Multiple Reinventions of Mating-type Switching during Budding Yeast Evolution

Highlights

- Mating-type switching by flip-flopping arose at least 10 separate times
- Mating-type switching by copy-and-paste arose only once in budding yeasts
- Transitions toward homothallism outnumber transitions away from homothallism
- Heterothallic species can become homothallic by DNA introgression

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In Brief

Krassowski et al. find that mating-type switching originated at least 11 times independently during the evolution of budding yeasts, pointing to the adaptive value of self-fertility to unicellular fungi.
Multiple Reinventions of Mating-type Switching during Budding Yeast Evolution

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SUMMARY

Cell type in budding yeasts is determined by the genotype at the mating-type (MAT) locus, but yeast species differ widely in their mating compatibility systems and life cycles. Among sexual yeasts, heterothallic species are those in which haploid strains fall into two distinct and stable mating types (MATa and MATα), whereas homothallic species are those that can switch mating types or that appear not to have distinct mating types [1, 2]. The evolutionary history of these mating compatibility systems is uncertain, particularly regarding the number and direction of transitions between homothallism and heterothallism, and regarding whether the process of mating-type switching had a single origin [3–5]. Here, we inferred the mating compatibility systems of 332 budding yeast species from their genome sequences. By reference to a robust phylogenomic tree [6], we detected evolutionary transitions between heterothallism and homothallism, and among different forms of homothallism. We find that mating-type switching has arisen independently at least 11 times during yeast evolution and that transitions from heterothallism to homothallism greatly outnumber transitions in the opposite direction (31 versus 3). Although the 3-locus MAT-HML-HMR mechanism of mating-type switching as seen in Saccharomyces cerevisiae had a single evolutionary origin in budding yeasts, simpler “flip/flop” mechanisms of switching evolved separately in at least 10 other groups of yeasts. These results point to the adaptive value of homothallism and mating-type switching to unicellular fungi.

RESULTS AND DISCUSSION

Infering Mating Compatibility Systems from Genome Sequences

Shen, Opulente, Kominek, Zhou, et al. [6] recently reported a phylogeny of budding yeasts, based on the genome sequences of 332 species. We analyzed these sequences to infer the “mating compatibility system” of each species, by which we mean its thallism state (i.e., whether it is heterothallic or homothallic) and, for each homothallic species, the molecular basis of its homothallism [2–5, 7].

To identify MAT-like loci, we searched for genes coding for the four canonical MAT proteins (a1, a2, α1, and α2) in a reference genome sequence (one strain) from each species [6], by using automated TBLASTN searches with a diverse set of MAT protein sequences as queries. Genomic regions containing MAT genes were then examined by eye to validate and classify the loci. We classified each species as having one of seven possible mating compatibility systems (Figure 1) based on its genome’s content of MAT genes and the presence or absence of repeated sequences near them, as summarized below. Detailed descriptions of each species’ status are given in STAR Methods.

HET

In heterothallic species, strains occur as two different mating types, and the mating types are stable. We classified species as heterothallic (HET; Figure 1A) if their genome sequences contain only MATa genes or only MATα genes (presumed heterothallic haploids) or if they contain both MATa and MATα genes on separate contigs that appear to be allelic (presumed heterothallic diploids).

In homothallic species, strains do not fall into two distinct mating types. Instead, any strain can mate with any other strain. Two major forms of homothallism are recognized—primary and secondary—with mating-type switching occurring only in secondary homothallics [2, 3, 8]. In primary homothallic species, it is thought that any cell can mate with any other cell, though this has not been investigated in detail [5]. In secondary homothallic species, cells have distinct mating types and mating always occurs between a MATa cell and a MATα cell, but strains do not maintain stable mating types because cells can switch their mating type. We classified species into three distinct genomic categories of secondary homothallism (3LOC, FF1, and FF2) and two distinct genomic categories of primary homothallism (PHC and PHN), as explained below, based on their inferred molecular mechanisms. These terms describe categories of genomic organization, not phylogenetic groups.

3LOC

Among the secondary homothallics, we refer to species such as Saccharomyces cerevisiae as having a three-locus system.
They have an active MAT locus and two or more silent loci (called HML and HMR in *S. cerevisiae*) that contain non-expressed a and α sequence information [9, 10]. They switch mating types by a copy-and-paste mechanism, copying DNA from the silent loci and pasting it into the MAT locus. Exchange of DNA between MAT and HML/HMR is facilitated by DNA repeat sequences called X and Z that flank these three loci (Figure 1B). HO endonuclease, which cleaves the MAT locus to initiate mating-type switching in *S. cerevisiae* [10], has a narrow phylogenetic distribution and is only present in a subset of the genera that use the 3LOC system [5].

In contrast to 3LOC species, secondary homothallic species with flip/flop systems (FF1 and FF2) switch their mating types by inverting a section of chromosome, exchanging MAT genes between an expression site and a repression site near a centromere or telomere [7, 13, 14]. The best-characterized species with a flip/flop system is *Ogataea polymorpha*, which we categorize as FF1 (Figures 1C and S1B) because there is one inverted repeat (IR) sequence flanking its MAT genes. Recombination between the sequences that form the IR inverts the whole region containing the MAT genes in *O. polymorpha*. The FF2 category (Figure 1D) describes species such as *Komagataella phaffii* that also use a flip/flop mechanism to switch mating types but have two IRs, one on each side of their MAT locus [13].
We classified species as primary homothallics if they contain both $\text{MAT}_a$ and $\text{MAT}_a$ genes at non-allelic positions but lack any DNA repeats near these genes. The absence of repeats means that there is no apparent mechanism by which they could switch mating type, in contrast to the secondary homothallics.

We defined one group (PHC, primary homothallic contiguous; Figure 1E) as those in which the $\text{MAT}_a$ and $\text{MAT}_a$ genes are close to each other in the genome (<20 kb apart), as previously seen in species such as $\text{Debaryomyces hansenii}$ and $\text{Scheffersomyces stipitis}$, both of which are considered to be primary homothallics [5, 15–17]. Other genomes in which $\text{MAT}_a$ and $\text{MAT}_a$ genes are both present on the same contig but far apart (all examples are >90 kb apart), or on different non-allelic contigs, were categorized as PHN (primary homothallic non-contiguous; Figure 1F).

NOMAT

Two of the 332 genomes contained no identifiable $\text{MAT}$ genes and were classified as NOMAT (Figure 1G; STAR Methods)—$\text{Lodderomyces elongisporus}$ [18, 19] and $\text{Candida sojae}$ [20]. The molecular mechanisms that these species use to control cell type and mating are completely unknown.

A Single Origin of the Three-Locus System of Mating-type Switching in Budding Yeasts

Phylogenomic analysis has grouped the 332 budding yeast species into 12 major clades [6], and most clades include species that differ in their mating compatibility systems (Figure 2; Data S1). Approximately half the species in the dataset are heterothallic (192 of 332), and most clades include both heterothallic and homothallic species (Figure 2).

The 3LOC system of mating-type switching is present in almost all the studied species of the family Saccharomycetaceae (66 of 71 species) but occurs nowhere else in the phylogenetic tree of budding yeasts. It is therefore inferred to have originated during the early evolution of Saccharomycetaceae, prior to the deepest divergence within this family, which is the split between the $\text{Kluyveromyces}$ lineage and the $\text{Saccharomyces}$ lineage. The five Saccharomycetaceae species that do not have a 3LOC system are $\text{Lachancea kluyveri}$, which is HET and has lost the $\text{HML}$ and $\text{HMR}$ loci [21, 22], and four species of $\text{Kazachstania}$. The four $\text{Kazachstania}$ species are not monophyletic and represent one transition from the 3LOC system to HET and two separate transitions from 3LOC to primary homothallism (PHN) (Data S1). The best characterized of these PHN $\text{Kazachstania}$ species is $\text{K. africana}$, in which a chromosomal breakage at the ancestral $\text{MAT}$ locus, together with the loss of $\text{HML}$ and $\text{HMR}$, created a species with non-allelic $\text{MAT}_a$ and $\text{MAT}_a$ loci on two different chromosomes [23]. These four $\text{Kazachstania}$ species have all lost the $\text{HO}$ endonuclease gene, which indicates that they do not switch mating types, whereas the 13 other sequenced $\text{Kazachstania}$ species retain $\text{HO}$.

