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Minireview

MINIREVIEW

Clade- and species-specific features of genome evolution in the Saccharomycetaceae

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ABSTRACT

Many aspects of the genomes of yeast species in the family Saccharomycetaceae have been well conserved during evolution. They have similar genome sizes, genome contents, and extensive collinearity of gene order along chromosomes. Gene functions can often be inferred reliably by using information from *Saccharomyces cerevisiae*. Beyond this conservative picture however, there are many instances where a species or a clade diverges substantially from the *S. cerevisiae* paradigm—for example, by the amplification of a gene family, or by the absence of a biochemical pathway or a protein complex. Here, we review clade-specific features, focusing on genomes sequenced in our laboratory from the post-WGD genera *Naumovozyma*, *Kazachstania* and *Tetrapisispora*, and from the non-WGD species *Torulaspora delbrueckii*. Examples include the loss of the pathway for histidine synthesis in the cockroach-associated species *Tetrapisispora blattae*; the presence of a large telomeric *GAL* gene cluster in *To. delbrueckii*; losses of the dynein and dynactin complexes in several independent yeast lineages; fragmentation of the *MAT* locus and loss of the *HO* gene in *Kazachstania africana*; and the patchy phylogenetic distribution of RNAi pathway components.

Keywords: evolution; comparative genomics; Kazachstania; Naumovozyma; Tetrapisispora; Torulaspora

INTRODUCTION

Yeast species provide remarkable opportunities to study genomic evolution. In the two decades since the sequence of *Saccharomyces cerevisiae* was first reported (Goffeau *et al.* 1996), three major areas of research into yeast genome evolution have developed. First, studies such as mutation accumulation experiments have provided a view of mutational processes and rates at unprecedented resolution, both in *Saccharomyces* (Lynch *et al.* 2008; Nishant *et al.* 2010; Zhu *et al.* 2014) and in other yeasts (Polakova *et al.* 2009; Friedrich *et al.* 2015). Second, population genomics

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studies are providing extensive detail about genetic variation, both in *S. cerevisiae* and in its undomesticated relative *S. paradoxus*, which has led to inferences about the natural life cycle, the strength and direction of selection, and the nature of quantitative genetic variation of yeast phenotypes (Tsai *et al.* 2008; Liti *et al.* 2009; Schacherer *et al.* 2009; Fay 2013; Bergstrom *et al.* 2014; Liti 2015). Third, the comprehensive annotation and wealth of experimental information about the functions of *S. cerevisiae* genes has provided a solid starting point for the exploration of the genomes of related fungi (Sunnerhagen and Piskur 2006; Dujon 2010; Rozpedowska, Piskur and Wolfe 2011; Zarin and Moses 2014). This third area—yeast comparative genomics—is the focus of this review.

Saccharomyces cerevisiae is a member of the family Saccharomycetaceae (Fig. 1), which in turn is part of the subphylum Saccharomycotina (Kurtzman 2011). The most striking evolutionary event in the family was the occurrence of a whole-genome duplication (WGD) approximately 100–200 million years ago (Wolfe and Shields 1997). This event defines a clade called the post-WGD species, whose genomes contain evidence of this shared event, whereas outgroup species that diverged from the *S. cerevisiae* lineage before the WGD occurred are called non-WGD species. The ancestral organism that underwent WGD contained about 5000 genes. The WGD increased this number transiently to about 10 000 genes, but most of the extra copies of genes were not retained and instead they became 'lost'—that is, one of the two genes in each pair became deleted, usually without any other rearrangements in the local area of chromosome (Byrnes, Morris and Li 2006; Scannell, Butler and Wolfe 2007a). Post-WGD species now typically contain about 5500 genes, which includes 500 pairs of genes (ohnologs) that were formed by the WGD; the other 4500 loci were not retained in duplicate and became single-copy again.

The order of genes along chromosomes (synteny) is strongly conserved within the family Saccharomycetaceae, although it is relatively poorly conserved in more distant comparisons between different families within Saccharomycotina (Dujon 2010). We developed a database and browser interface, the Yeast Gene Order Browser (YGOB—http://ygob.ucd.ie), as a resource for exploring synteny relationships among Saccharomycetaceae species (Byrne and Wolfe 2005). Synteny comparisons, both within genomes and between post-WGD and non-WGD genomes, provided the main evidence for WGD (Wolfe and Shields 1997; Dietrich *et al.* 2004; Dujon *et al.* 2004; Kellis, Birren and Lander 2004). Synteny conservation also enabled our laboratory, in 2009, to infer the approximate gene content and genome organization of the ancestral yeast organism that underwent WGD, and hence to study the chromosomal rearrangements, gene losses and gains that occurred in *S. cerevisiae* and other post-WGD species in their hundred-million-year descent from this ancestor (Gordon, Byrne and Wolfe 2009). We refer to this inferred genome as the ancestral genome. It is a description of the state of the genome immediately before the WGD occurred. Our ancestral genome reconstruction was inferred manually, whereas other groups used computer-assisted methods and obtained highly congruent results (Sankoff 2009).

The ancestral genome reconstruction provides a convenient reference point for studying the evolution of gene content and chromosome organization in Saccharomycetaceae. The ancestral genome consists of eight lists, each of which is the deduced order of genes along one of the eight ancestral chromosomes that later became duplicated by the WGD. Its genes are named according to their chromosome and position, for example, Anc_2.345 indicates the 345th gene along ancestral chromosome 2. In any non-WGD species in the Saccharomycetaceae, the gene order along any section of chromosome is usually similar to the ancestral order. Discontinuities correspond to genomic rearrangements, either in the non-WGD species or on the lineage of the ancestor (i.e. the post-WGD lineage prior to the occurrence of the WGD). In any post-WGD species, there are two genomic regions corresponding to each chromosomal region in the ancestor. Each of these regions contains a subset of the ancestral genes, usually without rearrangement of gene order, and some ancestral genes remain in duplicate (ohnologs) and thus appear on both chromosomal regions of the post-WGD species.

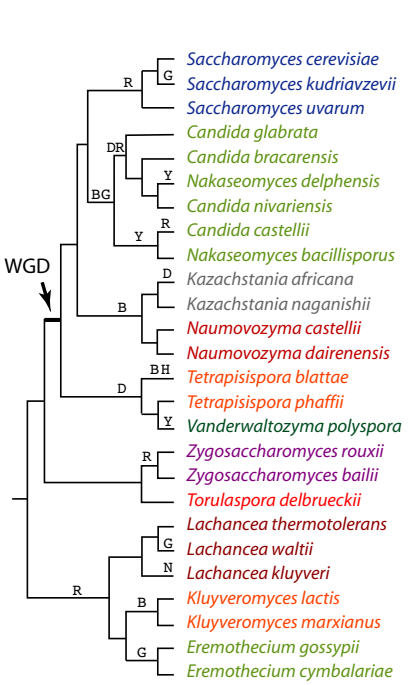
We now have a genome sequence from at least one species in almost every known genus of the family Saccharomycetaceae as defined by Kurtzman (2011) (Fig. 1). The only genera classified in this family that have not yet been sequenced are *Zygotorulaspora* (a sister clade to *Torulaspora* and *Zygosaccharomyces*; Kurtzman 2003) and *Cyniclomyces* whose phylogenetic position is uncertain and which may be basal to the family (Boundy-Mills and Miller 2011). Outside this family (Kurtzman 2003; Kurtzman and Robnett 2013), we have relatively limited genomic data from the closest outgroup genera such as *Hanseniaspora/Kloeckera* (Giorello *et al.* 2014), *Saccharomycodes*, *Wickerhamomyces* (Schneider *et al.* 2012a,b) and *Cyberlindnera* (Tomita *et al.* 2012; Freel *et al.* 2014).

