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REVIEW

Pheromone Response and Mating Behavior in Fission Yeast

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SUMMARY Most ascomycete fungi, including the fission yeast *Schizosaccharomyces pombe*, secrete two peptidyl mating pheromones: C-terminally modified and unmodified peptides. *S. pombe* has two mating types, plus and minus, which secrete two different pheromones, P-factor (unmodified) and M-factor (modified), respectively. These pheromones are specifically recognized by receptors on the cell surface of cells of opposite mating types, which trigger a pheromone response. Recognition between pheromones and their corresponding receptors is important for mate discrimination; therefore, genetic changes in pheromone or receptor genes affect mate recognition and cause reproductive isolation that limits gene flow between populations. Such genetic variation in recognition via the pheromone/receptor system may drive speciation. Our recent studies reported that two pheromone receptors in *S. pombe* might have different stringencies in pheromone recognition. In this review, we focus on the molecular mechanism of pheromone response and mating behavior, emphasizing pheromone diversification and its impact on reproductive isolation in *S. pombe* and closely related fission yeast species. We speculate that the "asymmetric" system might allow flexible

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adaptation to pheromone mutational changes while maintaining stringent recognition of mating partners. The loss of pheromone activity results in the extinction of an organism's lineage. Therefore, genetic changes in pheromones and their receptors may occur gradually and/or coincidently before speciation. Our findings suggest that the M-factor plays an important role in partner discrimination, whereas P-factor communication allows flexible adaptation to create variations in *S. pombe*. Our inferences provide new insights into the evolutionary mechanisms underlying pheromone diversification.

KEYWORDS fission yeast, pheromone, mating, mate choice, G-protein-coupled receptor, reproductive isolation, fission yeast

INTRODUCTION

Homothallism and Heterothallism

Most yeasts exist in both haploid and diploid forms (1, 2). While the budding yeast *Saccharomyces cerevisiae* and many other yeasts normally multiply as diploid cells, the fission yeast *Schizosaccharomyces pombe* basically propagates nutritionally as haploid cells (Fig. 1A). *S. pombe* has two mating types, minus (h^-) and plus (h^+) types (corresponding to *MAT***a** and *MAT*α cells of *S. cerevisiae*, respectively) (3–5). When the nitrogen source is depleted, opposite heterothallic haploid cells mate to form diploid zygotes (6). Subsequently, the diploid zygote undergoes meiosis to form a four-spore zygote (known as the "ascus") (3, 4, 7, 8) (Fig. 1A). The resulting spores germinate and propagate as haploid cells again when the environment becomes nutrient-rich. Thus, the life cycle of fission yeast is usually haploid, but diploid strains can also be selected.

Most wild fission yeasts in nature exhibit homothallism, which allows efficient switching between the P and M mating types at the expressed mating-type locus (9). The homothallic strain of fission yeast is known as h^{90}, including that it forms 90% of spores in pure culture. Previously, a strain known as h^{40}, which produced 40% spores, also existed but seems to have been lost. Mating switching in *S. pombe* occurs by replacing the genetic information of the transcriptionally active *mat1* locus with sequences copied from either the silent donor locus *mat2-P* or *mat3-M* (10). h^+ cells express two *mat2-P* genes (*mat-Pc* and *mat-Pi*), and h^- cells express two *mat3-M* genes (*mat-Mc* and *mat-Mi*) (11). *mat-Pc* and *mat-Mc* initially regulate the expression of mating-related genes, such as those for mating pheromones, while *mat-Pi* and *mat-Mi* are required for the initiation of meiosis after mating (12, 13). Overall, these genes are involved in the expression of mating type-specific genes.

In addition to the homothallic strain (L968), Leupold and Hottinguer (14) also isolated heterothallic strains and designated them h^+ (L975) and h^- (L972). Heterothallic strains that can no longer switch mating types exhibit heterothallism due to the irreversible loss of one of the silent cassettes at the mating-type locus from the homothallic wild-type strain. Such heterothallic strains can be widely used, for example, (i) to select for desired phenotypes by spore dissection, (ii) to investigate mating-type specific genes, and (iii) to verify reproductive isolation between closely related species. In the fission yeast *Schizosaccharomyces japonicus*, heterothallic strains have been isolated through spontaneous mutations and are available as NIG2017/NIG2025 (h^+) and NIG2028 (h^-) strains (15). In the fission yeast *Schizosaccharomyces octosporus*, stable heterothallic strains have been produced by artificially deleting the *mat2-P*/*mat3-M* silent region and are currently available as TS162 (h^+) and TS161 (h^-) (16). Thus, these three fission yeast species allow for pheromone response testing and mating experiments using heterothallic strains.

Life Cycle and Characterization of Fission Yeast Species

The yeasts of the genus *Schizosaccharomyces* are characterized by a mitotic mode of growth (division by medial fission to produce two daughter cells). The laboratory strain of *S. pombe*, which was isolated from Swiss grape juice, was identified by Leupold (17). *S. pombe* has promising homologs in higher organisms, including humans, and is amenable

