

1 Rapid selection response to ethanol in *S. eubayanus* emulates the domestication 2 process under brewing conditions

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20

21 **ABSTRACT**

22 Although the typical genomic and phenotypic changes that characterize the evolution of
23 organisms under the human domestication syndrome represent textbook examples of rapid
24 evolution, the molecular processes that underpin such changes are still poorly understood.
25 Domesticated yeasts for brewing, where short generation times and large phenotypic and
26 genomic plasticity were attained in a few generations under selection, are prime examples.
27 To experimentally emulate the lager yeast domestication process, we created a genetically
28 complex (panmictic) artificial population of multiple *Saccharomyces eubayanus* genotypes,
29 one of the parents of lager yeast. Then we imposed a constant selection regime under a
30 high ethanol concentration in 10 replicated populations during 260 generations (six months)
31 and compared them with evolved controls exposed solely to glucose. Evolved populations
32 exhibited a selection differential of 60% in growth rate in ethanol, mostly explained by the
33 proliferation of a single lineage (CL248.1) that competitively displaced all other clones.
34 Interestingly, the outcome does not require the entire time course of adaptation, as four
35 lineages monopolized the culture at generation 120. Sequencing demonstrated that *de novo*
36 genetic variants were produced in all evolved lines, including SNPs, aneuploidies, INDELs,
37 and translocations. In addition, the evolved populations showed correlated responses
38 resembling the domestication syndrome: genomic rearrangements, faster fermentation
39 rates, lower production of phenolic-off flavors and lower volatile compound complexity.
40 Expression profiling in beer wort revealed altered expression levels of genes related to
41 methionine metabolism, flocculation, stress tolerance and diauxic shift, likely contributing to
42 higher ethanol and fermentation stress tolerance in the evolved populations. Our study
43 shows that experimental evolution can rebuild the brewing domestication process in “fast
44 motion” in wild yeast, and also provides a powerful tool for studying the genetics of the
45 adaptation process in complex populations.

46 **INTRODUCTION**

47 Living organisms are continually adapting to changing environments by natural selection,
48 latently harboring the raw genetic variation required for such responses. When new
49 conditions arise, adaptation to almost every environmental scenario is possible (e.g.,
50 temperature, oxygen and nutrients) [1, 2]. In this context, the genomic analysis of human-
51 made populations (i.e., population genomics of domesticated species) is a relatively new
52 matter, and constitutes a promising research approach for the experimental study of
53 evolutionary processes [3]. Nevertheless, studies that search for the causal factors shaping
54 the genetic structure of yeast and fungal populations, such as small nucleotide
55 polymorphisms (SNP), insertions or deletions (INDELS), copy number variation (CNV) and
56 structural variants (SV), are still insufficient to fully characterize the integrated adaptation
57 process to new environments [4].

58 Adaptive evolution in microorganisms is a process that occurs ubiquitously, including in
59 artificial settings where micro-environments are created, and allows the adaptation of
60 populations to defined conditions, driving the evolution process (domestication) [5].
61 Domestication is a stereotyped adaptive process (a “domestication syndrome”, see [6, 7])
62 within a human-created environment, where several characteristics can be tracked and
63 defined as ‘domestication signatures’. These signatures are present in different fungal
64 species, including *Aspergillus oryzae* in soy sauce [8], *Penicillium* molds associated with
65 cheese [9] and *S. cerevisiae* [10, 11] together with *S. pastorianus* [12], responsible for beer
66 fermentation. In this context, spore production and viability, metabolic remodeling, changes
67 in volatile compound production, transcriptional re-wiring and faster growth rates are
68 considered key traits and goals of microbe domestication. In the case of brewing, the yeast
69 re-utilization process led to new spontaneous mutations generated during cell division,
70 which, together with selective environmental pressures, such as high ethanol
71 concentrations, selected fitter individuals [13]. Genomic analysis in beer yeast domesticated

72 strains demonstrated the presence of common genetic patterns, such as large genomic
73 rearrangements, aneuploidies, high heterozygosity levels and infertility, all of which are
74 hallmarks of the adaptation process [11, 14-16].

75 Two main types of yeasts suffered domestication under different brewing settings; *S.*
76 *cerevisiae* that ferments ale beers at temperatures near 20 °C, and *S. pastorianus* that
77 produce lager beers fermented at lower temperatures (8-15 °C) [17]. *S. pastorianus* is an
78 interspecific hybrid from the cross between *S. cerevisiae* and the cryotolerant wild yeast *S.*
79 *eubayanus* [18]. The hybrid nature of *S. pastorianus* confers a series of competitive
80 advantages in the fermentation environment, likely due to the combination of performance
81 at relatively cold temperatures, efficient sugar uptake and metabolic switching between
82 sugar sources [19]. During an intense domestication process over approximately 500 years,
83 lager beers have evolved reduced organoleptic complexity, mainly characterized by the
84 presence of ester compounds and the absence of phenolic off-flavors [20]. This is reflected
85 in the absence of *PAD1* and *FDC1* in *S. pastorianus*, genes which are responsible for the
86 synthesis of such off-flavors [21, 22] and present in *S. eubayanus*. Lager yeast
87 domestication is characterized by a reduced lag phase in the switch from glucose to maltose,
88 and regulatory cross-talk between *S. cerevisiae* and *S. eubayanus* sub-genomes, which
89 complement each other in terms of the genes required for maltose/maltotriose metabolism
90 [23, 24].

91 Given the recent discovery of *S. eubayanus*, its puzzling origin and apparently co-
92 evolutionary association with *Nothofagus* trees, several authors have analyzed the
93 worldwide distribution of *S. eubayanus*, together with its genetic, phenotypic, and
94 fermentative diversity [24-27]. Patagonian isolates of *S. eubayanus* exhibit the most
95 extensive genetic diversity, and the presence of the most significant number of lineages
96 compared to Northern hemisphere populations, including five different lineages and a large

97 group of admixed isolates [26, 27]. To date, there is no evidence of *S. eubayanus* isolates
98 in Europe, where the original *S. pastorianus* hybrid likely originated. Interestingly,
99 fermentation capacity varies significantly between *S. eubayanus* isolates, possibly due to
100 differences in maltose consumption and diauxic shift capacity, resulting in two opposite
101 outcomes: successful or stuck fermentations [27]. These isolates produce fruit and floral
102 flavors in beer [28], but high levels of 4-vinyl guaiacol, considered a phenolic off-flavor that
103 provides a clove-like aroma, which is not preferred among consumers [21, 28, 29] [30].

104 Although different reports have provided insights into the genomic and phenotypic changes
105 responsible for the brewing capacity of *S. pastorianus*, particularly the *S. cerevisiae* genome
106 portion, we know little about the process of *S. eubayanus* domestication before or after
107 hybridization. Thus, further evidence is needed to understand the molecular mechanisms
108 underpinning the *S. eubayanus* fermentative phenotype, which in turn will provide important
109 insights into the inherent evolutionary process represented by directional selection for
110 domestication, and correlated responses. In this study, a genetically complex artificial
111 mixture of 30 different genotypes of *S. eubayanus* was continually exposed to high ethanol
112 levels, mimicking the domestication process in breweries. We measured their correlated
113 responses including their genomic, transcriptomic and phenotypic changes, and identified
114 candidate genes that confer ethanol tolerance. Our results demonstrate that a single genetic
115 background consistently overcomes the remaining strains, showing greater fermentation
116 performance, but also significantly higher fitness in oxidative and osmotic stress
117 environments. To an extent, we thus recreate the domestication process in the laboratory,
118 showing how this cryotolerant yeast adapted to the competitive beer environment of a
119 human industry and proved that experimental evolution can rebuild the brewing
120 domestication process in *S. eubayanus* in “fast motion”. This provides a powerful tool for

121 disentangling the molecular, physiological and biochemical processes that underlie the
122 domestication of domesticated microorganisms.

123

124 **MATERIALS AND METHODS**

125 **Microorganisms and culture media**

126 Thirty *S. eubayanus* strains isolated from bark samples obtained from *Nothofagus pumilio*
127 trees in south Chile were utilized for the experimental evolution assay, as listed in **Table S1**.
128 These strains were previously reported and belong to the Patagonia B cluster [27]. *S.*
129 *cerevisiae* L299 [31] and MTF2444 (EC1118 *hsp12::GFP*) [32] strains were used as growth
130 control and in the competition assays, respectively. Additionally, we used the *S. pastorianus*
131 Saflager W-34/70 (Fermentis, France) strain as a lager fermentation control. All isolates
132 were maintained in YPD agar media (yeast extract 1%, peptone 2%, glucose 2% and agar
133 2%) and stored at -80°C in 20% glycerol stocks.

134 **Experimental evolution**

135 Initially, one colony from each *S. eubayanus* strain was cultured in 0.67% yeast nitrogen
136 base (YNB) media (Difco, France) with 2% glucose at 20°C (hereinafter referred to as GLU)
137 and 150 rpm orbital shaking. Later, each pre-inoculum was utilized to prepare a co-culture
138 in a single 250 mL flask to obtain a final concentration of 1x10⁶ cells/mL of each strain. Ten
139 replicates were set up (parallel populations) in 5 mL GLU and ten supplemented with 0.67%
140 YNB media, 2% glucose and 9% ethanol (hereinafter referred to as EtOH). The inoculum
141 was resuspended and transferred to the 20 replicates to obtain a final concentration of 1x10⁶
142 cells/mL (**Figure 1A**). The adaptative evolution assays were performed at 20°C at 150 rpm
143 for 72 h. Subsequently, the cultures were used to inoculate fresh 5 mL cultures at an
144 inoculum density of 1 x 10⁶ cell/mL, and this procedure was sequentially repeated. The

145 number of generations was estimated using the "generations = log (final cells - log initial
146 cells)/log2" formula, summing up the number of cells/mL doublings between every culture
147 transfer during the adaptive evolution process.