We infer that there was a transition from HET to 3LOC at the base of the family Saccharomycetaceae because the sister clade Saccharomycodaceae contains only HET species, and the closest outgroup clade (Phaffomycetaceae) contains many HET species and appears to have been HET at its base (Figure 2; Data S1). Nevertheless, such a HET → 3LOC transition in a single step is difficult to envisage because it would require numerous changes to the genome, so unseen intermediate steps may have been involved ([13]; see below).

Although we infer a single origin for the three-locus switching system in budding yeasts, a 3LOC system also evolved in parallel in the fission yeast $\text{Schizosaccharomyces pombe}$, which is a member of a different subphylum ($\text{Taphrinomycotina}$) and lies...
quite young (nine of the 11 are <100 million years old [6]).

flip/flop clades are phylogenetically quite narrow and therefore S. pombe. The switching mechanism of completely outside the tree in Figure 2. The switching mechanism of S. pombe is analogous, not homologous, to the mechanism in S. cerevisiae and its mechanistic details are substantially different [5, 24, 25].

At Least 10 Independent Origins of Flip/Flop Mating-type Switching Systems

The most parsimonious interpretation of our data is that the flip/flop mechanism of mating-type switching, in which a section of chromosome becomes inverted, does not have a single evolutionary origin but arose independently 10–11 times in different lineages. There are eight independent clades with FF1 systems and two or three with FF2 systems (Data S1). Most of these flip/flop clades are phylogenetically quite narrow and therefore quite young (nine of the 11 are <100 million years old [6]).

The eight FF1 clades include three that have been reported previously, and five newly discovered ones. The previously known ones are clades containing Pachysolen tannophilus, Ascoidea rubescens, and Ogataea polymorpha [5, 7]. P. tannophilus and A. rubescens are both singleton FF1 species whose closest relatives are heterothallic (Data S1, S2A, and S2B). O. polymorpha lies within a clade of 12 Ogataea species that all switch by an FF1 mechanism [13, 14, 26], but the genus Ogataea also contains two other clades with newly discovered FF1 systems that we infer to have originated independently of the 12-species O. polymorpha FF1 group (Data S1; STAR Methods).

SLA2 and VPS75, is orthologous to the single MAT locus of C. jadinii (HET, diploid). Starmera quercuum (Phaffomycetaceae) contains a similar but larger invertible region of 61 kb, flanked by an IR that extends to the ends of the available contig, while in Kregervanrija (Pichiaceae), the two studied species both contain only the four canonical MAT genes on a 12-kb invertible region flanked by IRs (Data S2C). One set of MAT genes, located between SLA2 and VPS75, is orthologous to the single MAT locus of C. jadinii (HET, diploid). Starmera quercuum (Phaffomycetaceae) contains a similar but larger invertible region of 61 kb, flanked by an IR that extends to the ends of the available contig, while in Kregervanrija (Pichiaceae), the two studied species both contain only the four canonical MAT genes on a 12-kb invertible region flanked by IRs (Data S2C).

The two clades with unambiguous FF2 systems are species of Komagataella and Saturnispora, which are both in family Pichiaceae but only distantly related to each other. The Komagataella FF2 system has been characterized in detail in K. phaffii (Figure 3A) and is shared by two other studied species of Komagataella (Data S1). In these species, the repressed MAT locus is located near a telomere [13, 28]. The region that inverts is 138 kb long and includes about 72 genes and a centromere [27].

The Saturnispora FF2 system, illustrated by S. zaruensis (Figure 3B), is newly discovered. It is present in a clade of four species within this genus, whereas three outgroup Saturnispora species are heterothallic (Data S1). The S. zaruensis MATa and MATα genes are almost 200 kb apart and flanked by two IRs, one on each side of the MAT genes (Figure 3B). We infer that there is a centromere located asymmetrically inside the 194-kb invertible region, close to one end (STAR Methods). The S. zaruensis system appears to use centromeric repression of transcription, in contrast to the telomeric repression seen in K. phaffii. Recombination between the inner IR sequences

Other newly discovered FF1 systems occur in the genera Cyberlindnera, Starmera, and Kregervanrija, representing three additional independent origins of FF1 (Data S1). In Cyberlindnera (Phaffomycetaceae), a clade of three species with FF1 secondary homothallism is nested inside this otherwise heterothallic genus. These three species (C. saturus, C. mukaii, and C. suaveolens) contain an invertible region of approximately 49 kb spanning 13 genes, with MATa genes at one end and MATα genes at the other end, flanked by an IR (Data S2C). One set of MAT genes, located between

Figure 3. Comparison of MAT Locus Organization in Two Species that Use FF2 Systems of Secondary Homothallism

Purple and blue shading indicates inverted repeat (IR) sequences. Centromeres (CEN) and telomeres (TEL) are marked.

(A) Organization in Komagataella phaffii [13, 27]. Recombination in the outer IR (blue) is known to move the telomere from beside MATa genes to beside MATα genes, repressing their transcription.

(b) Organization in Saturnispora zaruensis, inferred from the scaffolds (nodes) in its genome assembly. Recombination between the two copies of the inner IR (purple) is inferred to move the centromere from beside MATa genes to beside MATα genes, repressing their transcription. The total length of the region between the inner IRs is 194 kb.

Other newly discovered FF1 systems occur in the genera Cyberlindnera, Starmera, and Kregervanrija, representing three additional independent origins of FF1 (Data S1). In Cyberlindnera (Phaffomycetaceae), a clade of three species with FF1 secondary homothallism is nested inside this otherwise heterothallic genus. These three species (C. saturus, C. mukaii, and C. suaveolens) contain an invertible region of approximately 49 kb spanning 13 genes, with MATa genes at one end and MATα genes at the other end, flanked by an IR (Data S2C). One set of MAT genes, located between
In it, the transition from FF1 to FF2 could be caused by a MAT growing distance between the two MAT loci, leading to selection for a second IR to restore colinearity of the chromosome. Subsequently, duplication of one of the two IR-flanked MAT loci would cause the centromere to move from primary to secondary homothallism. The existence of different types of secondary homothallism, nor any transitions of DIC1 close to the MATa locus is present in the middle of a large (612 kb) scaffold, or the relative orientations of the flip-flopping regions (Figure 1). We did not detect any transitions between different types of secondary homothallism, nor any transitions from primary to secondary homothallism. The existence of multiple independent FF2 clades naturally suggests a series of transitions that could lead to the emergence of a 3LOC system: HET → FF1 → FF2 → 3LOC, first postulated by Hanson et al. [13]. In it, the transition from FF1 to FF2 could be caused by a growing distance between the two MAT loci, leading to selection for a second IR to restore colinearity of the chromosome. Subsequently, duplication of one of the two IR-flanked MAT loci could create a species with three MAT loci in a genomic arrangement very similar to that of a functional 3LOC system. Nevertheless, the intermediate transitions required by this scenario are not observed in our data.

Conversion of Heterothallistics to Primary Homothallistics by Introggression of MAT Genes at Telomeric and rDNA Sites

We identified a clear case of a heterothallic ancestor producing a primary homothallic (PHN) descendant, in the genus Nadsonia (Figure 4; Data S2E). Two species in this genus, N. fulvescens var. fulvescens [6] and N. starkeyi-henricii [29], have a single, orthologous, MAT locus (with MATa and MATg genotypes, respectively) and so are classified as heterothallic. A third species, N. fulvescens var. elongata [7], has MATg genes at this locus but also has MATa genes on another scaffold, at a site that is close to a telomere (Data S2E). Comparison between the two N. fulvescens varieties indicates that a few kilobases of DNA, including the MATa genes, have been gained by var. elongata at this telomere, converting it from a heterothallic species to a primary homothallic (PHN) species. Consistent with these genome-based designations of mating compatibility systems, N. fulvescens var. elongata is homothallic (sporulation follows conjugation between a cell and its bud), whereas the other two species have no known sexual cycle and do not form spores [30].

