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Research Article

RESEARCH ARTICLE

Recombination sites on hybrid chromosomes in *Saccharomyces pastorianus* share common sequence motifs and define a complex evolutionary relationship between group I and II lager yeasts

Chandre Monerawela and Ursula Bond[†]

School of Genetics and Microbiology, Trinity College Dublin, Dublin 2, Ireland

*Corresponding author: School of Genetics and Microbiology, Trinity College Dublin, Dublin 2, Ireland. Tel: +353189626578; E-mail: ubond@tcd.ie
One sentence summary: The analysis of recombination sites on hybrid chromosomes of lager yeasts reveals common sequence motifs and uncovers a complex evolutionary relationship between group I and II lager yeasts.
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[†]Ursula Bond, http://orcid.org/0000-0003-2877-6460

ABSTRACT

Saccharomyces pastorianus, referred to as lager yeasts, are hybrids of *S. cerevisiae* and *S. eubayanus*. Isolates within the species are divided into two groups (I and II) based on chromosome structure and composition. Following the hybridisation, the parental chromosomes underwent homeologous recombination, generating a set of hybrid chromosomes unique to the species. Here, we assessed the recombination events in seven lager yeast genomes to more clearly define the evolutionary route of lager yeasts. Meta-analysis of the recombination epicentres, as well as a detailed analysis of recombination events at the *MAT* locus, reveals a more complex evolutionary relationship between the group I and II lager yeasts than previously considered and identifies several divergent routes of evolution leading to the current *S. pastorianus* strains. We show that recombination epicentres contain sequential runs of pyrimidines, often flanked by purines, on one strand of the DNA, and identify two common sequence motifs present in >80% of the recombination epicentres, indicating that a common mechanism might account for the recombination events. Taken together, the data support a sequential hybridisation model of evolution for the two types of lager yeasts and suggest that the genomes of this newly emerged species are highly dynamic and continually evolving.

Keywords: lager yeasts; hybrid chromosomes; recombination epicentres; common motifs; evolution

INTRODUCTION

Yeasts used in the production of lager beers belong to the species *Saccharomyces pastorianus*, an interspecies hybrid of *S. cerevisiae* and *S. eubayanus* (Querol and Bond 2009; Wendland 2014; Gibson and Liti 2015; Monerawela and Bond 2017). The hybridisation event occurred ∼500–600 years ago in Central Europe around the time when brewing practices were converting from a cottage industry to a skilled craft. The *S. cerevisiae* parent of the lager yeasts appears to be most closely related to yeast isolates associated with the production of Ale beer although the exact isolate/strain involved in the hybridisation remains to be identified (Legras *et al.* 2007; Dunn and Sherlock 2008; Monerawela *et al.* 2015). *Saccharomyces eubayanus* is a cold-tolerant yeast preferring to grow at temperatures of 8°C –15°C. The species does not appear to be indigenous to Europe and was first identified

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in Patagonia, South America (Libkind *et al.* 2011). Initially, it was proposed that *S. eubayanus* found its way to Europe as part of the trade route established by Conquistadors during the exploration of the New World. However, the recent discovery of wild isolates of *S. eubayanus* in Tibet, China, North America and New Zealand (Bing *et al.* 2014; Peris *et al.* 2014; Gayevskiy and Goddard 2016) expands the possible geographical origins of the lager yeast parent. Genome analysis identified an *S. eubayanus* isolate from Tibet as sharing the greatest identity with the non-*cerevisiae* portion of the lager yeast genome (Bing *et al.* 2014; Peris *et al.* 2014, 2016).

The emergence of the new species *S. pastorianus* may have been influenced by the Beer Purity law (*Reinheitsgebot*), introduced in Bavaria in 1516, which restricted the brewing of beer to the winter months. Thus, the happenstance of the hybridisation event created a novel species possessing the cryotolerance of *S. eubayanus* and the fermentative capacity of the mesophilic *S. cerevisiae*, ideally adapted to winter month fermentation.

At least two types of lager yeasts have been identified, which are referred to as groups I and II, or Saaz and Frohberg types, respectively (Dunn and Sherlock 2008). The chromosome structures and composition of the group I and II lager yeasts have been characterised by whole genome sequencing and by competitive genome hybridisation analyses (Bond *et al.* 2004; Dunn and Sherlock 2008; Nakao *et al.* 2009; Hewitt *et al.* 2014; Walther, Hesselbart and Wendland 2014). The group I strains have an approximate triploid DNA content, consisting of a haploid *S. cerevisiae* and a diploid *S. eubayanus* genome content. On the other hand, group II strains are approximately tetraploid, containing both diploid *S. cerevisiae* and *S. eubayanus* genome contents. Additionally, significant aneuploidy is observed in both groups with the prototypic group I strain CBS 1513 possessing a total of 47 chromosomes (3n-1) while the prototypic group II strain Weihenstephan 34/70 contains 66 chromosomes (4n+ 2)(Wendland 2014; Monerawela and Bond 2017).

In addition to the parental chromosomes, the lager yeast strains contain a unique set of hybrid chromosomes arising from recombination, at specific locations, between the homeologous parental chromosomes (Bond 2009). The majority of the recombination sites are intragenic giving rise to a unique set of hybrid genes composed of part *S. eubayanus* and part *S. cerevisiae* sequences. An analysis of recombination events in the genomes of two group I strains (CBS 1513 and 1503) and two group II strain (WS 34/70 and CBS 1260) revealed that the group I and II strains shared several recombination sites (Hewitt *et al.* 2014; Walther, Hesselbart and Wendland 2014). In addition to shared recombination sites, each lager yeast strain possesses its own unique set of recombination sites. Interestingly, recombination at some of the identified recombination sites can be induced by exposure of lager yeasts to extreme high temperature followed by growth on medium containing high maltose concentrations, suggesting that recombination events between homeologous chromosomes can be triggered by environmental stress (James *et al.* 2008).

The presence of shared recombination sites in group I and II strains led to the hypothesis that the two types of lager yeast arose from a single hybridisation event between *S. cerevisiae* and *S. eubayanus* (Wendland 2014). It was proposed that following the initial hybridisation, the two groups subsequently evolved independently through further recombination events and a dramatic reduction in the *S. cerevisiae* content in group I strains. However, several other lines of evidence such as the composition of subtelomeric regions and single nucleotide polymorphisms (SNPs) between group I and II strains suggest a greater divergence in the *S. cerevisiae* content of group I and II strains than would be expected from a single hybridisation event occurring 500–600 years ago (Baker *et al.* 2015; Monerawela *et al.* 2015). Analysis of the composition of subtelomeric regions of lager yeasts revealed distinct relationships between group I yeasts and *S. cerevisiae* strains used in Ale production and between group II yeasts and *S. cerevisiae* strains used in Stout production in the British Isles. As it is highly unlikely that the subtelomeric composition would occur randomly in many different Ale and Stout strains, Monerawela *et al.* (2015) proposed that the two groups of lager yeasts arose by separate independent hybridisation events between *S. eubayanus* and two distinct *S. cerevisiae* isolates.

The recent discovery that group II yeasts appear to contain two different *S. cerevisiae* subgenomes posits yet another evolutionary model involving sequential rounds of hybridisation between *S. cerevisiae* isolates and *S. eubayanus* leading to the formation of group I and II lager yeast strains, respectively (Okuno *et al.* 2016).

Given the central role played by homeologous chromosome recombination in the evolutionary pathway of lager yeasts, here we reassessed the recombination sites in a broader range of lager yeast genomes to more clearly define the relationship between the two groups of lager yeasts. Second, with a more accurate assignment of the recombination sites, we determined if the recombination sites shared common sequence motifs. We identified two sequence motifs found at many of the recombination sites suggesting that a common mechanism might account for the recombination events. Meta-analysis of the recombination sites in different strains of lager yeasts, as well as a detailed analysis of the recombination sites at the *MAT* locus, reveals a more complex evolutionary relationship between the group I and II lager yeasts than previously considered.

