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Minireview

MINIREVIEW

Insights on life cycle and cell identity regulatory circuits for unlocking genetic improvement in *Zygosaccharomyces* and *Kluyveromyces* yeasts

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One sentence summary: Understanding mating-type regulatory circuits in nontraditional yeasts will accelerate the development of new hybrid strains for biotechnology.

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ABSTRACT

Evolution has provided a vast diversity of yeasts that play fundamental roles in nature and society. This diversity is not limited to genotypically homogeneous species with natural interspecies hybrids and allodiploids that blur species boundaries frequently isolated. Thus, life cycle and the nature of breeding systems have profound effects on genome variation, shaping heterozygosity, genotype diversity and ploidy level. The apparent enrichment of hybrids in industry-related environments suggests that hybridization provides an adaptive route against stressors and creates interest in developing new hybrids for biotechnological uses. For example, in the *Saccharomyces* genus where regulatory circuits controlling cell identity, mating competence and meiosis commitment have been extensively studied, this body of knowledge is being used to combine interesting traits into synthetic F1 hybrids, to bypass F1 hybrid sterility and to dissect complex phenotypes by bulk segregant analysis. Although these aspects are less known in other industrially promising yeasts, advances in whole-genome sequencing and analysis are changing this and new insights are being gained, especially in the food-associated genera *Zygosaccharomyces* and *Kluyveromyces*. We discuss this new knowledge and highlight how deciphering cell identity circuits in these lineages will contribute significantly to identify the genetic determinants underpinning complex phenotypes and open new avenues for breeding programmes.

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INTRODUCTION

A wide range of different yeasts are naturally associated with foods, both as active agents of food fermentations and as spoilage organisms. Of course, *Saccharomyces cerevisiae* attracts the most attention because of its capacity to ferment sugars to ethanol (Dasko *et al.* 2014), but a plethora of other species are critically important for production of fermented dairy, vegetable and meat products (Hittinger, Steele and Ryder 2018). In many cases, yeasts involved in producing fermented foods, or indeed in spoiling foods, have unique traits with regard to tolerance to particular stress, utilization of specific sugars or physiological adaptions to their environment (Rodicio and Heinisch 2013; Hittinger, Steele and Ryder 2018). Advances in strain engineering, synthetic biology and whole-genome sequencing offer opportunities to understand yeast biodiversity—and to exploit it for the development of new strains for food or industrial biotechnology (Riley *et al.* 2016; Deaner and Alper 2019; Daran 2020; Libkind *et al.* 2020). More specifically, there are exciting developments and increased interest in yeast hybrids—both naturally occurring ones and synthetic hybrids that can be constructed in the laboratory (Van De Peer, Mizrachi and Marchal 2017; Peris *et al.* 2020; Naseeb *et al.* 2021). Hybrids are not a new phenomenon and hybrid organisms have played a major role in the development of human civilization, most notably when emergence of hybrid wheat varieties ~6500 years BCE transformed agriculture, and they continue to do so through managed plant breeding programmes (Venske *et al.* 2019; de Sousa *et al.* 2021). Yeast hybrids have also made a major contribution to human society, most famously through the lager yeast *Saccharomyces pastorianus*, which is a hybrid between *S. cerevisiae* and *Saccharomyces eubayanus* (Libkind *et al.* 2011). In fact, there are many natural hybrids formed by yeasts in the *Saccharomyces* genus, and these are now a major focus of research, especially with the aim of producing new strains for the beverage sector (Mertens *et al.* 2015; Gibson *et al.* 2017; Naseeb *et al.* 2021). More recently, it has been discovered that hybrids are quite common in other food-associated yeast, for example, in *Kluyveromyces marxianus*, which is associated with fermented dairy products (Lane *et al.* 2011); *Zygosaccharomyces rouxii*, used in the manufacture of soy sauce and balsamic vinegar (Solieri and Giudici 2008; Devanthi and Gkatzionis 2019); and *Zygosaccharomyces bailii* (Kuanyshev *et al.* 2016), often responsible for food spoilage under low pH conditions. In many cases, hybrid strains display features of heterosis and hybrid vigour, which can be beneficial and that may have been selected by domestication (Birchler, Yao and Chudalayandi 2006; Shapira *et al.* 2014; Bernardes, Stelkens and Greig 2017). By studying natural hybrids, we can try to understand the genetic and molecular processes that allow hybrid formation and fertility restoration, when two different organisms breach the species barrier to create a new species. This offers insights into evolution, enables the use of genetics to study traits and creates opportunities to develop new hybrids for specific applications. Sex is fundamental to formation of hybrid species since it is the process by which two different cells come together to form one entity. For this to occur, individual cells must identify as being of a specific sex, gender or mating type, and recognize a cell with the corresponding opposite identity. Cellular identity has been extensively investigated in *S. cerevisiae* (Madhani 2007) since it underlies classical genetics but far less is known in other yeasts. Over the past two decades, knowledge of other systems has advanced incrementally but the advent of whole-genome sequencing has revolutionized the field and huge advances have been made in the past five years. In this review, we focus on species in the *Kluyveromyces* and *Zygosaccharomyces* genera and discuss recent developments that offer insights into the natural ecology and biotechnological potential of these yeasts. This is put in context with explanations of comparable systems in *S. cerevisiae*, and similarities and differences are highlighted.

LIFE CYCLE OF THE SACCHAROMYCOTINA

The Saccharomycotina, also known as the budding yeast subphylum, contains many species of biomedical and biotechnological importance (Dujon and Louis 2017). As would be expected in a subphylum that emerged approximately one billion years ago, a huge amount of diversity, encompassing strains with very distinctive characteristics, is present. A recent comprehensive phylogeny based on whole-genome sequence analysis of 332 individual strains that divided the subphylum into twelve major clades (families) is proving very valuable to study yeast evolution (Shen *et al.* 2018). Much of what is known about this group of fungi comes from research with *S. cerevisiae*, though as more insights are gained from genomic and molecular studies, it becomes apparent that often the canonical species has evolved highly specialized features that are not representative of budding yeasts as a whole. This variation is increasingly relevant as we seek to use diverse yeasts for applications in the food, beverage and biotechnology sectors (Hittinger, Steele and Ryder 2018). Somewhat ironically, many of these yeasts that are described as 'nonconventional yeasts' (NCY) are more 'typical' than *S. cerevisiae*, though it is also important to recognize that each family, genus and species has its own unique traits. Indeed, that is what makes them interesting and attractive.

Ascomycetous budding yeasts are sexual organisms and possess cells of two distinct mating types (Herskowitz 1988; Madhani 2007). Cells of the opposite sex can mate to form a diploid, which then undergoes meiosis to produce haploid spores that germinate to produce vegetative cells that are designated **a** or α. Although the ancestral state is heterothallism, where each haploid vegetative culture maintains a stable cell (mating) type, studies investigating the evolutionary history of mating-type systems in budding yeast found that a conversion to homothallism is quite widespread and appears favoured in some cases (Krassowski *et al.* 2019). In homothallic species, cells can self-mate, either because each cell does not express a distinctive mating type (primary homothallism) or because a cell can switch from one mating type to the other (secondary homothallism) (Wilson *et al.* 2015). Of the 332 strains used to create the Saccharomycotina phylogeny (Shen *et al.* 2018), 140 were homothallic and the transition to homothallism independently occurred multiple times (Krassowski *et al.* 2019). Suggested benefits of homothallism include diploidization and the assurance of reproduction, the enabling of DNA repair and genomes renewal, as well as evasion of early cell death in case of too early germination of spores in unsuitable environment (Mortimer *et al.* 1994;

Knop 2006; Giraud *et al.* 2008; Hanson, Byrne and Wolfe 2014; Magwene 2014; Nieuwenhuis and Immler 2016; Krassowski *et al.* 2019).

SEXUAL CYCLE IN THE SACCHAROMYCETACEAE

Within the budding yeast, the family Saccharomycetaceae exhibits a specific form of secondary homothallism known as the 3-locus (3LOC) system (Krassowski *et al.* 2019). Sixty-six of seventy-one species assessed in this clade possess three mating-type (*MAT*) loci, one of which is actively expressed and two of which are transcriptionally silenced. The silenced loci provide the templates to switch the active locus between the *MAT***a** and the *MAT*α forms, thereby also switching the cells between the **a** and α cell types. The Saccharomycetaceae is of special interest because it contains multiple yeast genera that are important for food and biotechnology, including *Saccharomyces*, *Zygosaccharomyces*, *Torulaspora*, *Lachancea* and *Kluyveromyces* (Fig. 1). As our knowledge of the biology of these yeasts and our desire to develop new applications increases, it transpires that sex has been a powerful driver in the evolutionary history of human-associated species and is a very useful tool for the development of new strains for biotechnology.

We have a comprehensive understanding of the sexual cycle and cell types in the canonical *S. cerevisiae*, which is described as having a haplo-diplontic life cycle as all three cell types—haploid **a** or α, or diploid **a**/α, can grow mitotically under the right conditions (Fig. 2) (Fabre *et al.* 2005; Merlini, Dudin and Martin 2013; Hanson and Wolfe 2017). Diploids proliferate mitotically under nutrient rich conditions and undergo meiosis when encountering nutrient starvation. Subsequent sporulation results in tetrads of four haploid spores enclosed within an ascus, which can germinate and mate with each other to reestablish the diploid state when environmental conditions become favourable. Mating can occur only between haploid cells of the opposite mating type (Haber 2012), but this can be within same ascus (a type of inbreeding called intra-tetrad mating or automixis), from different asci of the same lineage (referred to as inter-tetrad mating or amphimixis), or between haploids cells from divergent lineages (outcrossing) (Fig. 2). Alternatively, spores can germinate and grow as haplonts, though, given opportunity, these will mate with cells of the opposite mating type and the diplontic form of growth is preferred. As *S. cerevisiae* practises mating-type switching (secondary homothallism), the change of mating type gives rise to compatible cells within the same colony (referred to as haplo-selfing) (Wilson *et al.* 2015). Although this description implies that in nature *S. cerevisiae* cells would almost always exist as homozygous diploids, recent analysis of wild *S. cerevisiae* populations suggests that natural variation and environmental conditions may have an impact with frequencies of heterozygosity and outcrossing higher than hitherto appreciated (Fisher, Liti and Llorente 2021). The *Saccharomyces* preference for diplontic growth is an evolved trait whereas most other yeasts in the Saccharomycetaceae ordinarily grown as haplonts (Gerstein *et al.* 2006; Sherwood *et al.* 2014). In these yeasts, as a response to nutrient limitation, haploid **a** and α cells mate to form an **a**/α diploid, but then, rather than growing mitotically, meiosis occurs immediately, generating haploid spores (Fig. 2) (Barsoum, Rajaei and Aström 2011; Bizzarri, Cassanelli and Solieri 2018). Thus, in both haplontic and diplontic yeasts, nutrient starvation is the stimulus for sporulation and the main biological role could be seen as a mechanism to generate robust spores to survive unfavourable environmental conditions (Hanson, Byrne and Wolfe 2017).

