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# Genetic basis for high population diversity in *Protea*-associated *Knoxdaviesia*

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ABSTRACT

Sexual reproduction is necessary to generate genetic diversity and, in ascomycete fungi, this process is controlled by a mating type (*MAT*) locus with two complementary idiomorphs. *Knoxdaviesia capensis* and *K. proteae* (Sordariomycetes; Microascales; Gondwanamycetaceae) are host-specific saprophytic fungi that show high population diversity within their *Protea* plant hosts in the Cape Floristic Region of South Africa. We hypothesise that this diversity is the result of outcrossing driven by a heterothallic mating system and sought to describe the *MAT1* loci of both species. The available genome assembly of each isolate contained only one of the *MAT1* idiomorphs necessary for sexual reproduction, implying that both species are heterothallic. Idiomorph segregation during meiosis, a 1:1 ratio of idiomorphs in natural populations and mating experiments also supported heterothallism as a sexual strategy. Long-range PCR and shot-gun sequencing to identify the opposite idiomorph in each species revealed no sequence similarity between *MAT1-1* and *MAT1-2* idiomorphs, but the homologous idiomorphs between the species were almost identical. The *MAT1-1* idiomorph contained the characteristic *MAT1-1-1* and *MAT1-1-2* genes, whereas the *MAT1-2* idiomorph consisted of the genes *MAT1-2-7* and *MAT1-2-1*. This gene content was similar to that of the three species in the Ceratocystidaceae (Microascales) with characterized *MAT* loci. The *Knoxdaviesia MAT1-2-7* protein contained and alpha domain and predicted intron, which suggests that this gene arose from *MAT1-1-1* during a recombination event. In contrast to the Ceratocystidaceae species, *Knoxdaviesia* conformed to the ancestral Sordariomycete arrangement of flanking genes and is, therefore, a closer reflection of the structure of this locus in the Microascalean ancestor.



## 1. Introduction

Sexual reproduction is universal across eukaryotic life, despite being more biologically costly than asexual propagation (Lehtonen et al., 2012; Ni et al., 2011; Otto, 2009; Stearns, 1987). Most fungi maintain both of these reproductive strategies that are controlled by diverse genetic mechanisms (Billiard et al., 2012; Ni et al., 2011). Evidence suggests that many fungi exploit the low cost of clonal reproduction during favourable environmental conditions, but switch to sexual reproduction under stress when adaptation becomes necessary (Ni et al., 2010; Nielsen and Heitman, 2007; Seymour et al., 2005). Novel allele combinations are essential for adaptation and the re-shuffling of genetic material enables selection against harmful or unfavourable genotypes that may be propagated through clonal reproduction (Lynch et al., 1993).

The mating type (*MAT*) genes of fungi control the recognition between sexual partners and the subsequent development of sexual progeny (Coppin et al., 1997; Perkins, 1987). In ascomycetes, mating type is determined by a single locus, *MAT1*, and two mating type idiomorphs (dissimilar alleles), *MAT1-1* and *MAT1-2* (Kronstad and Staben, 1997; Nelson, 1996; Turgeon and Yoder, 2000). Homothallic fungi have a *MAT1-1/2* genotype (Turgeon and Yoder, 2000); both *MAT1* idiomorphs occur in one genome, making them self-fertile. In heterothallic species, the absence of either idiomorph results in self-sterility that necessitates outcrossing

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between two individuals of opposite mating type for sexual reproduction (Kronstad and Staben, 1997; Nelson, 1996). Although it is widely accepted that the presence of both idiomorphs is necessary for sexual reproduction, fungal mating strategies are diverse and many exceptions to this rule have been observed (Heitman, 2015). For example, some species are self-fertile despite only possessing one *MAT1* idiomorph (unisexual reproduction; Alby and Bennett, 2011; Glass and Smith, 1994; Lin et al., 2005; Wilson et al., 2015).

The two idiomorphs of the ascomycete *MAT1* locus each contain at least one open reading frame (ORF) with a characteristic motif (Turgeon and Yoder, 2000). The *MAT1-1* idiomorph is defined by an ORF with an alpha domain (*MAT1-1-1*), although up to two additional "accessory" ORFs can occur in this idiomorph. The *MAT1-2* idiomorph generally has a single ORF (*MAT1-2-1*) with an HMG-box motif. The functions of each of these *MAT1* genes is not fully understood, but it is believed that each idiomorph encodes transcription factors (Herskowitz, 1989) that ultimately perform a dual function. Firstly, the transcription factors mediate a hormonal recognition mechanism between individuals by producing a pheromone and receptors for the pheromone of the opposite mating type (Coppin et al., 1997; Glass et al., 1990; Ni et al., 2011). Secondly, these genes are involved in the formation of sexual structures (Coppin et al., 1997). Molecular studies have shown that the *MAT1-1-1* gene alone is able to induce fertilization, but in *Podospora anserina*, the accessory genes analogous to *MAT1-1-2* and *MAT1-1-3* are needed for the sexual structures to develop fully (Debuchy et al., 1993), supporting the dual function. As the only consistently occurring ORF on the *MAT1-2* idiomorph, *MAT1-2-1* appears to be involved in ascomatal development and is the sole determinant of the necessary functions in this mating type (Coppin et al., 1997; Staben and Yanofsky, 1990).

Of the five Microascales (Sordariomycetes) families (Maharachchikumbura et al., 2015; Réblová et al., 2011) only three species in the predominantly plant-associated, agriculturally important Ceratocystidaceae (De Beer et al., 2014) have been studied extensively in terms of mating type genetics. The sweet potato pathogen, *Ceratocystis fimbriata s.s.*, is homothallic and undergoes unidirectional mating type switching whereby it loses its *MAT1-2-1* gene and becomes self-sterile (Harrington and McNew, 1997; Wilken et al., 2014). The other two Ceratocystidaceae species studied are members of the genus *Huntiella* that typically show a saprophytic association with tree wounds (Van Wyk et al., 2006). Both of the studied species in this genus are heterothallic (Wilson et al., 2015), although *H. moniliformis* is also capable of unisexual reproduction; since it contains a single *MAT1* idiomorph, yet produces ascomata (Wilson et al., 2015). One trait that unites the diverse mating strategies in Ceratocystidaceae is their deviation from the consensus gene order of the Sordariomycetes. The cytoskeleton assembly control (*SLA2*) and DNA lyase (*APN2*) genes that flank the *MAT* locus in almost all Sordariomycetes (Debuchy and Turgeon, 2006) have an altered order and orientation in the Ceratocystidaceae. The genes that typically flank the downstream region of *MAT1* loci have shifted to an upstream position in *C. fimbriata*. A similar shift is evident in the two studied *Huntiella* species, although the *APN2* gene has shifted to a genomic position far from the *MAT1* locus (Wilson et al., 2015).

The aim of this study was to describe the *MAT1* locus of two saprophytic, but host-specific species in the Gondwanamycetaceae, which is also a member of the Microascales (Réblová et al., 2011). These fungi (*Knoxdaviesia capensis* and *K. proteae*) occur in the seed cones of *Protea*, a keystone plant genus in the Cape Floristic Region of South Africa (Bergh et al., 2014; Cowling, 1992; Manning and Goldblatt, 2012). The arthropod, and possibly bird, vectors of these fungi disperse ascospores between *Protea* flower heads (Roets et al., 2011b). After flowering, the *Protea* inflorescence matures into an enclosed seed cone in which *K. capensis* and *K. proteae* are visible on decaying floral structures as ascomata that present spore droplets on long ostiolar necks (Wingfield and Van Wyk, 1993; Wingfield et al., 1988). Although conidiophores may also be present, *K. capensis* and *K. proteae* sexual structures are abundant within infructescences (Wingfield and Van Wyk, 1993; Wingfield et al., 1988), indicating that sexual reproduction is prevalent and likely the dominant mode of reproduction at this stage of their life-cycle. As ascomycete fungi, *K. capensis* and *K. proteae* are haploid during the vegetative state and sexual reproduction would thus only add genetic diversity if it is not a result of self-fertilization (Fincham and Day, 1963; Milgroom, 1996; Moore and Novak Frazer, 2002). High gene and genotypic diversity and random allele association within two populations of *K. proteae* (Aylward et al., 2014, 2015b) and nine populations of *K. capensis* (Aylward, unpublished) strongly suggest that sexual reproduction in these species is non-selfing. *Protea*-associated *Knoxdaviesia* individuals, therefore, regularly recombine to produce genetically novel offspring, but whether outcrossing is a prerequisite for sexual reproduction in these species (*i.e.* heterothallism) or whether it is optional (*i.e.* homothallism) remains unknown.

