Fermentation innovation through complex hybridization of wild and domesticated yeasts

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Abstract

The most common fermented beverage, lager beer, is produced by interspecies hybrids of the brewing yeast *Saccharomyces cerevisiae* and its wild relative *Saccharomyces eubayanus*. Lager-brewing yeasts are not the only example of hybrid vigor or heterosis in yeasts, but the full breadth of interspecies hybrids associated with human fermentations has received less attention. Here we present a comprehensive genomic analysis of 122 *Saccharomyces* hybrids and introgressed strains. These strains arose from hybridization events between two to four species. Hybrids with *S. cerevisiae* contributions originated from three lineages of domesticated *S. cerevisiae*, including the major wine-making lineage and two distinct brewing lineages. In contrast, the undomesticated parents of these interspecies hybrids were all from wild Holarctic or European lineages. Most hybrids have inherited a mitochondrial genome from a parent other than *S. cerevisiae*, which recent functional studies suggest could confer adaptation to colder temperatures. A subset of hybrids associated with crisp flavor profiles, including both lineages of lager-brewing yeasts, have inherited inactivated *S. cerevisiae* alleles of critical phenolic off-flavor genes and/or lost functional copies from the wild parent through multiple genetic mechanisms. These complex hybrids shed light on the convergent and divergent evolutionary trajectories of interspecies hybrids and their impact on innovation in lager-brewing and other diverse fermentation industries.

Introduction

Humans have been producing and consuming fermented beverages for thousands of years [1]. During this process, they have unwittingly shaped the evolutionary history of the microbes that are responsible for fermented products. The star of fermented beverage production is often *Saccharomyces cerevisiae*. Many studies have investigated the evolutionary impact of domestication in fermentation environments on the genomes of different lineages of this species [2–13]. These human-associated fermentation environments have also led to innovation through the hybridization of distantly related species.

Lager beers are made with hybrids between the distantly related species *S. cerevisiae* and *Saccharomyces eubayanus* [14–16]. These hybrids combine unique properties from each; *S. cerevisiae*'s carbon utilization and fermentation capabilities combined with *S. eubayanus*'s cryotolerance to produce yeasts that could ferment well in the cold [17–22]. Other interspecies hybrids of *Saccharomyces* have been associated, both favorably and unfavorably, with diverse fermentations. *S. cerevisiae* × *Saccharomyces kudriavzevii* hybrids are prized for their unique flavor profiles in beer and wine [23]. Conversely, hybrids and introgressed strains with large genomic contributions from *S. eubayanus* and *Saccharomyces uvarum*, are viewed as contaminants in breweries due to the production of off-flavors, while other strains have been associated with sparkling wine and cider fermentation [16,24,25]. Although these previous studies have hinted at the complexity of fermentation hybrids, their focus on a handful of strains or a handful of loci has only given us a fleeting glimpse of the diversity *Saccharomyces* hybrids, their total genomic compositions, and their evolution.

Here we identified, sequenced, and analyzed the genomes of 122 interspecies hybrids and introgressed strains in the genus *Saccharomyces* to understand their origins and evolutionary

innovations. This collection contains pairwise hybrids, as well as more complex hybrids and introgressed strains with three or four parent species. We show that all genomic contributions from *S. cerevisiae* have arisen out of three domesticated lineages of *S. cerevisiae*, while all other parents belonged to Holarctic or European wild lineages of their respective species. We also analyzed inheritance of the mitochondrial genome and the genetic events generating functional diversity in genes relevant to fermented beverages. The genomic complexity of these hybrids provides insight into their origins and evolutionary successes in human-associated fermentation environments.

Results

Summary of Interspecies Hybrid Types

Here, we analyzed the genome sequences of 122 interspecies hybrids and introgressed strains of *Saccharomyces*, 63 strains of which are newly sequenced here, more than doubling the number of previously published hybrid genomes. Collectively, industrial settings dominated the isolation origins of all hybrids; 86% (n=105) were from beer, wine, cider, a distillery, or other beverages (Figure 1b, Table S1, Supplementary Text). We identified four types of hybrids: 1) lager-like (*S. cerevisiae* (*Scer*) × *S. eubayanus* (*Seub*)) (n=56); 2) *S. cerevisiae* × *S. kudriavzevii* (*Skud*) (n=15); 3) *S. eubayanus* × *S. uvarum* (*Suva*) (n=41); and 4) more complex hybrids, with three or four parent species (n=11 more than doubling those previously identified [26]) (Figure 1a, Table S1, Supplementary Text). These more complex hybrids fell into three groups: 4A) *S. cerevisiae* × *S. kudriavzevii* × *S. eubayanus* × *S. uvarum* (n=5), 4B) *S. cerevisiae* × *S. eubayanus* × *S. uvarum* (n=4), and 4C) one *S. cerevisiae* × *S. kudriavzevii* × *S. eubayanus* (Table S1). The lager-like hybrids were almost exclusively associated with beer (Figure 1b) and have genomic

contributions that were consistent with previous observations in the two lineages (Saaz and Frohberg) [27]. The *S. cerevisiae* × *S. kudriavzevii* strains were associated with beer and wine (Figure1b). They had considerable differences in *S. kudriavzevii* genomic content, suggesting that these hybrids are of variable ages and evolutionary histories. The *S. eubayanus* × *S. uvarum* hybrids and introgressed strains were the most variable, both in isolation environment and genomic contributions (Figure 1, Table S1). The wide range in genomic contributions in these strains was likely influenced by their ability to backcross due to the low, but non-zero, spore viability of hybrids of these sister species [16]. These *S. eubayanus* × *S. uvarum* strains had the most total number of translocations (χ^2 = 1250.1, p_adj = 2.64 E-15), as well as the most translocations shared with other hybrid types (χ^2 = 15.964, p_adj = 0.0138) (Figure S2). The shared nature of some of these translocations in hybrids with more than two parents suggests that *S. eubayanus* × *S. uvarum* introgressed strains further hybridized to produce some of the complex three or four parent species hybrids. Thus, these four types of hybrids each show unique dynamics in genome evolution and are used for different products that range from several regional niche beverages to the globally dominant beer style, lagers.

Wild Parent Populations

Three out of four of the species contributing to these hybrids (*S. kudriavzevii*, *S. uvarum*, and *S. eubayanus*) have primarily been isolated from wild settings and have global distributions with populations that reflect their geography [28,29]. We used these established populations and phylogenomic and PCA approaches to evaluate the origins of these hybrids (Supplementary Text).

S. kudriavzevii has been isolated in Europe and Asia and consists of three described populations: Asia A, Asia B, and Europe [23,30,31]. The *S. kudriavzevii* sub-genomes of the hybrids all clustered with the European population as a monophyletic clade (Figure 2a, Figure S3, Table S2, File S1, Supplementary Text). These findings show that these hybrids were drawn from a closely related lineage of the European population of *S. kudriavzevii*.

In *S. eubayanus*, analysis of both large and small contributions, showed that these hybrids and introgressed strains clustered with the Holarctic lineage of *S. eubayanus* (Figure 2b, Figure S5, Table S2, File S3, Supplementary Text). Our vastly expanded dataset suggests that the Holarctic lineage is the closest known relative of all industrially relevant *S. eubayanus* hybrids and introgressed strains. The array of hybrids observed here requires that multiple hybridization events occurred between this lineage and other species. We also analyzed genetic diversity of the *S. eubayanus* contributions to industrial hybrids and introgressed strains (Supplementary Text). We found low nucleotide diversity in lager-like hybrids that shows that these widely used interspecies hybrids arose out of a narrow swath of *S. eubayanus* diversity, while the less frequently used hybrids and introgressed strains retained more nucleotide diversity.

S. uvarum has a parallel population structure to *S. eubayanus* [26,32], with the exception of its increased isolation frequency in the Northern Hemisphere and the presence of pure strains isolated from Europe. Here we found that all contributions from *S. uvarum* arose out of the *S. uvarum* Holarctic lineage [26]. In contrast to our *S. eubayanus* findings, the *S. uvarum* sub-genomes of these hybrids and introgressed strains were interspersed with pure wild strains (Figure 2c, Figure S7 & S7, Table S2, File S5 & S6). These findings suggest that there have been multiple hybridization events and extensive backcrossing with wild lineages of *S. uvarum*, integrating wild diversity into these hybrids and leading to a diverse set of introgressed strains.

137

138 Domesticated *S. cerevisiae* Parent Lineages

139 Of the species contributing to domesticated interspecies hybrids, *S. cerevisiae* has the
140 most extensive datasets, including industrial yeasts [5,8–11]. Through both phylogenomic and PCA
141 approaches, we recapitulated the previously described domesticated *S. cerevisiae* clades [8,9], and
142 our 81 interspecies hybrids with *S. cerevisiae* contributions fell into three domesticated lineages:
143 Wine, Ale/Beer1, and Beer2 (Figure 2d, Figure S9, Table S2, File S7).

144 The *S. cerevisiae* × *S. kudriavzevii* hybrids grouped with both Beer2 and Wine. Strains
145 with contributions from three or four parent species fell into both clades (Beer2 and Wine),
146 suggesting that these complex hybrids originated stepwise through iterative hybridization
147 (Supplementary Text).

148 Interestingly, the only hybrids we detected in the Ale/Beer1 group were the lager-
149 brewing yeasts (Figure 2d). The *S. cerevisiae* sub-genomes of the Saaz and Frohberg lager-
150 brewing lineages formed distinct clades, and although we identified more Frohberg strains,
151 Frohberg genetic diversity was lower (Supplementary Text). To determine if there was a
152 particular clade of Ale/Beer1 that was the closest known relative to lager-brewing hybrids, we
153 performed a targeted analysis of just the Ale/Beer1 *S. cerevisiae* strains and lager-brewing
154 hybrids, (Figure S10 & S10, Table S2, File S8, Supplementary Text). Our concatenated
155 phylogenomic analyses did not strongly support any recognized geographical clade of Ale/Beer1
156 *S. cerevisiae* strains as the closest outgroup to the lager-brewing yeasts. Our PCA analyses,
157 which make no assumptions about consistent genome-wide signals, suggested several Stout beer,
158 Wheat beer, and mosaic strains as sharing the most ancestry with lager-brewing yeasts, rather
159 than any clade affiliated with a geographic style (Figure S9). Overall, our analyses clearly show

that lager strains belong to the Ale/Beer1 lineage of *S. cerevisiae* and suggest affinity with a novel set of diverse beer yeasts, but they do not support any known extant strain as the sole closest relative.

Collectively, our data and analyses conclusively show that there have been multiple interspecies hybridization events between different domesticated lineages of *S. cerevisiae* and wild strains from three other *Saccharomyces* species (Figure 2d). The sheer number and diversity of hybrids analyzed here shows that evolutionary and industrial innovation through hybridization has happened on a scale and with a complexity beyond what previous smaller scale studies have suggested. In these diverse hybrids, the domesticated *S. cerevisiae* sub-genomes were likely preadapted with general industrial fermentation traits, while the wild parent likely contributed one or more traits advantageous in the specific new industrial fermentation niche being explored.

Mitochondrial Genome Inheritance

The classic example of yeast hybrid vigor comes from the cryotolerance of lager-brewing yeasts. *S. eubayanus*, *S. kudriavzevii*, and *S. uvarum* are all known to tolerate much colder temperatures [33,34], and recent functional experiments have shown that the mitochondrial genome (mtDNA) plays a pivotal role in the cryotolerance of interspecies hybrids [17,35]. Strikingly, in our comprehensive dataset, a majority (94%) of the hybrids inherited a mtDNA from another species, rather than the *S. cerevisiae* mtDNA (Figure 3a).

We tested if the parent that donated the mtDNA was also the parent that contributed the most nuclear gene content. We used a logistic regression to determine if the same parent species contributed both the mtDNA and the most complete set of orthologs. We found that this trend was generally true (p=8.0E-6, AIC= 83.75), but there were informative outliers (Figure 3b). In

particular, more than half of the hybrids with *S. kudriavzevii* nuclear contributions inherited the *S. kudriavzevii* mtDNA, despite the fact that the *S. kudriavzevii* nuclear contribution was never in the majority. This discrepancy could be due to a fitness advantage conferred by the *S. kudriavzevii* mtDNA in colder fermentations, or it could be due to a fitness advantage conferred by the *S. cerevisiae* or other nuclear genomes [36,37]. Indeed, all outliers in our logistic regression analysis were in the direction of inheriting a cryotolerant parent's mtDNA. These findings suggest that the inheritance of a cryotolerant mtDNA allowed these hybrids to thrive in colder environments where pure *S. cerevisiae* strains struggle, providing evolutionary and genetic innovation that enabled new fermentation techniques, such as lager brewing.

Hundreds of nuclear-encoded proteins localize to the mitochondria [38]. This interaction can be a source of genetic incompatibilities between the nuclear and mtDNAs, several of which have been characterized in *Saccharomyces* interspecies hybrids [39–41]. Therefore, we tested whether mitochondrially localized, nuclear-encoded genes were retained more often than other genes encoded in the nuclear genome matching the mtDNA parent. We found that more mitochondrially localized genes were retained in the same ratio as all other orthologs ($p = 0.8612$, odds ratio = 0.9653) (Table S3, Figure 3c). Although these results suggest that mitochondrial localization is not the main cause of the correlation between nuclear and mtDNA content, some nuance is warranted. First, only a small number of mitochondrially localized genes have been implicated in mito-nuclear incompatibilities [39–41], and other factors that do not rely on protein localization could also play a role (e.g. metabolite exchange between the mitochondria and cytoplasm). Perhaps more importantly, these hybrids have often lost whole chromosomes or regions containing hundreds of genes at a time through chromosome mis-segregation or mitotic recombination events [15]; this restriction imposed by genetic linkage may prevent fine-scale

retention or loss and obscure any signal driven by specific genes. Finally, some yet unmapped cryotolerant nuclear alleles might also be favored independently from the cryotolerant mtDNA. Overall, from this dataset, we conclude that there is a strong correlation between the amount of nuclear and mitochondrial DNA contributed by each parent species, but mitochondrially localized genes are not more affected than other genes.

Pan-Genome Analyses:

To characterize the core genome of these hybrids, we first analyzed the retention of 1:1:1:1 orthologs conserved in all four parent species and determined which parents contributed the least and most coding sequences to each hybrid. As few as 12 genes were retained in one strain, whereas some hybrids have retained almost complete sets of orthologs from all their parents (Figure S12, and Table S4). On average, these hybrids retained 56.2% of orthologs from the parent who contributed the least genomic material.

We preformed de novo genome assemblies to analyze the genomic content that was not present in the parent reference genomes (Figure S13). On average, these hybrids had 47.7 kbp of novel genomic content; the minimum was 2.2 kbp, and the maximum was 363.3 kbp. In addition to novel content that may come from the pan-genomes of other the *Saccharomyces* species, we detected previously characterized content from prior *S. cerevisiae* pan-genome analyses, including horizontally transferred genes (Supplemental Text) [5,12,42]. When we searched this material for *Saccharomyces*-like genes for which we could assign a function, we found an enrichment in genes associated with sugar transport, including the Gene Ontology [43,44] terms: transporter activity (corrected p-val = 4.67E-08), sugar:proton symporter activity (corrected p-val = 6.04E-08), cation:sugar symporter activity (corrected p-val = 6.04E-08), and sugar

transmembrane transporter activity (corrected p-val = 6.04E-08) (Table S5). The enrichment of sugar transport genes in the novel content of these hybrids and introgressed strains is consistent with strong selection for these activities in industrial fermentation environments.

Maltotriose Utilization Genes

We took a more detailed look at maltotrisoe utilizing genes because maltotriose is generally the second most abundant sugar in beer wort or malt extract, and *Saccharomyces* strains that utilize it are relatively rare outside of domesticated ale-brewing strains [45–48]. Our analyses of lager-brewing yeasts suggest that both *S. cerevisiae* and *S. eubayanus* contributed genes encoding functional maltotriose transporters to the hybrids, including alleles of *S. cerevisiae MTT1* and *S. eubayanus AGT1* previously shown to be functional [18] (Figure 5b, Supplementary Text). We also recovered other predicted maltose/maltotriose transporter homologs in other interspecies hybrids and their parent species, which have yet to be explored functionally (Table S6). We conclude that the complexity and diversity of maltose transporter genes across *Saccharomyces* species is extensive and may have provided a source of functional diversity to fermentation hybrids.

Phenolic Off-Flavor Genes

The introduction of genes from wild strains, especially the mitochondrial genome and *S. eubayanus AGT1*, may have been key to cold fermentations, but other genes likely negatively impacted products. 4-vinyl guaiacol (4VG) is perceived as a clove-like, phenolic, or smoky flavor and considered an undesirable off-flavor in most beers. Lager beers are known for their crisp flavor profiles that lack appreciable 4VG, while wild strains of *S. eubayanus* and other

species produce 4VG [49]. Two genes, *PAD1* and *FDC1*, are essential for the production of 4VG [50]. Studies in ale-brewing yeast show that this trait is under strong domestication selection (Supplementary Text), but the genotypes of *PAD1* and *FDC1* across diverse interspecies hybrids already in use by industry have not been investigated, nor have the evolutionary genetic events leading to these genotypes. In our large hybrid dataset, we analyzed both retention and predicted functionality of *PAD1* and *FDC1* alleles from their parent species (Figure 4).

In both *S. cerevisiae* × *S. kudriavzevii* and *S. eubayanus* × *S. uvarum* hybrids and introgressed strains, we found both *FDC1* and *PAD1* alleles that were predicted to be functional (Supplementary Text). These findings may reflect selection for diverse flavors, which are desirable in niche Trappist-style beers made with *S. cerevisiae* × *S. kudriavzevii*. In contrast *S. eubayanus* × *S. uvarum* are often viewed as contaminants in industrial brewing environments, and production of 4VG could contribute to this perception.

In the lager-brewing hybrids, we found that all strains have lost the ability to produce 4VG, but mechanism of this loss differed between Saaz and Frohberg (Supplementary Text). The Frohberg lager strains likely inherited a loss-of-function *FDC1* allele from their domesticated *S. cerevisiae* parent and functional *PAD1* and *FDC1* alleles from their *S. eubayanus* parent. These functional wild alleles were then lost through translocations, likely due to break-induced replication. In contrast, the Saaz lineage has completely lost both the *S. cerevisiae* and *S. eubayanus* alleles of these genes through aneuploidy, an evolutionary trajectory facilitated by the fact that these subtelomeric genes reside on different chromosomes in these two species. The end result is that both Saaz and Frohberg lagers lack substantial phenolic off-flavors and have a crisp flavor profile. Even though Saaz and Frohberg strains evolved this trait through different final mutations that removed functional *S. eubayanus* alleles, the pre-adaptation of the domesticated *S.*

cerevisiae parent, which already lacked functional genes, played a critical role by limiting the number of mutations needed. The contrast between Saaz and Frohberg strains highlights that there are many potential evolutionary trajectories open to interspecies hybrids to achieve a domestication trait.

Conclusions

Here, we characterized the genomes of 122 interspecies yeast hybrids and introgressed strains, the largest dataset of its kind to date. These hybrids have complex genomes with contributions from two to four species: *S. cerevisiae*, *S. kudriavzevii*, *S. uvarum*, and *S. eubayanus* (Figure 5a). The hybrids with *S. cerevisiae* contributions all arose out of three domesticated *S. cerevisiae* lineages: the wine lineage and two distinct beer clades. In contrast, all the *S. kudriavzevii*, *S. uvarum*, and *S. eubayanus* parents belonged to Holarctic or European wild lineages. Our results show how hybrid vigor also applies to microbes, with the domesticated *S. cerevisiae* parents providing genes and traits pre-adapted for industrial fermentations and the divergent species of *Saccharomyces* contributing new genes and traits that led to the successes of these hybrids in specific products. First, the frequent retention of mitochondrial genomes from cryotolerant parents likely conferred a fitness advantage during cold fermentation (Figure 5b). Second, although the *S. cerevisiae* genome is required for maltotriose utilization by hybrids, both *S. eubayanus* and *S. cerevisiae* contributed functional maltotriose transporter genes to lager-brewing yeasts. Third, phenolic off-flavor genes have been inactivated or eliminated from lager-brewing yeasts by multiple types of mutations (Figure 5b), while these genes have been retained in yeasts that ferment products where phenolic off-flavor is prized.