METHODS AND MATERIALS

Strains

Saccharomyces cerevisiae, *S. pastorianus* and *S. eubayanus* strains (Table S1, Supplemental Information) were acquired from various yeast culture collections including the Collection de Levures d'Interet Biotechnologique (CLIB, Paris France), the Belgium Lager Strains Collection (BLSC), European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF, Frankfurt, Germany), the Portuguese Yeast Culture Collection (PYCC, Lisbon, Portugal), VTT Technical Research Centre of Finland (VTT, Helsinki, Finland) and Guinness Brewery (Dublin, Ireland).

DNA extraction and polymerase chain reaction (PCR)

Yeast strains were cultured in YEP (3% yeast extract peptone) supplemented with 2% maltose at 30°C for 16–18 h. DNA was extracted using the phenol–chloroform method as previously described (James *et al.* 2002). PCRs were carried out as previously described (Monerawela *et al.* 2015). The DNA sequences of primers used in the PCR reactions are shown in Table S3 (Supplemental Information). PCR products were electrophoresed on a 1% agarose gels in Tris Acetate EDTA buffer and were visualised by staining with ethidium bromide (Canavan and Bond 2007).

Bioinformatics analysis of recombination sites

DNA sequences corresponding to the region surrounding previously identified recombination sites in *S. pastorianus*

Table 1. Genome databases.

Group	Strain	Genome size	Genbank accession number	Reference
I	CBS1503	17.2	BBYV01000000	Okuno *et al.* (2016)
I	CBS1513	19.5	AZCJ01000000	Walther *et al.* (2014)
		19.2	BBYW01000000	Okuno *et al.* (2016)
I	CBS1538	14.4	BBYX01000000	Okuno *et al.* (2016)
II	WS 34/70	25.0	ABPO01000000	Nakao *et al.* (2009)
		22.5	BBYU01000000	Okuno *et al.* (2016)
II	CBS1483	21.97	JTFI01000000	van den Broek *et al.* (2015)
II	CCY48–91	24.2	ALJS01000000	Kvasnicka *et al.* (2012)
II	790	22.7	LSMH01000000	De León-Medina *et al.* (2016)

were used as a query sequence in BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the corresponding homology region in *S. cerevisiae* from the *Saccharomyces* Genome Database (SGD; yeastgenome.org). The *S. cerevisiae* genome sequence generally consisting of the gene containing the recombination site as well as the adjacent intergenic region and the downstream gene was used to search for the homologous regions in *S. eubayanus* and *S. pastorianus* using the Genbank database (https://www.ncbi.nlm.nih.gov/genbank/). By limiting sequence identity to 90%–98% of the *S. cerevisiae* query sequence, hybrid chromosomes in *S. pastorianus* were identified as this approach eliminates the *S. cerevisiae*-like homologous sequence, which return sequence identity of >99% and the *S. eubayanus*-like sequences, which return <90% sequence identity. The identified sequences were aligned by ApE (http://biologylabs.utah.edu/) and/or ClustalW2/ClustalO (http://www.clustal.org/). Percentage sequence identity maps were generated directly from the Clustal alignments using a sliding window DNA sequence comparison algorithm with 100 nt increments (kindly provided by Dr Kenneth H. Wolfe University College Dublin). Recombination sites were identified as the genome regions where the *S. cerevisiae* and *S. eubayanus* percentage identity lines intersect on the map. Recombination epicentres were defined as the region between the last *S. cerevisiae*-like nt and the first *S. eubayanus*-like nt or vice versa in a 5′ to 3′ direction. The recombination site within *YPR160W* was previously identified in the group II CMBS strain (Usher and Bond 2009).

Recombination sites were previously identified in the lager yeast CBS1538; however, the chromosome locations were not resolved at the nucleotide level (Okuno *et al.* 2016). To identify the recombination sites, *S. cerevisiae* query sequences of up to 50 kb, spanning multiple ORFs, were used to locate the hybrid regions in CBS 1538. Sequences sharing 90%–98% identity to the *S. cerevisiae* query sequence were processed through the percentage sequence identity maps analysis as described above.

To search for recombination events in the *S. pastorianus* genomes CCY48–91 and 790, the acquired hybrid gene sequences from the above analysis were used to search for homologous regions showing greater than 99% identity in CCY48–91 and 790 using NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Autonomously replicating sequences (ARS, acquired from http://cerevisiae.oridb.org/) were identified in *S. cerevisiae* S-288c in SGD. Distances were calculated between the *S. cerevisiae* co-ordinates of the recombination epicentres and the nearest ARS sequence.

Spo11p-binding sites in the vicinity of the recombination epicentres were located using the genome browser in SGD (Pan *et al.* 2011) using the corresponding *S. cerevisiae* co-ordinates.

Motif identification

Identified recombination epicentres (36) and immediate surrounding DNA sequences (40–50 bp) as well as 36 randomly generated 40 bp DNA sequences generated at http://www.bioinformatics.org/sms2/random_dna.html were analysed for common motifs by inputting the sequences into the discriminative mode of MEME (http/::meme-suite.org:tools:meme). The minimum length of motif was set at 6 nt and the maximum at 20–30 nt with searches on one or both strands of the DNA. Motifs enriched in the epicentres and not in the randomly generated sequences, with P values < 0.001, were considered significant (Bailey and Elkan 1994).

Motifs identification was confirmed and extended by sequence alignment of recombination epicentres using the multiple sequence alignment programme MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/). The resulting alignment was manually assessed for common motifs and base composition between the recombination epicentres.

RESULTS

Assessment of recombination sites in lager yeast genomes

The recombination events between homeologous chromosomes in lager yeasts have been previously mapped at high resolution by genome sequencing in two group I strains (CBS 1503, CBS 1513) and two group II strains (WS 34/70, CBS 1260) (Nakao *et al.* 2009; Hewitt *et al.* 2014) and at a lower resolution in several other strains by competitive genomic hybridisation (Bond *et al.* 2004; Dunn and Sherlock 2008). In these initial studies, the genome of *Saccharomyces bayanus* CBS 7001 (synonym *S. uvarum*) was used to align the non-*S. cerevisiae* portion of the *S. pastorianus* genome as, at that time, this was considered the closest relative. Since these initial analyses, whole genome sequences for several additional lager yeast strains have been generated (Kvasnicka *et al.* 2012; Walther, Hesselbart and Wendland 2014; van den Broek *et al.* 2015; De León-Medina *et al.* 2016; Okuno *et al.* 2016) and a high-resolution whole genome sequence for *S. eubayanus* (FM1318) is now available (Baker *et al.* 2015).

