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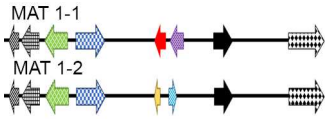

Heterothallism revealed in the root rot fungi *Berkeleyomyces basicola* and *B. rouxiae*

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Abstract

Berkeleyomyces basicola and *B. rouxiae,* two cryptic sister species previously treated collectively as *Thielaviopsis basicola,* reside in the *Ceratocystidaceae* (Microascales, Ascomycota). Both species are important plant pathogens that infect the roots of many important agricultural crops and ornamental plants. Although *T. basicola* has been known for more than 150 years, a sexual state has never been found and it has been assumed to be an asexual pathogen. The aim of this study was to determine the mating strategy of the two *Berkeleyomyces* species. Investigation of the genome sequences of two *B. basicola* isolates allowed for the complete characterization of the *MAT* locus. This revealed that it has a typical heterothallic mating system with the *MAT1-1* and *MAT1-2* idiomorphs occurring in different isolates. PCR amplification using mating type primers developed in this study, showed that the *MAT1-1-1* and *MAT1-2-1* genes were also present in different isolates of *B. rouxiae.* Pairing of isolates representing the two mating types of both *B. basicola* and *B. rouxiae* using a variety of techniques failed to produce sexual structures. Although we have found no direct evidence that they reproduce sexually, these fungi are clearly heterothallic with both mating types occurring in some countries suggesting that a cryptic sexual cycle could exist for them.

Keywords: Black root rot, Sexual reproduction, mating, plant pathogen, *MAT* locus

1. Introduction

Berkeleyomyces basicola and its sister species *B. rouxiae* are filamentous Ascomycete fungi that reside in the *Ceratocystidaceae* (Nel *et al.* 2018; De Beer *et al*. 2014). The two species are plant pathogens, causing a disease commonly known as black root rot on many important crops such as tobacco (Stover 1950), cotton (Pereg 2013) and carrots (AbdAllah *et al.* 2011). Despite being important pathogens of agricultural crops, both species are only known from their two asexual states: the first being the chlamydospore state that gives rise to distinct dark-coloured, club shaped spores only known to *Berkeleyomyces*; and the second being the endoconidial state characterized by phialides that give rise to endoconidia (Nel *et al.* 2018).

Until recently, these two pathogens were treated as a single species *Thielaviopsis basicola,* originally introduced by Ferraris (1912) based on morphological-characteristics. In a re-classification of the *Ceratocystidaceae,* De Beer *et al.* (2014) suggested that the generic level placement of the species required revision. This was because the isolate included in their study grouped outside *Thielaviopsis* and all other known genera in the Family. Based on phylogenetic and clear biological and morphological differences from other genera in the *Ceratocystidaceae*, Nel *et al.* (2018) described the new genus *Berkeleyomyces.* They also showed that isolates previously described as *T. basicola* represent two cryptic species. The epithet "*basicola"* was retained for the type species of the new genus and the second species was described as *B. rouxiae*.

Although [the former] *T. basicola* has been known for more than 150 years (Berkeley & Broome 1850) nothing is known regarding its mating strategy. Soon after the original description of the fungus, Zopf (1876) described a sexual state as *Thielavia (Th.) basicola*. However, during the early 1900's experiments involving single spore cultures were used to show that the sexual structures of *Thielavia* and the asexual structures of *Berkeleyomyces* belonged to two unrelated fungi (McCormick 1925, Lucas 1948, Lucas 1949, Stover 1950). There has subsequently been only a single report attempting to induce sexual structures in [the former] *T. basicola*. Johnson & Valleau (1935) performed co-culture experiments using multiple single spore isolates of the fungus but no sexual structures were observed over a period of more than one year. The absence of a sexual state has led to a view that the species represents an exclusively asexual pathogen (Tabachnik & DeVay 1980, Paulin & Harrington 2000, Paulin-Mahady *et al.* 2002).

Mating and sexual reproduction in filamentous Ascomycetes, including the *Ceratocystidaceae,* is controlled by a single region in the genome known as the mating type or *MAT* locus (Yoder *et al.* 1986, Wilken *et al.* 2017). This region can be present as two non-

homologous idiomorphs known as *MAT1-1* and *MAT1-2* (Yoder *et al.* 1986, Turgeon & Yoder 2000, Wilken *et al.* 2017). The *MAT1-1* idiomorph contains at least the *MAT1-1-1* open reading frame (ORF) encoding a protein with an α-box domain. Similarly, the *MAT1-2* idiomorph contains at least the *MAT1-2-1* ORF encoding a protein with an HMG-box domain (Butler 2007, Wilken *et al.* 2017). When both the *MAT1-1-1* and *MAT1-2-1* genes are found in the genome of a single isolate, the species is considered homothallic and sexual reproduction can take place independent of a second isolate (Butler 2007, Wilson *et al.* 2015a, Wilken *et al.* 2017). However, when the *MAT1-1-1* and *MAT1-2-1* genes occur in different isolates of a species, the species is considered heterothallic and at least two isolates, each containing one of the *MAT* idiomorphs, are required for sexual reproduction to take place. There are, however, exceptions to these norms. The first exception is known as mating type switching, whereby a single isolate contains both *MAT1-1-1* and *MAT1-2-1* genes in its *MAT* locus but this locus undergoes structural changes before sexual reproduction can occur (Wilken *et al.* 2014). The other exception is known as unisexuality, in which any isolate of a single mating type can undergo sexual reproduction without the need of an opposite mating type (Wilson *et al.*, 2015a, 2015b). Although the overall genetic organization at the *MAT* locus can vary among species, certain genes, such as those encoding the cytoskeleton assembly control protein (*SLA*), the AP endonuclease (*APN*), the anaphase promoting complex (*APC*), and subunit VIa of cytochrome C oxidase (*COX*), are frequently found flanking the *MAT* locus in species of the *Ceratocystidaceae* (Butler 2007, Wilken *et al.* 2017).

The *Ceratocystidaceae* includes examples of a wide variety of sexual reproductive strategies. These include species that are heterothallic (Wilson *et al.* 2015b, Wilken *et al.* 2018, Mayers *et al.* 2017) and primary homothallic (Mbenoun *et al.* 2014). Some species have secondary homothallic reproductive strategies including mating type switching (Harrington & McNew 1997, Witthuhn *et al.* 2000, Wilken *et al.* 2014) and unisexuality (Wilson *et al.* 2015b).