Similar to the situation we described for Nadsonia fulvescens var. elongata, many of the other species we classified as PHN also contain one old MAT locus at a site syntenic with the MAT loci of closely related HET species, and a new second MAT locus with the opposite MAT allele, at a location that is close to a telomere. The PHN species in this category are Blastobotrys proliferans (Trichomonascaceae), Kuraishia molsichiana (Pichiaceae), two Yamadazyma species (CUG-Ser1 clade), Wickerhamia fluorescens (CUG-Ser1 clade), Saccharomyces capsulatis (CUG-Ser2 clade), two Lipomyces species (L. oligophaga and L. suomienis; Lipomycesaceae), Kazachstania rosinii (Saccharomycesaceae), and Nadsonia fulvescens var. elongata. In four of these species, the new subtelomeric MAT locus is beside a pseudogene of SLA2. Thus, among the 12 transitions to PHN in our dataset (Figure 1), eight of them involved the gain of DNA containing the opposite MAT allele, at a site near a telomere. We hypothesize that these events are the result of DNA introgression between strains that were initially heterothallic. A related situation occurs in two Peterozyma species (CUG-Ala clade) that became PHN by gaining a second MAT locus at a site beside the rDNA array (Figure 4; Data S2B).
Genomic regions close to rDNA, telomeres, and centromeres can form heterochromatin that represses transcription, and it is striking that almost every example of PHN that we detected involves gain of a new MAT locus either near a telomere or beside the rDNA. Furthermore, the genomic rearrangement that converted Kazachstania africana from 3LOC to PHN left one of its two MAT loci in a subtelomeric region [23]. In fact, only two of the 12 PHN clades in our dataset have a second MAT locus that is not telomere or rDNA associated, and in both of these clades (Lipomyces japonicus and a pair of Ambrosiozyma species) there is a large, possibly heterochromatic, region of non-coding DNA beside the second MAT locus (STAR Methods).

The pattern of PHN species emerging by gaining a second MAT locus in a heterochromatic region of the genome (Figure 4) may indicate that these second MAT loci are silenced at some stage in the life cycle of the organisms that contain them. Cells of primary homothallic species contain both MATa and MATα genes, but the molecular details of how these genes control mating and sporulation are not understood (how does each cell know whether it should mate or sporulate?). We agree with previous speculations that primary homothallic species may use epigenetic or other regulatory mechanisms to ensure that each haploid cell expresses only MATa genes, but the molecular details of these mechanisms are not well understood. Unlinked MATa and MATα loci in these yeasts cannot be predicted. A third possibility is that the bias toward subtelomeric and rDNA sites may simply reflect the receptiveness of these sites toward introgressed DNA [6, 32].

**Evolutionary Transitions toward Homothallism**

In the entire dataset of 332 yeast species, we see 31 transitions from heterothallism to homothallism, but only three instances of transition in the opposite direction (Figure 1). The asymmetry between these numbers is striking, particularly considering the likely molecular mechanisms of transition. Naively, we would expect transitions from heterothallism to homothallism to be difficult, because they require a complex series of steps: introgression of MAT genes to create a primary homothallic species or relocations of MAT genes to silenced positions near the centromere or telomere, as well as DNA duplications to make repeats, to create a secondary homothallic species. In contrast, a homothallic cell can become heterothallic very easily, by just deleting some DNA [33].

The 31 transitions to homothallism comprise 19 to primary homothallism and 12 to secondary homothallism (Figure 1). The three transitions away from homothallism consist of two 3LOC → HET transitions that occurred in Lachancea kluyveri [22] and a pair of Kazachstania species, and a PHC → HET transition in Lipomyces doorenjorgii (Data S1 and S2F; STAR Methods).

Our analysis shows clearly that evolutionary pressure has repeatedly led to the emergence of homothallic descendants from heterothallic ancestors. The fact that homothallism was gained 31 times and lost only 3 times suggests that it has overwhelming evolutionary benefits, as has been postulated in the past [3, 13, 34, 35]. We have suggested that the evolutionary benefit of homothallism is that it gives a yeast species the ability to form new spores during the first few cell generations after spore germination, thereby removing the death penalty that too-early germination otherwise entails if the environment is inadequate [13, 36]. Alternatively, homothallism may have been favored by selection because it enables reproductive assurance [35, 37], diploidization and DNA repair [9], or genome renewal [38, 39]. The large number of independent origins of homothallism, in both its primary and secondary forms, points to DNA rearrangements of the MAT locus being an effective, albeit radical, way of increasing the fitness of a budding yeast species.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.06.056.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, K.H.W., T.K., and C.T.H.; Methodology, T.K. and K.H.W.; Software, T.K.; Investigation, T.K., K.H.W., J.K., D.A.O., X.-X.S., and X.Z.;
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DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Deposited Data      |        |            |
| The genomes used in the study | DDBJ/ENA/GenBank | Table S1 in [6] |
| Software and Algorithms | | |
| BLAST               | [40]   | RRID: SCR_004870 |
| Seaview             | [41]   | RRID: SCR_015059 |

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Kenneth Wolfe (kenneth.wolfe@ucd.ie). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We analyzed the same dataset of 332 budding yeast genome sequences as in Shen, Opulente, Kominek, Zhou, et al. [6], which combined previously published genomes of 112 species with 220 newly sequenced genomes (Data S1).

METHOD DETAILS

For each species we examined only one reference genome sequence assembly [6], which means that for heterothallic (HET) species we may have detected only one of the two possible MAT alleles, depending on the nature of the assembly. Because of the high sequence diversity of MAT genes, we used a strategy of multiple automated TBLASTN searches [40] against each genome sequence, with diverse sets of MAT protein sequences (a1, a2, a1, a2) as queries, retaining even very weak hits (TBLASTN E < 10). Genomic regions that were hit by more than one different MAT protein were then examined manually. As additional MAT genes were found, they were added to the query dataset. Automated searches were also used to annotate genes commonly located near MAT loci, such as SLA2 and DIC1, and to label repeated DNA sequences in the vicinity of MAT-like loci that could form parts of IR, X or Z regions. These repeats often contain duplicated parts of MAT genes, so care was taken to distinguish between intact genes and gene fragments. When annotating genomic regions, we interpreted genomic regions containing an intact a1 and/or a2 gene to be MATa loci, and regions containing an intact a1 and/or a2 gene to be MATα loci (i.e., we did not require both of the a genes or both of the a genes to be present). All cases of inferred absence of one but not both of the genes in a pair (e.g., if a1 is present but a2 is absent, etc), as well as all NOMAT cases, were verified by manual BLAST searches.

For some species, the structure of MAT-like regions was inferred from multiple contigs in the genome assembly. In genomes assembled using SPAdes [42], any repeat regions that exist as two highly similar copies in the real genome (such as IRs, X and Z regions) usually co-assemble into single contigs whose coverage is approximately twice the average coverage. These contigs often have short sequence overlaps with the contigs that flank them on each side; the overlaps are the same length as the longest k-mer used by SPAdes. By detecting these overlaps and comparing contig coverage, we were able to infer the overall organization of the MAT-like regions manually for most species.

We classified species into the 3LOC, FF1 and FF2 categories of secondary homothallism based on the presence of both MATa and MATα genes in the genome at non-allelic positions, the presence of repeat sequences (IRs or X/Z regions), the numbers of copies of MAT-like and repeat sequences, and the presence of truncated genes such as often occur in IRs and X/Z regions [36]. We classified genomes as primary homothallic if they contain both MATa and MATα genes at non-allelic positions that are not near repeated sequences, and classified them as PHC or PHN depending on whether the distance between MATa and MATα genes was less than 20 kb. All the examples of PHN that we report have their two MAT loci on different non-allelic contigs, or > 90 kb apart on the same contig.

Transitions between mating compatibility systems were inferred manually using parsimony, by reference to the genome structures at MAT-like loci and the phylogenomic tree [6] (Data S1). We considered parsimony to be the the most appropriate method, because the number of transitions is low relative to the number of species, and because we have no way of weighting a priori the probability of transitions between different systems.

Most (118) of the 220 genomes that were newly sequenced for the Y1000+ Project [6] were assembled using the SPAdes assembler [42]. We noticed that two genomes (Cyberlindnera saturnus and Starmera quercuum) that were assembled using a different assembler, DISCOVAR [43], each contain a very large inverted repeat (>100 kb) flanking the MAT genes (Data S2C). In each case
the two copies of the repeat differ by only 1 nucleotide, and extend to the ends of the assembled contig. The Illumina sequencing protocol used by Shen, Opulente, Kominek, Zhou, et al. [6] does not have power to resolve such large near-identical repeats, so we concluded that these structures are artifacts that occur when FF1 genomes are assembled using DISCOVAR. They do not occur with the SPAdes assembler.

