Doing it alone: Unisexual reproduction in filamentous ascomycete fungi

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ABSTRACT

Unisexuality in fungi is the result of sexual reproduction in a single isolate that harbors genes associated with only a single mating type. To date, unisexual reproduction has been described in only three genera of filamentous fungi. Consequently, our understanding of this unusual pathway is limited. In this critical review, we compare genetic, genomic and transcriptomic data from a variety of unisexual species to similar data from their primary homothallic and heterothallic relatives. These analyses show that unisexual reproduction is likely derived from heterothallism via the mutation of genes involved in the initiation of sexual reproduction. We show that significant changes in mating-type genes, pheromone precursor genes and pheromone receptor genes are common in unisexual species, but that similar changes are not evident in their primary homothallic or heterothallic relatives. These findings are particularly notable because the unisexual species are accommodated in unrelated genera, illustrating that a similar transition to unisexuality has likely occurred independently in their lineages.

KEYWORDS

1. Filamentous ascomycetes
2. Unisexual reproduction
3. Homothallism
4. Heterothallism
5. *MAT* genes
6. Mating pheromones

1. INTRODUCTION

Sexual reproduction in fungi usually occurs either between two isolates of opposite mating type that each harbor a different set of mating-type (*MAT*) genes (heterothallism) or within a single isolate that possesses all the necessary *MAT* genes (primary homothallism) (Blakeslee, 1904; Ni et al., 2011). In ascomycetous fungi, these *MAT* genes are classified as either of the *MAT1-1* or *MAT1-2* type, which confer the MAT1-1 and MAT1-2 mating identities, respectively. The two primary *MAT* genes, *MAT1-1-1* and *MAT1-2-1* each encode highly conserved proteins that are present in most of the Dikarya. The MAT1-1-1 protein harbours the alpha domain, while the MAT1-2-1 protein has an HMG box domain. Both of these conserved domains are known to bind DNA. It thus not surprising that these genes typically encode transcription factors that control the expression of several hundred sex-related genes, often in a *MAT*-dependent manner (Bidard et al., 2011; Böhm et al., 2013; Kronstad and Staben, 1997; Pöggeler et al., 2006). The MAT1-1-1 protein, for example, controls the expression of the α-factor mating pheromone precursor, while the MAT1-2-1 protein controls the expression of the **a**-factor mating pheromone precursor. Isolates of opposite mating type are thus able to recognize and respond to suitable mating partners (Bobrowicz et al., 2002; Shen et al., 1999; Wilson et al., 2018; Zhang et al., 1998). As a direct result, homothallic species that harbour both *MAT* genes are usually able to express both pheromone precursors (Lee et al., 2008; Pöggeler, 2000).

The form of homothallism described above is termed primary homothallism to differentiate it from other types of homothallism that also allow for independent sexual reproduction (Wilson et al., 2015b). Two types of secondary homothallism have also been described-pseudohomothallism and mating-type switching. In the former, two nuclei of opposite

mating type are found in a single cell, thereby allowing for self-fertile mating (Merino et al., 1996). In contrast, mating-type switching enables independent mating by allowing an isolate of one mating type to switch to the opposite mating type- thereby creating a single culture capable of sexual reproduction (Lin and Heitman, 2007). The most recent form of homothallism to be described is known as unisexual reproduction. In contrast to primary homothallism, unisexuality describes the ability of a single isolate to sexually reproduce independently despite having genes of only one mating type. These species thus express what would typically be considered a heterothallic genotype (Roach et al., 2014). Consequently, it is possible that species previously described as homothallic are in fact unisexual. This is due to the fact that they act as primary homothallic species in culture, but will genetically appear as heterothallic (Alby et al., 2009; Lin et al., 2005; Schuerg et al., 2016; Wilson et al., 2015a). Therefore, both sexual behavior and genome data are required to effectively describe the sexual strategy exhibited by a species. The discovery of unisexuality has overturned the paradigmatic view that sexual reproduction in fungi depends on the expression of the two primary *MAT* genes, *MAT1-1-1* and *MAT1-2-1* (Roach et al., 2014).

The two best-defined unisexual cycles are those in the basidiomycetous yeast, *Cryptococcus neoformans* and the ascomycetous yeast, *Candida albicans*. *Cr. neoformans* has a well-defined heterothallic cycle where isolates of the α and **a** mating types interact to produce sexual structures (Kwon-Chung, 1976). However, cultures representing a single mating type are able to engage in unisexual behavior under particular environmental conditions (Lin et al., 2005; Tscharke et al., 2003). Although this process was originally thought to be asexual and mitotic in nature (Wickes et al., 1996), it has been shown to involve strictly sexual

processes such as meiosis and genome diploidization (Lin et al., 2010; Wang et al., 2000). The sexual nature of this mating strategy was further confirmed when non-isogenic α-strains of *Cr. neoformans* were shown to undergo genetic recombination (Lin et al., 2005).

In order for isolates of *Cr. neoformans* to sexually reproduce, a single cell must convert from haploid to diploid phase (Hull and Heitman, 2002; Kwon-Chung, 1976; Sia et al., 2000). During unisexual reproduction, this can occur via one of two mechanisms (Fig. 1): endoreplication, where a single cell simply duplicates its own genome; or cell fusion, where two cells of either mating type fuse and undergo karyogamy (Lin et al., 2005). The frequency at which either of these mechanisms occurs can be enhanced by the presence of pheromones in the environment. A small number of **a** cells expressing **a**-factor pheromone, for example, can initiate unisexual reproduction between two cells of the α mating type (Hull et al., 2002; Lin et al., 2005; Wang et al., 2000). Similarly, mating between two **a** cells can be induced in the presence of α-factor-expressing cells.

Pheromone expression is also an important factor in the unisexual cycle of *Ca. albicans*. During heterothallic mating, **a** cells express both the **a**-factor as well as the α-factor pheromone precursor (Bennett and Johnson, 2003; Dignard et al., 2007). These cells also express a Bar1 protease, which degrades the α-factor, ensuring that **a** cells secrete only the **a**-factor pheromone and maintain their cell identity (Schaefer et al., 2007). However, some *Ca. albicans* **a** cells have mutations in the *Bar1* gene that renders the protein non-functional (Alby et al., 2009). In these isolates, the α-factor can thus be expressed and secreted, and is then recognized by the same cell in which it was expressed. This initiates the mating pathway via the autocrine pathway and results in unisexual reproduction (Fig. 2). A second

manner by which unisexual reproduction can be achieved in *Ca. albicans* relies on the presence of α cells in a population dominated by **a** cells and is termed the paracrine pathway (Alby et al., 2009; Alby and Bennett, 2011). Secretion of the α-factor by α cells initiates the sexual pathway in **a** cells, rendering them sexually competent. Due to the high number of **a** cells in the population, the sexually competent cells are then likely to fuse with other **a** cells and complete the sexual cycle (Fig. 2), similar to the unisexual cycle of *Cr. neoformans*.

Unisexuality has been described in only three genera of filamentous ascomycetes. The first of these was *Neurospora*, where MAT1-1 isolates of *Neurospora africana*, *Neurospora galapagosensis, Neurospora dodgei* and *Neurospora lineolata* were shown to reproduce unisexually (Glass et al., 1990b; Glass and Smith, 1994). MAT1-2 isolates of two *Huntiella* species, *Huntiella moniliformis* and *Huntiella fecunda* were subsequently described as unisexual (Liu et al., 2018; Wilson et al., 2015a). More recently, *Thermoascus aurantiacus* was shown to undergo a unisexual cycle despite possessing only the *MAT1-1* genes (Schuerg et al., 2016). Interestingly, isolates of the opposite mating type have not been identified in *N. africana* or *H. moniliformis* (Glass et al., 1990b; Glass and Smith, 1994; Wilson et al., 2015a). This has led to the assumption that these populations exhibit a highly skewed mating-type distribution and may even entirely lack the second mating type. The *T. aurantiacus* isolate included in this study is the first to be assessed for mating type and thus it remains possible that isolates of both mating type are present (Schuerg et al., 2016). In contrast to the two unisexual yeasts, the unisexual cycles of these filamentous fungi have not been investigated in any detail. Consequently, the genetic mechanisms underlying this capability remain to be discovered.

This critical review considers the genetic mechanisms that might make unisexuality possible in a group of distantly related filamentous ascomycetes possible. For this purpose, we investigated previously published genetic, genomic and transcriptomic data, with particular emphasis on the *MAT* loci and pheromone response pathways, in a variety of unisexual species. These were then compared with their homothallic and heterothallic relatives. In cases where such data had not previously been published, we utilized publicly available NGS data to run additional analyses (details provided in Supplementary File A). Our hypothesis was that unisexuality is derived from a heterothallic state via the mutation of genes involved in the initiation phase of sexual reproduction. We thus present data showing that unisexual species: 1) harbour significantly altered secondary *MAT* genes; and/or 2) exhibit major changes to their mating pheromone pathways when compared to their heterothallic and primary homothallic relatives. Collectively, these comparable differences likely result in the unisexual strategies exhibited by these unrelated species.

2. UNISEXUAL FILAMENTOUS ASCOMYCETES AND THEIR RELATIVES

Publicly available genome and, where available, transcriptome data from 14 different filamentous ascomycetes were obtained from either the National Centre for Biotechnology Information (NCBI) or the Joint Genome Institute (JGI) (Table A.1). These species included fungi that reside in three different groups: 1) *Neurospora* (six species), 2) *Huntiella* (three species), and 3) *Byssochlamys* and *Thermoascus* (five species collectively).