297 Hundreds of years ago, a *S. cerevisiae* strain meeting a *S. eubayanus* strain sparked the
298 cold-brewing revolution, and crisp refreshing lagers eventually overtook the global beer market.
299 This extensive genomic dataset reveals the genetic mechanisms and distinct evolutionary
300 trajectories followed by hybrid and introgressed strains associated with fermentation products.
301 These diverse hybrids and introgressed strains highlight how dynamic and complex fermentation
302 innovation has cascaded down divergent and convergent evolutionary trajectories.
303
304 Methods
305 Strain Selection and Sequencing
306 The strains newly published here are from wild or beverage isolations, the Agricultural
307 Research Service (ARS) NRRL collection (https://nrrl.ncaur.usda.gov), and commercially
308 available sources. Table S7 contains the full metadata for strains. Whole genome Illlumina
309 paired-end sequencing was done as previously described using either 2X100 or 2X250 reads [32,51].
310 This short-read data is available through the NCBI SRA database under the accession number
311 PRJNA522928. Short-read data for published genomes were downloaded from NCBI; Table S8
312 contains a full list of accession numbers and citations [8,9,11,16,26,30,32,42,52–72].
313
314 Hybrid Identification
315 We used sppIDer [73], a hybrid detection and analysis pipeline, to identify new hybrids,
316 pure species, and reconfirm the species and hybrid identities of published data. For sppIDer, we
317 used a combination reference genome that included all published genomes for all the
318 *Saccharomyces* species [63,72,74,75] (https://www.yeastgenome.org/,
319 www.saccharomycessensustricto.org). For *S. kudriavzevii*, we used the genome from the

Portuguese strain ZP591. As previously noted [72], the published *S. uvarum* genome has the labels for chromosomes X and XII swapped, so we manually corrected them. We ran sppIDer with parameters set to identify genomic contributions >1% of the total genome. As sppIDer is reference genome-based, inheritance of regions not in the reference genome was not analyzed. Therefore, interspecies hybrids with only minor or subtelomeric introgressions were missed with this method. We also detected some smaller introgressions through the pan-genome analyses (see below).

Hybrid isolation environment was classified based on marketed product type for commercial strains; for published strains or strains from the ARS NRRL collection, we used available metadata supplied by the authors or depositors. Full details on hybrid isolation environment classification can be found in Table S1. To determine if there was an association between hybrid type and isolation environment, we completed χ^2 analyses of hybrid by environment and of environment by hybrid with a Bonferroni multiple test correction in R. We limited this test to our most common (n>15) hybrid types (*S. cerevisiae* × *S. eubayanus*, *S. cerevisiae* × *S. kudriavzevii*, and *S. eubayanus* × *S. uvarum*) and the most common (n>8) origins (beer, wine, and fruit).

Whole Genome Sequence Assembly Pipeline

Alignment and single nucleotide polymorphism (SNP) calling were done as described previously [32]. Briefly, short reads were mapped with bwa "mem" to a concatenated reference genome of just the contributing parents. Reference genomes used for concatenation were the same as used for sppIDer. Samtools "view" and "sort" were then used to prepare the mapped reads with a mapping quality greater than 20 for SNP calling. PCR duplicates were removed with

picard "MarkDuplicates", and read groups were set with picard "AddOrReplaceReadGroups". SNPs were called with GATK's haplotype caller. Genome coverage per base pair was assessed with bedtools "genomeCoverageBed". Strain-specific FASTA files were created by replacing called SNPs in repeat-masked concatenated reference genomes. Variants called as indels were replaced with Ns. Regions of extremely high coverage, (i.e. the 99.9th percentile of genome-wide coverage) were masked as Ns. Regions that do not exist in hybrids were masked as Ns, and regions at low coverage (i.e. between 3X-10X, depending on where the 10^{th} percentile of the distribution of depth of coverage across the concatenated genomes fell) were masked as Ns. The strain-specific FASTAs for hybrid genomes were split into their component sub-genomes to be analyzed with pure strains.

Genomic completeness was estimated as the percent of the reference genome with coverage above the low-coverage masking threshold. Ploidy was estimated across the combination genome in 10-kbp windows. We used the R package *modes* (version 0.7.0) to analyze the distribution of depth of coverage and determine the antimodes, which correspond to a change in ploidy state. Some manual curation was needed for strains with "smiley patterns", a pattern of increased coverage at chromosome ends that has been noted in other depth-of-coverage analyses [8,76] and may be due chromatin structure [77]. For these strains, we used only the coverages that fell below the 95^{th} percentile to estimate the antimodes and then assigned the distal ends to the largest ploidy estimated. We also visually checked and corrected rare instances when a "smiley pattern" lowered the ploidy estimate for the middle of the chromosome. From this antimode analysis, we were able to assign each 10-kbp window a ploidy value. The total DNA base-pair content contributed by each parent could then be estimated as the sum of each ploidy value multiplied by 10k and the number of windows with that ploidy value. Correcting

this total DNA content per species by the total sum of all contributing species gave us a measure of total genomic content per species. Genomic contribution to a hybrid genome can be viewed as genomic content and genomic completeness. To estimate genomic completeness, we determined what percent of a total parent sub-genome had at least one haploid copy. To estimate genomic content, we took into account both completeness and ploidy across the combination of sub-genomes. Full details on hybrid genome contributions can be found in Table S1. For visualizations, we clustered the strains based on ploidy estimated across the combination genome using Ward's method in the R package *pvclust* (v. 2.0–0) [78].

For each strain, we calculated the number of sites called as heterozygous with GATK for each sub-genome. Strains with more than 20,000 heterozygous sites in any sub-genome were phased with GATK's "ReadBackedPhasing" command [79], which can phase short regions of the genome based on overlapping reads. We then split the output into two phases, one that retains more reference variants and one that contains more alternative variants in phased regions. This pseudo-phasing allowed us to investigate regions that are less similar to the published reference. We converted these phases into two strain-specific FASTA files and masked them for coverage as above. Both phases were included in all downstream analyses involving phased genomes, which are noted as "strainID 1" or "strainID 2".

1:1:1:1 Orthologs

We identified genes that are orthologous across all parent genomes based on the annotations in the published gff files for each reference genome, which yielded a list of 3,856 genes. We used the coordinates to determine the coverage for each ortholog. Gene presence was noted if the mean coverage for that ortholog was >3X.

389

390 De Novo Genome Assembly and Pan-Genome Analyses

391 We assembled the hybrid genomes with the meta-assembler iWGS [80] and choose the best
392 assembly based on the largest N50 score. All hybrids, except DBVPG6257, were successfully
393 assembled and are available under GenBank BioProject PRJNA522928.

394 We mapped the short-read data back to these assembled genomes and used the sppIDer
395 output to classify to which parent reference genome each short read mapped. With this analysis,
396 we determined which reads did not map to a parent reference genome but did assemble de novo
397 into a contig of 1.5-kbp or greater. We classified these regions as "unmapped" and used a
398 tBLASTx to search for *S. cerevisiae*-like genes using S288C ORFs and retaining hits with e-
399 value < 10^{-10}. To determine if this set of genes identified in these novel assembled regions were
400 enriched for any functions, we used GO Term Finder (Version 0.86) [43,44]. To determine the
401 potential origin of these novel regions, we used a BLASTn search of the NCBI nucleotide
402 database (v5). The output of this was then parsed for number of hits with an e-value < 10^{-10}. To
403 determine the number of hits to different species, we completed χ^2 analyses with a Bonferroni
404 multiple test correction in R.

405

406 Translocation Identification

407 To detect shared breakpoints and translocations, we use LUMPY [81] with the mapped
408 short-read data. We masked for repetitive regions by excluding regions with coverage above
409 twice the genome-wide mean. Each breakpoint call had to be supported by at least 4 reads to be
410 included in downstream analyses. We parsed this output for species sub-genome, hybrid type,
411 and the species pair between which the translocation was detected. We calculated the total

number of called breakpoints, breakpoints that were shared in at least two hybrids of the same type, and breakpoints that were shared in multiple hybrid types. We compared these different categories with χ^2 analyses and a Bonferroni multiple test correction in R.

We also identified translocations from the de novo assemblies. For this analysis, we used sppIDer results to assign regions of the de novo assemblies to a parent species. Some regions were unmapped with sppIDer, as noted above. Additionally, some regions had high coverage from multiple parents in the de novo assembly, where the donor species could not be unambiguously assigned; these regions are likely repetitive and difficult to assemble. Translocations were identified when regions that were >2-kbp came from different donor species and were assembled with <100-bp of unmapped or ambiguous data separating them. On average, we identified 17 translocations per strain. From this output, we counted the number of translocations identified in each hybrid type, the donor species, and the pair of species between which the translocations occurred. We compared hybrid type, species pair, and individual species with a χ^2 analyses with a Bonferroni multiple test correction in R.

Mitochondrial Genome Analysis Pipeline

We use mitoSppIDer [73] to determine the mitochondrial genome (mtDNA) parent for the hybrids. This analysis was done in a similar manner to the whole genome sppIDer analysis, except that mtDNAs for each *Saccharomyces* species were used [72,82,83], except *Saccharomyces jurei*. GenBank accessions lacking full manuscripts included *S. mikatae* (KX707788) and *S. kudriavzevii* (KX707787).

To determine if the mtDNA parent was associated with retention of the nuclear genes, we performed a logistic regression in R. We used the set of 1:1:1:1 orthologs to determine which

parent contributed the most complete set of orthologous genes. To determine if there was an enrichment for the retention of nuclear-encoded, mitochondrially interacting proteins, we used the set of genes products identified as localize to the mitochondria through the Yeast GFP Fusion Localization Database [38]. When we filtered for genes that were also 1:1:1:1 orthologs, our final list consisted of 459 genes. To determine if there was a linear relationship between retention of mitochondrially localized genes and all other orthologs, we performed a linear regression and to determine if there were more mitochondrially localized genes retained compared to all other genes, we used a Fisher's Exact Test with a Bonferroni correction. Tests were performed in R.

Since past work has shown that reticulate evolution, introgression, and horizontal gene transfers are widespread in *Saccharomyces* mtDNAs [84], we wanted to explore the inheritance of mitochondrially encoded genes in more depth. Due in part to their high AT content (~85%), mtDNAs are often poorly covered using Illumina sequencing. In particular, intergenic regions and coding sequencing with transposable elements (introns, homing endonucleases, and GC clusters) can be difficult to assemble. To explore the phylogenetic relationships of these mtDNAs, we used a bait-prey bioinformatic method to pull out the read sequences of coding sequences. We used HybPiper [85] to pull out reads from the hybrid Illumina libraries that mapped to those mitochondrial genes using gene sequences from reference strains used in mitoSppIDer as baits. These extracted Illumina reads were aligned to the reference genes in Geneious (v. 6.1.6) [86] and manually assembled. We successfully covered six mitochondrial genes (*COX2, COX3, ATP6, ATP8, ATP9,* and *15S rRNA*), which were used to construct the mitochondrial phylogenetic haplotype network. This unique set of unambiguously completed genes was concatenated (4.7-kbp) by strain to produce the haplotype for each pure *Saccharomyces* or hybrid strain (Figure S14). Haplotypes and haplotype frequencies for each strain were encoded

as a nexus-formatted file for PopART v1.7.2 [87]. The haplotype network was reconstructed using the TCS method [88]. Strains were assigned to each haplotype using DnaSP v5 [89]. For some strains, we could not assemble the *15S rRNA* gene because of low-coverage data. For these strains, we inferred their haplotype designation based on an analysis where we removed the*15S rRNA* gene. This information is not included in Figure S14 but can be found in Table S9.

Genes of Functional Interest Analysis Pipeline

To assemble the sequences of genes relevant to brewing, we again used HybPiper [85]. To be included for further analyses, the assembled length had to be at least as long as the bait gene and had to have a minimum 10X depth of coverage. For the baits, we used either gene sequences from the *S. cerevisiae* strain S288C found on the *Saccharomyces* Genome Database (https://www.yeastgenome.org); from the *S. eubayanus* type strain, CBS12357^{T} [72]; or the lager strain W34/70 [90]. For the *PAD1* analysis in *S. eubayanus* × *S. uvarum* hybrids, we used the *PAD1* gene sequence from the *S. uvarum* reference genome, CBS7001 [63]. To get precise gene locations for *PAD1* and *FDC1*, we used a tBLASTn search of the *S. eubayanus*, *S. kudriavzevii*, and *S. uvarum* reference genomes with the *S. cerevisiae* sequences for these genes as the query.

The assembled genes were aligned with MAFFT v.7 [91], allowing for reverse complementation. The alignments were manually trimmed to the protein-coding sequences. For *PAD1* and *FDC1*, the alignments were conceptually translated to amino acid sequences, and haplotype networks were built with a modified minimum-spanning network and visualized with *iGraph* [92] in R. The haplotype networks were split into communities as previously described [93].

Pairwise distances between sequences were calculated using the trimmed MAFFT nucleotide sequence alignments and the p-distance method as implemented in MEGA-X [94] with

481 the following parameters: substitutions to include Transitions + Transversions, assuming uniform
482 rates among sites, and using pairwise deletion of gaps. The percent identity of hits to the bait
483 sequence was organized by species, and hybrid status was recorded in Table S6, along with the
484 origin of the bait gene and tallies of sequences whose translations were visually identified as
485 being incomplete or containing premature stop codons.
486
487 Phylogenomic and Population Structure Analyses
488 We masked regions with no coverage as Ns, which is interpreted as missing data by most
489 tools; therefore, for downstream whole genome analyses, we only included sub-genomes that
490 were >50% complete (i.e. major contributions). To include the minor contribution hybrids in the
491 non-*S. cerevisiae* analyses, we used reduced genomes that were concatenations of the regions of
492 the genome that existed in at least one minor hybrid (Table S10). This procedure allowed us
493 include strains with minor introgressions and only use regions of the genome that had been
494 contributed by the minor parent. To balance some of our analyses for Saaz and Frohberg lager
495 strains, we used a random subset of Frohberg strains to match the number of Saaz strains.
496 Phylogenomic trees were built with RAxML v8.1 [95] using SNPs from the whole genome for the
497 major analyses or the reduced genome for the minor analyses. Trees were visualized with iTOL
498 [96]. The PCA analyses were done with the *adegenet* package in R [97] and visualized with *ggPlot2*
499 [98]. Estimates of adjusted π (π *100) were calculated with the *PopGenome* package in R [99].
500
501 Data and Code Availability
502 References and accession numbers for the published data used can be found in Table S8.
503 Short-read data newly published here is available through the NCBI SRA database under the

accession number PRJNA522928. Custom R and Python scripts used for this publication can be found on GitHub (https://github.com/qlangdon/hybrid-ferment-invent).

Author Contributions

QKL performed most analyses with assistance from DAO; DP and QKL performed mitochondrial genome analyses and drafted text; EPB and QKL analyzed genes of functional interest and drafted text; QKL, EPB, and DAO sequenced genomes; HVN, UB, PG, and JPS contributed key strains to study design; QKL, DP, EPB, DL, and CTH designed the study; and QKL and CTH wrote the manuscript with editorial input from all co-authors.

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Figure Legends

Figure 1. Summary of genomic contributions and isolation environments for interspecies hybrids. (a) Hybrids were clustered by genomic contributions. Lager strains are in the bottom half, *S. uvarum* × *S. eubayanus* strains are at the top, and most complex hybrids are in the

780 middle, except for the single *S. cerevisiae* × *S. eubayanus* × *S. kudriavzevii* hybrid (very bottom).
781 Individual hybrid strains are along the y-axis, and the genomes of the species contributing to
782 hybrids are along the x-axis. *S. cerevisiae (Scer)* is in red, *S. kudriavzevii* (*Skud*) is in green, *S.*
783 *uvarum* (*Suva*) is in purple, and *S. eubayanus* (*Seub*) is in pink. Dotted lines indicate
784 chromosomes. Ploidy estimates are indicated by opacity, where darker regions are higher ploidy.
785 (b) Counts of hybrids isolated from different environments. The lagers have been split into Saaz
786 and Frohberg lineages. Other is grouped with Unknown and represents one isolate from a
787 distillery. Tables S1 & S3 includes all isolation information and metadata.
788
789 Figure 2. Population and phylogenomic analyses of *S. cerevisiae*, *S. kudriavzevii*, *S. uvarum*, *S.*
790 *eubayanus*, and their hybrid sub-genomes.
791 All phylogenies were built with RAxML with pure strains of a species and any hybrids with
792 >50% complete sub-genome for given species. Bootstrap support values >70% are shown as
793 gray dots. Branches are colored by the origin of isolation for each strain. Each hybrid has a
794 stacked bar plot showing the genomic content for each species contributing to their genome;
795 species colors are the same as in Figure 1a. For the Principal Component Analyses (PCA), dot
796 colors represent strains' origins, and color clouds represent populations or lineages. The axes of
797 all PCAs are scaled to the same range. Phylogenies with strain names, Newick formatted files,
798 and data frames used to build PCAs are available as Figures S2, S4, S6, and S8; Table S2; and
799 Files S1, S3, S5, and S7. (a) Left: Phylogeny of *S. kudriavzevii* with 30 strains and 38,992 SNPs
800 from across the genome and rooted with an Asia B strain, IFO1803 (removed for clarity). Right:
801 Principal component projection for PC1 and PC2, excluding Asia B. (b) Phylogeny of *S.*
802 *eubayanus* with 92 strains and 18,878 SNPs from across the genome, rooted with Population A

(PopA). Right: Principal component projection of PC1 and PC2. (c) Phylogeny for *S. uvarum* with 82 strains and 18,652 SNPs from across the genome, rooted with the Australasian lineage (removed for clarity). Right: Principal component projection for PC1 and PC2, excluding the Australasian lineage. (d) Top: Phylogeny for *S. cerevisiae* with 612 phased (for strains with >20K heterozygous sites) or unphased haplotypes and 21,222 SNPs from across the genome, rooted with the Taiwanese strain EN14S01 (removed for clarity). Previously identified wild lineages from West Africa, Malaysia, North America, Japan, and the Philippines are included in the Wild Misc group [11,74]. The other lineages are named in a similar manner to previous studies on ale-brewing and Mediterranean Oak (MedOak) strains [8,9,71]. Bottom: Principal component projection for PC1 and PC2 (including EN14S01, which groups with Sake/Asian).

Figure 3. Mitochondrial genome inheritance in interspecies hybrids.