148 **Phenotyping assay**

149 The phenotyping assay was performed as previously described [27]. Briefly, isolates were
150 pre-cultivated in 200 μ L 0.67% YNB medium supplemented with glucose 2% for 48 h at
151 25°C. Next, strains were inoculated to an optical density (OD) of 0.03–0.1 (wavelength 630
152 nm) in 200 μ L growth media, where the following carbon sources were considered: Glucose
153 2%, Fructose 2%, Maltose 2%, Galactose 2%, Pilsner Beer Wort 12 °Plato (°P) and
154 incubated without agitation at 20°C for 24 h using a Tecan Sunrise absorbance microplate
155 reader. Additionally, several environmental stressors were assessed, including ethanol 9%,
156 Sorbitol 20%, H₂O₂ 3 mM, SDS 0.001% and high temperature (28 and 34°C) during 48 h.
157 For ethanol 9%, experiments were carried out for 96 h. The OD was measured every 30
158 minutes using a 630 nm filter. Each experiment was performed in triplicate. Maximum growth
159 rate, lag time and OD max parameters were obtained for each strain using the GrowthRates
160 software with default parameters [33].

161 Growth curves incorporating carbon source switching from glucose to maltose and galactose
162 were determined under micro-cultivation conditions in YP (1% yeast extract, 2% peptone)
163 media including either 5% glucose, 5% maltose or 5% galactose at 25°C for 48 h. Pre-
164 cultures were grown in YP with 5% glucose medium at 25°C for 24 h. Cultures were then
165 diluted to an initial OD_{600nm} of 0.1 in fresh YP 5% glucose medium for an extra overnight
166 growth. The next day, cultures were used to inoculate a 96-well plate with a final volume of
167 200 μ L YP with the disaccharide source at an initial OD_{600nm} of 0.1. The growth curves
168 were monitored by measuring the OD_{600nm} every 30 min as previously mentioned. All

169 experiments were performed in triplicate. Lag phase and maximum specific growth rate
170 (μ_{max}) were estimated as previously described [34] using the R software version 3.6.3.

171 **Fermentations in beer wort**

172 Fermentations were carried out as previously described [28, 29]. Briefly, fermentations were
173 performed in at least three biological replicates, depending on the experiment, in 12 °P using
174 a BrewFerm Pilsner commercial beer kit (Beringen, Belgium). For this, a colony was
175 transferred to 5 mL 6 °P pilsner beer wort supplemented with 0.3 ppm ZnCl₂ and incubated
176 at 20°C with orbital shaking at 150 rpm for 24 h. Then, the complete pre-inoculum was
177 transferred to 50 mL 12 °P pilsner beer wort and incubated in similar conditions for 24 h.
178 Cells were utilized to inoculate 50 mL fresh 12 °P pilsner beer wort to a final concentration
179 of 1.8×10^7 cell/mL. Cultures were maintained at 12°C for 14 days without agitation and
180 weighed every day to calculate the CO₂ released.

181 Larger volume fermentations for RNA extraction and metabolite production analysis were
182 carried out in 1.5 L 12 °P beer wort for 14 days at 12°C. At the end of the fermentation,
183 metabolites such as glucose, fructose, maltose, maltotriose, ethanol and glycerol were
184 estimated using HPLC [27]. Volatile compounds were detected using HS-SPME-GC-MS as
185 previously described [28].

186 **Competition Assays**

187 A total of 1×10^6 cells/mL of the evolved and *S. cerevisiae* MTF2444 (EC1118 *hsp12::GFP*)
188 strains were separately pre-incubated in 5 mL YNB media supplemented with 2% glucose
189 for 24 h. Evolved individuals were mixed in equal proportions with the *S. cerevisiae*
190 MTF2444 GFP expressing-mutant strain at a final concentration of 2×10^6 cell/mL in YNB
191 media supplemented with 2% glucose and 6% ethanol. Cultures were incubated in an orbital
192 shaker at 20°C and 150 rpm during 72 h, and 100 μ L samples from each culture were

193 extracted every 24 h. Aliquots were washed twice in PBS and stored in the same buffer.
194 Cultures were then analyzed in a BD FACScanto II Cytometer (Biosciences, USA). Finally,
195 the proportion of non-fluorescent/GFP-fluorescent cells was estimated. Experiments were
196 performed in triplicate.

197 **Sequencing of the evolved lines and identification of mutations**

198 DNA extraction was performed as previously described [27, 29]. Sequencing of three parallel
199 populations at final and intermediate stages of the evolution process was performed using
200 the Illumina HiSeq X ten platform (BGI sequencing, China). Overall, approximately 45 million
201 reads (paired-end) were obtained for each evolved line. The raw reads were processed to
202 remove adaptor sequences using the Fastp tool and filtered considering a 20 phred score
203 cut-off [35]. Reads were aligned against the *S. eubayanus* CBS12357^T reference genome
204 [36] using the Burrows-Wheeler Aligner [37]. Overall, 99% of the reads were aligned,
205 obtaining a mean coverage of 980X. Genome sequences of 27 parental strains were
206 previously sequenced [27], from which a list of SNPs that were unique for each of those
207 sequenced strains was obtained, using a custom R script. To estimate the proportion of the
208 parental genetic backgrounds in every evolved line, the alternative genotype coverage at
209 each unique SNP coordinate was obtained using bcftools mpileup [38] [39]. De novo SNP
210 calling in the evolved lines was performed using freebayes v 1.3.0
211 (<https://github.com/ekg/freebayes>). The total number of SNPs was calculated using
212 Freebayes [40] and the effect of each SNP was predicted with SnpEff [41] and the *S.*
213 *eubayanus* CBS12357^T reference genome [36]. Reads are available in the Biosample
214 Database Project PRJNA666059.

215 **Genome reconstruction of the CLEt5.1 mutant**

216 The genome of the CLEt5.1 mutant was reconstructed using Nanopore sequencing coupled
217 with Illumina sequencing. Nanopore sequencing was performed using a minION system
218 (Oxford Nanopore, Oxford, UK). For this, DNA extraction and sequencing proceeded as
219 previously described [29]. Overall, 26.1 million reads for Illumina and 96,000 reads for
220 Nanopore were obtained (**Table S2**). The raw fast5 files were transformed to fastq files and
221 debarcoded using Guppy 2.3.5 [42]. Barcode and adapter sequences were trimmed using
222 Porechop (<https://github.com/rrwick/Porechop>) and filtered with Filtlong
223 (<https://github.com/rrwick/Filtlong>) using a Phred score of 30. Genome assembly was
224 performed with Canu (<https://github.com/marbl/canu>) using default settings. Additionally,
225 two rounds of nanopolish (<https://github.com/jts/nanopolish>) and pilon
226 (<https://github.com/broadinstitute/pilon>) were carried out. Moreover, the raw assembly was
227 polished using the Illumina reads filtered with a Phred score of 20 (Burrows-Wheeler
228 Aligner). The genome assembly was annotated with the pipeline LRSDAY [43] using the *S.
229 eubayanus* CBS12357^T reference genome as model for training AUGUSTUS [44],
230 supported by the transcriptome assembly produced by TRINITY [45]. The completeness of
231 the genome assembly was evaluated using BUSCO [46]. The assembly was compared with
232 CBS12357^T using nucmer (Marçais et al, 2018) to evaluate the synteny, whilst specific
233 structural variants (SVs) were identified using MUM&Co [47]. All the parameters of the
234 pipeline were set up as default. The enrichment analysis of Gene Ontology (GO) terms and
235 KEGG pathways was performed using METASCAPE [48]. The identification of transcription
236 factor binding sites in the regulatory region 500 bp upstream of the upregulated genes of the
237 evolved strain was performed using CiiDER [49]. Reads are available in the Biosample
238 Database Project PRJNA666059.

239 **RNA-sequencing and differential expression analysis**

240 RNA was extracted using the E.Z.N.A.® Total RNA Kit I (Omega Bio-tek, USA). RNA was
241 DNase I treated (ThermoFisher, USA) and purified using the RNeasy MinElute Cleanup Kit
242 (Qiagen, Germany). The Illumina libraries and sequencing were performed as previously
243 described [29] in the BGI facilities (Hong Kong, China). Briefly, RNA integrity was confirmed
244 using a Fragment Analyzer (Agilent, USA). The RNA-seq libraries were constructed using
245 the TruSeq RNA Sample Prep Kit v2 (Illumina, USA). The sequencing was conducted using
246 paired-end 100-bp reads on an Illumina HiSeq X Ten in a single lane for the six samples.
247 Reads are available in the Biosample Database Project PRJNA666059. Reads were
248 mapped to the *S. eubayanus* CBS12357^T reference genome using RNAstar ver. 2.7.3 [50]
249 and analyzed using featurecounts in R [51]. Differential expression was analyzed statistically
250 using DESeq2 package in R [52]. Genes showing an adjusted *P*-value of 0.05 or less were
251 considered as differentially expressed genes (DEGs). Analysis of GO term enrichment was
252 performed with the R package enrichGO
253 (<https://www.rdocumentation.org/packages/clusterProfiler/versions/3.0.4/topics/enrichGO>).
254 Cytoscape was used to visualize transcription factor regulatory networks [53].