Based on the available data sets, comparisons were drawn between MAT1-1 isolates of the heterothallic *Neurospora crassa* (Shear and Dodge, 1927) and *Neurospora discreta* (Perkins

and Raju, 1986), the pseudohomothallic *Neurospora tetrasperma* (Dodge, 1927), and the unisexual *N. africana* (Glass et al., 1990b). The primary homothallic species, *Neurospora terricola* (Gochenaur and Backus, 1962) and *Neurospora pannonica* (Krug and Khan, 1991), were also included in these comparisons. The *Huntiella* comparison consisted of MAT1-2 isolates of the two heterothallic species, *Huntiella bhutanensis* and *Huntiella omanensis* (Wilson et al., 2015a), and the unisexual *H. moniliformis* (Wilson et al., 2015a). Lastly, the *Byssochlamys* and *Thermoascus* comparisons included MAT1-1 isolates of the heterothallic *Byssochlamys spectabilis* (Houbraken et al., 2008) and the unisexual *T. aurantiacus* (Schuerg et al., 2016). The primary homothallic *Byssochlamys fulva* (Hebert, 1972) was also included in this comparison. During the course of conducting these comparisons and analyses, we also discovered two additional unisexual species from this group; *Byssochlamys nivea* and *Thermoascus crustaceus*. Both species had previously been described as homothallic (Biango-Daniels and Hodge, 2018; Kwon-Chung et al., 1984; Samson et al., 2009; Stolk and Samson, 1971), but our genomic analyses showed that they harbor only *MAT1-2* genes and are thus unisexual. These species were consequently also included in the comparisons as additional unisexual representatives of this group.

3. STRUCTURE AND GENE CONTENT OF THE *MAT* LOCI

3.1 *Neurospora* Species

Heterothallic *Neurospora* species, such as *N. crassa* and *N. discreta*, as well as the pseudohomothallic species, *N. tetrasperma*, have well-characterized *MAT* idiomorphs (Fig. 3). MAT1-1 isolates harbor three *MAT* genes, *matA-1* (*MAT1-1-1*), *matA-2* (*MAT1-1-2*) and *matA-3* (*MAT1-1-3*) (Glass et al., 1990a; Wik et al., 2008). These proteins each have the typical conserved functional domains; the α-box (PF04769), the MAT1-1-2 superfamily

domain (PF17043), and the HMG box domain (PF00505), respectively. Isolates having the *MAT1-2* idiomorph harbor only a single *MAT* gene, *mata-1* (*MAT1-2-1*) that also encodes a protein with the HMG box domain (PF00505) (Staben and Yanofsky, 1990). In contrast, primary homothallic *Neurospora* species, such as *N. terricola* and *N. pannonica*, possess genes associated with both the *MAT1-1* and *MAT1-2* idiomorphs (Fig. 3). *N. terricola* thus expresses *MAT1-1-1, MAT1-1-2* and *MAT1-2-1* (Beatty et al., 1994); while *N. pannonica* has these three genes as well as *MAT1-1-3*. The MAT1-1 unisexual *N. africana* possesses all three *MAT1-1* genes (Gioti et al., 2012) (Fig. 3). To date, no *MAT1-2*-associated genes have ever been identified from *N. africana* (Gioti et al., 2012; Pöggeler, 1999; Wik et al., 2008) and this was confirmed in the present study.

3.2 *Huntiella* Species

Heterothallic *Huntiella* species also have well-defined *MAT* idiomorphs, conferring the two alternate mating types (Fig. 3) (Wilson et al., 2015a). In *H. omanensis* and *H. bhutanensis*, isolates with the *MAT1-1* idiomorph have the *MAT1-1-1* and *MAT1-1-2* genes with their associated functional domains (PF04769 and PF17043, respectively). MAT1-2 isolates also harbor two genes at their *MAT1-2* idiomorph, the primary *MAT* gene, *MAT1-2-1*, possessing the conserved HMG box domain (PF00505), as well as a secondary *MAT* gene, *MAT1-2-7*, which possesses no recognizable conserved domains (Wilson et al., 2015a). All studied isolates of the unisexual *H. moniliformis* possess only the *MAT1-2* idiomorph and its two respective genes (Fig. 3).

3.3 *Byssochlamys* and *Thermoascus* Species

MAT1-1 isolates of the heterothallic *B. spectabilis* harbor the *MAT1-1-1* gene, with its functional domain, as well as a secondary *MAT* gene (Houbraken et al., 2008) (Fig. 3). This gene displays similarity to the *MAT1-1-4* gene of *Trichophyton verrucosum*, which was renamed *MAT1-1-9* in a recent update of *MAT* gene nomenclature (Wilken et al., 2017) and has been annotated as such here. The primary homothallic *B. fulva* has the *MAT1-1-1*, *MAT1-2-1* and *MAT1-2-4* genes (Fig. 3). Both the primary genes contain the appropriate functional domains. The MAT1-2-4 protein has no known functional domains. MAT1-1 isolates of the unisexual *T. aurantiacus* harbor both the *MAT1-1-1* and *MAT1-1-9* genes (Fig. 3). MAT1-2 isolates of the unisexual species *B. nivea* and *T. crustaceus* both possess the *MAT1-2-1* and *MAT1-2-4* genes (Fig. 3).

4. MUTATIONS IN THE SECONDARY *MAT* GENES

The genes present at the *MAT* loci of the unisexual species considered in this study are broadly comparable with those of their heterothallic and primary homothallic relatives. This is particularly true for the primary *MAT* genes, *MAT1-1-1* and *MAT1-2-1*. In each case, the unisexual species and their relatives harbor the same primary *MAT* genes, that are of similar length and show high levels of sequence identity at both the nucleotide and amino acid level. These genes also encode proteins that have the relevant protein domains (Fig. 3).

The high degree of conservation found in the primary *MAT* genes does not extend to the secondary *MAT* genes of these species. In four of the five unisexual species, there have been significant changes to the secondary *MAT* genes, including both partial deletion and truncation events. One exception is the *MAT1-1-2* gene of *N. africana,* which, while shorter

than that of *N. crassa*, is still expressed and encodes a protein harboring the relevant conserved domain (Fig. 4A). The *N. africana MAT1-1-3* gene is significantly different from the *MAT1-1-3* genes in other species. In *N. crassa, N. discreta, N. tetrasperma* and *T. pannonica*, the *MAT1-1-3* gene is characterized by four exons that are interrupted by three introns (Fig. 4B). The functional domain, an HMG box, spans the third intron and is thus encoded by sequence from both exons three and four. In contrast, the *N. africana MAT1-1-3* gene has undergone a major internal deletion, with the majority of the third exon and third intron having been deleted. Consequently, the gene produces a protein without the HMG box functional domain (Fig. 4B). It is reasonable to speculate that this gene is no longer functional.

In the cases of *H. moniliformis* and *T. crustaceus*, the introduction of premature stop codons has significantly truncated their respective secondary *MAT* genes (Fig. 3). The stop codon in the *H. moniliformis MAT1-2-7* gene shortens it to less than 30% of the length of the *H. omanensis* homolog (Wilson et al., 2015a). Furthermore, while it is expressed during sexual development in *H. omanensis*, expression of this gene cannot be detected in vegetative or sexual tissues of *H. moniliformis* (Wilson et al., 2018). In *T. crustaceus*, the premature stop codon shortens the *MAT1-2-4* gene to less than 20% of the corresponding gene in *B. fulva*. Careful inspection of this region revealed no alternative start codons that could be used to produce a full-length protein.

The *MAT1-1-9* genes of *T. aurantiacus* and *B. spectabilis* present an interesting case. *De novo* predictions of these genes produce similar gene models that share some structural characteristics. The gene in *B. spectabilis* produces a 245 aa protein that is confirmed to be

expressed in culture. In contrast, the available RNA sequence data for *T. aurantiacus* does not support the gene model for this species. Instead, mapping the RNA data to the genomic region encoding for the *MAT1-1-9* gene in *T. aurantiacus* supports a gene model with fewer introns than originally predicted (Fig. 5). Exclusion of the first intron introduces a premature stop codon that significantly shortens the protein coding region to only 51 nt, less than 10% of the total coding region of the *B. spectabilis MAT1-1-9*. Closer inspection of this region, using the RNA mappings as a guide, revealed no alternative start codons or alternative splicing options that would allow for the expression of this gene in full.

Because the *MAT1-2-7, MAT1-2-4* and *MAT1-1-9* proteins do not possess any recognizable conserved domains, it is difficult to assess the effect of these truncations on the functional regions of the proteins. However, it is reasonable to assume that the significantly shortened length of each protein would render them non-functional.

5. MODIFICATIONS IN THE PHEROMONE RESPONSE PATHWAY

We were able to identify the genes encoding the pheromone precursors in the genomes of most the species considered in this study (Fig. 3, Tables A.2, A.3, A.4). The identified pheromone precursor exhibited the expected structural characteristics and possessed the relevant functional domains (Table A.3, A.4, Figs C.1, C.2). While the pheromone precursor expression patterns of the considered heterothallic species closely resemble those reported from other heterothallic species (Bobrowicz et al., 2002; Samils et al., 2013; Wilson et al., 2018), pheromone precursor expression in three of the unisexual species is unusual for isolates of a single mating type. Under this paradigm, the MAT1-1 isolates of *N. africana* and the MAT1-2 isolate of *H. moniliformis* should only express the α-factor or the **a**-factor

pheromone precursors, respectively. However, both *N. africana* and *H. moniliformis* (Wilson et al., 2018) express both pheromone factors. This suggests that a single isolate of either species is able to recognize itself, or another isolate of the same mating type, as a suitable mating partner. This allows for sexual reproduction to occur in the absence of an opposite mating-type partner.

The pheromone precursor expression profile of *T. aurantiacus* also exhibits various interesting deviations from that observed in other species. At the outset, we were able to identify the α-factor pheromone precursor genes from *B. spectabilis, B. fulva, B. nivea and T. crustaceus* (Table A.3). To achieve this, a microsynteny approach was used, exploiting the synteny between the α-factor pheromone precursor gene and its flanking regions in *Aspergillus flavus* and the four other species (Fig. 6). The predicted *B. spectabilis, B. fulva, B. nivea and T. crustaceus* α-factor pheromone precursor genes produce putative proteins with the structural characteristics typical of the ascomycete α-factor pheromone precursor (Table A.3). This synteny-based approach also enabled us to identify a significantly truncated α-factor pheromone precursor gene in the genome of *T. aurantiacus* (Fig. 6). This shortened gene would produce a protein of only 17 aa and does not possess the mature pheromone sequence.

Genes encoding the **a**-factor pheromone precursor could not be found in the genomes of the *Byssochlamys* and *Thermoascus* species. These species reside in the Eurotiomycetes, a class of fungi that also accommodates the model species *Aspergillus nidulans*. The **a**-factor pheromone has not been identified in any studied species of Eurotiomycetes, despite the fact that thorough genomic investigations have been undertaken (Dyer et al., 2003;

Pöggeler, 2002). In future, techniques such as comparative transcriptomics using isolates of opposite mating type could be useful to identify this elusive gene. Identification of this gene would shed light on the role of the pheromone response pathway in many homothallic species and particularly in the unisexual species that reside in the genera *Byssochlamys* and *Thermoascus*.