MATING-TYPE LOCI AND GENETIC SWITCHING

Mating-type loci in yeast are analogous to sex chromosomes that define sexuality of multicellular eukaryotes as they govern cell identity, establish mating competence and determine the spectrum of permissible partners for mating (Billiard *et al.* 2012). In the Saccharomycetaceae, the system comprises one expressed *MAT* locus and two silent (cryptic) loci termed *HML* (Hidden MAT Left) and *HMR* (Hidden MAT Right) loci (Fig. 1B). This 'left' and 'right' naming convention comes from *S. cerevisiae* where *MAT*, *HML* and *HMR* are all located on chromosome III (Haber 2012) although, in many other cases, including both *Kluyveromyces* and *Zygosaccharomyces*, *HMR***a** is located on a different chromosome to *MAT* and *HML*α (Fabre *et al.* 2005). Epigenetic silencing of the *HMR*/*HML* loci is a complex mechanism that requires the recruitment of the Sir complex and the presence of *cis*-acting silencers to assure that only the *MAT* locus is functionally active (Farris, Saxton and Rine 2021). The *MAT* locus contains one of two idiomorphs that comprises three regions Z, Y and X. For any given species, the Z and X regions are shared by the *MAT*, *HML* and *HMR* loci whereas idiomorph-specific versions of the Y region, Y**a** and Yα distinguish the *MAT***a** and *MAT*α loci and determine cell mating type. Mating-type switching in *S. cerevisiae* is very well understood and has been the subject of many comprehensive reviews so the more complex nuances will not be described in detail here (Haber 2012; Thon *et al.* 2019). The underlying principle is the same in all 3LOC species; namely, switching occurs when the Y region in the *MAT* locus is replaced by a copy of the Y region from either the *HML* or the *HMR* locus (Fig. 1C). This is a classic gene conversion event that is driven by the homology between the X and Z regions of the loci. The main differences between species arise in the frequency of switching and in the contribution of site-specific endonucleases.

In *S. cerevisiae* and some other species, a site-specific endonuclease, HO, introduces a DNA double-strand break (DSB) at the Y–Z junction of the *MAT* locus, starting the gene conversion process that uses the silent *HML* or *HMR* locus as a donor sequence. *Zygosaccharomyces* also carries *HO* and thus uses HO endonuclease to initiate switching but species in the earlier branching *Kluyveromyces/Lachancea/Eremothecium* (KLE) clade do not (Strathern *et al.* 1982). The evolutionary history of *HO* was recently resolved and it is proposed that *HO* is a variant of a class of genetic homing elements termed *WHO* elements (Coughlan *et al.* 2020). These mobile genetic elements began life as bacterial inteins, elements that can insert themselves into specific genes ('homing'), and entered the yeast genome by invading the *VMA1* gene to become a yeast intein termed *VDE1*. Later, *VDE1* 'mishomed' into a zinc finger protein that is the common ancestor of the *WHO* elements and *HO*. At some point *HO* changed target and was essentially repurposed or domesticated to facilitate more efficient mating-type switching. The event leading to formation of *HO* occurred post the divergence from the KLE clade and thus species in the KLE clade can possess *WHO*-like elements but lack *HO*. The presence of *WHO*-like genes or pseudogenes is probably the reason for earlier incorrect suggestions that a nonfunctional *HO* relic was present in *K. lactis* (Fabre *et al.* 2005). For mating-type switching to fulfil its core purpose of allowing self-mating, the entire process needs to be tightly controlled (Rusche and Rine 2010; Hanson and Wolfe 2017; Haber

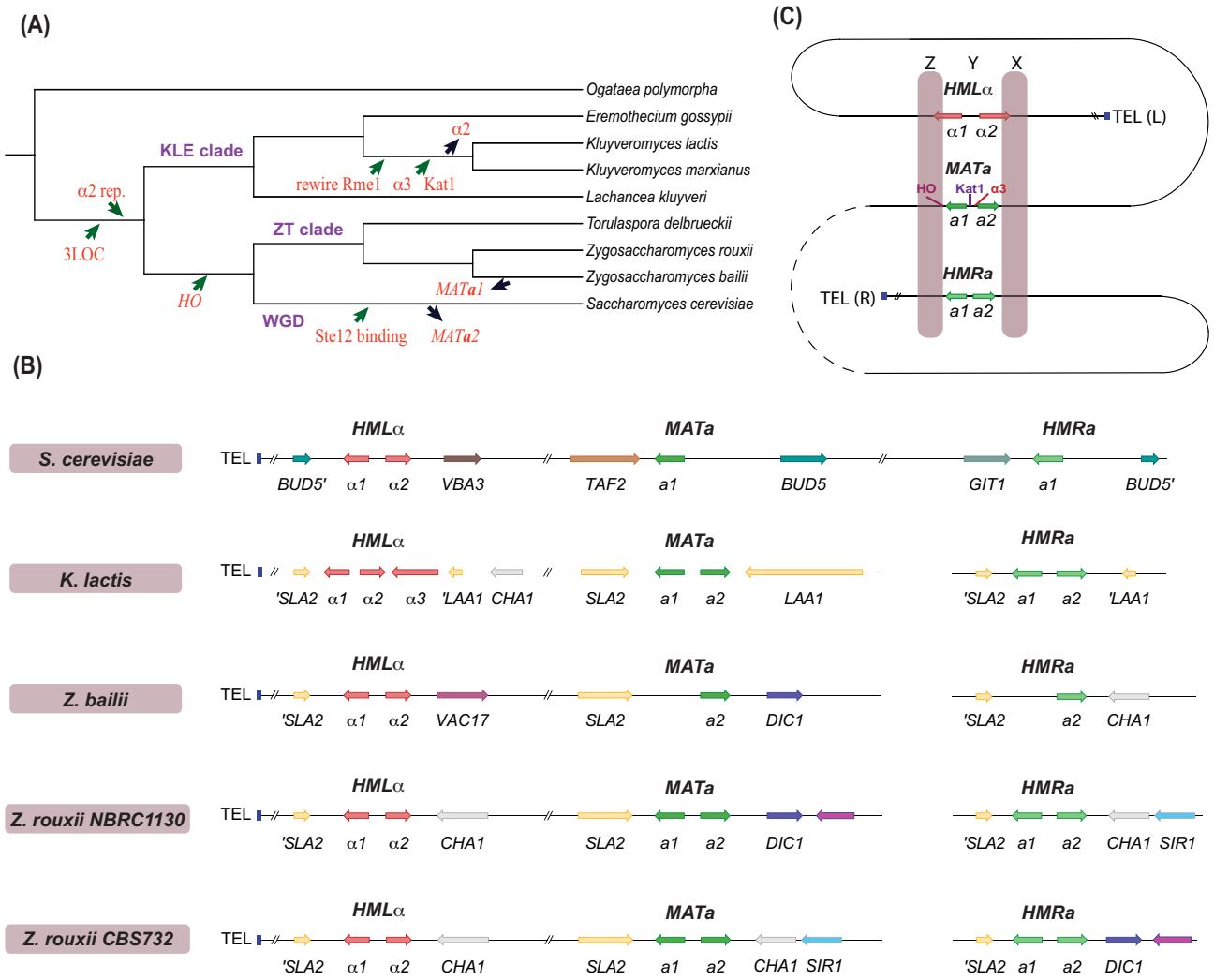

Figure 1. Evolution of mating-type systems in the Saccharomycetaceae. **(A)** The phylogeny of selected species in the family Saccharomycetaceae is shown with key evolutionary events marked. Arrows indicate the acquisition or loss of particular genes or traits. In most cases, these represent gain or loss of genes, the exceptions being 'α2 rep', the gain of α2 repression of **a**sg; rewire Rme1, the integration of Rme1 into control of hsg; and Ste12 binding, the gain of Ste12 binding sites at the promoters of **a**sg. The precise order of events on the *Kluyveromyces* branch is not certain and the position of Rme1 rewiring needs to be determined. Panel **(B)** shows the arrangement of the mating-type loci in representative species. The arrangement at the *MAT* locus in *Z. bailii* resembles that of the ancestor of the Saccharomycetaceae and other species, which have deleted or translocated genes flanking the locus (see main text for details). In *S. cerevisiae*, the *HML*, *MAT* and *HMR* loci are all on the same chromosome but in the other species shown *HMR* is on a separate chromosome. *Zygosaccharomyces rouxii* CBS732^T is isogenic with *Z. rouxii* NBRC1130^T other than a chromosomal translocation that exchanged the region 3′ of the *MAT* locus with the equivalent region 3′ of *HMR*. Panel **(C)** is a schematic cartoon showing the mechanism by which the X and Z regions of homology mediate recombination between the *HML/R* and the *MAT* locus. The position of genes relative to recombination sites varies by species, and in some cases *MAT***a** and *MAT*a genes extend into or even beyond the X and Z regions (Hanson and Wolfe 2017). In the case of all species with HO endonuclease, recombination is initiated by a DSB introduced by this enzyme, whereas in *Kluyveromyces* the DSB is made by either Kat1 or α3 (see main text).

2021). After germination, a haploid spore divides (by budding) to produce two cells of the same mating type. Then, both of these cells divide, with the original mother cell switching but the first daughter cell not. This results in two **a** and two α cells, which can mate to produce a clonal population of diploids. Transcription of *HO* is very tightly controlled to ensure that it is expressed only in a mother cell that has already budded a daughter cell, and even then only during the late G1 phase.

Although both *Saccharomyces* and *Zygosaccharomyces* possess *HO*, it is not certain that HO endonuclease has acquired equal importance in both genera (Solieri *et al.* 2014). For example, haploid *Z. rouxii* cells that constitutively transcribe *HO* rarely switch mating type (Bizzarri, Cassanelli and Solieri 2018), while *Z. rouxii* Δ*ho* cells only slightly decrease switching frequency compared with wild-type strains (Watanabe, Uehara and Mogi 2013). This contrasts with *S. cerevisiae*, where inactivating mutations in *HO* have made most laboratory strains heterothallic, consequentially, switching is almost nonexistent (Meiron, Nahon and Raveh 1995; Mortimer 2000; Ezov *et al.* 2006). These findings suggest that in *Z. rouxii*, *HO* escapes the regulation that assures a tight cell-cycle and cell-type control in *S. cerevisiae*, and that mating-type switching is not completely dependent on HO endonuclease. That raises an intriguing question as to whether *Saccharomyces* has coevolved its systems to simultaneously possess an efficient, tightly controlled, highly integrated *HO* on which it has become completely dependent for switching. In that case, genera like *Zygosaccharomyces* may represent an earlier stage in the evolutionary process. Determining what