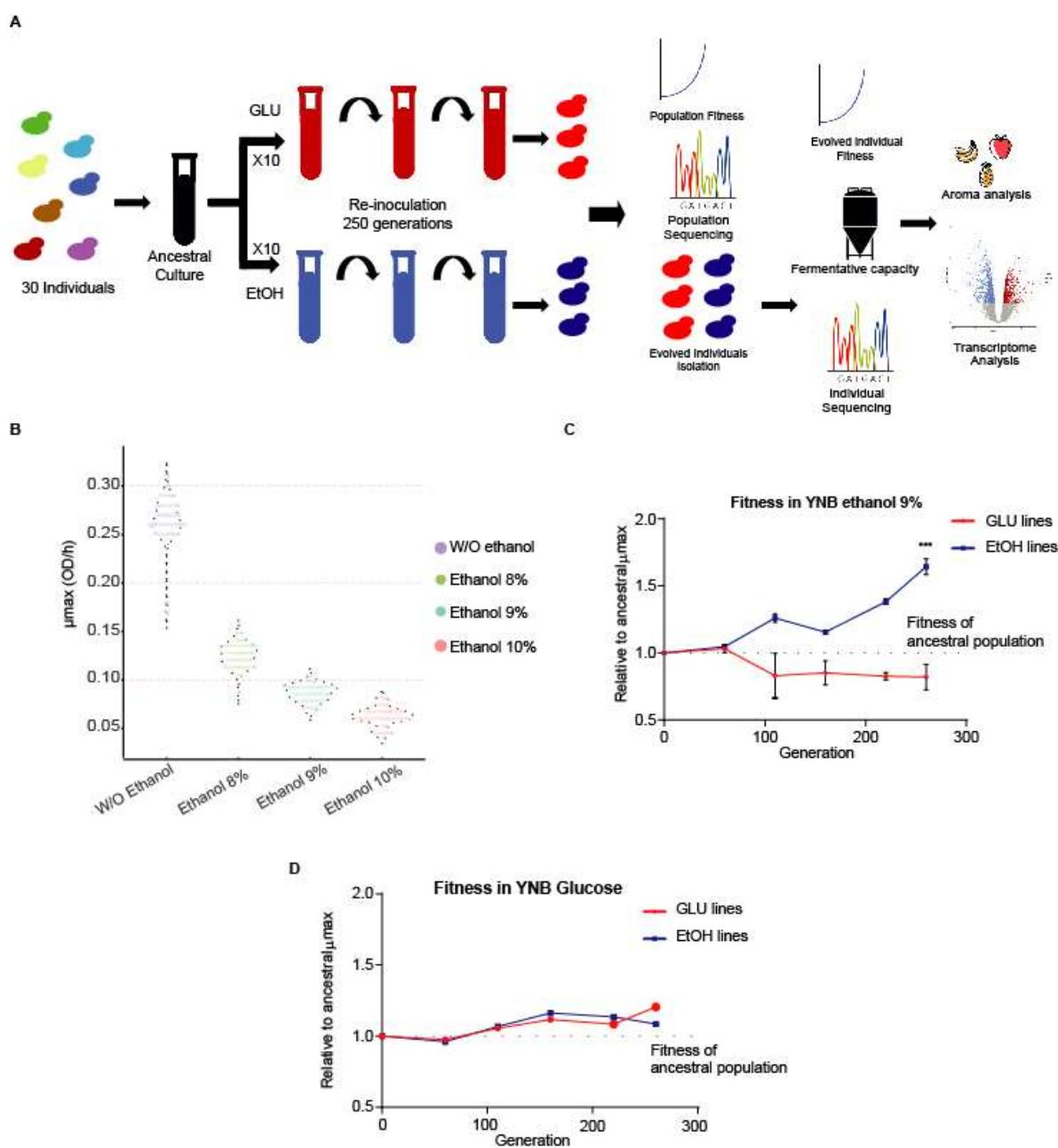
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256 RESULTS

257 ***S. eubayanus* fitness sensitivity under high ethanol conditions**

258 We performed a parallel population assay to obtain high ethanol-tolerant *S. eubayanus*
259 individuals (**Figure 1A**). For this, thirty *S. eubayanus* strains belonging to the PB-2 and PB-
260 3 lineages, previously isolated in southern Chile (Villarrica, Coyhaique and Puyehue; [27]),
261 were selected and characterized for microbial growth under different ethanol conditions.
262 Initially, we used micro-cultures to evaluate biomass generation in 8%, 9% and 10% ethanol.
263 Growth under these conditions showed long lag phases and low growth rates for all strains

264 in concentrations above 9% ethanol (**Figure 1B, Table S3**). This growth was significantly
265 lower compared to that of the L299 wine *S. cerevisiae* control strain (p -value < 0.05,
266 ANOVA), demonstrating a greater susceptibility of *S. eubayanus* to high ethanol
267 concentrations (**Table S3**). Furthermore, for all tested parameters, micro-culture assays
268 demonstrated significant phenotypic differences between strains (**Figure 1B**), representing
269 a genetically and phenotypically heterogeneous group of strains, ideal for the parallel
270 population assay. Based on the above, we chose 9% ethanol as our selective environment
271 for the experimental evolution procedure (from now on referred to as EtOH).



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276 **Figure 1. Fitness of the individual and evolved lines under ethanol.** (A) Experimental
277 evolution strategy in 10 replicated lines under YNB + glucose (GLU, red tubes) and YNB +
278 GLU + ethanol 9% (EtOH, blue tubes). From every line, individuals were isolated and
279 subjected to phenotyping, fermentation and sequencing analysis. (B) The growth rate
280 (μ_{max}) of the different parental strains used in this study was estimated under ethanol 8%,
281 9% and 10%. The fitness of the evolved lines under (C) ethanol and (D) glucose.

282

283 Our population assay began by mixing the thirty strains in equal proportions and subdividing
284 them into ten mock replicates (YNB-glucose media, from now on referred to as GLU) and
285 ten EtOH lines (**Figure 1A**). The ethanol fitness of each evolved line was evaluated at
286 different time points during the progression of the assay (**Figure 1C**). After 260 generations
287 (approximately six months), all GLU lines showed a significant decrease in ethanol fitness
288 compared to the ancestral culture (p -value < 0.05, ANOVA, **Figure 1C**). In contrast, the
289 EtOH-evolved lines showed higher maximum growth rates (μ_{max}) in ethanol compared to
290 the original mixed-culture, attaining a 60% greater μ_{max} (p -value < 0.05, ANOVA). These
291 differences were not observed in glucose micro-cultures (**Figure 1D**). Thus, demonstrating
292 that the evolved lines performed better in their selective environment compared to the
293 control condition. Interestingly, we did not detect major adverse phenotypic effects in beer
294 wort, suggesting a low accumulation of detrimental mutations (**Table S3b**).

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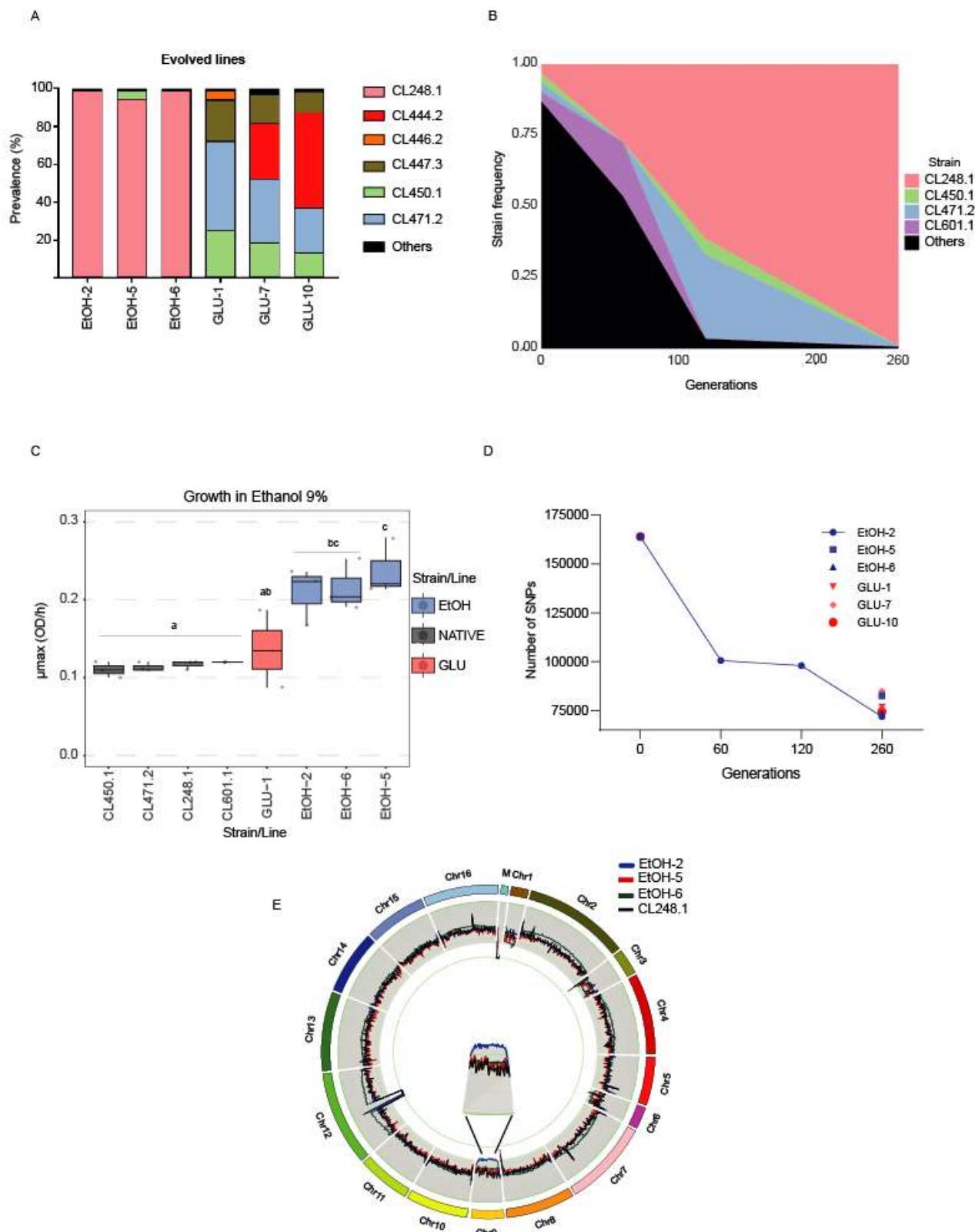
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298 **Genome sequencing reveals consistent strain selection in parallel populations**

299 Three GLU (GLU-1, 7 and 10) and three EtOH lines (EtOH-2, 5 and 6) were sequenced at
300 the end of the experiment to identify the genomic changes and the pervasiveness of the
301 different genetic backgrounds across the assay. Interestingly, all the EtOH sequenced lines
302 showed a sustained prevalence of strain CL248.1 (belonging to PB-2 and isolated in
303 northern Patagonia), reaching over 95% of the population's allele frequency by the end of
304 the experimental evolution assay (**Figure 2A**). That being said, CL248.1 did not show the
305 highest growth rate (μ_{max}) under ethanol 9% of the *S. eubayanus* strains considered in this
306 study, suggesting that selection did not occur solely due to ethanol tolerance (**Table S3**). In
307 contrast, we did not observe a consistent selection in the GLU lines, where different genetic
308 backgrounds were found depending on the evolved line (**Figure 2A**). These results likely
309 suggest a milder and different selection pressure in yeast when glucose is used as a
310 selection regime, and a particular competitive fitness advantage of CL248.1 solely under
311 EtOH selection, demonstrating a convergent phenomenon when ethanol and biotic stress
312 are applied together.