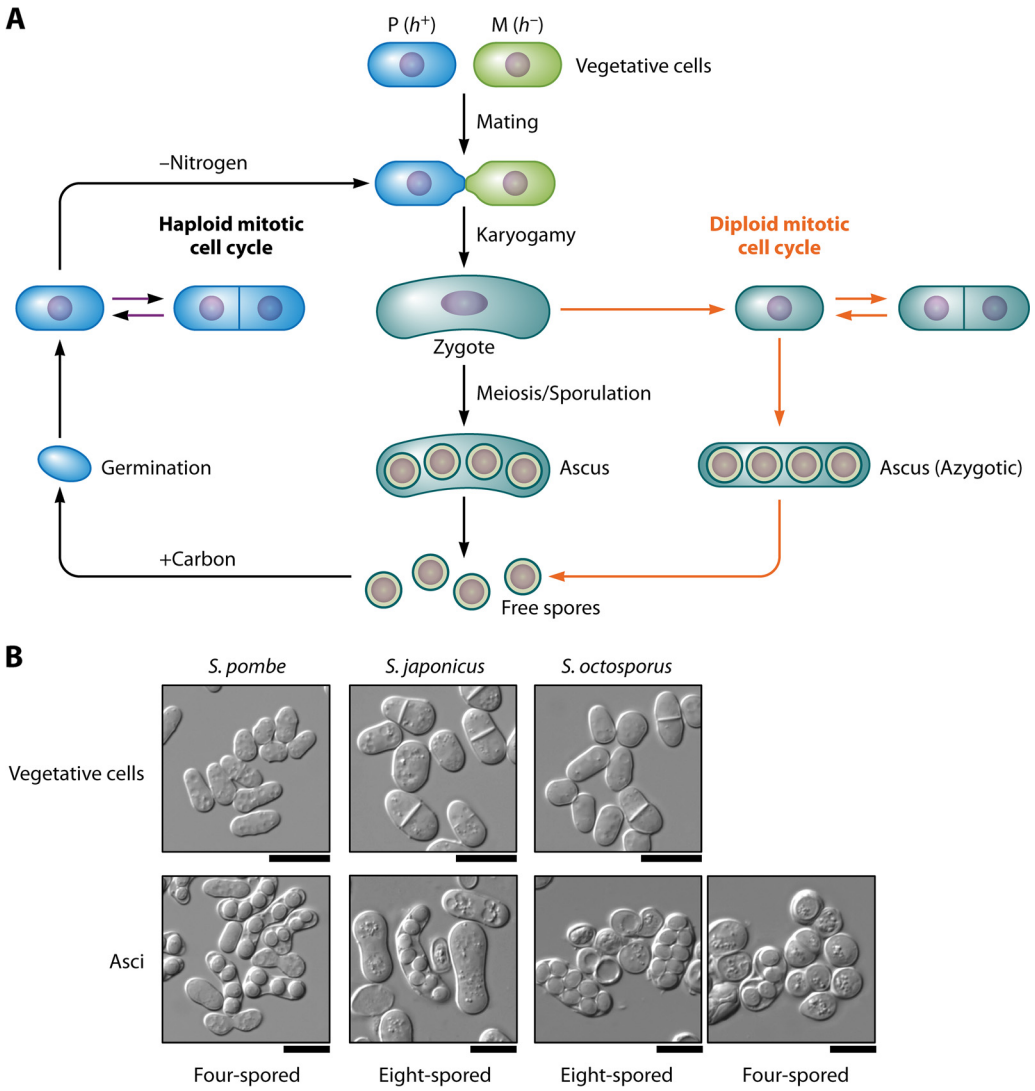

FIG 1 Life cycle of *S. pombe* and morphology of the cells of *Schizosaccharomyces* species. (A) *S. pombe* develops stably as haploid cells, propagating by mitotic cell division. When nitrogen is absent, cells of opposite mating types, P (h^+) and M (h^-), mate, after which the nuclei fuse. This is known as karyogamy. A diploid zygote is then formed, which undergoes meiosis and sporulation to produce a four-spore zygote, the ascus (zygotic). Acquisition of carbon leads to spore cell germination and the resumption of vegetative cell cycles. Following immediate resupply of nutrients, the diploid zygote can also grow vegetatively by mitotic cell division, where the diploid cells undergo meiosis and generate an azygotic ascus that is morphologically distinct from the zygotic ascus. (B) Morphology of vegetative cells and asci of *Schizosaccharomyces* species. *S. pombe* and *S. japonicus* asci contain four and eight spores, respectively, but *S. octosporus* asci contain up to eight spores depending on the nutrient conditions. Typical octad and tetrads are shown. Scale bar, 10 μm.

to genetic analysis, which has led to several studies on sexual reproduction (13, 18). In addition to *S. pombe*, *S. japonicus*, *S. octosporus*, *S. cryophilus*, and *S. osmophilus* have been isolated from strawberry fields at Kyushu University, Japan (19), from dried black currants and figs (20), as a contaminant of *S. octosporus* culture medium (21), and from honey (22), respectively. The complete genome sequences of all four species except *S. osmophilus* have already been published by the Broad Institute (23).

S. pombe produces four spores in their asci, while other fission yeast species basically produce eight spores (15, 24). On the other hand, *S. octosporus* has an interesting feature where the number of the 4 to 8 spores in its asci varies depending on nutritional conditions (16) (Fig. 1B). The evolution of mechanisms controlling spore production in fission yeast is interesting. For instance, *S. japonicus* is known to engage in mycelial growth, as