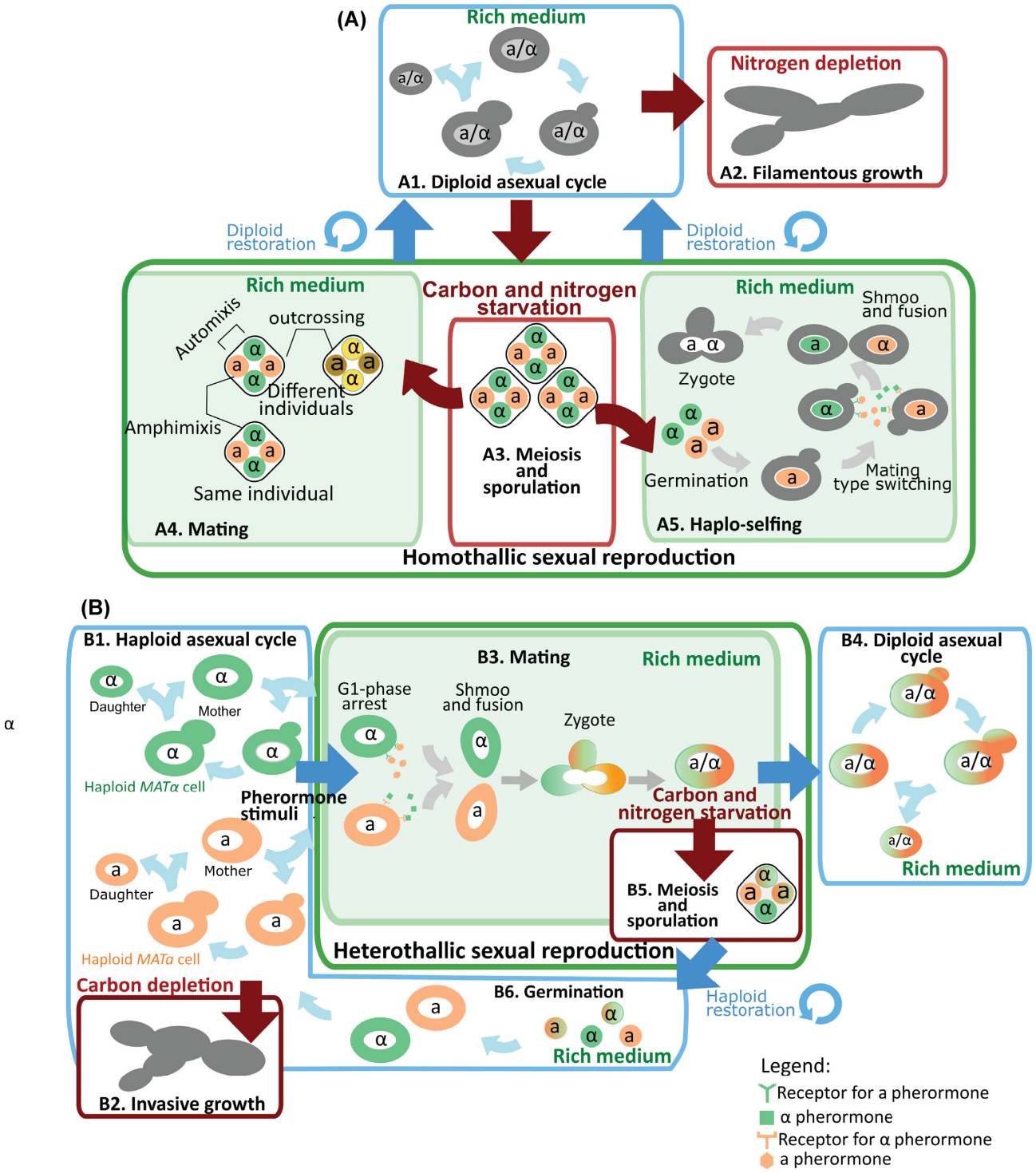

Figure 2. Life cycle in *S. cerevisiae*. **(A)** Homothallic diploid strains mainly reproduce asexually (A1) and activate the commitment towards filamentous growth in response to nitrogen depletion (A2). Carbon and nitrogen depletion induces cells to enter into meiosis and sporulation (A3). Mating can occur between opposite haploid gametes from different individuals (outcrossing), between gametes from distinct meiotic events of the same individual (arisen from two different asci in the same clonal population; amphimixis); and between gametes from the same meiotic event (within the same ascus; automixis) (A4). In addition, haploid gametes can germinate under favourable growth conditions. Mother cells after one round of mitosis can switch mating type (secondary homothallism) and mate daughter cell (haplo-selfing) (A5). **(B)** In absence of pheromone stimuli, heterothallic haploid cells with either *MAT***a** or *MATα* cells reproduce mitotically in rich medium (B1). Carbon depletion induces invasive growth (B2), while pheromone stimuli trigger the mating response via the pheromone MAPK module (B3). This signalling cascade pathway culminates with the Ste12-mediated cell cycle arrest in G1 and the remodelling of cell wall and cytoskeleton to assure shmoo protections and cellular fusion (B3). The resulting diploid zygote can either asexually reproduce (clonal diploid population incompetent for mating) (B4) or enter meiosis and sporulation in response to carbon and nitrogen starvation (B5). Favourable environmental conditions induce spore germination and restoration of haploid state (B6).

other genes play a role in mating-type switching in *Zygosaccharomyces* will be critical to understand the homothallic and heterothallic nature of strains that may be used in the construction of synthetic hybrids for biotechnology.

Kluyveromyces has acquired a completely independent system to introduce double stranded breaks in the *MAT* locus. Unlike *S. cerevisiae*, where a single endonuclease initiates the switch between *MAT***a** and *MAT*α, in *Kluyveromyces*, two domesticated transposases, α3 and Kat1, are required (Barsoum, Martinez and Åström 2010; Barsoum, Rajaei and Aström 2011; Rajaei *et al*. 2014). The *Kluyveromyces* hobo/Activator/Tam3 (hAT) transposase 1 (Kat1) drives the switch from *MAT***a** to *MAT*α, and the α3 transposase of the mutator-like (MULE) family the switch from *MAT*α to *MAT***a**. Mechanistic details were elucidated in *K. lactis* but bioinformatic and molecular studies confirm that *K. marxianus* uses an identical mechanism (Lane *et al*. 2011; Cernak *et al*. 2018; Lee *et al*. 2018; Ortiz-Merino *et al*. 2018b). The independent domestication of three different genetic elements in two major Saccharomycetaceae lineages demonstrates strong selective pressure to evolve efficient switching. In contrast, high frequency switching appears to be less common in other yeast species, perhaps indicating that there is something about the niche occupied by Saccharomycetaceae that requires this capacity. Since the main benefit for the yeast of switching is the capacity to form a diploid and then produce robust spores, it may help survival in changeable environments. The tight control that yeasts exhibit over expression of the endonucleases indicates that a balance needs to be struck as introducing double stranded breaks in a chromosome is an inherently risky activity. In *Saccharomyces*, *HO* expression is restricted to when a mother cell, having already budded a daughter cell, is undergoing mitosis, whereas in *Kluyveromyces*, expression of both *KAT1* and *α3* is under the control of the transcriptional regulator Rme1 (syn. Mts1), which is activated by nutrient limitation. Interestingly, in *Kluyveromyces*, there seem to be additional controls to dampen expression since active α3 is synthesized from *HML*α3, which is located in the largely silenced *HML* locus, rather than from *MAT*α3, and *KAT1* translation requires a programmed −1 frameshift (Rajaei *et al*. 2014). It can be inferred that the activity of the *Kluyveromyces* transposases α3 and Kat1 needs to be carefully tuned to ensure the same proportions of **a** and α cells are present for efficient mating in a population to occur (Rajaei *et al*. 2014). It is also surely not by chance that the agents promoting switching originated as mobile genetic elements as it is well established that these selfish genetic entities need the sexual life cycle of the host for their own reproduction and propagation (Rajaei *et al*. 2014; Coughlan *et al*. 2020; Rusche 2020).

DSBs in the genome are corrected by DNA repair systems and it is likely that such sites could become recombination hotspots. Indeed, examination of the genes that flank the *MAT* locus in different yeasts demonstrate that this region is prone to genomic rearrangements (Butler *et al*. 2004; Gordon *et al*. 2011). The ancestral location of the *MAT* locus in Saccharomycetaceae is between *DIC1* and *SLA2*, though over the course of evolution, genomic events have led to significant variation especially in the genes towards the left arm. It is proposed that recombination errors during mating-type switching can cause accidental deletion of the gene immediately adjacent to the *MAT* locus and, over time, this leads to progressive erosion of the region of the genome between the *HML* and *MAT* loci (Gordon *et al*. 2011). In some cases, this is accelerated by transposition of genes to other chromosomal locations, and it is more pronounced in post whole-genome duplication (WGD) species, presumably because the presence of a second allele alleviated the selection against gene loss. Thus, in *S. cerevisiae*, all the genes between *BUD5* and *MAT* have been deleted/translocated whereas in *Kluyveromyces*, just the genes between *LAA1* and *MAT* are lost. In *Zygosaccharomyces*, the ancestral organization is generally maintained, although substantial variation has been reported in *Z. rouxii*, with frequent errors and rearrangements promoted by ectopic recombination around the *MAT* loci (Watanabe, Uehara and Mogi 2013; Solieri *et al*. 2014). One very clear example is reciprocal translocation in strains NBRC1130^{T} and CBS732^{T} between the region flanking the *HMR* locus and the corresponding region on the left flank of the *MAT* locus, but others were also seen (Watanabe, Uehara and Mogi 2013; Bizzarri *et al*. 2016; Ogata *et al*. 2018) (Fig. 1B). The interesting thing about this translocation is that NBRC1130^{T} and CBS732^{T} were thought to be identical culture collection depositions of the same strain, so it is apparent that these translocations can happen anytime and are not merely evolutionary relics. These inter-chromosomal rearrangements are considered responsible for the karyotypic variation frequently observed in this species (Solieri *et al*. 2008). Recombination at the *MAT* locus could produce intra-strain genotype variation and lead to genetic instability and phenotypic novelties inside the progeny cells upon which selection can act (Bizzarri, Cassanelli and Solieri 2018). It appears that *MAT* loci in *Z. rouxii* are particularly prone to recombination events, escaping the general rule in biology that genomic regions determining sexual compatibility display recombination suppression extended beyond the genes determining sexes or mating types (Hartmann *et al*. 2021). Some limited *MAT* locus variation has also been seen in *K. marxianus* (Huff and Morrissey, unpublished data), but not to the extent reported in *Z. rouxii* and it remains to be determined whether the observations with *Z. rouxii* are due to intrinsic features and a high rate of aberrant rearrangement at these loci, or whether the environments from which these strains were isolated selected for such variants.

MULTIPLE ROUTES FOR CELL IDENTITY DETERMINATION

The genes expressed from the *MAT*loci encode four transcriptional regulators that between them determine cell identity (**a**, α or **a**/α) because they control the expression of the genes that are responsible for the phenotypic manifestation of cell type (Rusche and Rine 2010; Haber 2012). There are surprisingly few genes that are required to confer the functions that correspond to cell identity. Both *S. cerevisiae* and *K. lactis* have 12 genes defined as haploid specific (hsg), four of which are also in common with *C. albicans* and are involved in the conserved part of the pheromone response pathway (Booth, Tuch and Johnson 2010). Interestingly, given its divergent function, a fifth gene, *RME1* (Repressor of Meiosis 1), is also a core hsg in the Saccharomycetaceae. In the case of haploids, the **a**- and α-specific genes (**a**sg and αsg, respectively), which are mainly involved in synthesis and response to pheromones, define whether the cell identifies as **a** or α. There is not a set of genes that define the diploid cell identity as such, but genes involved in meiosis and sporulation are only expressed in diploids. The core *MAT***a** locus contains two genes, *MAT***a**1 and *MAT***a**2, whereas the core *MAT*α locus comprises *MAT*α1 and *MAT*α2 (Tsong *et al*. 2006; Sorrells *et al*. 2015; Hanson and Wolfe 2017). There are, however, variations, with *Saccharomyces* lacking *MAT***a**2 (Haber 1998, 2012), *Z. bailii* lacking *MAT***a**1 (Ortiz-Merino *et al*. 2017) and *Kluyveromyces* spp. possessing an extra gene, *MAT*α3 (Tsong *et al*.

