ELSEVIER
British Mycological Society promoting fungal science
journal homepage: www.elsevier.com/locate/funbio
Fungal Biology

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

P. Markus WILKEN[a,*], *Emma T. STEENKAMP*[b], *Tracy A. HALL*[a], *Z. Wilhelm DE BEER*[b], *Michael J. WINGFIELD*[a], *Brenda D. WINGFIELD*[a]

[a]*Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private Bag X20, Hatfield, Pretoria 0028, South Africa*
[b]*Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private Bag X20, Hatfield, Pretoria 0028, South Africa*

ARTICLE INFO

Article history:
Received 11 August 2011
Received in revised form
2 January 2012
Accepted 5 January 2012
Available online 14 January 2012
Corresponding Editor:
Michael Lorenz

Keywords:
Fungal mating
MAT1-1
MAT1-2
Ophiostoma

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.

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

* *Corresponding author*. Tel.: +27 12 420 3906; fax: +27 12 420 3960.
E-mail addresses: markus.wilken@fabi.up.ac.za, emma.steenkamp@fabi.up.ac.za, tracy.hall@fabi.up.ac.za, wilhelm.debeer@fabi.up.ac.za, mike.wingfield@fabi.up.ac.za, brenda.wingfield@fabi.up.ac.za
1878-6146/$ – see front matter
doi:10.1016/j.funbio.2012.01.002

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, *MAT1-1-1* (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-Ferráez 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 $MgCl_2$, 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 MEA cultures and subjecting the harvested mycelium to a salt-based DNA extraction method (Aljanabi & Martinez 1997). PCRs were performed on an Eppendorf

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

Isolate[a]		Host	Country	Collector	Mating group	Genbank acc. Nr.				Reference
CMW	Other					ITS	*MAT1-1-1*	*MAT1-1-3*	*MAT1-2-1*	
2520	CBS 116321	*Eucalyptus* chips	South Africa	ZW de Beer	A	AF493241[c]	**JQ319599**[d]	**JQ319598**[f,g]	**FJ865421**	(De Beer *et al.* 2003)
2521		*Eucalyptus* chips	South Africa	ZW de Beer	B	FJ441283[c]	**JN225450**[e]	**JQ319595**[f,g]	**FJ865420**	(De Beer *et al.* 2003)
14307		*Acacia mearnsii*	Uganda	J Roux	B	**FJ959044**	**JQ319600**[d]	**FJ865415**[g]	**FJ865425**	(Kamgan *et al.* 2008)
17256[b]		*A. mearnsii*	Uganda	J Roux	A	**FJ959042**	**JQ319601**[d]	**JQ319597**[f,g]	**FJ865422**	(Kamgan *et al.* 2008)
17257[b]		*A. mearnsii*	Uganda	J Roux	A	**FJ959045**	**JQ319602**[d]	**JQ319593**[f,g]	FJ865424	(Kamgan *et al.* 2008)
17258[b]		*A. mearnsii*	Uganda	J Roux	B	**FJ959043**	**JQ319603**[d]	**FJ865418**[g]	**FJ865423**	(Kamgan *et al.* 2008)
27845	H 2190	*Quercus* sp.	Canada	K Seifert	A	**JQ319592**	**JQ319604**[d]	**JQ319596**[f,g]	**FJ865426**	(Brasier & Kirk 1993)
27846	H 1039	*Quercus* sp.	UK	PT Scard	A	**JQ319591**	**JQ319605**[d]	**JQ319594**[f,g]	**FJ865427**	(Brasier & Kirk 1993; De Beer *et al.* 2003)
27847	H 920	*Quercus* sp.	UK	JN Gibbs	B	**JQ319590**	**JQ319606**[d]	**FJ865413**[g]	**FJ865429**	(Brasier & Kirk 1993)
27848	H 1042	*Quercus* sp.	UK	PT Scard	B	EF429089[c]	**JQ319607**[d]	**FJ865412**[g]	**FJ865428**	(Brasier & Kirk 1993; De Beer *et al.* 2003)

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.

thermocycler (Eppendorf AG, Mannheim, Germany) using the following conditions: one cycle of 5 min at 96 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 50 °C, and 1 min at 72 °C, followed by one cycle of 7 min at 72 °C. Products were visualized by agarose (LE Agarose, SeaKem, Rockland, USA) gel electrophoresis (Sambrook & Russell 2001), and purified using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). Individual products were then sequenced using the original PCR primers, a Big Dye cycle sequencing kit with Amplitaq DNA polymerase (Perkin-Elmer, Warrington, UK) and an ABI PRISM 3300 Genetic Analyser (Applied Biosystems, Foster City, USA). After analysis of chromatograms with Chromas Lite v. 2.01 (Technelysium Pty. Ltd; http://www.technelysium.com.au), the sequences of all isolates were compared to the ITS sequence of the ex-neotype culture for *O. quercus* (Grobbelaar *et al.* 2009).