In the remainder of this section we describe the genomic data supporting our inferences of mating compatibility systems, sorted by clade. For species in which we found only MATα genes or only MATβ genes, and no other evidence is available, we inferred that the species is heterothallic (HET) and do not describe it. In all other cases, the rationale for classifying each species into its category is provided below.

Saccharomycetaceae

71 species: genera Saccharomyces, Nakaseomyces, Kazachstania, Naumovozyma, Tetrapisispora, Vanderwaltozyma, Yueomyces, Torulaspora, Zigotorulaspora, Zygosaccharomyces, Lachancea, Eremothecium, Ashbya, Klyuyveromyces, and species Candida ni- variensis, Candida bracarenensis, Candida glabrata and Candida castellii.

The vast majority of species in family Saccharomycetaceae are capable of mating type switching using a system homologous to that of Saccharomyces cerevisiae and are classified as 3LOC. We identified three exceptions to this rule in the genus Kazachstania, and one exception in the genus Lachancea:

Whereas most species in the genus Kazachstania have a 3LOC organization, four of them do not. These four species fall into three separate clades within the genus: K. africana, K. rosinii, and the pair K. yukushimaensis/K. transvaalensis. The genome assemblies of each of these four species contain both MATα and MATβ genes. None of the 4 has an HO endonuclease gene; these are the only known post-WGD species that do not have an HO gene. Moreover, in these species the Yz region does not end at the HO cleavage site in the MATβ1 gene, as it does in HO-containing species [36]. Together, these observations indicate that these four Kazachstania species have lost the ability to switch mating type. The four are discussed below.

Kazachstania africana has sustained a genomic rearrangement that we have described in detail elsewhere [23], which was probably the result of chromosome breakage at the ancestral MAT locus. We classify K. africana as primary homothallic non-contiguous (PHN).

Kazachstania rosinii has a MATα1 gene located between CAN1 and RNH203, similar to most Kazachstania species [36], and a MATα1-MATα2 gene pair located between homologs of HYR1 and a flocculin (FLO) gene at a locus that appears to be subtelomeric. Since there are no repeated sequences in the vicinity of the MAT genes, we conclude that K. rosinii is primary homothallic non-contiguous (PHN).

The Kazachstania yakushimaensis assembly contains two 9-kb MAT contigs that appear to be alleles. One contains MATα1, the other contains MATα1-MATα2, and the two contigs are identical over their first 1 kb and last 1 kb. The K. yukushimaensis MAT contigs lie between CAN1 and RNH203 genes. The allele-specific regions (Ya and Yz) are 7 kb, which is unusually long for a Saccharomyces species, but they contain no additional genes. We therefore classified K. yukushimaensis as a heterothallic (HET) diploid.

Kazachstania transvaalensis is the closest relative of K. yukushimaensis. The K. transvaalensis assembly is rather fragmented and this genome has a high content of repeat sequences, but the available data are consistent with K. transvaalensis having the same organization as K. yukushimaensis, so we classified it as heterothallic (HET) diploid.

In Lachancea klyuyveri, synteny indicates that both of the silent loci (HML and HMR) have been lost [21, 22]. The sequenced strain is diploid, and the species is known to be heterothallic [44]. We therefore classified L. klyuyveri as heterothallic (HET).

In addition to the species mentioned above, 17 more Saccharomycetaceae assemblies have only two or fewer genomic loci containing MAT genes, instead of the three expected for a 3LOC species. Many of these assemblies are highly fragmented. In 6 assemblies (Kazachstania unispora, Kazachstania taiensis, Kazachstania broemiacearum, Tetrapisispora fleeti, Klyuyveromyces aestuari, and Klyuyveromyces dozhanskii), coverage data suggests the existence of additional loci that were collapsed onto one contig. For 9 other species (Saccharomyces mikatae [45], Saccharomyces kudriavzevii [46], Saccharomyces arboricola [47], Nakaseomyces bacillisporus [48], Torulaspora pretoriensis, Torulaspora franciscae, Torulaspora microellipsoides [49], Zygosaccharomyces bailii [50, 51], and Eremothecium cymbalariae [52]), synteny or the presence of X and Z regions suggests that the apparent absence of 3 loci in the assembly used in the Y1000 Project dataset is due to a misassembly rather than a real deletion of one of the loci. For most of these 9 species a standard 3LOC system has been reported in the literature, in some cases from a different strain than was analyzed in the Y1000 dataset. In the final 2 cases (Lachancea quebecensis [53] and Lachancea lanzarotensis [54]), synteny with their closest relatives (Lachancea thermotolerans and Lachancea fantastica, respectively) also suggests that the lack of 3 loci is due to misassembly. In all 17 cases, we conclude that the species in question are capable of mating type switching and should be classified as 3LOC.

Saccharomycodaceae

8 species: genus Hanseniaspora and species Klöeckera hatyaensis. Four species in this family (Hanseniaspora uvarum, H. pseudoguilliermondii, H. valbyensis, and H. osmophila) contain only a MATα locus or only a MATβ locus. We classify them as heterothallic (HET).

Three species (Klöeckera hatyaensis, Hanseniaspora singularis, and Hanseniaspora vineae) contain both a MATα and a MATβ locus and synteny suggests they are diploid assemblies. Hanseniaspora clementiae contains both a MATα and a MATβ locus in very short contigs. In all 4 cases, we classify these species as diploid assemblies of heterothallic species (HET).
Phaffomycetaceae

34 species: genera Cyberlindnera, Wickerhamomyces, Phaffomyces, Barnettozyma, Starmera, and species Candida mycetangi, Candida freyschussii, Candida orba, Candida montana, Candida stellimalicola, and Candida ponderosa.

Cyberlindnera jadinii and Cyberlindnera maclurae each have assemblies of MAT loci suggesting they are diploid assemblies of heterothallic species. Diploidy is suggested by the presence of flanking sequences around the loci and, in the case of C. maclurae, also by the coverage data.

Cyberlindnera saturnus is one of two species that we infer to be FF1 species whose genome assemblies were affected by an artifact caused by the DISCOVAR assembler. In the C. saturnus assembly, a 49 kb (13 gene) region containing MATa genes (a1 and a2) at one end and MATx genes (α1 and α2) at the other end is flanked by an inverted repeat (Data S2C), so we conclude that C. saturnus can switch mating types by a flip/flop mechanism using one IR (FF1). In the reported C. saturnus assembly [6], the IRs are 118 kb long and differ by only 1 nucleotide, and extend out to the ends of the contig that contains them (NCBI accession PPNR02000017.1). We suspect that the length of the IRs has been artfactually extended by the assembler (DISCOVAR) that was used for this genome, which came from the type strain of C. saturnus (NRRL Y-17396). In independent SPAdes assemblies of three other C. saturnus strains from the NCYC collection (NCYC22, NCYC23, and NCYC57) we found a similar 49-kb region flanked by short IRs that ran to the end of the SPAdes contigs. Unusually for an FF1 system, the genes SLA2 and DIC1 are located inside the invertible region in C. saturnus and do not form part of the IR. By synteny with C. saturnus, we infer that its close relatives Cyberlindnera mrakii and Cyberlindnera suaveolens are also FF1 species.

Starmera quercuum is the second species that we infer to be an FF1 species whose genome assembly was affected by an artifact caused by the DISCOVAR assembler (Data S2C). In the S. quercuum assembly, the MAT contig (NCBI accession PPIB01000006.1) contains a unique 61-kb (21 gene) region with MATa genes at one end and MATx genes at the other, flanked by IRs that were assembled as 195 kb long and differ by only 1 nucleotide. As with Cyberlindnera saturnus, we conclude that S. quercuum is an FF1 species with misassembled IRs of unknown length.