313

314



315 Figure 2. Genomic and phenotypic changes in the evolved lines.

316 (A) The presence of the prevalent genetic backgrounds in three glucose (GLU) and ethanol
317 (EtOH) evolved lines. (B) Prevalence (frequency) of the most prominent genetic
318 backgrounds during the evolution of line EtOH-2. (C) Ethanol 9% growth rates for the most
319 representative parental strains and evolved lines. (D) Total number of SNPs relative to the
320 CBS12357 reference genome at the beginning and end of the evolution assay for EtOH-5,
321 6 and GLU-1, 7 and 10 lines. In addition, the number of SNPs during the evolution assay is
322 shown for EtOH-2. (E) Chromosome number estimation across EtOH lines. Only the EtOH-
323 2 line showed an aneuploidy by the end of the evolution assay (chromosome 9).

324

325 Line EtOH-2 was sequenced at different time points (0, 60, 120 and 260 generations) to
326 identify the genotypic course of the assay and additional genotypes under selection (**Figure**
327 **2B**). We observed a predominance of CL248.1 and CL601.1 genotypes after 60
328 generations, demonstrating a competitive displacement of CL248.1 in the culture, together
329 with higher fitness over the other genetic backgrounds (**Figure 2B**). Interestingly, after 120
330 generations, four genotypes monopolized the culture, representing 96.6% of the EtOH-2
331 line. Nevertheless, none of these parental genotypes showed high ethanol growth rates
332 compared to the evolved lines (**Figure 2C**). A second genotype, CL471.1 reached significant
333 frequencies (maxima 29.3%) during intermediate periods of the evolution assay. However,
334 it was almost absent by the end of the experiment, being detected at a frequency of just
335 0.15% in the final population. Moreover, over time, we calculated the total number of SNPs
336 in evolved lines against the reference strain CBS12357^T. We found a decrease in the
337 number of SNPs over time across all lines relative to the ancestral culture, particularly in
338 EtOH-2, which exhibited the greatest decay compared to other lines (**Figure 2D**).

339 To identify de novo genetic variants with a potential effect on ethanol tolerance, we used the
340 EtOH-2 line and compared polymorphisms (SNPs and short INDELs identified using
341 freebayes) before and after selection. We chose this line because it showed the highest
342 homology to a single genetic background (CL248.1), allowing the identification of novel
343 genetic variants over the raw population's genetic variation. In this way, we arbitrarily
344 selected for polymorphisms with a putative moderate/high impact on the gene function and

345 found 34 impacted genes under these criteria (**Table S4**). Among others, we found
346 mutations in genes such as *YPS6* and *IMA1*, encoding for a putative GPI-anchored aspartic
347 protease [54] and a isomaltase [55], respectively. We also found a single aneuploidy in the
348 EtOH-2 line in chromosome IX, where an extra copy was found (**Figure 2E**). Altogether, our
349 results demonstrate how ethanol promotes a significant decrease in genetic variability due
350 to genotype selection coupled with the emergence of new adaptive mutations vital for
351 ethanol survival in biological processes such as stress damage and sugar metabolism.

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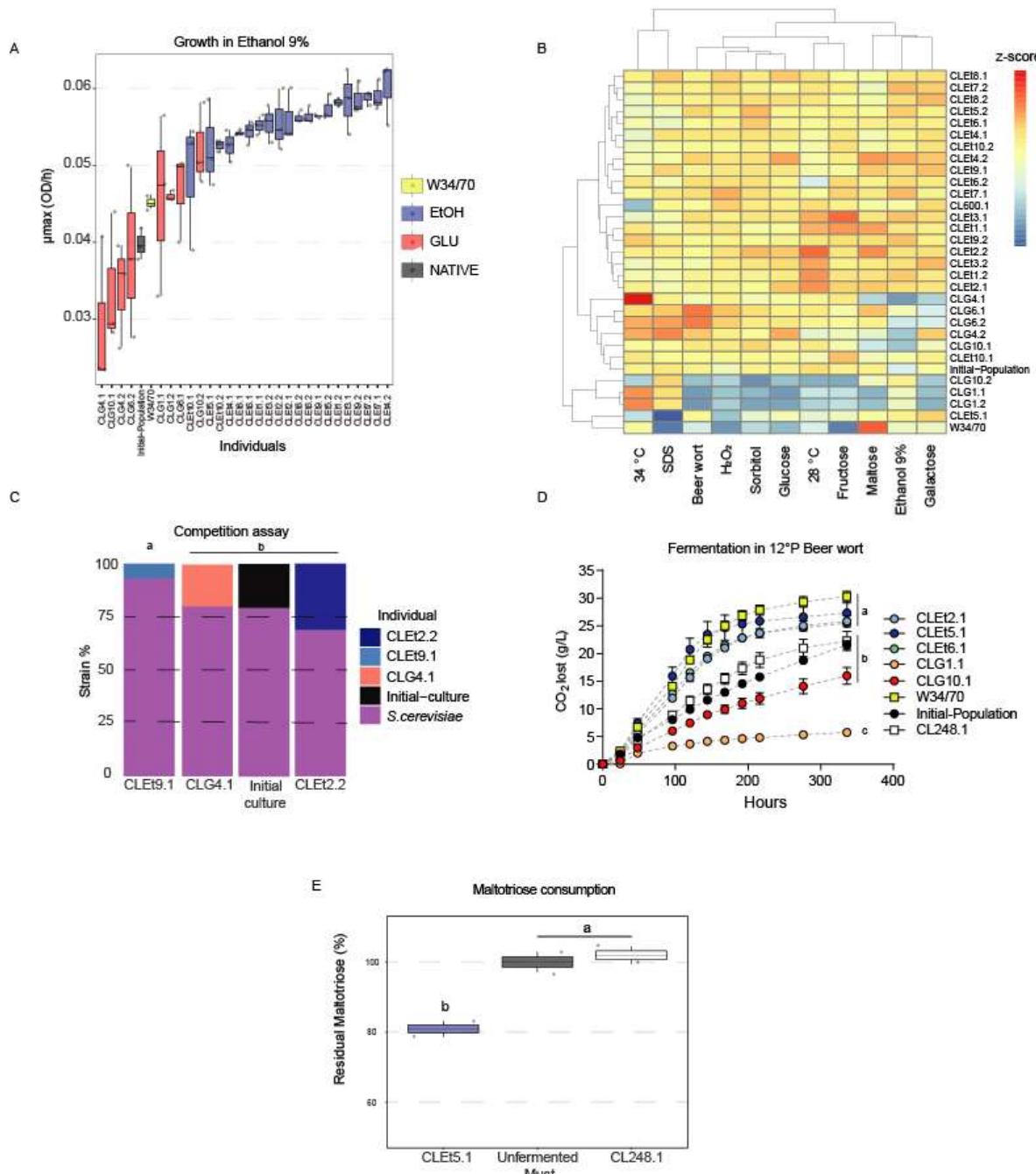
354 **Ethanol-evolved individuals have greater fermentation capacity and maltotriose**
355 **consumption**

356 We determined the fitness cost of ethanol adaptation in 24 different environmental
357 conditions for those EtOH adapted individuals isolated after 260 generations of selection.
358 For this, we randomly isolated two clones from each EtOH line and estimated growth rates
359 in micro-cultures considering diverse phenotypic growth conditions, including high
360 temperature, different carbon sources, and oxidative and osmotic stress (**Table S5**). To
361 control for adaptive mutations in YNB laboratory media, we also isolated two colonies from
362 three GLU lines. In general, individuals from EtOH-evolved lines showed higher μ_{max} in
363 ethanol (**Figure 3A**), and also for a greater number of conditions, compared to GLU-evolved
364 individuals and the ancestral culture (p -value < 0.05, ANOVA, **Table S5**). These conditions
365 included greater growth rates in sources such as glucose, maltose and fructose, together
366 with resistance to oxidative (H_2O_2) and osmotic stresses (sorbitol 20%) (**Figure 3B**),
367 suggesting that selection improved general stress tolerance in these evolved strains.
368 Interestingly, we found that one EtOH evolved individual (CLEt5.1, isolate n°1 from the
369 EtOH-5 line) exhibited greater ethanol tolerance, but a lower growth rate under high
370 temperature (34°C) and an ionic detergent (SDS 0.001%, **Figure 3B**), indicating the
371 existence of a trade-off.

372 To determine the relative fitness of EtOH and GLU evolved individuals, we carried out a
373 competition assay in YNB-glucose supplemented with 6% ethanol, against a recombinant
374 *S. cerevisiae* that constitutively expresses GFP (**Figure S1**). We observed that all tested
375 strains were unable to outcompete *S. cerevisiae*; however, significant differences were
376 found in the final proportion of the tested strains at the end of the experiment (p -value <
377 0.05, ANOVA). For example, strain CLEt9.1 was almost absent at the end of the competition
378 assay (relative frequency < 0.1), while CLEt2.2 was found to represent 31% of the cells

379 quantified in the final culture (**Figure 3C**). These results demonstrate fitness differences
 380 between EtOH and GLU isolated individuals.

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384 Figure 3. **Phenotypic profiling of evolved individuals.** (A) Growth rates under YNB-
385 glucose-ethanol 9% of different evolved individuals. (B) Phenotypic heatmap based on
386 micro-culture growth rates of EtOH and GLU-evolved individuals, evaluated in 11 different
387 conditions. (C) The evolved strains were challenged using a GFP-mutant *S. cerevisiae* in
388 YNB media supplemented with ethanol at 9%. The strain frequency of the evolved
389 individuals was evaluated using flow cytometry. (D) Fermentation in 12 °P beer wort of
390 different evolved individuals. The fermentative capacity was estimated from the CO₂ lost at
391 different time-points. The statistical differences were calculated after 216 h of fermentation
392 using ANOVA. (E) Maltotriose consumption in YNB maltotriose 2% micro-cultures.