2006; Barsoum, Martinez and Åström 2010; Lane *et al.* 2011; Hanson and Wolfe 2017). As already discussed, *MAT*α3 is derived from a transposon and encodes an endonuclease that promotes switching of cells from α to **a**. The mechanism by which the *MAT*-encoded transcription factors control expression of cell-type specific genes is conserved in principle in the Saccharomycetaceae but there are some fundamental differences where alternative regulatory wiring is used to achieve the same outcome (Baker *et al.* 2012; Sorrells *et al.* 2015). In *S. cerevisiae* **a** cells, **a**sg are constitutively expressed whereas in *K. lactis*, expression of **a**sg requires **a**2 as an activator (Coria *et al.* 2006; Sorrells *et al.* 2015). Both species need to recruit Ste12 as a coactivator and the difference arises because in *Kluyveromyces*, Ste12 is recruited as part of a complex with **a**2 and Mcm but in *Saccharomyces*, Ste12 binds directly to the promoter and so does not need **a**2. In α cells, αsg are induced by α1, which binds promoters as part of a complex with Mcm1, recruiting Ste12, activating expression. When it comes to the **a**sg in α cells, again a difference arises between *Saccharomyces* and *Kluyveromyces*. In *Saccharomyces*, an α2-Mcm1 complex inhibits expression of **a**sg but, in *K. lactis*, α2 plays no role in regulating these genes. Although achieved by different means, the end result is the same in both lineages: **a** cells only express **a**sg and α cells only express α-sg, while both lineages are competent to express hsg, which do not use these activators. In **a**/α cells, an **a**1-α2 complex represses expression of hsg and causes cells to behave as diploids. This repression is direct in *Saccharomyces* but indirect in *Kluyveromyces*, where the control is exerted via Rme1 (syn. *Mts1*), which is an activator of hsg (Fig. 3) (Booth, Tuch and Johnson 2010).

The situation in *Zygosaccharomyces* is complex and requires further investigation as current evidence is that some species lack the functional **a**1-α2 complex that is required for a stable diplophase. *Zygosaccharomyces bailii* completely lacks *MAT***a**1 and cannot form the **a**1-α2 complex (Ortiz-Merino *et al.* 2017), whereas in a *Z. rouxii* /*Z. pseudorouxii* hybrid, it was reported that the **a**1-α2 heterodimer formed with subunits from different parents was inactive (Bizarri *et al.* 2016). Data are not available for other *Zygosaccharomyces* species and there is a need for more studies on how lack of the **a**1-α2 complex affects other signalling pathways. Phylogenomic analysis offers some insights into how the cell identity regulatory systems evolved. Ancestrally, in the Saccharomycotina, the function of α2 was as one of the two subunits in the **a**1-α2 complex that represses expression of hsg in diploid cells. α2 did not have a repression function at the **a**sg and this was gained around the time that the 3LOC system emerged in the Saccharomycetaceae (Baker *et al.* 2012; Sorrels *et al.* 2015). From this, it can be inferred that ancestral Saccharomycetaceae used α2 to repress **a**sg, and **a**2 to activate their expression in α or **a** cells, respectively—there was not a default identity like in *S. cerevisiae* (Tsong *et al.* 2003, 2006). The loss of *MAT***a**2 in the *Saccharomyces* lineage only became possible when promoters of **a**sg gained independent Ste12 binding sites, an event that appears linked to the genome duplication since the phenomenon is present in all post-WGD species (Sorrells *et al.* 2015). Genera like *Zygosaccharomyces* are fascinating because they retain most of the ancestral regulatory apparatus and can be presumed to use the full capability of α2 as a repressor and **a**2 as an activator, though more studies are required in this area. It is also interesting to note that the variability that arises in the genes flanking the *MAT* locus in *Z. rouxii* (and derived hybrids) gives rise to multiple *MAT***a**2 alleles, raising questions as to whether these **a**2 variants differ in their ability to bind **a**sg promoters and to recruit Ste12 (Bizzarri, Cassanelli and Solieri 2018) (Fig. 4). Loss of the α2 repression appears to be specific to certain *Kluyveromyces* lineages as other genera in the KLE clade, and even *Kluyveromyces wickerhamii*, retain this function. It was shown by mutagenesis and complementation studies that a point mutation leading a single amino acid substitution is responsible for the loss of α2 repression of **a**sg in *K. lactis* (Tsong *et al.* 2006; Baker *et al.* 2012).

REWIRING THE RESPONSE NETWORKS

The primary function of meiosis in yeast is to produce robust spores that can survive adverse conditions and that will germinate under favourable conditions allowing the cell to resume growth. This involves the processes of mating, mating-type switching, meiosis and sporulation. The sequence of events must be different, however, in cells growing as haploids or diploids. In the diplont *S. cerevisiae*, starvation triggers meiosis and sporulation, and following germination under favourable conditions, mating-type switching and mating to form a stable diploid take place. In contrast, in the haplont *K. lactis*, starvation first triggers mating-type switching and mating, followed by meiosis and sporulation. Thus, for different species mating-type switching occurs under favourable or unfavourable conditions. Considering this fundamental difference, it is not surprising that the regulatory networks are wired differently across the subphylum where the same cast of regulatory proteins interact differently to achieve the desired outcome (Fig. 3) (Hanson, Byrne and Wolfe 2017). In *S. cerevisiae*, starvation leads to inhibition of Rme1, which is a transcriptional repressor of *IME1*, a global inducer of meiosis. The starvation signals are routed through PKA and TORC and the molecular mechanism by which Rme1 leads to an increase in transcription of *IME1* is quite complex involving regulatory ncRNAs (Van Werven *et al.* 2012; Moretto and van Werven 2017; Moretto *et al.* 2018) (Fig. 5). Nonetheless, the consequence is that starvation induces meiosis in diploid cells. In contrast, starvation in *K. lactis* leads to increased levels of active Rme1 by inducing expression of *RME1* (Booth, Tuch and Johnson 2010; Barsoum, Rajaei and Aström 2011) (Fig. 5). Again, the signal comes via PKA, but it appears that the direct connection to PKA is absent and the Msn2 transcriptional activator is used to create an inverse relationship between PKA activity and transcription of *RME1*. Rme1 directly activates expression of *STE12*, *MAT*α3 and *KAT1*, the latter two of which then trigger mating-type switching. Induction of *STE12* expression creates sufficient Ste12 so that in the presence of pheromone, mating is activated. Rme1, therefore, serves a critical function in a coordinated response under starvation conditions to stimulate mating-type switching and the formation of diploids. Although less detail is available on all the molecular interactions in *Zygosaccharomyces*, it was recently shown that *Z. rouxii* cells can mate under nitrogen starvation conditions and that Ste12 is connected to both starvation response and mating (Ogata and Kuroki 2021). Interestingly, in strain CBS732^T a frameshift mutation prematurely truncated the *STE12* ORF, resulting in haploid cells unable to mate opposite cells, while in the coisogenic strain NBRC1130^T a functional Ste12 is responsible for the mating response under nitrogen starvation (Ogata and Kuroki 2021). It remains to be established whether, in addition to mating, mating-type switching is also under the control of nutritional cues in *Z. rouxii*.

The different regulatory wiring used to produce spores in response to starvation is striking and reflects the different needs of a haploid or diploid species. As already discussed, in hap-

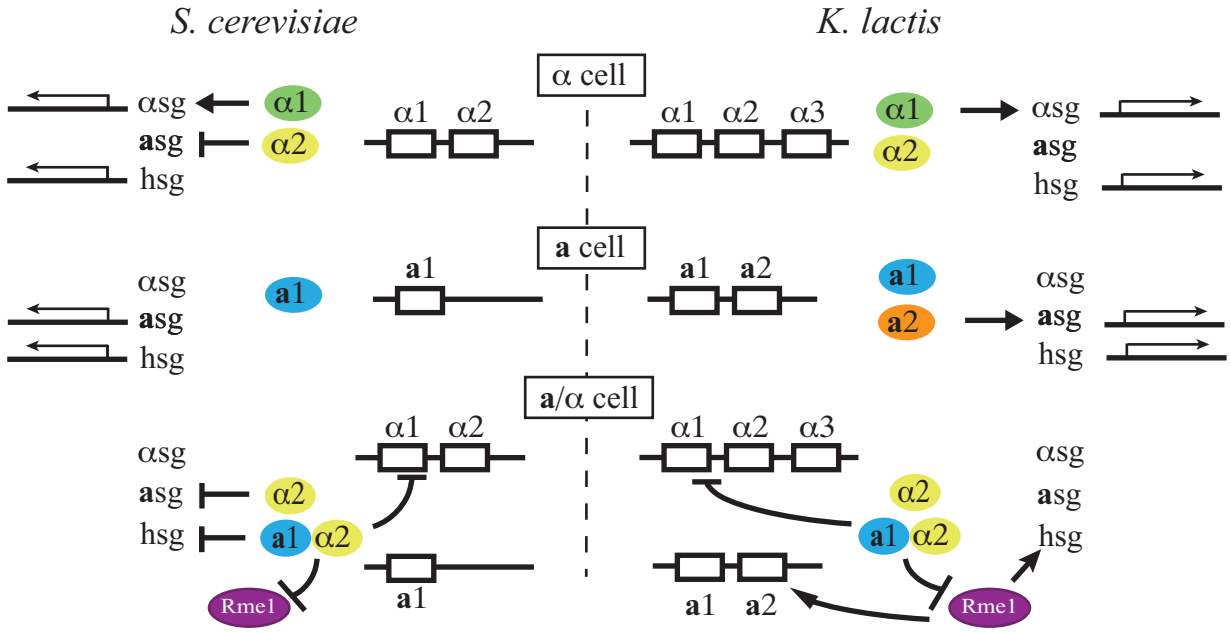

Figure 3. Transcriptional control of cell identity. The molecular mechanisms by which cell identity is controlled are best understood in *S. cerevisiae* and *K. lactis*. For clarity, genes are indicated by the protein they encode. It is possible to make inferences for other species based on comparative genomics and some limited experimental data. In *S. cerevisiae*, the **a**1, α1 and α2 transcription factors work as indicated to control expression of genes required for a cell identity (**a**sg), α cell identity (αsg) and haploid-specific genes required by both **a** and α cells (hsg). All these genes are repressed in diploid cells (**a**/α). *Saccharomyces cerevisiae* does not have **a**2 and **a**sg are expressed by default as they have acquired independent Ste12 binding sites (not shown, see main text). In *K. lactis*, **a**2 is required to activate expression of **a**sg, which are also not repressed by α2. Loss of α2 repression seems to be due to lineage-specific polymorphisms in MATα2. In diploid **a**/α cells, hsg are not directly repressed by the **a**1-α2 heterodimer. Instead, hsg have lost binding sites for **a**1-α2 but require the activator Rme1, which has come under the negative control of **a**1-α2. Expression of αsg and **a**sg in *K. lactis* **a**/α cells is supressed by the **a**1-α2 heterodimer, which directly (MATα1) or indirectly (MAT**a**2) prevents the expression of the α1 and **a**2 activators.