Barnettozyma hawaiiensis, B. populi, B. californica, and B. salicaria each have a MAT locus assembly suggesting that they are primary homothallic contiguous (PHC). All four canonical MAT genes are found next to one another, and in all these species, except B. populi, it is in the context of a longer contig, with no repeats present. It is worth noting that another closely related species, B. pratensis, shares synteny with these four but has lost its MATa genes and is classified as heterothallic (HET).

The MAT locus of Wickerhamomyces hampshirensis suggests it has a primary homothallic contiguous (PHC) arrangement. All four canonical MAT genes are found in the middle of a long contig very close to one another with no apparent repeats that would allow the species to flip-flop.

In Wickerhamomyces canadensis, MATa genes are present between full-length SLA2 and DIC1 genes in the middle of large (612 kb) contig, and MATx genes are present on a short (7 kb) contig (Data S2D). The MAT genes are flanked by two sequences that are repeated on the two contigs: a 1.7 kb repeat containing the 3′ end of SLA2 (97% DNA sequence identity between the copies), and a 213 bp repeat containing the 5′ end of DIC1 (100% identity). Both contigs extend beyond these repeats, and in the small contig the available 1 kb of sequence upstream of the truncated SLA2 appears to include a telomere, with multiple tandem repeats of the 11 bp sequence ATGGTGTTCTG (Data S2D). We interpret these data as indicating that W. canadensis is secondary homothallic with an FF2 mechanism and telomeric silencing, because its genomic organization resembles that in K. phaffii, although we do not know whether its MATa and MATx genes are on the same chromosome.

Another possibility is that W. canadensis is secondary homothallic but uses a mechanism other than inversion to exchange DNA between the expression locus (between the complete SLA2 and DIC1 genes) and the silenced locus (beside the telomere). In any case, the existence of repeats flanking the W. canadensis MAT genes shows that it is not primary homothallic. The duplicated SLA2 region maintains an intact open reading frame, unlike the PHN species we report that have SLA2 pseudogenes near a subtelomeric MAT locus. In the closely related Wickerhamomyces sp. NRRL YB-2243 (Data S2D), there is only one set of MAT genes, in the arrangement SLA2–MATx1–MATx2–ORF–DIC1, so Wickerhamomyces sp. NRRL YB-2243 is heterothallic and opposite in mating type at the ancestral MAT locus to the sequenced W. canadensis strain.

The remaining 22 sequenced species in family Phaffomycetaceae either have only MATa genes or have only MATx genes and we conclude they are heterothallic (HET).

CUG-Ser2 clade

4 species: genera Ascoidea and Saccharomycopsis. Ascoidea asiatica has a MAT locus flanked by ADR1 and MHP1 (Data S2A). The genes AGEl and STE20 have become integrated into the allele-specific (Ya/Yx) regions of its MAT locus, similar to the way that extra genes (PIK, PAP, OBP) are integrated into the MAT (MTL) locus of Candida albicans [55]. One of the A. asiatica MAT alleles contains MATa2–AGE1a–STE20a–MATa1, and the other contains AGE1a–MATx1–STE20x. We did not find a MATu2 gene in A. asiatica. The a and x versions of AGE1 and STE20 are in different orientations in the two alleles, with approximately 50% amino acid sequence identity in both of the protein pairs. We classify A. asiatica as a heterothallic diploid (HET).

Ascoidea rubescens (Data S2A) is a flip-flopping species previously described [7]. It has a single MAT region with MATa1–MATa2 and MATx1–MATx2 genes separated by 44 kb of non-coding DNA, flanked by 2-kb inverted repeats that include the 5′ end of the MATa1 gene. One of the copies of the IR is right beside a telomere, so telomeric silencing of transcription is probable. Riley et al. [7] showed that different strains of A. rubescens contain the invertible region in different orientations. We classify A. rubescens as a flip-flopper with one set of inverted repeats (FF1).
Comparison of the two Ascoidea species A. rubescens (FF1) and A. asiatica (HET) reveals some details of how flip/flop switching originated in this genus (Data S2A). In the derived FF1 organization seen in A. rubescens, the MAT genes have moved about 200 kb from the ADR1-AGE1-MHP1 region and are now located beside a telomere and flanked by an IR (Data S2A). Moreover, STE20 has been lost from the A. rubescens genome, and its role as a mating-specific PAK kinase may have been taken over by an A. rubescens-specific duplication of the kinase CLA4. The single AGE1 gene of A. rubescens appears to be derived from an AGE1a gene, because it is more closely related to A. asiatica’s AGE1a than its AGE1x, and in the same orientation relative to MHP1 (Data S2A).

Saccharomyces capsularis has MATx1 and MATx2 genes located between SLA2 and YJR098C in the middle of a large (398 kb) contig, and MATa1 and MATa2 beside a SLA2 pseudogene near one end of a short contig (27 kb) that contains almost no other intact protein-coding genes, from which we conclude that S. capsularis is primary homothallic (PHN). Saccharomyces malanga has only a MATa2 gene, located between SLA2 and YJR098C, and we classify it as heterothallic (HET).

**CUG-Ser1 clade**

93 species: genera Metschnikowia, Hyphopichia, Daniellozyma, Lodderomyces, Spathaspora, Scheffersomyces, Suhomyces, Kodamaea, Aciculococonidium, Teunomyces, Wickerhamia, Priceomyces, Debaromyces, Millerozyma, Yamadazyma, Meyerzyma, Kurtzmania, Babjeviella, Cephaloascus, Babjeviella, and 28 species of the genus Candida.

Two species, both in the CUG-Ser1 clade, were classified as NOMAT because we could not identify any of the four canonical MAT genes in their genomes. These species are *Lodderomyces elongisporus* and *Candida sojae*. The *L. elongisporus* genome was Sanger sequenced and assembled into 11 large scaffolds (145 contigs; contig N50 = 261 kb; accession number AAP001000000.1) by Butler et al. [18], who reported that there are no MAT genes in the sequenced strain. In addition, multiple other *L. elongisporus* isolates were examined by PCR and sequencing, but no MAT genes were identified in them [18, 19]. The *Candida sojae* genome was sequenced by Borelli et al. [20], accession number LMTL00000000.1 (48x coverage Illumina; contig N50 = 56 kb). Using query sequences from its close relative *C. tropicalis*, we were unable to find any MAT genes in the *C. sojae* assembly, and we were also unable to find many of the genes that are normally near the MAT locus in CUG-Ser1 clade species such as orf19.3202, PAP1, RCY1, and orf19.3204. The sequenced strain may therefore have lost a large region of DNA spanning the MAT locus.

*Spathaspora passalidarum* is a species previously discussed in the literature [7, 17]. It has contiguous MAT genes of both types (a1, x1 and x2) close to one another with no repeats indicating the ability to switch mating type. We classify it as a member of the PHC category. (The MATa1 of *S. passalidarum* was overlooked in [7], leading to an incorrect classification as heterothallic. This error was corrected in [17], leading to recognition as PHC.) The assemblies of *Spathaspora hagerdaliae* and *Spathaspora gorwiae* contain both MATa and MATx genes, but they are highly fragmented, and we classify them as heterothallic diploids (HET) as there is no clear evidence for homothallism in these species. Another species from the genus, *Spathaspora arboriae*, has MATa1 and MATa2 genes but no MATx genes [17] and is therefore heterothallic (HET). *Spathaspora girioi* is syntenic with *S. arboriae* at the MATa locus, and its MATx sequence seems to be allelic to MATa, so we infer that *S. girioi* is a heterothallic diploid (HET).

*Scheffersomyces stipitis* has a single MAT locus with contiguous MATa1, MATa2 and MATx1 genes close to each other [17] and no repeats indicating the ability to switch mating type, so we characterize it as primary homothallic contiguous (PHC).

In *Wickerhamia fluorescens*, MATx1 genes are located near PK1, PAP1, and OBP genes, as in other members of the CUG-Ser1 clade. However, it also contains MATa genes 220 kb away, at a site that is probably subtelomeric (33 kb from one end of a 1.6 Mb scaffold), so we classified this species as PHN.

*Candida tropicalis* has MATa and MATx genes on separate contigs [17]. Repeated sequences at the ends of them indicate that they are allelic. We conclude that it is a diploid assembly of a heterothallic species (HET).