Against the background of this diverse array of reproductive strategies in the *Ceratocystidaceae*, we asked what the mating type strategy could be for the *Berkeleyomyces* species, and would the mating type locus suggest the absence of a sexual cycle as has previously been speculated. The aim of this study was thus to characterize the mating locus in these fungi and to consider their possible mating strategy. We also attempted to induce sexual reproduction of these species by carrying out various mating crosses.

2. Materials and Methods

2.1. Isolates

Cultures used in this study were obtained from various collections including the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, and the International Collection of Microorganisms from Plants (ICMP), New Zealand. Cultures (**Table 1**) were maintained on 2 % Malt Extract Agar (MEA – 2 % malt extract, 2 % Difco® agar Biolab, Midrand, South Africa).

2.2. Characterization of the *MAT1-2* idiomorph

The genome of *B. basicola* isolate CMW49352 (Wingfield *et al*. 2018) was investigated to identify the contig containing the *MAT* locus. The tBLASTx algorithm in CLCbio Genomics Workbench 9.5 (CLCbio, QIAGEN, Aarthus, Denmark) was used to screen the assembled genome for *MAT* idiomorph sequences using reference sequences for *MAT* genes from *Ceratocystis fimbriata* (GenBank accession number: APWK02000000; Wilken *et al*. 2013). The scaffold containing the greatest similarity to the reference sequences was extracted and annotated using Augustus 3.3.2 (Stanke *et al.* 2004) using the pre-optimized species model of *Fusarium graminearum*.. A BLASTp analysis was made using the NCBI database to identify each of the predicted protein sequences.

2.3. Genome sequencing and characterization of the *MAT1-1* idiomorph

2.3.1. Identification of putative *MAT1-1* idiomorphic isolates

CLCbio Genomics Workbench 9.5 (CLCbio, QIAGEN, Aarthus, Denmark) was used to design primers for the *MAT1-2-1* and *MAT1-2-7* genes identified in section 2.2, along with two additional *MAT* locus associated genes (*SLA* and a gene of unknown function [*UNK*]) (**Table 2**). Thirteen isolates of *B. basicola* and 26 isolates of *B. rouxiae* were screened by PCR, using these primers. Isolates that do not give positive PCR amplification for these *MAT1-2* genes were presumed to have *MAT1-1* idiomorph. DNA extraction and PCR amplification were conducted using the methods described by De Beer *et al.* (2014) and the annealing temperatures for the different primer pairs are indicated in **Table 2**.

2.3.2. Genome sequencing

Based on the amplification results for the different isolates, *B. basicola* isolate CMW25440 (putatively identified as containing the *MAT1-1* idiomorph) was selected for additional genome sequencing. A single spore culture of isolate CMW25440 (= CBS142829 collected in Indonesia from *Styrax benzoin*) was grown in YM broth (2 % malt extract, 0.5 % yeast extract, Biolab, Midrand, South Africa) for 3-5 days. Mycelium was collected in 1.5 mL Eppendorf tubes by centrifugation and lyophilized. The tubes containing the lyophilized

mycelium was submerged in liquid nitrogen and the mycelium ground to a fine powder using a sealed sterile pipette tip. Total genomic DNA was extracted from the powdered mycelium using the method described by Duong *et al.* (2013). A paired-end library (350 bp average insert size) was prepared and sequenced using the Illumina HiSeq2500 Platform at Macrogen Inc. (Seoul, Korea). The 101 bp pair-end reads obtained were assembled into contigs using SPAdes 3.9 (Bankevich *et al.* 2012) using default settings and k-mer sizes of 21, 33, 55 and 77. Completeness of the assembly was assessed using the Benchmarking Universal Single-Copy Orthologs (BUSCO 1.1b1) tool using the Ascomycota dataset (Simão *et al*., 2015).

2.3.3. Characterization of the *MAT1-1* idiomorph

The newly assembled genome of *B. basicola* isolate CMW25440 was investigated to identify the *MAT1-1* idiomorph using the same method described in section 2.2. In this case, the genes identified flanking the *MAT* locus in the genome of isolate CMW49352, as well as the *MAT1-1-1* gene from *C. fimbriata,* was used to screen the genome of isolate CMW25440 for the *MAT1-1* idiomorph. The scaffold containing the greatest similarity to the reference sequences was extracted and annotated again as described in section 2.2. and the putative genes were identified by BLASTp.

2.4. Development of multiplex PCR-based mating type markers for *Berkeleyomyces*

A primer set targeting the *MAT1-1-1* gene was designed based on the *MAT1-1* sequences identified from the genome of *B. basicola* isolate CMW25440 (**Table 2**). The primer parameters were selected to be compatible with multiplex PCR amplification together with the *MAT1-2-1* primer set designed in section 2.3.1. The *MAT1-1-1* primer pair was selected to produce a longer amplicon size but with the same annealing temperature as the *MAT1-2-1* primer pair. These primers were combined in a multiplex PCR reaction following the protocol described by Duong *et al.* (2013) with an annealing temperature of 56 °C for all isolates of *B. basicola* and *B. rouxiae*. From the PCR result, three putative *MAT1-1-1* and three *MAT1-2-1* amplicons from *B. rouxiae* isolates were selected for Sanger sequencing to confirm the identity of the amplified products. Sequencing reactions were carried out following the protocol described in De Beer *et al.* (2014)

2.5. Comparison and phylogenetic analyses of the HMG and α-box amino acid sequences

The *MAT* locus and its flanking regions were extracted and annotated from the available genomes of various species in the *Ceratocystidaceae* (**Table 3**) in the same manner as

described in section 2.2. The amino acid sequences of the HMG and α-box were identified using the conserved domain function of BLASTp. Datasets were compiled and aligned to the HMG and α-box amino acid sequences of *B. basicola* and *B. rouxiae* using the online version of MAFFT v.7.0 (Katoh & Standley 2013). Neighbour-joining analyses were performed on the amino acid alignments using MEGA v.6.0 (Tamura *et al.* 2013) using the poisson method with 1000 bootstrap replicates.