The recombination sites on hybrid chromosomes were assessed in three group I and four group II lager yeast strains (Table 1). For two of the strains (group I, CBS 1513, and group II, WS 34/70), newly generated genome sequences were compared to previous genome databases to (i) determine the accuracy of recombination site identification by different methods and (ii) confirm the provenance of the lager yeast strains. The genome regions, encompassing previously annotated recombination sites, were used to create a query *S. cerevisiae* sequence to

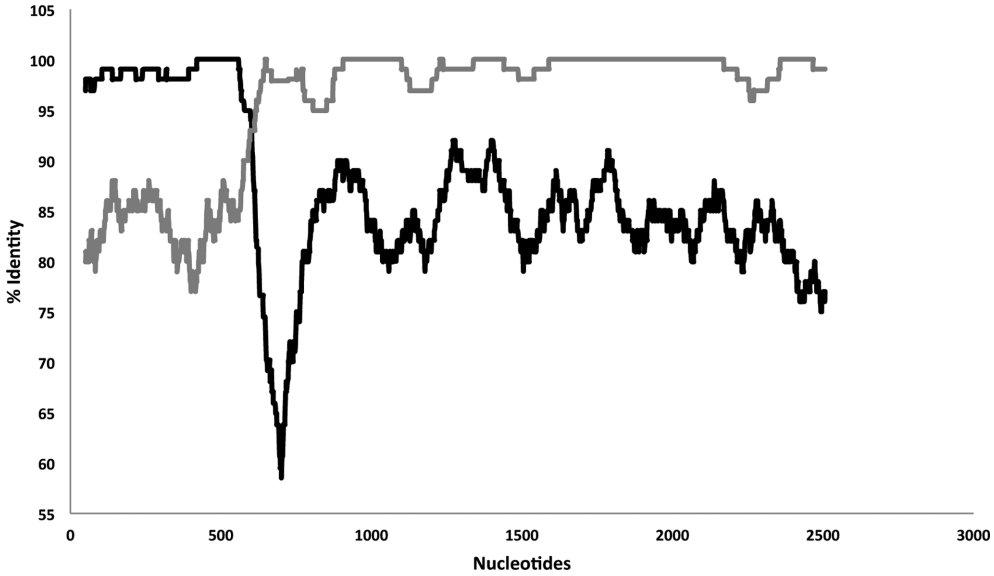

Figure 1. *Percentage sequence identity map of the hybrid gene YMR302C*. The percentage sequence identity of the *S. pastorianus* nucleotide sequence to the *S. cerevisiae* strain S288C (grey) and *S. eubayanus* strain FM1318 (black) is shown. The recombination epicentre is identified from the region where the percentage sequence identities to the individual parental species intersect.

identify the homologous regions in the *S. eubayanus* and *S. pastorianus* genomes. A percentage sequence identity map of the hybrid *S. pastorianus* sequence relative to the *S. cerevisiae* and *S. eubayanus* sequences was created using a sliding window analysis (Fig. 1). The recombination site is defined as the location on the map where the *S. cerevisiae* and *S. eubayanus* lines intersect. The DNA region surrounding each recombination sites was aligned to the *S. cerevisiae* and *S. eubayanus* DNA, and a recombination epicentre was defined as the minimum nucleotide distance between the last *S. cerevisiae*-like base and the first *S. eubayanus*-like base or vice versa of the hybrid region. Using this approach, 37 recombination sites were identified in the seven lager yeast genome sequences (Table 2). The recombination epicentres ranged in size from 2 to 50 bp. The chromosomal coordinates for the majority of the recombination epicentres identified by this bioinformatic approach differ slightly at the nucleotide level, by ~1–47 bp, to those previously mapped (Hewitt *et al.* 2014), and several sites such as those at *YKL080W, YKL045W, YML073C* and *YMR287C* deviate significantly from previously assigned locations (Table S2, supplemental material). These discrepancies most likely arose as previous analyses used the *S. bayanus* CBS 7001 genome rather than *S. eubayanus* as the query genome.

A comparison of the recombination sites in the genomes identifies common and unique sites between and within both groups of lager yeasts. Two recombination sites (*S. cerevisiae*-like hybrid *YGL173C/XRN1* and *YGR285C/ZUO1*) are conserved at a nucleotide level between *all* group I and II lager yeasts (Table 2). Four unique recombination sites (*YER164W, YKL203C, YKL080W* and *YMR306W*) are common to group I strains CBS 1503 and CBS 1538 but these sites are not found in the other group I strain CBS 1513. The latter strain has 10 unique recombination sites not shared with the other group I strains or the group II strains (Table 2). The group II strains share five common recombination sites at *YJR009C Hybrid 1, YMR302C, YPL240C, YPR160W* and *YPR191W* (Table 2). Interestingly, several genes (*YDR324C, YGL173C, YHR165C, YJR009C* and *YPR160W*) contain more than one recombination site (Table S2, supplemental material). The recombination events within these hybrid genes occurred at different sites in different strains. For example, in addition to the hybrid form of *YGL173C* found in all strains, CBS 1513 contains a second hybrid version of *YGL173C* (for co-ordinates see Table S2, supplemental material). The latter is designated as *S. eubayanus*-like hybrid *YGL173C* as the majority of the gene derives from *S. eubayanus* as distinct from the *S. cerevisiae*-like hybrid *YGL173C* found in all other strains, which contains mostly *S. cerevisiae* sequences. Additionally, strain CBS 1513 contains hybrid versions of *YDR324C* and *YHR165W*, which differ from the hybrid versions of the same gene found in strains CBS 1503 and WS 34/70, respectively (Table S2, supplemental material). It should be noted that previous genome analysis had suggested that the recombination site at *YPL240C* was common to group I and II strains (Hewitt *et al.* 2014; Wendland 2014). While we confirm that the site is present in all group II strains as well as in the group I strains CBS 1503 and CBS 1513, it was not observed in the group I strain CBS 1538.

To determine if the recombination sites identified by bioinformatic analysis were present in other lager yeast strains, PCRs were carried out on DNA from eight group II strains, for which genome sequences are not available, and the three group I strains using primers specific to the hybrid genes conserved between group I and II strains and within the group II strains (Table S3, supplemental material). The PCR amplification patterns confirm the assignation of recombination sites by bioinformatic analysis, including the absence of a recombination site at *YPL240C* in CBS 1538 (Fig. 2).

Recombination sites at the *MAT* locus

Previous genome analysis had uncovered a recombination site within the *MAT* locus that was shared in both the group II strain WS 34/70 and the group I strain CBS 1513 (Wendland 2014). To determine if this common recombination event extends to other group I and II strains, we re-evaluated recombination event(s) at the *MAT* locus in WS 34/70 and CBS 1513 as well as in other group I and II strains. The percentage sequence identity analysis reveals subtle but distinct differences between the recombination event(s) at the *MAT* locus (Fig. 3). The *MAT*

Table 2. Recombination epicentres in group I and II lager yeast strains.

		Group I			Group II			
Chr	ORF/Gene	CBS 1503	CBS1538	CBS 1513	WS 34/70	CBS1483	CCY 48-91	790
I	*YAL054C/ASC1*							
II	*YBR289W/SNF5*							
III	*MAT/Se MATα*		NH		ND	ND	ND	ND
III	*MAT/Se MATa*	*	NH					
III	*MAT/Sc MATa*		NH			ND	ND	ND
IV	*YDR324C/UPT4*							
V	*YER074W-YER074C-A*							
V	*YER164W/CHD1*							
VII	*YGL173C/SC-like XRN1*							
VII	*YGL173C/SE-like XRN1*							
VII	*YGR285C/ZUO1*							
VIII	*YHR165C/PRP8*							
VIII	*YHR173W-YHR174C*							
X	*YJL197W/UBP12*							
X	*YJL139C-YJL138C*							
X	*YJR009C/TDH2 Hybrid 1*							
X	*YJR009C/TDH2 Hybrid 2*							
XI	*YKL203C/TOR2*							
XI	*YKL080W/VMA5*							
XI	*YKL045W/PRI2*							
XII	*YLR410W/VIP1*							
XII	*YLR413C-YLR414C*							
XIII	*YML073C/RPL6A*							
XIII	*YML051W/GAL80*							
XIII	*YMR287C/DSS1*							
XIII	*YMR302C/YME2*							
XIII	*YMR306W/FKS3*							
XV	*YOR092W/ECM3*							
XV	*YOR109W/INP53*							
XV	*YOR133W/EFT1*							
XVI	*YPL240C/HSP82*							
XVI	*YPL036W/PMA2*							
XVI	*YPR160W/GPH1*							
XVI	*YPR184W-YPR185W*							
XVI	*YPR191W/QCR2*							

Grey, recombination site detected; white, recombination site not detected; ND, not detected in published genome sequence; NH, no hybrid chromosome detected
*detected by PCR only

locus in WS 34/70 (Fig. 3A) contains *S. eubayanus*-like sequences in the W region and *S. cerevisiae*-like sequences in the Y region and beyond into the downstream gene, *YCR042C*. The Y-region contains an *S. cerevisiae MAT*(a) cassette. Since the X-region in the *S. cerevisiae* and *S. eubayanus* genomes are highly identical at a nucleotide level, it is not possible to pinpoint the exact recombination epicentre. A possible location for the recombination event is nucleotide positions 92–114 of the X region where there is 9 bp insertion in the *S. eubayanus* genome, which presents a region of mispairing with the *S. cerevisiae* genome (Fig. S1, Supporting Information).