(a) The bar plots show proportion of 1:1:1:1 ortholog content for each sub-genome for each hybrid grouped by the mitochondrial genome (mtDNA) parent, which are labeled across the top. Colors represent different parent species and are that same as in of Figure 1a. (b) Analysis of concordance between which mtDNA was inherited and which parent contributed the most complete set of orthologous genes. “True” includes hybrids that inherited the most nuclear gene content from the same species as the mtDNA. “False” includes hybrids with mtDNA that did not match the species that contributed the most nuclear gene content. Colors represent the mtDNA parent, and shapes represent the largest nuclear genome contributor. The middle of the box plot corresponds to the median, the upper and lower limits are the 75^{th} and 25^{th} percentiles respectively, and the whiskers extend to the largest or smallest value no greater than 1.5 × the differences between the 75^{th} and 25^{th} percentiles. There was a significant correlation between the

826 mtDNA parent and the largest nuclear genomic contributor (logistic regression p=3.58E-8, AIC=
827 118.21). Notably, the *S. eubayanus* × *S. uvarum* hybrids, which have often undergone many
828 backcrossing events, follow this trend and are both cryotolerant species. (c) Linear relationship
829 of the number of 1:1:1:1 orthologs versus the number of nuclear-encoded, mitochondrially
830 localized genes present in the sub-genome that matches the mtDNA (linear regression p=2.0E-
831 16, AIC= 1151.5). The inset shows the mean proportion of mitochondrially localized versus all
832 other nuclear genes present in the sub-genome that matches the mitochondrial parent (p =
833 0.8612, odds ratio = 0.9653).
834
835 Figure 4. Hybrid inheritance and functionality of genes responsible for 4-vinyl guaiacol (4-VG)
836 production.
837 Retention of the regions where the adjacent *PAD1* and *FDC1* genes, which are both required for
838 4-VG production, are located in each parent species (a-c), shown as 10-kbp windows of ploidy
839 estimates over last 100-kbp of the chromosome. Gene locations are represented by black dotted
840 lines. Higher opacity represents higher ploidy. Species colors are that same as in Figure 1a. *Scer*
841 *= S. cerevisiae*, *Spar = Saccharomyces paradoxus, Smik = Saccharomyces mikatae, Skud = S.*
842 *kudriavzevii*, *Suva = S. uvarum*, and *Seub = S. eubayanus*. (a) *Scer* × *Skud* hybrids: all strains
843 inherited versions of both *PAD1* and *FDC1* from *Scer* that are predicted to be functional, + | +,
844 but they have lost the *Skud* alleles. (b) *Suva* × *Seub* hybrids: all strains inherited versions of
845 *PAD1* and *FDC1*, from either *Suva* or *Seub*, that are predicted to be functional, + | +. (c) All
846 lager strains have completely lost the region in the *Seub* genome where these genes reside.
847 Additionally, all Saaz strains have also completely lost the *Scer* versions of these genes, Δ | Δ.
848 All but two Frohberg strains have retained versions of *PAD1* from *Scer* that are predicted to be

functional, but inherited *Scer* alleles of *FDC1* that are predicted to be inactive due to a frameshift mutation, + | Ψ. Haplotype networks were built for the amino acid sequences for Fdc1 (d) and Pad1 (e). Colored pies correspond to *Scer* lineages, hybrids, or wild species with size representing the number of strains with that haplotype. Non-*Scer* nodes or groups of nodes are labeled by the species to which they correspond. Colored clouds correspond to communities: red is mostly *Scer*, blue is mostly non-*Scer* (including *Seub* and *Suva*), yellow is mostly *Spar* and *Smik*, green is mostly *Skud*, and gray is mostly loss-of-function alleles. Pseudogenes are marked as Ψ with additional information about the loss-of-function nucleotide and amino-acid changes. Dotted connections represent >100 amino acid differences.

Figure 5. Summary of hybrids and origin of lager traits.

(a) Simplified summary of parents and resulting hybrids. On the left is a cladogram of just the *Saccharomcyes* species that have contributed to fermented beverage hybrids. Three distinct lineages of *S. cerevisiae* (*Scer*) have contributed to hybrids; for the wild parents (*S. kudriavzevii* (*Skud*), *S. uvarum* (*Suva*), and *S. eubayanus* (*Seub*)), Holarctic or European lineages gave rise to the hybrids. Gray lines point from each parent to the resulting hybrid. The order of secondary or tertiary hybridization events was inferred from genome composition. This simplified view does not show when multiple lineages of *Scer* have contributed to different hybrid types (e.g. *Scer* × *Skud* hybrids), backcrossing (e.g. *Seub* × *Suva* hybrids), or minor subtelomeric contributions (e.g. small *Scer* contributions to some *Seub* × *Suva* hybrids). (b) Summary of how lager-brewing yeasts acquired their unique trait profile. The two lager-brewing lineages, Saaz and Frohberg, arose out of hybridizations between domesticated *Scer* ale strains and wild *Seub* strains. The *Scer* strains could utilize maltotriose (+), did not produce phenolic-off-flavor (POF^-), and preferred

warmer temperatures (☼), while the *Seub* strains tolerated colder temperatures (❄), could not use maltotriose (-), and produced phenolic-off-flavors (POF^+). The two lager-brewing lineages inherited the *Seub* mitochondrial genome (pink circle), which partly conferred cryotolerance. Both lineages also inherited maltotriose transporter genes from both parents (*MTT1* from *Scer* and *SeAGT1* from *Seub*). Finally, both lineages convergently became POF^- through multiple distinct mechanisms, including pre-adaptation in the *S. cerevisiae* ale-brewing parent due to a mutated pseudogene (*PAD1* | *fdc1Ψ* in red), aneuploidy removing functional S. *eubayanus* genes (*pad1Δ* | *fdc1Δ* in pink), and translocations in all Saaz strains and some Frohberg strains (*pad1Δ* | *fdc1Δ* in red).

Figure S1. Genomic contribution comparison of Muri and WLP351.

Modified sppIDer plot, where the y-axis is estimated ploidy, rather than coverage, for the *S. cerevisiae* (50%) × *S. eubayanus* (5%) × *S. uvarum* (45%) strains Muri[61] and WLP351.

Figure S2. Summary of total genomic coverage and shared translocations.

The minimum and maximum normalized coverage of all strains that contain each chromosome are shown as colored bars. Darker chromosomes mean that chromosome is present in more strains. Vertical dotted lines represent translocations that are shared in at least four strains, including between hybrid types. The color of the line represents the reciprocal species. (a) Only lager strains and translocations found only in lagers. (b) All 122 hybrids and interspecies translocations.

Figure S3. Phylogenomic trees for *S. kudriavzevii* with strains labeled.

(a) Phylogeny identical to Figure 2a with strains labeled. (b) Phylogeny identical to Figure S4 with strains labeled. Newick files are available as Files S1 & S2.

Figure S4. Phylogenomic and population placement of hybrids with minor *S. kudriavzevii* contributions.

(a) Phylogenomic tree built with 36 strains and 12,424 SNPs from regions of the genome that exist in at least one minor contributing hybrid. Bootstrap support values >70% are shown as gray dots. Branch colors represent origin of isolation. The inner colors correspond to origin or population. Outer stacked bar plots show the genomic content for each of the hybrids; species colors match Figure 1a. (b) PCA using whole genome data for European *S. kudriavzevii* strains and all major contributor hybrids. (c) PCA using a reduced genome (67%) but including additional minor hybrids. Phylogenies with strain names, Newick formatted files, and data frames used to build PCAs are available as Figure S3, Table S2, and File S2.

Figure S5. Phylogenomic trees for *S. eubayanus* with strains labeled

(a) Phylogeny identical to Figure 2b with strains labeled. (b) Phylogeny identical to Figure S6 with strains labeled. Newick files available as Files S3 & S4.

Figure S6. Phylogenomic and population placement of hybrids with minor *S. eubayanus* contributions.

(a) Phylogenomic tree built with 112 strains and 69,631 SNPs from regions of the genome that exist in at least one minor contributing hybrid. Bootstrap support values >70% are shown as gray dots. Branch colors represent origin of isolation. The inner colors correspond to origin or

population. Outer stacked bar plots show the genomic content for each of the hybrids; species colors match Figure 1a. Long branches are biased by the extensive missing data in hybrids with very small contributions from *S. eubayanus*. (b) PCA using whole genome data for Holarctic *S. eubayanus* strains and all major contributor hybrids. (c) PCA using a reduced genome (25%) but including additional minor hybrids. Phylogenies with strain names, Newick formatted files, and data frames used to build PCAs are available as Figure S5, Table S2, and File S4.

Figure S7. Phylogenomic trees for *S. uvarum* with strains labeled.
(a) Phylogeny identical to Figure 2c with strains labeled. (b) Phylogeny identical to Figure S8 with strains labeled. Newick files are available as Files S5 & S6.

Figure S8. Phylogenomic and population placement of hybrids with minor *S. uvarum* contributions.
(a) Phylogenomic tree built with 69 strains and 36,541 SNPs from regions of the genome that exist in at least one minor contributing hybrid. Bootstrap support values >70% are shown as gray dots. Branch colors represent origin of isolation. The inner colors correspond to origin or population. Outer stacked bar plots show the genomic content for each of the hybrids; species colors match Figure 1A. (b) PCA using whole genome data for Holarctic *S. uvarum* strains and all major contributor hybrids. (c) PCA using a reduced genome (84%) but including additional minor hybrids. Phylogenies with strain names, Newick formatted files, and data frames used to build PCAs are available as Figure S7, Table S2, and File S6.

Figure S9. Phylogenomic tree for full *S. cerevisiae* analysis with strains labeled.

941 Phylogeny identical to Figure 2d with strains labeled. A Newick file is available as File S7.

942

943 Figure S10. Phylogenomic and population placement of lagers within the Ale/Beer1 clade.
944 (a) Phylogenomic tree built with 267 strains and 21,953 SNPs from the whole genome. The total
945 number of Frohberg strains was down-sampled to match the same number of Saaz strains. The
946 tree was rooted with the Wine strain DBVPG1106. Bootstrap support values >70% are shown as
947 gray dots. Branch colors represent origin of isolation. The inner colors correspond to origin or
948 population. Outer stacked bar plots show the genomic content for each of the hybrids; species
949 colors match Figure 1a. (b) PCA using whole genome data for Ale/Beer1 strains, all Saaz strains,
950 and the down-sampled set of Frohberg strains. The two lineages of lager strains form separate
951 groups, but they do not cluster with any described geographical lineage of the Ale/Beer1 clade.
952 Pure *S. cerevisiae* Ale/Beer1 strains outside of the labeled lineages are unplaced, including a
953 cluster of Stout strains, Wheat strains, and mosaic strains that our analyses suggest share the
954 most ancestry with lager-brewing yeasts. (c) PCA using all lager strains. The low diversity in the
955 Frohberg lager strains drives PC1, which led us to balance the dataset by down-sampling this
956 lineage. Phylogenies with strain names, Newick formatted files, and data frames used to build
957 PCAs are available as Figure S11, Table S2, and File S8.

958

959 Figure S11. Phylogenomic tree for Ale/Beer1 *S. cerevisiae* analysis with strains labeled.
960 Phylogeny identical to Figure S10 with strains labeled. A Newick file is available as File S8.

961

962 Figure S12. 1:1:1:1 orthologs present in hybrid genomes.

(a) Stacked bar chart of all 1:1:1:1 orthologs present in hybrids. Strains are sorted from most to least ortholog content. Completeness of the ortholog set from the species that contributed the most (b) or least (c) orthologs to the strains. Strains are ordered independently in all panels.

Figures S13. Complete de novo genome assembly for all strains.

Total assembled genome for each strain. Regions are colored by which parent could be assigned in the de novo assembly based on the sppIDer results. "Multi" are regions where reads from many species mapped at high coverage. "Unmapped" are novel regions assembled from reads that do not map to parent reference genomes. For each assembly, contigs are ordered from largest to smallest from left to right.

Figure S14. Mitochondrial genome haplotype network.

Six mitochondrial genes were concatenated in 364 wild *Saccharomyces* strains and interspecies hybrids and used to build a TCS[88] phylogenetic network. Haplotype classification is provided in Table S9. Haplotypes are represented by circles, and circle size is scaled according to the haplotype frequency. Pie charts show the frequency of haplotypes based on species or hybrid designation. The number of mutations separating each haplotype are indicated by lines on the edges connecting the haplotype circles.

Figure S15.

Labeled (in turquoise) haplotype networks for *PAD1* and *FDC1*. Edge numbers are the number of amino acid changes. Networks correspond to those used in Figure 4 for the amino acid sequences of (a) Fdc1 and (b) Pad1. (b) A different haplotype network orientation of Figure 4E

that increases the visibility of each community and haplotype. Table S9 contains the key to which strains belong to which haplotype.

Table S1. All hybrids and their parent contributions.

Table S2. PCA analyses.

Percent explained by each principal component included in column headers.

Table S3. Results of Fisher's Exact Test and Bonferroni correction of mitochondrially localized genes.

mtInteracting = nuclear-encoded but mitochondrially localized gene.

Table S4. Summary of number of 1:1:1:1 orthologs present in each sub-genome.

Table S5. GO term results of genes found in novel regions of the de novo assembled genomes.

Table S6. Brewing relevant gene summaries.

"-" Indicates when HybPiper failed to recover and assemble genes for this group or that these assemblies failed our length and coverage cutoffs.

Table S7. Metadata for all strains newly sequenced in this study.

The "New hybrid" column denotes hybrid genome sequences that are newly published in this study.

Scer = S. cerevisiae, *Spar = Saccharomyces paradoxus*, *Smik = Saccharomyces mikatae*, *Skud = S. kudriavzevii*, *Suva = S. uvarum*, and *Seub = S. eubayanus*.

Table S8. Published data accession information.

Table S9. Haplotype key for mitochondrial genomes, *PAD1*, and *FDC1*.

Dataset A only includes strains where *15S rRNA* could be assembled, while Dataset B has *15S rRNA* removed.

1009 Table S10. Regions used for minor contribution analyses.

1010

1011 File S1. Newick formatted file of the *S. kudriavzevii* phylogeny with major hybrids.

1012 File S2. Newick formatted file of the *S. kudriavzevii* phylogeny with minor hybrids.

1013 File S3. Newick formatted file of the *S. eubayanus* phylogeny with major hybrids.

1014 File S4. Newick formatted file of the *S. eubayanus* phylogeny with minor hybrids.

1015 File S5. Newick formatted file of the *S. uvarum* phylogeny with major hybrids.

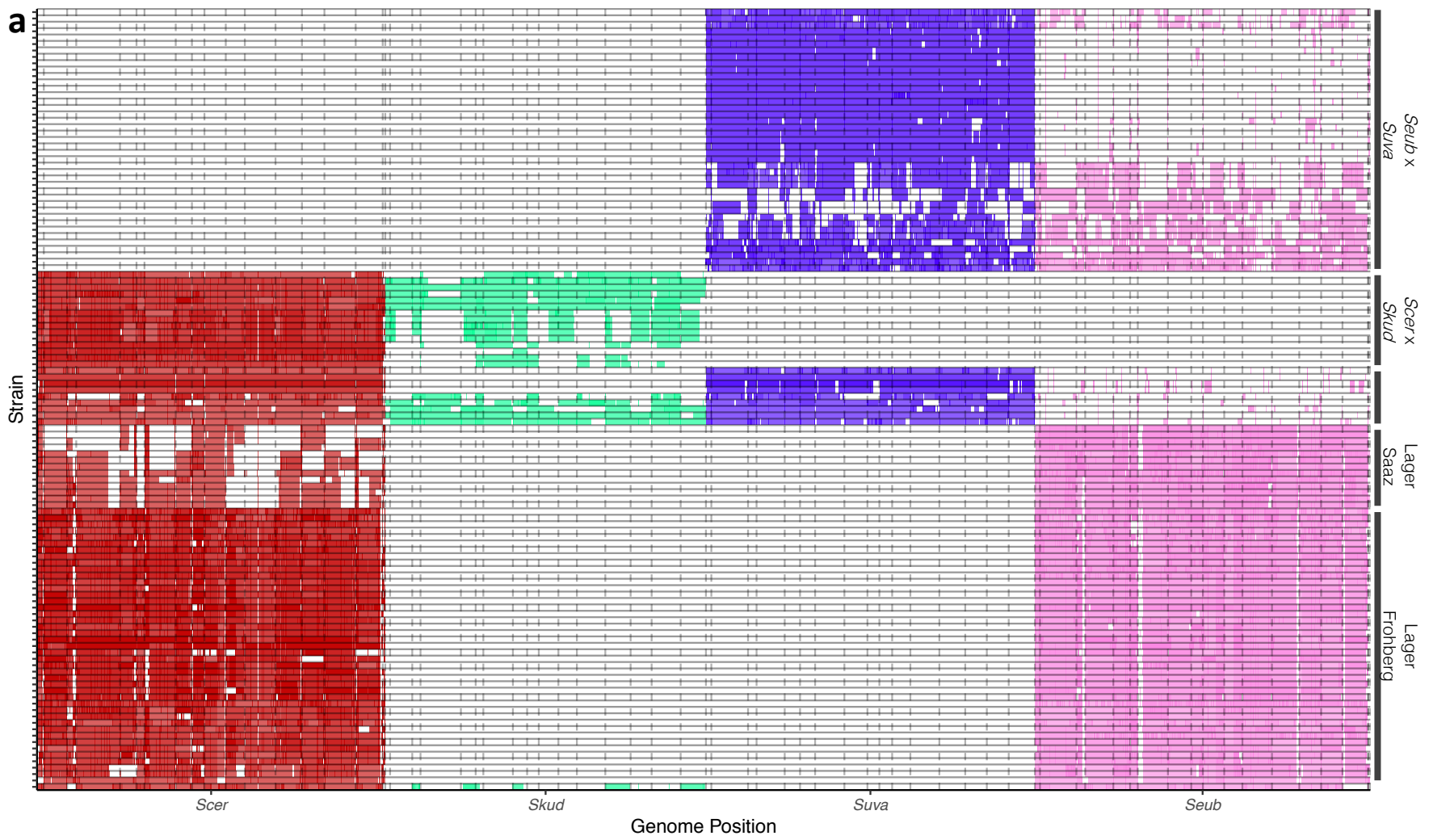
1016 File S5. Newick formatted file of the *S. uvarum* phylogeny with minor hybrids.