2.6. Mating crosses

2.6.1. On carrots

The mating types of *B. basicola* and *B. rouxiae* isolates were determined using the newly developed multiplex PCR primers. Isolates, of opposite mating type, were selected and paired on carrot slices in an attempt to induce mating. Thirteen isolates of *B. basicola* (three *MAT1-1* and 10 *MAT1-2*) and 26 isolates of *B. rouxiae* (18 *MAT1-1* and 8 *MAT 1-2*) were paired in all possible combinations on carrot pieces. The experiment was conducted in triplicate for each pair of isolates.

Whole carrots were surface disinfected using 70 % ethanol and these were cut into 5 cm thick pieces. These pieces were halved lengthwise and rinsed in a solution of streptomycin (0.3 g Streptomycin, 500 mL distilled water). The pieces were air dried after which isolates representing either the *MAT1-1* or *MAT1-2* were inoculated on the surface of one half approximately 2 cm apart. The uninoculated half carrot slice was then placed against the inoculated half and the pieces were wrapped with parafilm to form a tight “sandwich”. The carrot “sandwiches” were placed in a sealed sterile plastic container and incubated for two weeks at ± 20 °C, after which the halves were separated and inspected for the presence of sexual structures. Once inspected, the carrot halves were returned to the plastic containers and incubated for a further two weeks after which they were again inspected for sexual structures.

Because sexual structures were not observed during the initial incubation period, the inoculated carrot slices were transferred to a 4 °C incubator for 12 weeks after which the experiment was terminated.

2.6.2. In culture

Isolates, of opposite mating type, were selected and paired in culture in an attempt to induce mating. Thirteen isolates of *B. basicola* (three *MAT1-1* and 10 *MAT1-2*) and 15 isolates of *B. rouxiae* (nine *MAT1-1* and six *MAT1-2*) were paired in all possible combinations on 0.2 % MEA, 10 % Carrot agar (900mL MEA and 100 mL organic carrot juice, Rugani, South Africa), and 2 % MEA supplemented with Thiamine (200 mg/L, Sigma Aldrich). The

experiment was conducted in triplicate for each pair of crossed isolates. A total of 90 combinations of *B. basicola* and 162 combinations of *B. rouxiae* were tested on each medium.

Inoculum for the pairing experiment was taken from a two-week-old culture. Squares (3mm x 3mm) of agar bearing mycelium of the test isolates were inoculated 25 mm apart from one another in the centre of Petri dishes. The paired isolates were incubated for one month at 20 °C (± 2 °C) and inspected regularly for the presence of sexual structures. After one month, the plates were transferred to a 10 °C incubator to induce a stress response that might give rise to sexual structures. The paired isolates were inspected monthly for the appearance of sexual structures for six months.

3. Results

3.1. Characterization of the *MAT1-2* idiomorph of *B. basicola*

tBLASTx searches using the reference *Ceratocystis fimbriata MAT1* gene sequences identified highly similar sequences to *MAT1-2-1* in scaffold 18 of the genome of isolate CMW49352 (Wingfield *et al.* 2018). Annotation of the 2 Mb scaffold using Augustus 3.3.2 identified 1001 putative ORFs. BLASTp of the predicted protein sequences identified the structure of the *MAT1-2* idiomorph. A *MAT1-2-1* and a *MAT1-2-7* encoding ORF was identified, flanked at one side by *COX*, *APN*, *APC*, and *SLA* encoding genes, and at the other side by a gene of unknown function (*UNK*) and an importin gene (*IMP*) (**Fig. 1A**).

3.2. Genome sequencing and characterization of the *MAT1-1* idiomorph of *B. basicola*

Illumina sequencing of the genome of the putative *MAT1-1* isolate (CMW25440), produced approximately 39.6 million paired reads. *De novo* assembly of the genome using SPAdes 3.9 produced 841 scaffolds longer than 500 bases. The assembly produced a genome of approximately 25.1 Mb in size with an N50 of 90Kb and a GC content of 52%. This draft assembly was similar in size to that available for *B. basicola* (Wingfield *et al.* 2018). BUSCO analysis predicted an assembly completeness of 97%. The assembly contained 1276 complete single-copy BUSCOs, 1 complete and duplicated BUSCO, 10 fragmented BUSCOs and 28 missing BUSCOs from a total of 1315 BUSCO groups searched.

tBLASTx searches using the identified *MAT* flanking genes from isolate CMW49352 and *MAT1-1-1* from *C. fimbriata* identified highly similar sequences in scaffold 63 of the genome of isolate CMW25440. Annotation of the 100Kb scaffold identified 41 putative ORFs. BLASTp of the predicted protein sequences allowed for the identification of the *MAT1-1* idiomorph. The organization of the genes flanking the *MAT1-1* from CMW49352 and *MAT1-2*

from CMW25440 were almost identical (**Fig. 1B**) and only the presence of the different *MAT* genes allowed for delineation between the two isolates.

In both the *MAT1-1* and *MAT1-2* isolates, the region from the *COX* encoding gene to the *IMP* encoding gene spanned about 31Kb and closely resembled that of the *MAT* flanking regions in other *Ceratocystidaceae* species (**Supplementary figure 1**). The predicted *MAT1-2-1* gene was 909 bases long, composed of three exons with a combined sequence length of 798 bases and two introns (53 bases and 55 bases). The predicted protein sequence included 265 amino acids and harboured the characteristic HMG-box domain. The predicted *MAT1-2-7* gene was 857 bases long, composed of three exons with a sum sequence length of 603 bases and two introns (53 bases and 201 bases). The predicted *MAT1-2-7* protein sequence included 200 amino acids and contained no known conserved structural domains. The predicted *MAT1-1-1* gene was 1233 bases long, composed of two exons with a total sequence length of 1176 bases with a single intron of 56 bases. The predicted protein sequence included 391 amino acids and harboured the characteristic α-box. The predicted *MAT1-1-2* gene region was 1518 bases long, composed of five exons of 1284 bases in total and four introns (59 bases, 57 bases, 141 bases and 59 bases). The predicted protein sequence included 427 amino acids and contained the HPG/PFF pfam domain (accession pfam17043). The scaffold sequences, together with their *MAT* gene annotations, were deposited in NCBI GenBank (**Table 1**).

3.3. Multiplex and other PCRs

The *MAT1-1-1* and *MAT1-2-1* primer pairs were successfully used in multiplex amplification of the *MAT* genes from both *Berkeleyomyces* species. Multiplex amplification of 13 *B. basicola* isolates identified three isolates containing the *MAT1-1-1* gene region and ten isolates containing the *MAT1-2-1* gene region (**Fig. 2A**). Multiplex amplification of 26 *B. rouxiae* isolates identified 18 isolates containing the *MAT1-1-1* gene region and eight isolates containing the *MAT1-2-1* gene region (**Fig. 2B**).