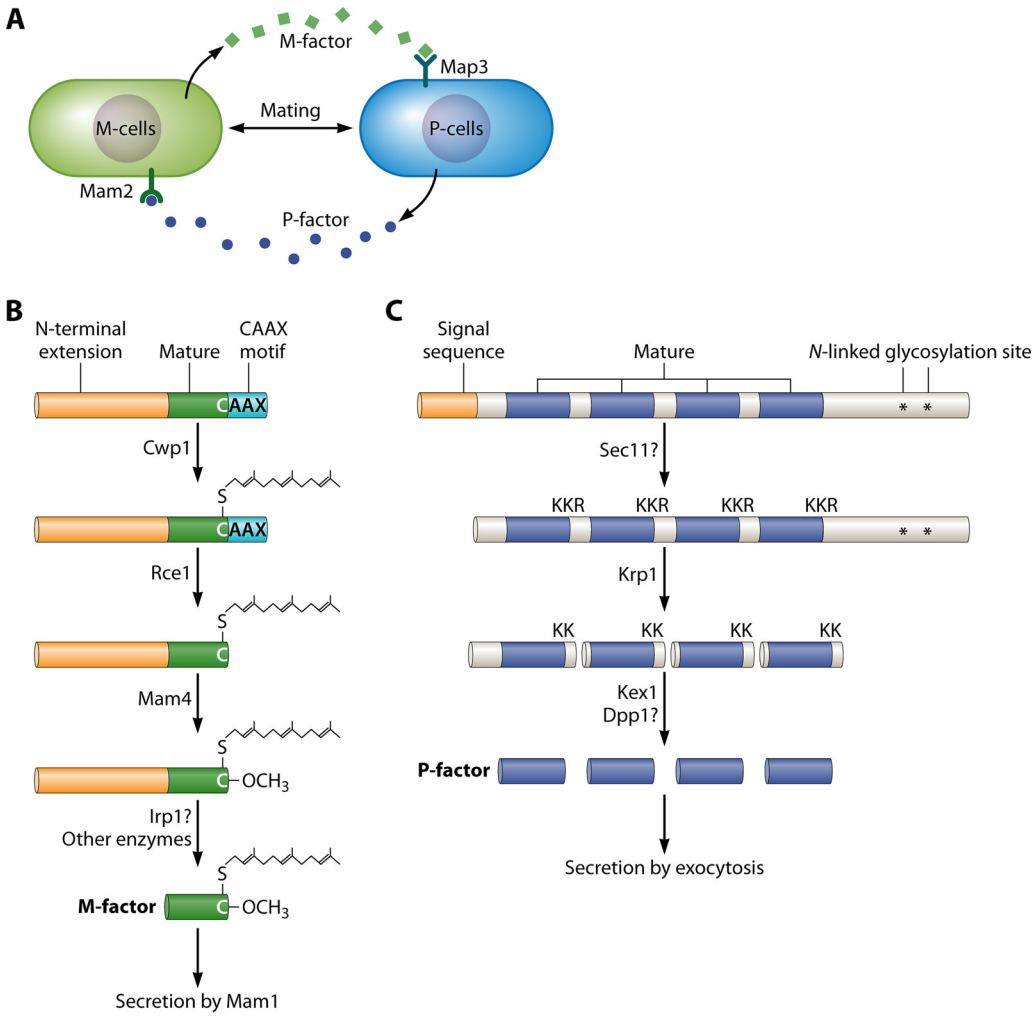

FIG 2 Pheromone signaling and biosynthetic pathway in *S. pombe*. (A) Illustration of mating pheromone signaling in *S. pombe*. M-factor, a modified peptide secreted from M cells, is recognized by the M-factor receptor Map3 on the surfaces of P cells, whereas P-factor, an unmodified peptide secreted from P cells, is recognized by the P-factor receptor Mam2 on the surfaces of M cells. (B) Biosynthetic pathway of M-factor. The CAAX modifications and the processing are carried out by integral membrane proteins localized in the ER membrane: Cwp1, farnesyltransferase; Rce1, CAAX prenylprotease; and Mam4, methyltransferase. The N-terminal precursor is cleaved by Irp1 (probably) and other enzymes. A single mature M-factor with farnesyl group and the *o*-methylated group is produced by the *mfm*$^+$ genes and is secreted by the transporter Mam1. (C) Biosynthetic pathway of P-factor. The two *N*-linked glycosylation sites are indicated by asterisks. The signal peptide from precursors is removed by Sec11 (probably), and the remaining precursors are sequentially cleaved by several enzymes: Krp1, endopeptidase; Kex1, carboxypeptidase; and Dpp1 (probably), dipeptidyl peptidase. Finally, mature P-factors are secreted by exocytosis. Some of the proteins involved in the processing are not still identified in *S. pombe*. Putative enzymes functioning in M- and P-factor biogenesis have been deduced based on the homology with the corresponding enzymes of *S. cerevisiae*.

well as spore formation (25, 26). Fission yeast species differ in cell size and do not mate between different species (Fig. 1B). Genetic hybridization between *S. pombe* and *S. octosporus* using protoplast fusion has been performed in the past (27, 28), but these two species are usually reproductively isolated (29, 30).

STIMULATION BY MATING PHEROMONES

Pheromone Signaling Pathway in *S. pombe*

When fission yeasts face nitrogen deficiency, M- and P-type cells mate by way of mating pheromones (Fig. 2A). M-type cells secrete M-factor, a C-terminal, S-farnesylated, and *o*-methylated nonapeptide (31, 32). M-factor is synthesized from three redundant genes—*mfm1*$^+$, *mfm2*$^+$, and *mfm3*$^+$ (32, 33)—that generate a precursor polypeptide of 41 to 44

amino acids, each producing one copy of the same M-factor (YTPKVPYMC^{Far}-OCH$_3$). The pathway is similar to that of *S. cerevisiae* a-factor (34) (Fig. 2B). The precursor synthesized from the three *mfm* genes in *S. pombe* has an elongation at the N terminus and a CAAX (Cys, followed by two aliphatic amino acids and an arbitrary residue) motif at the C terminus, which serves as a signal for prenylation and carboxylation (35). Posttranslational modifications of M-factor are carried out by three transmembrane enzymes, Cwp1, Rce1, and Mam4, which are presumed to localize to the endoplasmic reticulum (ER) membrane (36, 37). As a result, M-factor undergoes S-farnesylation and *o*-methylation modifications, which are essential for its activity and extracellular secretion (32, 38). The precursor undergoes several N-terminal cleavages through the action of protease Irp1 (probably) and an unidentified enzyme to synthesize M-factor. Finally, the mature M-factor is secreted extracellularly by the ATP-binding cassette (ABC) transporter Mam1 (39). Although a single *mfm* gene has been shown to be sufficient for hybridization, M cells lacking all three *mfm* genes cannot produce M-factor and never mate (33). Secreted M-factor is specifically recognized by a seven transmembrane G-protein-coupled receptor (GPCR), Map3, expressed on the plasma membrane of P-type cells (40).

In contrast to M-type cells, P-type cells secrete P-factor, an unmodified simple peptide of 23 amino acids (41), synthesized from a single *map2*$^+$ gene and processed from a precursor polypeptide (41). The P-factor biosynthetic pathway is similar to that of α-factor in *S. cerevisiae* (42) (Fig. 2C). The *map2*$^+$ gene contains a signal sequence that carries the polypeptide to the ER, two *N*-linked glycosylation sites, and four tandemly aligned coding sequences of the mature pheromone separated by a spacer region (41, 43). The N-terminal signal sequence is cleaved by the signal peptidase Sec11 (probably). In the Golgi apparatus, the kexin-related endopeptidase Krp1 cleaves the KKR (Lys-Lys-Arg) motif, and the remaining product is cleaved by the serine carboxypeptidase Kex1 and the putative dipeptidyl protease Dpp1 (probably) to yield the mature P-factor (43). The mature P-factor of laboratory strain L968 is a mixture of three different peptides since the sequence of the part encoding the pheromone is slightly different (41) and is secreted as a simple peptide by exocytosis (not by transporters) (41). P cells lacking the *map2*$^+$ gene cannot produce P-factor, resulting in complete sterility. Secreted P-factor is specifically recognized by GPCR Mam2, expressed on the plasma membrane of M-type cells (44).

In *S. pombe*, both Map3 and Mam2 pheromone receptors bind to the monomeric form of the G-protein alpha subunit Gpa1 (45, 46). When the pheromone binds to the receptor, the G-protein subunit dissociates, and activation of the released Gpa1 transmits signals through the MAPK cascade, consisting of Byr2/Ste8 (MAPKKK), Byr1/Ste1 (MAPKK), and Spk1 (MAPK) (45, 47, 48), ultimately inducing the transcription of pheromone-regulated genes (37, 49–51). Notably, P- and M-type cells are thought to share signaling pathways downstream of activated pheromone GPCRs.