The genomes of *K. capensis* and *K. proteae* have recently been sequenced (Aylward et al., 2016). In this study, we used these genomes to investigate the genetic basis of mating in *Knoxdaviesia*. In so doing, we tested the hypothesis that the genetic diversity observed in natural populations of these species is due to outcrossing driven by a heterothallic mating system. As a secondary aim, we compared the identified *Knoxdaviesia MAT1* loci to the three species in the Ceratocystidaceae with characterized *MAT1* loci.

## 2. Materials and methods

### 2.1. Fungal isolates and genome sequences used

The genomes of *K. capensis* CBS139037 (LNGK00000000.1) and *K. proteae* CBS140089 (LNGL00000000.1) were sequenced in a previous study (Aylward et al., 2016) and are available in GenBank® (Benson et al., 2013). The *MAT1* locus of *C. fimbriata* CMW14799, previously characterized from its sequenced genome (Wilken et al., 2014), was also obtained from GenBank (KF033902.1; KF033903.1). Other than the *Knoxdaviesia* genome isolates, three additional strains of *K. capensis* (CMW40886, CMW40889, CMW40892) and *K. proteae* (CMW40879, CMW40882, CMW40883) were used in this study to perform crossing experiments. All isolates were routinely cultured on Potato Dextrose Agar (PDA; Merck, Wadeville, South Africa) for approximately seven days at 25 °C and maintained at 4 °C.

### 2.2. Identification of MAT loci from genome sequences

*Ceratocystis fimbriata s.s.* is currently the species most closely related to *Knoxdaviesia* that has a characterized *MAT1* locus with available gene models. The predicted proteins of the *MAT1* locus of *C. fimbriata* CMW14799 (AHV84683-84701) were used to search for the *MAT1* locus in the genomes of *K. capensis* and *K. proteae* by performing local BLASTx searches in CLC Genomics workbench 6 (CLC Bio, Denmark). Preliminary analysis identified a single *MAT1* idiomorph from each *Knoxdaviesia* genome and revealed that the two genomes contained opposite *MAT1* idiomorphs. Subsequently, we mapped the raw sequence reads from the *K. capensis* genome (GenBank Accession: SRX1453186, SRX1453795 and SRX1453796) to the *K. proteae MAT1* region in CLC Genomics workbench to identify the terminal ends of the *MAT1* locus in *K. proteae*.

The procedure was repeated using the *K. proteae* raw sequence reads (GenBank Accession: SRX1453891, SRX1453905 and SRX1453906) and the *K. capensis MAT1* region.

### 2.3. Identification of the opposite MAT idiomorph in each species

Since each *Knoxdaviesia* genome contained a single *MAT1* idiomorph, the opposite idiomorph of each species had to be determined from a strain with the opposite mating type. Primers were designed in the conserved flanking regions of the *MAT1* locus in both *Knoxdaviesia* genomes (Table 1; Fig. 1) and used, in conjunction with internal primers (Table 1; Fig. 1), to amplify the opposite idiomorph in *K. capensis* CMW40886 and *K. proteae* CMW40882. The 50 µl reactions contained 1.75 mM $MgCl_2$, 0.3 mM of each dNTP, 0.5 µM of each primer, approximately 250 ng template DNA, 2 µl DMSO, 10 µl 5× KAPA LongRange Buffer and 1.25 units of KAPA LongRange HotStart DNA Polymerase (KAPA Biosystems, Inc., Wilmington, MA). Initial denaturation was performed at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 55.5 °C for 30 s and 68 °C for one minute per kb to be amplified (Table 1). The final extension step was at 72 °C for 10 min. The products were submitted for paired-end shotgun sequencing on the Illumina MiSeq platform at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa.

The next generation reads were trimmed with Trimmomatic (Bolger et al., 2014), discarding the first 10 bases, those with an average phred score below 25 (using a sliding window of four base pairs), and reads <30 bp in length. The quality of the sequences was confirmed with FastQC 0.11.4 (Babraham Bioinformatics, Babraham Institute, Cambridge). A *de novo* assembly of the trimmed sequences of each idiomorph was performed in CLC Genomics workbench. Subsequently, the sequence reads were mapped back onto the assembly of each idiomorph to detect potential errors. In order to compare the two idiomorphs in each species and the two pairs of homologous idiomorphs, dotplot comparisons of the nucleic acid sequences were computed with YASS (Noé and Kucherov, 2005) using the default parameters. Since repeat structures have been found in the *MAT1* locus of *C. fimbriata* (Wilken et al., 2014), the *Knoxdaviesia MAT1* loci were interrogated for repeat clusters with RepFind (Betley et al., 2002).

### 2.4. Knoxdaviesia MAT genes

WebAUGUSTUS (Hoff and Stanke, 2013) was used to predict open reading frames (ORFs) in the *MAT1* loci obtained from the *Knoxdaviesia* genomes and the amplified idiomorphs, using gene models from the closest available relative, *Fusarium graminearum*. The putative identities of the predicted ORFs were determined by DELTA-BLAST protein searches (Boratyn et al., 2012) at the National Centre for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Conserved domains in putative *MAT1* proteins were identified with searches at Interpro (Mitchell et al., 2015) and NCBI's Conserved Domains Database (CDD; Marchler-Bauer et al., 2015).

**Table 1**
Summary of the *Knoxdaviesia MAT1* primers used in this study.

| Number | Primers | | Products | |
|---|---|---|---|---|
| | Name | 5′-3′ | Alpha domain | Product size (bp) |
| 1a | KcM1-F | CCG CAC TGT ACA TCA CAA CA | 1a ↔ 2 | 835 |
| 1b | KpM1-F | CCT CGT CTC GAA TGA AGG AG | 1b ↔ 2 | 357 |
| 2 | KcM1-R | GGT CAC CGA AAA GAA GAC CA | **HMG-box domain** | |
| 3 | KpHMG-F | ATC CTC ATG CCA CAA TAC CC | 3 ↔ 4 | 516 |
| 4 | KpHMG-R | GAA GTT GAA GTC CGC TTT GC | **Long range *MAT1-1*** | |
| 5 | KxMAT-F | TGG TTG AAA GGG AAA TGA GG | 5 ↔ 2 | 3914 |
| 6a | KxMAT-R | AAG ACA AAG GAC GGC CTA GC | 1c ↔ 6a | 6268 |
| 1c | LR-KpM1F | GAG TGG CCT TGT CTT GAC CT | **Long range *MAT1-2*** | |
| 6b | Apn-R | ATC TGT GCC GGT ACT TCA ACC | 5 ↔ 4 | 5751 |
| | | | 3 ↔ 6b | 2825 |