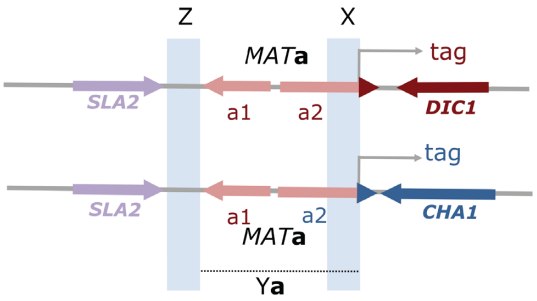

Figure 4. Variable *MAT* flanking genes generate novelty in *Z. rouxii* MAT**a**2 variants. Two possible arrangements of MAT**a** loci are reported. In *Z. rouxii*, Y**a** exceeds X region. Consequently, when *MAT/HMR* loci undergo reciprocal translocation or mating-type switching, the 3′ flanking region changes together with the final portion of MAT**a**2 ORF (tag), leading to variable MAT**a**2 alleles.

lontic yeasts like *Kluyveromyces* and *Zygosaccharomyces*, mated diploids rapidly undergo meiosis and sporulation. The detailed mechanism that governs this is not yet known, but clues may come from more distant yeasts. For example, in *Candida lusitaniae* and *Schizosaccharomyces pombe*, expression of genes governing mating and meiosis are tightly coupled thus ensuring that cells, once mated, immediately enter meiosis and return to haploid state (Sherwood *et al.* 2014). In *C. lusitaniae* and *S. pombe*, Ste12 was implicated in this process but deletion of *STE12* in *K. lactis* did not have the same phenotype, so this is another example of evolutionary divergence to achieve a comparable outcome. The frequent appearance of Ste12 in these processes is surely not coincidental and points to its role as a critically important transcriptional regulator in the cell. Although molecular mechanisms are not identical, the finding of nutritional control of mating competence in *Kluyveromyces*, *Zygosaccharomyces*, *Ogataea* and *Komagataella* suggests a higher degree of module interconnectivity than that occurs in *S. cerevisiae* and illustrates that regulatory networks governing sexual reproduction have been extensively rewired during yeast evolution (Hanson and Wolfe 2017). In such scenarios, the usual assumption is that the non-*Saccharomyces* yeast co-opted and reprogrammed an existing regulatory system. In some cases, this must be true since *KAT1* and *MATα3* genes were acquired in *Kluyveromyces* and brought under the control of Rme1. But for other parts, for example the links between *PKA*, *MSN2/4* and *RME1*, it is possible that it was *Saccharomyces* that carried out the rewiring. Answers to questions like these will come from future experiments that address these pathways in other yeasts, most notably in lineages like *Lachancea*, *Zygosaccharomyces* and *Torulaspora*.

FORMATION OF HYBRIDS IN THE SACCHAROMYCES GENUS

Speciation arises when lineages or subpopulations become reproductively isolated, either because of ecological or biological separation. In the case of yeasts, genetic incompatibility is the main force that maintains separate species within a genus. Despite this, there is ample evidence that species barriers have been breached multiple times with far-reaching consequences. When discussing yeast evolution, the WGD, first described in 1997, always features strongly as this has had an enormous impact on how *S. cerevisiae* and its close relatives evolved (Wolfe and Shields 1997). More recent analysis based on additional genome sequences offers a new perspective as it is now believed

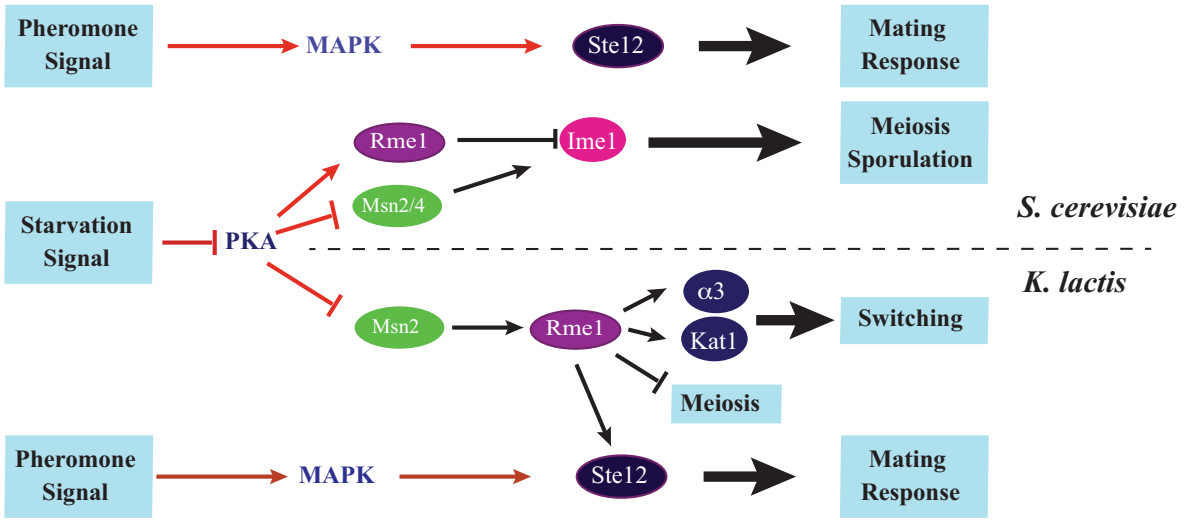

Figure 5. Rewiring signals in the pheromone and starvation response pathways. Both *S. cerevisiae* and *K. lactis* respond to pheromone by activating a highly conserved MAPK cascade that culminates in activation of the transcription factor Ste12. Similarly, both species respond to starvation by triggering the Ras-cAMP pathway and inhibiting protein kinase A (PKA) activity. There is a fundamental difference, however, at the Rme1 (syn. Mts1 in *Kluyveromyces*) node. In *S. cerevisiae*, Rme1 is a direct target of PKA and the consequence of carbon starvation is a reduction in active Rme1. There is a complex control system but reducing Rme1 activity ultimately leads to transcription of *IME1*, thereby triggering meiosis. There is no direct connection between pheromone signalling, which is important during haploid growth, and meiosis, which only occurs in diploid cells. In *K. lactis*, Msn2 (the single orthologue of Msn2 and Msn4) has acquired transcriptional control of *RME1*. Inhibition of PKA (i.e. starvation) increases activity of Msn2, leads to more transcription of *RME1* and more Rme1. Rme1 is a transcriptional activator of the *MATα3* and *KAT1*, two transposon-derived genes encoding endonucleases important for mating-type switching. Rme1 also inhibits meiosis and has taken over the function of **a**1-*α*2 in inhibiting hsg (not shown). Rme1 also activates expression of *STE12*, thereby linking starvation and pheromone responses. Red lines in the figure depict responses at the level of protein activity, whereas black lines indicate transcriptional regulation. For clarity, some components of the signalling pathways are omitted, and further details may be found in the references cited in the main text.

that the WGD was, in fact, a hybridization between a strain from the ZT clade, and thus related to *Zygosaccharomyces*, and a strain from the KLE clade, thereby related to *Kluyveromyces* and *Lachancea* (Fig. 1A) (Marcet-Houben and Gabaldón 2015; Wolfe 2015). The footprint of the ZT parent often seems to be stronger, explaining the conservation of features like *HO*. It may well be the case, however, that some aspects of *Saccharomyces* biology might resemble KLE yeasts, or it may have been possible to combine features to create new processes and networks. This was an ancient hybridization, estimated to have taken place 100 million years ago. But much more recent unions have also been found and the mechanisms by which hybridization can give rise to new yeast lineages was the topic of comprehensive recent reviews (Gabaldón 2020; Ono, Greig and Boynton 2020).

As is generally the case for yeast studies, far more is known about sex, mating and formation of hybrids involving *S. cerevisiae* than in any other system. Although this is probably a reflection of our experimental bias, these studies still offer very good models for understanding the molecular and evolutionary processes that are at play. *Saccharomyces cerevisiae* and its close relatives can be found in the same fermentations, and readily mate with each other with few prezygotic reproductive barriers. It is not surprising, therefore, that hybrids can arise in mixed populations, and, if at a selective advantage due to heterosis or a combination of advantageous traits from the parent species, can dominate fermentations. The prime example of this is the brewing hybrid, *S. pastorianus* used in lager fermentation. The combination of the ability to grow and ferment at low temperatures and utilize maltotriose, one of the major sugars in wort, is one reason it is more fit for the brewing environment than the two parent species, *S. cerevisiae* and *S. eubayanus* (Libkind *et al.* 2020). There are other examples from the wine and cider industries where hybrids between *S. cerevisiae* and *S. kudriavzevii* or *S. uvarum* are found (Gonzalez *et al.* 2006; Gallone *et al.* 2019). In nature, hybrids between *S. cerevisiae* and *S. paradoxus* are found though these are not used in domestic situations (D'Angiolo *et al.* 2020). These hybrids are sterile however, having postzygotic reproductive isolation, and are therefore evolutionary dead ends in general. Some hybrids, such as *S. pastorianus*, have unbalanced genomes that contribute to sterility but also to their fitness in the domestic environment. Indeed, most of the 'natural' hybrids found have mosaic/chimeric genomes with unbalanced genome content due to various mechanisms of genome reduction in meiotic and/or mitotic reproduction (Sipiczki 2018). This genome instability is also observed in artificial hybrids created in the lab (Sipiczki 2018; Marsit *et al.* 2021). This does not necessarily mean hybrids cannot contribute to continued evolution, indeed the whole post-WGD clade is derived from a hybrid between two species that was initially sterile. The hybrid that generated the WGD must have been sterile yet extant *Saccharomyces* species are fertile and evolve as any diploid sexual species does. To get to this point, the hybrid or its descendants underwent several drastic changes including loss of 85% of the duplicated genome (Wolfe and Shields 1997; Wolfe 2015). In addition to this loss, in order to behave like a haploid of a new species derived from the hybridization event, there must be loss of one of the mating-type systems along with its mating-type switching mechanism. Euploidy must also be restored as the initial genome instability of hybrids results in unbalanced chromosomal content, aneuploidy and segmental aneuploidy, which contributes to sterility. Restoration of fertility and euploid behaviour after hybridization takes several evolutionary steps and these provide lessons for the creation of synthetic hybrids (Marcet-Houben and Gabaldón 2015).