Gene Expression in Sexual Reproduction

Pheromone signaling elicits various responses in cells of opposite mating types. Once cells receive a pheromone, they arrest the cell cycle at the G_1 phase (38, 41, 52) and then induce the expression of the transcription factor Ste11 (49, 53). Next, Ste11 interacts with Spk1 downstream of the MAPK cascade, inducing its activation (54) and the expression of key pheromone signaling genes (37, 49), which ultimately induces mating type-specific gene transcription (37, 49).

In *S. pombe*, no Gβ or Gγ subunits were identified to function in the pheromone signaling pathway. Unlike in *S. cerevisiae*, *S. pombe* does not have any scaffold proteins that G$\beta\gamma$ binds to (55), implying that yeasts have undergone evolutionary changes in the presence and role of scaffold proteins to coordinate the expression of genes required for mating and regulating polarity and the cell cycle (56). In *S. pombe*, the activation of Byr2/Ste8 upstream of the MAPK cascade requires the interaction of a MAPK cascade adaptor protein, Ste4 (48, 57, 58), and Ras1-GTP activated by a Ras guanine nucleotide exchange factor (GEF), Ste6. Ras1-GTP promotes the activation of Scd1, a GEF of the Rho family GTPase Cdc42, which is important for cell polarity (55) and induces

the expression of genes required for cell polarity. Thus, the MAPK cascade and transcription factor Ste11 induce the expression of a series of genes involved in various sexual differentiations, such as sex cell aggregation (59, 60) and morphogenesis by polar cell elongation (31, 61, 62).

BIOSYNTHETIC PATHWAY AND STRUCTURE OF PHEROMONES IN FISSION YEAST

Asymmetry in Chemical Modification and Secretion of Mating Pheromones

Mating pheromones secreted by the mating-type cells of *S. pombe* and *S. cerevisiae* have several differences. M-factor (*S. pombe*) or a-factor (*S. cerevisiae*) is a "hydrophobic" lipid peptide farnesylated and carboxymethylated at the C-terminal CAAX motif (63) and secreted extracellularly via a plasma membrane transporter, while P-factor (*S. pombe*) or α-factor (*S. cerevisiae*) is a "hydrophilic" unmodified simple peptide secreted by exocytosis (64). This asymmetry in the modification and secretion system found in mating pheromones is widely conserved throughout ascomycete fungi (65). In contrast, basidiomycete fungi such as mushrooms express only lipid-modified a-factor class-like pheromones, one of the distinguishing features between ascomycetes and basidiomycetes (66). A previous study using *S. cerevisiae* reported that asymmetry in pheromone chemical modification is not absolutely necessary for mating (67). This same study showed that two yeast cells could mate if only one cell could secrete a pheromone that stimulates the pheromone receptor expressed on the other cell; that is, the ability of the pheromone and receptor to bind is the requirement for mating (67). Nevertheless, the mating efficiency between two yeast cells that have broken the asymmetry in the chemical modification of the pheromone is low (67, 68). Therefore, there may be some biological significance to the mating pheromone asymmetry observed in ascomycetes, but the reasons for this asymmetry are not fully understood.

Conservation and Diversity of Pheromone Genes in Other Ascomycete Fungi

Cell-to-cell recognition by pheromones and receptors is primarily essential for mating in ascomycetes (18, 67, 69, 70). Because pheromone gene sequences are relatively diverse among yeasts (65), it is highly likely that such differences in pheromones play a role in reproductive isolation and speciation in yeasts. Precursors of the unmodified pheromone α-factor class have several peptide sequences arranged in tandem, and the number of repeats is variable among yeast strains. For example, intra- and interspecific variation in repeat number has been reported in yeasts of the genera *Schizosaccharomyces* (Fig. 3A) and *Saccharomyces* (29, 71–73). This perturbation seems to be a result of continuous duplication and deletion of repeats rather than coordinated evolution (65). Evolution by duplication and deletion is known as "birth-and-death" evolution and is a common phenomenon in such tandem repeats (74). Peptide sequences may be completely identical, or multiple peptides may be produced simultaneously (Fig. 3A). In general, pheromone peptide sequences are relatively similar within a species, suggesting that peptide sequences are affected by selection pressure (75). Thus, the similarity of mature peptide repeat sequences is adaptive and reflects the need for sequence specificity in pheromone reception.

The analysis of the modified pheromone a-factor class is falling behind that of the α-factor class pheromones because the precursors are extremely small and most contain introns in their genes, the presence of which is overlooked (76). However, several studies reported that modified pheromones also diversify among species (65, 77) (Fig. 3B). It has been discussed that some homothallic strains may have required only one pheromone pathway (only the α-factor class) and the other was eventually lost (78–80). The evolution of pheromone diversity may have contributed to speciation in ascomycete fungi, but the role of species recognition by peptide pheromones requires further detailed analysis.