1017 File S7. Newick formatted file of the *S. cerevisiae* phylogeny with all strains analyzed.

1018 File S8. Newick formatted file of the *S. cerevisiae* phylogeny of just the Ale/Beer1 clade.

1019

Figure 1


a
Strain
Seub x Suva
Scer x Skud
Lager Saaz
Lager Frohberg
Scer
Skud
Suva
Seub
Genome Position

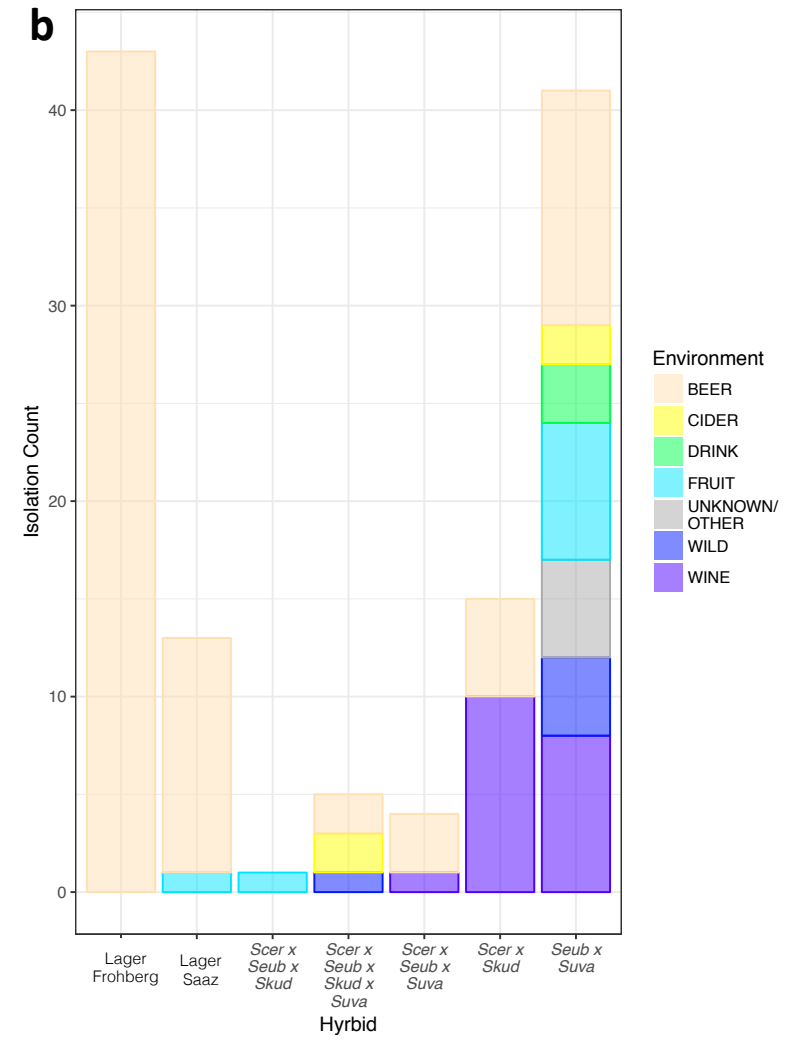

b
Isolation Count
0
10
20
30
40
Lager Frohberg
Lager Saaz
Scer x Seub x Skud
Scer x Seub x Skud x Suva
Scer x Seub x Suva
Scer x Skud
Seub x Suva
Hyrbid
Environment
BEER
CIDER
DRINK
FRUIT
UNKNOWN/ OTHER
WILD
WINE

Figure 2

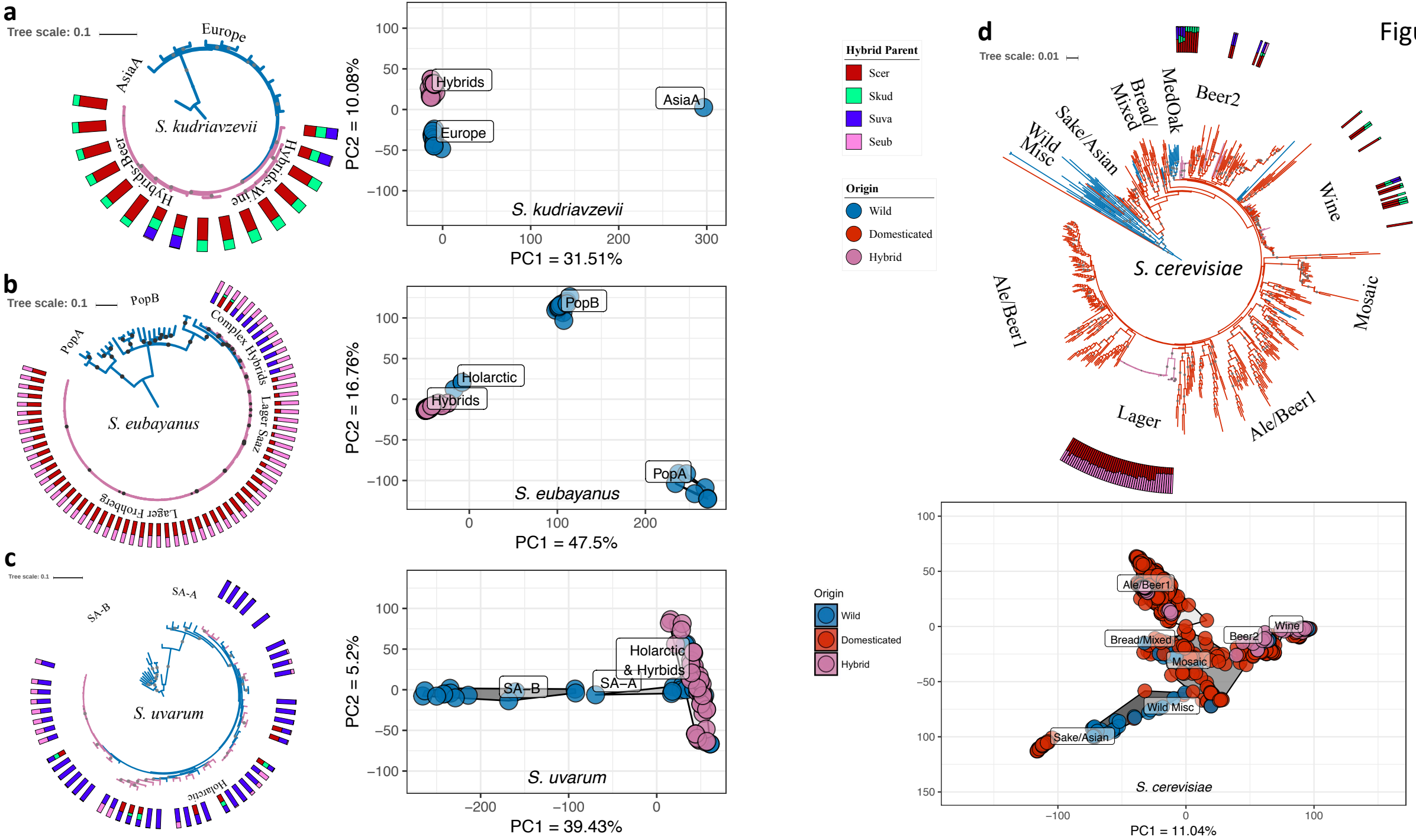

a
Tree scale: 0.1
Europe
AsiaA
S. kudriavzevii
Hybrids-Beer
Hybrids-Wine
PC2 = 10.08%
Hybrids
AsiaA
Europe
S. kudriavzevii
PC1 = 31.51%
b
Tree scale: 0.1
PopB
PopA
Complex Hybrids
Lager Saaz
Lager Frohberg
S. eubayanus
PC2 = 16.76%
PopB
Holarctic
Hybrids
PopA
S. eubayanus
PC1 = 47.5%
c
Tree scale: 0.1
SA-A
SA-B
S. uvarum
Holarctic
PC2 = 5.2%
Holarctic & Hyrbids
SA–B
SA–A
S. uvarum
PC1 = 39.43%
Hybrid Parent
Scer
Skud
Suva
Seub
Origin
Wild
Domesticated
Hybrid
d
Tree scale: 0.01
Beer2
MedOak
Bread/ Mixed
Sake/Asian
Wild Misc
Wine
Mosaic
Ale/Beer1
Lager
Ale/Beer1
S. cerevisiae
Origin
Wild
Domesticated
Hybrid
Ale/Beer1
Bread/Mixed
Beer2
Wine
Mosaic
Wild Misc
Sake/Asian
S. cerevisiae
PC1 = 11.04%

Figure 3

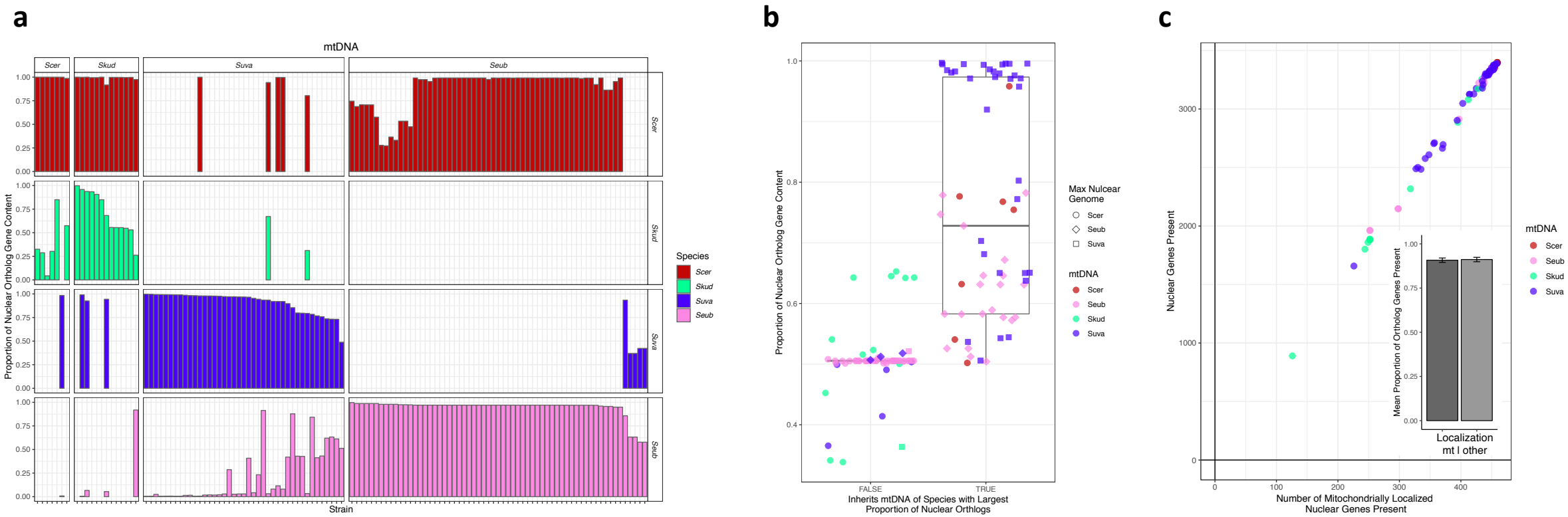

a
mtDNA
Scer
Skud
Suva
Seub
Proportion of Nuclear Ortholog Gene Content
Strain
Species
Scer
Skud
Suva
Seub
b
Proportion of Nuclear Ortholog Gene Content
FALSE
TRUE
Inherits mtDNA of Species with Largest Proportion of Nuclear Orthlogs
Max Nulcear Genome
Scer
Seub
Suva
mtDNA
Scer
Seub
Skud
Suva
c
Nuclear Genes Present
Number of Mitochondrially Localized Nuclear Genes Present
Mean Proportion of Ortholog Genes Present
Localization
mt I other
mtDNA
Scer
Seub
Skud
Suva

Figure 4

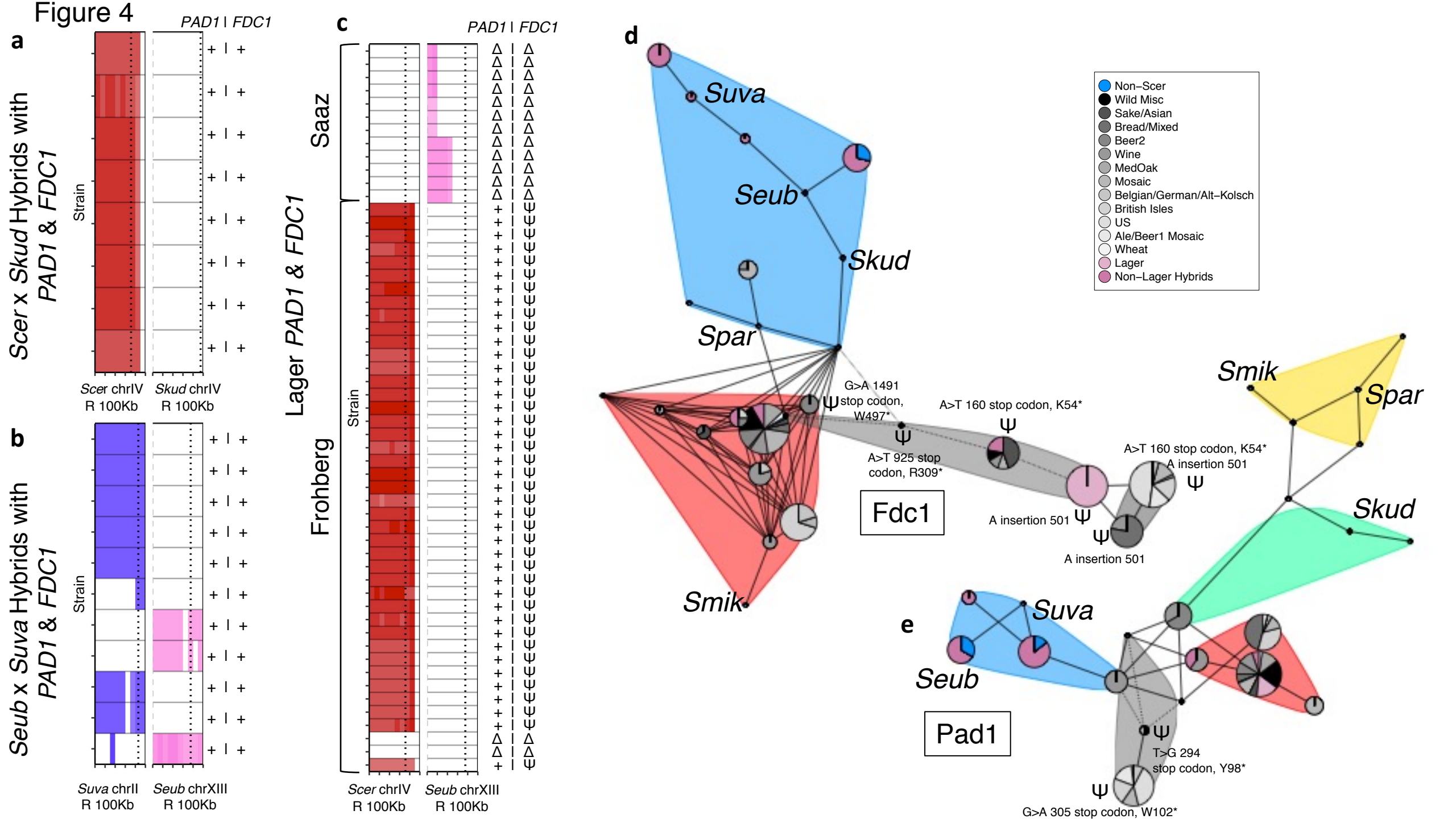

a
Scer x Skud Hybrids with PAD1 & FDC1
PAD1 | FDC1
Strain
Scer chrIV R 100Kb
Skud chrIV R 100Kb
b
Suva x Seub Hybrids with PAD1 & FDC1
Strain
Suva chrII R 100Kb
Seub chrXIII R 100Kb
c
PAD1 | FDC1
Saaz
Lager PAD1 & FDC1
Frohberg
Strain
Scer chrIV R 100Kb
Seub chrXIII R 100Kb
d
Suva
Seub
Skud
Spar
Smik
G>A 1491 stop codon, W497*
A>T 925 stop codon, R309*
A>T 160 stop codon, K54*
A insertion 501
Fdc1
Non−Scer
Wild Misc
Sake/Asian
Bread/Mixed
Beer2
Wine
MedOak
Mosaic
Belgian/German/Alt−Kolsch
British Isles
US
Ale/Beer1 Mosaic
Wheat
Lager
Non−Lager Hybrids
e
Suva
Seub
Smik
Spar
Skud
Pad1
T>G 294 stop codon, Y98*
G>A 305 stop codon, W102*

Figure 5

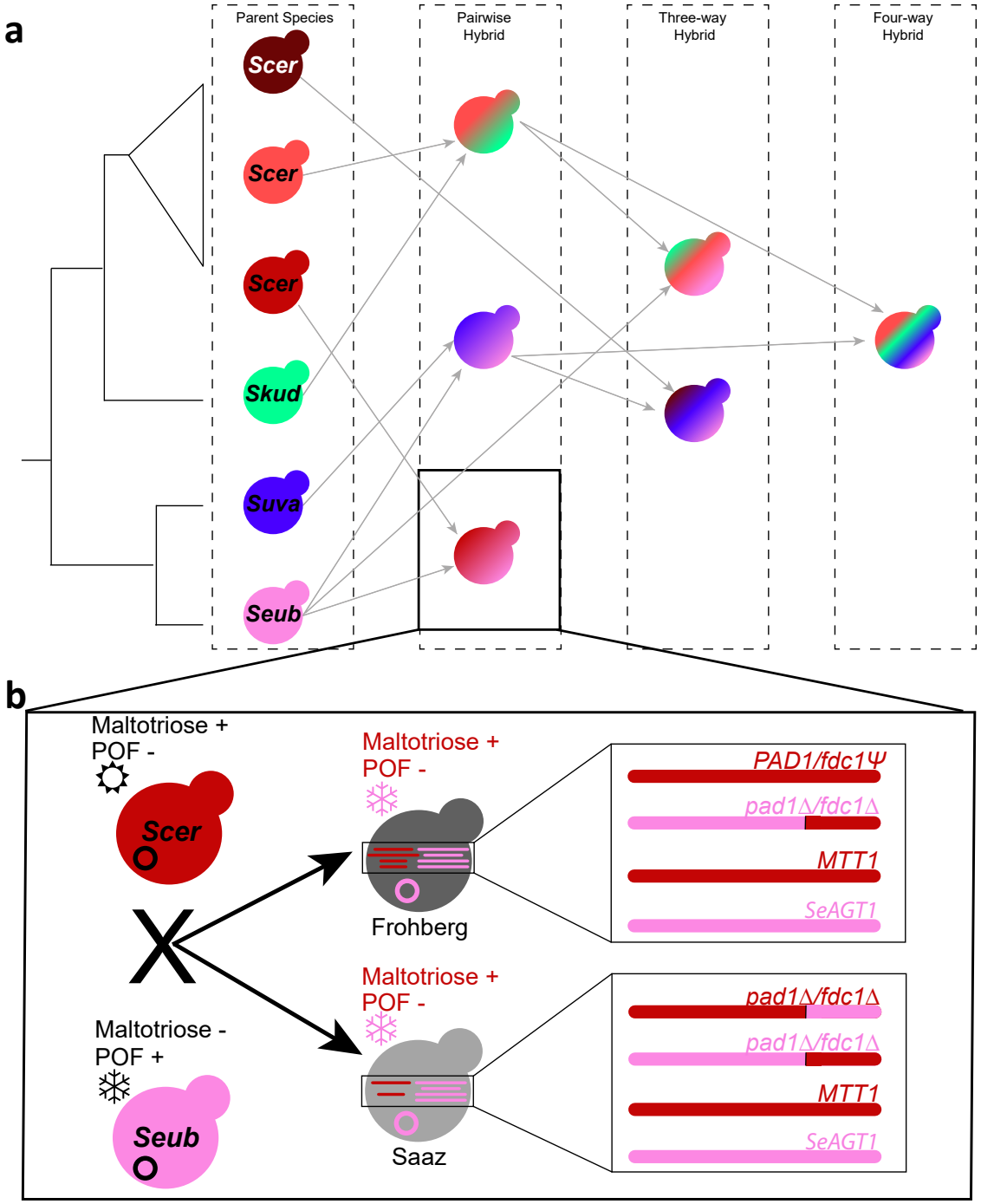

a
Parent Species
Pairwise Hybrid
Three-way Hybrid
Four-way Hybrid
Scer
Scer
Scer
Skud
Suva
Seub
b
Maltotriose +
POF -
Scer
Maltotriose +
POF -
Frohberg
PAD1/fdc1Ψ
pad1Δ/fdc1Δ
MTT1
SeAGT1
Maltotriose -
POF +
Seub
Maltotriose +
POF -
Saaz
pad1Δ/fdc1Δ
pad1Δ/fdc1Δ
MTT1
SeAGT1