To confirm the mating types of all ten isolates, mating tests were used as described previously by De Beer *et al.* (2003) and Brasier & Gibbs (1975). In the mycelial mating test (De Beer *et al.* 2003), isolates were paired in all possible combinations on sterilized *Quercus* twigs that were placed on 1.5 % (w/v) agar (Biolab, Merck) medium. Control crosses were included where each isolate was paired with itself. A mating interaction was scored as negative if no perithecia were formed, if the perithecia contained no ascospores or if the ascospores were not viable when incubated on MEA medium. Positive mating responses were recorded only when perithecia containing viable ascospores were formed. The entire mating study was performed twice.

A second mating study using liquid medium (Brasier & Gibbs 1975) was conducted where sterilized *Quercus* twigs were dipped into liquid cultures of each isolate in 2 % malt-extract broth (ME; 20 g/L^{-1} malt extract [Biolab, Merck]) grown at 25 °C for 3–5 d. The twigs were placed onto the agar surface in Petri dishes containing 2 % MEA for 1 week before being dipped into a liquid culture of the second mating-type isolate. Isolates were paired in all combinations, with self-pairings not being dipped a second time following the example of Brasier & Gibbs (1975). All twigs were then transferred to the agar surface in Petri dishes containing 2 % MEA for up to a month and examined regularly for the formation of perithecia. Positive and negative mating reactions were scored as described above.

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

To study the *MAT* idiomorphs of *Ophiostoma quercus*, published (Jacobi *et al.* 2010; Paoletti *et al.* 2005, 2006) and publicly available sequences (National Centre for Biotechnology Information; http//:www.ncbi.nih.gov) for *Ophiostoma novo-ulmi* were used. The software packages Primer3 v. 0.4.0 (Rozen & Skaletsky 2000) and CLC Main Workbench v. 5.5 (CLC Bio, Aarhus, Denmark) were used to design primers that would allow the amplification of *MAT* sequences (Table 2). To amplify the *MAT1-2* idiomorph gene *MAT1-2-1*, a set of primers was designed based on *O. novo-ulmi* sequence data. In an attempt to extend *MAT1-1* sequences, two strategies were followed. In the first strategy, the *MAT1-1-1* and *MAT1-1-2* sequences were targeted by designing primers based on sequence information for *O. novo-ulmi*. In the second strategy, the *O. quercus MAT1-1-3* gene and 3′ non-coding region were targeted using a primer set that was based on previously published (Paoletti *et al.* 2005) sequence data (Table 2). After a single round of PCR and sequencing, the resulting *O. quercus* sequence data were used to design two new primer sets. The first set included the newly designed primer Mt3cF, which was used together with primer Mt3cR, also based on unpublished *O. novo-ulmi* sequence data, to target the coding region of *MAT1-1-3*. The second set targeted a section of the *MAT1-1-1* coding region using primers 11aF and 11cR designed on *O. quercus* sequence data (Table 2).

Amplification reactions of the mating-type regions and purification of PCR products were carried out as described above. Purified products were cloned using the pGem®-T Easy cloning

Table 2 – Primers used in this study.

Primer name	Sequence (5′–3′)	Primer binding region in *O. novo-ulmi* *MAT1-1* and *MAT1-2* idiomorphs		Region amplified
OqMt1F	TGGCAAGAAAGGAAGACTGG	1653	1672[b]	*MAT1-1* idiomorph
OqMt1R	GCGTTATTGGGAGACAGGAA	1493	1512[b]	
OqMt2	GCACACAACTTTGCCAGGTA	119	138[c]	*MAT1-2* idiomorph
Seq9[a]	GGGGATGTAAAAGGAAC	1188	1204[c]	
Mt1aF	CCCAGGTCCTCAAATAATAA	4622	4641[b]	*MAT1-1-1* gene
Mt1aR	GAAACTCCCCACCGATAA	5324	5341[b]	
11aF	TCCTTCTTCCGTCCTTCT	4817	4834[b]	
11cR	CGATGCTTTCTTGGTTATTG	5076	5095[b]	
Mt2aF	GAGTCATCTTACCGAAACA	2961	2934[b]	*MAT1-1-2* gene
Mt2aR	CGGCGGATCATAGTACTTA	2916	2934[b]	
Mt2bF	AATGCGAGTCATCTTACC	2911	2928[b]	
Mt2bR	TGTGTTTCTAGGTGGCTG	3597	3614[b]	
Mt3cF	CTCCCAGTCTTCCTTTCT	1650	1667[b]	*MAT1-1-3* gene
Mt3cR	GAAATTCATTGTCGTCATCC	2291	2310[b]	