393

394 Additionally, we evaluated the fermentative capacity in small-scale lager wort fermentations
395 at low temperature (12 °C) of three EtOH and two GLU evolved individuals. The selected
396 strains were monitored for 15 days, and their fermentative capacity was estimated by
397 measuring CO₂ loss and sugar consumption throughout the fermentative process (**Figure**
398 **3D**). Surprisingly, all the EtOH-evolved individuals showed a similar fermentative profile
399 compared to the commercial strain, where no significant differences were found in terms of
400 total CO₂ loss (*p*-value < 0.05, ANOVA). Furthermore, the best-evolved isolate (CLEt5.1)
401 showed a 22.6% increase in loss of CO₂ compared with the ancestral culture after 14 days
402 of fermentation (**Figure 3D** and **Figure S2A**, *p*-value > 0.05, ANOVA), and also exceeded
403 the fermentative performance, in terms of fermentation rate, of its parental genetic
404 background CL248.1 (**Figure 3D, S2B, and S2C**). Moreover, sugar consumption differed
405 between the W34/70 commercial strain and the evolved individuals. Although the isolates
406 were able to consume all the glucose, maltose, and fructose found in the wort (**Figure S2D**),
407 no maltotriose consumption was observed (*p*-value < 0.05, ANOVA, **Table S6**) in the
408 evolved strains. We only detected maltotriose consumption under fermentation conditions
409 in the lager commercial strain, in agreement with the inability of *S. eubayanus* to use this
410 carbon source (**Figure S2D**, [23]). To further analyze maltotriose consumption, we
411 quantified the remaining maltotriose concentration after a 5-day incubation period of the
412 evolved individuals in YNB synthetic media supplemented with 2% maltotriose as the sole
413 carbon source (**Figure 3E**). Interestingly, we detected 19.1% maltotriose consumption in the

414 evolved strain CLEt5.1, while no consumption was found in CL248.1 (**Figure 3E**). These
415 results suggest genomic and molecular changes leading to maltotriose metabolism in this
416 genetic background that only arise when maltotriose is used as the sole carbon source.

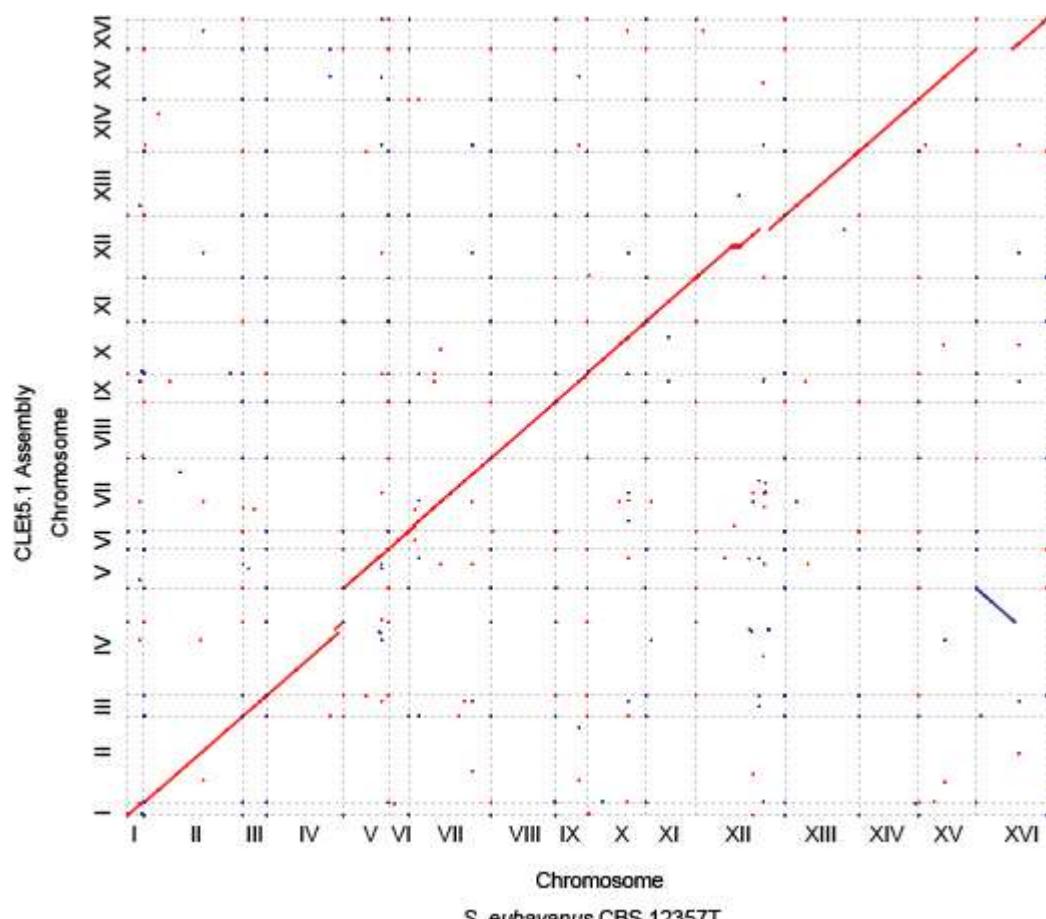
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418

419 **Identification of *de novo* genetic variants in the EtOH evolved strain CLEt5.1**

420 The genome of the EtOH-evolved individual CLEt5.1 was sequenced by coupling Nanopore
421 and Illumina technologies to elucidate the genetic origin of the phenotypic changes acquired
422 through the evolution process. We obtained a high-quality assembly and identified 5,946
423 genes in the final genome annotation, organized in 37 scaffolds (**Figure 4, Table S2 and**
424 **Table S7a**). The completeness analysis using BUSCO showed that the *de novo* assembly
425 contained almost all the expected set of genes for a member of the *Saccharomyces* genus
426 (97.5%). By comparing the scaffolds of the assembly against the CBS12357^T reference
427 genome, high synteny between genomes was observed, except for an evident translocation
428 between chromosomes IV-R and XVI-L (**Figure 4**). Therefore, we proceeded to identify
429 structural variants between CLEt5.1 and its parental background (CL248.1) using MUM&Co
430 [47]. In this way, we identified 100 structural variants (Deletions: 47, Insertions: 41,
431 Duplications: 10, Inversions: 0 and Translocations: 2, **Table S7b**), primordially INDELs and
432 confirming the translocation between chromosomes IV-R and XVI-L of 980 kb. Additionally,
433 we found a 47 kb deletion in chromosome XII, and two 24 kb and 39 kb duplications in
434 chromosomes VII and IV, respectively. Among the genes present in the chromosome VII
435 duplication, we found *VID30*, which is involved in the regulation of carbohydrate metabolism
436 and the balance of nitrogen metabolism towards glutamate production, and *HAP2*, a
437 transcription factor which is predicted to regulate many of the proteins induced during the
438 diauxic shift [56] (**Table S7c**). SNP calling using freebayes detected 1,006 high quality SNPs.

439 To better understand the molecular basis of ethanol adaptation, we searched for
440 polymorphisms across the CLEt5.1 genome that could generate moderate or high impact
441 mutations on the gene function (based on snpeff predictions). We found 11 genes with
442 significant polymorphisms between CLEt5.1 and the native CL248.1 strain (**Table S7d**). For
443 example, we found a missense variant in *PUT4*, which encodes for a proline permease
444 essential in proline assimilation during fermentation [57]. Similarly, we found a frameshift in
445 *IRA2*, which encodes for a GTPase-activating protein, and previously related to high-
446 temperature fermentation [58] and low glucose-growth defect rescue [59]. These results
447 demonstrate that this relatively short period of ethanol adaptation promoted punctual, small
448 and large rearrangements, which, taken together may be responsible for the phenotypic
449 differences between the CLEt5.1 and CL248.1 strains.

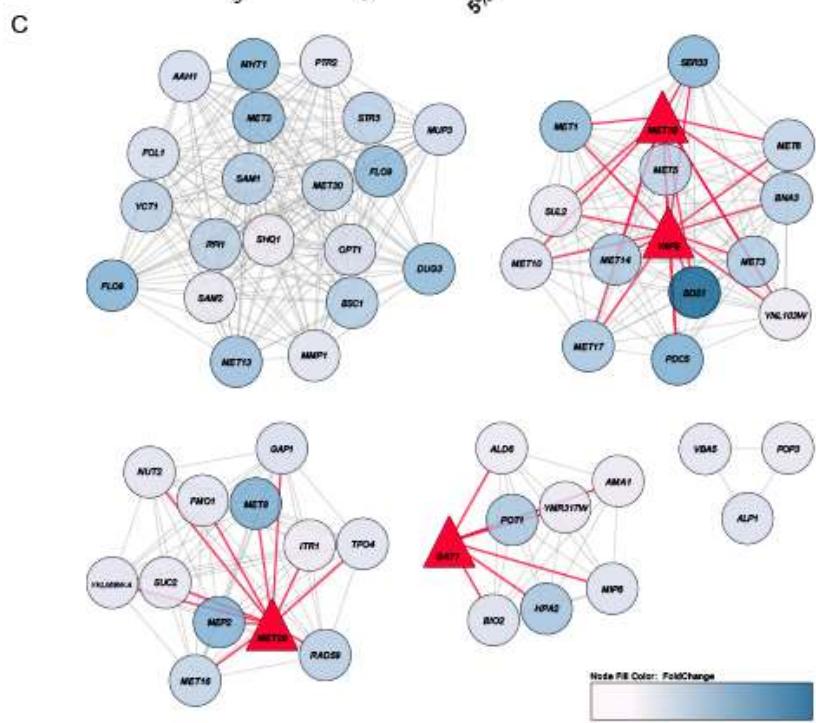
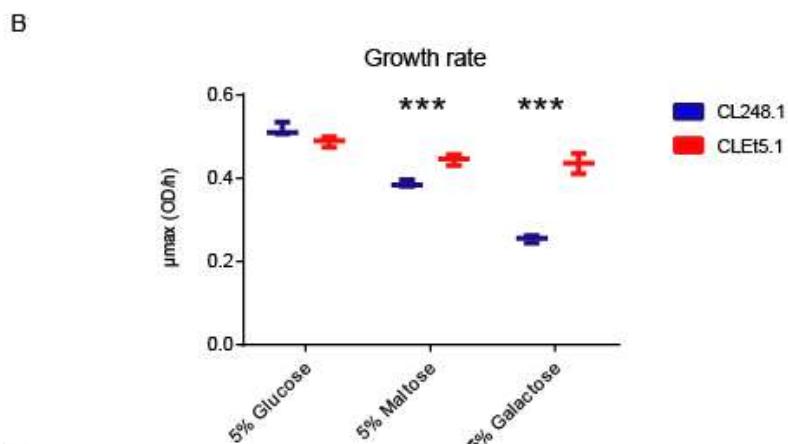
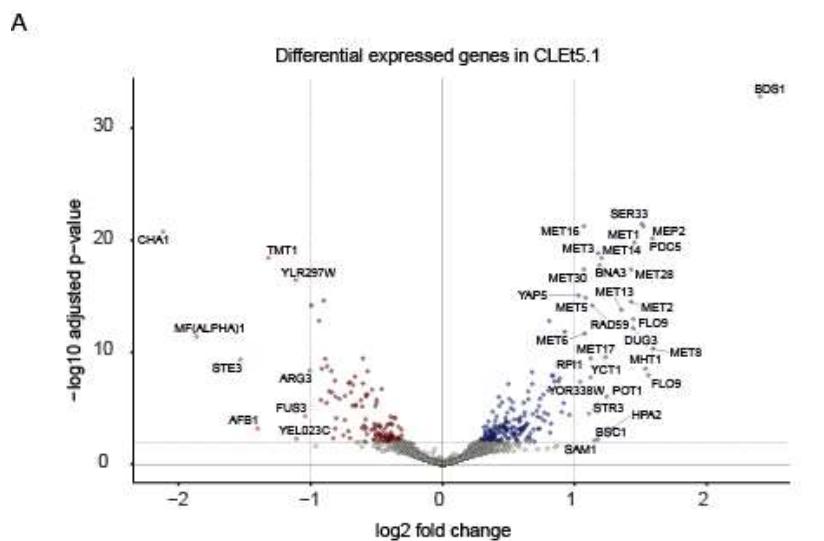


451 **Figure 4. Genome synteny analysis of the EtOH evolved CLEt5.1 strain.** Dot plot
452 representation of DNA sequence identity between the *S. eubayanus* CBS12357^T strain and
453 the EtOH evolved CLEt5.1 strain. A single translocation was found between chromosome
454 IV and XVI.