Sterility of hybrids is not a complete barrier to contribution to future generations as we see evidence of introgressions from one species into another in many populations of different *Saccharomyces* species. Here, we mean introgressions as the observed

replacement of syntenic genomic regions from one species into a related species rather the process of such replacement, which could be due to various mechanisms as discussed below. We are not discussing the acquisition of more distant foreign DNA segments, for example, the Horizontal Gene Transfers into the wine yeast EC1118 (Novo *et al*. 2009), which come from more distant species where hybridization with *Saccharomyces* does not occur. One of the earliest found after genomes of relatives of *S. cerevisiae* were sequenced was an introgression of a subtelomeric segment from *S. cerevisiae* into the European population of *S. paradoxus* (Liti, Barton and Louis 2006). Since then, numerous examples of introgressions between different species in the clade have been found and are analogous to the introgressions of Neanderthal and Denisovan DNA into the human genomes of today. These introgressions are generally thought to arise from rare fertile backcrosses to one of the parent species. In *Saccharomyces*, such fertility can be restored in a single generation via return to growth of sporulating hybrids (D'Angiolo *et al*. 2020). That study found the abortion of meiosis prior to completion of meiotic recombination and chromosome segregation results in loss of heterozygosity, through homozygosis rather than genome segment loss, at numerous locations in the hybrid diploid genome, which then allows proper meiotic recombination and chromosome segregation in a subsequent meiosis. The resulting haploid spores have chimeric genomes of the two parent species, with a majority from one, allowing successful backcrossing to that parent species. Segments retained from the other species are now introgressions. These strains with introgressions still form sterile hybrids with the donor parent species. Even aneuploid hybrids such as *S. pastorianus*, can give rise to rare viable meiotic products (Gjermansen and Sigsgaard 1981), which can subsequently be used in breeding, at least for one generation. Sterility can be overcome by other mechanisms where genome reduction due to instability leads to loss of heterozygosity of the mating-type locus and generally loss of chromosomes and segments leading to aneuploidy (Sipiczki 2018) but the introgressions observed in many cases are in euploid diploids that are still members of one of the parental species.

DOMESTICATION OF NON-*SACCHAROMYCES* HYBRIDS

The frequency of hybrid strains associated with food and industrial fermentations has led to questions as to whether formation of hybrids is favoured, or even promoted, in these anthropogenic settings. The current consensus, however, is that formation of hybrids occurs completely naturally but the pressure imposed by these processes could select for more robust strains over time. Indeed, this is another facet of the domestication of nature that humans commenced ~12 000 years ago. Domestication refers to the controlled breeding of an organism that becomes genetically distinct from its wild relatives in ways making it more useful to humans (Diamond 2002). Thanks to domestication, agricultural production and animal husbandry have sustained the still-occurring human demographic expansion from an estimated 10 million in the Neolithic to 6.9 billion today (Driscoll, Macdonald and O'Brien 2009). Like plants and animals, yeasts underwent domestication through unconscious human activities aimed at selecting microbes suitable to properly carry out fermentative transformation and conservation of food stuff (Almeida *et al*. 2015, 2017; Gallone *et al*. 2016; Gonçalves *et al*. 2016; Duan *et al*. 2018; Legras *et al*. 2018; Peter *et al*. 2018). In addition to domestication, human environments drove quasi-domestication events in which microbial adaptive phenotypes evolved without the emergence of traits recognized as useful for humans (Barbosa *et al*. 2018; Pontes *et al*. 2019). Overall, these domestication processes are under the umbrella term of 'domestication syndrome' (De Chiara *et al*. 2020).

Dairy strains of *Kluyveromyces* display evidence of domestication dating right back to the origins of farming and agriculture. *Kluyveromyces lactis* and *Kluyveromyces marxianus* have long been associated with traditional dairy fermented foods and beverages, a capacity that is reliant on their ability to use the milk sugar lactose. This is a rare trait in yeast and evidence in recent years demonstrated that efficient uptake of lactose is an acquired characteristic, found only in dairy lineages (Varela *et al*. 2017; Ortiz-Merino *et al*. 2018b). Population genetics show that environmental isolates of *K. marxianus* reveals are sexually active (both mating types common and strains can switch, mate and sporulate in laboratory conditions), grow haplontically and are relatively poor assimilators of lactose, whereas dairy isolates are diploid or triploid, often exhibit aneuploidy and loss of heterozygosity (LOH), and encode a variant of the Lac12 lactose transporter that has improved uptake kinetics (Fasoli *et al*. 2015, 2016; Tittarelli *et al*. 2018; Ortiz-Merino *et al*. 2018b). Intriguingly, diploid dairy isolates contain two distinct genomes, termed the 'A' and 'B' haplotypes, the B version of which provides the improved *LAC12* allele. The majority of environmental isolates are of haplotype A, with some being of a third haplotype, 'C'. It appears that the dairy AB diploids formed by the mating of strains of the A and B haplotypes but, intriguingly, but no haploid isolates with haplotype B have been identified. *Kluyveromyces marxianus* AB diploids can be considered a type of intraspecies hybrid, occupying an intermediary space where lineages are similar enough to allow mating, but differences must prevent implementation of the normal meiosis and sporulation. It should be noted, however, that it is believed that sporulation is possible in at least some of these diploids therefore there are still questions regarding how and why these isolates remain as diploids in what is essentially a haploid species. The answers might, in part, relate to the benefits of heterosis and it is also notable that polyploidy and LOH are considered footprints of domestication in yeast (Peter *et al*. 2018). The role of domestication in the evolution of *K. lactis* has recently been uncovered (Varela *et al*. 2019). *Kluyveromyces lactis*, as a species, came into being when a section of a *K. marxianus* chromosome was acquired by an ancestor of *K. lactis* (*K. lactis* var. *lactis*) very closely related to *K. drosophilarum* (*K. lactis* var. *drosophilarum*). This region of DNA carried the improved *LAC12* allele and thus conferred enhanced lactose utilization of this new yeast. The most likely route by which this DNA was acquired was by introgression from a B haplotype *K. marxianus* strain following an illegitimate mating between that strain and an insect-derived ancestor of *K. drosophilarum*. This mating and subsequent resolution would have taken place in a fermented dairy environment and so *K. lactis* is really the product of a double domestication.

There is substantial evidence that hybridization is also frequent in the *Zygosaccharomyces* genus, which comprises 12 formally described species (Hulin and Wheals 2014), at least three of which are interspecies hybrids (Fig. 6). *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* are the two most studied species because of notable tolerance to adverse environmental conditions and their close association with food. The observation that tolerance to weak organic acids at low pH in the case of *Z. bailii* (Kuanyshev *et al*. 2016), and highly osmotic/halophilic conditions in the case of *Z. rouxii* (Solieri 2021), seems espe-

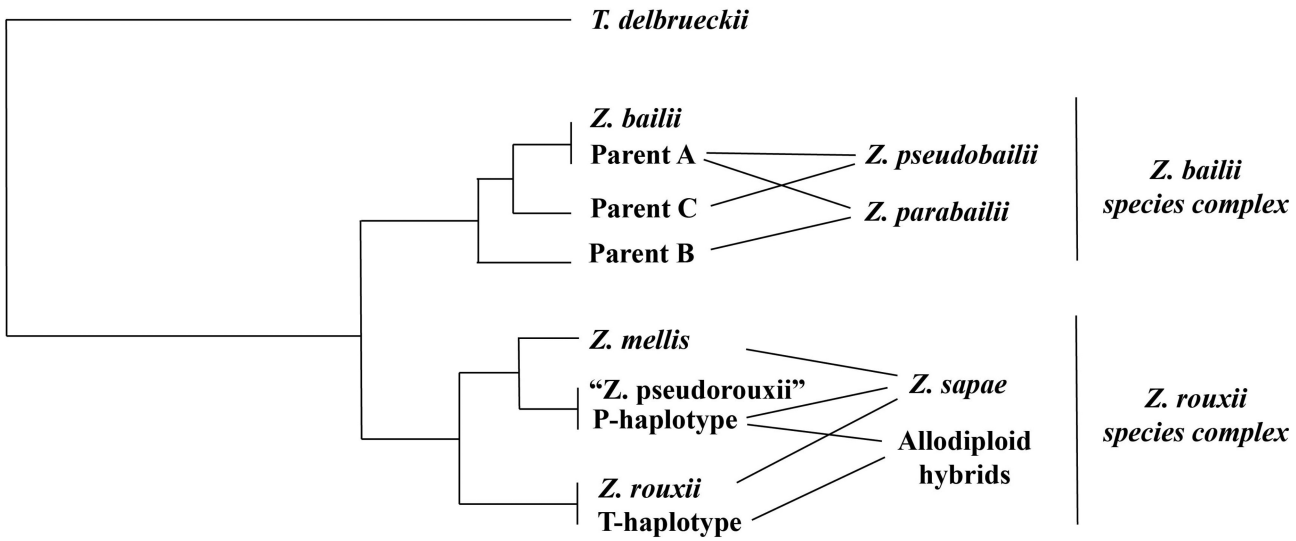

Figure 6. Population structure of the *Zygosaccharomyces* species complex. The phylogenetic tree (not to scale) shows the relationships between the strains in the *Z. bailii* and *Z. rouxii* species complex. *Zygosaccharomyces bailii* and two unknown related species that diverge by ~7% form the parents for the allodiploid hybrid species *Z. pseudobailii* and *Z. parabailii*. *Zygosaccharomyces rouxii* (T-haplotype) and *Z. pseudorouxii* (P-haplotype), which has only one isolate represented and is not formally recognized as a species, are the parents of a heterogeneous set of allodiploid strains. *Zygosaccharomyces sapae* is a trihybrid, with contribution from a third haplotype similar to *Z. mellis* in addition to the T- and P-haplotypes.

cially pronounced in hybrid strains suggests the evolutionary rationale for their association with certain fermented foods. The *Z. bailii* species complex comprises the haploid species *Z. bailii* and two hybrid species *Z. parabailii* and *Z. pseudobailii* (Galeote *et al.* 2013; Suh *et al.* 2013; Mira *et al.* 2014; Ortiz-Merino *et al.* 2017; Braun-Galleani *et al.* 2018; Palma, Guerreiro and Sá-Correia 2018). *Zygosaccharomyces bailii* is one parent of each hybrid but the other parent is unknown. In each case, there is 7–10% divergence between the parental genomes, which are labelled 'A' (coming from *Z. bailii*), 'B' (the second parent of *Z. parabailii*) and 'C' (the second parent of *Z. pseudobailii*) (Ortiz-Merino *et al.* 2017; Braun-Galleani *et al.* 2018). The *Z. rouxii* complex comprises two formally recognized strains, *Z. rouxii* and *Z. sapae* (Solieri, Dakal and Giudici 2013), a presumptive species called *Z. pseudorouxii* that is represented by a single cultured strain (NCYC3042) and a collection of allodiploid hybrid strains (Solieri *et al.* 2008, 2013). These hybrids are described as having genomes composed of a T-haplotype and a P-haplotype, which correspond to the *Z. rouxii* and *Z. pseudorouxii* genomes, respectively (James *et al.* 2005; Gordon and Wolfe 2008; Solieri *et al.* 2013; Bizzarri *et al.* 2016, 2018; Watanabe *et al.* 2017). The parental species diverged several million years ago and the genomes of the P and T haplotypes differ by ~15% (Gordon and Wolfe 2008). *Zygosaccharomyces sapae* is also a hybrid of T-haplotype and P-haplotype parents, but compared with these allodiploids, also carries introgressed sequences from a third haplotype that resembles *Z. mellis* (Bizzarri *et al.* 2018; Cassanelli, unpublished results).