Figure S1

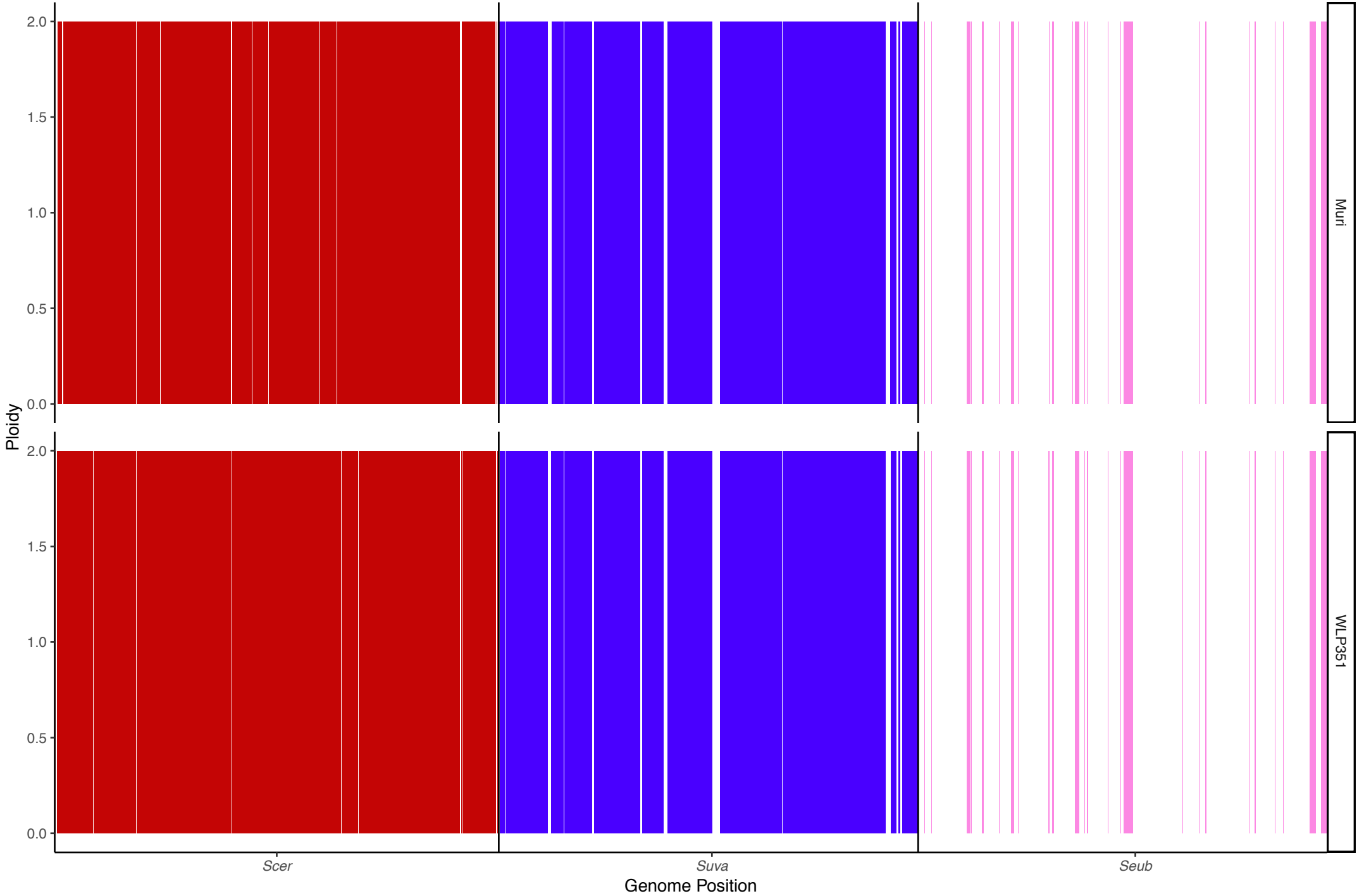
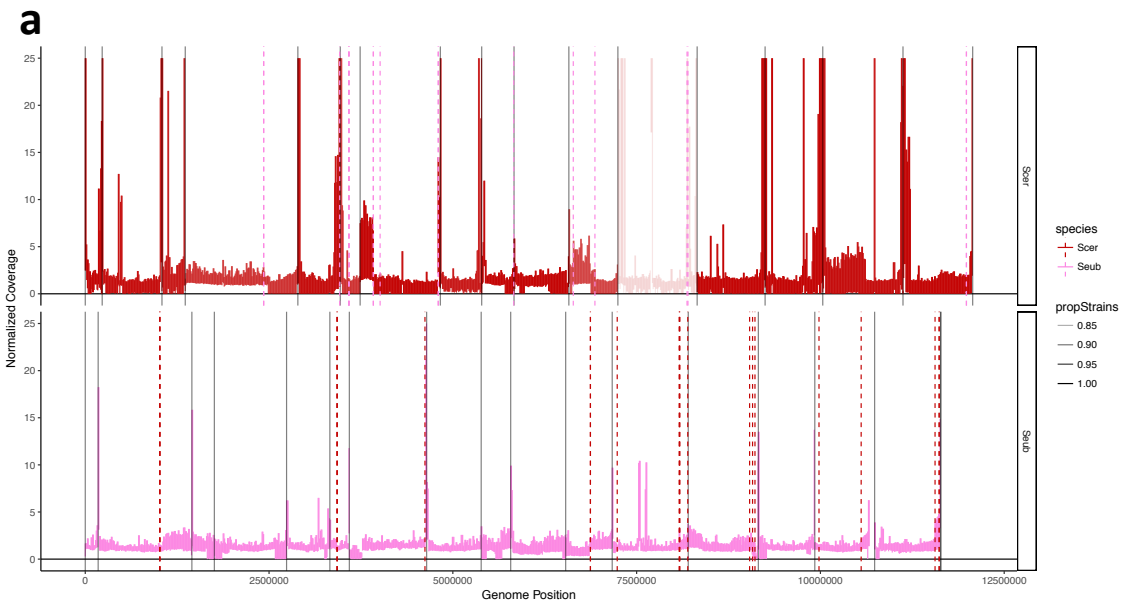

Ploidy
2.0
1.5
1.0
0.5
0.0
Muri
WLP351
Scer
Suva
Seub
Genome Position

Figure S2


a
Normalized Coverage
Genome Position
Scer
Seub
species
Scer
Seub
propStrains
0.85
0.90
0.95
1.00

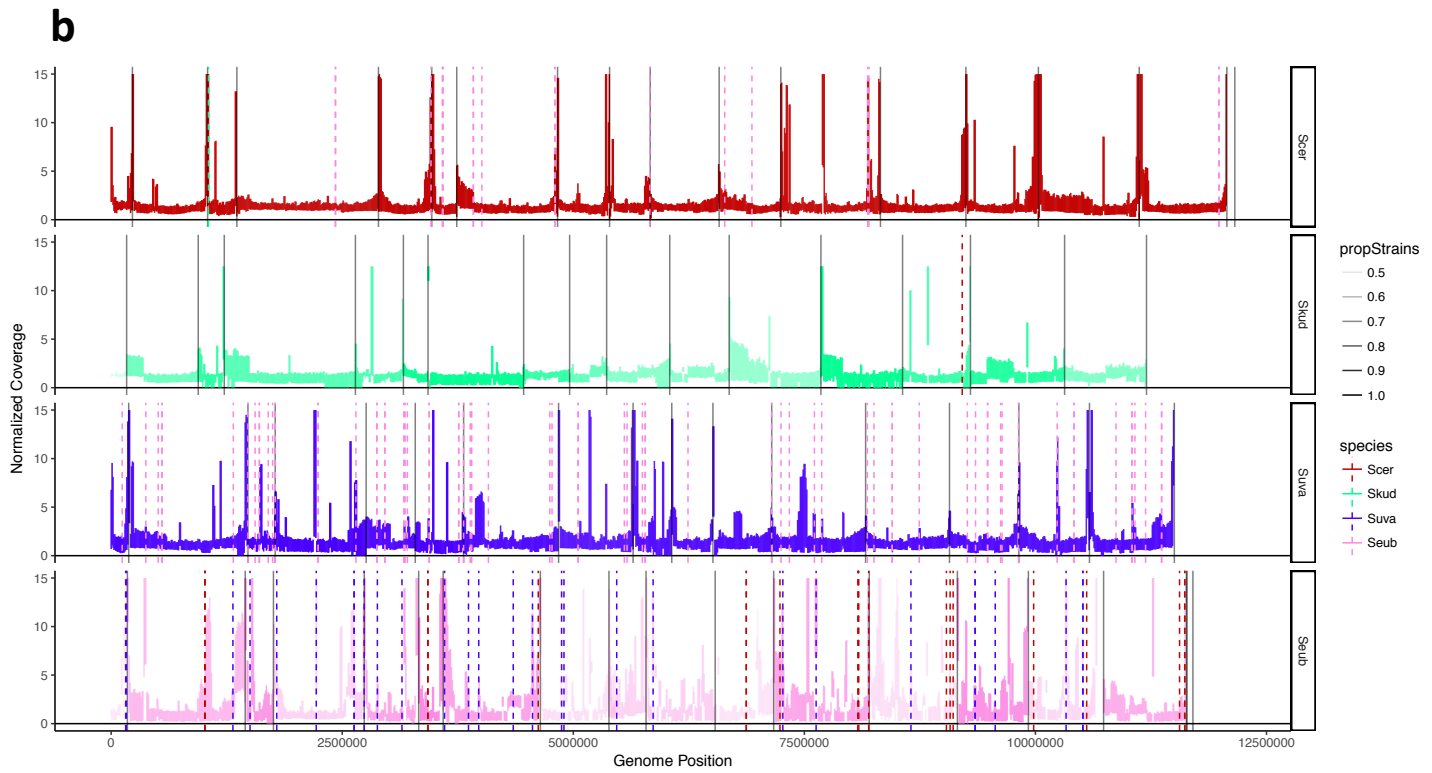
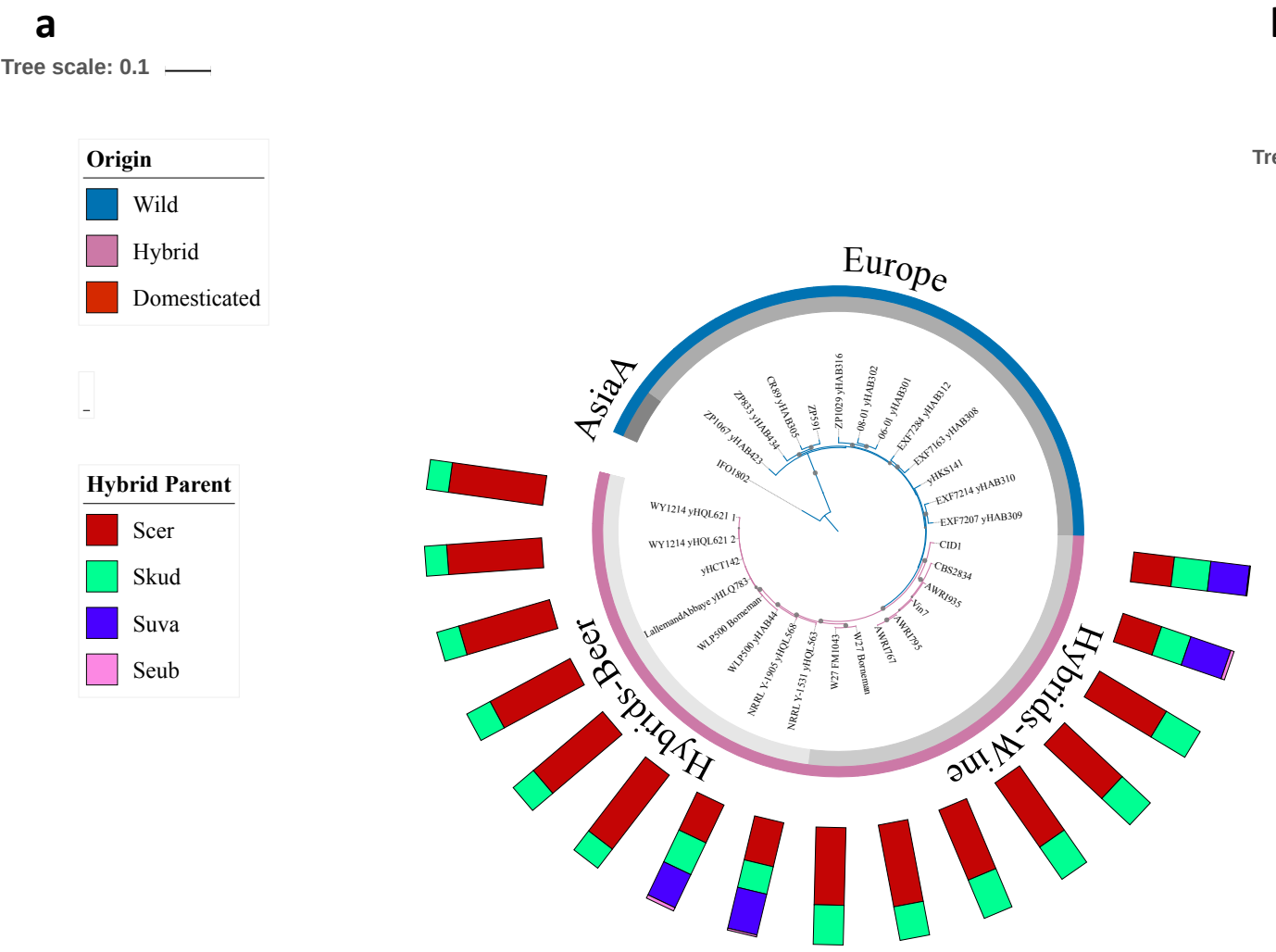

b
Normalized Coverage
Genome Position
Scer
Skud
Suva
Seub
propStrains
0.5
0.6
0.7
0.8
0.9
1.0
species
Scer
Skud
Suva
Seub

Figure S3


a
Tree scale: 0.1
Origin
Wild
Hybrid
Domesticated
Hybrid Parent
Scer
Skud
Suva
Seub
Europe
AsiaA
Hybrids-Beer
Hybrids-Wine
ZP1029 yHAB316
08-01 yHAB302
06-01 yHAB301
EXF7284 yHAB312
EXF7163 yHAB308
yHKS141
EXF7214 yHAB310
EXF7207 yHAB309
CID1
CBS2834
AWRI935
Vin7
AWRI795
AWRI767
W27 Boeneman
W27 FM1043
NRRL Y-1531 yHQL563
NRRL Y-1905 yHQL568
WLP500 yHAB44
WLP500 Boeneman
LallemandAbbaye yHLQ783
yHCT142
WY1214 yHQL621 2
WY1214 yHQL621 1
IFO1802
ZP1067 yHAB423
ZP833 yHAB434
CR89 yHAB305
ZP591

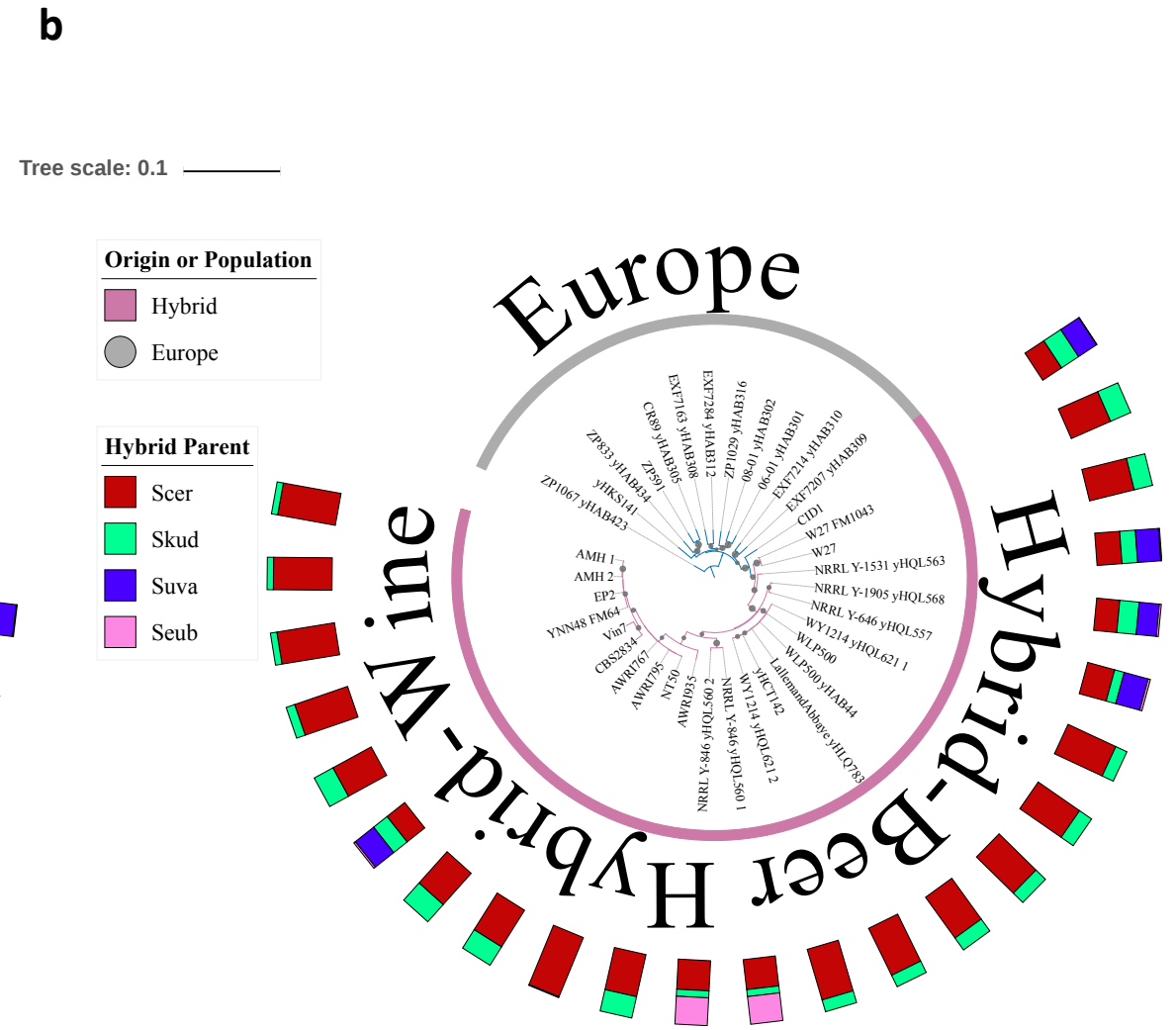

b
Tree scale: 0.1
Origin or Population
Hybrid
Europe
Hybrid Parent
Scer
Skud
Suva
Seub
Europe
Hybrid-Beer
Hybrid-Wine
EXF7163 yHAB308
EXF7284 yHAB312
ZP1029 yHAB316
08-01 yHAB302
06-01 yHAB301
EXF7214 yHAB310
EXF7207 yHAB309
CID1
W27 FM1043
W27
NRRL Y-1531 yHQL563
NRRL Y-1905 yHQL568
NRRL Y-646 yHQL557
WY1214 yHQL621 1
WLP500
WLP500 yHAB44
LallemandAbbaye yHLQ783
yHCT142
WY1214 yHQL621 2
NRRL Y-846 yHQL560 1
NRRL Y-846 yHQL560 2
AWRI935
NT50
AWRI795
AWRI767
CBS2834
Vin7
YNN48 FM64
EP2
AMH 2
AMH 1
ZP1067 yHAB423
yHKS141
ZP833 yHAB434
ZP591
CR89 yHAB305