455

456 **Transcriptome and organoleptic analysis of the CLEt5.1 evolved strain under beer**
457 **fermentation**

458 To determine the impact of genetic changes in metabolic processes during wort fermentation
459 in EtOH adapted individuals, we used a transcriptome approach. This allowed us to identify
460 differentially expressed genes (DEGs) between the CLEt5.1 and the CL248.1 parental strain
461 after 24 h of fermentation in a 1.5 L fermenter. Overall, we observed 92 DEGs (Fold change
462 > 0.7 and FDR < 0.05, **Figure 5A** and **Table S8**), of which 59 and 33 were up- and down-
463 regulated in the CLEt5.1 strain, respectively. Enrichment analysis of GO terms in up-
464 regulated genes revealed that diverse biological and molecular pathways, including sulfur
465 compounds, methionine metabolism, and several cellular amino acid metabolic processes
466 were enriched in the evolved strain (**Table S8**). In contrast, down-regulated genes were
467 significantly enriched in alpha-amino acid metabolism and pheromone response
468 metabolism, together with cofactor and vitamin binding molecular functions (**Table S8**).
469 Similarly, KEGG enrichment analysis highlighted that genes within several pathways were
470 differentially expressed between genotypes. For example, assimilatory sulfate reduction,
471 cysteine and methionine metabolism, seleno-compound metabolism and biosynthesis of
472 antibiotics pathways were enriched in the up-regulated genes set (**Table S8**). In contrast,
473 we found a significant enrichment of the amino acid biosynthesis pathway among down-
474 regulated genes (*p*-value < 0.01, hypergeometric test). Interestingly, these two analyses
475 highlight that several DEGs were related to nitrogen and amino acid uptake, stress
476 tolerance, and faster diauxic shift, suggesting that nitrogen uptake and a rapid stress
477 response play essential roles during fermentation in this evolved strain.



479

480 **Figure 5. Differential gene expression analysis between the EtOH evolved CLEt5.1**
481 **strain and its native parental strain under beer wort fermentation conditions.** The
482 transcriptome of the CLEt5.1 EtOH evolved strain was evaluated and compared against the
483 CL248.1 native strain under beer wort fermentation conditions. (A) The volcano plot depicts
484 differentially expressed genes between CLEt5.1 and CL248.1 (B) Relative growth rates of
485 CLEt5.1 and CL248.1 strains shifted from two 24 hours 5% glucose pre-cultures to 5%
486 maltose and 5% galactose media. (C) Network analysis in upregulated genes in CLEt5.1
487 depicting the most relevant hubs differently regulating genes between CLEt5.1 and CL248.1.
488 Transcription factors are shown in red triangles, while TF-gene connections are shown in
489 red lines.

490

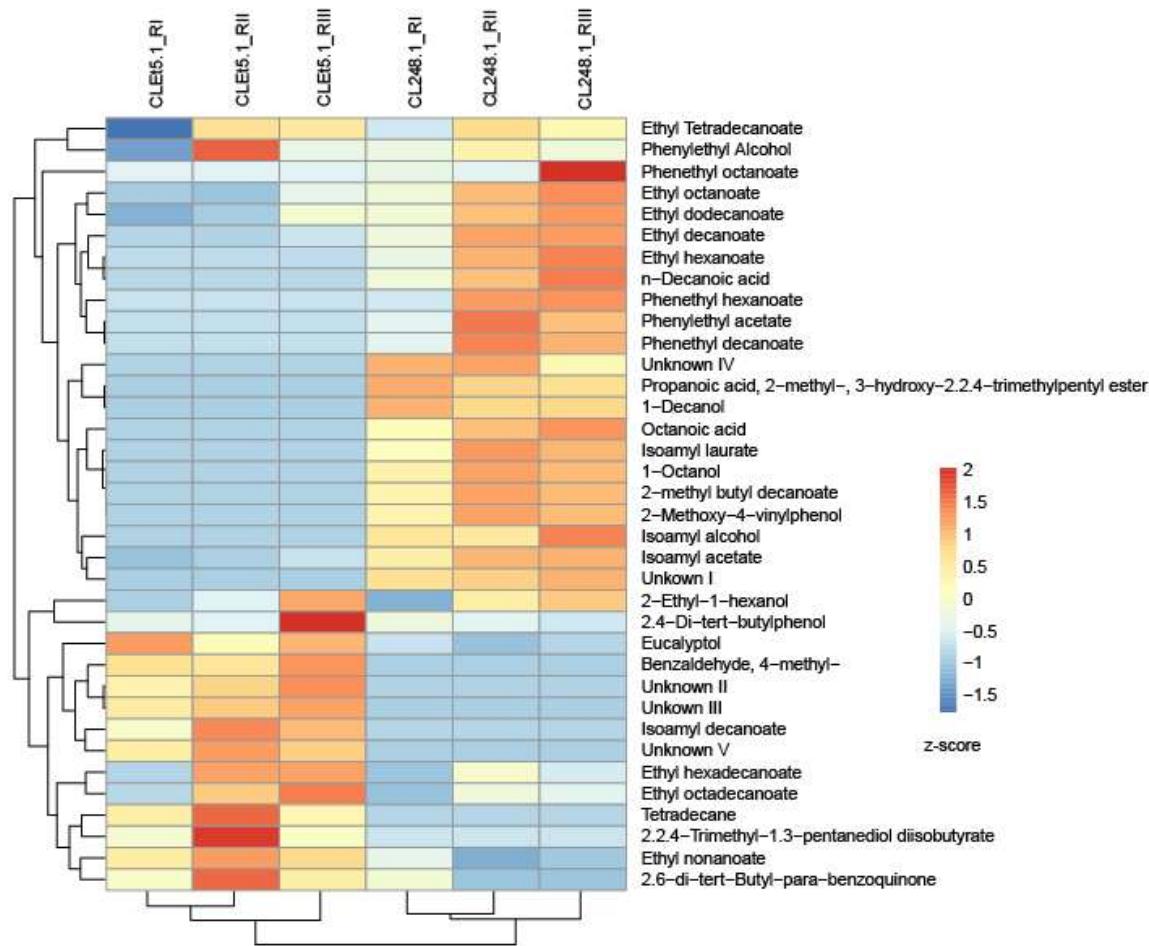
491 To evaluate the fast diauxic shift and the capacity of these two strains to switch from glucose
492 to other disaccharides, we estimated their growth capacity under maltose and galactose
493 after two 24 h pre-cultures in 5% glucose. In agreement with our transcriptome results, the
494 evolved strain showed a significantly greater growth rate compared to CL248.1 under 6%
495 maltose and 6% galactose concentrations after long glucose incubation periods (**Figure 5B**).

496 Additionally, to identify possible common regulatory elements of the up-regulated genes, we
497 analyzed their promoter sequences (500 bp upstream of the transcription start site), and
498 found a significant enrichment of transcription factor binding sites (p -value < 0.05 , Fisher's
499 exact test) for transcription co-activators of the Cbf1-Met4-Met28p complex (methionine
500 metabolism), Dal80p and Uga3p (activators of nitrogen metabolism), Tye7p (glycolytic
501 genes activator) and Sfl1p (repression of flocculation-related genes, and activation of stress
502 responsive genes, **Table S9**). Additionally, we used Cytoscape to visualize the resulting
503 network predicting regulatory interactions from the set of upregulating genes (**Figure 5C**).

504 According to our network model, we found four transcription factors: Met28p, Met32p, Gatp
505 and Yap5p modulating the expression of these up-regulated genes in CLEt5.1. Interestingly,
506 Yap5p is known to be involved in the diauxic shift [60]. These results highlight a
507 transcriptional rewiring in CLEt5.1 for genes related with nutrient acquisition, stress

508 tolerance and methionine metabolism during the evolution of tolerance to fermentation
509 stress.

510 During the fermentation process, we subjectively perceived that the organoleptic properties
511 of the beers produced by the evolved strain differed from those of the parental native strain.
512 Therefore, to determine how the transcriptional rewiring and genomic changes impacted the
513 production of volatile compounds and the beer profile in the CLEt5.1 evolved strain, we
514 quantified volatile compound production using HS-SPME-GC/MS at the end of fermentation
515 (day 15). As expected, we found significant differences in the composition of volatile
516 compounds produced in beer between the evolved and parental strains (p -value < 0.05,
517 paired t-test, **Figure 6A, Table S10**). In general, the evolved clone showed lower levels of
518 ester compounds, such as isoamyl acetate and ethyl octanoate (p -value < 0.05, ANOVA).
519 Additionally, we detected high levels of benzaldehyde 4-methyl (aromatic aldehyde) and
520 ethyl hexadecanoate in the evolved strain compared to the native genetic background, which
521 could confer a fruity aroma to the beer similar to those found in lager beers. The most
522 interesting differences were found in terms of off-flavors. We detected a significantly lower
523 production of 2-Methoxy-4-vinylphenol (4-vinyl guaiacol) in the evolved strain, likely
524 reducing its clove-like flavor, which is typically found in fermented beverages by wild strains
525 (p -value < 0.05, ANOVA, **Figure 6A**). Interestingly, we did not find mutations in the *FDC1*
526 and *PAD1* coding regions, or a significant difference in gene expression for *FDC1* ($\log_{2}FC$
527 = -0.038, p -value adjusted = 0.838) and *PAD1* ($\log_{2}FC$ = -0.0095, p -value adjusted = 0.965)
528 between both strains. However, a series of mutations in the regulatory regions of both genes
529 were found in CLEt5.1, which could alter expression levels in later fermentation stages.
530 These results suggest that the evolution process significantly impacted the volatile
531 compound profile of beers produced by CLEt5.1, emulating the domestication process that
532 modified several commercial yeasts.