a From Paoletti *et al.* (2005).
b Sequence positions corresponding to sequence from *O. novo-ulmi* isolate H327 – Accession number FJ858801.
c 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 + Mt3cR 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

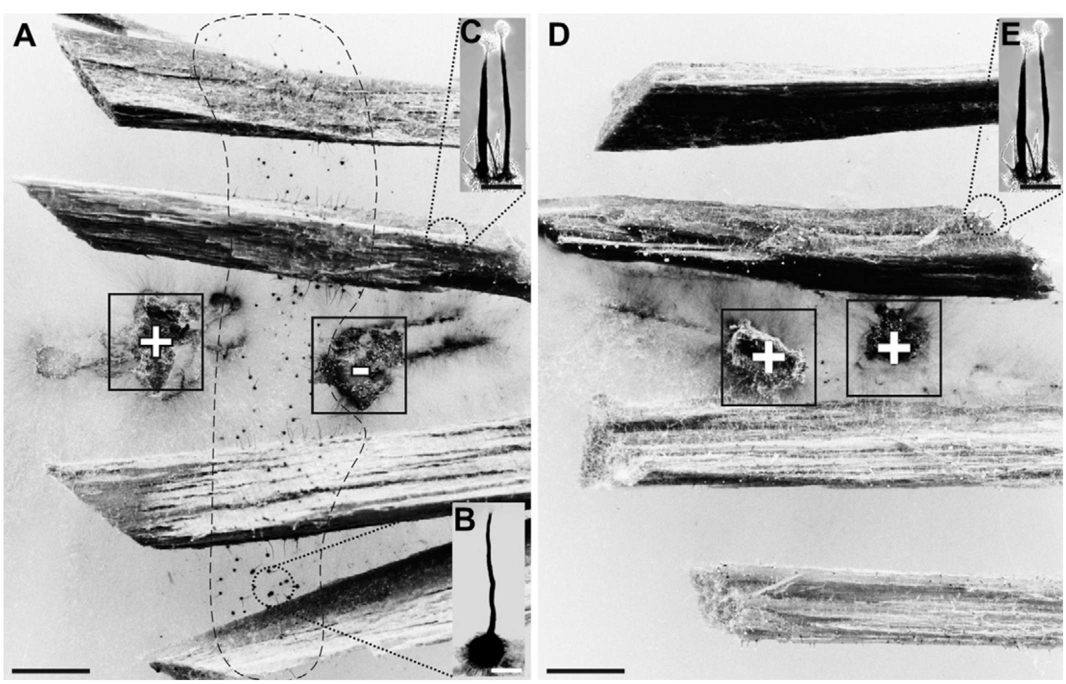

Fig 1 – Mating in *Ophiostoma quercus*.
(A) Cross between two *O. quercus* isolates of opposite mating types [CMW 2520 (MAT A) × CMW 2521 (MAT B)] inoculated onto agar with wood pieces. Inocula indicated with squares. Abundant sexual ascomata (B) are produced all along the interaction zone (dashed line) 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.

		A										B	
		2520		17256		17257		27845		27846		2521	
	2521	++	-	++	-	--	-	--	-	--	-	--	-
	17258	--	-	--	+	--	-	+-	-	--	+	--	+
B	14307	--	-	++	-	--	-	--	-	++	-	--	-
	27847	--	+	--	-	--	+	+-	+	--	-	--	+
	27848	++	-	--	-	++	-	+-	-	--	-	--	-

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.