Figure S4

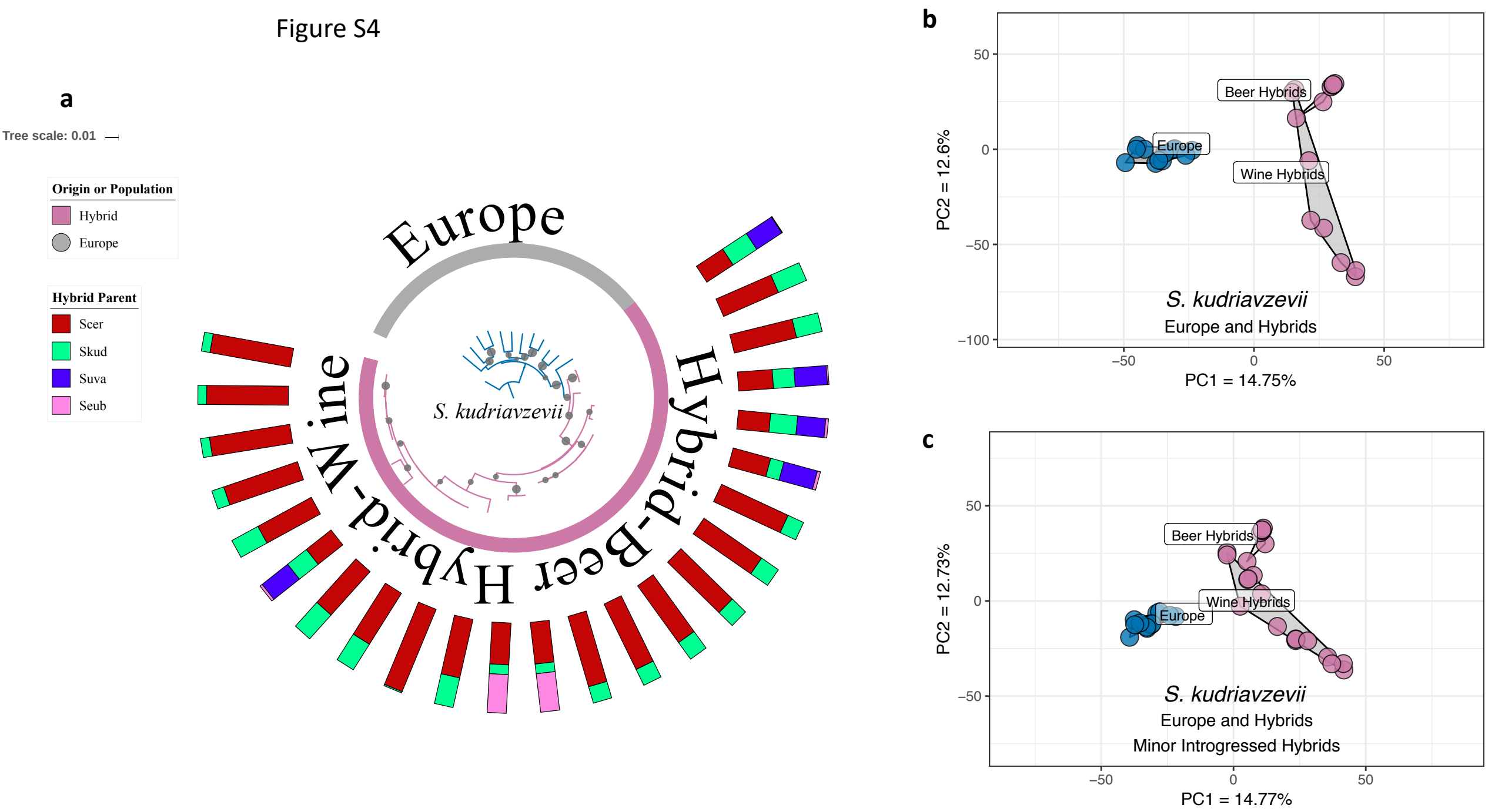
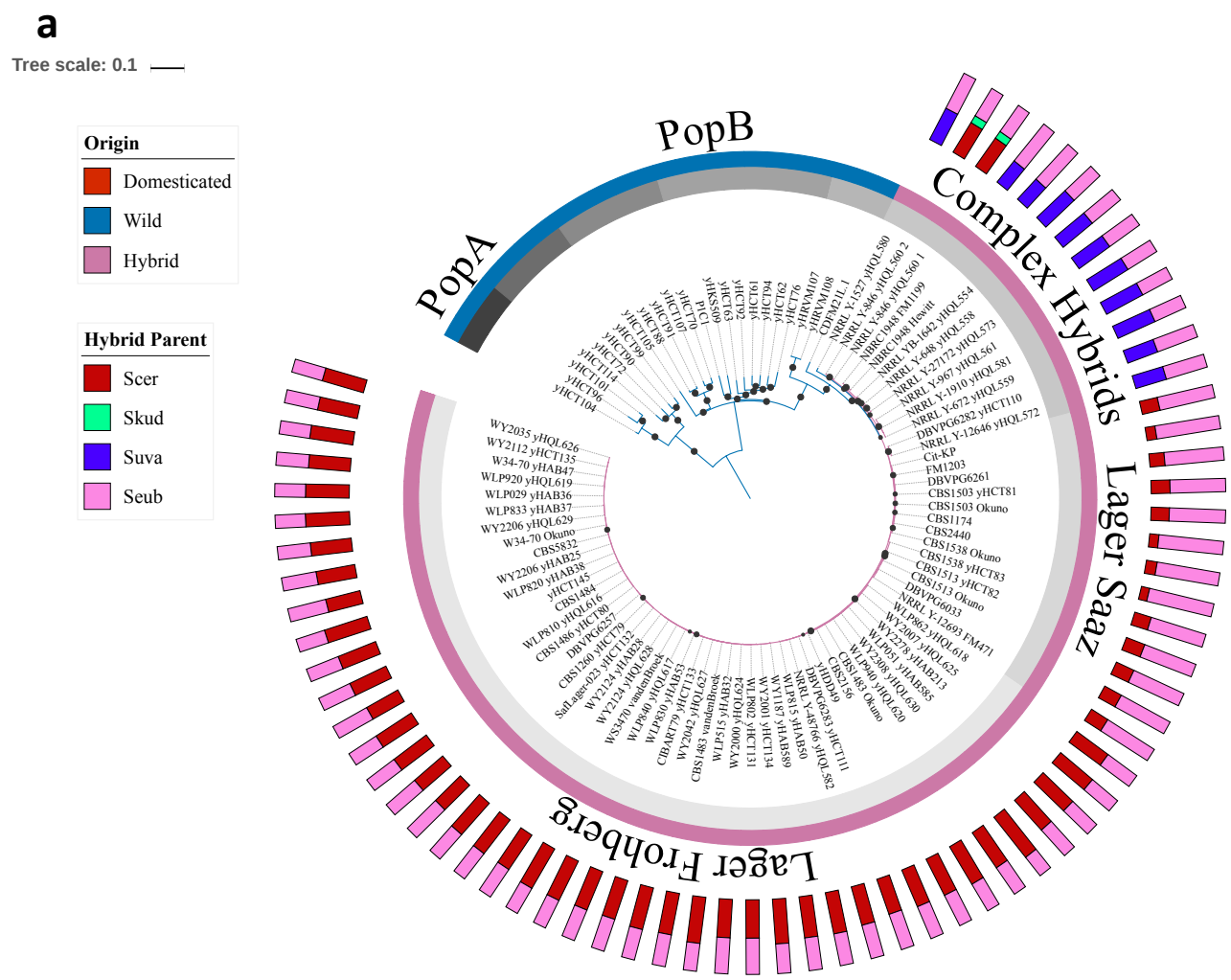

a
Tree scale: 0.01
Origin or Population
Hybrid
Europe
Hybrid Parent
Scer
Skud
Suva
Seub
Europe
Hybrid-Beer
Hybrid-Wine
S. kudriavzevii
b
Beer Hybrids
Europe
Wine Hybrids
PC2 = 12.6%
PC1 = 14.75%
S. kudriavzevii
Europe and Hybrids
c
Beer Hybrids
Wine Hybrids
Europe
PC2 = 12.73%
PC1 = 14.77%
S. kudriavzevii
Europe and Hybrids
Minor Introgressed Hybrids

Figure S5


a
Tree scale: 0.1
Origin
Domesticated
Wild
Hybrid
Hybrid Parent
Scer
Skud
Suva
Seub
PopA
PopB
Complex Hybrids
Lager Saaz
Lager Frohberg

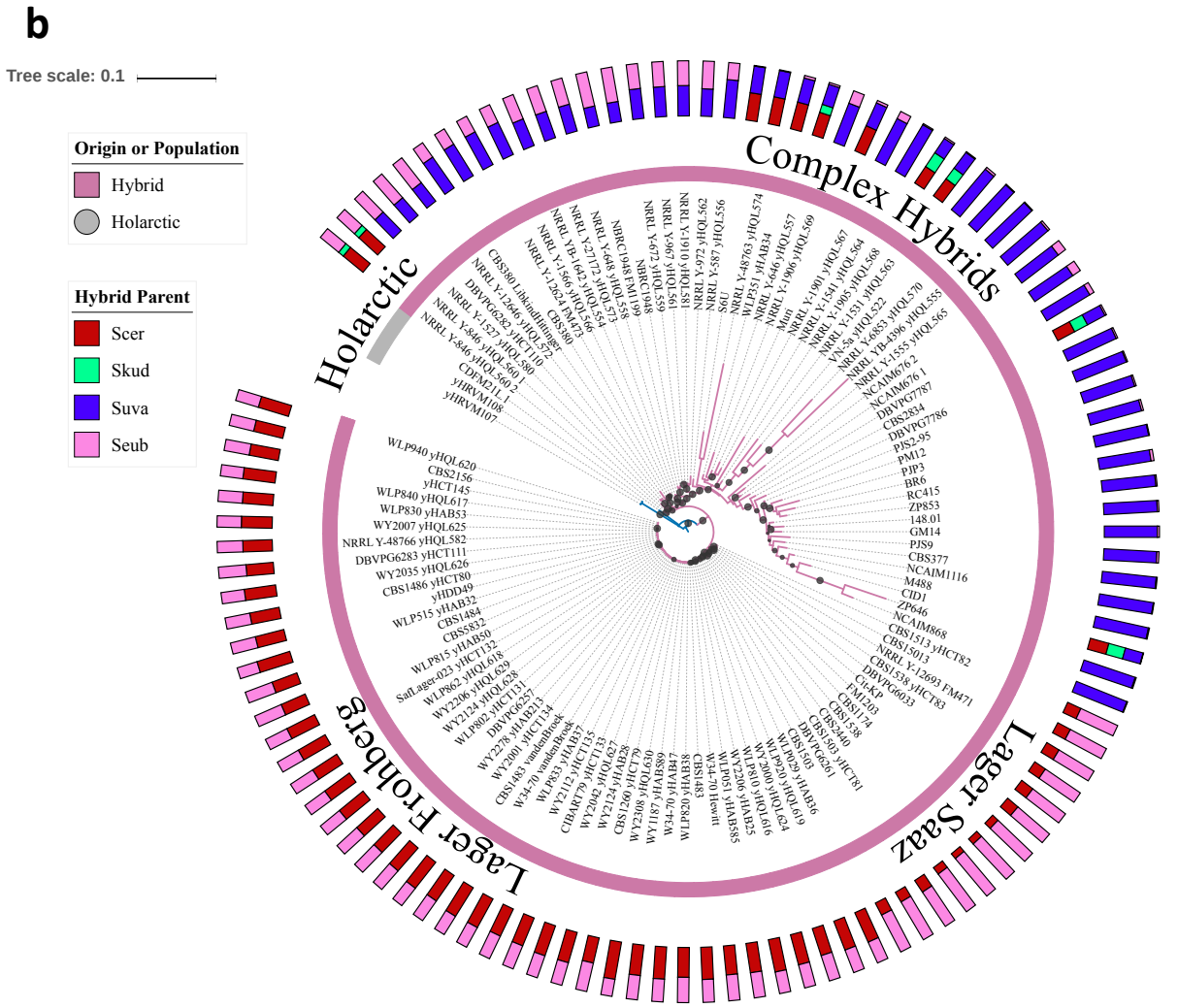
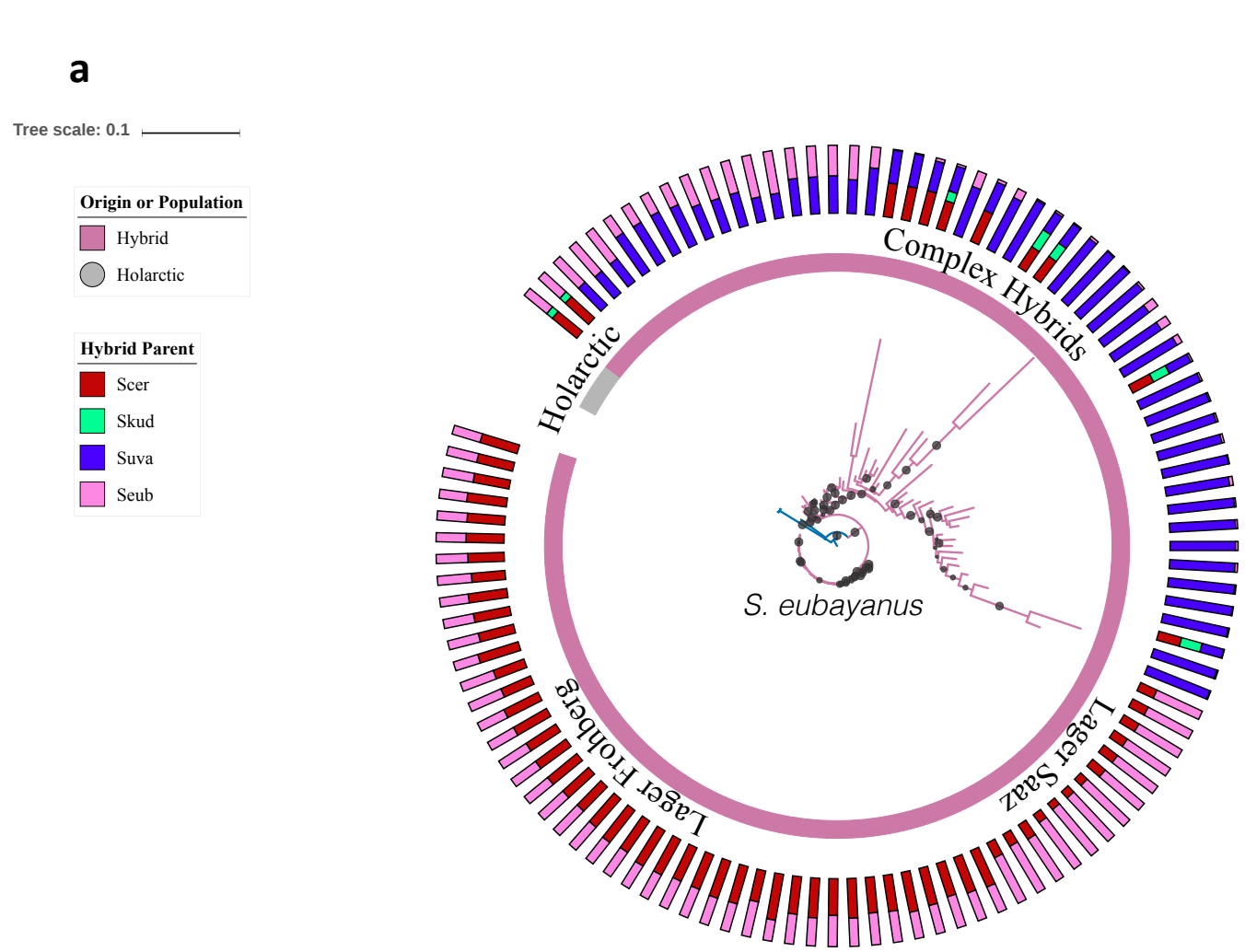
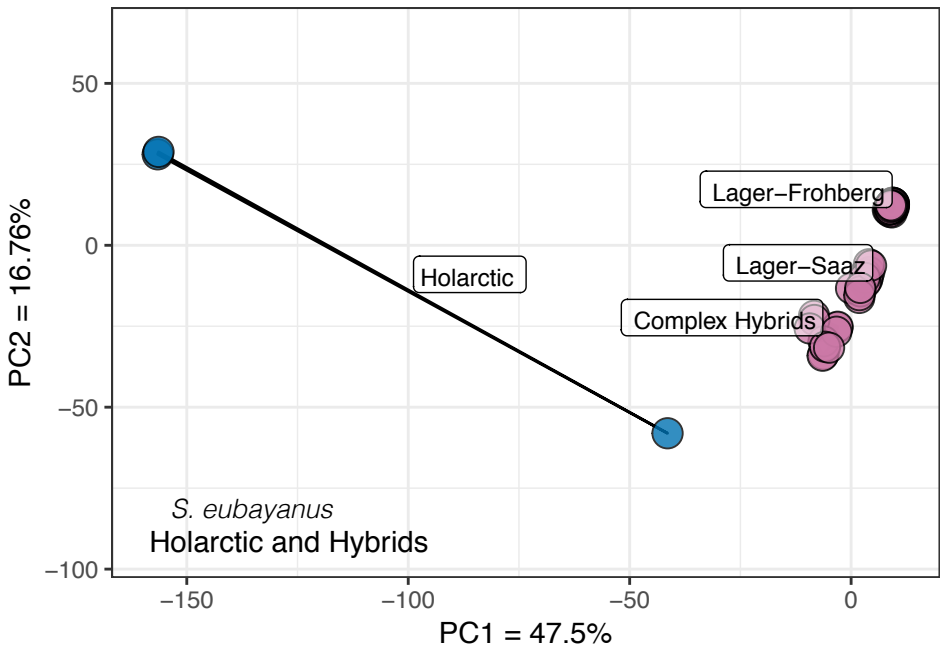

b
Tree scale: 0.1
Origin or Population
Hybrid
Holarctic
Hybrid Parent
Scer
Skud
Suva
Seub
Holarctic
Complex Hybrids
Lager Saaz
Lager Frohberg

Figure S6


a
Tree scale: 0.1
Origin or Population
Hybrid
Holarctic
Hybrid Parent
Scer
Skud
Suva
Seub
Holarctic
Complex Hybrids
Lager Frohberg
Lager Saaz
S. eubayanus


b
50
0
−50
−100
PC2 = 16.76%
Lager−Frohberg
Holarctic
Lager−Saaz
Complex Hybrids
S. eubayanus
Holarctic and Hybrids
−150
−100
−50
0
PC1 = 47.5%

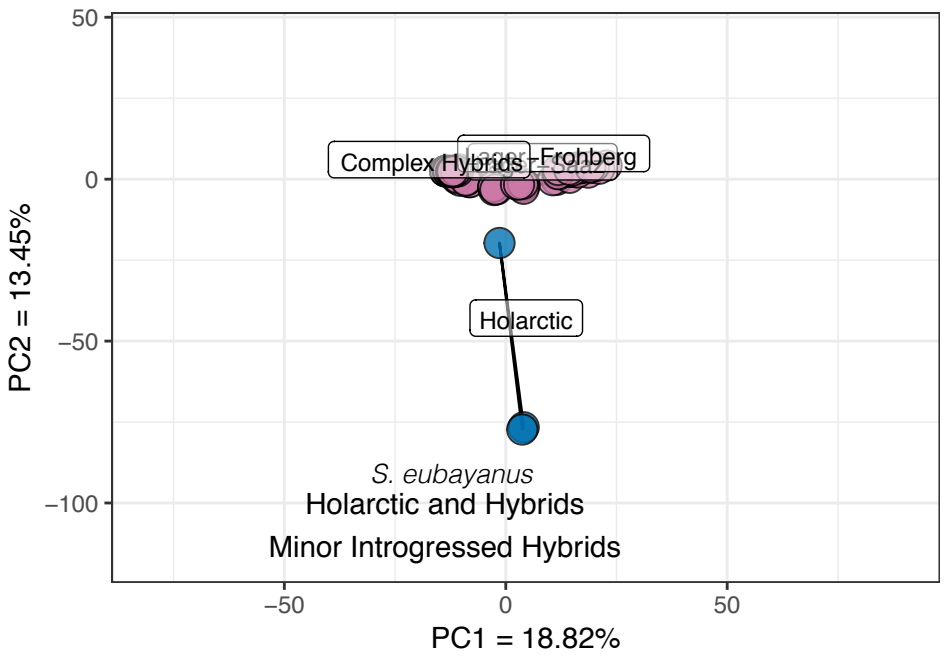

c
50
0
−50
−100
PC2 = 13.45%
Complex Hybrids
Lager−Frohberg
Holarctic
S. eubayanus
Holarctic and Hybrids
Minor Introgressed Hybrids
−50
0
50
PC1 = 18.82%