In the group I strain CBS 1513, the hybridisation event occurred at the different location within the *MAT* locus (Fig. 3B): here the W and Y regions of the *MAT* locus are *S. eubayanus*-like and the Y region contains an *S. eubayanus MATα* cassette. The region downstream of the Y region is missing in the genome sequence, however, the adjacent gene *YCR042C* is *S. cerevisiae*-like (data not shown). Based on the DNA sequence identity within the *MAT* locus, we predict that the recombination event occurred downstream of the Y region within the Z1 or Z2 regions (Fig. S2, Supporting Information). The HO endonuclease recognition site, which initiates mating type switching in *S. cerevisiae*, is located in the Z1 region. Thus, the recombination events at the *MAT* locus occur at different locations in the group I and II yeasts WS 34/70 and CBS 1513, and furthermore each contains a different mating type cassette from a different species at the *MAT* locus.

We also evaluated the *MAT* locus in the genomes of the other group I and II strains. In the group I strain CBS 1503, the hybrid chromosome III switches from *S. cerevisiae*-like to *S. eubayanus*-like, which is the opposite orientation to the hybrid chromosome III found in the group I CBS 1513 and in the group II strains (data not shown, but see Fig. 4). The sequences are *S. cerevisiae*-like in the W region; however, sequence coverage in the X and Y regions of the *MAT* locus is poor and therefore it was not possible to assign a specific mating type cassette at *MAT* locus by bioinformatics analysis. The downstream gene *YCR042C* is *S. eubayanus*-like. The other group I strain CBS 1538 does not contain a hybrid chromosome III and instead only contains *S. eubayanus* copies of this chromosome. Due to poor sequence coverage of the *MAT* locus in the group II strains CBS 1483, CCY48–91 and 790 (Table 1), it was not possible to examine recombination events in this region nor to assign a mating type cassette by bioinformatics analysis. Instead, and in light of the significant differences at the *MAT* locus in the group I and II strains, we examined this region by PCR analysis in the three group I strains and in eight group II strains for which genome sequences are not available (Fig. 4).

The PCR analysis identified three types of chromosome III in the group II strains, specifically, (i) *S. cerevisiae*-like with a *MAT* (a) cassette, (ii) a hybrid *S. eubayanus*–*S. cerevisiae* with an

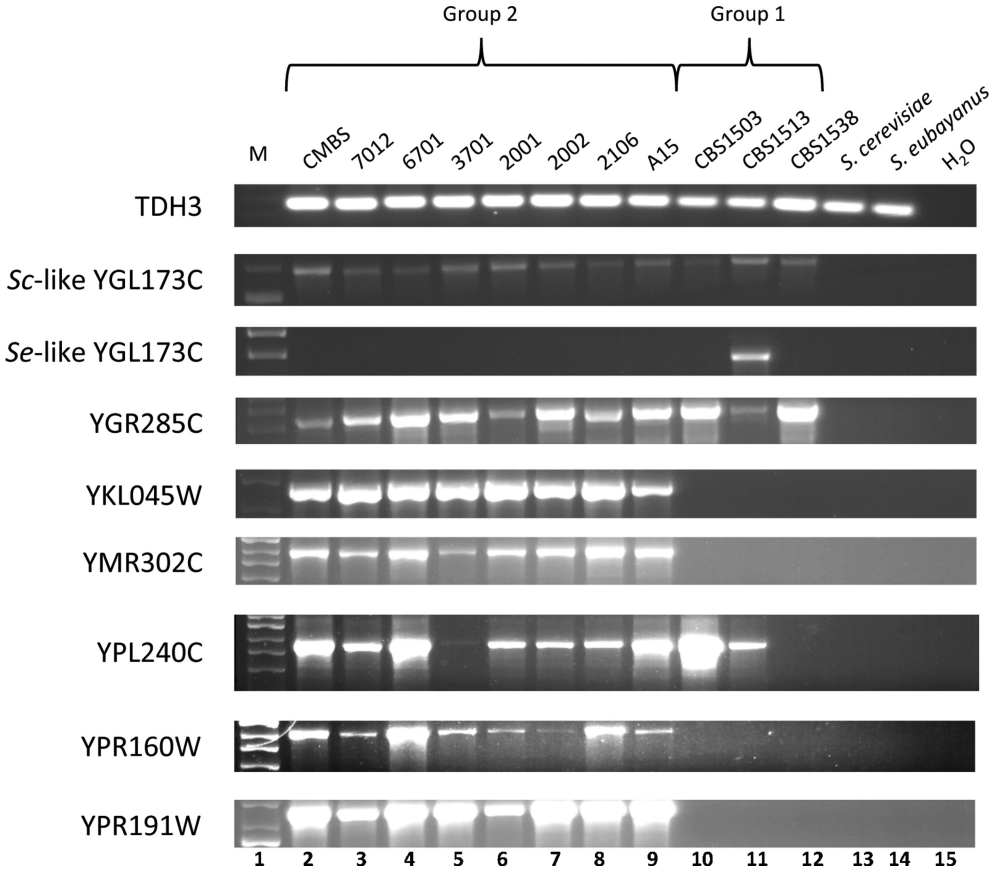

Figure 2. *Identification of hybrid genes in group I and II lager yeasts*. Primers flanking the recombination epicentres of the hybrid genes listed on the left were used to amplify DNA from the yeast strains listed above the gel panel. Lane 1; M: molecular weight marker, lanes 2–9; group II strains; lanes 10–12, group I strains, lane 13, *S. cerevisiae* S-150, lane 14; *S. eubayanus* FM1318, lane 15, negative control, no DNA. The gene *TDH3* was used as a positive control.

S. eubayanus MAT (α) cassette and (iii) a second hybrid chromosome, a hybrid *S. eubayanus*–*S. cerevisiae* with an *S. cerevisiae MAT* (a) cassette (Fig. 4, lanes 2–9). The group I strain CBS 1513 shares an *S. eubayanus*–*S. cerevisiae*/*S. eubayanus MAT* (α) hybrid chromosome and an *S. cerevisiae*-like *MAT* (a) chromosome with the group II strains (Fig. 4, lane 11). The group I strain CBS 1503 contains an *S. cerevisiae*–*S. eubayanus* hybrid chromosome III with an *S. eubayanus MAT* (a) cassette as well as an *S. eubayanus* MAT (a) chromosome III (Fig. 4, lane 10), while strain CBS 1538 contains only *S. eubayanus*-like copies of chromosome III with both *MAT* (a) and (α) cassettes (Fig. 4, lane 12). The *S. eubayanus* strain FM1318 contains two copies of chromosome III, each containing opposite mating type cassettes at the *MAT* locus (Fig. 4, lane 14). Thus, all three group I strains contain different types of chromosome III and furthermore, the group II strains contain two hybrid chromosomes III arising from distinct recombination events at the *MAT* locus, one of which is conserved in the group I strain CBS 1513.