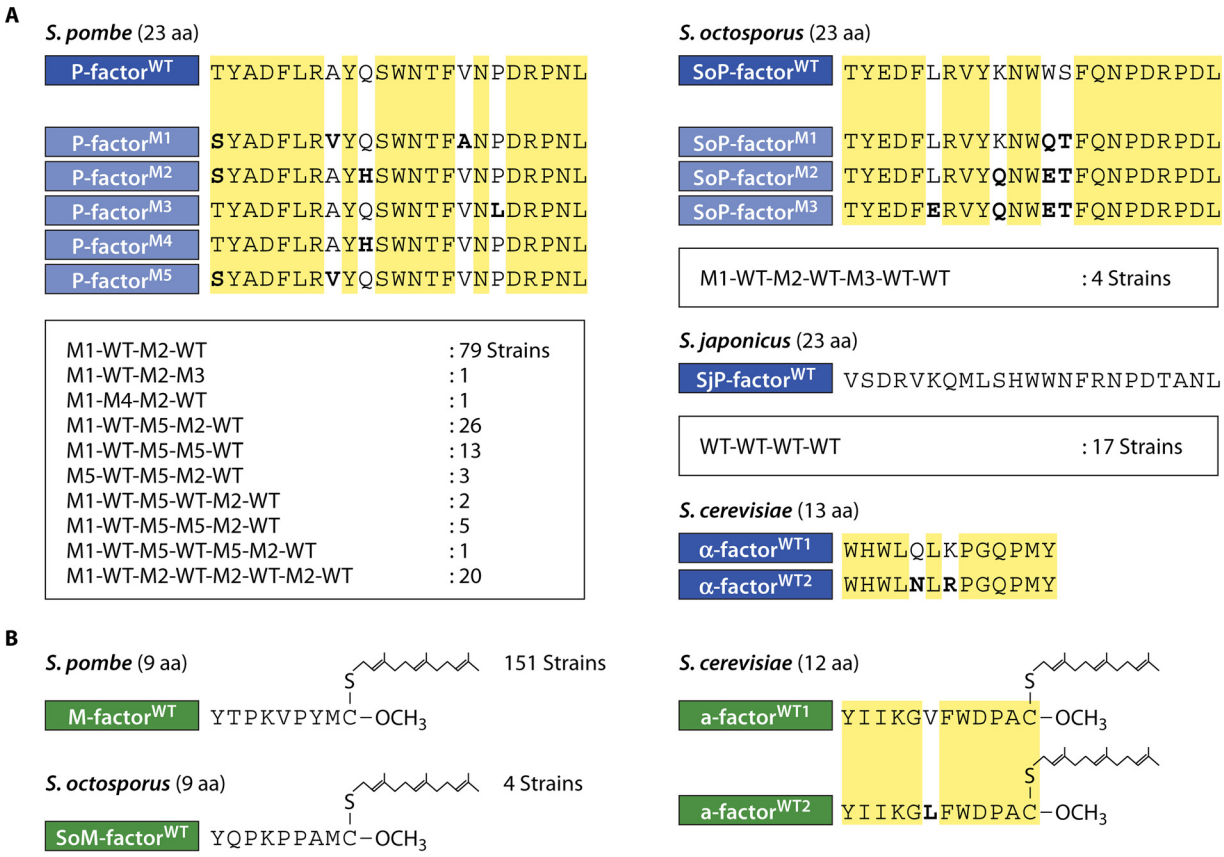

FIG 3 Amino acid sequences and polymorphism of mating pheromones in *Schizosaccharomyces* species and *S. cerevisiae*. (A) The six different P-factors found in 151 natural *S. pombe* isolates are shown. P-factor^{WT} is the standard P-factor of *S. pombe*. Diversified pattern (four to eight repeats) of the *map2* gene in different strains, including the laboratory strain (L968), which carries four tandem P-factor-encoding repeats (M1-WT-M2-WT). The four different P-factors found in four natural *S. octosporus* isolates are shown. SoP-factor^{WT} is the standard P-factor of *S. octosporus*. SjP-factor of *S. japonicus* and α-factors of *S. cerevisiae* that differ in amino acids among P-factors in intraspecies are indicated in boldface, and identical amino acids are indicated in yellow. (B) M-factor^{WT} of *S. pombe* and SoM-factor^{WT} of *S. octosporus* have shown no variant M-factors. M-factor of *S. japonicus* is not identified. a-factors of *S. cerevisiae* that differ in amino acids are indicated in boldface, and identical amino acids are shown in yellow.

DIVERSIFICATION OF MATING PHEROMONES IN NATURE

Polymorphisms of Pheromone Genes in Wild *S. pombe* Strains

Little is known about the evolutionary history and ecology of *S. pombe* (81), but there are some reports of its isolation from various fruits, plants, and beverages worldwide (29, 81–83). In addition, a recent study of large samples shows that *S. pombe* and *S. octosporus* appear to be abundant in honey bees (84). We have previously examined the sequence diversity of M- and P-factor genes in 150 wild strains of *S. pombe* isolated from more than 22 countries worldwide (29). Results indicated that laboratory strain L968 of *S. pombe* has *mfm1*$^+$, *mfm2*$^+$, and *mfm3*$^+$ as the genes encoding M-factor, and all wild strains also possessed three *mfm* genes (it is not known whether any strain possesses more than four). Interestingly, there were no missense mutations in the peptide sequence that would alter the amino acids, and all produced the same M-factor (Fig. 3B), although some mutations were found in the precursor and intron portions. This is a surprising result since any one of the three M-factor genes is sufficient for mating (33). Perhaps, under more severe nutrient-limiting conditions in nature, an efficient mating frequency is highly dependent on the number of M-factor genes, possibly limiting consequent M-factor variation.

In contrast, the gene *map2*$^+$ encoding P-factor varied significantly among wild strains. Laboratory strain L968 has four tandem peptide repeat sequences within the *map2* gene (41), but in the wild strain, the repeat sequences varied between four and eight (Fig. 3A). We also found three peptide sequences absent from the laboratory strain. We determined

that there were no strains with fewer than three repeats or with the same peptide sequence only. Furthermore, strains with fewer than three repeats had significantly reduced mating ability (29). Similar results have been found for *S. cerevisiae*, where a decrease in the number of peptide sequence repeats causes a decrease in pheromone production (85). Furthermore, secretory transport by exocytosis requires a certain length of precursor (86). Taken together, in *S. pombe*, at least four repeat sequences appear to be essential for mating. Although the number of repeat mature pheromone sequences affects pheromone production, an increased number of repeats does not necessarily increase pheromone production. In *S. cerevisiae* experiments, strains with eight repeat sequences have less chance of mate choice by different mating-type cells than strains with six repeat sequences, resulting in reduced mating ability (73). This may be due to a reduction in the translation rate caused by precursor lengthening, leading to decreased pheromone production. Changes in P-factor repeats in *S. pombe* may affect various factors involved in mating, but the physiological and evolutionary significance of the simultaneous production of diverse P-factors is not well understood.

Pheromone Genes in Other Fission Yeast Species

Genome analysis revealed the presence of six putative M-factor genes (*Somfm1*$^+$–*Somfm6*$^+$) in *S. octosporus* and five M-factor genes (*Scmfm1*$^+$ to *Scmfm5*$^+$) in *S. cryophilus* (29). However, BLAST (Basic Local Alignment Search Tool) searches are difficult due to the low similarity with closely related species and the small size of the genes themselves. The respective *mfm* genes of *S. octosporus* and *S. cryophilus* ultimately produce M-factor peptides with identical amino acid sequences (87) (Fig. 3B) compared to the M-factor of *S. pombe* (some wild *S. octosporus* strains have at least six *mfm* genes as well). Although there are some mutations in the precursor, as in *S. pombe*, there are no amino acid variations in the peptide sequence, making the *S. octosporus* M-factors (SoM-factors) completely identical within the species.