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 *MAT1-2* 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 17256) 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

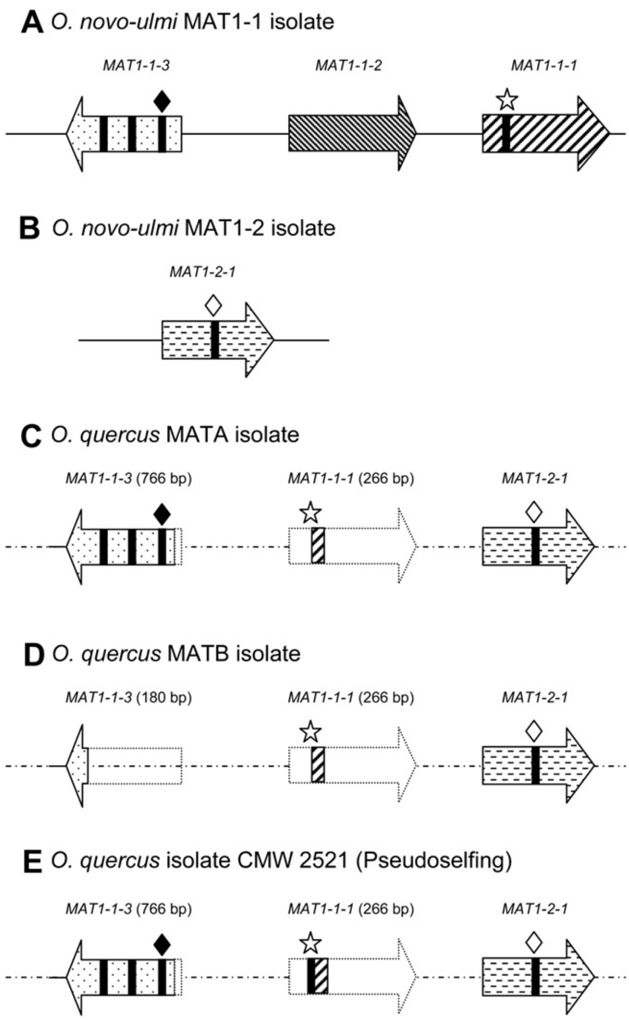

Fig 3 – Diagrammatic representation of the gene information currently available for *Ophiostoma*.
For *O. novo-ulmi*, three *MAT* genes are predicted for a MAT1-1 isolate (NCBI accession number FJ858801) (A), while only a single gene is present in a MAT1-2 isolate (B). In this study, the *MAT1-2-1* gene 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 α-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 11aF + 11cR 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 11aF + 11cR mapped to a 266 bp region coding for the *MAT1-1-1* α-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 α-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 11aF + 11cR also showed high amino acid homology to the *O. novo-ulmi* α-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 (Paoletti *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 *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* idiomorph of *Ophiostoma* species such as *Ophiostoma ulmi*, *Ophiostoma novo-ulmi*, and *Ophiostoma himal-ulmi* encodes the *MAT1-2-1* gene (Paoletti *et al.* 2005). For the *MAT1-1* idiomorph, 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* idiomorph 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* idiomorphs were found in all the *O. quercus* isolates examined. Although the typical heterothallic mating system was