Figure S7

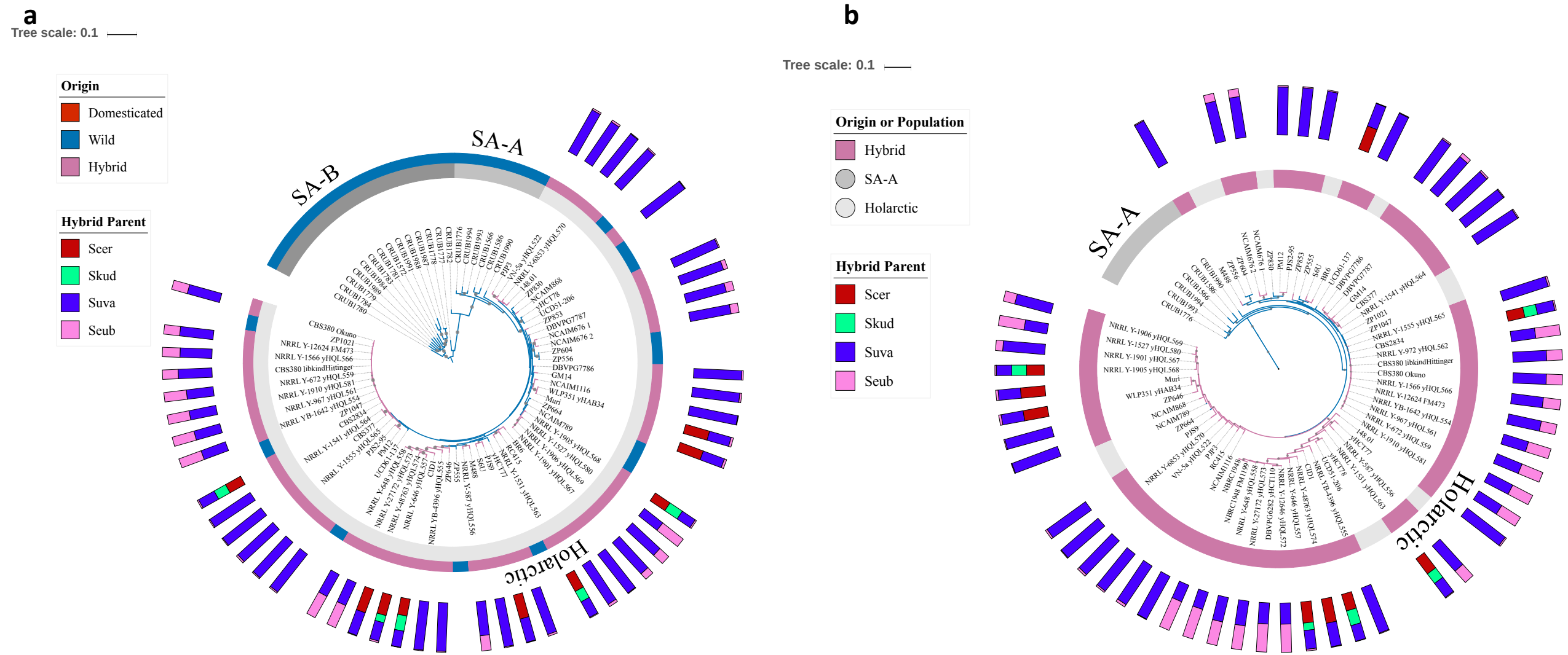
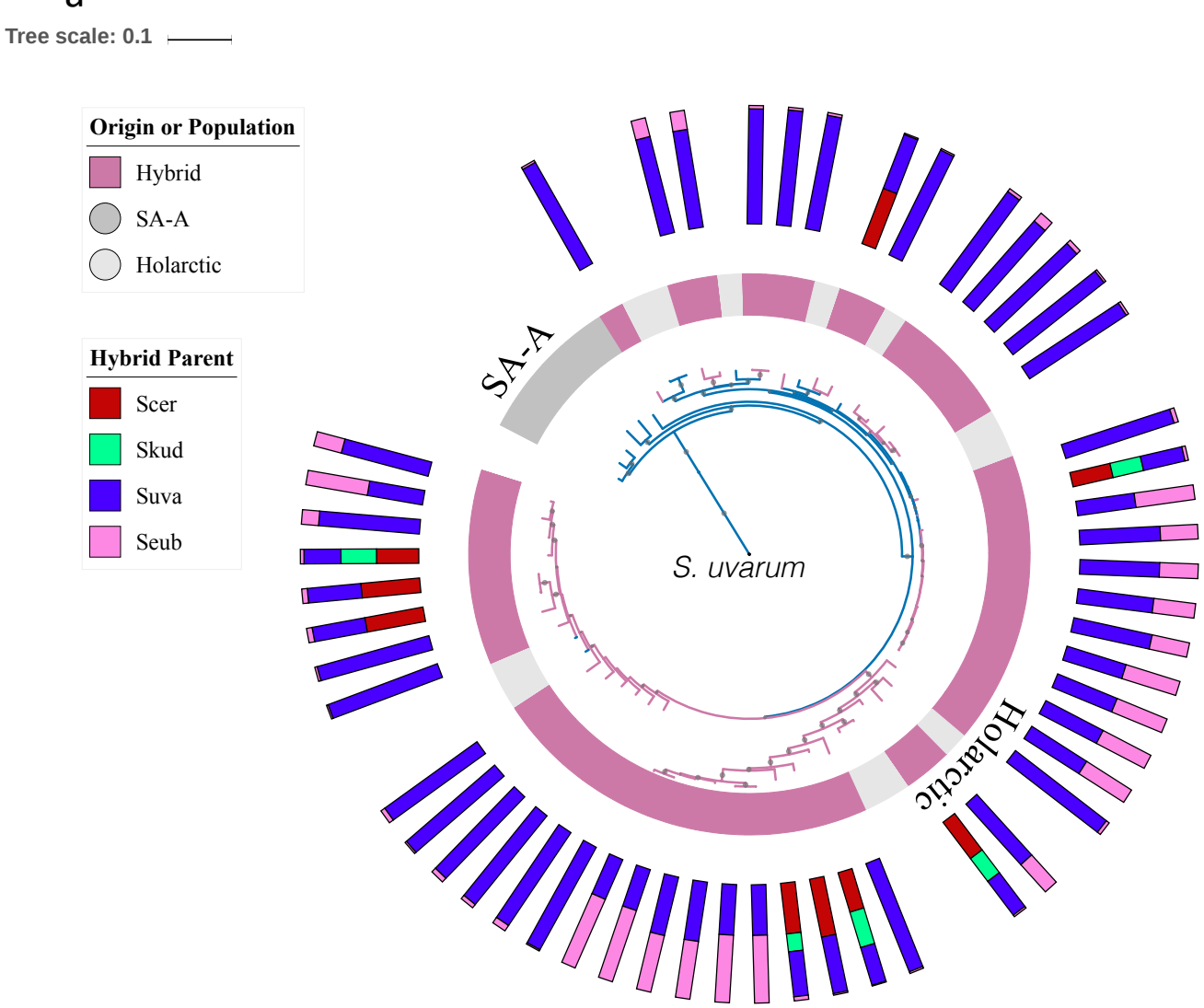
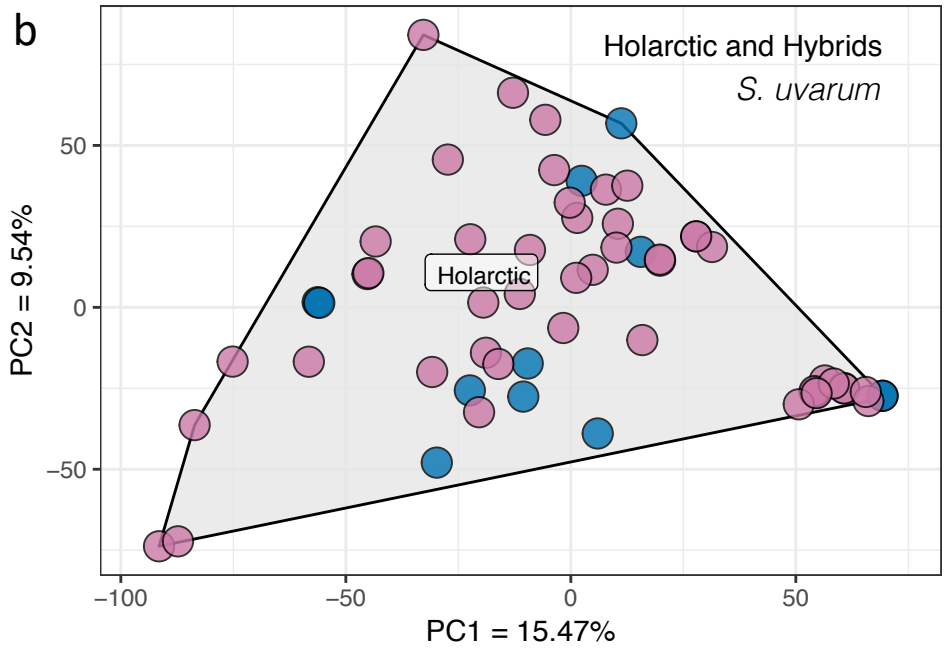

a
Tree scale: 0.1
Origin
Domesticated
Wild
Hybrid
Hybrid Parent
Scer
Skud
Suva
Seub
SA-A
SA-B
Holarctic
b
Tree scale: 0.1
Origin or Population
Hybrid
SA-A
Holarctic
Hybrid Parent
Scer
Skud
Suva
Seub
SA-A
Holarctic

Figure S8


a
Tree scale: 0.1
Origin or Population
Hybrid
SA-A
Holarctic
Hybrid Parent
Scer
Skud
Suva
Seub
SA-A
Holarctic
S. uvarum


b
Holarctic and Hybrids
S. uvarum
Holarctic
PC2 = 9.54%
PC1 = 15.47%
50
0
−50
−100
−50
0
50

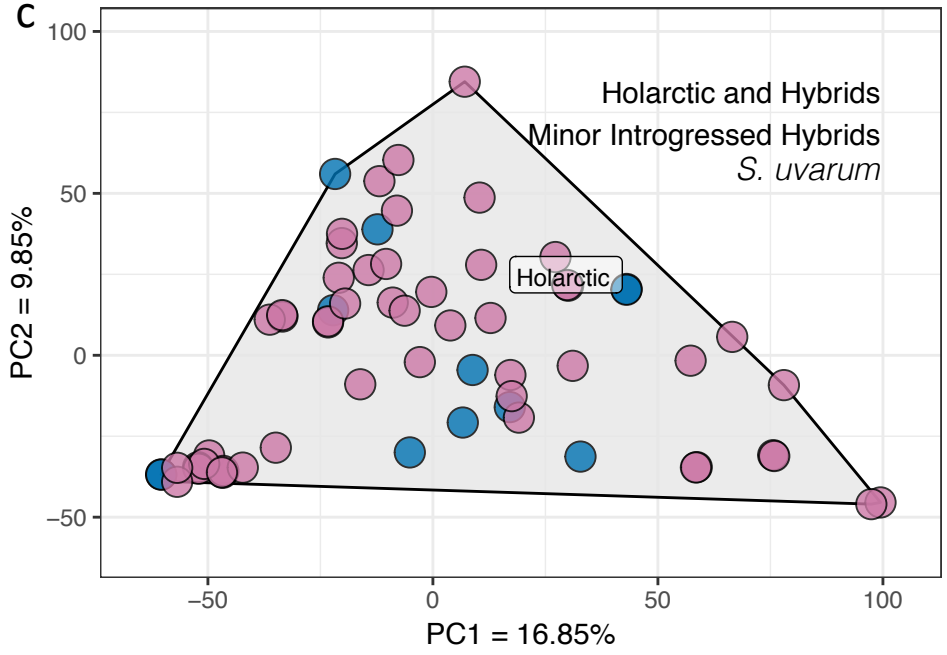

c
Holarctic and Hybrids
Minor Introgressed Hybrids
S. uvarum
Holarctic
PC2 = 9.85%
PC1 = 16.85%
100
50
0
−50
−50
0
50
100

Figure S9

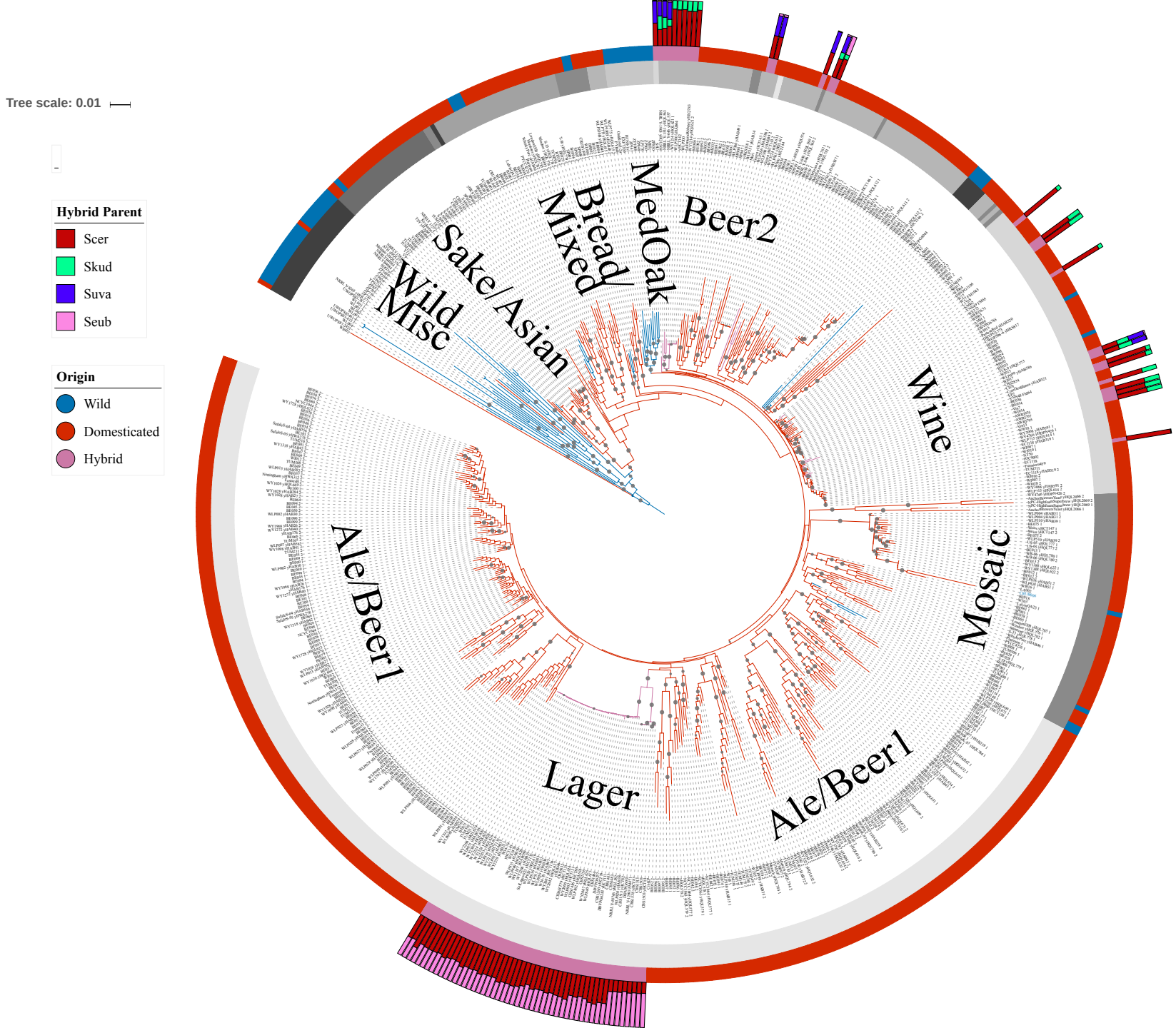

Tree scale: 0.01
Hybrid Parent
Scer
Skud
Suva
Seub
Origin
Wild
Domesticated
Hybrid
Wild Misc
Sake/Asian
Bread/ Mixed
MedOak
Beer2
Wine
Mosaic
Ale/Beer1
Lager
Ale/Beer1

Figure S10

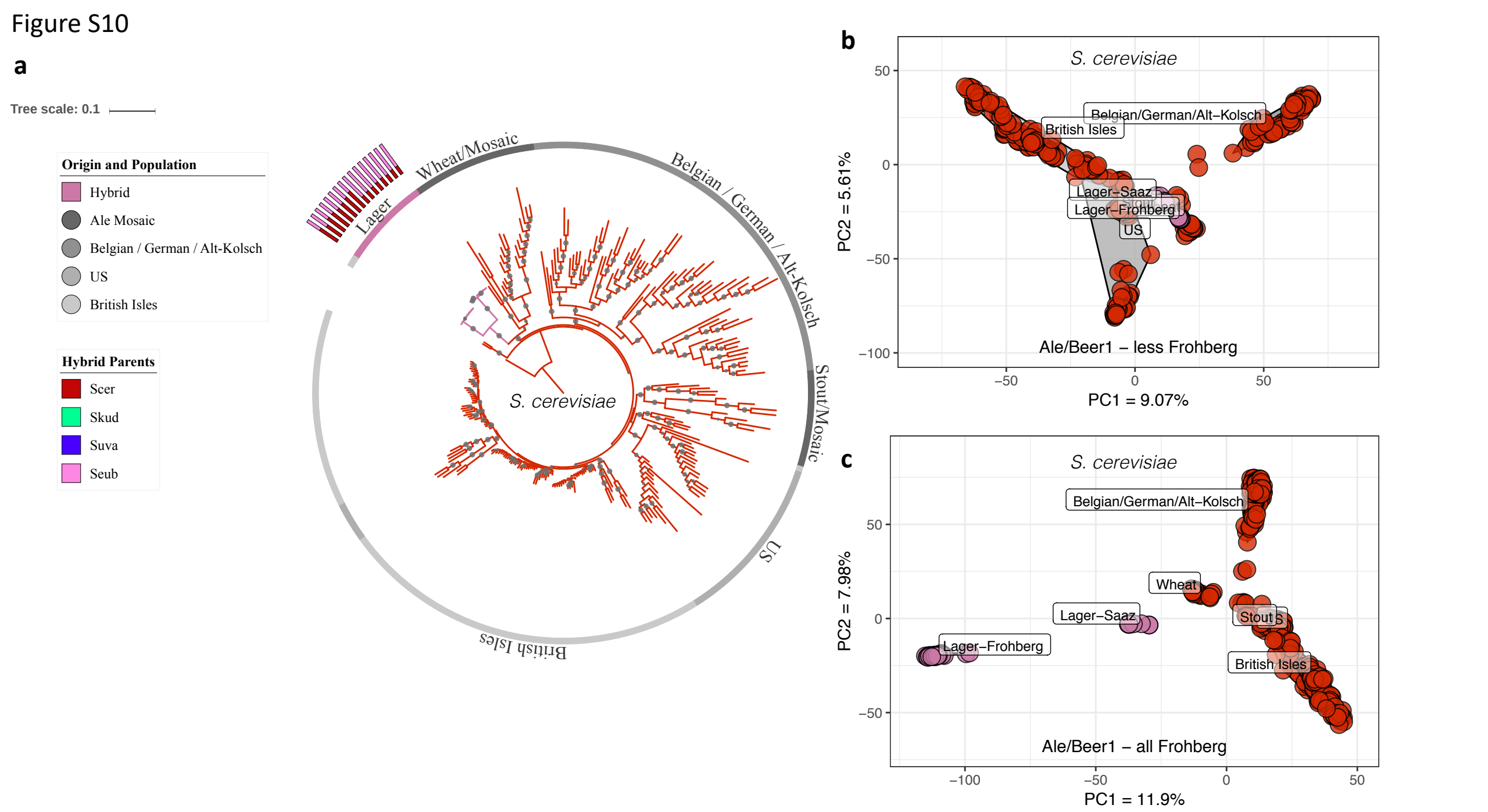

a
Tree scale: 0.1
Origin and Population
Hybrid
Ale Mosaic
Belgian / German / Alt-Kolsch
US
British Isles
Hybrid Parents
Scer
Skud
Suva
Seub
Lager
Wheat/Mosaic
Belgian / German / Alt-Kolsch
Stout/Mosaic
US
British Isles
S. cerevisiae
b
S. cerevisiae
Belgian/German/Alt−Kolsch
British Isles
Lager−Saaz
Lager−Frohberg
US
Ale/Beer1 − less Frohberg
PC2 = 5.61%
PC1 = 9.07%
c
S. cerevisiae
Belgian/German/Alt−Kolsch
Wheat
Lager−Saaz
Stout S
Lager−Frohberg
British Isles
Ale/Beer1 − all Frohberg
PC2 = 7.98%
PC1 = 11.9%