Additional support for the identity of *MAT1-1* and *MAT1-2* idiomorph isolates was provided by PCR amplification of the *MAT1-2-7* gene region. This region was successfully amplified in all the isolates where the *MAT1-2-1* region was amplified, and no amplification was observed in isolates where the *MAT1-1-1* region was amplified (**Supplementary figure 2A**). The *SLA* gene and the conserved *UNK* region were successfully amplified for all *B. basicola* and *B. rouxiae* isolates included in this study (**Supplementary figure 2B & 2C**).

From the multiplex PCR results, we selected three *MAT1-1-1* and three *MAT1-2-1* containing isolates for MAT amplicon sequencing. Sequencing of the *MAT1-1-1* region produced a product of around 810 base-pairs spanning the entire α-box region (**Fig. 2A**). Sequencing of

the *MAT1-2-1* region produced a product of around 500 base-pairs containing the intron region and encoding 53 of the 81 amino acids of the HMG-box domain (**Fig. 2B**).

3.4. Comparison and phylogenetic analyses of the HMG and α-box amino acid sequences

Neighbour joining analyses of the HMG-box and α-box amino acid sequences for various isolates of the *Ceratocystidaceae* produced phylogenetic trees with similar topology (**Fig. 4 A & B**). In both trees, *B. basicola* and *B. rouxiae* grouped together with high bootstrap support. The included *Ceratocystis* species formed a strongly supported sub-group to the *Berkeleyomyces* species, except for *C. adiposa* that grouped together with *Huntiella* species in both trees.

3.5. Mating experiments

None of the pairings between the identified *MAT1-1* and *MAT1-2* idiomorph isolates of *B. basicola* and *B. rouxiae* resulted in the production of sexual structures under any of the conditions tested. All isolates produced abundant mycelium, asexual endoconidia and chlamydospores. In many cases a zone of vegetative incompatibility developed between the isolates preventing the mycelium of the different isolates colonizing a plate from making contact.

4. Discussion

Whole genome sequencing and investigation of the genomes of two *B. basicola* isolates allowed for the identification and characterization of the *MAT* locus for *Berkeleyomyces.* From the genome of *B. basicola* isolate CMW49352 (Wingfield *et al.* 2018) we were able to identify and characterize the *MAT1-2* idiomorph. From genome sequencing results of *B. basicola* isolate CW25440, we were further able to identify and characterize the *MAT1-1* idiomorph. The presence of the *MAT1-1* and *MAT1-2* idiomorphs in different isolates of *B. basicola* is similar to that of other species of *Ceratocystidaceae* and other ascomycete fungi that have a heterothallic mating system. This heterothallic mating systems was found in both *B. basicola* and *B. rouxiae*. Although no sexual state is currently known for these closely related species, the presence of an apparently functional mating-type locus in these species suggest that cryptic sexual reproduction could occur in nature (Kuck & Poggeler 2009, Dyer & Kuck 2017). However, more in-depth sampling of natural populations followed by various population genetic analyses are needed to determine if mating is taking place in nature.

Characterization of the genes located at the *MAT* locus of two isolates of *B. basicola* allowed us to elucidate the structure of both the *MAT1-1* and *MAT1-2* idiomorphs for the fungus. Each idiomorph was flanked on one side by the *COX APN*, *APC*, and *SLA* genes and on the

other side by a gene of unknown function (here referred to as *UNK*) and an *IMP* gene. This arrangement is very similar to that of *Ceratocystis fimbriata* (Wilken *et al.* 2014) and appears to be conserved among the various genera of the *Ceratocystidaceae* and *Gondwanamycetaceae* (Aylward *et* al. 2016, Wilken *et al.* 2018, Wilson *et al*. 2015b). Our results also showed that *B. rouxiae,* the cryptic sister species of *B. basicola*, is also heterothallic. Although the *MAT* locus of *B. rouxiae* was not fully characterised, using PCR and sequencing it was possible to confirm that the *MAT1-2* idiomorph in *B. rouxiae* contains homologs to the *MAT1-2-1* and *MAT1-2-7* genes and that the *MAT1-1* idiomorph contains a homolog of the *MAT1-1-1* gene. We also confirmed the presence of homologs to the *SLA* and *UNK* genes in *B. rouxiae*.

The *MAT1-1* idiomorph of *B. basicola* included the additional *MAT* gene, *MAT1-1*-2 originally described from *Neurospora crassa* (Turgeon & Yoder 2000). Like many other heterothallic species in the *Ceratocystidaceae* the gene was located adjacent to the *MAT1-1-1* gene. We were able to identify the conserved HPG/PFF domain in this gene. Like the *MAT1-1-2* gene, its conserved domain was also first identified in *Neurospora crassa* (Turgeon & Yoder 2000). The *MAT1-2* idiomorph included the *MAT1-2-7* originally described in *Huntiella omanensis* (Wilson *et al*. 2015b). This gene was located adjacent to *MAT1-2-1*, but is in the opposite orientation. Prior to the present investigation, a full length *MAT1-2-7* gene had been characterized only in *Huntiella omanensis* and a truncated version of this gene had been characterized in *Huntiella moniliformis* (Wilson *et al.* 2015b). The present study revealed homologs of this gene in both *Berkeleyomyces* species, as well as putative homologs to this gene in other species of *Ceratocystidaceae* where they had not previously been identified.

Alignment of the α-box amino acid sequences in *B. basicola, B. rouxiae* and various other genera in the *Ceratocystidaceae* showed relatively low conservation of this region. On the other hand, alignment of the HMG-box amino acid sequences showed that the region is more conserved than that of the α-box region, but overall conservation between the genera was low. A similar situation was seen when aligning the HPG/PFF domain amino acid sequences (results not shown). The low level of conservation of these conserved domains among genera was not unexpected and has for example been found in the *Ophiostomatales* (Duong *et al.* 2013) and *Pleosporales* (Yun *et al.* 2013).