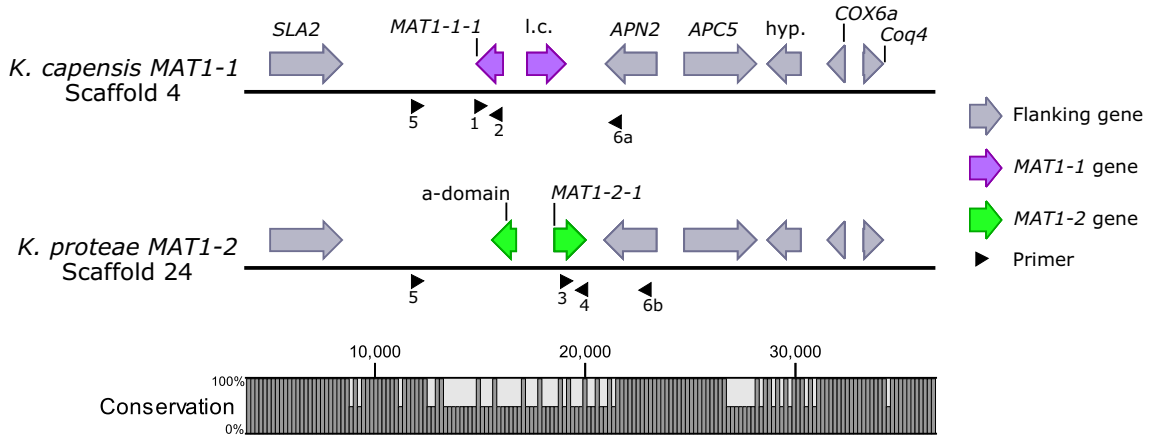


**Fig. 1.** The *MAT1* mating type locus identified from the genomes of *Knoxdaviesia capensis* and *K. proteae*. The graph indicates nucleotide sequence conservation between the loci of the two fungi. Open reading frames are indicated by blocked arrows. The lack of conservation in the *APC5* gene is due to missing sequence data in the *K. proteae* genome sequence. l.c. = low complexity region protein, hyp. = hypothetical protein, a-domain = alpha domain-containing protein. See Table 1 for primer names and sequences. Primers 1a, 1b and 1c lie in the same approximate location.

The *Knoxdaviesia MAT1* proteins were compared with those of *C. fimbriata* (KF033902), *H. moniliformis* and *H. omanensis* (obtained from A. Wilson). In addition, we used the *MAT1-1-1*, *MAT1-1-2* and *MAT1-2-1* proteins of six other Sordariomycetes also used by Wilken et al. (2014): *Cryphonectria parasitica* (AF380365/AF380364), *Cordyceps takaomontana* (AB096216/AB084921), *Fusarium fujikuroi* (AF100925/AF100926), *Magnaporthe grisea* (AB080670/AB080671), *Neurospora crassa* (M33876/M54787) and *Podospora anserina* (X73830/X64194/X64195). These were aligned to the *Knoxdaviesia* proteins and to the consensus sequences of the alpha (PFAM04769) and HMG-box (PFAM00505) domains, using the accurate alignment algorithm in CLC Genomics workbench. The consensus sequences were obtained from the protein families (Pfam) database (Finn et al., 2014).

### 2.5. Distribution of MAT idiomorphs in natural populations and ascomata

To determine the abundance of each *MAT1* idiomorph in natural populations, DNA previously extracted from 94 strains of *K. capensis* isolated from six different hosts (Aylward et al., 2015a; Aylward, unpublished) and 184 strains of *K. proteae* from a single host (Aylward et al., 2014, 2015b) in the Cape Floristic Region, were used (Table 2). Primers KcM1-F (1a in Fig. 1) and KcM1-R (2 in Fig. 1), that flank the conserved alpha domain of *MAT1-1-1*, and KpHMG-F (3 in Fig. 1) and KpHMG-R (4 in Fig. 1), that flank the HMG-box of *MAT1-2-1* in the two *Knoxdaviesia* species were designed with Primer3 (Table 1; Untergasser et al., 2007). A new forward primer, Kp-M1-F (1b in Fig. 1), was later designed to bind to the *K. proteae* alpha domain region with greater specificity (Table 1). The sizes of the amplicons for these two conserved regions were calculated to differ by at least 150 bp, in order to be distinguished on an agarose gel and, therefore, to identify the *MAT1* idiomorph using a single multiplex PCR. The 20 µl PCRs contained 10 µl KAPA Taq ReadyMix (Kapa Biosystems, Inc., Boston, USA), $MgCl_2$ at a final concentration of 2.75 mM, 0.4 µM each of the alpha domain and HMG-box primer pairs and approximately 150 ng template DNA. PCR cycling conditions were 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1.15 min, and a final extension step of 72 °C for 10 min. The hypothesis of a 1:1 distribution of *MAT1-1* and *MAT1-2* idiomorphs within different sampling localities and from different host species was tested with Pearson's Chi-Square and two-sided binomial tests in R version 3.2.3 (R Core Team, 2014).

The distribution of *MAT1* idiomorphs in ascospores derived from a single parent was also investigated. For both *K. capensis* and *K. proteae*, single ascospore progeny were cultured from a spore droplet taken from a single ascoma following the protocol of Wilson et al. (2015). Since *Knoxdaviesia* species do not readily from sexual structures in culture, a spore droplet from an ascoma on *P. coronata* (for *K. capensis*) and *P. repens* (for *K. proteae*) flowers were transferred to 20 µl Soltrol 130 oil (Chemfit, Gauteng, South Africa), vortexed and streaked out onto half-strength PDA (19.5g PDA/L; Merck, Wadeville, South Africa). For *K. proteae*, ascospores germinated only if an equal volume (20 µl) of sterile water was mixed with the Soltrol oil prior to streaking. Plates were incubated at 25 °C and 30 germinating ascospores from each species were transferred onto fresh half-strength PDA after approximately 48 h. After approximately 10 days at 25 °C, DNA from the single ascospore progeny was extracted and amplified with the Extract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, Steinham, Germany) according to the manufacturer's instructions. PCR reaction conditions and protocols followed those described above. The hypothesis of a 1:1 distribution of *MAT1-1* and *MAT1-2* idiomorphs within the spore drops of each species was again tested with Pearson's Chi-Square and two-sided binomial tests in R.

### 2.6. Knoxdaviesia mating type experiments

*Knoxdaviesia* species rarely produce sexual reproductive structures in culture and then only when an ascospore mass is cultured directly from the host (Wingfield and Van Wyk, 1993; Wingfield et al., 1988). Subsequent propagation in culture is exclusively asexual. We attempted to induce the formation of ascomata in culture by reciprocally pairing two *MAT1-1* and two *MAT1-2* strains from each species in all six possible combinations on half-strength PDA. The pairings, therefore, included four *MAT1-1* vs. *MAT1-2* crossings and two negative controls (*MAT1-1* vs. *MAT1-1* and *MAT1-2* vs. *MAT1-2*). All six pairings were made in triplicate by (1) sub-culturing two strains approximately two cm apart (Wilson et al., 2015), (2) streaking two conidial suspensions on opposite sides of plates (Gilgado et al., 2010) and (3) combining equal volumes of two conidial suspensions and streaking the mixture. We also paired *K. capensis* and *K. proteae* to observe whether recognition and subsequent ascomatal development would take place. Plates were incubated at room temperature and in the dark to simulate the infructescence environment. They were monitored for up to four months for the presence of ascomata.

**Table 2**
*Knoxdaviesia* strains and populations used in this study.

| Species | Host | Location[a] | Number of strains | Reference |
|---|---|---|---|---|
| *Knoxdaviesia capensis* CMW40890 | *Protea longifolia* Andrews | Hermanus | 1 | Aylward et al. (2016) |
| " | *P. coronata* Lam. | Ataraxia | 15 | Aylward (unpublished) |
| " | " | Du Toits Kloof | 11 | " |
| " | " | Greyton | 9 | " |
| " | " | Kleinmond | 5 | " |
| " | " | Helderberg | 9 | " |
| " | *P. neriifolia* R. Br. | Betty's Bay | 6 | " |
| " | " | Kogelberg | 8 | " |
| " | *P. lepidocarpodendron* L. | Betty's Bay | 7 | " |
| " | *P. longifolia* | Kogelberg | 9 | " |
| | *P. repens* L. | Gouritz | 7 | Aylward et al. (2015b) |
| | " | Franschoek | 10 | " |
| *Knoxdaviesia proteae* CMW40880 | *P. repens* | Stellenbosch | 1 | Aylward et al. (2016) |
| | " | Gouritz | 83 | Aylward et al. (2014) |
| | " | Franschoek | 101 | Aylward et al. (2015a) |

[a] All localities are in the Western Cape Province, South Africa.

