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 allopolyploids 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; Deane 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 Kluyveromycetes 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, Steikens 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 Saccharomycoitina, 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 (Krasowski 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 (Krasowski 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 MATa and the MATu forms, thereby also switching the cells between the a and u 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 u, or diploid a/α, can grow mitotically under the right conditions (Fig. 2) (Fabre et al. 2005; Merlino, Dulin 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 asc of the same lineage (referred to as inter-tetrad mating or amphi mixis), 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 diplo mictic form of growth is preferred. As S. cerevisiae practices 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 homoyzogous 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 are as an alternation of life that would almost always exist as homoyzogous diploids, Saudi Arabia. In these yeasts, as a response to nutrient limitation, haploid a and u 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, HMRa is located on a different chromosome to MAT and HMLa (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, Ya and Yα distinguish the MATa and MATu 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 ‘mis-homed’ 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 fulfill 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 hsg, rewire Rme1, the integration of Rme1 into control of hsg, and Ste12 binding, the gain of Ste12 binding sites at the promoters of hsg. The precise order of events on the chromosomes varies by species, and in some cases the same chromosome but in the other species shown on HO endonuclease. That raises an intriguing question as to whether 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 Δho 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 MATα 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 MATa and MATα in Kluyveromyces, two domesticated transposases, α3 and Kat1, are required (Barsoum, Martinez and Aström 2010; Barsoum, Rajaei and Aström 2011; Rajaei et al. 2014). The Kluyveromyces hobo/Activator/Tam3 (hAT) transposase 1 (Kat1) drives the switch from MATα to MATa, and the α3 transposase of the mutator-like (MULE) family the switch from MATa to MATα. 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 Saccharomyces 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 Saccharomyces 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 HMLα3, which is located in the largely silenced HML locus, rather than from MATa3, 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 recognized 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 Saccharomyces 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 NBRC1130T and CBS732T 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 NBRC1130T and CBS732T 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 MATloci encode four transcriptional regulators that between them determine cell identity (α, 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 (hsp), 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 hsp in the Saccharomyces. In the case of haploids, the a- and α-specific genes (asg 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 MATa locus contains two genes, MATα1 and MATα2, whereas the core MATα locus comprises MATα1 and MATα2 (Tsong et al. 2006; Sorrells et al. 2015; Hanson and Wolfe 2017). There are, however, variations, with Saccharomyces lacking MATα2 (Haber 1998, 2012), Z. bailii lacking MATα1 (Ortiz-Merino et al. 2017) and Kluyveromyces spp. possessing an extra gene, MATα3 (Tsong et al. 2014).
When considering all these factors, it is clear that the regulatory networks are wired differently across the species, and these differences can be traced back to the genome duplication event that occurred in the ancestor of all Saccharomyces species. The consequence is that starvation triggers 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 Åström 2011), 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; Moreto and van Werven 2017; Moreto 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 Åström 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, MATα3 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 Zygospachromyces, 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 CBS732T a frameshift mutation prematurely truncated the STE12 ORF, resulting in haploid cells unable to mate opposite cells, while in the coisogenic strain NBR1130 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 *α1, α2* and *α2* transcription factors work as indicated to control expression of genes required for a cell identity (asg), α cell identity (hsg) and haploid-specific genes required by both α and ω cells (hsg). All these genes are repressed in diploid cells (α/ω). Saccharomyces cerevisiae does not have α2 and asg are expressed by default as they have acquired independent Ste12 binding sites (not shown, see main text). In *K. lactis*, α2 is required to activate expression of asg, which are also not repressed by ω2. Loss of ω2 repression seems to be due to lineage-specific polymorphisms in MATα2. In diploid α/ω cells, hsg are not directly repressed by the α1-ω2 heterodimer. Instead, hsg have lost binding sites for α1-ω2 but require the activator Rme1, which has come under the negative control of α1-ω2. Expression of asg and asg in *K. lactis* α/ω cells is suppressed by the α1-ω2 heterodimer, which directly (MATω1) or indirectly (MATω2) prevents the expression of the α1 and α2 activators.

Figure 4. Variable MAT flanking genes generate novelty in *Z. rouxii* MATa2 variants. Two possible arrangements of MATa loci are reported. In *Z. rouxii*, Ya 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 MATa ORF (tag), leading to variable MATa2 alleles.

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 MATω3 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
leads to transcription of hybrids between \textit{S. cerevisiae} and \textit{K. lactis} (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 \textit{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 \textit{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. \textit{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, \textit{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, \textit{S. cerevisiae} and \textit{S. eubayanus} (Libkind et al. 2020). There are other examples from the wine and cider industries where hybrids between \textit{S. cerevisiae} and \textit{S. kudriavzevii} or \textit{S. uvarum} are found (Gonzalez et al. 2006; Gallone et al. 2019). In nature, hybrids between \textit{S. cerevisiae} and \textit{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 \textit{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 \textit{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 \textit{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 humans commenced Indeed, this is another facet of the domestication of nature that these processes could select for more robust strains over time.

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. drosophilum). 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-
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/a heterozygote at the MAT locus, had four silent HMRa/HMLa 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.
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 ATCCA2981 and JCM22060. ATCCA2981 retains two active MATα/MATα loci, while the coisogenic strain JCM22060 lacks an active MATα 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 CBS7327 (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 α1-α2 heterodimer. This issue presumably only arises in Z. rouxii hybrids as MATα1 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. paraballii and Z. pseudobailii has never been isolated, only a single isolated of Z. pseudorouxi 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. paraballii 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 homoeolog 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 ZYGOSACCAROMYCES

Genetic crossing also can be used to uncover the molecular basis of traits and to breed new strains, including hybrids, in nonSaccharomyces 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 Wheeler 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, CBS732T is homothallic and can switch in culture (de Montigny et al. 2000). In fact, pure MATa and MATa cultures derived from CBS732T 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; MATa) and CBS4838 (NBRC1877; MATa) (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 Sykrova 2007) and allodiploid (Watanabe et al. 2017) strains. Furthermore, recent finding that nitrogen starvation triggers mating in Zygosaccharomyces establishes a foundation for the potential recovery of gametes in future outcrossing experiments (Ogata and Kuroki 2021). However, two main drawbacks still hamper the possible application of extensive breeding programs. As the HO endonuclease is probably not essential in 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 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 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. pastorius 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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