Common sequence motifs at recombination sites

Having defined the recombination sites in the seven lager yeast genomes, we next set out to determine if the identified recombination sites share any common sequence motifs or similarities in base composition. A region of ~40 bp, encompassing 36 recombination epicentres, together with 36 randomly generated 40 bp DNA sequences which act as a control, was input into the motif discovery programme, MEME. Two motifs were identified in the recombination epicentres that were not observed in the random sequences (*P* value < 0.001) (Fig. 5). Motif 1 consists of two thymidine-rich regions flanking a weaker purine-rich sequence on one strand of the DNA. The second motif (motif 2) contains a pyrimidine-rich sequence followed by a stronger purine-rich region. One or other of the three motifs were observed in 18 of the 36 epicentres (50%).

The motifs were identified using stringent criteria in the MEME programme, therefore, to determine if submotifs might be present at the other recombination sites, the recombination epicentres were aligned using the multiple alignment programme MAFFT (Table 3). The alignment confirms the pyrimidine-rich nature of the recombination epicentres: in general, the regions have an average CT content of 59% $\pm$ 0.9 (range 44%–85%) which is significantly higher than the expected CT content (50%) of the *S. cerevisiae* and *S. eubayanus* genomes. Based on this analysis, the recombination sites fall into two broad categories: (i) sites with very high T content (n = 7, %CT> 60%) and (ii) sites with one or more purine-rich regions ($\geq$4 nts) flanked by pyrimidine-rich regions ($\geq$ 4 nts, n = 18), on one strand of the DNA. Several other sites contain the pyrimidine-purine-pyrimidine arrangement but with weaker purine-rich regions ($\leq$3 nts, n = 5). In total, up to 83% of epicentres contain overall base compositions with similarity to the motifs predicted by MEME. For genes that contain more than one recombination site (Table S2, supplemental material), the recombination sites within the gene appear to be highly conserved; for example, in *YPR160W*,

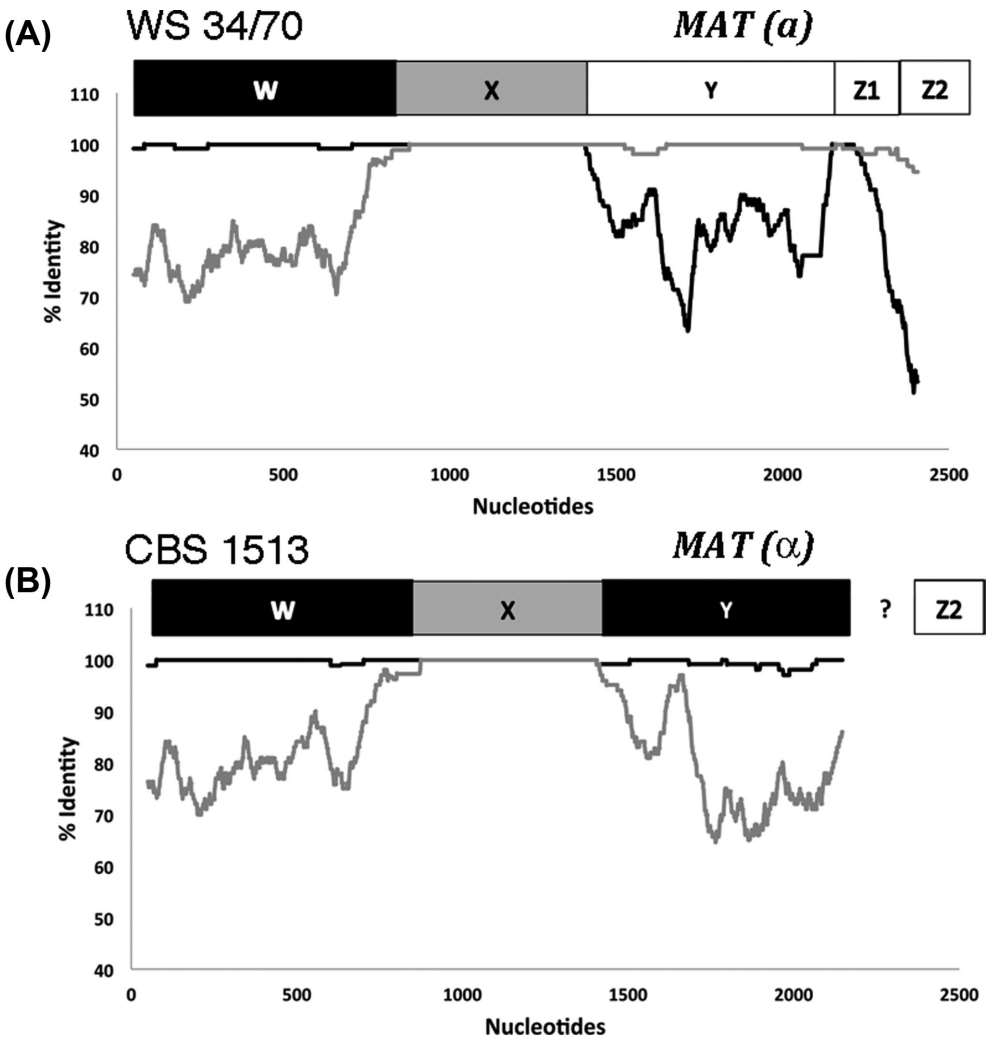

Figure 3. *Recombination sites at the MAT locus in CBS1513 and WS 34/70.* The *MAT* locus on chromosome III consists of a W, X, Y, Z1 and Z2 (Z) regions. The percentage sequence identity of *S. pastorianus* nucleotides to the *S. cerevisiae* strain S288C (grey) and *S. eubayanus* strain FM1318 (black) in group II WS 34/70 (**A**) and from group I lager yeast CBS1513 (**B**). The Y region contains a MAT(a) cassette in (A) and a MAT(α) cassette in (B). The DNA sequence in the Z1 region in CBS 1513 (B) is underrepresented, indicated here a by (?).

the sequences at the two sites are TCTCTGGAGAGTTTTCTTC and TTTTTGCTGGGTTTTGTTC, respectively, each complying with the pyrimidine-purine-pyrimidine motif (Table 3). Furthermore, the epicentres defined by the percentage identity maps (Fig. 2) are embedded within or beside the motifs (Table 3, boxed areas).

As the majority of non-reciprocal recombination events in yeasts are initiated by double-strand breaks (DSBs) in the genome as a result of replication errors during mitosis, crossover events in meiosis or within repetitive DNA elements (Prado *et al.* 2003), we addressed the question of whether there was a correlation between the location of recombination epicentres and sites of replication origins (ARS sites), meiosis-induced DSB sites or repetitive DNA elements such as Ty elements and tRNA gene clusters. A total of 23 of the 37 recombination epicentres are located closer to an ARS sequences than the average gene (mean distance to ARS = 7051nts) but only by <300 nts (mean distance = 6774 nts). Seven recombination epicentres are <1000 bp away from an ARS sequence, 16 lie between 1000–6774 bp while 14 are more than 6774 bp from an ARS sequence (Table S4, supplemental material). However, there appears to be no correlation between the distance from an ARS sequence and the location of recombination sites.