Figure S11

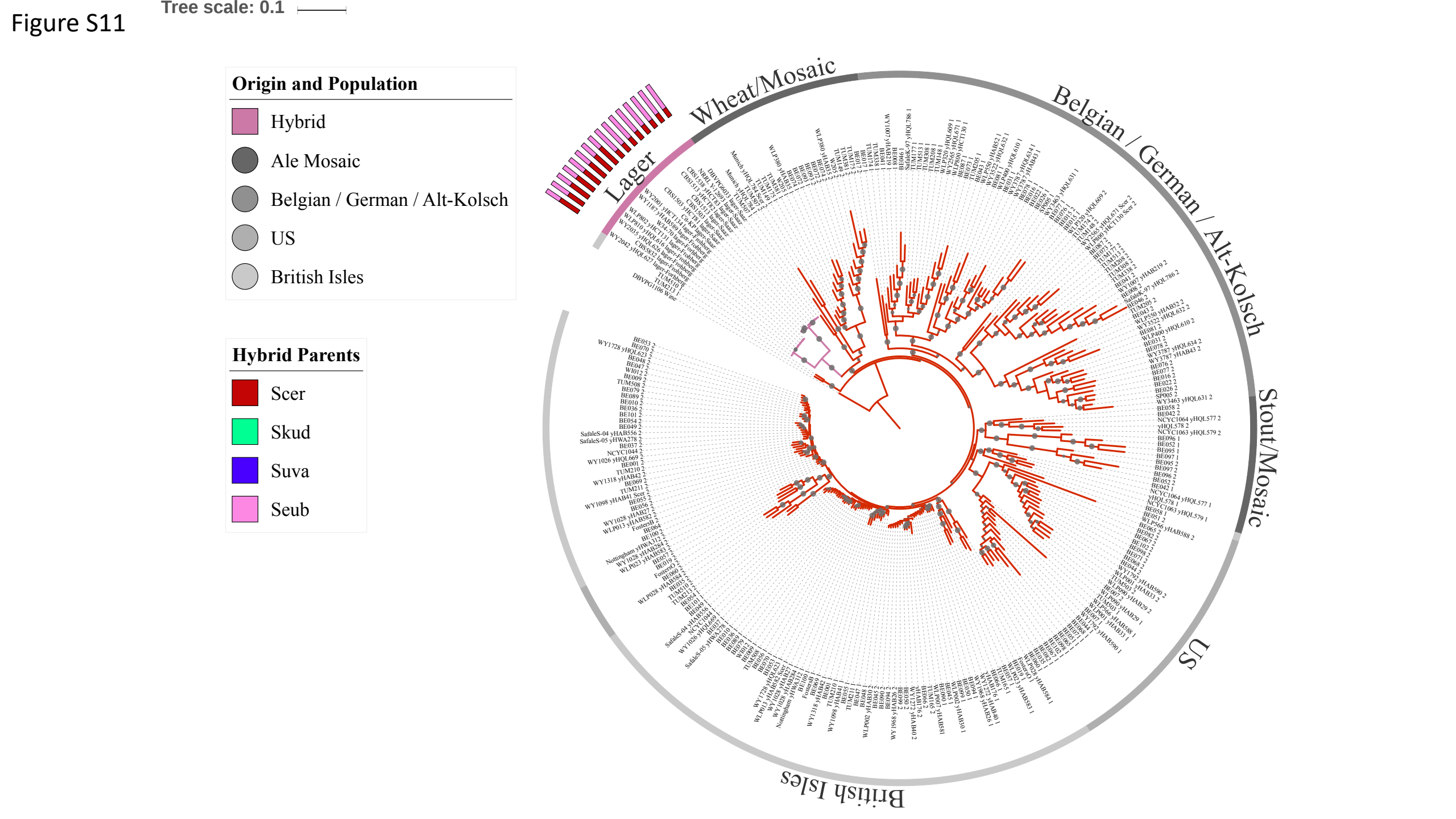
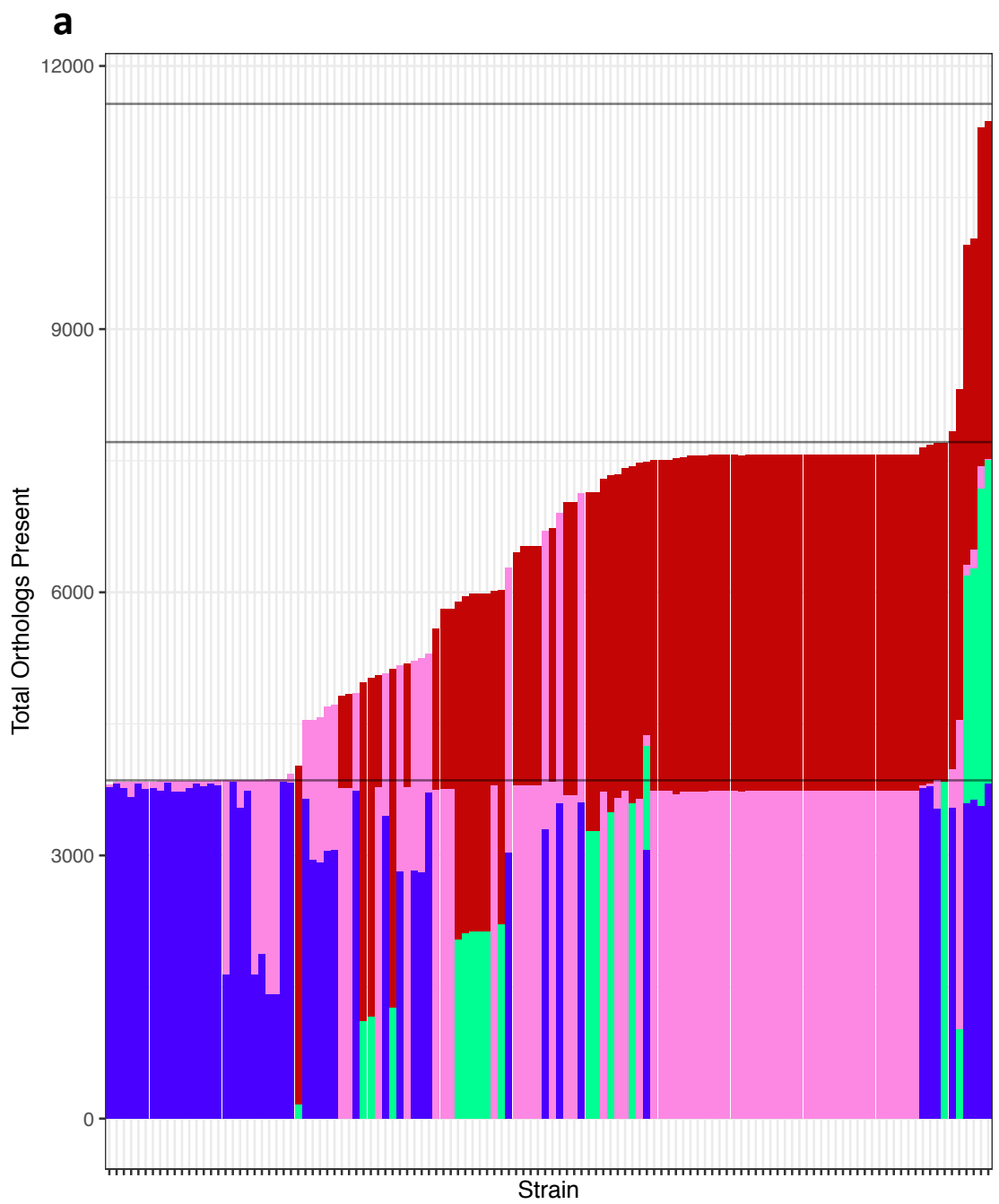
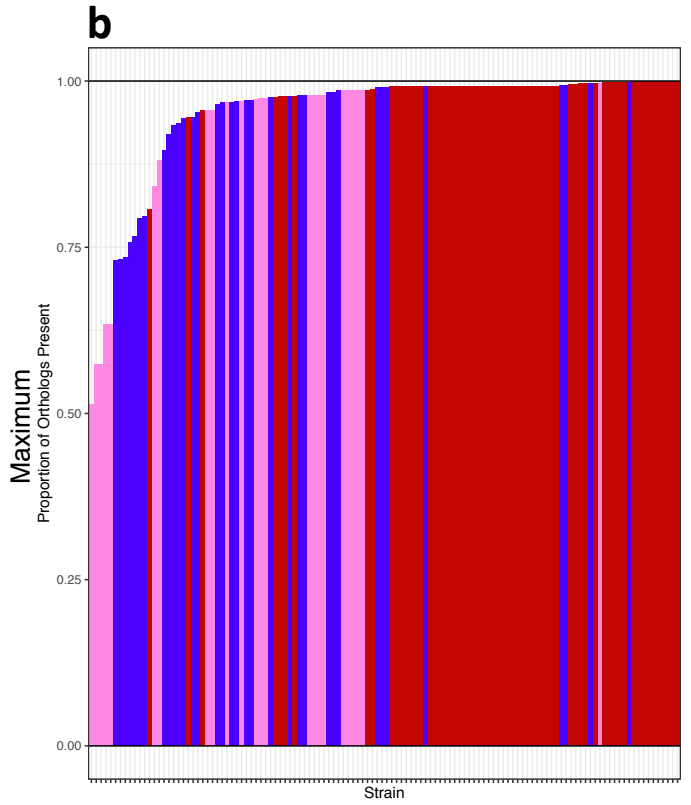

Tree scale: 0.1
Origin and Population
Hybrid
Ale Mosaic
Belgian / German / Alt-Kolsch
US
British Isles
Hybrid Parents
Scer
Skud
Suva
Seub
Lager
Wheat/Mosaic
Belgian / German / Alt-Kolsch
Stout/Mosaic
US
British Isles

Figure S12


a
12000
9000
6000
3000
0
Total Orthologs Present
Strain


b
1.00
0.75
0.50
0.25
0.00
Maximum
Proportion of Orthologs Present
Strain

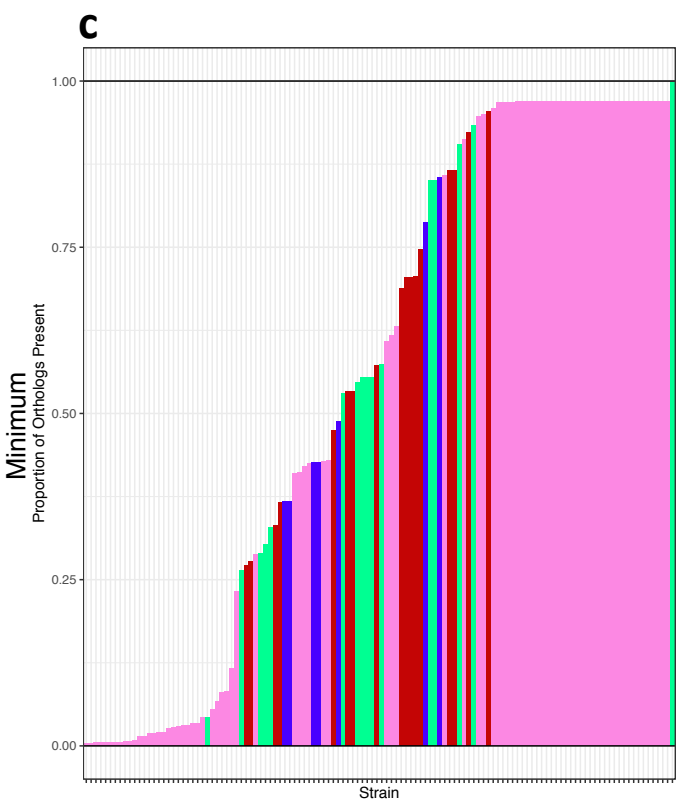

c
1.00
0.75
0.50
0.25
0.00
Minimum
Proportion of Orthologs Present
Strain

Figure S13

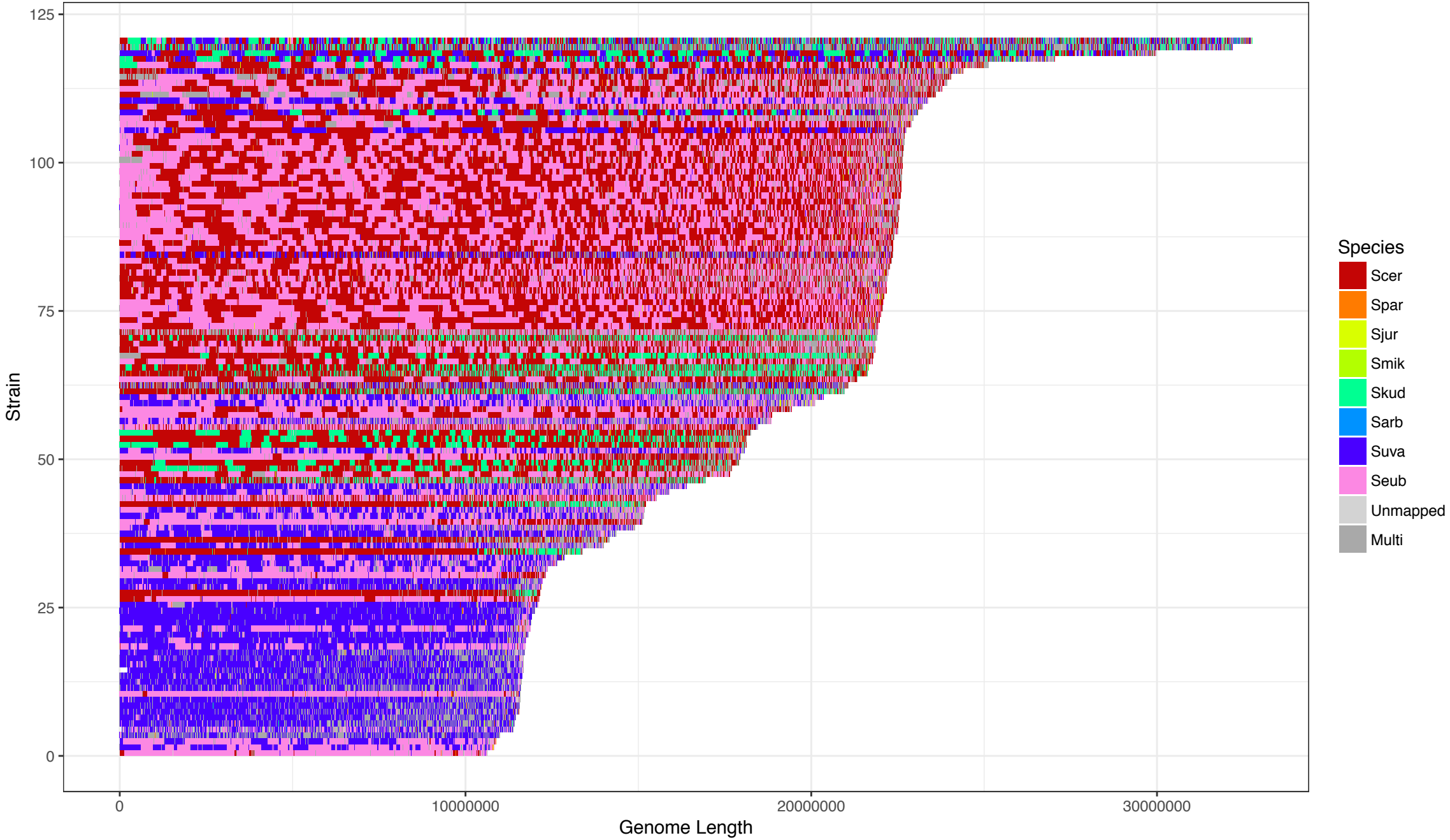

125
100
75
50
25
0
Strain
0
10000000
20000000
30000000
Genome Length
Species
Scer
Spar
Sjur
Smik
Skud
Sarb
Suva
Seub
Unmapped
Multi

Figure S14

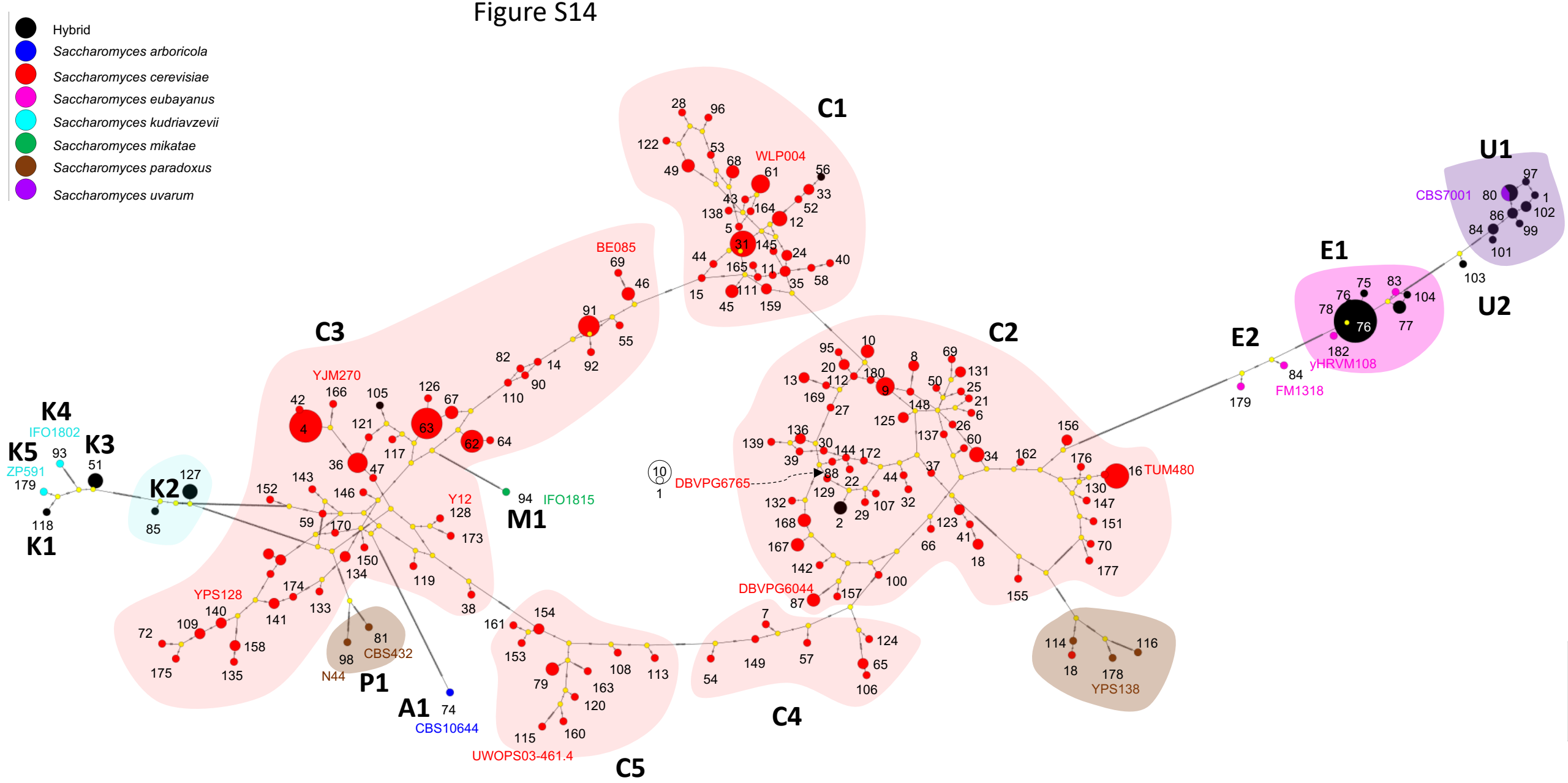

Hybrid
Saccharomyces arboricola
Saccharomyces cerevisiae
Saccharomyces eubayanus
Saccharomyces kudriavzevii
Saccharomyces mikatae
Saccharomyces paradoxus
Saccharomyces uvarum
C1
C2
C3
C4
C5
U1
U2
E1
E2
K1
K2
K3
K4
K5
P1
A1
M1
WLP004
BE085
YJM270
IFO1802
ZP591
IFO1815
Y12
YPS128
CBS432
N44
CBS10644
UWOPS03-461.4
DBVPG6765
DBVPG6044
TUM480
CBS7001
yHRVM108
FM1318
YPS138

Figure S15