Although genome evolution in the sequenced Saccharomycetaceae species has largely been conservative, with similar genes being arranged in similar ways along the chromosomes of each species, the exceptions to this rule—the differences between the species—can often be of interest and can point to differences in the biology of the species that own the genomes. In this review, we focus on aspects that are unique to the genome of a particular species or genus. We focus in particular on seven genomes that we sequenced in 2011, from three post-WGD genera (*Naumovozyma*, *Kazachstania* and *Tetrapisispora*) and one non-WGD genus (*Torulaspora*). We have previously described the evolution of the mating-type (*MAT*) loci of these species (Gordon *et al.* 2011a), but here we comment on some of their other features. Summary statistics of these genomes is given in Table 1.

Saccharomycetaceae genomes

We sampled three post-WGD genera that had not previously been extensively studied: *Naumovozyma*, *Kazachstania* and *Tetrapisispora* (Kurtzman 2003). Together with the recent sequencing of genomes in the *Nakaseomyces* clade (including *Candida glabrata* and its asexual relatives) by Gabaldon *et al.* (2013), these data mean that we now have a genome sequence for every known post-WGD genus, and multiple genomes for all post-WGD genera except *Vanderwaltozyma* (Fig. 1). The genome of *Naumovozyma castellii* had been sequenced to draft level by Cliften *et al.* (2003, 2006) and it had been shown to be a post-WGD species with differential gene loss as compared to *S. cerevisiae* (Langkjaer *et al.* 2003; Scannell, Butler and Wolfe 2007a). We completed the genome sequence of the type strain of *N. castellii* (CBS 4309; this species was previously also called *Saccharomyces castellii* and *Naumovia castellii*), and also sequenced its congener *N. dairenensis* (CBS 421). Until recently, these were the only two species known in the genus *Naumovozyma* (Liu *et al.* 2012a).

The genus *Kazachstania* is much more species rich and we sequenced two representatives, *Kazachstania naganishii* (CBS 8797) and *K. africana* (CBS 2517), that span the phylogenetic breadth of this genus (Vaughan-Martini, Lachance and Kurtzman 2011). *Kazachstania naganishii* was previously known as *S. exiguus* strain Yp74L-3, before it was realized to be a separate species distinct



	Chrs.	*DAL* genes	*GAL* genes	*BNA1-7*	*HIS1-7*	Argonaute	Dicer	NHEJ	Dynein pathway losses
Saccharomyces cerevisiae	16	clustered	+	+	+	0	0	+	-
Saccharomyces kudriavzevii	16	clustered	none	+	+	0	0	+	-
Saccharomyces uvarum	16	clustered	+	+	+	0	1	+	-
Candida glabrata	13	none	none	*BNA3*	+	0	0	+	*ARP10, NDL1, KIP1*
Candida bracarensis	12	none	none	*BNA3*	+	0	0	+	*ARP10, NDL1, KIP1*
Nakaseomyces delphensis	10	none	none	*BNA3*	+	0	0	+	11 losses
Candida nivariensis	13	none	none	*BNA3*	+	0	0	+	*ARP10, NDL1, LDB18, KIP1*
Candida castellii	8	clustered*	none	*BNA3,6*	+	0	1	+	12 losses
Nakaseomyces bacillisporus	15	clustered*	none	*BNA3*	+	1	1	+	12 losses
Kazachstania africana	12	none	+	*BNA3*	+	1	2	+	-
Kazachstania naganishii	13	clustered*	+	*BNA3*	+	1	2	+	*LDB18, KIP1*
Naumovozyma castellii	10	clustered	+	*BNA3*	+	1	1	+	-
Naumovozyma dairenensis	11	clustered	+	*BNA3,6*	+	2	3	+	-
Tetrapisispora blattae	10	none	+	none	*HIS6* only	2	1	+	*NDL1*
Tetrapisispora phaffii	16	none	+	+	+	1	1	+	-
Vanderwaltozyma polyspora	14	none	+	+	+	1	1	+	11 losses
Zygosaccharomyces rouxii	7	dispersed	+	+	+	0	0	+	*ARP10*
Zygosaccharomyces bailii	?	dispersed	+	+	+	0	0	+	*ARP10*
Torulaspora delbrueckii	7	dispersed	large cluster	+	+	1	1	+	*PAC1, NDL1*
Lachancea thermotolerans	8	dispersed	+	+	+	0	0	+	*KIP1*
Lachancea waltii	8	dispersed	none	+	+	0	0	+	*KIP1*
Lachancea kluyveri	8	dispersed	+	+	+	0	0	absent	-
Kluyveromyces lactis	6	dispersed	+	*BNA3*	+	0	0	+	*NDL1, KIP1*
Kluyveromyces marxianus	7	dispersed	+	*BNA3*	+	0	0	+	*NDL1, KIP1*
Eremothecium gossypii	7	dispersed	*GAL4* only	+	+	0	0	+	-
Eremothecium cymbalariae	8	dispersed	none	+	+	0	0	+	-

Figure 1. Phylogenetic relationships of species in the family Saccharomycetaceae, and summary of some gene content differences. Letters above branches indicate inferred points of loss of the *DAL* (D), *GAL* (G), *BNA* (B), *HIS* (H), RNAi (R; loss of all Argonaute genes or all Dicer genes), NHEJ (N) and dynein (Y) pathways. 'Chrs', number of chromosomes. '*DAL* genes' refers to *DAL1,2,3,4,7* and *DCG1*. Asterisks indicate *DAL* clusters that also include *DAL5*. '*GAL* genes' refers to *GAL1/3*, *GAL4*, *GAL7*, *GAL10* and *GAL80* (Hittinger, Rokas and Carroll 2004). The topology of the cladogram is from (Kurtzman 2003; Hedtke, Townsend and Hillis 2006; Gordon *et al.* 2011a; Gabaldon *et al.* 2013). Different colors indicate different genera. Note that *Tetrapisispora* does not appear to be monophyletic (Gordon *et al.* 2011a).

Table 1. Properties of the sequenced genomes.

	Naumovozyma castellii CBS 4309	*Naumovozyma dairenensis* CBS 421	*Kazachstania africana* CBS 2517	*Kazachstania naganishii* CBS 8797	*Tetrapisispora phaffii* CBS 4417	*Tetrapisispora blattae* CBS 6284	*Torulaspora delbrueckii* CBS 1146
Protein-coding genes	5648	5548	5378	5321	5253	5389	4972
Ancestral[a]	5199	5100	5018	4987	4991	4895	4611
Singletons[b]	365	368	314	297	237	479	256
Median length (a.a.)	412	428	400	410	419	439	405.5
tRNA genes	270	264	267	159	205	327	193
Chromosomes	10	11	12	13	16[c]	10	8
Genome size (Mb)	11.2	13.5	11.1	10.8	12.1	14.0	9.2

[a]Number of protein-coding genes with an ortholog in the ancestral genome reconstruction.
[b]Number of protein-coding genes without a syntenic ortholog in any other species.
[c]Excluding a short unmapped 17th scaffold.

from *S. exiguus* (which itself is now called *K. exigua*). Extensive methods for genetic manipulation of *K. naganishii* have been developed by Hisatomi, Kubota and Tsuboi (1999a,b) and Sugihara *et al.* (2011).