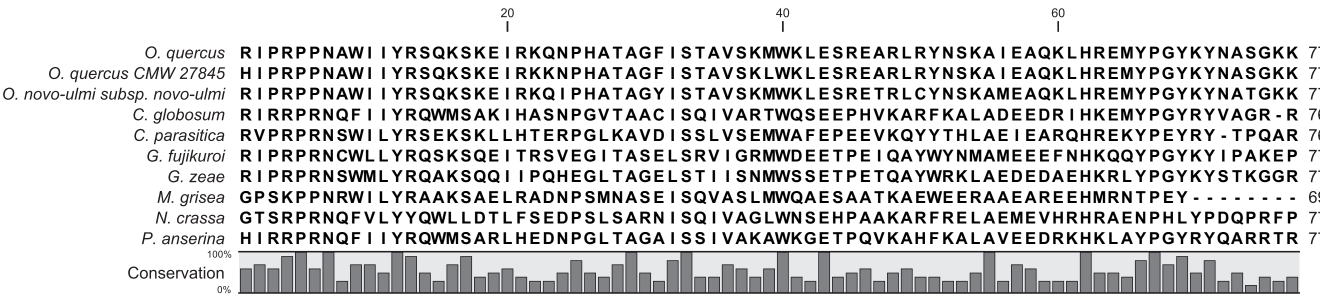

Fig 4 – Alignment of the HMG-box domain of the MAT1-1-3 protein.
An alignment of the HMG-box conserved domain characteristic of the MAT1-1-3 protein was done. A plot showing the conservation across the protein fragment is presented at the bottom of the alignment. The *O. quercus* sequence is representative of the MAT1-1-3 protein sequences produced for all but isolate CMW 27845. Accession numbers: *O. quercus* sequences – Table 1, *O. novo-ulmi* subsp. *novo-ulmi* – ACZ53925; *Chaetomium globosum* – EAQ89965; *Cryphonectria parasitica* – AF380365_1; *Gibberella fujikuroi* – AAC71053; *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 (De Beer *et al.* 2003) produced only ten of a possible 25 positive matings between opposite mating-type isolates (Fig 2). This low number of successful crosses was not unexpected, and similar results have been observed in other studies (De Beer *et al.* 2003). Variation in fertility between isolates 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 isolates 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

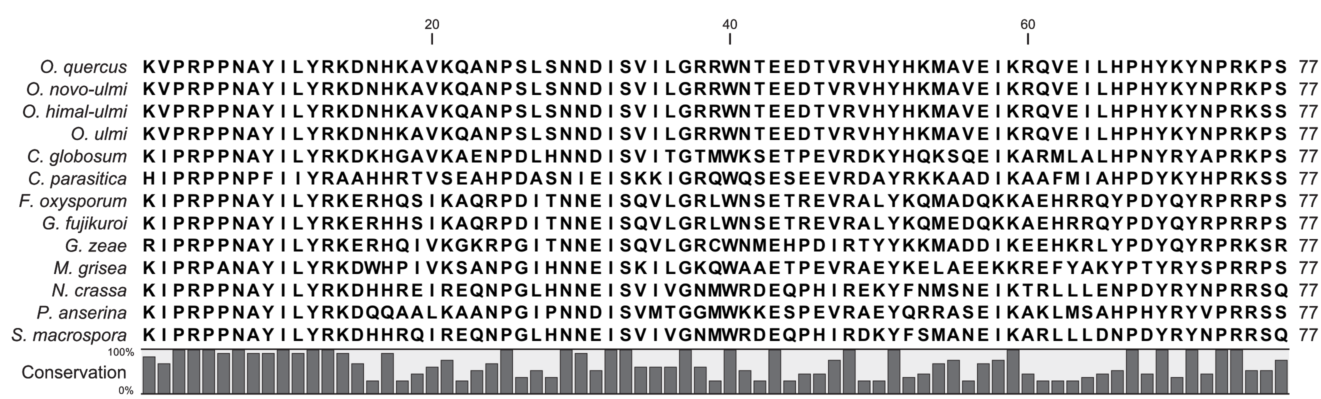

Fig 5 – Alignment of the HMG-box domain of the MAT2-1 protein.
An alignment showing the conservation of amino acids across the HMG-box domain of the mating type protein MAT1-2-1 (MAT2-1). The last row included a conservation plot indicating the conservation in amino acid sequence across the fragment. The listed *O. quercus* sequence is representative of the MAT1-2-1 HMG region for all isolates used in this study. Accession numbers: *O. quercus* sequences – Table 1, *O. novo-ulmi* – AAX83067; *O. himal-ulmi* – AAX83073; *O. ulmi* – AAX83065; *Chaetomium globosum* – EAQ91645; *Cryphonectria parasitica* – AF380364_1; *Fusarium oxysporum* – BAA28611; *Gibberella fujikuroi* – AAC71056; *G. zeae* – AAG42810; *Magnaporthe grisea* – BAC65090; *Neurospora crassa* – AAA33598; *Podospora anserina* – CAA45520; *Sordaria macrospora* – CAA71624.

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 tester types. 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 *Cryphonectria 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* 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* 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*, *Cryphonectria 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*.

Role of the funding sources

Funding for this study was provided by the University of Pretoria, the National Research Foundation (NRF) and the DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB) South Africa. The funding sources played no role in the study design; in the collection, analyses or interpretation of the data. Neither were these entities involved in writing the manuscript nor in the decision to submit it to be considered for publication.

Acknowledgements

The University of Pretoria, the National Research Foundation (NRF) and the DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB) South Africa provided financial support that made this study possible.

Supplementary material

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.funbio.2012.01.002.

REFERENCES

Aljanabi SM, Martinez I, 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research* **25**: 4692–4693.

Arie T, Christiansen SK, Yoder OC, Turgeon BG, 1997. Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved *MAT* HMG box. *Fungal Genetics and Biology* **21**: 118–130.