The entire Priceomyces genus including *P. haplophilus*, *P. castillae*, *P. medius* and *P. carsonii* has a single MAT locus with contiguous MATa and MATx genes close to one another and shared synteny. We infer that the genus is primary homothallic contiguous (PHC).

The MAT locus of *Debaryomyces Hansenii* was previously described [17] and contains neighboring MATa1, MATa2 and MATx1 genes. The five other sequenced *Debaryomyces* species (*D. maramus*, *D. nepalensis*, *D. prosopidis*, *D. fabryi* and *D. subglobosus*) all have MAT loci syntenic with *D. hansenii*, and with the same gene content. We classify all these species as members of the PHC category.

Two monophyletic species of the genus Yamadazyma (*Y. nakazawae* and *Y. philogaea*) have non-contiguous MATa and MATx loci that are non-allelic. They both have MATx1 and MATx2 genes located near PK1, PAP1, and OBP genes similar to other members of the CUG-Ser1 clade, but they also have MATa1 and MATa2 genes. The MATa genes are located at the ends of large contigs (142 kb in *Y. nakazawae*: 215 kb in *Y. philogaea*), and are close to homologs of the gene CANTEDRAFT_101864, which in the closely related species *Yamadazyma tenuis* (formerly called *Candida tenuis*) is a member of a subtelomeric gene family [56]. Because there are no repeat sequences near the MAT genes of *Y. nakazawae* and *Y. philogaea*, there is no evidence that they can switch mating type, and we conclude they are primary homothallic without non-contiguous MAT genes (PHN).

*Cephaloascus fragrans* has a single MAT locus with contiguous MATa and MATx genes close to one another. We conclude that it is primary homothallic contiguous (PHC).

**Pichiaceae**

61 species: genera *Ambrosiozyma*, *Brettanomyces*, *Citeromyces*, *Dekkera*, *Komagataella*, *Kregervanrija*, *Kuraishia*, *Martiniozyma*, *Ogataea*, *Pichia*, *Saturnispora* and species *Candida arabinofermentans*, *Candida boidinii*, *Candida sorbyoxyla*, and *Candida succiphila*.
The *Pichia kudriavzevii* genome in the Y1000 Project dataset is misassembled at the MAT locus. From our laboratory’s reference genome sequence for this species [57], which is diploid and has both alleles of the MAT locus, we deduce that it is a heterothallic species (HET). *Pichia nakasei, Pichia occidentalis, Pichia membranifaciens,* and *Candida sorboxylosa* all have assemblies with two MAT loci with flanking sequences indicating that they are diploid assemblies of heterothallic species (HET).

The assemblies of MAT regions of *Saturnispora* species are fairly fragmented. By manually merging a few contigs of the assembly of *Saturnispora zaruensis* (Figure 3B), we conclude that it is a flip-flopper with two sets of repeats (inner and outer) around its two MAT loci (FF2). There is a centromere in the region between the two MAT loci, which is an arrangement similar to that of *Komagataella phaffii* [13, 27] (Figure 3A). However, *S. zaruensis* differs from *K. phaffii* in that (i) the centromere is very close to one set of MAT genes, and (ii) none of the MAT genes are close to a telomere. We propose that proximity to the centromere is the mechanism used by *S. zaruensis* to repress transcription of the silent MAT locus, and that the entire 194-kb region between the two MAT loci inverts during switching, by recombination between the inner pair of repeats. Under this hypothesis, the inner IR is necessary for switching, while the function of the outer IR remains unknown, which is the opposite of what we know about the IRs in switching, by recombination between the inner pair of repeats. Under this hypothesis, the inner IR is necessary for switching, and (ii) none of the MAT loci (FF2). There is a centromere in the region between the two *Saturnispora zaruensis* species (HET).

The published assembly of the *Kregervanrija fluxuum* genome [6] was assembled using the SPAdes assembler. It has a single MAT scaffold with MATa1-MATa2 and MATa1-MATa2 genes, respectively, at opposite ends of a 12 kb contig that contains no other genes. No repeats are present. However, in an independent DISCOVAR assembly of the same *Kregervanrija fluxuum* Illumina data, the 12 kb region is flanked by two identical 15-kb sequences that form a large IR extending to the ends of the contig (Data S2C). This situation resembles the DISCOVAR assemblies of the *Cyberlindnera saturnus* and *Starmera quercuum* genomes, and we believe that, in each case, an IR is present (i.e., it is an FF1 species) but the length of the IR has been artificially inflated by DISCOVAR.

*Kregervanrija delfensis* (SPAdes assembly) has an identical MAT locus organization to *K. fluxuum*. We conclude that *K. delfensis*, and *K. fluxuum* are both FF1 secondary homothallic species.

*Ambrosiozyma ambrosiae* and *A. philentoma* each have two MAT loci (a and z) that are not contiguous. In both species, the MATa genes are in the ancestral context SLA2–MATa1–MATa2–DIC1–ASA1, and the MATz genes are in the non-ancestral context YLR001C–IZH3–MATz1–MATz2–EFM5. There are no flanking sequences that would indicate that they are allelic, and no repeat sequences that would indicate a mating-type switching mechanism. Both of the MAT loci in both species are in the middle of large contigs and therefore not subtelomeric. There is a large gene-free region between the MATz genes and the next gene, IZH3 (11 kb in *A. ambrosiae*, 19 kb in *A. philentoma*). In *Saccharomyces cerevisiae*, YLR001C and EFM5 are genes located immediately next to centromeres. We infer that *A. ambrosiae* and *A. philentoma* are primary homothallic non-contiguous (PHN).

*Ambrosiozyma oregonesis* has two MAT loci (a and z) on very short contigs with flanking repeated sequences around them. We infer that it is a diploid assembly of a heterothallic species (HET).

*Brettanomyces anomalus* has two MAT loci that have flanking sequences suggesting they are alleles of each other. We conclude that it is a diploid assembly of a heterothallic species (HET).

Only 5 species in the *Ogataea* genus clade were classified as heterothallic (HET): *O. methylivorae,* *O. ramenticola,* *Candida succiphila,* *O. nitratovaersa,* and *Candida arabinofermentans.*

*Ogataea naganishii* (PHC) has the gene organization SLA2–MATa1–MATa2–MATz1–MATz2–DIC1. There are no gaps in the assembly in this region, and no repeated sequences that would suggest the ability to flip-flop. The MATa2 and MATz1 genes are separated by 7 kb of noncoding DNA. We conclude that it is primary homothallic contiguous (PHC).

12-species *Ogataea* FF1 clade: *Ogataea polymorpha,* *O. parapolymorpha,* and *O. nonfermentans* have contiguous MATa and MATz genes in a flip-flop-like arrangement with an inverted repeat (part of the SLA2 gene) at the ends. We infer they are secondary homothallic flip-flopers with a single IR (FF1). Switching by inversion in *O. polymorpha* has been reported [13, 14]. *Ogataea philodendri,* *O. kodamae,* *O. minuta,* *O. henricii,* *O. pinii,* *O. glycozyma,* *O. zsoliti,* *O. trehaloaestinensis,* and *O. populiaibaevae* have broken assemblies at their MAT loci, but they share synteny with *Ogataea polymorpha* and we conclude they are also homothallic flip-flopers with a single IR (FF1). Switching in *O. minuta* has been reported [26].

*Ogataea trehalophila* and *O. methanolica* form a pair of sister FF1 species. They each have non-contiguous MATa and MATz loci, that are flanked by a repeated sequence derived from SLA2. We infer that these two species also use a flip/flop switching mechanism (FF1), but the invertible region appears to be much larger than in *O. polymorpha* (several hundred kb). The *O. methanolica* assembly contains two very large duplications, one of a 23-kb region that ends at SLA2, and one of an 8-kb region, but we suspect that the length of these duplications may be artifactually inflated, because the *O. methanolica* genome was sequenced using a library with DNA inserts approximately 3 kb long [6], so in principle it is unlikely that identical repeat sequences, 23 kb or 8 kb long, as are present in the assembly (AbySS assembler), were correctly resolved.
Ogataea pilisensis (FF1) has a 7-kb contig that contains genes SLA2-SU11-CWC25 and is present at 2x sequence coverage compared to the rest of the assembly. The 3’ end of SLA2 overlaps with the ends of two other contigs, one containing MATα-MATα and the other containing MATα1-MATα2. We therefore classified O. pilisensis as a secondary homothallic (FF1) with a 7-kb IR. Ogataea nitroaversa and Candida arabinofe rentans are closely related to O. pilisensis and show conserved synteny with it at the MAT locus, but they both appear to be heterothallic (HET). O. nitroaversa contains only MATα1-α2 genes, and C. arabinofe rentans contains only MATα1-α2 genes [7], located in both cases between SLA2 and DIC1.