In addition to the pheromone factors, the pheromone receptors also play an integral role in the pheromone response pathway and have been shown to be important for sexual reproduction in a variety of both heterothallic and homothallic species (Kim et al., 2012; Kim and Borkovich, 2004; Lee et al., 2008; Seo et al., 2004). Genes encoding the receptors for both pheromones can be found in the genomes of all 14 species considered in this study (Fig. 3). Both receptors in the *Neurospora* and *Huntiella* species as well as in *B. spectabilis*, *B. fulva* and *T. crustaceus* harbor the recognizable protein domains associated with the α-factor and **a**-factor receptors (PF02116 and PF02076, respectively). They also possess the typical seven transmembrane domain structure required for receptor function. The same is true of the **a**-factor receptor in the MAT1-1 *T. aurantiacus* and the α-factor receptor in the MAT1-2 *B. nivea*. In contrast, the *T. aurantiacus* α-factor receptor possesses the relevant functional domain, but only three of the transmembrane domains (Fig. 7). The *B. nivea* **a**-factor receptor is significantly truncated and does not possess the functional domain nor the seven transmembrane domains. Thus, the pheromone response pathway appears to have been significantly altered in the unisexual *T. aurantiacus* and *B. nivea*.

6. THE TRANSITION TO UNISEXUALITY

A long-held debate in the field of fungal biology concerns the ancestral sexual strategy of fungi (Billiard et al., 2012; Lin and Heitman, 2007). It is thought, for example, that homothallism is the ancestral state in genera such *Sclerotinia* and *Botrytis* (Amselem et al., 2011), and that numerous transitions to heterothallism have taken place during their evolutionary trajectory. In contrast, the ancestral strategy for fungi in the genera *Aspergillus* (Ojeda-López et al., 2018)*, Neurospora* (Gioti et al., 2012) and *Cochliobolus* (Yun et al., 1999) is thought to be heterothallism, with various independent transitions towards homothallism having taken place. The ancestral state for all fungi, however, continues to be debated (Gioti et al., 2012; Heitman, 2015; Lin and Heitman, 2007).

Transitions between homothallism and heterothallism have occurred frequently across the fungal Kingdom (Lin and Heitman, 2007). It is thought that these shifts are predominantly the result of changes in the selective pressures imparted by the environment (Lee et al., 2010). Harsh environmental conditions that favour the generation of high genetic diversity and environmentally-resistant spores, for example, may lead to the transition towards heterothallism in an attempt to enhance outcrossing and increase genetic diversity (Heitman, 2006). In contrast, considering the major cost of obligate mate seeking, environments where the probability of encountering a mate is limited might select for some form of homothallic behavior (Billiard et al., 2011).

In this review, we posit that unisexuality has its origins in heterothallism. This is certainly possible for *Cr. neoformans*; the most extensively investigated of the known unisexual species (Wang and Lin, 2011). While this yeast has a well-characterized heterothallic cycle

(Kwon-Chung, 1976), the vast majority of naturally-occurring isolates collected are of the α mating type (Kwon-Chung and Bennett, 1978). Given that both **a** and α cells are capable of unisexual reproduction (Tscharke et al., 2003), the unequal mating-type ratio is most likely not the result of a fitness advantage conferred to the unisexually-reproducing cells. Rather, it appears more likely that unisexuality evolved as a mechanism to ensure sexual outcrossing in a population dominated by a single mating type. In this case, the heterothallic lifestyle represents the ancestral state, while unisexuality is likely a derived state. A similar transition may have occurred in the unisexual species investigated in this study. For example, all isolates of *H. moniliformis* that have been collected to date are of the MAT1-2 mating type (Wilson et al., 2015a). This suggests either a dominance of this mating type in nature or the extinction of the MAT1-1 mating type. A similar case is found in naturally occurring isolates of *N. africana*, where only those that possess the *MAT1-1* genes have been collected (Glass and Smith, 1994). Thus, while the uneven mating-type ratio could be attributed to unisexual reproduction, unisexuality likely evolved in response to this mating-type inequality.

Our results suggest that the transition from heterothallism to unisexuality is the result of relatively minor changes to the genes that control the initiation of sexual reproduction, ie: the *MAT* genes and/or the pheromone response pathway genes. It has been shown in several species that the genes and pathways involved in sexual reproduction in heterothallic species are similar to those involved in homothallic mating (Beatty et al., 1994; Ferreira et al., 1998; Lin et al., 2010). In fact, homothallic species such as *A. nidulans*, *F. graminearum* and *Sordaria macrospora*, retain the pheromone response pathway, a pathway that is primarily involved in partner attraction and recognition (Dyer et al., 2003; Lee et al., 2008;

Pöggeler, 2000). This conservation strongly suggests that, while the sexual processes in homothallic and heterothallic mating differ in the initiation phase, the downstream pathways and networks remain the same (Galagan et al., 2005). This further adds to the ease with which a species can transition between the two mating strategies, as small changes to the initiation processes should be sufficient.

Known transitions from heterothallism to primary homothallism have been associated with changes in the regulation of genes that were previously under the control of mating-type genes. This is true in *A. nidulans* (Paoletti et al., 2007), where despite being important for the sexual cycle, genes such as those involved in the pheromone response pathway are not controlled by the MAT proteins. Since unisexual reproduction is considered a form of homothallism, it is not surprising that there are potentially similar changes observed in the unisexual species considered here. In *H. moniliformis*, for example, it is clear that the **a**-factor pheromone is no longer under the control of the MAT1-1-1 protein (Wilson et al., 2018), whilst the same is true of the *N. africana* MAT1-2-1 protein and the **a**-factor pheromone. The key difference is that primary homothallism relies on the presence and expression of both the *MAT1-1-1* and *MAT1-2-1* genes, while unisexual fungi, by definition, harbour only one or the other (Roach et al., 2014; Wilson et al., 2015b).

In four of the five unisexual species considered in this study, major disruptions were evident in the secondary mating-type genes: *MAT1-1-3 (N. africana)*, *MAT1-1-9 (T. aurantiacus)*, *MAT1-2-4 (T. crustaceus)* and *MAT1-2-7 (H. moniliformis)*. Given that these species retain their capacity for sexual reproduction, these genes are clearly not essential for the sexual pathway. We hypothesize that these genes are important for mating in strictly heterothallic

species, and this is certainly true for the *N. crassa MAT1-1-3* (Ferreira et al., 1998) and the *H. omanensis MAT1-2-7* (Wilson et al., 2020). However, they may predominantly act by suppressing the activation of the sexual pathway in the absence of a suitable mating partner. Their inactivation in unisexual species might thus result in a loosening of the tight regulation that usually controls the initiation of sexual reproduction.

Four of the unisexual species included in this study exhibited changes to their pheromone response pathways. Unexpected pheromone precursor expression patterns were observed in three of these species. The MAT1-1 *N. africana* was capable of **a**-factor pheromone precursor expression, the MAT1-1 *T. aurantiacus* was unable to express a functional α-factor pheromone precursor or its cognate receptor and the MAT1-2 *H. moniliformis* was capable of expressing the α-factor pheromone precursor. Lastly, *B. nivea*, possessed a severely truncated **a**-factor pheromone receptor. Taken collectively, this suggests that changes to the pheromone response pathway may also play a role in the transition from heterothallism to unisexuality.

The fifth unisexual species considered in this study, *T. crustaceus,* appears to harbor an intact and normal pheromone pathway. Unfortunately, there are no transcriptome data available for this species and thus the expression of this pathway cannot be confirmed. Yet on the basis of our other results, it seems likely that this isolate would be capable of α-factor pheromone precursor expression even though it is of the MAT1-2 mating type. The generation of transcriptomic data from sexually reproducing cultures of this species could further support the hypothesis being posited in this study.

The results of this study strongly suggest that unisexuality is the result of modifications to genes involved in the initiation of sexual reproduction. In some cases, unisexual species exhibit changes to both the *MAT* genes as well as the pheromone response pathway; while in other cases, disruptions are present in only one set of genes. In either case, it is evident that mating identities have been affected and the result is sexual reproduction in single mating type cultures. Importantly, this sexual pathway appears to be different from and independent of the primary homothallic system. In fact, the *MAT* genes and pheromone pathways in the homothallic species included here seem to be intact and fully functional. This is with the exception of *MAT1-1-3* in *N. terricola* and *MAT1-1-9* in *B. fulva*, which are both entirely absent and yet cause no change to the sexual capabilities of these species.

It is important to note that the model being proposed here suggests that gene loss or truncation, at least in part, resulted in unisexual reproduction. It is, however, also possible that the opposite is true and that the transition to unisexuality resulted in subsequent gene loss due to these genes no longer being required for sexual development. It is, for example, possible that pheromone signaling is not important in unisexual reproduction and thus, once *T. aurantiacus* began unisexually reproducing, the α-factor pheromone gene was lost. It is possible to differentiate between these two different possibilities using a variety of experimental techniques, as outlined below.

This study provides a model upon which further experimentation can be conducted to test the hypothesis that minor mutations in the *MAT* genes and pheromone response pathway allow for the transition from heterothallism to unisexuality. One approach would be to conduct genome editing experiments that result in the truncation of secondary *MAT* genes

in heterothallic species of *Neurospora*, *Huntiella*, *Thermoascus* and *Byssochlamys*. Results from such studies could add experimental evidence to support this model if unisexual behaviour can be induced in typically heterothallic species. This kind of experimentation has already been conducted in *H. omanensis* (Wilson et al., 2020) and could be followed up with further genome-editing experiments that target the pheromone precursor genes. Alternatively, genome-editing could be used to replace the truncated *MAT* genes in unisexual species with full-length homologs to determine if the presence of intact *MAT* genes abolishes unisexual behaviour. In both cases, the relevant, genus-specific changes to the pheromone precursors, their cognate receptors, and their expression patterns may also need to be taken into account.