Phylogenetic analyses of the α-box amino acid sequences and HMG-box amino acid sequences grouped *B. basicola* and *B. rouxiae* distinct from the other *Ceratocystidaceae* genera. This region had little resolution and the two species could not be separated in these analyses. The trees generated from these sequences were also not congruent, which is consistent with the findings of other studies (Goodwin *et al.* 2003, Yokoyama *et al.* 2006,

Bihon *et al.*, 2014). Mating type genes are under selective and evolutionary pressure (Duong *et al.* 2013, Bihon *et al.* 2014), which makes them unsuitable for phylogenetic analyses (Schmitt *et al.* 2009, Stielow *et al.* 2015).

It was not possible to induce sexual structures in cultures of either *B. basicola* or *B. rouxiae,* even though isolates of known opposite mating type were paired in many different combinations and grown under a relatively wide range of conditions. While these experiments provide some evidence that sexual reproduction does not occur in *B. basicola* or *B. rouxiae*, many different biological, ecological and environmental factors could influence the ability of these fungi to reproduce sexually. Various other fungi known to include isolates of two different mating types typical of heterothallism have yet to reveal sexual states (Gordon 2017). In some cases such as *Aspergillus fumigatus,* many years passed before sexual structures were found even though the fungus was known to be heterothallic (Paoletti *et al.* 2005, O'Gorman *et al.* 2009). For the present, it is not possible to know whether sexual reproduction can occur in the two cryptic species of *Berkeleyomyces*.

PCR amplification of the *MAT* genes using the mating type markers developed in this study identified three *MAT1-1* and ten *MAT1-2* isolates of *B. basicola,* and 18 *MAT1-1* and eight *MAT1-2* isolates of *B. rouxiae* in our collection of isolates. Our collection of isolates of the two species showed that both *B. basicola* and *B. rouxiae* occur in South Africa and the Netherlands. Available isolates of *B.* basicola from these countries included only a single mating type. In contrast, the collection of isolates of *B. rouxiae* from South Africa and New Zealand included both *MAT1-1* and *MAT1-2* isolates. This implies that sexual reproduction could occur in *B. rouxiae* in these countries. A population genetics study of a sufficiently large collection of isolates would shed light on this question.

Conflict of interest

The authors report no conflict of interest.

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Figures Captions

Figure 1: The structure of the *MAT1-2* and *MAT1-1* idiomorphs of *B. basicola* elucidated from its genomes. (**A**) The structure of the *MAT1-2* idiomorph and its flanking genes was identified from the genome of CMW49352; (**B**) The structure of the *MAT1-1* idiomorph and its flanking genes was identified from the genome of isolate CMW25440.

Figure 2: Results obtained from a multiplex PCR assay to determine the *MAT-1* genotype of isolates of *B. basicola* and *B. rouxiae.* (**A**) Electrophoresis gel for 13 isolates of *B. basicola;* (**B**) Electrophoresis gel for 13 isolates of *B. rouxiae.* The molecular weight maker used was a GeneRuler 100 bp DNA Ladder (Fermentas). Fragments were separated on a 2 % agarose gel stained with GelRed and visualized under UV light. The band sizes of 1 kb, 500 and 100 bp are indicated for the molecular weight marker lane. Amplification of the *MAT1-1-1* gene region produced an amplicon size of 845 bp and amplification of the *MAT1-2-1* gene region produced an amplicon size of 530 bp.

Figure 3: Alignment of the amino acid sequences of the (**A**) α-domain and the (**B**) HMG-box domain from *B. basicola, B. rouxiae* and other *closely related* species..

Figure 4: Neighbour joining trees derived from phylogenetic analyses of the (**A**) α-domain and (**B**) HMG-box amino acid sequences for various *Ceratocystidaceae* species including *B. basicola* and *B. rouxiae*. Bootstrap values above 75 % are indicated at nodes.

Tables

Table 1: Isolates included in this study with the GenBank accession numbers of the respective *MAT* gene sequences obtained for the different isolates

Current name	Collection number	Other collection numbers	Country	Idiomorph	GenBank accession numbers
					MAT scaffolds
Berkleyomyces basicola	CMW4098[a]		Ecuador	*MAT1-2*	
	CMW5896[a]		Uganda	*MAT1-2*	
	CMW6714[a]		Australia	*MAT1-2*	
	CMW7065[a]	CBS341.33/MUCL9545	Netherlands	*MAT1-2*	
	CMW7067[a]	CBS487.48/MUCL9542	Belgium	*MAT1-2*	
	CMW7069[a]		Netherlands	*MAT1-2*	
	CMW49352[a]	CBS142796	Netherlands	*MAT1-2*	MH522765
	CBS414.52[a]	MUCL8363	Netherlands	*MAT1-2*	
	CBS430.74[a]	CMW7071	Netherlands	*MAT1-2*	
	CMW25439[a]		Indonesia	*MAT1-1*	
	CMW25440[a]	CBS142829	Indonesia	*MAT1-1*	MH522766
	CMW26479[a]		Indonesia	*MAT1-1*	
	CMW51564[a]		South Africa	*MAT1-2*	
					MAT1-1-1 or *MAT1-2-1*
Berkeleyomyces rouxiae	CBS118120[a]		South Africa	*MAT1-2*	
	CMW5472[a]	CBS117825	Ethiopia	*MAT1-2*	MH522760
	CMW7064[a]	CBS194.26/MUCL9544	Unknown	*MAT1-1*	MH522756
	CMW7066[a]	CBS342.33/MUCL9456	Netherlands	*MAT1-1*	
	CBS413.52[a]		Netherlands	*MAT1-1*	
	CBS150.67[a]	IHEM3832	Switzerland	*MAT1-1*	
	CMW7622	CBS117826	South Africa	*MAT1-1*	
	CMW7623	CBS118119	South Africa	*MAT1-1*	MH522757
	CBS117827		South Africa	*MAT1-1*	
	CMW7625[a]	CBS117828	South Africa	*MAT1-1*	
	CMW14219[a]		Chile	*MAT1-2*	
	CMW14220		Chile	*MAT1-2*	
	CMW14221	CBS142830	Chile	*MAT1-2*	MH522761
	CMW14222		Chile	*MAT1-2*	
	CMW14223		Chile	*MAT1-2*	