533 Figure 6

534

535 Figure 6. **Volatile compound production on beer wort.** The final beer from EtOH evolved
536 CLEt5.1 and its parental strain CL248.1 was analyzed using HS-SPME-GC/MS. The relative
537 abundance of each compound detected was evaluated and a heatmap was constructed.
538 The compounds were grouped in accordance to their relative abundance.

539

540 DISCUSSION

541 Human-driven selection associated with yeast domestication in fermentative environments
542 has been extensively reported in *S. cerevisiae* and related hybrids [14, 15]. However, the
543 genetic basis and molecular changes in other *Saccharomyces* genomes associated with
544 alcoholic beverages is still unclear. In our study, we have reconstructed the putative

545 domestication history of the yeast *S. eubayanus* under biotic and abiotic stresses, using a
546 panmictic founding population that simulated the natural process of adaptive evolution, and
547 using an ethanol environment as the selective agent. We used dozens of wild genotypes in
548 a single culture, in order to replicate the natural genetic variability of these organisms. We
549 observed that a single genetic background, CL248.1, systematically outcompetes the
550 others, acquiring *de novo* mutations and improving basal ethanol tolerance. Interestingly,
551 the time-course of this competitive displacement was complex, involving genotype selection
552 and innovations throughout the assay (key adaptive mutations) that were constantly
553 replaced by others during the "fast-motion" evolution time-course. Thus, the evolved
554 lineages derived from our founding genetic background exhibited higher ethanol growth
555 rates compared to their ancestors, demonstrating a rapid response to selection, and so
556 adapted successfully to their new environment. However, CL248.1 was not the best ethanol-
557 tolerant strain, suggesting that pre-existing variants, together with *de novo* mutations,
558 combined to positively-affect fitness in this strain. In this sense, it has been demonstrated
559 that pre-existing and *de novo* genetic variants can both drive long term adaptation to
560 environmental changes in yeast [61]. This indicates that not only a fitness advantage related
561 to a given environmental selection pressure is essential for directional selection to occur in
562 populations [62], but also that a combination of standing genetic variation with some
563 genomic plasticity for beneficial mutations are essential [63]. In this way, the success of an
564 individual is established in such a competitive environment [64]. Our results show that both
565 pre-existing genetic variation and *de novo* mutations of a range of effects were important in
566 explaining rapid evolution in this ecological context [65, 66]. Importantly, the *Saccharomyces*
567 "make-accumulate-consume (ethanol)" life strategy is fundamental for withstanding the
568 antimicrobial effects of ethanol in a complex population [67, 68]. Thanks to this, multiple
569 *Saccharomyces* genotypes were selected, domesticated, and used over centuries in the
570 beer industries, including the *S. pastorianus* hybrid [14, 15].

571 Domestication signatures in yeast, as a result of the human-domestication syndrome,
572 included genomic changes in the *S. cerevisiae* and *S. eubayanus* genomic portions leading
573 to faster fermentation rates under low temperatures, a more moderate organoleptic
574 complexity, and the absence of off-flavors in beers [14, 20]. Under the premise that
575 evolutionary experiments can lead to unexpected and somewhat counterintuitive results
576 [69], we evaluated the beer fermentation performance of *S. eubayanus* evolved individuals.
577 Interestingly, evolved individuals exhibited a similar fermentation performance compared to
578 lager yeast, suggesting in turn that ethanol, together with competitive displacement, could
579 be the leading drivers of yeast domestication in brewing environments. This persistent
580 directional selection involved correlated selection of other traits, such as osmotic stress
581 tolerance and efficient nitrogen uptake [70]. In general, domesticated fungi used in
582 fermented foods exhibit genomic rearrangements, fewer spores and produce desirable
583 volatile compounds [9]. These domestication signatures have been reported in other
584 systems, such as *Aspergillus* and *Penicillium*, where a transition to environments rich in
585 carbon and nitrogen sources led to extensive metabolism remodeling when used to produce
586 cheese [8, 9].

587 Ethanol-evolved individuals presented a series of genomic changes related to yeast
588 domestication, such as aneuploidy and chromosomal rearrangements [16]. Furthermore,
589 signatures of trait domestication are evident in evolved individuals showing improved stress
590 resistance, fast fermentation rates, lower organoleptic complexity and a lower production of
591 phenolic off-flavors [14]. *S. cerevisiae* beer strains are characterized by strong
592 domestication signatures in their genomes, including polyploidies, the decay of sexual
593 reproduction, and maltotriose consumption [16]. Interestingly, one of our strains was able to
594 consume maltotriose, which is another key domestication hallmark. In terms of the molecular
595 mechanisms that explain their increased fermentative capacity, we observed that some

596 stress response genes were either mutated or up-regulated in the ethanol-evolved line
597 compared to its parental genetic background. In this way, the mutations and genomic
598 rearrangements found in the CLEt5.1 evolved individual could explain the transcriptional
599 rewiring and improved fermentative profile. Indeed, ethanol exposure leads to the
600 recruitment of error-prone DNA polymerases, causing DNA replication stress and increased
601 mutation rates [71]. Accordingly, we found that *RAD59* (involved in DNA double-strand break
602 repair) was overexpressed in the evolved strain CLEt5.1, likely indicative of a mechanism
603 that counteracts the mutagenic effect of ethanol [72]. Other overexpressed genes could also
604 be directly related to an increased fermentative capacity, such as *SUC2*, *YAP5* and *MET*,
605 which could promote glucose uptake, a dynamic diauxic shift, and the accumulation of S-
606 Adenosylmethionine, respectively [73, 74 2013]. In this context, genomic rearrangements,
607 such as the duplication found in chromosome VII containing *HAP2*, which is involved in
608 promoting the diauxic shift, are in agreement with these findings. Furthermore, previous
609 reports in lager yeast demonstrated that the accumulation and exogenous supplementation
610 of S-Adenosylmethionine promotes an increase in the fermentative capacity of yeast under
611 high-gravity wort [75].

612 **Concluding remarks**

613 In summary, the results found in our study could be applied to determine the domestication
614 dynamics of the *S. eubayanus* genomic portion in the lager strain, given the occurrence of
615 similar desirable traits for beer. Based on multiple analyses, we provide evidence of the
616 intermediate evolutionary changes in *S. eubayanus*, which have direct implications in the
617 generation of novel yeasts for the industry. In this way, genomic changes promote a
618 transcriptional rewiring that induces a favorable response in a fermentative environment. For
619 the first time, these findings provide novel insights into the genomic and phenomic changes

620 in wild *S. eubayanus* leading to faster wort fermentation rates and desirable organoleptic
621 complexity, demonstrating its broad feasible use in the beer industry.

622

623

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632

633 **SUPPLEMENTARY MATERIAL**

634 **SUPPLEMENTARY FIGURES**

635 Figure S1. **Competition assay of evolved individual in ethanol 9%.**

636 Figure S2. **Fermentative capacity of the evolved individuals.** (A) The fermentative
637 capacity is indicated as a percentage of the capacity of the *S. pastorianus* control strain
638 (W34/70) at 7 days. The fermentative capacity was estimated from the loss of CO₂ over
639 time. All assays were performed in triplicate. (B) The fermentative capacity was also
640 determined at 14 days. (C) The velocity of the fermentation was estimated and (D) the
641 residual sugars and metabolites in the wort were evaluated using HPLC.

642

643 **SUPPLEMENTARY TABLE LEGENDS**

644 **Table S1. Native *S. eubayanus* strains used in the experimental evolution assay.** The
645 strain ID and the location of isolation site are indicated.

646 **Table S2. Bioinformatics Summary statistics**

647 **Table S3. Growth kinetic parameters in glucose and ethanol of the native parental**
648 **strains used for the ancestral culture.** Growth parameters μ_{max} (OD/hr), OD max (OD)
649 and lag phase (1/hr).

650 **Table S4. SNPeffect analysis of the novel polymorphisms in EtOH-2.** Snpeffect analysis
651 of the novel/fixed polymorphisms in EtOH-2 after 260 generations

652 **Table S5. Phenotype data of evolved individuals.** The data shows the average μ_{max}
653 across three replicates and the standard deviation (SD) for diverse growth conditions,
654 including high temperature (28°C and 34°C), different carbon sources (glucose, fructose,
655 maltose, galactose, xylose), and oxidative (ethanol 9%, 3 mM H₂O₂) and osmotic stress
656 (beer wort, SDS 0.001%, Sorbitol 20%).

657 **Table S6. Sugar consumption and metabolite production of the evolved individuals**
658 **from fermentations in beer wort.** Sugar consumption (g/L) and metabolite production (g/L)
659 are informed.

660 **Table S7. Structural variants identified in CLEt5.1 using MUM&Co.** A. CLEt5.1 genome
661 assembly and annotation statistics. The genome assembly of CLEt5.1 using Nanopore and
662 Illumina sequencing technology was used to calculate several assembly statistics. B. All
663 structural variants. C. Duplicated genes present in the chromosome IV – chromosome XVI
664 duplication in CLEt5.1. D. High/moderate SNPeff prediction of SNPs and short INDELs in
665 CLEt5.1

666 **Table S8. Differential gene expression between CL248.1 and CLEt5.1 under beer wort.**

667 A. Gene expression results. B. Upregulated and C. Downregulated genes in CLEt5.1. R1,
668 R2 and R3 represent the three biological replicates for each genotype.