7. CONCLUSIONS

Unisexual reproduction is a largely understudied sexual strategy in the filamentous fungi. Given the importance of sexual reproduction for the evolutionary longevity and survival of species, unisexual reproduction may have evolved as a mechanism to ensure genetic recombination under environmental conditions not conducive for typical sexual reproduction. We have provided evidence in this review to suggest that the transition from heterothallism to unisexuality may occur via relatively minor mutations in a suite of genes and could thus represent a fairly common state. Although the results presented here are based on findings from species accommodated in only four filamentous fungal genera, we believe the rapidly growing availability of whole genome sequences, coupled with classical fungal physiology, will reveal a greater number of unisexual fungal species. The difference between primary homothallism and unisexuality is, by definition, strictly based on the presence or absence of both mating types and therefore on the *MAT* locus structure and

gene content. It is thus likely that more unisexual species will be described in the future. Indeed, this was the case for *B. nivea* and *T. crustaceus*, both of which were previously thought to be homothallic and were shown to be unisexual in this study.

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Figure Titles:

Fig. 1: The two mechanisms of unisexuality in *Cryptococcus neoformans*. A) The genome of a single cell of either mating type can undergo endoreplication, thereby forming a diploid cell which can consequently undergo sexual reproduction. B) Two non-isogenic cells of the same mating type can fuse, thereby forming a diploid cell which can then unisexually reproduce. Both of these mechanisms are enhanced in the presence of pheromone peptides expressed by cells of the opposite mating type.

Fig. 2: The two mechanisms of unisexuality in *Candida albicans*. A) Autocrine mode: A single cell of the **a**-mating type expresses both pheromones in the absence of a functional Bar1 protease. This cell can then respond to endogenously produced α-factor and produce sexual structures. B) Paracrine mode: An α-cell expresses the α-factor which binds to the receptors of **a**-cells in the environment and stimulates sexual reproduction between two cells of the **a**-mating type.

Fig. 3: Schematic diagram of the *MAT*, pheromone and pheromone receptor genes in each species. In each of the unisexual species, significant changes are evident in the *MAT* locus and/or the pheromone pathway. In *N. africana*, the functional domain of *MAT1-1-3* has been deleted and both pheromone precursors are expressed. In *H. moniliformis*, *MAT1-2-7* is significantly truncated and both pheromone precursors are expressed. *In B. nivea*, the **a**-pheromone receptor is significantly truncated. In *T. aurantiacus*, the *MAT1-1-9* gene is significantly truncated, the **α**-pheromone precursor is significantly truncated, and the α-pheromone receptor is truncated, possessing only three transmembrane domains. In *T. crustaceus*, *MAT1-1-9* is significantly truncated.

[a] Genes that are expressed are outlined in yellow.

[b] Species for which raw RNA data was available are indicated in blue text.

[c] In *N. tetrasperma*, *N. terricola* and *N. pannonica*, gene expression data was obtained from previously published articles.

[d] Genes for which no expression data exists but are likely expressed are outlined in orange.

[e] The darker bars and triangles on the gene models illustrate the various functional domains characteristic of gene/protein. (*MAT1-1-1*: alpha box, *MAT1-1-2*: MAT1-1-2 superfamily domain, *MAT1-1-3*: HMG box, *MAT1-2-1*: HMG box, α-pheromone precursor: signal peptide, **a**-pheromone precursor: CaaX domain, α- and **a**-receptors: seven transmembrane domains).

[f] The grey gene models indicate genes that are absent. Note: gene models are not drawn to scale.

Fig. 4: The structure of the *N. africana* and *N. crassa MAT1-1-2* and *MAT1-1-3* genes. A) Truncation in the *Neurospora africana MAT1-1-2* gene does not affect the functional domain of the MAT1-1-2 protein. A protein alignment between the MAT1-1-2 proteins of *N. africana* (top, dark green) and *N. crassa* (bottom, light green) shows that the *N. africana* protein is shorter than that of *N. crassa*, but that the functional domains (dark and light blue) are of similar length and position. B) A deletion within the *N. africana MAT1-1-3* results in the deletion of the HMG box functional domain. The blue bar represents the sequence of the *MAT1-1-3* gene from *N. crassa*, while the orange connected bars represent the coding sequence of the gene. The maroon bar represents the region coding for the HMG domain. The green line represents the regions within the *N. africana* genome where significant similarity to the *N. crassa MAT1-1-3* gene was found. While the 5' and 3' regions of the *N. crassa* gene show significant similarity to that of *N. africana*, large parts of the

third exon and intron, have been entirely deleted in *N. africana*. This results in the deletion of a significant part of the functional domain as well as the inclusion of a premature in frame stop codon shortly after the second intron *in N. africana* (indicated by the green arrow).

Fig. 5: Predicted and supported gene models of the *T. aurantiacus MAT1-1-9* gene. The predicted gene model is indicated in orange, and harbours a total of five introns which interrupt six exons. Indicated in blue and green are the two alternate models supported by the RNA-seq data. Neither model supports the first or second introns, but does suggest alternative splicing which results in one of two splice variants. However, in both splice variants, the first intron is removed, thereby introducing a premature stop codon. This gene consequently encodes a truncated protein.

Fig. 6: Identification of the *B. spectabilis*, *B. fulva*, *B. nivea*, *T. crustaceus* and *T. aurantiacus* α-factor pheromone genes. A microsynteny approach was used to identify the α-factor pheromone precursor genes, using the α-factor precursor gene and its flanking genes from *A. flavus*. By identifying the precursor gene flanking genes in three *Byssochlamys* species and the two *Thermoascus* species, it was possible to manually annotate the intergenic region and identify the α-pheromone precursor gene in four of these species. A full-length gene could not be found in the *T. aurantiacus* genome.

Fig. 7: Transmembrane domains of the two pheromone receptor proteins. In the heterothallic *B. spectabilis*, there are seven transmembrane domains in both receptors, as expected from these proteins. The same is true for the *T. aurantiacus* **a**-factor receptor and

the *B. nivea* α-factor receptor. In contrast, the α-factor receptor from the unisexual *T. aurantiacus* harbours only three transmembrane domains and the **a**-factor receptor in *B. nivea* is significantly truncated and does not possess any functional or transmembrane domains. The x-axis represents the length of the protein, while the y-axis represents the posterior label probability, a measure of the likelihood that a region of the protein is hydrophobic. The output from Phobius was used to create these graphs using the ggplot2 package in R.

SUPPLEMENTARY FILE A: METHODS

1. Genome assemblies and annotation

All of the genome assemblies used in this study are publicly available from either the National Centre for Biotechnology Information (NCBI) or the Joint Genome Institute (JGI) (Table S1). All 14 genomes were subjected to gene annotation using the web-based gene annotator AUGUSTUS (Stanke and Waack, 2003). For the *Neurospora* genomes, the *N. crassa* species-specific gene model parameters were used. The *Fusarium graminearum* parameters were used for all three *Huntiella* genomes, while the *Byssochlamys, Thermoascus* and *A. flavus* genomes were annotated using *Aspergillus nidulans* specific parameters. These species-specific parameters were chosen based on the closest relative that could be found in the AUGUSTUS gene model parameter library. In addition to these genome-wide annotations, the contigs on which the target genes (*MAT*, pheromone and pheromone receptor genes) were identified were also subjected to Fgenesh annotation (Salamov and Solovyev, 2000). The RNA sequence mapping described below was also used to confirm or correct the predicted gene models.

2. RNA-seq data

The majority of the RNA sequence data used in this study were obtained as raw reads from the Sequence Read Archive (SRA) and Gene Expression omnibus (GEO) databases of the NCBI (Table A.1).

Table A.1: The species used in this study, their sexual strategies, the genome assemblies used and, where relevant, the raw RNA reads used.

Species	Sexual Strategy	Isolate	Source	Accession Numbers	Reference / Submitter
Neurospora crassa	Heterothallic	OR7A FGSC4200 / 2489	NCBI	*DNA:* AABX00000000.3 *RNA:* GSE41484	Galagan et al., 2003 Wang et al., 2014
Neurospora discreta	Heterothallic	FGSC 8579	NCBI	RANX00000000.1	University of California, Berkeley
Neurospora tetrasperma	Pseudohomothallic	FGSC 2508	NCBI	AFBT00000000.1	Ellison et al., 2011
Neurospora terricola	Primary homothallic	FGSC 1889	NCBI	CAPR000000000.2	Gioti et al., 2013
Neurospora pannonica	Primary homothallic	FGSC 7221	NCBI	CAPQ000000000.2	Gioti et al., 2013
Neurospora africana	Unisexual (MAT1-1)	FGSC 1740	NCBI	*DNA:* CAPO000000000.2 *RNA:* ERS213531	Gioti et al., 2013 European Bioinformatics Institute
Huntiella bhutanensis	Heterothallic	CMW 8217	NCBI	MJMS00000000.1	Wingfield et al., 2016
Huntiella omanensis	Heterothallic	CMW 11056	NCBI	*DNA:* JSUI00000000.1 *RNA:* SRP108437	van der Nest et al., 2014 Wilson et al., 2018
Huntiella moniliformis	Unisexual (MAT1-2)	CMW 10134 CMW 36919	NCBI	*DNA:* JMSH00000000.1 *RNA:* SRP108437	Van Der Nest et al., 2014 Wilson et al., 2018
Byssochlamys spectabilis	Heterothallic	NBRC 109023	NCBI	RCHW00000000.1 SRP138004	Prefecture, 2014 Joint Genomes Institute
Byssochlamys fulva	Primary homothallic	BYSS01	NCBI	NIXA00000000.1	University of Dayton Research Institute
Byssochlamys nivea	Unisexual (MAT1-2)	CO 7	NCBI	QEIL00000000.1	Cornell University
Thermoascus aurantiacus	Unisexual (MAT1-1)	ATCC 26904	JGI NBCI	*DNA:* 405671 *RNA:* Pending	Singer, n.d. This study
Thermoascus crustaceus	Unisexual (MAT1-2)	JCM 12817	NGBI	BCIC00000000.1	RIKEN Center for Life Science Technologies
Aspergillus flavus	Primary homothallic	NRRL 3357	NCBI	AAIH00000000.2	Yin et al., 2018

2.1 RNA-seq data for *Thermoascus aurantiacus*

T. aurantiacus ATCC 26904 was grown in a modified glucose-preculture medium as previously described (Schuerg et al., 2017). Accordingly, ascospores from 7-day-old potato dextrose agar plates were harvested and 1 x 10^6 spores per ml were used to inoculate glucose pre-cultures (2% glucose [w/v], McClendon, pH = 5.25, 0.8% soy meal peptone [w/v]). 50 mL pre-cultures were incubated for 48 h in 250 mL Erlenmeyer flasks at 50 °C and 180 rpm. These cultures were filtered under vacuum, using a Buchner funnel and Whatman paper #1, each washed with 100 mL defined McClendon's medium without peptone (McClendon's salts pH = 5.25, 0.25 mM ammonia nitrate) (Mcclendon et al., 2012) and shifted to four new flasks containing the same medium and either 2% beechwood xylan (Megazyme) or no carbon source. All cultures were grown under constant incandescent light for 48 hours in a rotary shaker at 50 °C and 180 rpm. From these shift cultures, samples were taken 1, 4, 6 and 8 hours after shifting. The sampling procedure involved taking an aliquot of 8 ml culture and vacuum filtering and washing it on a Whatman paper with immediate freezing in liquid nitrogen.