Current name	Collection number	Other collection numbers	Country	Idiomorph	GenBank accession numbers
	CMW44562		South Africa	*MAT1-1*	
	CMW44563		South Africa	*MAT1-1*	
	CMW44564		South Africa	*MAT1-1*	
	CMW44565		South Africa	*MAT1-1*	
	CMW44566[a]		South Africa	*MAT1-1*	MH522758
	CMW44567		South Africa	*MAT1-1*	
	CMW44568		South Africa	*MAT1-1*	
	CMW44569		South Africa	*MAT1-1*	
	ICMP2460[a]		New Zealand	*MAT1-1*	
	ICMP13276[a]		New Zealand	*MAT1-2*	MH522762
	CBS178.86[a]	MUCL40417	Canada	*MAT1-1*	

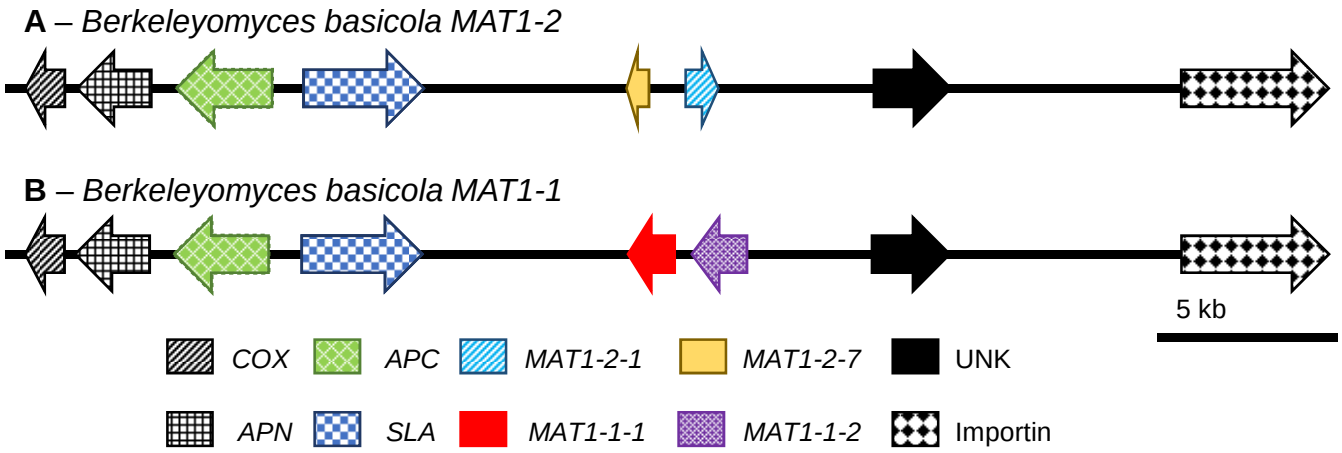
[a]Isolates used in single mating crosses

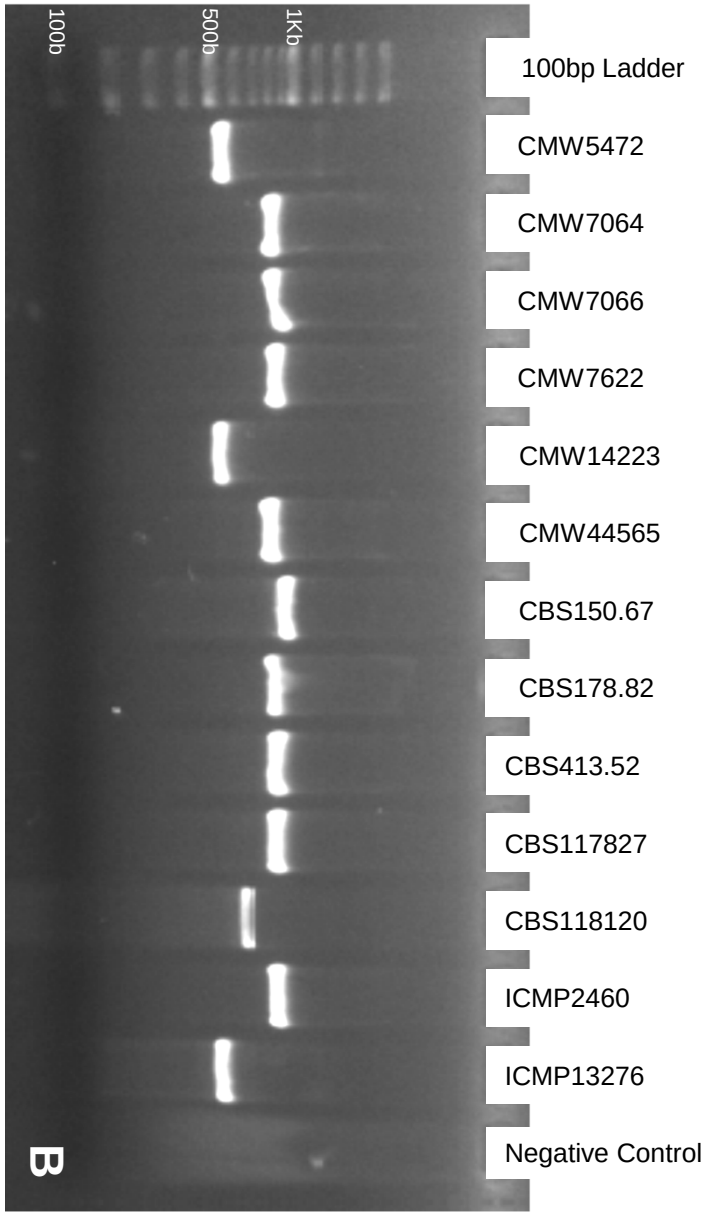
Table 2: Newly designed primer sets for amplification of *Berkeleyomyces* mating genes