We sequenced two *Tetrapisispora* species because this genus was known to be sister to *Vanderwaltozyma*, representing the post-WGD lineage most distantly related to *S. cerevisiae*. Analysis of the genome of *Vanderwaltozyma polyspora* (obsolete name: *Kluyveromyces polysporus*) showed that it had undergone extensive loss of duplicate genes independently of the post-WGD gene losses in *S. cerevisiae* (Scannell *et al.* 2007b). We again chose two species spanning the phylogenetic breadth of the genus: *Tetrapisispora phaffii* (CBS 4417) and *T. blattae* (CBS 6284). A transformation system for *T. phaffii* has been developed (Oro *et al.* 2014). *Tetrapisispora blattae* is an outgroup to all other known species of *Tetrapisispora* (Lachance 2011). Surprisingly, we found by phylogenomic analysis that the genus *Tetrapisispora* is not monophyletic: *T. phaffii* and *V. polyspora* are more closely related to each other than either of them is to *T. blattae* (Gordon *et al.* 2011a). This relationship is shown in Fig. 1.

At 14.0 Mb excluding the rDNA locus, the *T. blattae* genome is relatively large for a post-WGD species. The genome contains some unusually large regions of non-coding DNA (for example, flanking the *MAT* genes; Gordon *et al.* 2011a). It is also characterized by the presence of regions of amino acid repeats internal to many proteins. An extreme example is its ortholog of *S. cerevisiae* Yel1 (a GEF required for localization of Arf3 to the bud neck), which is more than twice the length of the orthologs in all other Saccharomycetaceae (2001 residues compared to 687 in *S. cerevisiae*). Across the whole proteome, *T. blattae* proteins have a longer median length than any of the other species discussed here (Table 1). But at the same time, the *T. blattae* genome lacks

orthologs of some well-known large genes: it has *IRA1* but not *IRA2*, *TOR1* but not *TOR2*, *SPH1* but not *SPA2*. In each of these cases, the lost gene is one member of an ohnolog pair in *S. cerevisiae*.

We also sequenced *Torulaspora delbrueckii* (CBS 1146), a stress-tolerant yeast that is associated with winemaking (Albertin *et al.* 2014). *Torulaspora*, *Zygosaccharomyces* and a third genus *Zygotorulaspora* comprise the clade of non-WGD species that is the closest outgroup to the post-WGD species (Fig. 1) (Kurtzman 2003). The genomes of two *Zygosaccharomyces* species, *Z. rouxii* and *Z. bailii*, were sequenced by Souciet *et al.* (2009) and Galeote *et al.* (2013), respectively. So far, no *Zygotorulaspora* genome has been sequenced. Outside this small clade, there is a larger clade of better known non-WGD genera: *Kluyveromyces*, *Lachancea* and *Eremothecium* (*Ashbya*).

Clade-specific gene families, amplifications and transposable elements

Clade-specific gene amplifications are of interest because they can indicate recent directional evolutionary pressure on genomes. *Saccharomyces cerevisiae*, for example, contains more hexose transporters than other Saccharomycetaceae, and this may indicate adaptation to the 'fermentative lifestyle' (Piskur *et al.* 2006; Merico *et al.* 2007; Lin and Li 2011). In many cases, however, the amplifications are of 'orphan' genes that lack homologs in other species and whose functions are unknown. There is at least one such orphan family in *S. cerevisiae* itself: a family of five highly divergent but related genes including *ABM1*. These genes are of unknown function and appear as recent insertions into the *Saccharomyces* genome when compared to the ancestral genome (Gordon, Byrne and Wolfe 2009).

Yeasts other than *S. cerevisiae* also contain species-specific or clade-specific gene amplifications. In many of these cases, however, it is difficult to say anything about the functions of the amplified genes because they are completely unknown and the genes lack homologs or even recognizable protein domains. It is often not even clear whether the amplified genes have 'normal' cellular roles or if they are mobile genetic elements. For example, the *N. castellii* genome has two large orphan gene families, as first described by Cliften *et al.* (2006). Neither of these families has homologs in the congeneric species *N. dairenensis*. One of these families, exemplified by *NCAS0A14090*, has 19 members. It was recently identified as a transposase of the hAT family, part of a mobile element that was named *Roamer* (Sarilar, Bleykasten-Grosshans and Neuveglise 2015). The other orphan family, exemplified by *NCAS0G03840*, has 24 members and lacks any identifiable protein domains. Despite being species-specific, this family is highly divergent in sequence with only 27% amino acid sequence identity between its most diverse members. Similarly *T. phaffii* has a diverse nine-member family, again completely species-specific, exemplified by *TPHA0N00100*. Amino acid sequence identity between the two most divergent members is only 24%. This *T. phaffii* family is telomeric whereas the *N. castellii* one is not. The existence of such diverse gene families within a single species raises some interesting (but currently unanswerable) evolutionary questions about how they originated and how the members of the family became so different in sequence. In other cases orphan gene families are genus-specific, for example a family with 5 members in *N. castellii* (e.g. *NCAS0G01740*) and 15 members in *N. dairenensis* (e.g. *NDAI0G00110*). In this last example, many of the *N. dairenensis* members of the family are telomeric, whereas the five *N. castellii* genes are not telomeric but are all clustered within a 40-kb region on chromosome 7.

We identified a singleton gene in *T. blattae* (*TBLA0D02000*, 1032 amino acids) with similarity to DNA transposases of the MULE (Mutator-like element) family. MULEs are members of the Mutator superfamily of DNA transposons (class II transposable elements) (Neuveglise *et al.* 2005; Wicker *et al.* 2007). The *T. blattae* gene has highest similarity to the α3 gene of *K. lactis*, which functions in mating-type switching in that species (Barsoum, Martinez and Astrom 2010; Rajaei *et al.* 2014) and is the only other MULE-like transposase in the family Saccharomycetaceae. Complete MULE elements have terminal inverted repeats (TIRs) as well as a transposase gene, but the only described MULE in subphylum Saccharomycotina that is complete and active in transposition is the *Mutyl* element of *Yarrowia lipolytica* (Neuveglise *et al.* 2005). Neither *TBLA0D02000* nor *K. lactis* α3 is flanked by TIRs. We also found truncated non-syntenic homologs of *TBLA0D02000* in other Saccharomycetaceae: *TPHA0G02110* in *T. phaffii* (270 residues lacking the conserved transposase domain), and several possible pseudogene fragments in *N. castellii* and *N. dairenensis*.

Mating genes, pheromones and receptors

The *MAT*α1 gene of *N. dairenensis* contains two identical tandem copies of a 20-bp repeat sequence, which cause a frameshift by comparison to the α1 genes of other species. It is therefore possible that α1 is a pseudogene in this species. Losses of genes from the *MAT* locus have previously been identified in the CTG clade of *Candida* species (Logue *et al.* 2005; Butler 2010), but are uncommon in the Saccharomycetaceae where all other species have intact α1, α2 and **a**1 genes. The **a**2 gene, coding for an HMG-domain activator of transcription of **a**-specific genes (Tsong *et al.* 2003, 2006), is present in all non-WGD Saccharomycetaceae but was lost in the common ancestor of all post-WGD species, more or less concurrently with the WGD (Butler *et al.* 2004).