Astell CR, Ahlstrom-Jonasson L, Smith M, Tatchell K, Nasmyth KA, Hall BD, 1981. The sequence of the DNAs coding for the mating-type loci of *Saccharomyces cerevisiae*. *Cell* **27**: 15–23.

Bennett RS, Yun SH, Lee TY, Turgeon BG, Arseniuk E, Cunfer BM, Bergstrom GC, 2003. Identity and conservation of mating type genes in geographically diverse isolates of *Phaeosphaeria nodorum*. *Fungal Genetics and Biology* **40**: 25–37.

Brasier CM, 1984. Inter-mycelial recognition systems in *Ceratocystis ulmi*: their physiological properties and ecological importance. In: Jennings D, Rayner ADM (eds), *The Ecology and Physiology of the Fungal Mycelium*. Cambridge University Press, Cambridge, pp. 451–497.

Brasier CM, 1990. China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* **39**: 5–16.

Brasier CM, 1993. The genetic system as a fungal taxonomic tool: gene flow, molecular variation and sibling species in the '*Ophiostoma piceae–Ophiostoma ulmi*' complex and its taxonomic and ecological significance. In: Wingfield MJ, Seifert K, Webber JF (eds), *Ophiostoma and Ceratocystis: taxonomy, biology and pathology*. American Phytopathological Society, St. Paul, Minnesota, pp. 77–92.

Brasier CM, Gibbs JN, 1975. Highly fertile form of the aggressive strain of *Ceratocystis ulmi*. *Nature* **257**: 128–131.

Brasier CM, Kirk SA, 1993. Sibling species within *Ophiostoma piceae*. *Mycological Research* **97**: 811–816.

Brasier CM, Mehrotra MD, 1995. *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch elm disease fungus endemic to the Himalayas. *Mycological Research* **99**: 205–215.

Britz H, Wingfield MJ, Coutinho TA, Marasas WFO, Leslie JF, 1998. Female fertility and mating type distribution in a South African population of *Fusarium subglutinans* f. sp. *pini*. *Applied and Environmental Microbiology* **64**: 2094–2095.

Butler ET, Chamberlin MJ, 1982. Bacteriophage SP6-specific RNA polymerase. I. Isolation and characterization of the enzyme. *Journal of Biological Chemistry* **257**: 5772–5778.

Carlier FX, Decock C, Jacobs K, Maraite H, 2006. *Ophiostoma arduennense* sp. nov. (Ophiostomatales, Ascomycota) from *Fagus sylvatica* in southern Belgium. *Mycological Research* **110**: 801–810.

Cherif M, Chilvers MI, Akamatsu H, Peever TL, Kaiser WJ, 2006. Cloning of the mating type locus from *Ascochyta lentis* (teleomorph: *Didymella lentis*) and development of a multiplex PCR mating assay for *Ascochyta* species. *Current Genetics* **50**: 203–215.

Chung WH, Kim JJ, Yamaoka Y, Uzunovic A, Masuya H, Breuil C, 2006. *Ophiostoma breviusculum* sp. nov. (Ophiostomatales, Ascomycota) is a new species in the *Ophiostoma piceae* complex associated with bark beetles infesting larch in Japan. *Mycologia* **98**: 801–814.

Conde-Ferráez L, Waalwijk C, Canto-Canché BB, Kema GHJ, Crous PW, James AC, Abeln ECA, 2007. Isolation and characterization of the mating type locus of *Mycosphaerella fijiensis*, the causal agent of black leaf streak disease of banana. *Molecular Plant Pathology* **8**: 111–120.

Coppin E, Debuchy R, Arnaise S, Picard M, 1997. Mating types and sexual development in filamentous ascomycetes. *Microbiology and Molecular Biology Reviews* **61**: 411–428.

De Beer ZW, Wingfield BD, Wingfield MJ, 2003. The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycological Research* **107**: 469–476.

Debuchy R, Turgeon BG, 2006. Mating-type structure, evolution, and function in euascomycetes. In: Kües U, Fischer R (eds), *The Mycota: growth, differentiation and sexuality*, 2nd edn. Springer, Berlin Heidelberg, pp. 293–323.

Dunn JJ, Studier FW, Gottesman M, 1983. Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *Journal of Molecular Biology* **166**: 477–535.

Dyer PS, Furneaux PA, Douhan G, Murray TD, 2001. A multiplex PCR test for determination of mating type applied to the plant pathogens *Tapesia yallundae* and *Tapesia acuformis*. *Fungal Genetics and Biology* **33**: 173–180.