## 3. Results

### 3.1. Identification of the MAT1 locus

The BLASTx searches conducted on the *K. capensis* and *K. proteae* genomes highlighted regions of *K. capensis* scaffold 4 as comprising sequences with homology to *MAT1-1-1* and common *MAT1* flanking regions. BLAST searches could not detect the commonly occurring *MAT1-1-2* gene at this locus or in the rest of the genome. Scaffold 24, 30 and 37 of *K. proteae* contained ORFs with an HMG-box domain, but only the predicted protein in scaffold 24 had homology to fungal *MAT1-2-1* proteins (Table S1): *Ustilaginoidea virens* (KDB17701), *Metarhizium brunneum* (KID72395) and *Metarhizium majus* (KID83388). Mapping the raw sequence reads of the *K. capensis MAT1-1* genome against the *K. proteae MAT1-2* genome, and *vice versa* (Fig. S1) illustrated that the *MAT1-1* idiomorph (7744 bp) is slightly larger than the *MAT1-2* idiomorph (4968 bp).

The opposite *MAT* idiomorph for each *Knoxdaviesia* species was amplified in two parts using the primers designed from the conserved *MAT1* flanking regions in the *Knoxdaviesia* genomes and internal primers designed from the *MAT1* loci. These overlapping sequences were pooled before constructing Illumina paired-end libraries for each idiomorph. The *MAT1-1* locus of *K. proteae* was amplified with KxMAT-F (5) and KcM1-R (2), and LR-KpM1F (1c) and KxMAT-R (6a), respectively yielding amplicons of approximately 3.9 kb and 6.3 kb. The *K. capensis MAT1-2* locus was amplified with KxMAT-F (5) and KpHMG-R (4), and KpHMG-F (3) and Apn-R (6b), yielding 5.8 and 2.8 kb amplicons, respectively.

The *MAT1-1* idiomorph of *K. proteae* could be assembled into a 9374 bp contig and the *MAT1-2* idiomorph of *K. capensis* into a 6908 bp contig. For *K. capensis* and *K. proteae*, respectively, 93.8% and 95.1% of the trimmed sequence reads mapped back to the assembly. Both of these assemblies were larger than the estimated size of each idiomorph, reflecting the upstream and downstream flanking regions that were included in the long-range PCR. The idiomorph sizes were again estimated by mapping the genome reads of the opposite mating type to each idiomorph. The *K. proteae MAT1-1* idiomorph was estimated at 7795 bp and the *K. capensis MAT1-2* idiomorph at 4988 bp.

### 3.2. Knoxdaviesia MAT1 genes

#### 3.2.1. MAT1-1 idiomorph

WebAUGUSTUS predicted two ORFs in each *MAT1* idiomorph. In the *K. capensis* genome, a conserved motif search identified an alpha-box (PS51325)/*MAT* alpha 1 domain (IPR006856; PFAM04769) at amino acid residues 57-196 in the first *MAT* ORF (Kc1_g2). The second *MAT* ORF (Kc1_g3) contains a low complexity region and could not be matched to any protein on the NCBI protein database. After masking the low complexity region, a BLASTp search revealed poor similarity to the *MAT1-1-2* gene of *Cordyceps militaris*. Similar results were obtained for the two ORFs predicted from the *K. proteae MAT1-1* locus. Detailed BLAST results are given in supplementary Table S1.

The predicted *MAT1-1-1* genes of both *Knoxdaviesia* species contained a single intron occurring within the conserved alpha domain (Fig. 2A). The position of this intron in the conserved protein

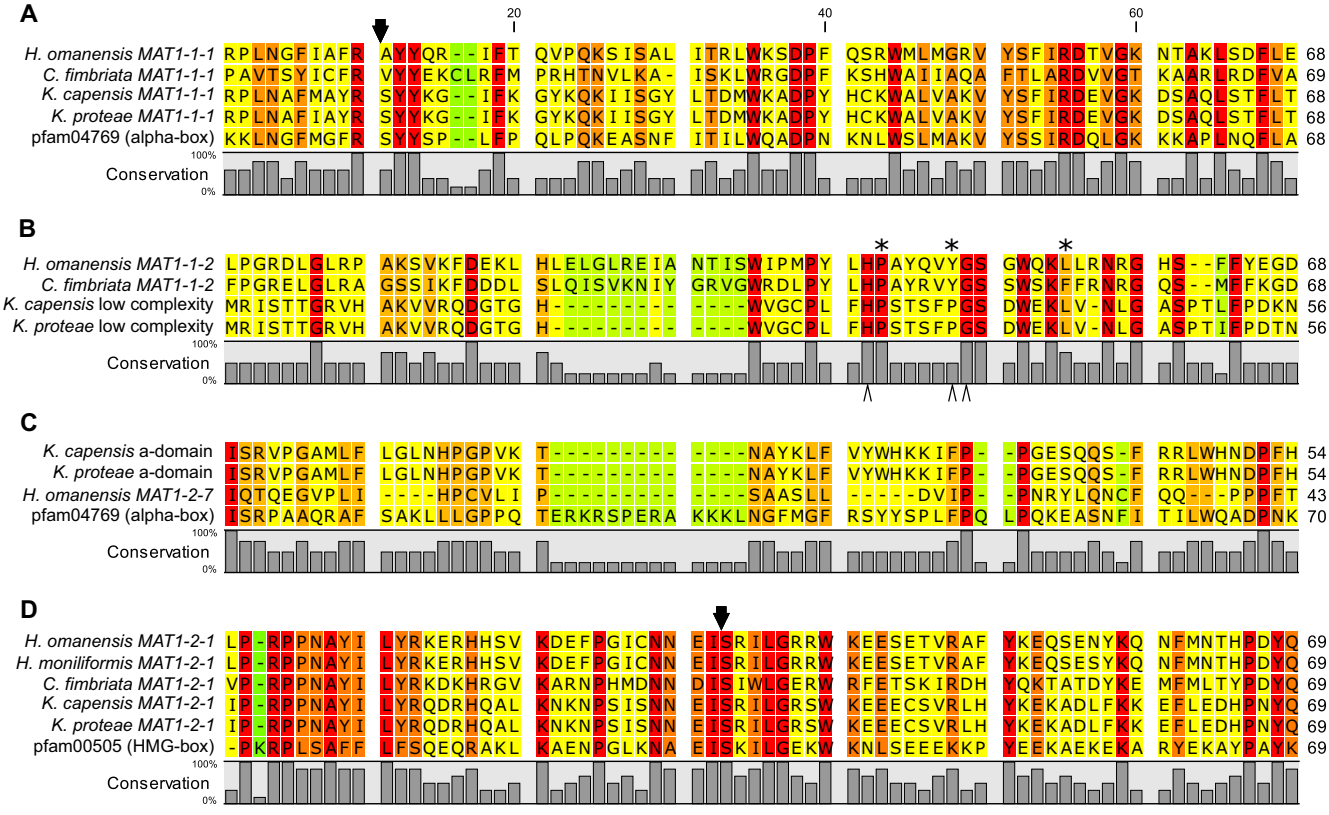


**Fig. 2.** Alignment of conserved regions of the *MAT1* locus of *Knoxdaviesia*, *Huntiella* and *Ceratocystis fimbriata*. (A) Alpha domain of *MAT1-1-1*, (B) proposed PPF (*) and HPG (^) domains of *MAT1-1-2*, (C) similarity between *Knoxdaviesia* alpha domain-containing proteins and *Huntiella MAT1-2-7* and (D) HMG-box domain of *MAT1-2-1*. The conservation of residues is illustrated with a spectrum from green to red, with green representing < 30%, yellow < 65%, orange < 100% and red 100% amino acid identity. Conserved intron positions are indicated with a black arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