In *K. naganishii*, the *HMR* locus, containing a silenced copy of the mating-type **a** information, is not located near a telomere as in all other species. Instead, it is located between orthologs of the *S. cerevisiae* genes *YKR011C* and *TOF2*, more than 300 kb from the nearest telomere. Comparison of the *MAT*, *HML* and *HMR* structures in this species (Fig. 2) shows that the copy of the **a**1 gene at *HMR* is truncated at the 5′ end, which is unusual. The way these loci are organized in *K. naganishii* makes silencing of transcription at *HMR* unnecessary, because the copy of the **a**1 gene at *HMR* has no promoter or start codon. During mating-type switching, a copy of the 3′ part of the **a**1 gene from *HMR* is inserted beside the complete 5′ part of the gene at the *MAT* locus to assemble a full-length and functional **a**1 gene. The lack of requirement for silencing has apparently removed the need for *HMR* to be located beside a telomere, allowing *HMR* to move to a new chromosomal site in this species. However, in *S. cerevisiae* chromatin modification at *HMR* has a dual role: as well as silencing transcription, it also prevents the Ho endonuclease cleaving *HMR* (Haber 2012). Therefore, it is unclear what prevents *K. naganishii* Ho endonuclease from cleaving its *HMR*, and laboratory experiments will be necessary to discover whether there are chromatin modifications at *K. naganishii HMR*.

Similarly, in *T. blattae* the *HML* locus is not beside a telomere. This change is the result of a rearrangement that occurred between the *HML* locus and the ancestral telomere. The *T. blattae HML* genes (*HML*α1 = Anc_1.1 = *TBLA0A07590* and *HML*α2 = Anc_1.2 = *TBLA0A07600*) retain linkage to the end of ancestral chromosome 1 on one side (they are beside Anc_1.4 and Anc_1.5), but on the other side, where a telomere is found in most other species, they are instead adjacent to genes Anc_7.365 (*PEX13*)

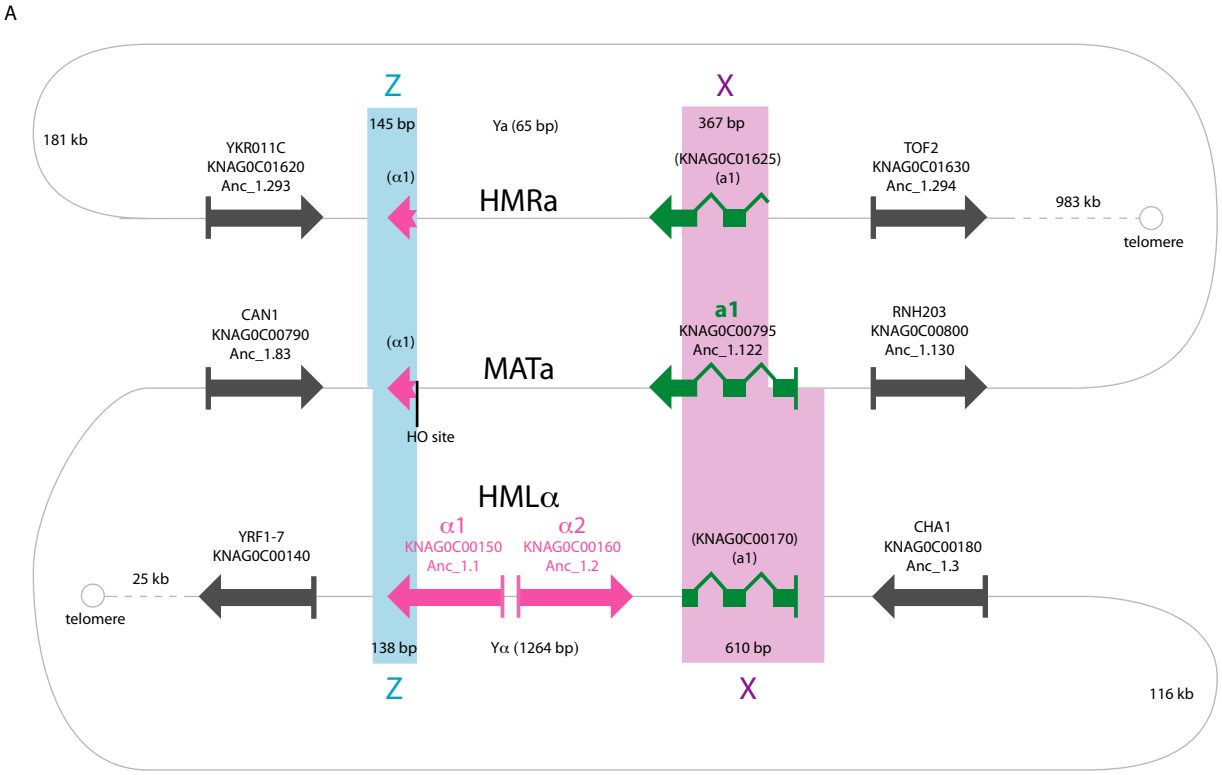


Figure 2. Non-telomeric *HMR* locus in *K. naganishii*. The organization of the *MAT*, *HML* and *HMR* loci and the triplicated X and Z repeat regions is shown schematically. *HMR* is 322 kb from one telomere and 983 kb from the other. The copy of the **a**1 gene at *HMR* lacks exon 1 and therefore does not need to be transcriptionally repressed by chromatin modification. *HML* is close to a telomere and transcription of *HMLα*1 and *HMLα*2 is probably repressed by Sir proteins. Gene copies with names in parentheses are incomplete.

and Anc_7.366 (*MMR1*) from the middle of ancestral chromosome 7. Consequently, *T. blattae HML* is more than 800 kb from the nearest telomere. It is still on the same chromosome as the *MAT* locus as seen in all Saccharomycetaceae (Gordon *et al.* 2011a). *Tetrapisispora blattae* has a low number of chromosomes for a post-WGD species (10 chromosomes; Fig. 1), and this example of a telomere fusion is one of several that have occurred in the *T. blattae* genome. Unlike the situation in *K. naganishii*, Sir proteins are probably still required for silencing of the non-telomeric *HML* in *T. blattae* because the copy of the *α*1 gene at this locus is full length and intact (*HMLα*2 is truncated at the 3′ end).

In *K. africana*, the ability to switch mating types appears to have been lost. This species has no *HO* endonuclease gene. Instead of the usual arrangement of three *MAT*-like loci (*MAT*, *HML* and *HMR*), this species has only two *MAT*-like loci which we refer to as *MAT***a** and *MATα* (Fig. 3). Neither of these loci appear to be an *HML* or *HMR*-type silent cassette because they have no X or Z repeat sequences that could allow DNA exchange to occur between the two loci during mating-type switching. The situation in *K. africana* appears to have arisen by chromosome breakage and rearrangement; its *MATα* and *MAT***a** loci share synteny with the left and right sides, respectively, of the *MAT* locus in the related species *K. naganishii* which has an organization resembling *S. cerevisiae*. We are confident that our assembly of the *K. africana* genome is structurally correct across the *MAT***a** and *MATα* loci because our sequencing strategy generated pairs of sequence reads from the ends of clones in four genomic libraries with large insert sizes (averaging 3, 7, 19 and 20 kb). The chromosome 4 and 7 rearrangements are supported by 134 and 160 unique pairs of reads, respectively, with no pairs supporting an unrearranged configuration. The type strain of *K. africana* (CBS 2517), which is the strain we sequenced, has been described as diploid and capable of sporulation (Vaughan-Martini, Lachance and Kurtzman 2011). However, we have not examined any other strains of *K. africana*.