Total nucleic acids were extracted by grinding the frozen culture samples with a mortar and pestle in liquid nitrogen and resuspending this in TRIzol® Reagent (Thermo Fisher Scientific). RNA was isolated with Phenol:Chloroform:Isoamyl Alcohol, ethanol precipitation and subsequent DNAseI treatment. RNA quantity was determined on a Qubit (Thermo Fisher Scientific) and RIN values were determined using a Bioanalyzer (Agilent RNA 6000 Nano). Samples of suitable quality (RIN values

> 8) from all time points of xylan and no carbon cultures were taken for cDNA synthesis and library construction with the TruSeq® Stranded Total RNA Library Kit (Illumina®). For this study, only one biological replicate for each condition and time point was sequenced on a MiSeq (Illumina®).

3. Gene identification

3.1 *MAT* genes

MAT genes for which reference sequences were available were downloaded from NCBI (Table A.2) and used in BLASTn queries against their respective genomes in order to identify the *MAT* loci. For example, the sequences for *matA-1* (*MAT1-1-1*), *matA-2* (*MAT1-1-2*) and *matA-3* (*MAT1-1-3*) from *N. crassa* were downloaded and used as a BLASTn query against the *N. crassa* genome. In order to identify the *B. spectabilis MAT* locus, the MAT1-1-1 protein sequence was downloaded from NCBI and used as a tBLASTn query against the *B. spectabilis* genome. For species whose *MAT* genes were not available from NCBI (Table A.2), various BLASTn and tBLASTn searches were conducted against their genomes using *MAT* genes from closely related species. For example, to search for the *MAT* locus of *N. discreta*, the three *MAT* genes from *N. crassa* were used in BLASTn searchers.

Table A.2: The accession numbers or Gene IDs for all genes used in this study.

Species	Gene	Accession Number/Gene ID
N. crassa	*matA-1 (MAT1-1-1)*	3880391
	matA-2 (MAT1-1-2)	3880488
	matA-3 (MAT1-1-3)	3880489
	*mfa-1 (***a**-factor pheromone)	XM_011396530.1
	*ccg-4 (*α-factor pheromone)	XM_960001.3
	*pre1 (***a**-factor receptor)	3872329

	*pre2 (*α-factor receptor)	3875871
N. discreta	*matA-1 (MAT1-1-1)* [a]	Appendix B
	matA-2 (MAT1-1-2) [a]	Appendix B
	matA-3 (MAT1-1-3) [a]	Appendix B
	*mfa-1 (***a**-factor pheromone) [a]	Appendix B
	*ccg-4 (*α-factor pheromone) [a]	Appendix B
	*pre1 (***a**-factor receptor) [a]	Appendix B
	*pre2 (*α-factor receptor) [a]	Appendix B
N. tetrasperma	*matA-1 (MAT1-1-1)*	XM_009857139.1
	matA-2 (MAT1-1-2)	XM_009857138.1
	matA-3 (MAT1-1-3)	XM_009856640.1
	*mfa-1 (***a**-factor pheromone) [a]	Appendix B
	*ccg-4 (*α-factor pheromone)	XM_009858490.1
	*pre1 (***a**-factor receptor)	AM749905.1
	*pre2 (*α-factor receptor)	AM904749.1
N. terricola	*matA-1 (MAT1-1-1)*	HE600070.1
	matA-2 (MAT1-1-2)	HE600070.1
	mata-1 (MAT1-2-1)	HE600070.1
	*mfa-1 (***a**-factor pheromone) [a]	Appendix B
	*ccg-4 (*α-factor pheromone) [a]	Appendix B
	*pre1 (***a**-factor receptor) [a]	Appendix B
	*pre2 (*α-factor receptor)	HE861764.1
N. pannonica	*matA-1 (MAT1-1-1)*	HE600067.1
	matA-2 (MAT1-1-2)	HE600067.1
	matA-3 (MAT1-1-3)	HE600067.1
	mata-1 (MAT1-2-1)	HE600067.1
	*mfa-1 (***a**-factor pheromone) [a]	Appendix B
	*ccg-4 (*α-factor pheromone) [a]	Appendix B
	*pre1 (***a**-factor receptor)	HE861745.1
	*pre2 (*α-factor receptor)	HE861760.1
N. africana	*matA-1*	HE600066.1
	matA-2	HE600066.1
	matA-3	HE600066.1
	*mfa-1 (***a**-factor pheromone) [a]	Appendix B
	*ccg-4 (*α-factor pheromone) [a]	Appendix B
	*pre1 (***a**-factor receptor)	HE861735.1
	*pre2 (*α-factor receptor)	HE861751.1
H. bhutanensis	*MAT1-2-1* [a]	Appendix B
	MAT1-2-7 [a]	Appendix B
	a-factor pheromone [a]	Appendix B
	α-factor pheromone [a]	Appendix B
	*pre1 (***a**-factor receptor) [a]	Appendix B
	*pre2 (*α-factor receptor) [a]	Appendix B
H. omanensis	*MAT1-2-1*	KU950302.1
	MAT1-2-7	KU950303.1
	a-factor pheromone [a]	Appendix B

	α-factor pheromone [a]	Appendix B
	*pre1 (***a**-factor receptor) [a]	Appendix B
	*pre2 (*α-factor receptor) [a]	Appendix B
H. moniliformis	*MAT1-2-1*	KU950299.1
	MAT1-2-7	KU950298.1
	a-factor pheromone [a]	Appendix B
	α-factor pheromone [a]	Appendix B
	*pre1 (***a**-factor receptor) [a]	Appendix B
	*pre2 (*α-factor receptor) [a]	Appendix B
B. spectabilis	*MAT1-1-1*	GAD92179.1 [b]
	MAT1-1-9 [a]	Appendix B
	ppgA (α-factor pheromone) [a]	Appendix B
	preA (**a**-factor receptor) [a]	Appendix B
	preB (α-factor receptor) [a]	Appendix B
B. fulva	*MAT1-1-1* [a]	Appendix B
	MAT1-2-1 [a]	Appendix B
	MAT1-2-4 [a]	Appendix B
	ppgA (α-factor pheromone) [a]	Appendix B
	preA (**a**-factor receptor) [a]	Appendix B
	preB (α-factor receptor) [a]	Appendix B
B. nivea	*MAT1-2-1* [a]	Appendix B
	MAT1-2-4 [a]	Appendix B
	ppgA (α-factor pheromone) [a]	Appendix B
	preA (**a**-factor receptor) [a]	Appendix B
	preB (α-factor receptor) [a]	Appendix B
T. aurantiacus	*MAT1-1-1* [a]	Appendix B
	MAT1-1-9 [a]	Appendix B
	ppgA (α-factor pheromone) [a]	Appendix B
	preA (**a**-factor receptor) [a]	Appendix B
	preB (α-factor receptor) [a]	Appendix B
T. crustaceus	*MAT1-2-1* [a]	Appendix B
	MAT1-2-4 [a]	Appendix B
	ppgA (α-factor pheromone) [a]	Appendix B
	preA (**a**-factor receptor) [a]	Appendix B
	preB (α-factor receptor) [a]	Appendix B
Aspergillus flavus	*ppgA* (α-factor pheromone)	7919755
	preA (**a**-factor receptor)	7912518
	preB (α-factor receptor)	7914252

[a] This study
[b] Protein sequence

3.2 Pheromone genes

3.2.1 *α-factor pheromone*

Pheromone genes for which reference sequences existed were downloaded from NCBI (Table A.2) and used in BLASTn queries against their respective genomes in order to identify the gene's location as previously described for the *MAT* genes. For species where the pheromone genes were not available from NCBI (Table A.2), tBLASTn searches were conducted against their genomes using pheromone genes of closely related species. This method successfully identified all the α-factor pheromone genes in the *Neurospora* and *Huntiella* species.

Identification of the α-factor pheromone genes in the *Byssochlamys* and *Thermoascus* genomes was achieved using a microsynteny approach based on the location of the α-factor pheromone gene in the *Aspergillus flavus* genome. The proteins encoded by the genes directly flanking the *A. flavus* α-factor gene were used in local tBLASTn searches against the genomes of the *Byssochlamys* and *Thermoascus* species. The region between the identified flanking genes in these species was subjected to Fgenesh (Salamov and Solovyev, 2000) and manual annotation in order to identify the pheromone gene.

The predicted α-factor pheromone genes of all species considered in this study were translated and analyzed using the hydrophobicity and signal peptide predicting software programs, ExPASy ProtScale (Gasteiger et al., 2005) and Phobius (Krogh et al., 2007). Hydrophobicity was measured using the Kyte & Doolittle measure, with a window size of 9, a relative weight of window edges compared to window centers of

100%, a linear weight variation model and no scale normalization. Probability outputs from both programs were used to generate plots using the *ggplot2* package in R (Wickham, 2010; Wilkinson et al., 2005) (Figs S1 and S2). The potential mature α-factor repeats were manually annotated (Table A.3).

Table A.3: Structure and repeat sequence of the α-pheromones from each species.

[a] The gene sequence for the α-pheromone from *N. pannonica* was split across two contigs and had to be manually assembled. It is likely that sequence data is missing and thus there may be a peptide repeat missing from this schematic.