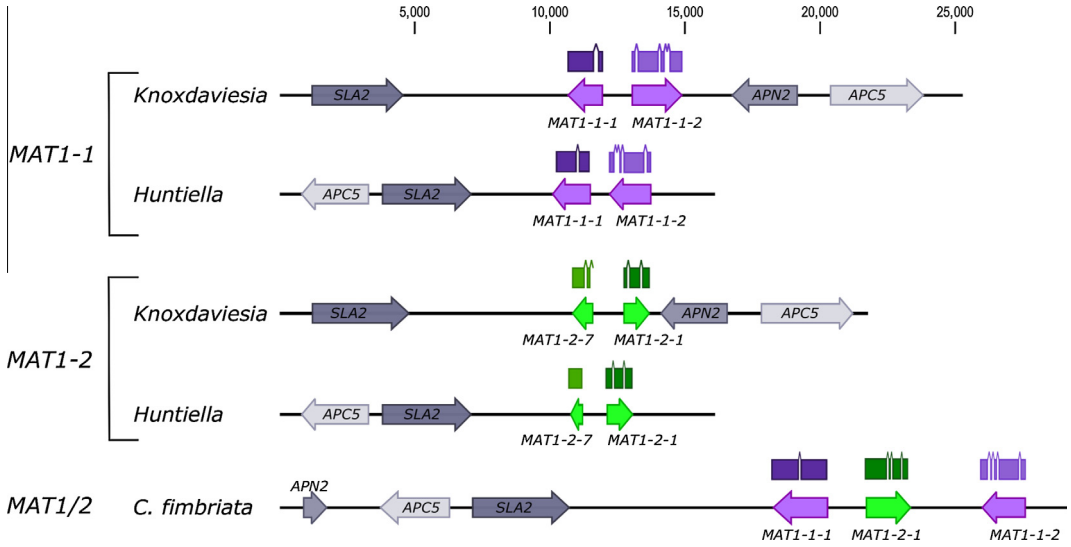


**Fig. 3.** Comparison between the *MAT1* locus organisation of *Knoxdaviesia*, *Huntiella* and *Ceratocystis fimbriata*. Gene models are indicated above the *MAT1* genes as boxes (exons) and gaps (introns). *APN2* is not present in the regions flanking *MAT1* of *Huntiella*.

domain was identical to the position of the intron in the *MAT1-1-1* proteins of other Sordariomycetes (Debuchy and Turgeon, 2006), including *C. fimbriata* and *H. omanensis* (Wilken et al., 2014; Wilson et al., 2015). This domain was well conserved between *Knoxdaviesia* and the Ceratocystidaceae species, although *C. fimbriata* displays several deviations from amino acids that remain conserved in *Knoxdaviesia* and *Huntiella* (Fig. 2A).

Alignment of the *Knoxdaviesia* low complexity-region proteins to the Ceratocystidaceae (Fig. 2B) and other Sordariomycete *MAT1-1-2* proteins (Fig. S2) showed that they contain the conserved HPG (Histidine-Proline-Glycine) domain (PFAM17043) that has been proposed for this protein by Debuchy and Turgeon (2006). In the Ceratocystidaceae this motif is, however, HYG (Histidine-Tyrosine-Glycine; Wilken et al., 2014). Kanematsu et al. (2007) proposed a conserved PPF (Proline-Proline-Phenylalanine) motif, but in *Knoxdaviesia* only the two Prolines were present, whereas only the first Proline is conserved in the Ceratocystidaceae species. The gene models predicted for these low complexity-region proteins also agree with the *MAT1-1-2* gene model of *C. fimbriata* (Fig. 3), providing additional evidence that they represent the *MAT1-1-2* gene in *Knoxdaviesia*. These proteins were longer (548 in *K. capensis* and 547 in *K. proteae*) than any of the other *MAT1-1-2* proteins considered here, partly owing to the low complexity region of approximately 24 amino acids at the C-terminal ends.

### 3.2.2. MAT1-2 idiomorph

The first *MAT* ORF (Kp2_g2) in the genome of *K. proteae* is an ORF smaller than the putative *Knoxdaviesia MAT1-1-1* genes, but with homology to the *MAT* alpha 1 domain. The second *K. proteae* ORF (Kp2_g3) contained a HMG-box domain (IPR009071; PFAM00505) at residues 156–230, analogous to *MAT1-2-1* proteins. As with the *K. proteae MAT1-1* idiomorph, the ORFs predicted from the assembled *K. capensis MAT1-2* idiomorph were also identified as an alpha domain-containing protein and the *MAT1-2-1* gene (Table S1).

Whereas the *Knoxdaviesia MAT1-2-1* proteins showed a high level of conservation with known *MAT1-2-1* proteins (Fig. 2d, S2), including a conserved intron position (Debuchy and Turgeon, 2006), the first ORF in the *Knoxdaviesia MAT1-2* idiomorphs was unusual. These alpha domain-containing proteins were almost half the size of the *Knoxdaviesia MAT1-1-1* proteins (208 vs. 401 amino acids) and alignment indicated that the conserved domain is the only common region (Fig. S3). The conserved domain in these proteins also showed more deviation from the consensus *MAT* alpha 1 domain than *MAT1-1-1* proteins. Surprisingly, however, the alpha domain proteins had a predicted intron at the same position as the *MAT1-1-1* proteins (Fig. S3), suggesting that the two have a common origin.

Wilson et al. (2015) recently described a new *MAT1* gene from the *MAT1-2* idiomorph of *H. omanensis*. This gene, named *MAT1-2-7*, lies upstream of *MAT1-2-1* and does not have a known conserved domain, neither does it contain an intron. Alignment between the *Knoxdaviesia* alpha domain-containing proteins, the *H. omanensis MAT1-2-7* protein and the consensus *MAT* alpha 1 domain (Fig. 2C) showed that the *Knoxdaviesia* and *Huntiella* proteins are similar. This suggests that the *Huntiella MAT1-2-7* protein may also have arisen from an alpha domain-containing protein. We thus propose that these genes are homologous and therefore consider the *Knoxdaviesia MAT1-2* alpha domain-containing genes as *MAT1-2-7*.

### 3.2.3. Genes flanking the MAT locus

BLAST searches identified the ORFs flanking the *MAT1* locus in both *Knoxdaviesia* species as the *SLA2* and *APN2* genes (Fig 1; Table S1). Other genes are commonly associated with the *MAT* locus (*e.g.* the cytochrome C oxidase VIa subunit (*COX6a*), anaphase promoting complex subunit 5 (*APC5*) and coenzymeQ biosynthesis (*Coq4*) genes) and were also detected downstream of *APN2*. Unlike the Ceratocystidaceae, the *Knoxdaviesia MAT1* locus conformed to the ancestral Sordariomycete arrangement of flanking genes (Fig 3; Debuchy and Turgeon, 2006).