Elliott CG, 1994. *Reproduction in Fungi: genetical and physiological aspects*, 1st edn. Chapman & Hall, London.

Foster SJ, Fitt BD, 2003. Isolation and characterisation of the mating-type (*MAT*) locus from *Rhynchosporium secalis*. *Current Genetics* **44**: 277–286.

Fraser JA, Heitman J, 2004. Evolution of fungal sex chromosomes. *Molecular Microbiology* **51**: 299–306.

Gardes M, Bruns TD, 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Glass NL, Grotelueschen J, Metzenberg RL, 1990. *Neurospora crassa* A mating-type region. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 4912–4916.

Glass NL, Nelson MA, 1994. Mating-type genes in mycelial ascomycetes. In: Wessels JGH, Meinhardt F (eds), *The Mycota. I. Growth, Differentiation and Sexuality*, 1st edn. Springer-Verlag, Berlin, Germany, pp. 295–306.

Gorton C, Webber JF, 2000. Reevaluation of the status of the bluestain fungus and bark beetle associate *Ophiostoma minus*. *Mycologia* **92**: 1071–1079.

Grobbelaar JW, Aghayeva DN, de Beer ZW, Bloomer P, Wingfield MJ, Wingfield BD, 2009. Delimitation of *Ophiostoma quercus* and its synonyms using multiple gene phylogenies. *Mycological Progress* **8**: 221–236.

Harrington TC, McNew D, Steimel J, Hofstra D, Farrell R, 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111–136.

Hausner G, Reid J, Klassen GR, 1993. On the phylogeny of *Ophiostoma*, *Ceratocystis* s.s., and *Microascus*, and relationships within *Ophiostoma* based on partial ribosomal DNA-sequences. *Canadian Journal of Botany* **71**: 1249–1265.

Jacobi V, Dufour J, Bouvet GF, Aoun M, Bernier L, 2010. Identification of transcripts up-regulated in asexual and sexual fruiting bodies of the Dutch elm disease pathogen *Ophiostoma novo-ulmi*. *Canadian Journal of Microbiology* **56**: 697–705.

Kamgan NG, Jacobs K, De Beer ZW, Wingfield MJ, Roux J, 2008. *Pesotum australi* sp. nov. and *Ophiostoma quercus* associated with *Acacia mearnsii* trees in Australia and Uganda, respectively. *Australasian Plant Pathology* **37**: 406–416.

Kanematsu S, Adachi Y, Ito T, 2007. Mating-type loci of heterothallic *Diaporthe* spp.: homologous genes are present in opposite mating-types. *Current Genetics* **52**: 11–22.

Katoh K, Misawa K, Kuma K, Miyata T, 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.

Li W, Metin B, White TC, Heitman J, 2010. Organization and evolutionary trajectory of the mating type (*MAT*) locus in dermatophyte and dimorphic fungal pathogens. *Eukaryotic Cell* **9**: 46–58.

Linde CC, Liles JA, Thrall PH, 2010. Expansion of genetic diversity in randomly mating founder populations of *Alternaria brassicicola* infecting *Cakile maritima* in Australia. *Applied Environmental Microbiology* **76**: 1946–1954.

Mandel MA, Barker BM, Kroken S, Rounsley SD, Orbach MJ, 2007. Genomic and population analyses of the mating type loci in *Coccidioides* species reveal evidence for sexual reproduction and gene acquisition. *Eukaryotic Cell* **6**: 1189–1199.

Marra RE, Cortesi P, Bissegger M, Milgroom MG, 2004. Mixed mating in natural populations of the chestnut blight fungus, *Cryphonectria parasitica*. *Heredity* **93**: 189–195.

Marra RE, Milgroom MG, 2001. The mating system of the fungus *Cryphonectria parasitica*: selfing and self-incompatibility. *Heredity* **86**: 134–143.

Martin SH, Wingfield BD, Wingfield MJ, Steenkamp ET, 2011. Structure and evolution of the *Fusarium* mating type locus: new insights from the *Gibberella fujikuroi* complex. *Fungal Genetics and Biology* **48**: 731–740.

McDonald BA, Linde C, 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology* **40**: 349–379.

Metzenberg RL, Glass NL, 1990. Mating type and mating strategies in *Neurospora*. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology* **12**: 53–59.