Species	Pheromone Structure	Repeat Sequence
N. crassa	---■-■-■-■-■---	■ QWCRIHGQSCW
N. discreta	---■-■-■-■-■---	■ QWCRIHGQSCW ■ QWCHIHGQSCW
N. tetrasperma	---■-■-■-■-■---	■ QWCRIHGQSCW
N. terricola	---■-■-■-■-■---	■ QWCRIHGQSCW ■ QWCQIHGQSCW
N. pannonica [a]	---■-■-■-■-■---	■ QWCRIHGQSCW ■ QWCQLHGQSCW
N. africana	---■-■-■-■----	■ QWCRIHGQSCW ■ QWCRIRGQSCW
H. bhutanensis	---■-■-■-■-■-■-■-■-■-■---	■ DSNGGLPGELL ■ NSNGGLPGELL
H. omanensis	---■-■-■-■-■-■-■-■---	■ DSNGGLPGELL ■ NSNAGLPGELL ■ YSNAGLPGELL
H. moniliformis	---■-■-■-■-■-■---	■ DANGGLPGELF ■ DAWGGLPGELF
B. spectabilis	---■---	■ WCRRPGQPC
B. fulva	---■---	■ WCVDPGQVC
B. nivea	---■---	■ WCRRPGQPC
T. aurantiacus	------	N/A

T. crustaceus	---■---	■ WCSLNGQGC

3.2.2 ***a**-factor pheromone*

In order to identify the **a**-factor pheromone genes from the *Neurospora* and *Huntiella* genomes, we used the same method as was used for the α-factor pheromone gene identification (Table A.2). These genes were all translated into their predicted proteins to confirm the presence of the conserved C-terminal CaaX domain (Table A.4)

Table A.4: Sequence of the a-pheromones from the *Neurospora* and *Huntiella* species. The C-terminal CaaX domains have been indicated in red.

Species	Pheromone Sequence
N. crassa	MPSTAASTKVPQTTMNFNGYCVVM
N. discreta	MPSTAASTKVPQTTMNFNGYCVVM
N. tetrasperma	MPSTAASTKVPQTTMNFNGYCVVM
N. terricola	MPSTAASTKVPQTTMNFNGYCVVM
N. pannonica	MPSTAASTKVPQTTMNFNGYCVVM
N. africana	MPSTAASTKVPQTTMNFNGYCVVM
H. bhutanensis	MAAIKNITSSKNAARGVDQSNPCNVMRGVDQSNPCAVMRGVDQSNPCTVM
H. omanensis	MAAIKNTTTSKNAARGVDQSNPCAVMRGVDQSNPCAVMRGVDQSNPCTVM RGVDQSNPCTLM
H. moniliformis	MPSIKNHTPSTKTSGNETIQPPTSNAGRGAIQSPINPITRGVTQAPPCNV MRGVTQAPPCNVM

A similar approach was not conducted for the *Byssochlamys* and *Thermoascus* genomes, because no **a**-factor pheromone has been identified in the genomes of any other Eurotiomycetes species and thus no pheromone sequences were available from closely related species. Thorough tBLASTn searches using all the **a**-factor

pheromone sequences from (Dyer et al., 2003; Pöggeler, 2002) yielded no significant results. A method similar to the microsynteny-based approach used above also failed to produce usable results. Instead, all of the predicted coding sequences in the *Byssochlamys* and *Thermoascus* genomes were translated into putative protein products. These proteins were then filtered for those harboring the terminal CaaX domain. These proteins were then screened to identify proteins with other similarities to previously identified **a**-factor pheromones.

3.3 Pheromone receptor genes

The receptor genes were identified using the same method as was used to identify the *MAT* genes (Table A.2). Thus, where sequences were available from NCBI, they were used to identify the genes in the genomes. Where sequences were not available, sequences of closely related species were used in BLASTn and tBLASTn searches as necessary. The **a**- and α-factor receptor genes from each of the 14 species were translated and subjected to functional domain discovery using the NCBI Conserved Domain Search (Marchler-bauer et al., 2015) to confirm their identity. These proteins were also subjected to hydrophobicity analysis using Phobius (Krogh et al., 2007) in order to identify putative transmembrane domains. Probability outputs from both programs were used to generate plots using the *ggplot2* package in R (Wickham, 2010; Wilkinson et al., 2005).

4. Gene, protein and functional domain comparisons

All nucleotide and amino acid comparisons were conducted using the *Create Alignment* and *Create Pairwise Comparison* functions in CLC MainWorkbench V8.1 (CLC bio, Aarhus,

Denmark). The gene, protein and functional domain alignments were conducted using the default settings, which includes gap open and gap extension costs of 50 and the "Very accurate (slow)" alignment setting. Pairwise comparisons were also conducted using the default settings and included gap, differences, distance, percent identity and identity comparisons.

5. RNA-seq mapping

Expression analysis was conducted using CLC Genomics Workbench V7.5 (CLC bio, Aarhus, Denmark). The raw data were filtered to retain only reads with a Phred score of at least 20 ($Q \geq 0.01$). The filtered reads were then mapped to the contigs containing the various genes of interest using the NGS Core Tool *Map Reads to Reference*. No masking was included, and the mapping settings were maintained at default. To ensure maximum mapping, the minimum length fraction and minimum similarity fraction values were set at 0.5 and 0.8, respectively. These relatively lenient mapping parameters ensured that reads spanning introns could successfully be mapped. This also ensured that successful mapping occurred despite the genome and transcriptomes originating from different isolates, as is the case for the majority of the species considered in this study. The resulting gene mappings were used to: 1) confirm or correct the gene models predicted by the various gene annotation programs and, 2) determine whether the various genes of interest are expressed in the two different sexual systems.