### 3.3. Knoxdaviesia capensis and K. proteae MAT1 comparison

Dotplot comparisons between the opposite *MAT* idiomorphs in each of the species indicated that the two idiomorphs share little sequence similarity, with the only exception being two similar 160 bp fragments in the *K. proteae* idiomorphs (Fig. 4A and B). The size difference between the *MAT1-1* and *MAT1-2* idiomorphs can be ascribed to smaller ORFs in the *MAT1-2* idiomorph and a smaller intergenic region (386 bp in *MAT1-1* vs. >1.8 kb in *MAT1-2*) separating the *MAT1-2-1* and *APN2* genes. In contrast, the homologous *MAT1-1* and *MAT1-2* idiomorphs shared 84.3%

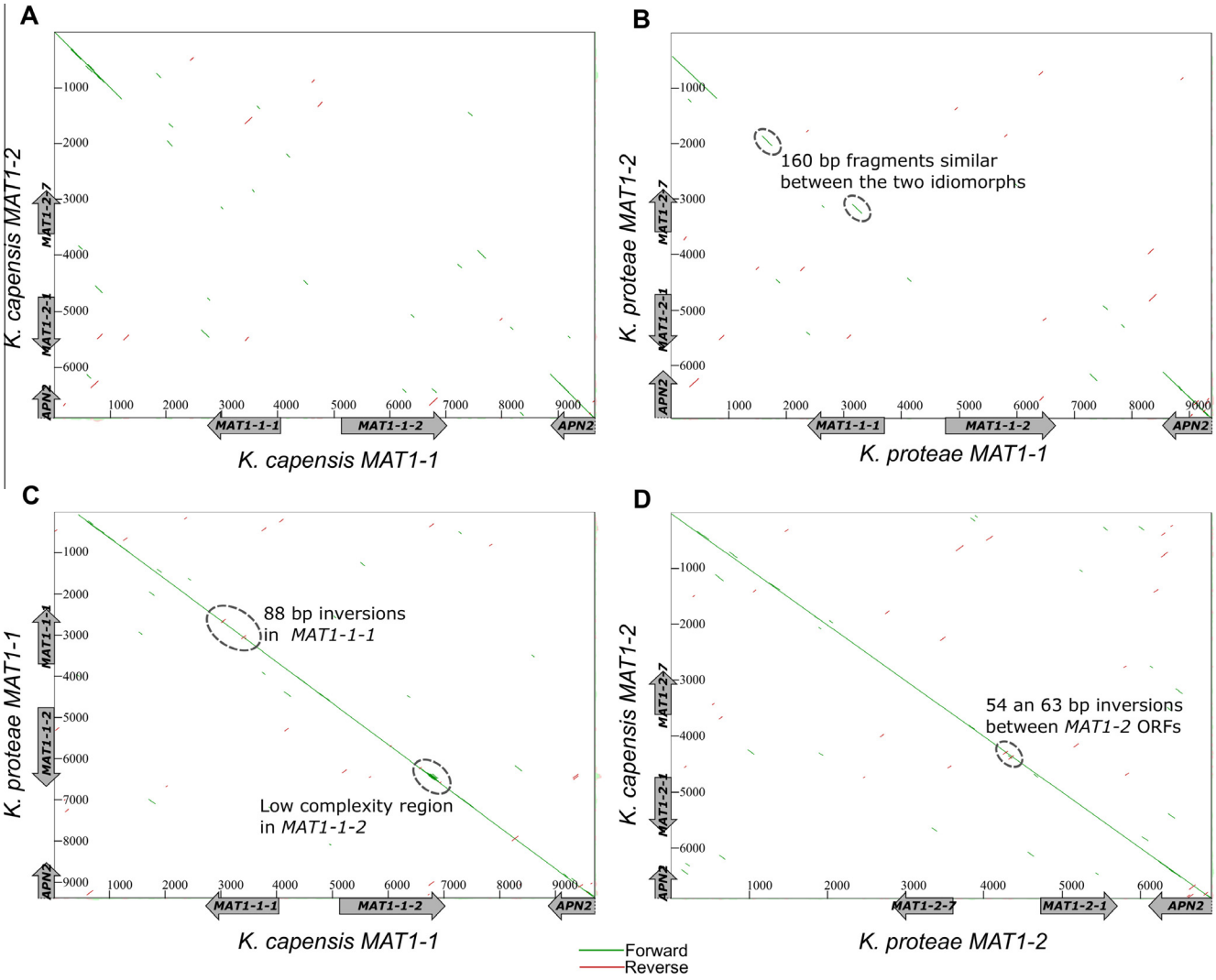


**Fig. 4.** Pairwise dotplot comparisons between the *MAT1* idiomorphs of *Knoxdaviesia capensis* and *K. proteae*. Intra-specific comparisons between the (A) *K. capensis* and (B) *K. proteae* idiomorphs, as well as inter-specific comparisons between the homologous (C) *MAT1-1* and (D) *MAT1-2* idiomorphs are shown. The positions of the *MAT1-1*, *MAT1-2* and *APN2* genes are indicated on each plot.

and 86.4% sequence identity at the nucleotide level. The dotplot indicated two small inversions between each of the idiomorphs in *K. capensis* and *K. proteae* and illustrated the low complexity region of the *MAT1-2-7* gene (Fig. 4C and D). Significant repeats, as are present in the unswitched *C. fimbriata MAT1* locus (Wilken et al., 2014), could not be identified in the *Knoxdaviesia* species. The *MAT1* idiomorphs of both *Knoxdaviesia* species have been deposited in GenBank: *K. capensis MAT1-1* (KX832965) and *MAT1-2* (KX832968); *K. proteae MAT1-1* (KX832967) and *MAT1-2* (KX832966).

### 3.4. Distribution of MAT idiomorphs in natural populations and ascomata

The Chi-Square and two-tailed binomial tests were conducted with the null hypothesis of a 1:1 ratio of mating types. The probability of obtaining either one of the idiomorphs was therefore set as 0.5. The P-values and 95% confidence intervals of the tests (Table S2) indicated that almost all populations had a ratio not significantly different from 1:1 (Fig. 5) Confidence intervals were smaller in populations with large ($\geqslant$30) sample sizes. The only population that deviated from the 1:1 ratio was a small population (Franshoek; n = 10) of *K. capensis* sampled from *P. repens*, which is a non-preferred host of this fungus (Roets et al., 2011a). This deviation also affected the overall distribution of *K. capensis* on this host (Fig. 5). The *P. longifolia* population appears to be skewed towards *MAT1-2* individuals, although this was not statistically significant. If we consider testing a single hypothesis multiple times as grounds for applying a multiple testing correction procedure, the new critical value, as defined by Bonferroni (Noble, 2009), would be P = 0.003. According to the binomial test, the Franschoek *K. capensis* population would not be significantly different from zero at this critical value, although it remains significant based on the Chi-Square test.

### 3.5. Knoxdaviesia mating experiments

Only the pairing methods that involved conidial suspensions of opposite mating types showed any sign of a mating reaction. Strains sub-cultured close together did not form an interaction zone or give rise to ascomata. Ascomatal development was observed in spore mixture pairings of *K. proteae* (Fig. 6A), although ascomata were sparse and surrounded by prolific vegetative growth. Ascomata were observed in one culture of *K. capensis*

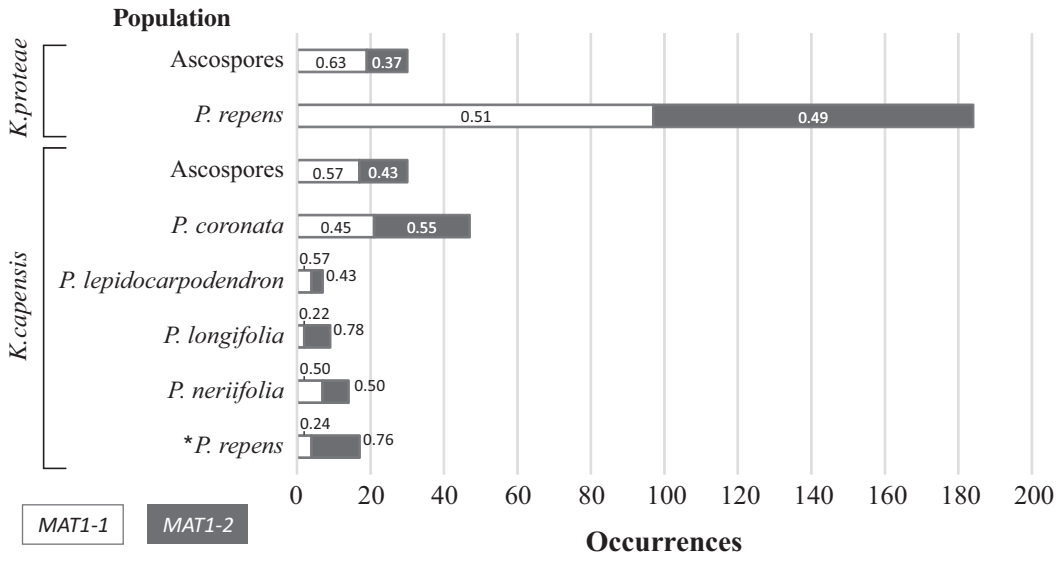


**Fig. 5.** Distribution of the *MAT1* idiomorphs in ascospore droplets and *Protea* populations of *Knoxdaviesia capensis* and *K. proteae*. White bars represent the number of occurrences of *MAT1-1* and grey the occurrences of *MAT1-2*. The frequency of each idiomorph is indicated on the bars. The population that showed a significant deviation (P = < 0.003) from a 1:1 distribution is indicated with an asterisk.