Zygosaccharomyces rouxii and *Zygosaccharomyces bailii* have typical haploid genomes (~10 MB) with seven (*Z. rouxii*) or eight (*Z bailii*) chromosomes, whereas the allodiploid hybrids of both species have double the number of chromosomes, genome sizes of close to 20 MB and two homologous copies of most genes. Although there have been some chromosomal rearrangements and translocations, the low level of gene inactivation suggests that these are relatively recent hybridization events that probably took place within the last 1000 years (Ortiz-Merino *et al.* 2017). Remarkably, these allodiploid strains have managed to overcome sterility barriers that are generally present in hybrids. While some aspects about the molecular elements involved in the sensing and pheromone signalling cascade still remain to be unveiled, the mechanism for overcoming the barriers of infertility now appears clear for these hybrids. Hybrid sterility is well studied in *S. cerevisiae* where it is found that there are two major barriers impairing fertility; namely, mispairing of the homologous chromosomes in prophase-I of meiosis and the inability of gametes that arise from rare sporulation to mate because of mating-type heterozygosity (Pfliegler, Antunovics and Sipiczki 2012). Experimental studies using large numbers of interspecies *Saccharomyces* hybrids have identified ways by which these barriers can be overcome (Karanyicz *et al.* 2017; Sipiczki, Antunovics and Szabo 2020) and it is striking to note that *Zygosaccharomyces* hybrids use some similar mechanisms. The first sterility barrier can be surmounted if the diploid self-mates to form a tetraploid as this generates two copies of each homologous chromosome that can then pair during prophase-I, allowing the completion of meiosis and the formation of viable diploid progeny. Both *Z. parabailii* and *Z. pseudobailii* display loss of heterozygosity at the *MAT* locus, which causes them to behave as though they were haploid cells, being able to carry out mating-type switching and competent to undergo mating (Fig. 7). The initial strain following hybridization would have had been an **a**/α heterozygote at the *MAT* locus, had four silent *HMRa/HMLα* loci and two *HO* genes. In both *Z. parabailii* and *Z. pseudobailii*, only the *MAT* locus derived from the *Z. bailii* parent is active and it is proposed that the second *MAT* locus was damaged during unproductive attempts to switch mating types (Ortiz-Merino *et al.* 2017; Braun-Galleani *et al.* 2018). Following a cleavage at the *MAT* locus, either a translocation (*Z. parabailii*) or a deletion (*Z. pseudobailii*) led to inactivation of one *MAT* locus causing the cell to become hemizygous for mating type. The HO endonuclease is implicated in both events and one intriguing observation is that the *Z. bailii*-derived A haplotype copy of *HO* was independently inactivated by point mutations in both types of hybrids. Few other genes are inactivated and the significance of lineage-specific *HO* mutation is not known.

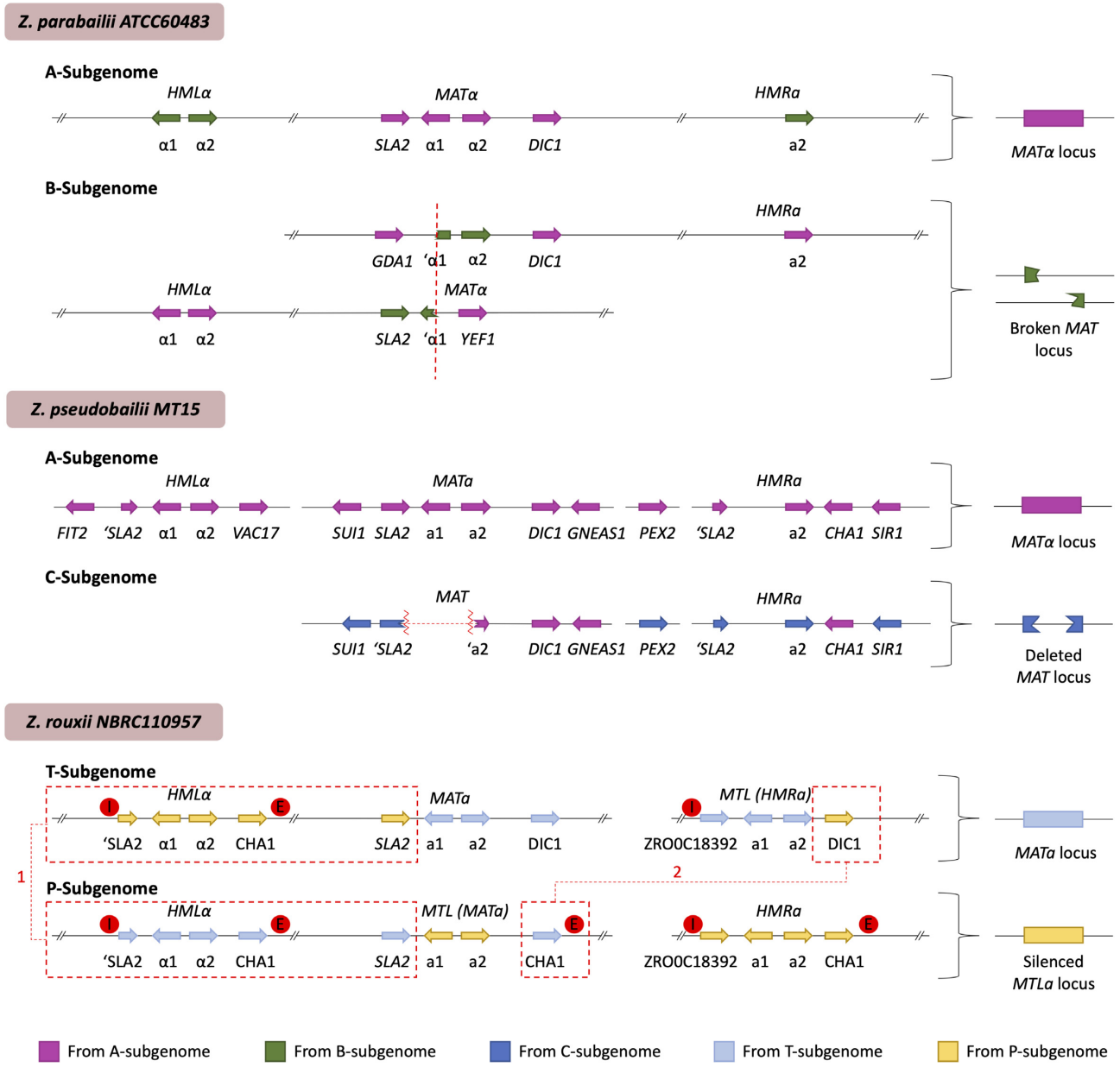

Figure 7. Restoration of fertility in the *Zygosaccharomyces* species complex. Hybrid species formed in *Zygosaccharomyces* have naturally restored fertility by arriving at a situation where the diploid strain expresses genes from a single *MAT* locus and is capable of switching mating types and thus behaving like a haploid. Both *Z. parabailii* and *Z. pseudobailii* achieved this by structural inactivation of one *MAT* locus. In the case of *Z. parabailii*, the *MAT* locus was broken by a failed mating-type switch, and in the case of *Z. pseudobailii*, one *MAT* locus carries a substantial deletion. The *MAT* loci of *Z. rouxii* allodiploids are intact but reciprocal translocations (red boxes 1 and 2) create exchanges between different *MAT* and *HMR* loci. This is shown for strain NBRC110957 and other strains have comparable rearrangements. The consequence of these is to bring the *MAT* genes under the control of the *HMR* silencers (I and E in red circles) that flank the original *HMR* and *HML* loci and thereby epigenetically silence expression. Genes are represented by arrows (not to scale) where each colour represents its subgenome origin. The prime symbol before the name of the genes indicates truncated genes. For simplicity, some details are omitted and further details may be found in the original studies from which this figure was adapted (Ortiz-Merino *et al.* 2017; Watanabe *et al.* 2017; Braun-Galleani *et al.* 2018).

Restoration of fertility was also described in the *Z. rouxii* complex but the mechanism is different and involves epigenetic silencing of the second *MAT* locus, thereby causing the cell to express genes from a single *MAT* locus (Watanabe *et al.* 2017). Mechanistically, reciprocal translocations between different *MAT* and *HML/R* loci result in a situation where one of the two *MAT* loci comes under the influence of the *HMR* silencer (Fig. 7). As it is not always clear from sequences which loci are silent and which are expressed, the general term 'mating-type like' (*MTL*) is often used to describe these loci. *Zygosaccharomyces rouxii* hybrids appear to be quite heterogeneous and there have been some contradictory reports in the literature regarding the fertility of specific hybrids. For example, while one study showed that the allodiploid strain ATCC42981 is not competent for mating (Bizzarri *et al.* 2016), the supposedly identical strain JCM22060 readily mated in a different study (Watanabe *et al.* 2017). Subsequent analysis showed that the propensity for *Z. rouxii* to perform illegitimate translocations between *MTL* loci

resulted in different patterns of active *MAT* loci in ATCC42981 and JCM22060. ATCC42981 retains two active *MAT***a**/*MATα* loci, while the coisogenic strain JCM22060 lacks an active *MAT***a** locus and behaves like haploid *MATα* cells (Bizzarri *et al.* 2019). A representative example of these inter-*MAT*/*HM* translocations is shown for strain NBRC110957 in Fig. 7, but the fluidity of translocation pattern is illustrated by the report that strain NBRC1876, apparently derived from the same parental hybrid, used different translocations to silence the second *MAT* locus (Watanabe *et al.* 2017). These frequent translocations are the reason why hybrid *Z. rouxii* strains are sometimes considered to be a complex of related strains rather than a single species. It is not known why reciprocal translocations occur so readily in *Z. rouxii* strains, but, as was discussed earlier, there are some very well-documented cases, not least where a reciprocal translocation has taken place within the type strains of the haploid *Z. rouxii*, NBRC1130^{T} and CBS732^{T} (Fig. 1B). It is certainly plausible that strains believed to be identical have undergone similar translocations that affect phenotypes with regard to switching and mating and this can explain some contradictions in the field.