One possible scenario to explain the rearrangement in *K. africana* is that a *MAT* chromosome broke into two pieces during an attempt to switch mating types. One piece, consisting of the right-hand part of the *MAT* chromosome (as drawn in Fig. 3) and the *MAT***a**1 gene, gained a new telomere to form *K. africana* chromosome 7. The other piece, consisting of the left-hand part of the *MAT* chromosome and the *MATα* genes, became fused end to end with another chromosome corresponding to *K. naganishii* chromosome 12 (Fig. 3). After this rearrangement occurred, the species was unable to switch mating types so the *HML*, *HMR* and *HO* loci all became unnecessary and were lost. Other scenarios, such as loss of *HO* before loss of *HML* and *HMR* and rearrangement of the *MAT* locus, are also plausible. Importantly, in the absence of any experimental data from *K. africana* it remains unclear how cell types are specified in this species, particularly whether *MAT***a** and *MATα* genes are both transcribed in haploid cells.

All species of Saccharomycetaceae contain homologs of the *S. cerevisiae* genes for mating pheromones (*α*-factor MF*α* and **a**-factor MF**a**) and their receptors (Ste2 and Ste3). The **a**-factor genes seem to be remarkably mobile: among 23 species we examined, MF**a** genes were found at 19 different genomic locations (Fig. 4A; see also OhEigeartaigh *et al.* 2011). Some of this diversity is due to high levels of gene duplication (for example *N. castellii* and *T. blattae* each have five MF**a** genes). The diversity of

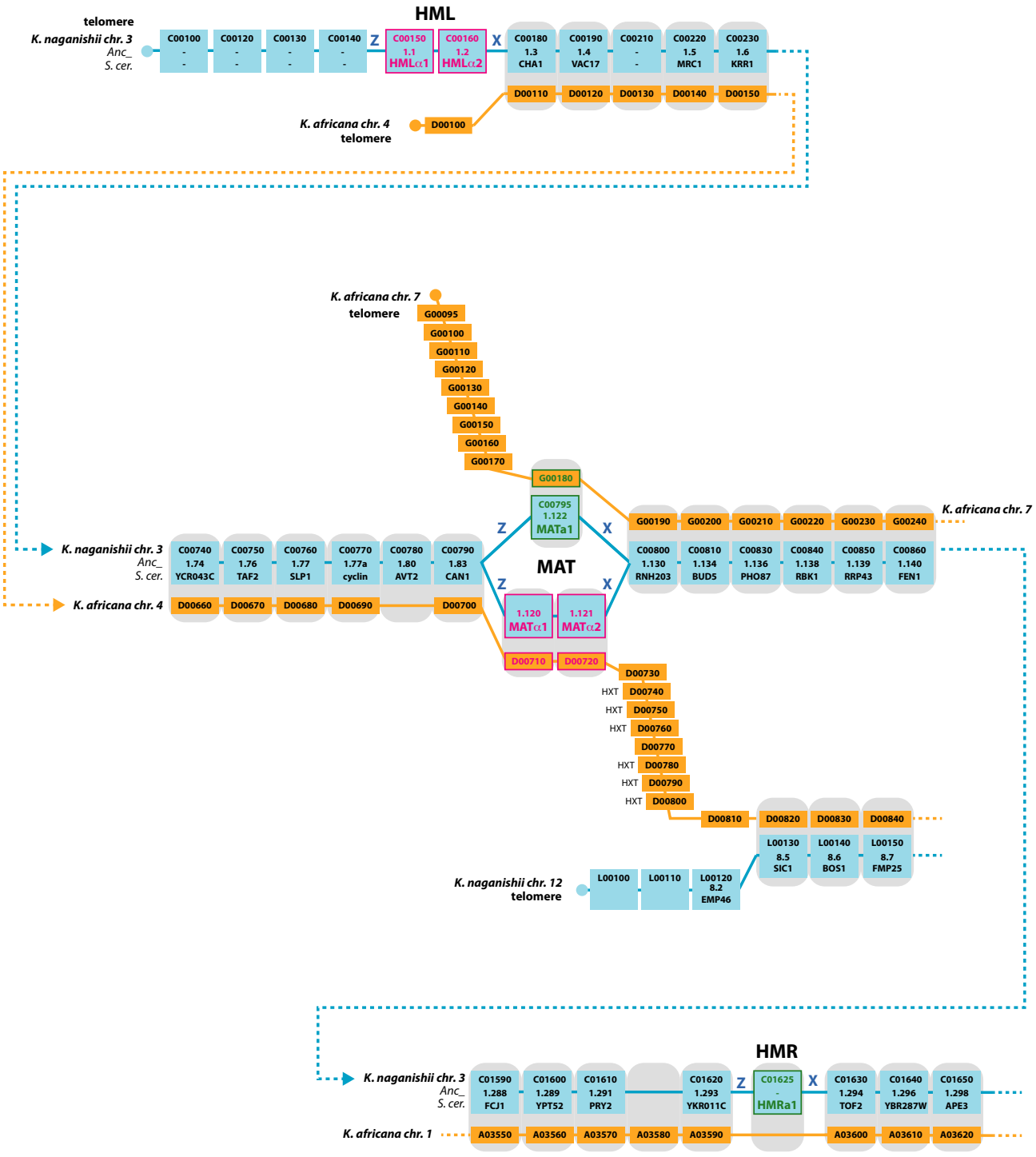


Figure 3. Evolutionary rearrangement at the *K. africana MAT* locus. *Kazachstania africana* (orange) has a MAT**a**-like locus on chromosome 7 and a MAT*α*-like locus on chromosome 4. It has no apparent *HML* or *HMR* loci, no Z or X repeats, and no *HO* endonuclease gene. For comparison, both idiomorphs (MAT*α* and MAT**a**) of the *MAT* locus in the related species *K. naganishii* are shown (blue). HXT indicates a cluster of hexose transporter genes.

locations seen even in species with only one *MF***a** gene may be due to cycles of gene duplication and loss from the original location. The *S. cerevisiae MFA2* gene is at a position (Anc_2.114) that appears to be the ancestral *MF***a** site for all post-WGD species and their close non-WGD relatives *Z. rouxii* and *To. delbrueckii*. Other non-WGD species show *MF***a** genes at four different sites and it is unclear which of these sites is ancestral to the non-WGD clade (Fig. 4A). Notably, in many cases where an *MF***a** gene has a location unique to a single species or genus, the location is also a site of rearrangement in that clade relative to the ancestral gene order (indicated by two 'nearest ancestral' genes in Fig. 4A). This suggests that the duplication of *MF***a** genes may have occurred at the same time as the chromosomal rearrangement. The high mobility of *MF***a** genes may be related to their