Nelson MA, 1996. Mating systems in ascomycetes: a romp in the sac. *Trends in Genetics* **12**: 69–74.

Paoletti M, Buck KW, Brasier CM, 2005. Cloning and sequence analysis of the *MAT-B* (*MAT-2*) genes from the three Dutch elm disease pathogens, *Ophiostoma ulmi*, *O. novo-ulmi*, and *O. himal-ulmi*. *Mycological Research* **109**: 983–991.

Paoletti M, Buck KW, Brasier CM, 2006. Selective acquisition of novel mating type and vegetative incompatibility genes via interspecies gene transfer in the globally invading eukaryote *Ophiostoma novo-ulmi*. *Molecular Ecology* **15**: 249–262.

Rozen S, Skaletsky H, 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds), *Bioinformatics Methods and Protocols: methods in molecular biology*, 1st edn. Humana Press, Totowa, New Jersey, pp. 365–386.

Sambrook J, Russell DW, 2001. *Molecular Cloning – a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Solla A, Dacasa MC, Nasmith C, Hubbes M, Gil L, 2008. Analysis of Spanish populations of *Ophiostoma ulmi* and *O. novo-ulmi* using phenotypic characteristics and RAPD markers. *Plant Pathology* **57**: 33–44.

Stanke M, Tzvetkova A, Morgenstern B, 2006. AUGUSTUS at EGASP: using EST, protein and genomic alignments for improved gene prediction in the human genome. *Genome biology* **7** (Suppl. 1): S11.11–S11.18.

Steenkamp ET, Wingfield BD, Coutinho TA, Zeller KA, Wingfield MJ, Marasas WF, Leslie JF, 2000. PCR-based identification of *MAT-1* and *MAT-2* in the *Gibberella fujikuroi* species complex. *Applied and Environmental Microbiology* **66**: 4378–4382.

Strandberg R, Nygren K, Menkis A, James TY, Wik L, Stajich JE, Johannesson H, 2010. Conflict between reproductive gene trees and species phylogeny among heterothallic and pseudohomothallic members of the filamentous ascomycete genus *Neurospora*. *Fungal Genetics and Biology* **47**: 869–878.

Turgeon BG, 1998. Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology* **36**: 115–137.

Turgeon BG, Yoder OC, 2000. Proposed nomenclature for mating type genes of filamentous ascomycetes. *Fungal Genetics and Biology* **31**: 1–5.

Uzunovic A, Seifert KA, Hwan KS, Breuil C, 2000. *Ophiostoma setosum*, a common sapwood staining fungus from western North America, a new species of the *Ophiostoma piceae* complex. *Mycological Research* **104**: 486–494.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Snindky JJ, White TJ (eds), *PCR Protocols: a guide to methods and applications*, 1st edn. Academic Press Inc., San Diego, California, pp. 315–322.

Wingfield MJ, Seifert KA, Webber J, 1993. *Ceratocystis and Ophiostoma: taxonomy, ecology, and pathogenicity*. American Phytopathological Society, St. Paul, Minnesota.

Yokoyama E, Yamagishi K, Hara A, 2004. Development of a PCR-based mating-type assay for *Clavicipitaceae*. *FEMS Microbiology Letters* **237**: 205–212.

Yun S-H, Arie T, Kaneko I, Yoder OC, Turgeon BG, 2000. Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella/Fusarium* species. *Fungal Genetics and Biology* **31**: 7–20.

Yun SH, Berbee ML, Yoder OC, Turgeon BG, 1999. Evolution of the fungal self-fertile reproductive life style from self-sterile ancestors. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 5592–5597.

Zaffarano PL, Duò A, Grünig CR, 2010. Characterization of the mating type (*MAT*) locus in the *Phialocephala fortinii* s.l. – *Acephala applanata* species complex. *Fungal Genetics and Biology* **47**: 761–772.

Zhan J, Kema GHJ, Waalwijk C, McDonald BA, 2002. Distribution of mating type alleles in the wheat pathogen *Mycosphaerella graminicola* over spatial scales from lesions to continents. *Fungal Genetics and Biology* **36**: 128–136.

Zhang Z, Schwartz S, Wagner L, Miller W, 2000. A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* **7**: 203–214.

Zhou X, de Beer ZW, Cibrian D, Wingfield BD, Wingfield MJ, 2004. Characterisation of *Ophiostoma* species associated with pine bark beetles from Mexico, including *O. pulvinisporum* sp. nov. *Mycological Research* **108**: 690–698.