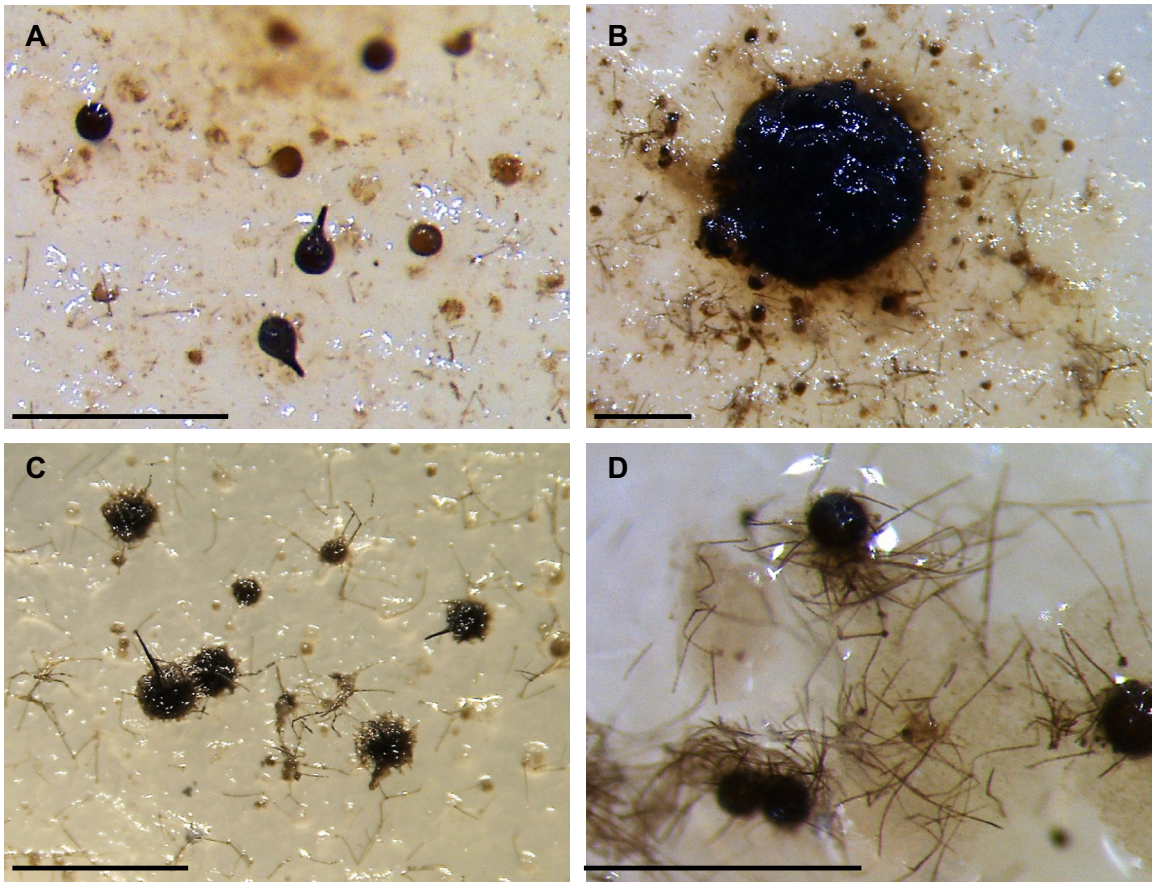


**Fig. 6.** Mating reactions between opposite idiomorphs of *Knoxdaviesia capensis* and *K. proteae* in culture. (A) Ascomata and developing ascomata of *K. proteae* (CMW408879 × CMW40883). (B) Aggregations of *K. capensis* (CMW40886 × CBS139037) likely representing developing ascomata. (C) Ascomata of *K. capensis* in a culture grown from an ascospore mass. (D) Aggregations formed during an inter-specific cross between *K. capensis* and *K. proteae*. Scale bars = 0.5 mm.

grown from a droplet containing a mass of ascospores, but crosses between isolates representing opposite idiomorphs resulted only in large masses of what could have been an aggregation of developing ascomata (Fig. 6B and C). The *K. capensis* "aggregations" produced spore droplets containing conidia, whereas the *K. proteae* ascomata did not exude spore droplets.

Although attempts to hybridise *K. capensis* and *K. proteae* were unsuccessful, black aggregations, similar to those observed in the developing ascomata of *K. proteae*, were apparent in these interspecific crosses (Fig. 6D). It is, therefore, possible that some recognition occurs between the two species, although further ascomatal development is impeded.

## 4. Discussion

### 4.1. Knoxdaviesia: outcrossing via heterothallism

Only one *MAT1* idiomorph was identified in each of the two *Knoxdaviesia* genomes examined, but the opposite idiomorphs could be amplified in different strains of the species. *MAT1-1* and *MAT1-2* individuals occur at similar proportions in the *Knoxdaviesia* populations from which high genetic diversity was previously detected (Aylward et al., 2014, 2015b; Aylward, unpublished), supporting the hypothesis that heterothallism underlies this diversity. The single *K. capensis* population that deviated from the 1:1 ratio was collected from *P. repens*, a non-preferred host (Roets et al., 2011a) on which it occurs infrequently and likely only survives until *K. proteae* is introduced (Aylward et al., 2015a). Furthermore, approximately half of the progeny of haploid crosses contained the *MAT1-1* and the other half the *MAT1-2* idiomorph.

Although the production of ascomata could be induced only poorly in some intra-specific crosses, single-spore isolates of *Knoxdaviesia* species do not form ascomata and a mating reaction could only be induced between strains containing opposite *MAT1* idiomorphs. The overall results of this study, therefore, show that both *K. capensis* and *K. proteae* have a heterothallic mating system, thereby requiring outcrossing between two individuals of opposite mating type for sexual reproduction.

*Knoxdaviesia* species are vectored by arthropods (Roets et al., 2011b), and possibly passerine birds, and may spend a considerable amount of time on these animals before being deposited in a suitable habitat. Since most fungi are capable of switching between sexual and asexual reproduction in response to environmental conditions (Billiard et al., 2012), the prevalence of sexual reproduction in *Protea*-associated *Knoxdaviesia* suggests that this form of reproduction is beneficial in the *Protea* environment. Various trade-offs exist for sexual reproduction (Billiard et al., 2012). Disadvantages include the energy cost of developing sexual structures, the risk of failing to find a suitable mate (in the case of non-selfing species) and the possibility that locally adapted allele combinations may be broken through recombination. In *Knoxdaviesia*, the cost of outcrossing is likely offset by, amongst others, preventing the accumulation of harmful alleles and enabling diversification through recombination, a trait especially important for adaptation. Other than the creation of genetic diversity, production of dispersal propagules may be an essential factor underlying the prevalence of sexual reproduction in *Protea* infructescences. This is because ascospores are usually more resilient, and therefore suitable for dispersal, than their conidial counterparts (Aanen and Hoekstra, 2007).