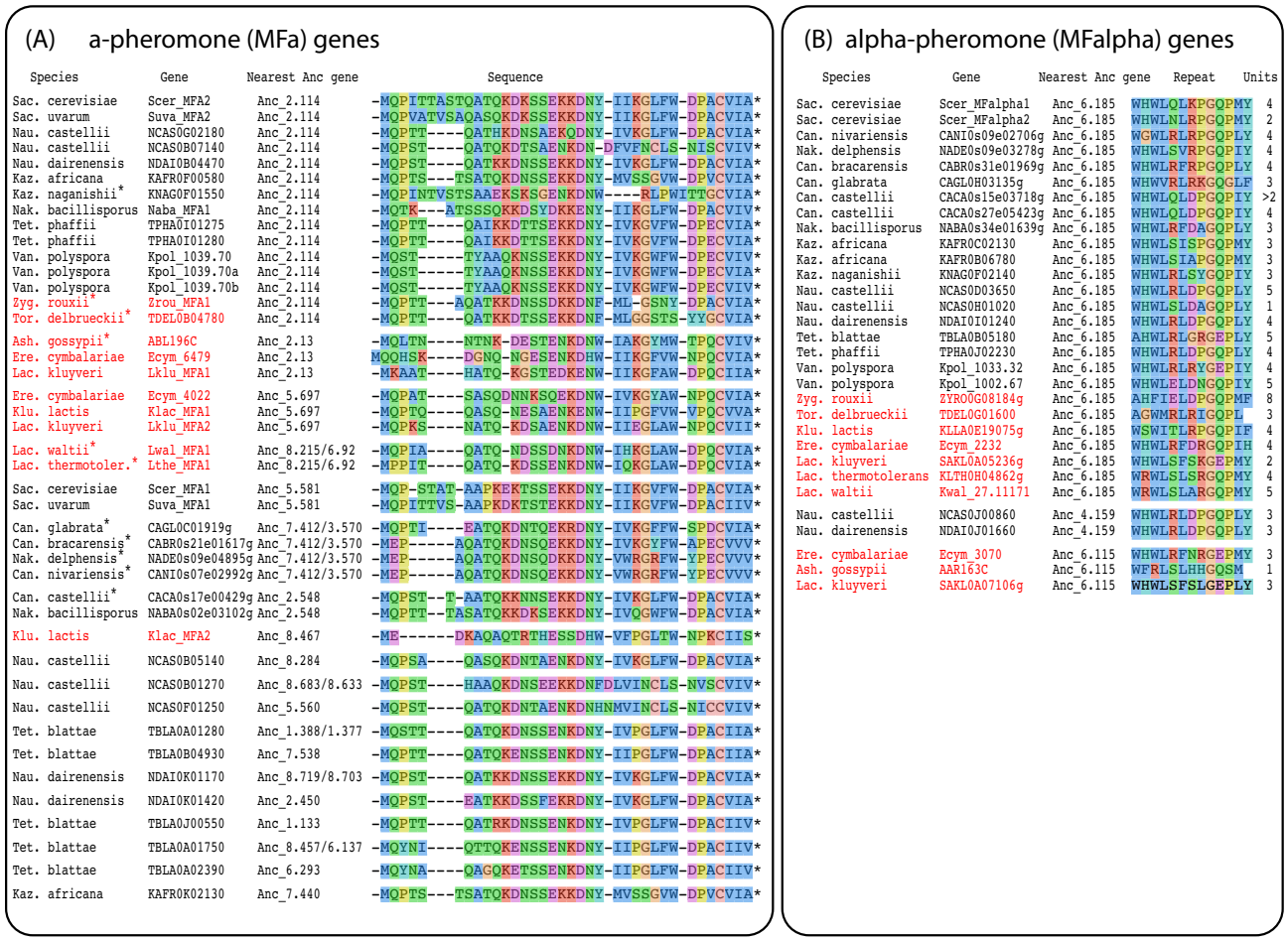


Figure 4. Pheromone genes in Saccharomycetaceae species. Species named in red are non-WGD species. (**A**) *MFa* genes. Complete **a**-factor proteins sequences are aligned and arranged into groups of orthologs according to their genomic location. For each gene, the nearest 'Anc' gene in the ancestral numbering system (Gordon *et al.* 2009) is shown. Genes with two Anc numbers are located at points of rearrangement relative to the ancestral genome. Species marked with asterisks have only one *MFa* gene. (**B**) *MFα* genes. For each gene, the number of mature pheromone repeat units and the amino acid sequence of the most common repeat unit is shown.

small size, with a coding region of only ~100 bp (OhEigeartaigh *et al.* 2011).

In contrast to the *MFa* genes, genes for α-pheromone and the Ste2/Ste3 pheromone receptors show more sedate modes of evolution. The α-pheromone gene(s) code for precursor proteins containing one to eight repeats of an active peptide that is 13 amino acid residues long in most species (Fig. 4B). Exceptions to this pattern are Ashbya *gossypii* whose *MFα* gene (*AAR163C*) codes for a single copy of a 12-mer peptide that has been shown to be functional (Wendland, Dunkler and Walther 2011), and *To. delbrueckii* whose *MFα* gene (*TDEL0G01600*) appears to code for three copies of a 12-mer peptide (Fig. 4B). Only two duplications of *MFα* genes can be inferred, apart from the retention of two ohnologs of the gene at its ancestral location (Anc_6.185) in many species after WGD. One duplication produced a second copy in an ancestor of *Naumovozyma* species, and the other produced a second copy in an ancestor of the *Eremothecium/Lachancea* clade (Wendland, Dunkler and Walther 2011). Interestingly, neofunctionalization of extra copies of pheromone genes appears to have occurred, separately in *A. gossypii* and *N. dairenensis*, after these two duplications. In each case, a gene was formed (*AFL062W* and *NDAI0F02280*) that codes for an N-terminal secretion signal similar to a pheromone precursor protein, but the gene does not code for any pheromone-like repeats. The current functions of these genes are unknown. The *STE2* and *STE3* pheromone receptor genes are single copy in all species, except *V. polyspora* which retained two *STE2* ohnologs after WGD, and they have not moved from their ancestral locations in any species.

Gene clusters

The *DAL* cluster of six genes, coding for the allantoin catabolism pathway, is the largest metabolic gene cluster in *S. cerevisiae* (Wong and Wolfe 2005; Naseeb and Delneri 2012). We previously showed that there is also a *DAL* cluster in *N. castellii*, but not in any non-WGD species, and that this cluster was assembled by relocation of genes after the WGD (Wong and Wolfe 2005). *Naumovozyma dairenensis* contains a *DAL* cluster with identical gene content and order to *N. castellii*. In *K. naganishii*, the cluster has expanded even more by the incorporation of a seventh gene, the allantoin transporter *DAL5/KNAG0D03140*, into the cluster. Seven-gene *DAL* clusters including *DAL5* have also been found in *Nakaseomyces bacillisporus* and *C. castellii* (Gabaldon *et al.* 2013). The phylogenetic distribution of the seven-gene cluster, and the fact that *DAL5* is at a different place in the clusters, suggests that *DAL5* was recruited into the cluster by two separate events in the *Kazachstania* and *Nakaseomyces* genera.

The *DAL* cluster in *K. naganishii* is not located at a site orthologous to the *DAL* clusters in *S. cerevisiae* and *N. castellii*, but instead

is at a site that corresponds to a point of rearrangement between an ancestral telomere (Anc_3.581) and an internal chromosomal site (Anc_4.55). In stark contrast to the *K. naganishii* cluster, the *DAL* genes are completely absent from the genomes of its congener *K. africana* and both of the *Tetrapisispora* species, as well as the previously reported absences from *V. polyspora* (Scannell *et al.* 2007b) and four species in the *C. glabrata/Nakaseomyces* clade (Gabaldon *et al.* 2013) (Fig. 1). Thus, the *DAL* genes are either tightly clustered in the genome or completely absent from the genome, in all post-WGD species, whereas they are scattered around the genome in all non-WGD species. It appears that the duplication of the gene pairs *DAL7/MLS1* and *DAL4/FUR4*, which occurred as part of the WGD, facilitated a major reorganization of the *DAL* pathway, enabling both the formation of a cluster and multiple subsequent losses of that cluster.

