidence of this occurring.

The occurrence of an atypical MAT locus in an apparently strictly heterothallic species is not unique to O. quercus. Two heterothallic Diaporthe species were recently shown to harbour unusual MAT1-2 idiomorph structures while having a normal MAT1-1 idiomorph structure with the three expected genes (Kanematsu et al. 2007). The Diaporthe MAT1-2 idiomorph contained three genes, one which represents the MAT1-2 idiomorph-specific gene MAT1-2-1. The other two apparently represent homologues of the MAT1-1 idiomorph genes MAT1-1-2 and MAT1-1-3. The authors suggested that this arrangement might have come about after a duplication event and that the ancestral type contained a MAT locus with three genes, MAT1-1-2, MAT1-1-3 and another gene similar to either MAT1-1-1 or MAT1-2-1 (Kanematsu et al. 2007). In the same manner, gene duplications could potentially explain the existence of the MAT1-2-1, MAT1-1-1, and MAT1-1-3 genes (possibly MAT1-1-2 although this was not detected) in a single isolate of O. quercus.

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Supplementary material

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References


Supplementary material

436 P.M. Wilken