We also examined the recombination epicentres for Spo11p-binding sites, which demarcate sites of meiosis-induced DSBs (Pan *et al.* 2011). With the exception of the intergenic region between *YPR184W-YPR185W* which contains multiple Spo11p sites and the epicentres at *YKL203C* and *YAL054C*, which have Spo11p sites lying immediately adjacent, the remainder of the recombination sites did not contain any Spo11p (n = 26) sites or had a low density of Spo11p sites (n = 8 sites) within 20 bp of the recombination site (Table S5, supplemental material). Thus, there appears to be no significant correlation with the chromosomal location of the recombination epicentres to meiosis-induced sites of DSBs. Likewise, there appears to be no repetitive elements close to the recombination sites with the exception of a tRNA gene close to *YML051W/GAL80* and *YOR133W/ETF1*. Interestingly, the recombination sites at *YLR410W/VIP1* and *YLR413C-YLR414C*, which together flank an ~6 kb *S. eubayanus* insertion into a *S. cerevisiae* chromosome XII in some group II lager yeasts, specifically in CBS 1483, CCY48–91 and 790, are in close vicinity to a Ty2 element (data not shown).

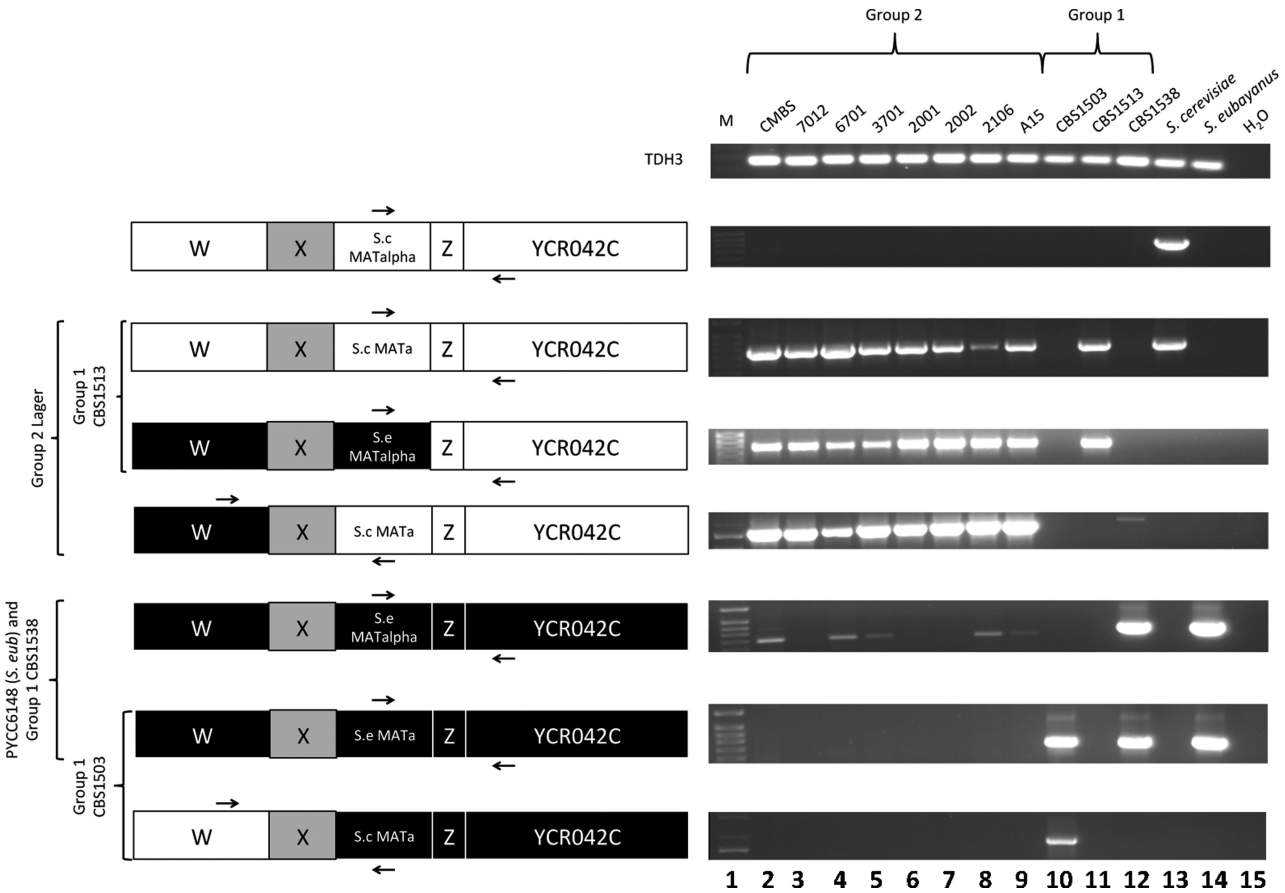

Figure 4. *Identification of mating-type cassettes at the MAT locus in group I and II lager yeasts*. The *MAT* locus was amplified in the yeast strains shown above the gel panel using the primers shown in the *MAT* locus schema on the left panel. Lane 1; molecular weight marker, 2–9; group II, 10–12; group I, 13, *S. cerevisiae*, 14, *S. eubayanus* FM1318, 15, negative control H_2O only. The gene *TDH3* was used as a positive control.

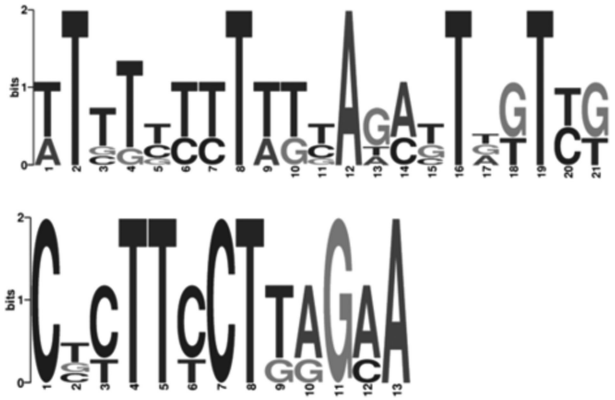

Figure 5. *Common motifs at recombination epicentres in lager yeasts*. The y-axis measures the frequency of the appearance of a nucleotide in bits. The x-axis indicates the residue number. Figures were generated by meme-suite.org.

DISCUSSION

The evolution of lager yeasts has been influenced by social and cultural developments of the human race, by geography and by ancient customs and practices of brewing. The happenstance of the arrival of the cold-tolerant yeast *Saccharomyces eubayanus* in Central Europe coupled with new laws that restricted brewing between St Michael's Day (29 September) to St George' Day (23 April) created the ideal conditions for the emergence of a new yeast species, *S. pastorianus*, which was adapted to fermentation at low temperatures.

Several hypotheses have been advanced to explain the evolutionary pathways that led to the emergence of the group I and II lager yeasts. First, the fact that the group I and II strains, CBS 1513 and WS 34/70, respectively, shared three common recombination sites (*MAT*, *YGL173C* and *YPL240C*) provided a compelling argument that the two groups share a common ancestor (Wendland 2014). Contradicting the common ancestor hypothesis is the finding that group I strains lack subtelomeric regions of *S. cerevisiae* chromosomes IV and XIII that are found in all group II strains. Furthermore, the same subtelomeric regions are absent in most *S. cerevisiae* strains used in the production of Ales but are present in *S. cerevisiae* strains used in stout production in the British Isles (Monerawela *et al.* 2015). Recent genome analysis of hundreds of *S. cerevisiae* strains used in beer production indicates that British-type Stout yeasts cluster in a distinctive clade within the Ale-type yeasts clade (Gallone *et al.* 2016; Gonçalves *et al.* 2016). These findings led Monerawela *et al.* (2015) to propose that the groups I and II lager yeasts arose by separate hybridisation events between *S. eubayanus* and two distinct types of *S. cerevisiae*. The independent origin hypothesis was supported by data that showed a 10-fold higher rate of synonymous substitutions in the *S. cerevisiae* subgenomes compared to the *S. eubayanus* subgenomes of the lager yeasts. The difference in the rate provides evidence for divergence of the *S. cerevisiae* subgenomes in group I and II lager yeasts prior to the hybridisation event (Baker *et al.* 2015). Recently, genome analysis revealed that the

Table 3. Sequence motifs in recombination epicentres.