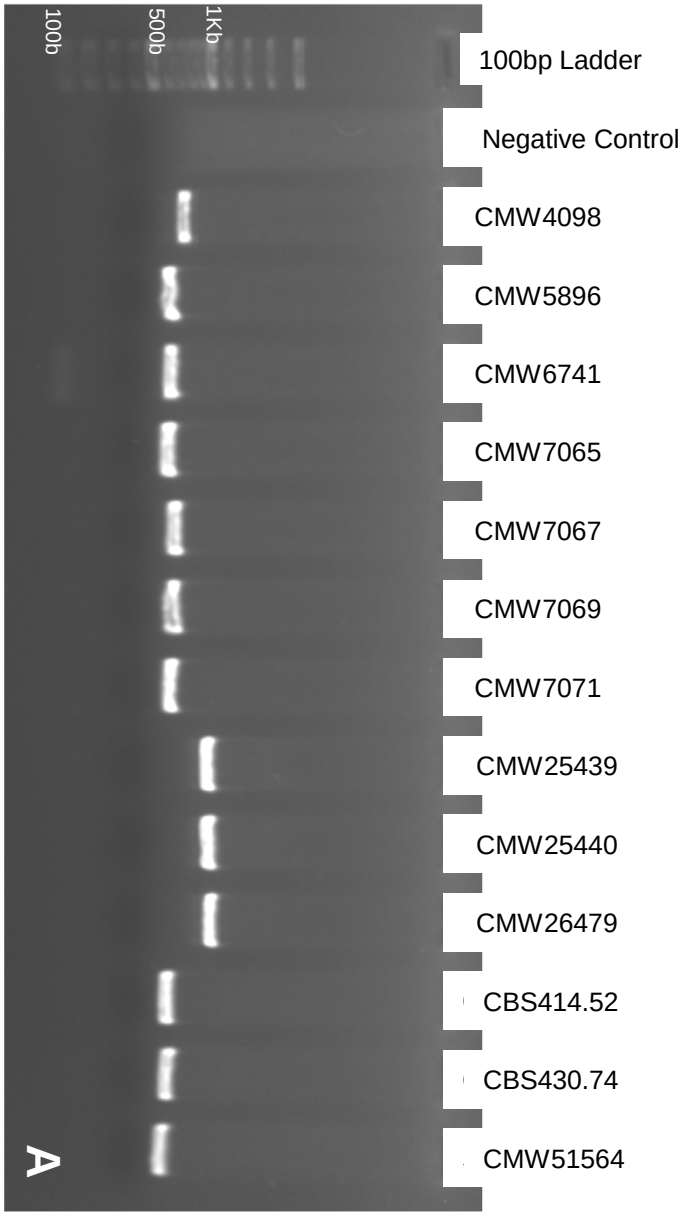
Name	Sequence (5' → 3')	Annealing Temperature (°C)	Fragment length (bp)
Tbas_SLA_F	CAACACCAAGGGTCTACTCCG	59	887
Tbas_SLA_R	TCCACCTGCTGCTCCATCTC		
Tbas_M127_F	TGAAGGAAGTAAGTCCGCACAG	59	445
Tbas_M127_R	GGGAAACTCAACCCAGAAGC		
Tbas_Unk_F	GACTGCCTACATCGCCTACC	57	1088
Tbas_Unk_F	TTGCCGTCACTACCAACCTG		
Tbas_M121_F	AAGACTTTACTCCGTGACTTTAGG	56	530
Tbas_M121_R	CCAATTCTTGATAGTGGGTGC		
TBAS_M111_F	GCTGAAATGGGTGGTGTT	56	845
TBAS_M111_R	CTTGGTTTTGGTTGGGTTG		

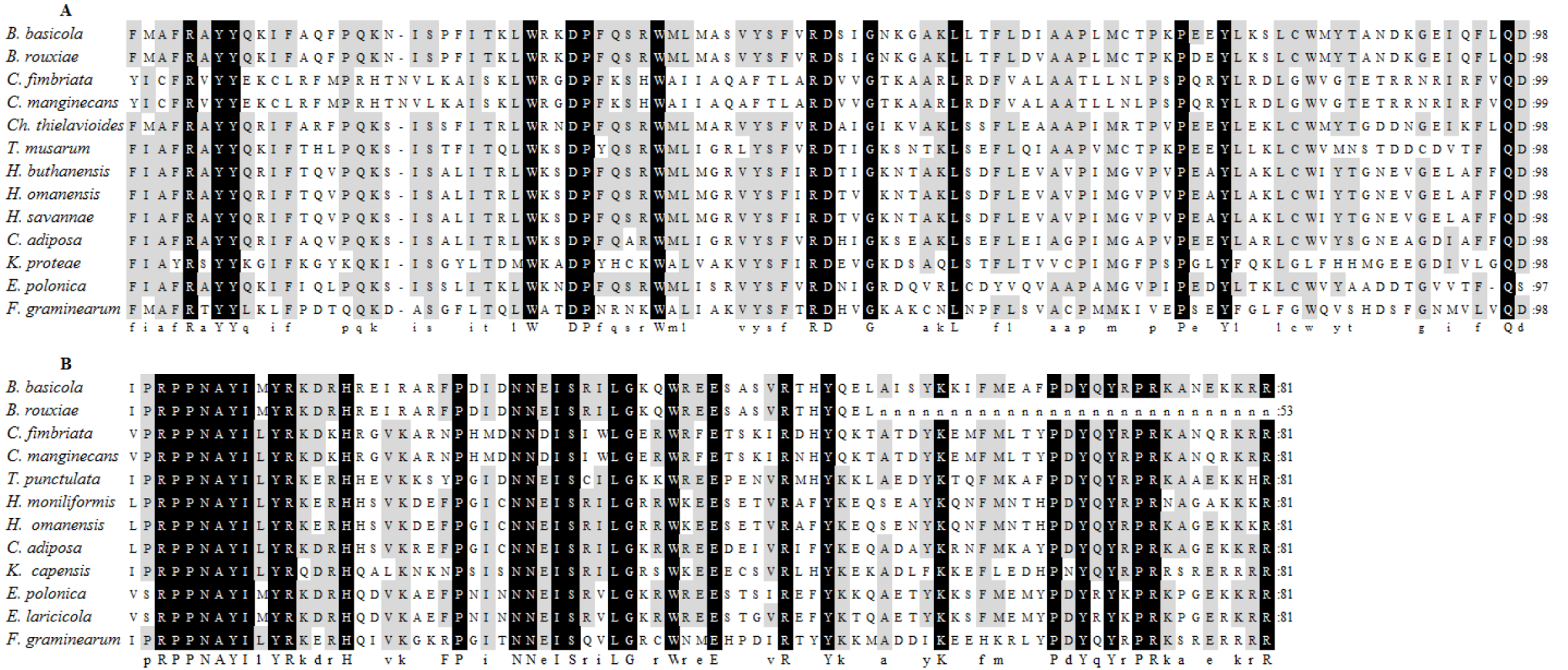
Table 3: Accession numbers of Ceratocystidaceae and Gondwanamycetaceae genomes used in this study