The *S. octosporus* laboratory strain yFS286 has only one *map2*$^+$ gene (*Somap2*$^+$) encoding P-factor, and cells lacking this gene are completely sterile (29). The *Somap2*$^+$ gene of *S. octosporus* contains seven repeating peptide sequences, resulting in the production of four slightly different P-factors (23, 29). On average, 8 of the 23 amino acids in the *S. octosporus* P-factor (SoP-factor) differ from the *S. pombe* P-factor (Fig. 3A). The amino acid sequence is partially identical to that of *S. octosporus*. The *S. japonicus* laboratory strain NIG2008 also has one *map2*$^+$ gene (*Sjmap2*$^+$) encoding P-factor and contains four repeat sequences. However, unlike other fission yeast genera, the peptide sequences are completely identical and produce an identical P-factor (SjP-factor) (Fig. 3A) (88). Interestingly, *S. japonicus* var. *versatilis*, a subspecies of *S. japonicus*, and other *S. japonicus* strains that we recently isolated from *Drosophila* have nucleotide mutations in the precursor of the *Sjmap2*$^+$ gene (88).

Stringency and Flexibility of Mating Pheromones

The wild *S. pombe* strain produces identical M-factor peptides as far as we determined, and there are at least six different polymorphisms of P-factors (Fig. 3A). Most of these six peptides have the ability to bind to Mam2 *in vitro* (29). This may suggest that recognition of M-factor, a lipid peptide, is stricter, whereas that of P-factor, a simple peptide, is relatively flexible. Indeed, although *S. pombe* genetically engineered to produce SoM-factor completely lost its mating ability to P cells, the chemically synthesized SoM-factor did not function on *S. pombe* P cells (29, 30, 89). In contrast, *S. pombe* engineered to synthesize SoP-factor showed sufficient mating ability, and almost all chemically synthesized SoP-factors acted on *S. pombe* M cells (29). Furthermore, *S. octosporus* was also able to accept *S. pombe* P-factors. In other words, the specificity of P-factor recognition is not strict, and cross-reactivity among closely related species can occur. Based on the above, the prezygotic isolation of *S. pombe* and *S. octosporus* may be a problem in the compatibility of M-factors. It has also been reported that in *S. cerevisiae*, pheromones of the a-factor class either promote mating efficiency completely or not at all, while those of the α-factor class act in a stepwise manner (77). Although this experimental observation suggests that lipid peptides play a more important role in

mate discrimination, Ste2 interactions that show specificity can also be obtained from the unmodified pheromone (90). Further studies with various yeast species are necessary to investigate the specificity between receptors and pheromones.

ASYMMETRIC BEHAVIOR IN MATE CHOICE

Distal and Proximal Mode of Action in Mating Induction

In nature, fission yeasts are thought to inhabit semiaquatic environments. Since yeasts are nonmotile and cannot actively move toward cells of the opposite sex, cell aggregation by cell-to-cell contact is important to increase the chance of encountering cells of the opposite mating type. Sexual cell aggregation in *S. pombe* is achieved by two mating type-specific agglutinin glycoproteins, Mam3 and Map4 (37, 91, 92), which are specifically expressed on the cell surface of M- and P-type cells, respectively. Before cell fusion, cell aggregation occurs between opposite mating-type cells to form macroscopic aggregates (59), enabling the cells to find mating partners (60). *S. pombe* can aggregate even in the absence of P-factor signaling (60). Mam3 expression is induced by nitrogen source depletion to some extent (37), suggesting that M-factor signaling is responsible for the pheromone regulation of mating. Because cell fusion rarely occurs in agglutinin-deficient mutants, agglutinin-mediated physical cell-to-cell contact is required for cell fusion between cells of opposite mating types regardless of the liquid environment.

Since the pheromone gradient is unlikely to form as stably in liquid environments as it does in solid environments, cell polarity may be regulated by a different mechanism. Initially, peptide pheromones are secreted by both cells in response to environmental cues such as nutrient starvation. On the one hand, P-factor, a simple hydrophilic peptide, diffuses easily into the surroundings, reaching distant cells and allowing the cells to rapidly recognize the presence of a suitable mating partner (68). On the other hand, uniform M-factor secreted by M-type cells, at relatively low concentrations, induces the production of P-factor agglutinin (Map4), which is necessary for cell-to-cell contact (distal action) (Fig. 4). This is especially true in liquid cultures where M-type cells are often found in the presence of M-factors. This is essential for fixing the relative positions of M- and P-type cells, especially in liquid culture. The establishment of polarity is thought to be achieved by the more direct action of M-factors secreted by neighboring cells (proximal action) (Fig. 4); the M-factor transporter Mam1 localizes to polar sites on M cells, while the M-factor receptor Map3 also localizes on P cells (or is locally activated) near Mam1. M-factor may be secreted mainly at the contact site (68, 93). The concentration of M-factor, a hydrophobic lipid peptide, may be relatively high in the vicinity of P cells because of the short distance that M-factor diffuses into the surrounding medium. The local concentration of M-factor stably establishes the polarity of the P-cell. Eventually, pheromone receptors and the MAPK cascade become enriched at the fusion focus, promoting successful mating between opposite mating-type cells (94). Thus, the two pheromones of *S. pombe* have these two distinct modes of action during mating (60).

Cell Polarization by Cdc42

Once *S. pombe* receptors sense pheromones, an activated Cdc42 zone is formed, which then moves around the plasma membrane (95). The Cdc42 zone colocalizes with its GEF Scd1 and a scaffolding protein, Scd2 (95, 96), and contains myosin Myo52, the two pheromone receptors Mam2 and Map3, and M-factor transporter Mam1 (55, 93). In *S. pombe*, these patches of Cdc42 repeatedly assemble and disassemble near the plasma membrane until the concentration of pheromones around cells is high enough to stabilize them (93, 95, 96). Control of Cdc42 and Ras1 GTPases plays a central role in regulating the dynamics and polar growth of these patches. Ras1 is activated by the mating-specific GEF Ste6 (97) and can overcome its strong inhibition by the Ras GAP Gap1 (96). Inhibition of Ras1 activity is important for both promoting polarity for cell fusion and preventing premature cell fusion (96). Gap1-dependent inhibition of Ras1 only occurs when the local pheromone signal is sufficiently high,