Similar to the *DAL* genes, the *GAL* genes for galactose catabolism form a cluster in many yeast species but are completely absent in others (Hittinger, Rokas and Carroll 2004; Slot and Rokas 2010) (Fig. 1). When present in Saccharomycetaceae, this cluster is usually found in a conserved syntenic location corresponding to the position of *S. cerevisiae GAL1/3*, *GAL7* and *GAL10* (Anc_3.217–3.219). In contrast, we found that *To. delbrueckii* has no *GAL* genes at this ancestral location but instead has a large cluster of *GAL* genes near the telomere of chromosome 5 (Fig. 5A). The telomeric cluster spans 22 kb and contains two *GAL1* genes (74% amino acid sequence identity), two *GAL10* genes (79% identity) and one *GAL7* gene. In addition, it contains one copy each of the genes *GAL4* (transcription activator) and *GAL2* (galactose permease) which do not form part of the cluster in other Saccharomycetaceae. The *To. delbrueckii* cluster also contains genes for additional enzymatic steps in the Leloir pathway: it has homologs of *S. cerevisiae MEL1* (secreted alpha-galactosidase, which converts extracellular melibiose into galactose and glucose), and *PGM1* (phosphoglucomutase, the glycolytic step immediately downstream of the Gal7 step). The cluster also has a homolog of *K. lactis HGT1* (high-affinity glucose transporter; Billard *et al.* 1996). The 10-gene cluster therefore contains all the genes necessary for conversion of extracellular melibiose into glucose-6-phosphate. Although its telomeric location might suggest that the cluster was gained by horizontal gene transfer into *To. delbrueckii*, phylogenetic analysis does not provide clear support for this hypothesis (Fig. 5B); the *To. delbrueckii* genes are of Saccharomycetaceae origin and its *GAL7* and *GAL4* genes group with *Zygosaccharomyces* (which has a *GAL1-GAL10-GAL7* cluster at the ancestral location) as expected in the absence of horizontal transfer. *Torulaspora delbrueckii* also contains a third *GAL10* gene (*TDEL0G04910*) near another telomere; all three Gal10 proteins contain the fused epimerase and mutarotase domains typical of Saccharomycetaceae (Slot and Rokas 2010). The multiple *GAL10* and *GAL1* genes appear to have originated by duplications that occurred within the genus *Torulaspora* (Fig. 5B). It is also notable that *To. delbrueckii* has no homolog of *GAL80* (an inhibitor of *GAL4*).

RNAi pathway genes

There is no RNA interference pathway in *S. cerevisiae*, but there is one in *N. castellii* and *V. polyspora* (Drinnenberg *et al.* 2009). The primary actors in this pathway are Dicer and Argonaute proteins. The genes coding for these proteins have a patchy phylogenetic distribution (Fig. 1), and it has been proposed that the presence of an RNAi system is inversely correlated with the presence of double-strand RNA killer viruses (Drinnenberg, Fink and Bartel 2011). Argonaute and Dicer genes are present in only 1 species (*To. delbrueckii*) out of the 11 non-WGD Saccharomycetaceae species whose genomes have been sequenced, although they are present in *C. albicans* and many other outgroup fungi (Alexandersson and Sunnerhagen 2005). Among the post-WGD species, Argonaute is present in all studied species of two very divergent clades—the *Vanderwaltozyma/Tetrapisispora* clade and the *Naumovozyma/Kazachstania* clade. Argonaute has been lost in the *Saccharomyces* genus and in all studied species of the *Nakaseomyces/C. glabrata* clade (Fig. 1) with the sole exception of *Na. bacillisporus*. It is also of interest to note that two species retained both of the ohnolog copies of the Argonaute gene after the WGD, which suggests that some functional divergence or specialization within the RNAi pathway may have occurred in these species (*N. dairenensis* and *T. blattae*). The phylogenetic distribution of Dicer genes closely follows that of Argonaute, except that there are two species that retain Dicer but not Argonaute (*C. castellii* and *S. uvarum*, the sole member of the genus *Saccharomyces* that has any RNAi component).

Missing genes and pathways

Comparison of the genome sequences to the ancestral genome reveals some examples of losses of complete biochemical pathways or protein complexes. The most dramatic of these is the absence of the histidine biosynthesis pathway in *T. blattae*, which lacks six genes (*HIS1, HIS2, HIS3, HIS4, HIS5* and *HIS7*) from this seven-gene pathway. *Tetrapisispora blattae* is known to be unable to grow on minimal media without histidine (Lachance 2011). The only gene it retains in the pathway is *HIS6*. It is possible that this auxotrophy reflects the natural environment of *T. blattae* because the only two known strains of this species were isolated from cockroaches, but it is unclear whether *T. blattae* is an intestinal symbiont of cockroaches or merely cockroach associated (Lachance 2011).

Almost all genes of the dynein/dynactin pathway (Winey and Bloom 2012), which in *S. cerevisiae* controls movement of the nucleus into daughter cells during budding, are missing in four species: *V. polyspora* (first reported by Scannell *et al.* 2007b), *Na. delphensis*, *Na. bacillisporus* and *C. castellii*. The 11 missing genes are *DYN1* (=*DHC1*), *DYN3*, *ARP1*, *ARP10*, *JNM1, NIP100, PAC1*, *PAC11*, *NDL1*, *LDB18* and *KIP1*. The latter two species are also missing a 12th gene, *KIP2*. The topology of the species tree (Fig. 1) shows that loss of 11–12 genes from this pathway has occurred independently three times. Some of the pathway components, particularly *NDL1* and *KIP1*, have also been lost repeatedly in many other Saccharomycetaceae species without complete collapse of the pathway (Fig. 1).

Losses of *BNA* genes in the seven-gene pathway for *de novo* NAD synthesis have previously been reported in *C. glabrata* and other members of the *Nakaseomyces* genus (Domergue *et al.* 2005; Gabaldon *et al.* 2013). The *BNA* pathway is also missing in the genera *Kluyveromyces, Kazachstania* and *Naumovozyma*, and in *T. blattae* (Fig. 1). Most species that have lost the pathway retain *BNA3*. However *T. blattae* has lost *BNA3* as well as the other six genes. Two species (*N. dairenensis* and *C. castellii*) retain *BNA6* as well as *BNA3*, but have lost the other five genes. *Candida castellii BNA6* is present at the gene's ancestral location (Anc_3.563), whereas *N. dairenensis BNA6* has transposed to a site of species-specific genomic rearrangement. Phylogenetic analysis indicated accelerated evolution in both these *BNA6* genes, but no evidence of horizontal gene transfer (data not shown). These gene distributions indicate that *BNA1,2,4,5,7* have been lost on a minimum of four independent occasions (marked by