6. References

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Fig 1

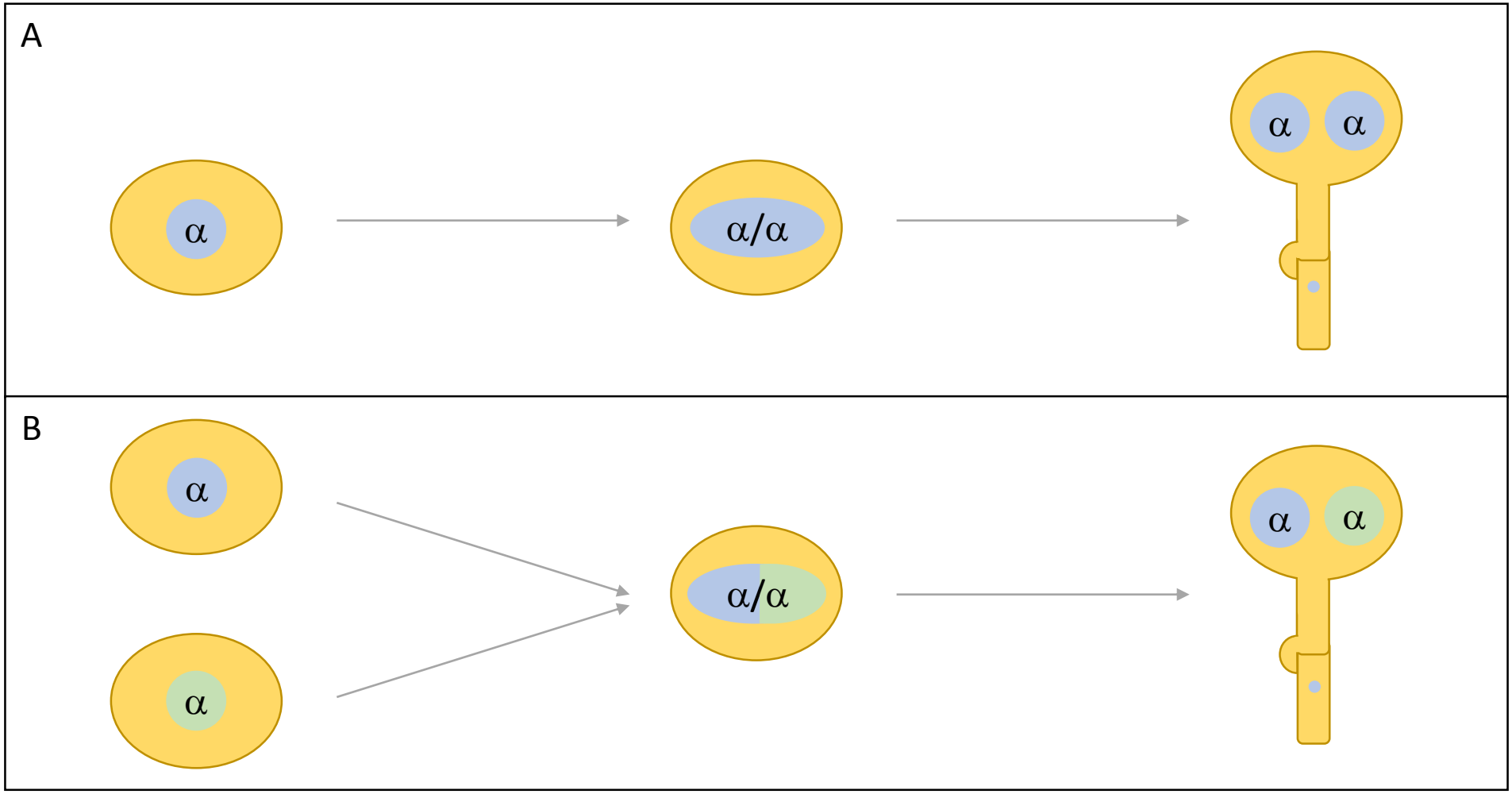


Fig 2

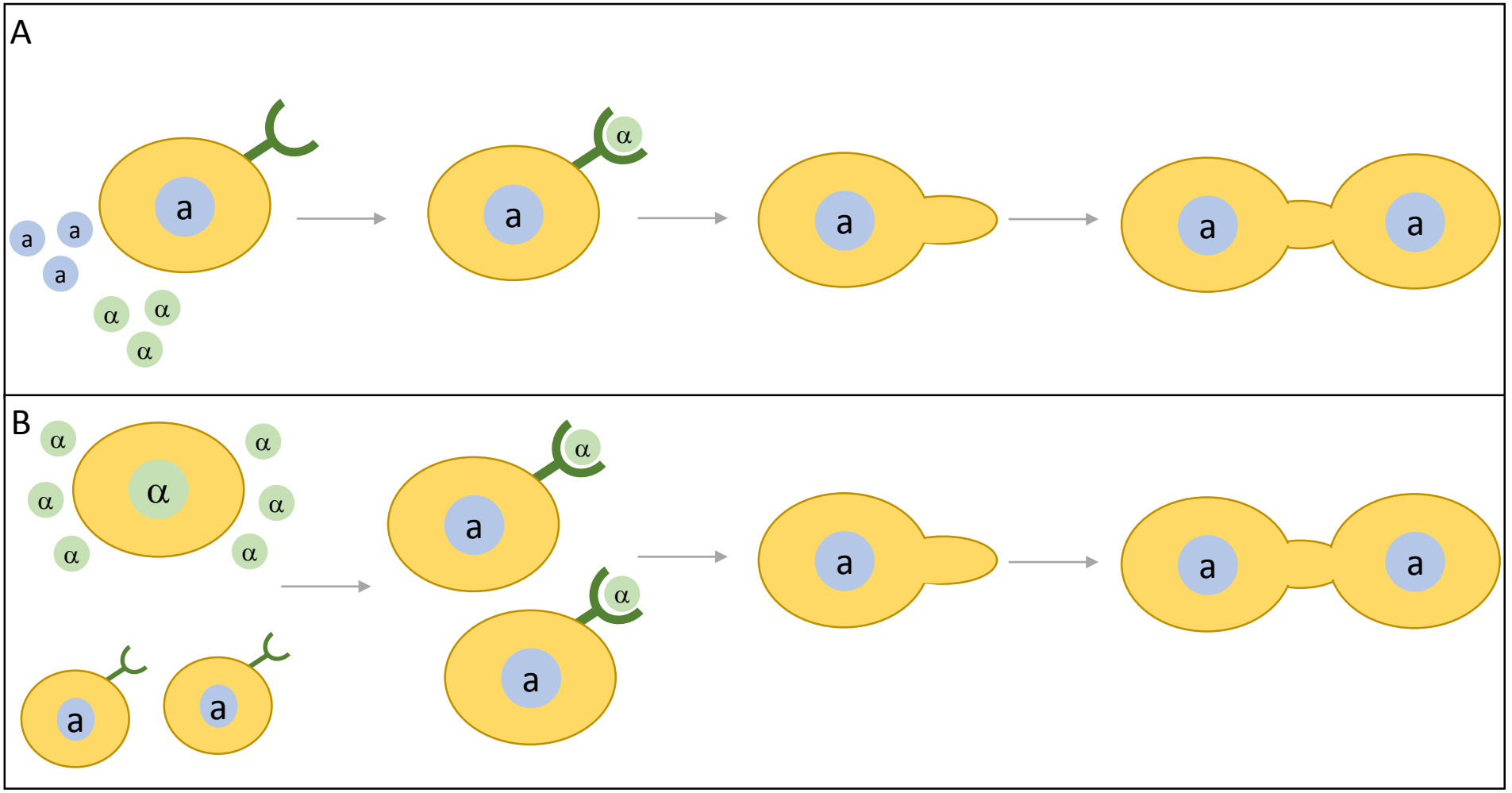
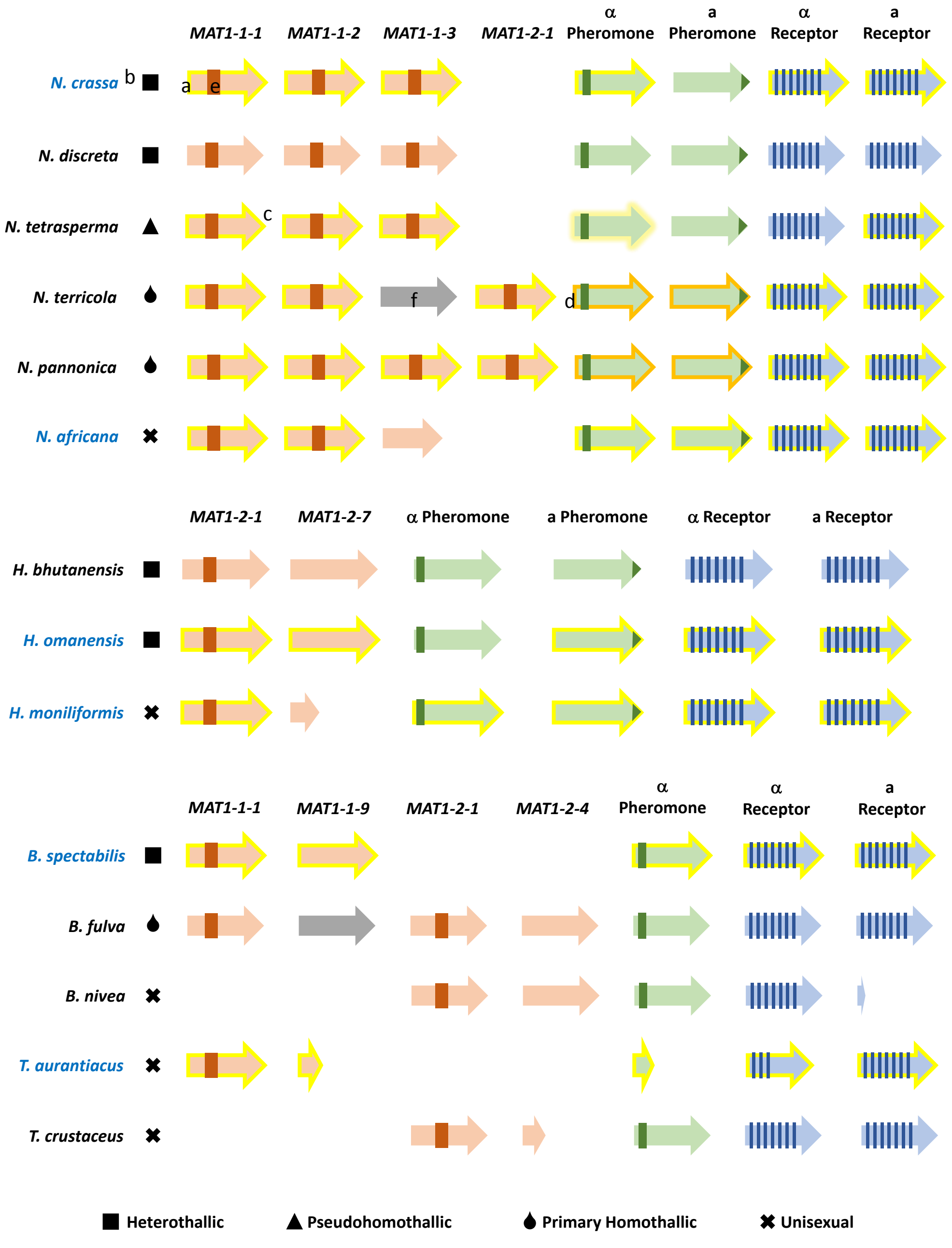


Fig 3


MAT1-1-1
MAT1-1-2
MAT1-1-3
MAT1-2-1
α Pheromone
a Pheromone
α Receptor
a Receptor
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N. discreta
N. tetrasperma
N. terricola
N. pannonica
N. africana
a
b
c
d
e
f
MAT1-2-1
MAT1-2-7
α Pheromone
a Pheromone
α Receptor
a Receptor
H. bhutanensis
H. omanensis
H. moniliformis
MAT1-1-1
MAT1-1-9
MAT1-2-1
MAT1-2-4
α Pheromone
α Receptor
a Receptor
B. spectabilis
B. fulva
B. nivea
T. aurantiacus
T. crustaceus
Heterothallic
Pseudohomothallic
Primary Homothallic
Unisexual