The FF1 species of Ogataea described above form three distinct clades within the genus. There are two equally parsimonious hypotheses about the transitions that led to this situation (either three independent gains of FF1, or one ancestral gain, followed by two losses). We conclude that three gains of FF1 is a more probable explanation, based on the gene orders that exist at the MAT loci. The main reason is that the region between the IRs is very different (in size and endpoints) among the three FF1 clades of Ogataea, so if the whole genus Ogataea was ancestrally FF1, it would be necessary to also postulate multiple reorganizations of the FF1 system within the genus. Specifically:

In the O. polymorpha FF1 group (12 species) the invertible region between the IRs is only 19 kb long, with DIC1 at one end and TPK3 near the other end.

In the pair of FF1 species O. trehalophila and O. methanolica, the invertible region is much larger (> 416 kb and > 700 kb, respectively), with a GCN4-like gene at one end and REC102 at the other end, in both species, and an IR that terminates in SLA2. Candida arabinofe rentans is a heterothallic outgroup to these two species, but the C. arabinofe rentans MAT locus is found in the context SLA2-MAT-DIC1 which is an ancestral and commonly observed context. If the heterothallism of C. arabinofe rentans was a derived situation (i.e., an FF1 → HET transition from an ancestor resembling O. trehalophila/O. methanolica), we would have expected its MAT locus to be in the context SLA2-MAT-GCN4 or SLA2-MAT-REC102. It is more parsimonious to propose that the C. arabinofe rentans arrangement is ancestral, and the O. trehalophila/O. methanolica arrangement is derived (i.e., a HET → FF1 transition).

Similarly, the FF1 species O. pilisensis has a large invertible region of at least 159 kb, with SLA2-MAT1-DIC1 at one end and SLA2-MAT2-LRS4 at the other, where SLA2 forms part of the IR. Its sister heterothallic species O. nitroaversa has the ancestral context SLA2-MAT-DIC1. Since the arrangement SLA2-MAT-LRS4 is not seen in any other species, either (i) this arrangement originated de novo when an FF1 system emerged in O. pilisensis, after it had diverged from O. nitroaversa (i.e., HET → FF1 transition); or (ii) this arrangement originated in O. pilisensis by reorganization of one endpoint of an existing FF1 system, and separately O. nitroaversa lost one of its two MAT loci, which happened to be the one at the non-ancestral location (i.e., FF1 → HET transition). We consider the first scenario to be more parsimonious.

Kuraishia molischiana has a MATα locus that is syntenic with the single MAT locus of the heterothallic species K. capsulata, but K. molisciana also has a MATα1 at one end of a large (458 kb) contig. The MATα1 gene is located beside a badly degraded pseudogene of SLA2. There are no repeats around the MAT genes, so we infer that K. molisciana is primary homothallic with non-contiguous MAT loci (PHN).

Citeromyces matritensis has two non-contiguous MAT loci (α and γ) with flanking sequences suggesting that it is a diploid assembly of a heterothallic species (HET). Citeromyces hawaiiensis partly shares synteny with it, but is missing the flanking sequences, possibly due to the contigs being broken at them. Citeromyces siamensis has a more fragmented assembly at the MAT loci. We conclude that Citeromyces hawaiiensis and C. siamensis are both diploid assemblies of heterothallic species (HET).

Komagataella phaffii (formerly called Pichia pastoris) is a flip-flopping secondary homothallic species with two sets of inverted repeats (FF2) around both the MATα and the MATα genes [13] (Figure 3A). The invertible region is approximately 138 kb. The assemblies of Komagataella pseudopastor and K. populi are broken around their two MAT loci, but flanking repeats are present. From their phylogenetic relationship with K. phaffii, we infer they are also secondary homothallic species using the flip-flopping system with two sets of inverted repeats (FF2).

CUG- Ala clade

5 species: genera Peterozyma and Nakazawaeae, and species Pachysolen tannophilus. Peterozyma xylosa and Peterozyma toletana (Data S2B) were classified as primary homothallic non-contiguous (PHN). Both of these species contain both MATα and MATα genes, at non-contiguous sites. Their MATα genes are located between SLA2 and SPC3, syntenic with the MAT loci of several heterothallic Pichiaceae species. These MATα genes are approximately 100 kb from the rDNA array. The MATα genes are present on the opposite side of the rDNA array, immediately beside the rDNA. We classified these Peterozyma species as PHN because there are no repeat sequences that could catalyze exchange between the MATα and MATα loci.

Nakazawaeae pelata and Nakazawaeae holstii both have only a MATα locus. We conclude that they are heterothallic (HET).

Pachysolen tannophilus is a species whose MATα locus was previously described [7]. It has a single invertible 8-kb-long MAT region with MATα and MATα genes at its ends, flanked by 2-kb inverted repeats (Data S2B). Riley et al. [7] experimentally confirmed that the region can be induced to invert by growth of a culture of P. tannophilus in nitrogen-depleted media. However, it is unclear how orientation-specific silencing of one of the two MAT genes is achieved in this species [7, 17]. We classify it as a member of the FF1 category.

Sporopachydermida

2 species: Sporopachydermia quercuum and S. lactativiora. Sporopachydermia quercuum has contiguous MATα and MATα genes very close to each other and does not have inverted repeats around them. We classify it as primary homothallic contiguous (PHC). Sporopachydermia lactativiora has only MATα genes and therefore is heterothallic (HET).
Dipodascaceae/Trichomonascaceae

37 species: genera Arxula, Blastobotrys, Deakozya, Diddensiella, Dipodascus, Nadsonia, Geotrichum, Groenewaldozyma, Magnusiomyces, Middelhovenomyces, Saprochaete, Spencermartinsiella, Starmerella, Sugiyamaella, Wickerhamiella, Yarrowia, Zygoascus; and species Candida hispaniensis and Candida incommissus.

Starmerella bombicola is classified as HET. The genome of the type strain of this species (JCM 9596 / NRRL Y-17069 / NBRC 10243) has been sequenced three times, including a high contiguity genome sequence by RIKEN (contig N₉₀ = 2.9 Mb) [5]. It contains an ORF with no identifiable domains, in the context SLA2-ORF-TFC1. The corresponding genomic region in two closely related species (Candida apicola and Wickerhamiella domercqiae) contains the series of genes SLA2-MATx1-MFₓ-TFC1, where MFₓ is the gene for alpha-pheromone (which is not commonly present at the MAT locus of ascomycetes). Similarly, a second strain of S. bombicola (PYCC 5882) contains the series SLA2-MATx1-MFₓ at the end of one contig (PEOC01000491 [58]), though TFC1 is elsewhere in the genome. It is therefore possible that the ORF in S. bombicola codes for a determinant of MATa mating type, but we are unable to detect any sequence relationship between it and known a₁ or a₂ proteins. We classified S. bombicola as heterothallic (HET) on the basis of the MATx1 gene present in strain PYCC 5882.

Blastobotrys proliferans has a MATx1 gene located between full-length SLA2 and APN2 genes, syntenic with the single MAT locus in other Blastobotrys species such as B. adeninivorans [59]. The MATx2 gene is absent throughout this genus. However, B. proliferans also has a MATa2 gene, located between pseudogenes of SLA2 and APN2, at a site that appears to be subtelomeric. The site is at the end of a 280 kb contig, and near a homolog of the S. cerevisiae gene SGS1, which is repeated on the subtelomeric regions of the well-assembled B. adeninivorans genome [59]. We infer that B. proliferans is primary homothallic non-contiguous (PHN).

Nadsonia fulvescens var. elongata has separate MATa and MATx loci that are not allelic, and there are no repeats that would constitute evidence of the ability to switch mating type. The MATa genes are near a telomere (Data S2F). We conclude that it is primary homothallic non-contiguous (PHN).