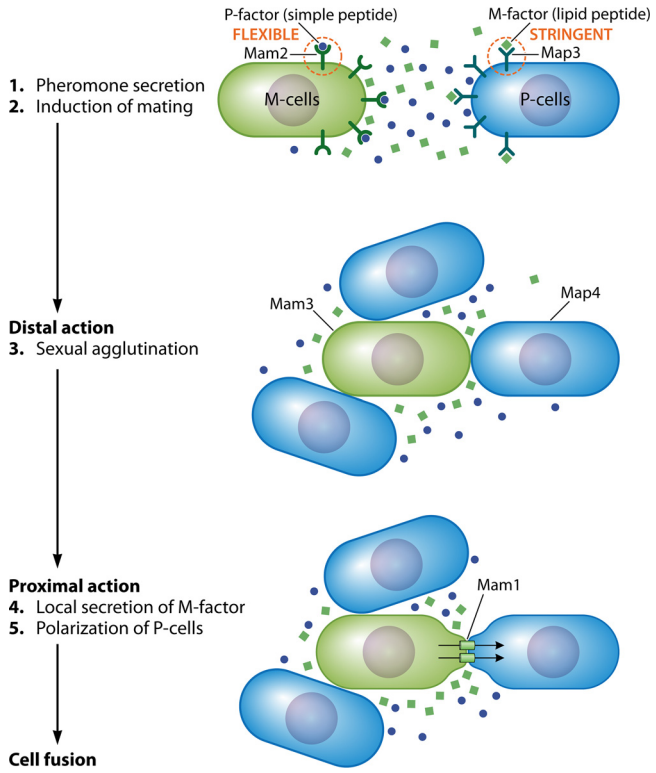

FIG 4 Steps of mating hypothesis regulated by the distal and proximal actions of two mating pheromones in *S. pombe*. The two chemically different mating pheromones may reflect their differential roles in the mating of *S. pombe*. First, when M cells and P cells are mixed in the nitrogen-free liquid medium, they secrete uniform pheromones, which facilitates the expression of pheromone-inducible genes to induce mating. Subsequently, sexual agglutination occurs, which leads to stable cell-to-cell contact (distal action). Polarized growth occurs at the contact site after agglutination. Possibly, hydrophobic M-factors are temporarily concentrated around P cells. Lastly, from the contact site, these locally secreted M-factors establish the polarity of P cells (proximal action), leading to cell fusion.

setting the threshold for cell-to-cell distance so that stabilization occurs, resulting in cell fusion (96).

When pheromone concentrations increase and Cdc42 patches stabilize, cells undergo polar growth (shmoo) toward opposite mating partners. Shmoo development, like polarized growth during the mitotic cycle, is thought to occur through a local supply of cell wall remodeling enzymes by Myo52, which locally degrades the cell wall to be driven by osmotic pressure (95, 98). Myo52 localizes at the shmoo tip and remains in a dynamic state until a mating-specific formin Fus1 is recruited. A positive-feedback loop between Myo52 and Fus1 is required for Myo52 focalization and ensures the formation of a fusion focus (98).

Mate Choice between Opposite Mating Types

Yeast pheromones are deeply involved in the selection of appropriate mating partners. For example, *S. cerevisiae* cells select mates that produce the strongest pheromone signals among potential mating partners (99, 100). This may be due to the formation of the Cdc42 polarization complex at the highest pheromone concentration, from which polarized growth begins (95). In fact, in *S. pombe*, adding an exogenous pheromone to cells that cannot produce their own pheromones does not restore their mating ability (33, 60). When M cells that do not produce M-factor are cultured with wild-type M cells, both M cells are incorporated into cell aggregates with P cells (60). The wild-type M cells help the mutant M cells by supplying their own M-factor, indicating that wild-type cells are altruistic and mutant cells behave as "impostors" in terms of animal sociology. However, to avoid such cheating behavior, P cells mate only with wild-type M cells (60). Cell fusion absolutely requires local secretion of M-factor by Mam1, essential for mating selection by P cells in *S. pombe*.

Pheromone-degrading enzymes may also play an important role in mate choice; the M cells of *S. pombe* secrete a serine carboxypeptidase, Sxa2, which specifically degrades extracellular P-factor from P cells (101–103). The terminal Leu residue of P-factor is removed by Sxa2 (102), and the resulting P-factor lacking Leu, comprising 22 amino acids, is inactive and not recognized by Mam2 (104). Yeasts sense a gradient of pheromones secreted by opposing cells and extends their shmoos toward the pheromone source (105). Degradation of the pheromone by peptidases is thought to make the gradient more stable (93). On the other hand, no enzyme that degrades extracellular M-factor has yet been found. It is possible that fine-tuning *mfm* gene expression could control M-factor production. In *S. cerevisiae*, *MAT***a** cells secrete a peptidase, Bar1p, that degrades α-factor (106–109). Bar1p has been shown to improve mate discrimination (99) and identification, with α-factor degradation by Bar1p limiting the diffused α-factor form forming a sharper gradient that improves mate discrimination ability (105, 110, 111). Thus, "cheater" cells are not selected as mating partners (112, 113). Taken together, the spatial arrangement of pheromone distribution is likely to be important for mate choice in yeast.

EVOLUTION OF PHEROMONE AND RECEPTOR SYSTEMS

Pheromone Recognition by GPCRs

Pheromone receptors of ascomycetes are Class D GPCRs. On the one hand, there is no sequence homology between the Map3 and Mam2 of *S. pombe*, a feature also observed between the a-factor receptor Ste3p (114) and α-factor receptor Ste2p (115) in *S. cerevisiae*. On the other hand, even though these yeast species are thought to have diverged between 1 and 300 million years ago, Map3 and Mam2 have significant sequence homology to Ste3p and Ste2p, respectively. For example, *S. pombe* Mam2 and *S. cerevisiae* Ste2p have approximately 70% amino acid sequence homology across the 5 to 7 TM helix (116), suggesting an evolutionary relationship between the two GPCRs subtypes, Ste3p/Map3 and Ste2p/Mam2. Genetic changes in pheromones and their receptors affect mate choice and cause prezygotic reproductive isolation (18, 77). Many studies on genetic analysis of pheromone receptors have been conducted in *S. cerevisiae* (114, 117–120), and recently, some have focused on the evolution of pheromone specificity in GPCRs (121, 122). However, information on genetic analysis and specificity of pheromone receptors in *S. pombe* is scarce.