Fig 4

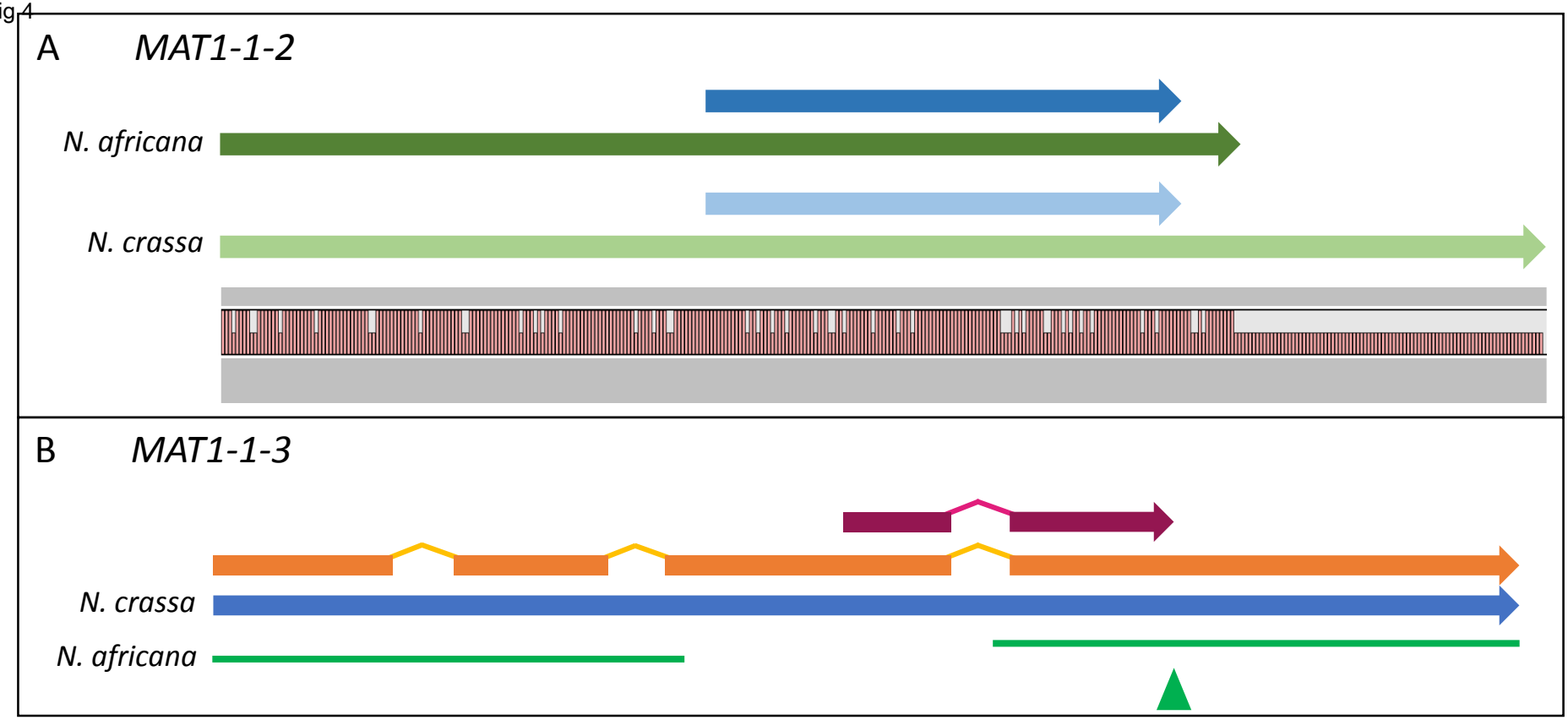


Fig 5

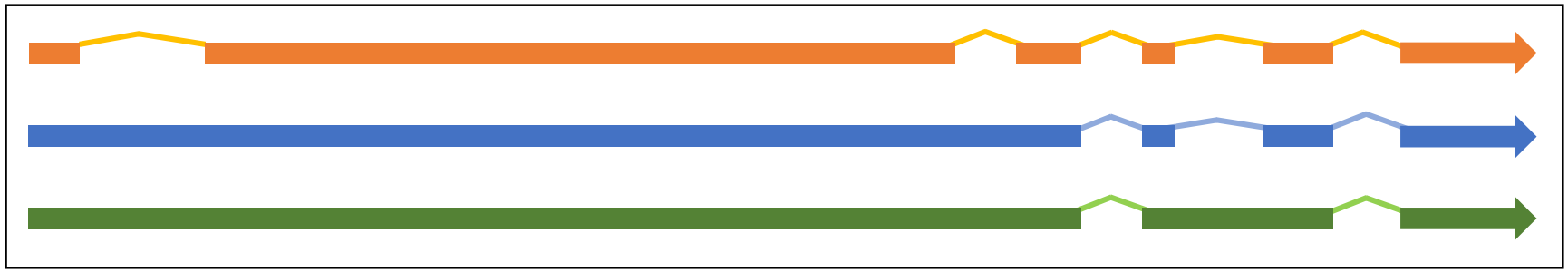


Fig 6

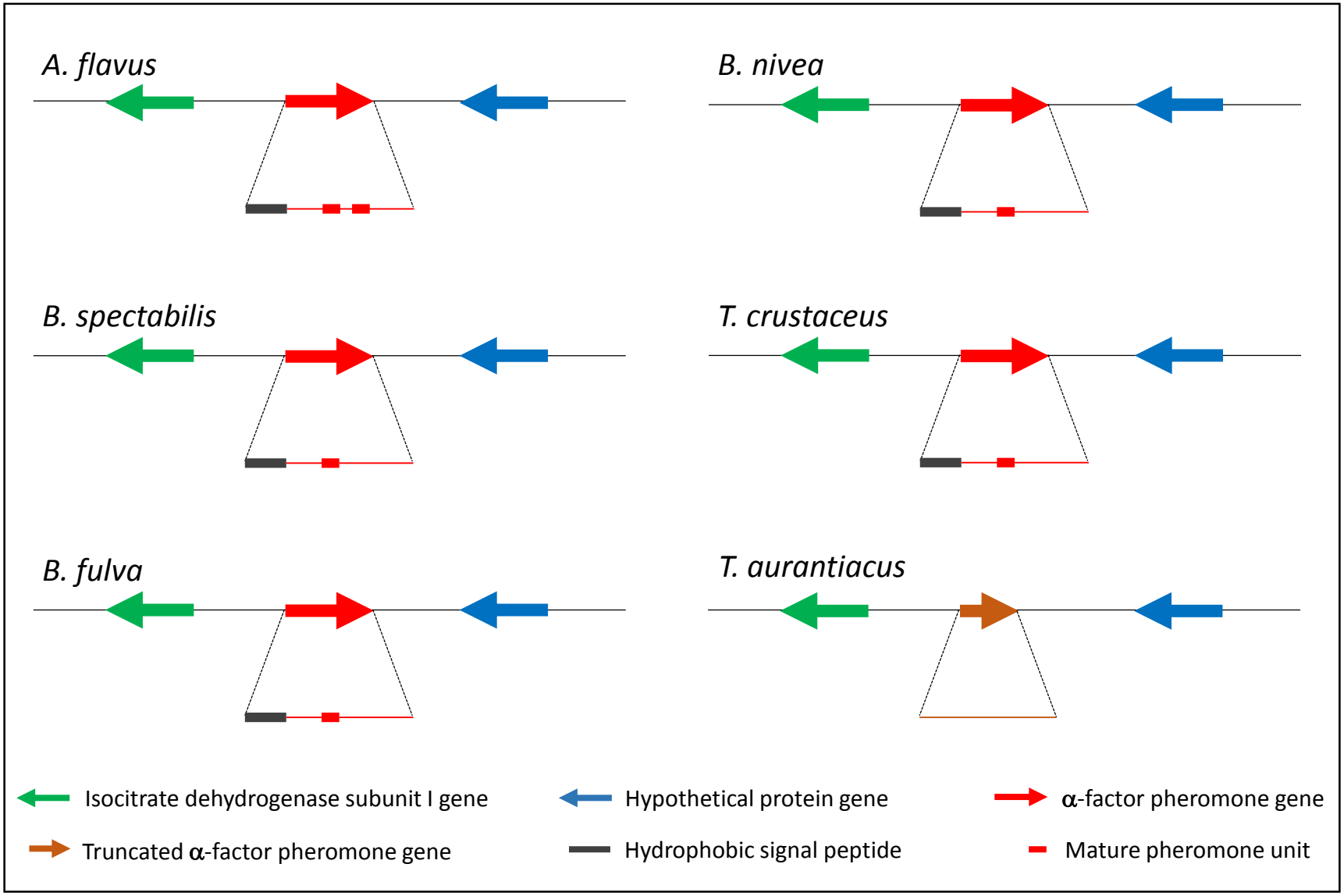
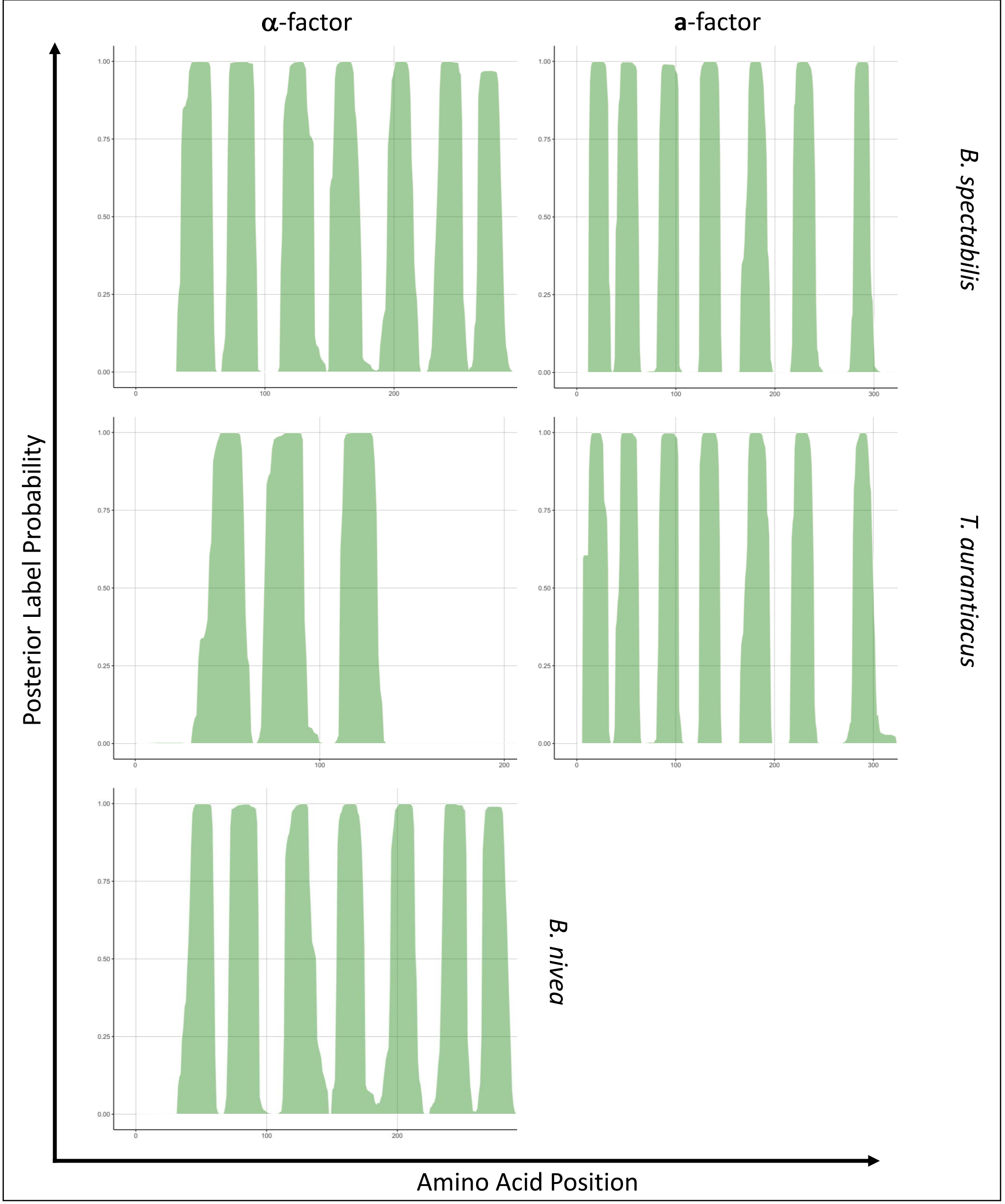

A. flavus
B. nivea
B. spectabilis
T. crustaceus
B. fulva
T. aurantiacus
Isocitrate dehydrogenase subunit I gene
Hypothetical protein gene
α-factor pheromone gene
Truncated α-factor pheromone gene
Hydrophobic signal peptide
Mature pheromone unit

Fig 7


α-factor
a-factor
B. spectabilis
T. auranticus
B. nivea
Posterior Label Probability
Amino Acid Position
1.00
0.75
0.50
0.25
0.00
0
100
200
300