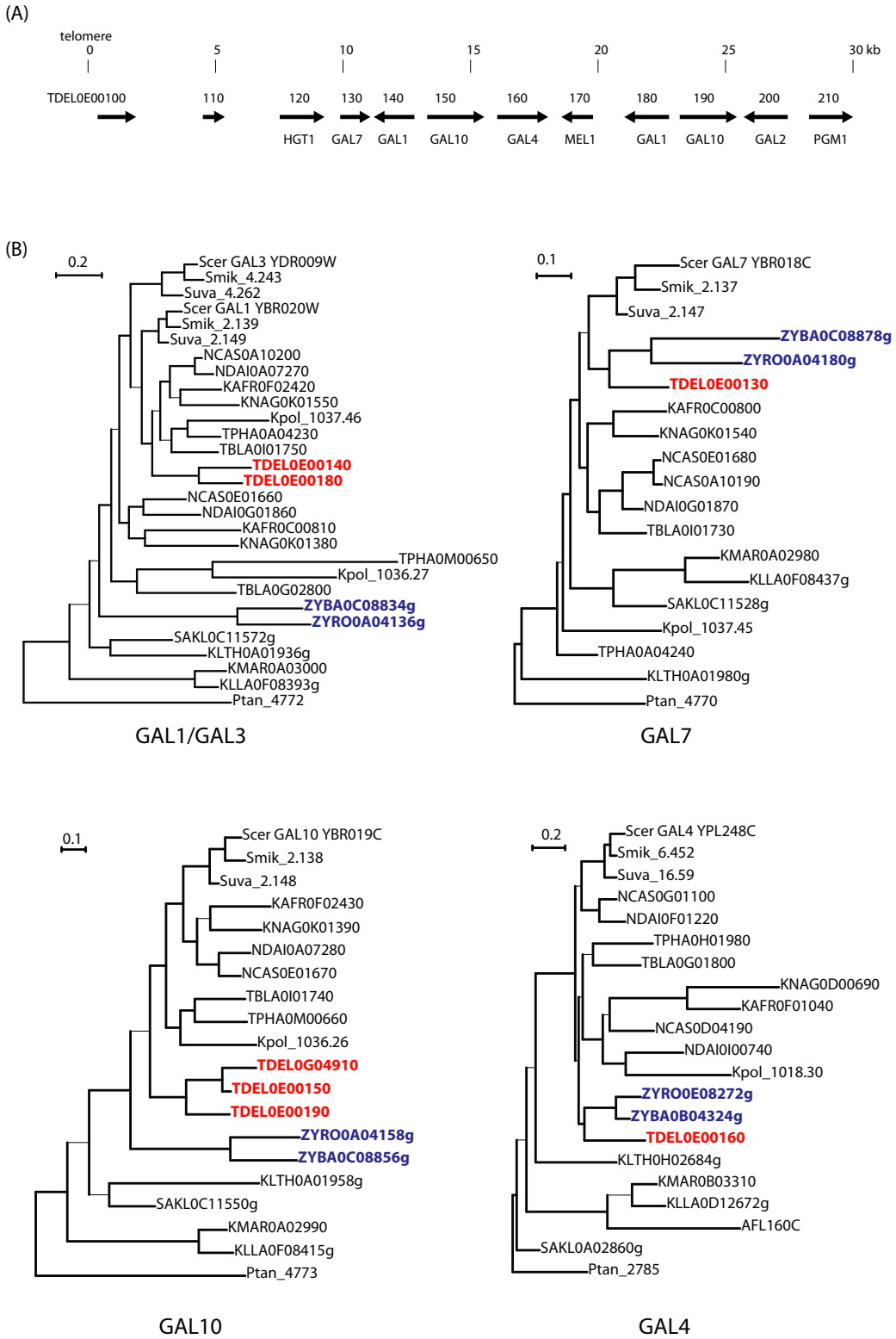


Figure 5. (**A**) Large *GAL* gene cluster near the telomere of *To. delbrueckii* chromosome 5. (**B**) Phylogenetic trees of *GAL1/GAL3*, *GAL7*, *GAL10* and *GAL4* genes. Sequences from *To. delbrueckii* (TDEL) are highlighted in red. Sequences from *Zygosaccharomyces* (ZYRO—*Z. rouxii* and ZYBA—*Z. bailii*; Souciet *et al.* 2009; Galeote *et al.* 2013), the sister taxon to *Torulaspora*, are highlighted in blue. Amino acid sequences were aligned using Clustal Omega, filtered with Gblocks and trees were constructed using PhyML, all as implemented in Seaview (Gouy, Guindon and Gascuel 2010). Thin lines indicate branches with aLRT (approximate likelihood ratio test) support values below 80%. Trees were rooted using *Pachysolen tannophilus* (Ptan) *GAL* genes (Liu *et al.* 2012b).

'B' in Fig. 1), and *BNA6* has been lost at least six times, during Saccharomycetaceae evolution.

As previously noted (Gordon, Byrne and Wolfe 2011b), *L. kluyveri* lacks four genes essential for non-homologous end joining (NHEJ), a DNA repair pathway that is normally used to repair double-strand DNA breaks in situations where homologous recombination is not possible, such as in haploid cells. These genes are *DNL4* (DNA ligase IV), *POL4* (DNA polymerase IV), *LIF1* (ligase interacting factor) and *NEJ1* (a regulator of NHEJ) (Deshpande and Wilson 2007). The absence of these genes is possibly a factor in the low level of rearrangement seen in the *L. kluyveri* genome as compared to other Saccharomycetaceae (Gordon, Byrne and Wolfe 2011b). In contrast, the NHEJ pathway is intact in all 25 other species shown in Fig. 1, including two other *Lachancea* species.

In summary, the tree in Fig. 1 indicates that there have been multiple independent losses, in different clades, of many groups of functionally related genes: three independent losses of the *DAL* genes, four losses of *GAL* genes, four losses of *BNA* genes, one loss of *HIS* genes, five losses of the RNAi pathway, one loss of NHEJ and three losses of the dynein/dynactin complex. Almost every species in the tree has been affected by one or other of these losses, which are dramatic but evidently not catastrophic.

CONCLUSION

These examples of interspecies functional variation highlight the shortcomings of *S. cerevisiae*, or indeed any single species, as a model for the biology of a wider taxonomic clade such as the Saccharomycetaceae. The RNAi pathway serves as a case in point: the functions of the Argonaute and Dicer genes in *N. castellii* would never have been discovered if *S. cerevisiae* was the only model organism, no matter how intensively *S. cerevisiae* was studied. These genes were saved from the anonymity of being labeled as 'conserved hypothetical' genes because they had homologs in organisms such as *Caenorhabditis* and *Schizosaccharomyces* in which RNAi had already been discovered. By extension, it is very probable that some other important biochemical pathways, protein complexes and even biological processes that are widely conserved across many yeast species remain undiscovered because they are both (i) absent in the model organism *S. cerevisiae*, and (ii) yeast specific so their functions cannot be inferred from other eukaryotes.

Our understanding of Saccharomycetaceae genome evolution is also incomplete because for most clades except *Saccharomyces* we only have one genome sequence per species. Population genomics has revealed that there is extensive intraspecies polymorphism of gene content, particularly at telomeric regions, leading to the concept of the pan-genome as the complete set of genes that exists within a species even if no individual member of the species contains them all (Song *et al.* 2015). *Saccharomyces kudriavzevii* provides a spectacular example of intraspecies polymorphism, with the *GAL* genes being intact in Portuguese isolates but pseudogenized in Japanese isolates, the result of a balanced polymorphism at multiple separate genomic loci (Hittinger *et al.* 2010). It is possible that a similar situation of intraspecies presence/absence polymorphism could pertain to other sets of genes that appear to have multiple independent losses in Fig. 1, such as the *DAL* and *BNA* genes, and that we simply have not yet sampled enough individuals from the lineages showing apparent losses.

Just as the lab strain S288c has turned out to be an imperfect representative of the species *S. cerevisiae*, with unrepresentative alleles at loci such as *FLO8* and *MKT1* (Liu, Styles and Fink 1996; Lewis *et al.* 2014), the species *S. cerevisiae* is also an imperfect representative of the family Saccharomycetaceae. That does not however mean that any other species could be a better model organism. Instead, we may benefit by extending the concept of the pan-genome to higher taxonomic levels to describe the complete set of biological molecules, complexes and processes that exists within Saccharomycetaceae, even if no single species contains the whole set. Ideally, we would then like to ask the evolutionary reasons why particular parts of the pan-genome are missing in particular lineages. However, our ability to answer such questions is severely limited by the elephant in the room: our lack of knowledge about the natural environments in which the different yeast species have evolved and the niches (if any) to which they are adapted (Goddard and Greig 2015).

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