There are still other crucial questions to be addressed. Further clarity is needed on the molecular events that are important for hybrid phenotypes; for example, how the activity of activator and repressor proteins affects the expression of hsg. For a hybrid to behave as a haploid, it should express hsg but these are ordinarily repressed in diploids by the **a**1-α2 heterodimer. This issue presumably only arises in *Z. rouxii* hybrids as *MAT***a**1 is absent in *Z. bailii*. One explanation may be impaired functionality in cases where the subunits of the heterodimer come from different parental subgenomes (Bizzarri *et al.* 2016) and there has also been speculation that epigenetic regulation via silencing may cause different patterns of expression in genotypically identical cells within a population (Watanabe *et al.* 2017). It is also striking that one of parents in many hybrids is rarely isolated. One parent of both *Z. parabailii* and *Z. pseudobailii* has never been isolated, only a single isolated of *Z. pseudorouxii* has been cultured, and the B haplotype of *K. marxianus* has not been found in the haploid state. One conclusion could be that an absent parent is very rare or no longer extant, but another is that it occupies a niche that has been under-sampled. This could be analogous to the situation with *S. pastorianus*, which has a missing parent for many years—until *S. eubayanus* was isolated and identified in South America (Libkind *et al.* 2011). This latter scenario should encourage further sampling of diverse environments to find these rare or unknown parents. A thorough analysis of the haplotype genome of the missing parent could be useful to offer some insights as to what ecological niches that parental species might occupy.

SYNTHETIC HYBRIDS

Given the benefits obtained from the use of hybrids found in domestic fermentations, there is a desire to create new hybrids with improved properties (Mertens *et al.* 2015; Gibson *et al.* 2017; and reviewed in Sipiczki 2018). In general, standard genetic improvement through breeding is not feasible due to the hybrid sterility. Two species can be crossed to generate a hybrid with new traits, and this has been done with *Saccharomyces* (Mertens *et al.* 2015), but this is limited to a single generation as these hybrids are sterile. The sterility of the diploid hybrid can be overcome by duplication leading to a tetraploid, which is fertile and produces diploid hybrid spores (Greig *et al.* 2002). Duplications leading to tetraploid intermediates have been generated in a variety of ways, including the controlled expression of *HO* in a hybrid diploid (Schwartz *et al.* 2012). This could be applied sequentially to overcome the one generation limitation. Allotetraploids undergo successful meiotic recombination and chromosome segregation leading to diploid hybrid spores. Those spores that contain one copy of each mating type from each parental species will now be sterile hybrid diploids hitting the second sterility barrier (Sipiczki 2018; Sipiczki, Antunovics and Szabo 2020). However, 50% of diploid spores from such allotetraploid meioses will inherit the same mating type from each parent. These will be acted upon by HO resulting in switching and regeneration of the tetraploid state that is fertile; therefore, the allotetraploid overcomes the second sterility barrier simply through independent segregation of the mating-type alleles from the two parental species. A recent example of this can be found in *S. cerevisiae* × *S. eubayanus* hybrids (Krogerus *et al.* 2021) where two of four spores from a single ascus were able to sporulate and yield viable spores. With continuous rounds of fermentation, genome instability was observed as previously described (Sipiczki 2018; Marsit *et al.* 2021). These regenerated tetraploid spores are now homozygous across the two parental genomes, except for mating type, and being homothallic are not easily used in a continuous breeding strategy. An alternative approach mimics the natural situation where mating-type loci and mating-type switching are manipulated (Naseeb *et al.* 2021). Here, *HO* is deleted in both parent species and diploids of the parent species or hybrid diploids between them have one of the two mating-type alleles deleted. When combined, these diploid maters create tetraploid hybrids with only two mating-type alleles at *MAT*. Following meiosis, these tetraploids generate diploid spores that only have one mating-type allele and therefore behave as haploids, allowing sexual reproduction over many generations. If the parent species each have two different strains with genetic variation between them, then genetic improvement through breeding is possible and the hybrid essentially becomes a new fertile species, amenable to all classical genetic methods, including quantitative trait locus analysis. These new synthetic hybrids, which can be considered *bona fide* species by the biological species definition—self-fertile but sterile when crossed to other 'species', open the door to exploring trait improvement beyond recent practices. For example, studying the contribution to robustness of the similar but not identical copies of genes deriving from the two original parental strains can open to novel knowledge, but at the same time poses challenges, especially for -omic studies. Recently, the response to lactic acid stress was investigated in the *Z. parabailii* hybrid ATCC60483 by differential transcriptomic analysis: the authors applied stringent mapping of RNA-seq reads to the genome to evaluate possible differences in the expression of the homologous gene pairs (Ortiz-Merino *et al.* 2018a). Remarkably, by studying the distribution of expression ratio values under control and stress conditions they found that one of the two genes in each homeolog pair was predominantly expressed. A study in the *Saccharomyces* hybrid, *S. pastorianus*, also found a similar phenomenon, with homologous genes differentially expressed in response to cold stress (Timouma *et al.* 2021). These findings highlight the need to study expression of individual genes but there is also increasing interest in protein complexes and whether complexes derived from different parental genomes may be functional different. While at one level this could be problematic if heterozygous complexes are inactive, it also creates opportunities to construct, and perhaps then evolve, complexes with altered or enhanced activity.

POTENTIAL TO USE GENETICS WITH *KLUYVEROMYCES* AND *ZYGOSACCHAROMYCES*

Genetic crossing also can be used to uncover the molecular basis of traits and to breed new strains, including hybrids, in non-*Saccharomyces* yeasts. In *Kluyveromyces*, the potential for classical genetics was demonstrated by isolation of auxotrophic mutants of different strains, their crossing to form diploids and sporulation of these diploids to obtain haploid spores (Yarimizu *et al*. 2013). The homothallic nature of the species means that it is difficult to control such matings since switching and self-mating frequently occur. The development of CRISPR genome editing and other molecular tools offer new possibilities, however, as it is now possible to make the species heterothallic by inactivating mating-type switching (Löbs, Schwartz and Wheeldon 2017; Cernak *et al*. 2018; Juergens *et al*. 2018; Lee *et al*. 2018). The use of heterothallic strains offers a route to determine the genetic basis of some of the more interesting *K. marxianus* traits, for example, thermotolerance, as well as to generate progeny with new characteristics. Regarding *Z. rouxii*, the type strain, CBS732^T is homothallic and can switch in culture (de Montigny *et al*. 2000). In fact, pure *MATα* and *MAT***a** cultures derived from CBS732^T have been successfully isolated, however, these strains appear unable to undergo mating under standard environments despite the mating-type genes being transcribed (Bizzarri, Cassanelli and Solieri 2018). Mating between heterothallic vegetative cells has been described between strains CBS4837 (NBRC1876; *MATα*) and CBS4838 (NBRC1877; *MAT***a**) (Wickerham and Burton 1960; Mori and Onishi 1967; Ogata and Kuroki 2021), although it is noted that we now know that these are actually allodiploid hybrids rather than pure *Z. rouxii*. Auxotrophic mutants have been successfully obtained both from haploid (Prybilova, de Montigny and Sychrova 2007) and allodiploid (Watanabe *et al*. 2017) strains. Furthermore, recent finding that nitrogen starvation triggers mating in *Zygosaccharomyces* establishes a foundation for high-throughput recovery of gametes in future outcrossing experiments (Ogata and Kuroki 2021). However, two main drawbacks still hamper the possible application of extensive breeding program. As the HO endonuclease is probably not essential to mediate mating-type switching, it is not possible to artificially create heterothallic haploid lineages with stable cell identities by CRISPR genome editing or conventional gene knockout techniques, without a more complete knowledge of the mechanistic effectors of mating-type switching. In addition, frequent translocations around *MTL* loci perturb *MAT* gene expression and can, in some cases, mask the effect of deletion of one *MAT* locus, preventing hemizygous F1 allodiploid cells from regaining fertility (Bizzarri *et al*. 2019). In other cases, translocations induce the opposite effect such that F1 allodiploids are unstable and can immediately mate with an isogenic cell of the opposite mating type, establishing tetraploids (Watanabe *et al*. 2017). Mechanisms governing silencing at the *HMR/HML* loci need to be clarified in the future to understand how to create F1 and F2 hybrids.

PERSPECTIVES

The genetics of mating in *S. cerevisiae* has been studied for over half a century and is extremely well understood. Whilst it remains an active area of study as there are still some molecular nuances to be dissected, explanations of mating, mating-type switching, sporulation and so forth have achieved stable textbook status (Madhani 2007). The realization that other yeasts in the Saccharomycetaceae have a similar 3LOC system with the potential to switch the active *MAT* locus with silent *HMR/HML* idiomorphs initially led to the fallacious assumption that yeasts genera like *Lachancea*, *Torulaspora*, *Zygosaccharomyces* and *Kluyveromyces* operate a mechanism that is largely identical, though perhaps less optimized by evolutionary pressures. In fact, what we now see is that there have been multiple modifications and amendments to the 3LOC system over the course of the last several hundred million years. In some cases, *Saccharomyces* adapted and optimized the system for its own niche, whereas in other lineages, for example *Kluyveromyces*, despite starting with the same components, completely different solutions were found. An obvious question that could be drawn from Fig. 1A when one considers the branches with no apparent gene gains or losses is 'what do we not know?' Given the selective pressures in natural environments, and the range of solutions that evolution throws up, it seems improbable that we have uncovered the full extent of pathway rewiring, acquisition and loss of genes, and other changes. It is more likely that there are still many interesting and important features to be discovered and dissected in the less explored lineages and there is a strong case to continue to amass genomic data and undertake molecular studies in all Saccharomycetaceae lineages (Lacerda, Oh and Eckert 2020; Libkind *et al*. 2020). A second mistaken view was that many of the idiosyncrasies seen in *S. cerevisiae* and related species were a direct consequence of domestication. In fact, most major features and mechanisms predate domestication by many millions of years—though there has been selection for certain features, most notably hybrids with favourable characteristics. Similarly, in genera like *Zygosaccharomyces* and *Kluyveromyces*, anthropogenic activity has selected—but not created—particular hybrid species. The time lines of formation of different known hybrids are noteworthy. The WGD event that derived from a hybrid between strains from the KLE and the ZT clade occurred ∼100 million years ago (Wolfe 2015), *Zygosaccharomyces* hybrids are probably <1000 years old (Mira *et al*. 2014; Kuanyshev *et al*. 2016; Ortiz-Merino *et al*. 2017), *Kluyveromyces* hybrids have not been dated but the low sequence divergence between the A and B haplotypes might suggest <1000 years (Ortiz-Merino *et al*. 2018b), and *S. pastorianus* was born ∼500 years ago (Monerawela and Bond 2017). The inference from this is that while hybridization is an ancient process, it should still be possible to take advantage of the systems and tools that nature has given to manipulate processes to select new hybrids with specialized functionality. Of course, to nature's intrinsic systems, we can add the synthetic tools that we have made and thus it is possible to include an aspect of design and creation into the generation of new species. In this regard, we have learned very useful lessons from *S. cerevisiae*. For instance, much of what was accomplished with *S. cerevisiae* from the heyday of genetics right up to recent years would not have been possible without stable heterothallic strains and this is a definite requirement for work on non-*Saccharomyces* yeasts. Furthermore, knowledge brings enlightenment and there is much still to be discovered about very fundamental processes like meiosis, wiring of signal transduction pathways and patterns of gene expression right across the Saccharomycetaceae. Experience tells us that we should use prior knowledge from *S. cerevisiae* as a guide, but not necessarily as a blueprint, for other species. The potential is enormous, the future is exciting and advances in genome sequencing, bioinformatics, molecular analysis and synthetic tools position us to realize this potential as we seek to take advantage of the fantastic diversity that exists in the budding yeasts.

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