Trigonopsidaceae

6 species: genera Botryozya, Tortispora and Trigonopsis. The genome assembly of Botryozya nematodaphila consists of 17.3 Mb in almost 11,000 scaffolds with average nuclear coverage only 4x and N₅₀ = 2575 bp. This high level of fragmentation makes synteny analysis impossible. However, we detected part of a MATx2 gene downstream of the 3' end of SLA2 in this species, so we tentatively classified B. nematodaphila as heterothallic (HET).

Tortispora ganteri has MATa and MATx genes on contigs with repeated flanking sequences, suggesting that these are allelic. We conclude that it is a diploid assembly of a heterothallic species (HET). The two other Tortispora species studied were also classified as haploid heterothallics (HET), with only MATx genes in T. caseinolytica [7], and only MATa genes in T. starmeri.

Trigonopsis variabilis has only MATa genes, and Trigonopsis vinaria has only MATx genes, so we classified these two species as heterothallic (HET).

Lipomyctaceae

9 species: genus Lipomyces. The MAT loci of all the sequenced species in the genus Lipomyces are illustrated in Data S2F and discussed below. In addition to the canonical MAT genes, some Lipomyces species have an extra gene at their MAT locus, coding for an HMG domain protein (i.e., a DNA-binding protein distantly related to the a₂ and a₁ proteins) whose function is unknown [7]. We refer to this gene here as HMGX. It is present in 6 of the 9 sequenced Lipomyces species (Data S2F).

Lipomyces starkeyi, L. arxii, L. mesembrius, and L. kononenkoe form a 4-species clade that we classified as PHC. Each of them has all four canonical MAT genes as direct neighbors of one another at a single genomic site (Data S2F). This site appears to be the ancestral MAT site, as it is situated between the gene SLA2 on one side and APN2 and MLH3 on the other. These four species have the HMGX gene, whose location in the MAT locus is conserved among L. starkeyi, L. arxii and L. mesembrius. However, in L. kononenkoe HMGX is at a different location in the genome, away from the MAT region. For all four species we conclude that they belong in the PHC category.

The fairly fragmented assembly of Lipomyces doorenjorgii (Data S2F) has two short contigs, containing a MATa locus (a₁ and a₂ genes) and a MATx locus (a₁ and a₂ genes) respectively. By manually identifying their overlaps with other contigs, we infer they are parts of a diploid assembly, and that L. doorenjorgii is a heterothallic diploid whose MAT locus is at the ancestral location between SLA2 and MAM3 on one side, and MLH3 on the other. Coverage data show that one of the contigs (NODE_122) has double coverage and can also be connected to a different contig (NODE_120), which is not ancestrally at a MAT-related position (Data S2F). The presence of NODE_120 raises the possibility that there could be a second MAT-like locus in the genome. Nevertheless, we decided to classify this species as HET (rather than PHN), making it the only heterothallic genome in the genus Lipomyces and one of the few (three) examples of an evolutionary transition toward heterothallism. This decision is conservative, because if L. doorenjorgii is PHN, the ratio between transitions toward and away from homothallism would be 31:2 rather than the 31:3 ratio we report.

Lipomyces japonicus has two sets of MAT genes, one located at the ancestral position (between SLA2 and APN2–MLH3), and the other located on a different contig (Data S2F). The two MAT loci must be at least 28 kb apart in the genome. The ancestral site contains MATa1, MATa2, and HMGX, while the non-ancestral site contains MATx1, MATx2 and another HMGX gene (46% amino acid sequence identity between the two HMGX genes). The contig (23 kb) containing the non-ancestral site contains no other genes apart
from the MAT\textsubscript{a} genes and HMGX, except for some pseudogenes of retrotransposons and transposases, so it may be heterochromatic but we have no indication that it is subtelomeric. We classify \textit{L. japonicus} as a member of the PHN category.

\textit{Lipomyces lipofer} has a single MAT site containing \textit{MAT\textsubscript{a}1, MAT\textsubscript{a}2, MAT\textsubscript{a}1, MAT\textsubscript{a}2}, and a homolog of \textit{HMGX}. Although both \textit{SLA2} and \textit{APN2} are located at other genomic loci, \textit{MLH3} is present in the neighborhood of the MAT genes, and so are some other genes (for example an RNA-dependent RNA polymerase (RdRP) on one side and \textit{FOL3} on the other) associated with the ancestral MAT locus. We classify this species as a member of the PHC category.

\textit{Lipomyces suomiensis} has two MAT loci. One is at the ancestral location, between \textit{MLH3} and \textit{SLA2} on one side and \textit{APN2} on the other, and contains \textit{MAT\textsubscript{a}1} and \textit{MAT\textsubscript{a}2}. The second MAT location contains \textit{MAT\textsubscript{a}1} and \textit{MAT\textsubscript{a}2} flanked by pseudogenes of \textit{SLA2} and \textit{APN2}. We infer that this second location is subtelomeric because the MAT\textsubscript{a} genes are 15 kb from one end of a contig, which terminates in multiple tandem copies of the sequence (TTTTAGTAGGG)\textsubscript{n} which resembles known yeast telomeres [60] and also occurs at the ends of several very large contigs in the \textit{L. suomiensis} assembly. The MAT\textsubscript{a} genes are in the center of a 27 kb region that contains no other intact genes. \textit{L. suomiensis} has no HMGX genes. We conclude that \textit{L. suomiensis} belongs in the PHN category.

\textit{L. oligophaga} forms a clade with \textit{L. suomiensis} and also has two MAT loci, one ancestral and one subtelomeric. However, the genotypes of the two MAT loci in \textit{L. oligophaga} are the opposites of those in \textit{L. suomiensis} (Data S2F). In \textit{L. oligophaga}, the ancestral MAT locus has the arrangement \textit{MLH3–SLA2–MAT\textsubscript{a}1–APN2}. We were unable to detect any MAT\textsubscript{a}2 gene in this species. The second MAT locus in \textit{L. oligophaga}, containing \textit{MAT\textsubscript{a}1} and \textit{MAT\textsubscript{a}2} genes, is at one end of a 547 kb contig that terminates in multiple tandem copies of a telomeric repeat (TTTGAGGG)\textsubscript{n} which also occurs at the ends of other large contigs in this species. The distance between the end of the \textit{MAT\textsubscript{a}2} gene and the first telomeric repeat is only 47 bp. \textit{L. oligophaga} has no HMGX genes. We conclude that \textit{L. oligophaga} belongs in the PHN category.

We infer that the genus \textit{Lipomyces} is ancestrally PHC, and that there have been two transitions to PHN, and one to HET, in this genus as explained below.

Because \textit{L. lipofer} and the 4-species \textit{L. starkeyi} clade have all four canonical MAT genes (\textit{a1, a2, a1, a2}) present at the ancestral MAT site, we infer that the most recent ancestor of the 7-species clade that includes these five also had this gene arrangement, and therefore would have been classified as PHC. Therefore, we infer a PHC \rightarrow PHN transition in \textit{L. japonicus}, and a PHC \rightarrow HET transition in \textit{L. doorenjongii}.

We hypothesize that it may be easier to undergo a transition from PHC to PHN than the other way round, because the situation in which some genes are removed from the ancestral MAT locus to some other part of the genome is more likely than one in which genes from two separated MAT loci become co-localized at a single site (the ancestral one). Another reason is that we already see such a transition in \textit{L. japonicus}. Therefore we classify the ancestor of the \textit{Lipomyces} genus as PHC, and infer a PHC \rightarrow PHN transition in the common ancestor of \textit{L. oligophaga} and \textit{L. suomiensis}. However, \textit{L. oligophaga} and \textit{L. suomiensis} have opposite alleles at the ancestral MAT locus, so it is possible that each of them represents a separate PHC \rightarrow PHN transition, but for reasons of parsimony we inferred a single transition in the common ancestor of this pair of species.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The data analyses reported in this manuscript do not use any statistical tests.

**DATA AND CODE AVAILABILITY**

Automated and manual sequence similarity searches were performed using TBLASTN [40]. The Python code used for automating the searches is available at GitHub at https://github.com/tadekkr/Multiple-reinventions-of-mating-type-switching-during-budding-yeast-evolution. Phylogenetic trees in Data S2 were made using Seaview [41].