Genes for pheromone receptors have been identified in four species of the genus *Schizosaccharomyces*. *S. pombe* Map3 has 65 and 43% homology with the Map3 of *S. octosporus* (SoMap3) and *S. japonicus* (SjMap3), respectively, and *S. pombe* Mam2 has 67 and 59% homology with the Mam2 of *S. octosporus* (SoMam2) and *S. japonicus* (SjMam2), respectively (30). The Map3 and Mam2 of *S. octosporus* and *S. cryophilus* are very similar. To test receptor specificity for pheromones, we exchanged genes between *S. pombe* and *S. octosporus* and found that SoMap3 was not functional in *S. pombe* cells, but SoMam2 was slightly functional, restoring *S. pombe* mating (30). This difference in pheromone recognition by the two GPCRs is striking because they share a downstream signaling pathway via G proteins. Studies in *S. cerevisiae* have shown that *STE3* and *STE2* genes are reportedly differentially regulated at both the transcriptional and posttranscriptional levels by cryptic polyadenylation (123). Thus, it is possible that ascomycete fungi, including *S. cerevisiae*, adopt different strategies for regulating gene expression of the two GPCRs for pheromones. Even though the set of genes that regulate downstream signaling of pheromone receptors might be similar in cells of different mating types, some characteristics, such as expression and regulation, may differ, resulting in different pheromone strengths observed in yeast. In *S. pombe*, differences in the specificities of Map3 and Mam2 may trigger the asymmetric diversification of pheromones (29). It is unclear why such an asymmetric system would be convenient for yeast, but simulations and computational modeling could shed light on this issue in the future.

Artificial Reproductive Isolation *In Vitro*

Reproductive isolation, which restricts gene flow between sympatric populations, is one

important mechanism of speciation (124). Animals employ various methods, including pheromones in insects and amphibians (125–128), body color in fish (129–131), and song in birds (132, 133), to properly recognize the opposite sex in breeding between closely related species. Such mate choice has been well studied in higher organisms but is less well known in fungi (134).

In ascomycete fungi, mating between partners critically depends on molecular recognition by pheromone receptors (67, 77, 135–138). In our recent study, we successfully created a novel *S. pombe* reproductive group that is reproductively isolated from the wild-type by artificially altering the primary structure of both M-factor and Map3 (18). We comprehensively mutated every amino acid of the eight amino acids comprising M-factor to generate a library of 152 variants (8 amino acids $\times$ 19 possible mutations) and screened for failure to mate. After identifying 35 missense M-factor mutations that conferred sterility, we then attempted to rescue the sterile phenotype of some by random Map3 mutagenesis. Ultimately, we found a single pair of M-factor/Map3 mutants that were highly fertile, but none of which were able to mate with cells that express the wild-type M-factor and Map3, thus resulting in incipient speciation. This success substantiated the hypothesis that pheromone and receptor coevolution is one of the mechanisms of prezygotic isolation in yeast. Based on the "biological species concept," this reproductive population is considered a new species. As far as we know, this is probably the first report of the artificial creation of a new species in the history of biological evolutionary studies. Thus, genetic changes in pheromones and their receptors are likely to be important in promoting yeast speciation. Similar differences (or variation) of sex pheromones exist between the newts *Cynops pyrrhogaster* and *Cynops ensicauda* (127, 128), indicating that slight differences in pheromones may prevent proper recognition of opposite sexes in nature.

Potential Mechanism of Genetic Changes in Mating Systems Resulting in Incipient Speciation

There is no doubt that pheromone and receptor systems promote reproductive isolation through very subtle changes in nature. More generally, however, changes in pheromones and receptors must occur gradually or accidentally before speciation can occur since pheromone activity loss leads to the extinction of an organism's lineage. Although this hypothesis is an attractive explanation for yeast speciation, the unknown processes by which natural selection could lead to the origin of new species from previously interbreeding individuals remain unsolved.

The fact that many yeasts have multiple pheromone genes or duplicate peptide sequences within a single pheromone gene may be convenient for creating pheromone diversity. *S. pombe* has three M-factor genes, and P-factor has at least four tandem repeats within a single gene. These redundancies enable yeasts to employ a strategy where if the receptor is mutated, the cell can modify one pheromone copy to successfully adapt to the changed receptor while leaving the other copies unchanged. Such pheromone gene duplication allows unlimited changes in pheromone structure while retaining the original version of the pheromone gene, allowing for flexibility in adapting to different mutations in the receptor protein. Coevolution of pheromones and their corresponding receptors is likely to be gradual, with mutational changes causing a slight decrease in pheromone activity or pheromone receptor activity. A second suppressor mutation may occur to restore the initial defect before such a mutant is completely lost. Multiple subtle changes may repeatedly occur in the actual course of pheromones and receptor coevolution until speciation occurs. Such gradual coevolution is likely to be the actual mechanism of the process of prezygotic isolation associated with the pheromone system. We hope that pheromone response and mating behavior in yeast will be elucidated through future large-scale studies of wild yeasts.

CONCLUSIONS

The ability of one yeast species to recognize another depends on the interaction between a pheromone and its associated receptor. In *S. pombe*, M-factor is strictly

conserved, while P-factor is relatively diversified. The observation suggests that reproductive isolation has benefited from an asymmetric environment during evolution. To test this hypothesis, we are currently conducting some experiments to investigate this hypothesis and observe to what extent the interaction of pheromones and their associated receptor can be altered while maintaining recognition. The two pheromones and their receptor genes were randomly mutagenized, and cell populations expressing the mutant genes are mixed to mate. Diploid zygotes that were able to mate by binding the pheromone to the receptor are collected, and then the combinations of the pheromone and receptor genes that zygotes possessed will be comprehensively determined using a next-generation sequencer. These experiments may provide a molecular mechanism for distinct stringencies in pheromone recognition by the two types of receptors. Furthermore, we intended to work on evolutionary simulations that take into account the reasons why an asymmetric environment is beneficial in *S. pombe*.

Perhaps the pheromone recognition mechanism of various organisms has both a "strict" part that is important for mate discrimination and an "ambiguous" part that can flexibly respond to changes, and such a mechanism creates diversity. Although the ecology of *Schizosaccharomyces* species is unknown, further attention should be paid to the mating behavior of natural yeasts in different ecological niches. Using *S. pombe* as a model, we hope that these approaches will provide valuable insights into the evolutionary mechanisms underlying the diversification of pheromones.

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We declare there are no conflicts of interest.

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