Gene Name		%CT
YGL173C-2	----------acttccctccatttggaatcgttagaaattttgtcatgaat---	59
YPL036W	cagccgctccacgtaaggccgcagccgcttctgccgctgat----------	59
YAL054C	----------gaagaacttagcagggtcttcaatggactgcttgtacag----------	44
YKL203C	----------gatagaaacaatttttttattagactggtcgttctcaagaa----	49
YHR173W-YHR174C	----------ttatttattttttcttttatcttagtttctttcataacacca	76
YPL240C	----------tccttctctctttcagctttttcttcgtcagtttcttcca--------	85
R_YPR184W-YPR185W	----------gtctctcacttttcacgtgtgttccctttatttcttttatt-	80
YML073C	----------ttccttcttggtcaacttttccttagcaaagtattcgaca--------	65
YGL173C	---------tgaatgaaatactgtaaattttttggtcaatttggccataaa----------	46
R_YPR160W	----------cgtggtgaattttttgctgggttttgttccagtcaatgacc-	58
YDR324C	----------gaatttacccatctgttgttcttttgagatttgttgctatt---	66
R_YHR165C	----------aaatagattacttcgtctgattgtagatcctaatattgct---	55
R_YJL139C-YJL140C	------atatgtacaataatttaaataatttttctctgattatttatcc----------	59
R_YPR160W-2	-----tctgatctgtctctctggagagttttctcgatgatggat----------	60
R_YPR191W	----------cttttcaaatcggcctcaacaatatttttca	63
YHR165C-2	----------gcatcatacatgttataagccagatcgataccaatcataa-----	48
YJR009C	----------gtttccttgttcaacttgacagtcaagtcaacaacg	56
MAT	----------tattttgttatgtatatgtgtatatatatatat	60
YER074W-YER074C	----------atatataaatatgtgtatctttttccaataaaaaatataatcaataattt	50
YLR413C-YLR414C	----------agtggatttttattccaacaacaatatattttaatcata	54
YKL045W	----------accaattctgaatcatcttcgtcaggatacacaattgca--------	55
R_YGR285C	---------gatttgtatgctgctatgggtttgtctaagttgcgtttcagagc-------	55
R_YMR287C	----------cttctttgtttccagaaagcactaatgttgatattg	58
R_YKL080W	-tgctctaaacttgcagcagagtcatgctctttttcaa----------	61
YMR306W	----------tttttggcaaaatacattgagtcctatttttt	80
R_YOR133W	--------ttcgaccaattttggcaagtcgttagcgttcttgacttc----------	62
YMR302C	--cccaatggttctaaggaagcgtcaattttcctgtaattcc----------	56
R_YBR289W	gtgattccgttcccgtatcctgagtaaccatttccccaaat----------	61
R_YOR109W	----------ggaataagttttctgacaccggaaaaccccttcctgagaagt	46
R_YJL197W	----------gccaacttttttaatcacattccaatcattcacgtcatca	64
YJR009C-2	----aacccccatggcaagttagctgggtctctttcttggaaagtg----------	51
R_YML051W	----------gatttaacaggccttttcgtagccataccaaccgccgtttcc---	61
R_YER164W	----------tttatagtactcagtctgtacgtcggatagttcgactctc--------	60
YDR324C-2	----------ctcatcaaaatagtatcgtgttccaagaca	50
R_YOR092W	----------gtgcattccatctgaacgggttcagtttcgtcctca	61
R_YLR410W	----------atcttcggaatacagctaatataatgcctgtcatctaattg	54

Boxed area: recombination epicentres. Pyrimidine regions underlined, purine regions bold italics

S. cerevisiae subgenome of group II lager yeasts displays significant SNP heterozygosity over a region of ~16% of the genome (Okuno *et al.* 2016). In these regions, one of the hetero SNPs is identical to a corresponding SNP in the group I genome, suggesting that group II lager yeasts contain two *S. cerevisiae* subgenomes, one of which is shared with the group I genome. Taken together, the data can be reconciled by an evolutionary model involving sequential rounds of hybridisation between *S. eubayanus* and different *S. cerevisiae* isolates (Fig. 6). The sequential hybridisation model for evolution of group I and II lager yeasts assumes that the initial hybridiation event occurred between a diploid *S. eubayanus* and a haploid *S. cerevisiae*. This assumption is based on the fact that the *S. eubayanus* type strain PYCC6148 is reported to be a diploid (Baker *et al.* 2015; Hebly *et al.* 2015). However, it is also possible that the first interspecies hybrid arose as a result of a hybridisation between haploid *S. cerevisiae* and *S. eubayanus* isolates or alternatively as a result of a rare interspecies mating between haploids of the opposite mating type followed by backcrossing or a subsequent hybridisation event to a different *S. eubayanus* isolate. Presently, we cannot distinguish between these hypotheses.

We reanalysed the recombination sites in two independently generated genome databases for each of the two prototypic group I and II strains (WS 34/70 and CBS 1513) and also expanded the analysis to five newly sequenced lager yeast genomes to gain insight into the evolutionary relationships between the different lager yeast strains and to determine if the pattern of conserved recombination sites applied to the expanded set of