Species	Genome accession number	Reference
Ceratocystis adiposa	LXGU01000000	Wingfield *et al.* 2016a
C. fimbriata	APWK00000000	Wilken *et al.* 2013
C. manginecans	JJRZ00000000	Van der Nest *et al.* 2014b
Chalaropsis thielavioides	BCGU00000000	Unpublished
Endoconidiophora laricicola	LXGT00000000	Wingfield *et al.* 2016a
E. polonica	LXKZ01000000	Wingfield *et al.* 2016a
Huntiella buthanensis	MJMS00000000	Wingfield *et al.* 2016b
H. moniliformis	JMSH00000000	Van der Nest *et al.* 2014b
H. omanensis	JSUI00000000	Van der Nest *et al.* 2014a
H. savannae	LCZG00000000	Van der Nest *et al.* 2015
Thielaviopsis musarum	LKBB00000000	Wingfield *et al.* 2015a
T. punctulata	LAEV00000000	Wingfield *et al.* 2015b
Knoxdaviesia capensis	LNGK00000000	Aylward *et al* 2016
K. proteae	LNGL00000000	Aylward *et al* 2016

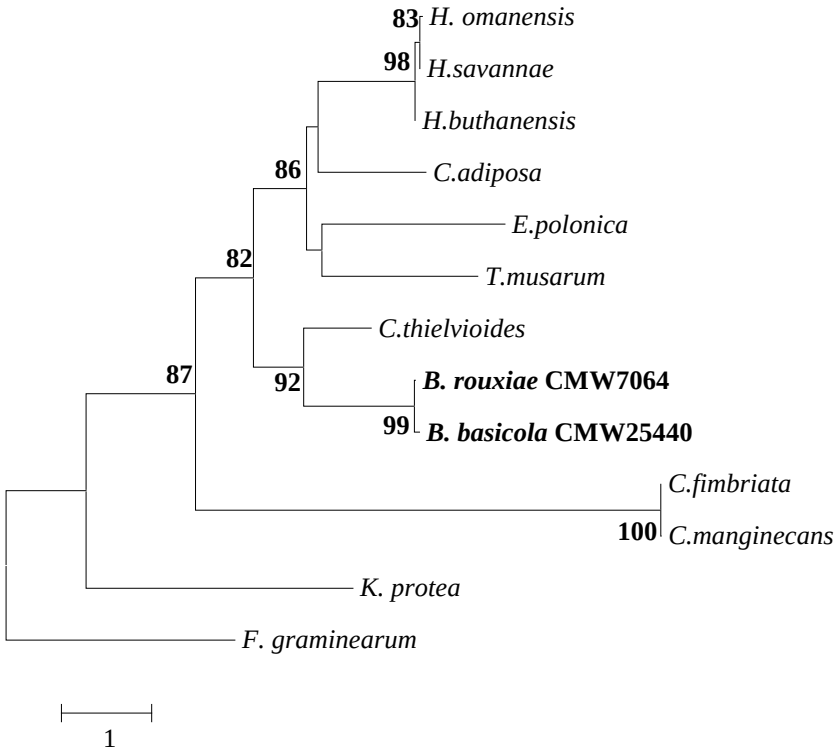




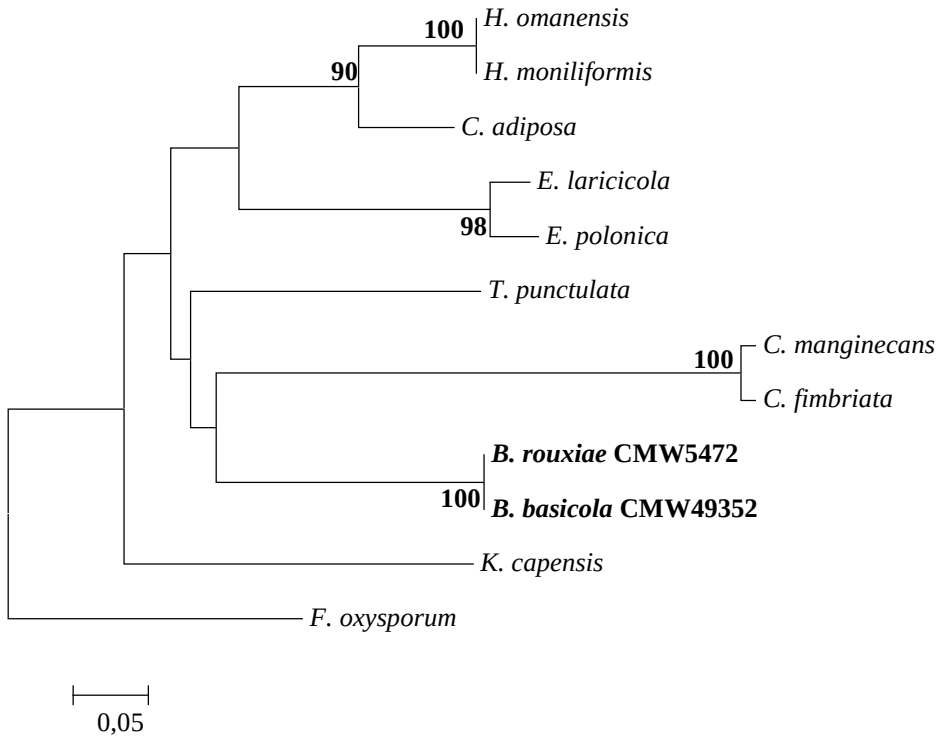



A
B. basicola
B. rouxiae
C. fimbriata
C. manginecans
Ch. thielavioides
T. musarum
H. buthanensis
H. omanensis
H. savannae
C. adiposa
K. proteae
E. polonica
F. graminearum
B
B. basicola
B. rouxiae
C. fimbriata
C. manginecans
T. punctulata
H. moniliformis
H. omanensis
C. adiposa
K. capensis
E. polonica
E. laricicola
F. graminearum

A
***MAT1-1-1* α-domain**
Neighbour-Joining
1000 bootstraps



B
***MAT1-2-1* HMG-box**
Neighbour-Joining
1000 bootstraps



- The mating type loci of *Berkeleyomyces basicola* and *B. rouxiae* were characterized.
- Both species were found to have the genes required for heterothallic mating.
- The sexual states of neither species have been observed in nature and could not be induced in laboratory crosses.
- Primers were designed that can distinguish the two mating types in multiplex PCR.