### 4.2. Evolutionary conservation between Knoxdaviesia MAT1 loci

The homologous *MAT1* idiomorphs of *K. capensis* and *K. proteae* were highly similar and their mating type proteins almost identical. Our interspecific mating type experiments also suggested that some determinants of recognition in strains of opposite mating type remain conserved between *K. capensis* and *K. proteae*. In contrast, the Sordariomycete *MAT1-1-1* and *MAT1-2-1* proteins used as a basis for comparison had low amino acid identities to each other and as well as to their *Knoxdaviesia* homologs (between 7.9% and 38.5%). Studies on the mating type loci of fungi have revealed great diversity in size and gene content. Despite their conserved domains, homologous *MAT1* genes in different species vary greatly and are believed to evolve more rapidly than other genes in the genome (Turgeon, 1998; Wik et al., 2008). The high level of similarity between the *Protea*-associated *Knoxdaviesia MAT1* loci is, therefore, likely a reflection of the close phylogenetic relationship between these species (Roets et al., 2009).

Despite the recognition that may occur between *K. capensis* and *K. proteae* and the sequence similarity in their *MAT1* regions, these species appear to be reproductively isolated. The initial prevention of gene flow in the common ancestor was likely facilitated by host association. *Knoxdaviesia proteae* occurs exclusively in *P. repens* (Wingfield et al., 1988), whereas *K. capensis* is found in the infructescences of nine different *Protea* host species (Aylward et al., 2015a; Roets et al., 2009; Wingfield and Van Wyk, 1993). The occurrence of *K. capensis* in *P. repens* infructescences was discovered only recently, this being due to the low level at which it occurs on this host (Aylward et al., 2015a). Therefore, despite the clear disparity in host range between *K. capensis* and *K. proteae*, host association does not completely isolate the two species, although the historical distribution of hosts may have facilitated isolation. We speculate that the *Protea*-associated *Knoxdaviesia* ancestor occupied a range of host species, but became isolated on *P. repens*. *Protea repens*, or at least one population of this host, may have been geographically separated from the other *Protea* hosts historically. Fire, a common occurrence in Fynbos vegetation in which *Protea* species occur (Cowling, 1992), may clear vast areas, possibly providing a mechanism for isolating host populations. Should the *Knoxdaviesia* ancestor have been isolated in *P. repens* by such means, sufficient time without gene flow would have resulted in genetic drift between the *Knoxdaviesia* populations on *P. repens* and the *Knoxdaviesia* populations on other hosts such that speciation has occurred (Slatkin, 1985, 1987).

### 4.3. Knoxdaviesia MAT1 characteristics

The most obvious difference between the *MAT1* loci of the Gondwanamycetaceae and Ceratocystidaceae species is the order of genes flanking the *MAT1* locus. Although we expected that the rearranged order in the Ceratocystidaceae might be a phenomenon common to the Microascales, the *Knoxdaviesia* flanking genes follow the ancestral Sordariomycete arrangement where *SLA2* and *APN2* flank the *MAT1* locus (Debuchy and Turgeon, 2006). Since *Knoxdaviesia* represents the sister family of *Ceratocystis* and *Huntiella* in the Microascales (Réblová et al., 2011), we suspect that this specific rearrangement is limited to the Ceratocystidaceae and probably occurred in an early ancestor of this family.

Although the *MAT1* genes in the two *Knoxdaviesia* species are virtually identical, the diversity of *MAT1* genes was exemplified in the comparison with the three Ceratocystidaceae species. Differences in protein length and amino acid sequence were apparent, yet regions associated with protein function, such as the conserved domains and intron positions, remain uniform. The accessory genes (*MAT1-1-2* and *MAT1-2-7*) in the *Knoxdaviesia* idiomorphs appeared particularly divergent in comparison to known *MAT1* genes, necessitating future expression analysis to determine whether they are transcribed. These could not be identified with BLAST searches, although alignments revealed similarity to Ceratocystidaceae accessory *MAT1* proteins. Until recently, *MAT1-1-2*

genes lacked a universally conserved PFAM domain and the low complexity region in the *Knoxdaviesia MAT1-1-2* gene also hampered identification. An HPYG domain is conserved in the studied Ceratocystidaceae *MAT1-1-2* proteins (Wilken et al., 2014), but in *Knoxdaviesia* the Tyrosine (Y) residue is replaced by a Proline. As with the flanking gene arrangement, the *MAT1* genes of *Knoxdaviesia* thus conform to the general pattern of the Sordariomycetes rather than to the derived state of the Ceratocystidaceae.

### 4.4. MAT1-2-7 gene descended from MAT1-1-1

To the best of our knowledge, the putative *Knoxdaviesia MAT1-2-7* genes represent the first report of Ascomycete *MAT1-2* idiomorph proteins containing an alpha domain. The presence of a truncated *MAT1-1-1* gene at the same position on the *MAT1-2* idiomorph is a recognised pattern in the Ophiostomatales (Comeau et al., 2015; Duong et al., 2013; Tsui et al., 2013), but this truncated *MAT1-1-1* gene lacks the alpha domain. Other than the *MAT1-1-1* gene, *Ophiostoma quercus* is also known to harbour fragments of the *MAT1-1-3* gene in its *MAT1-2* idiomorph (Wilken et al., 2012) and, conversely, *Aspergillus fumigatus* has part of the *MAT1-2-1* gene in its *MAT1-1* idiomorph (Paoletti et al., 2005). In all of these cases, the various fragments are thought to have originated from one or more unequal recombination/crossover events between the two *MAT1* idiomorphs in a common heterothallic ancestor (Gioti et al., 2012). However the truncated *MAT1-1-1* gene of the Ophiostomatales may be functional rather than a mere remnant of ancestral recombination. Tsui et al. (2013) showed that this gene is expressed in *Grosmannia clavigera* and suggested that the alpha domain had been lost after the crossover event.

Although the alpha domain is not readily detectable in the *MAT1-2-7* gene of *H. omanensis*, it is nevertheless similar to its *Knoxdaviesia* counterpart. The intron in *Knoxdaviesia MAT1-2-7*, which lies at the identical position as the *MAT1-1-1* intron, provides compelling evidence for an evolutionary link between the two genes. *MAT* alpha 1 domains have a DNA-binding/bending function (Gene Ontology: 0008301) and act as transcriptional activators for pheromone and receptor genes involved in the mating process (Bender and Sprague, 1987; Casselton, 2002). If the *MAT1-2-7* genes of *Knoxdaviesia* and *Huntiella* had arisen from the *MAT1-1-1* gene via recombination and, therefore, contained a MAT alpha 1 domain, the constraint to conserve this domain would be negated by the presence of a functional *MAT1-1-1* protein during the mating reaction. Conversely, this domain may have mutated to prevent self-fertility, reflecting the essential benefits that outcrossing confers in these species. Expression analysis is, however, needed to verify whether the *Huntiella* and *Knoxdaviesia MAT1-2-7* genes are functional.

## 5. Conclusions

This study is the first to investigate fungal reproduction in the Cape Floristic Region and it revealed how the *Protea*-associated *Knoxdaviesia* species maintain high levels of genetic diversity on their native hosts. Additionally, it provides information on the extent to which the *MAT1* locus has diversified in the Ceratocystidaceae, a family that gave rise to many economically important pathogens. The *Knoxdaviesia* species considered here were shown to have a classic heterothallic *MAT1* locus with strong resemblance to the ancestral gene organisation and conserved domains of Sordariomycete fungi. As such, the *Knoxdaviesia MAT1* locus is probably a more accurate representation of the ancestral *MAT1* locus of Microascalean fungi than the *MAT1* loci of Ceratocystidaceae species.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2016.10.002.

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