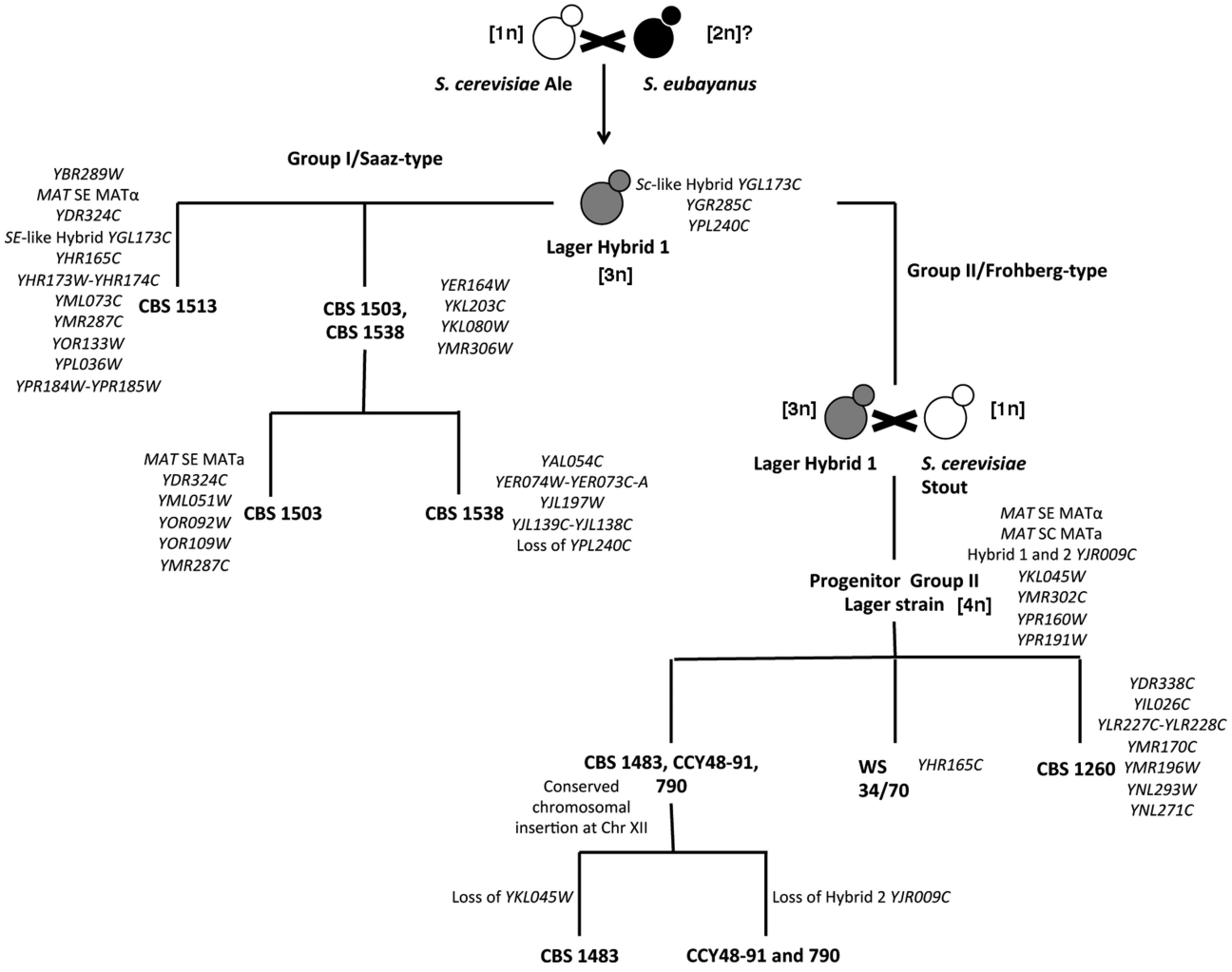

Figure 6. *Evolutionary relationships of group I and II lager yeasts*. Proposed evolutionary pathways for the different group I and II *S. pastorianus* strains based on the conservation, acquisition and loss of recombination sites. Recombination events are listed beside diverging events. The recombination sites listed for strain CBS1260 are from Hewitt *et al.* (2014)

sequenced genomes. The compiled analysis identified 37 recombination sites and reveals a complex relationship between the lager yeast strains. Taken together, the pattern of recombination events supports the sequential model of hybridisation (Fig. 6) and furthermore reveals several divergent routes of evolution leading to the current *S. pastorianus* strains.

The extended analysis of recombination sites confirms that one site at *YGL173C* is conserved in all strains along with a site at *YGR285C*. These recombination events must have occurred after the first hybridisation event between an Ale-like *S. cerevisiae* and *S. eubayanus* (Fig. 6). The recombination site at *YPL240C* which was previously identified as being common to groups I and II is shared in six of the seven strains, but is absent in group I CBS 1538. It is possible that this strain may have subsequently lost the hybrid chromosome XVI.

Recombination events at the *MAT* locus reveal a complex relationship between the group I and II strains. First, we identified two different types of hybrid chromosome III in group II strains, arising from homeologous recombination at different sites within the *MAT* locus. The hybrid chromosomes contain opposite mating type cassettes (a and α) derived from *S. cerevisiae* and *S. eubayanus*, respectively. The recombination event generating the hybrid chromosome containing the *S. eubayanus MATα* cassette is conserved between the group I strain CBS 1513 and the group II lager yeasts but not the other two group I strains. Instead strain CBS 1503 contains a hybrid chromosome of the opposite orientation containing an *S. eubayanus MAT* (a) cassette while strain CBS 1538 contains no hybrid chromosome III. The presence of two types of hybrid chromosome III is consistent with the finding of two distinct types of *S. cerevisiae* genomes in the group II strains.

Analysis of the remaining recombination sites identifies recombination events common to subgroups of strains as well as events unique to each strain, indicative of the dynamic and evolving nature of the lager yeast genomes. The group I strains show the greatest divergence and there is evidence of at least two distinct subgroups. Strains CBS 1503 and 1538 share four unique common recombination sites that are absent in strain CBS 1513, which appears to have evolved independently of the other two strains with its own unique sites (Fig. 6). The three group I strains were originally isolated by Emil Hansen at the Carlsberg brewery between 1883 and 1904: CBS 1513 is a descendent of the first pure yeast isolate from a beer fermentation, initially designated as Unterhefe Nr. I in 1883 and laterally, renamed *S. carlsbergensis* by Hansen in 1908. Hansen subsequently identified two other yeast isolates from beer,

Unterhefe Nr. II which he designated as *S. monacensis* (CBS 1503) and a third isolate, CBS 1538, which was named *S. pastorianus* Reess ex Hansen 1904, when it was deposited into the Centraalbureau voor Schimmelcultures (CBS) in 1935. The species name *pastorianus* was assigned in deference to the original classification by Max Reese of lager yeasts as *S. pastorianus* in 1870 (Barnett 2000; Wendland 2014). Based on the analysis here, strain CBS 1513 appears to have been under different selective pressures than strains CBS 1538 and 1503 and thus evolved independently of these latter strains. Alternatively, CBS 1513 may have arisen from an independent hybridisation between *S. eubayanus* and yet another *S. cerevisiae* isolate and subsequently underwent convergent evolution with the other hybrids. The divergent lineage of CBS1513 from CBS1503 and 1538 has previously been observed in a phylogenetic study of *S. cerevisiae* strains using microsatellite gene loci as query sequences (Nguyen *et al.* 2011). In that study, CBS1538 and 1503 clustered with two beer strains CLIB277 and 276 into an *S. monacensis* group, defined by having a lower *S. cerevisiae* gene content. CBS1513, on the other hand, clustered with rum and distillery strains. However, phylogenetic analysis using both subtelomeric and non sub-telomeric sequences as comparators reveals very low levels of sequence diversity between the three group I strains (0.07–0.11 SNPs kbp^{-1}) suggestive of a common lineage (Monerawela, unpublished).

Unlike the group I strains, group II strains are much more homogeneous sharing seven recombination sites; however, yet again, several of the strains contain unique sites not shared by other strains, suggestive of continued divergent evolution (Fig. 6).

Several approaches were taken to determine if the recombination sites share any common sequence motifs and base composition. We found that, in general, recombination epicentres have a significantly higher percentage of pyrimidines at the epicentres than expected in *S. cerevisiae* or *S. eubayanus* genomes. Second, two sequence motifs were identified within the recombination epicentres. The motifs contain pyrimidine-rich regions flanking a purine central motif or a pyrimidine region adjacent to a purine-rich sequence, on one of strand of the DNA. Further analysis of the recombination sites by multiple sequence alignment indicated that 30 of the 36 sites (83%) contain the identified motifs or submotifs. For genes that contain multiple recombination events, for example, *YJR009C* and *YPR160W* (Table S2, supplemental material), the recombination sites within the gene show a remarkable level of conservation. Interestingly, recombination at some of the recombination sites can be induced by exposure of lager yeasts to a combination of thermal and high specific gravity stress (James *et al.* 2008) and recombination at least one site, *YGR285C*, has also been observed in other interspecies hybrids of *S. eubayanus* (Libkind *et al.* 2011). Thus, recombination sites are not randomly situated in the genome but rather may represent specific 'fragile' chromosomal locations that share a common susceptibility to double-strand breakage leading to recombination, perhaps in response to environmental stress.

While we did not observe any correlation between the location of the recombination sites to the proximity of sites of replication origin or to meiotically induced DSBs, it is interesting to note that mitotically induced DSBs identified in humans have been shown to contain pyrimidine-rich motifs (Ohtsuka *et al.* 2016). While further research is required to understand the molecular mechanisms of homeologous recombination in lager yeasts, it is clear that recombination events confer evolutionary advantages to lager yeasts, as the majority of events are intragenic resulting in uninterrupted ORFs thus generating novel hybrid alleles that are truly unique to lager yeasts.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflict of interest. None declared.

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