669 **Table S9. Enrichment analysis of Transcription Factor binding sites in regulatory
670 regions of upregulated genes using CiiDER.**

671 **Table S10. Volatile compound production in CL248.1 and CLEt5.1 in beer wort.**

672

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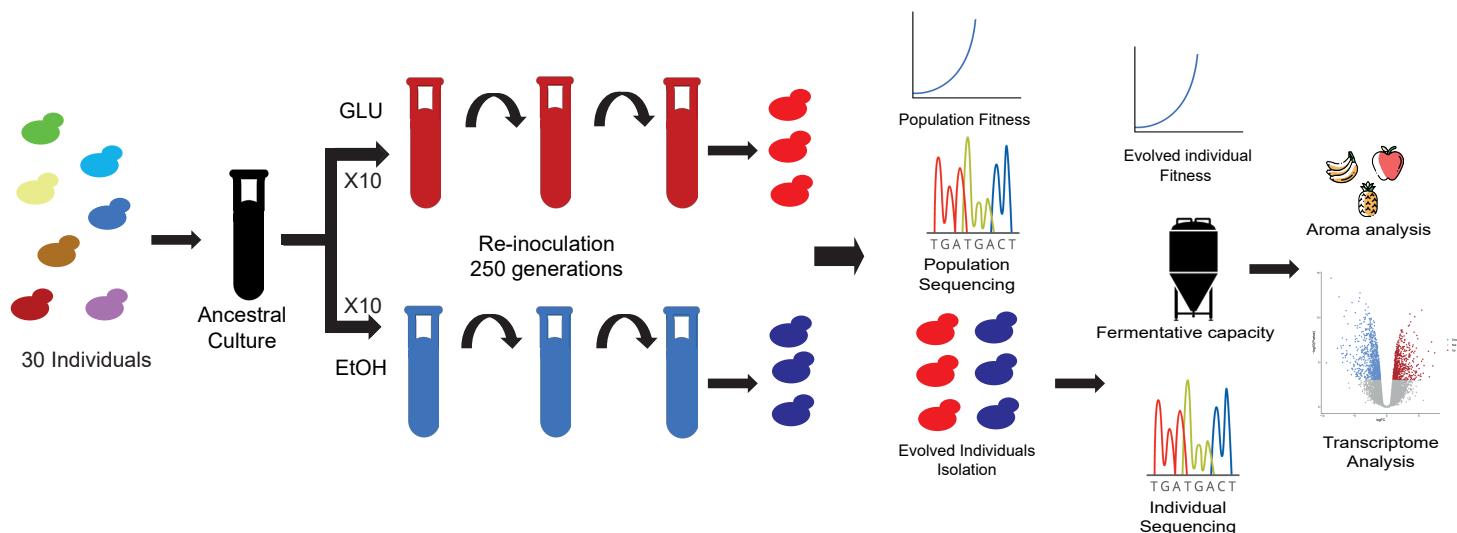
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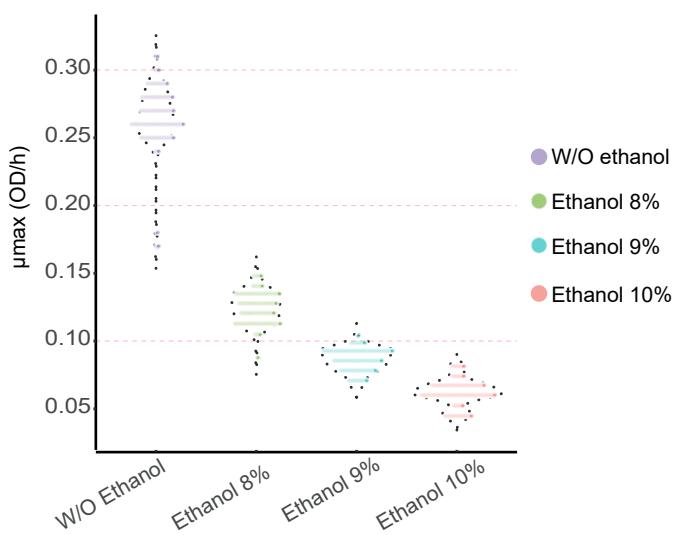
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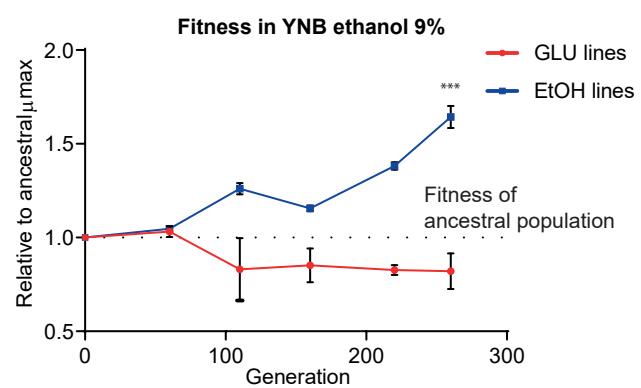
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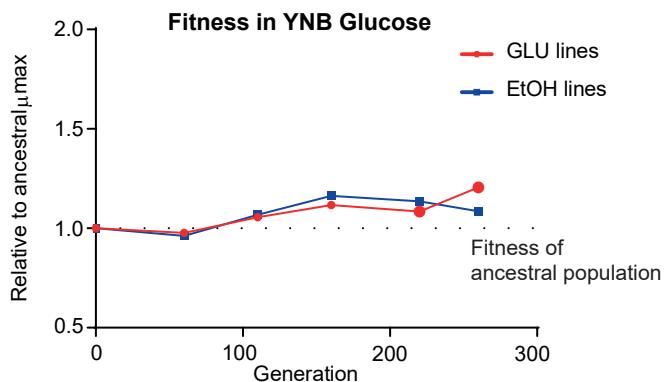
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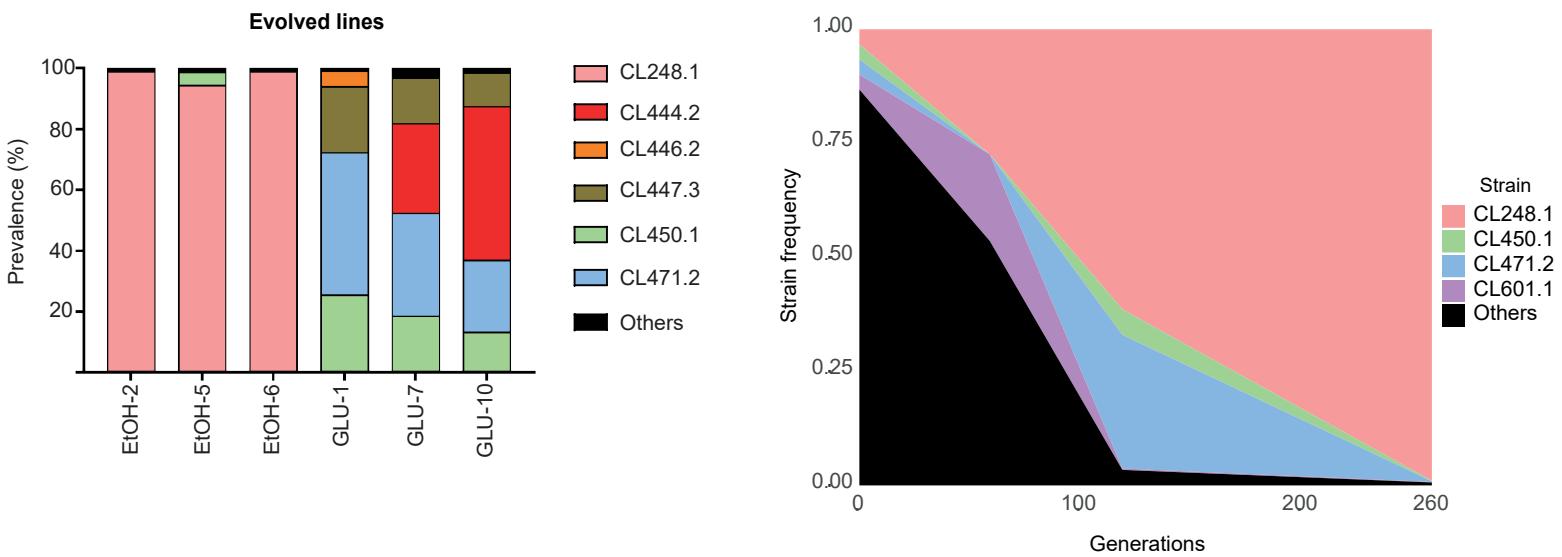
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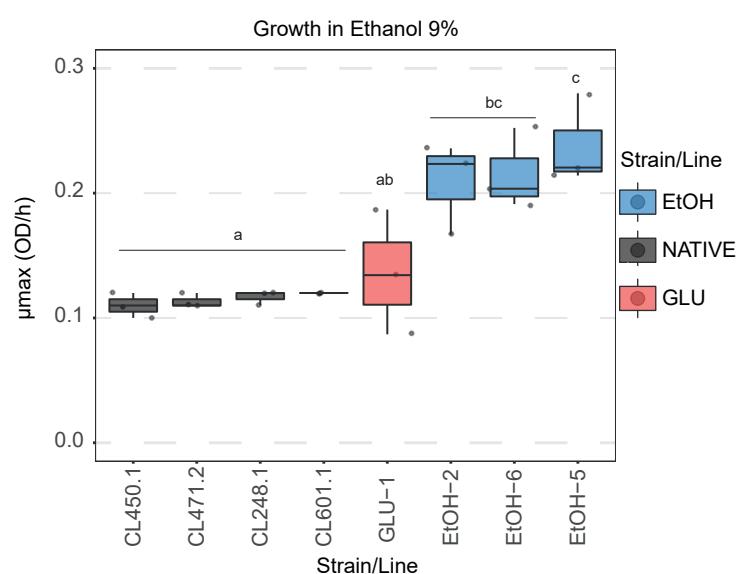
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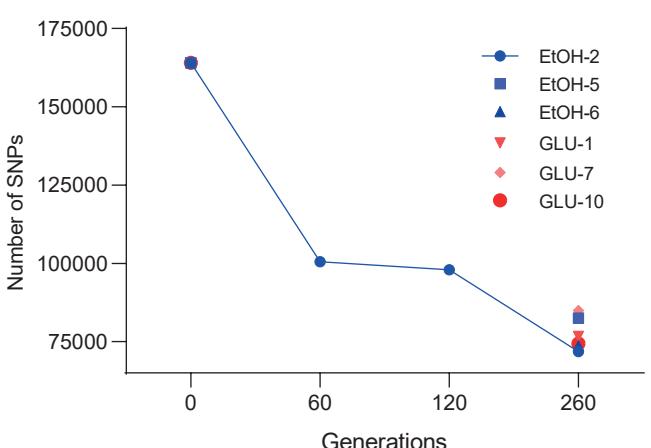
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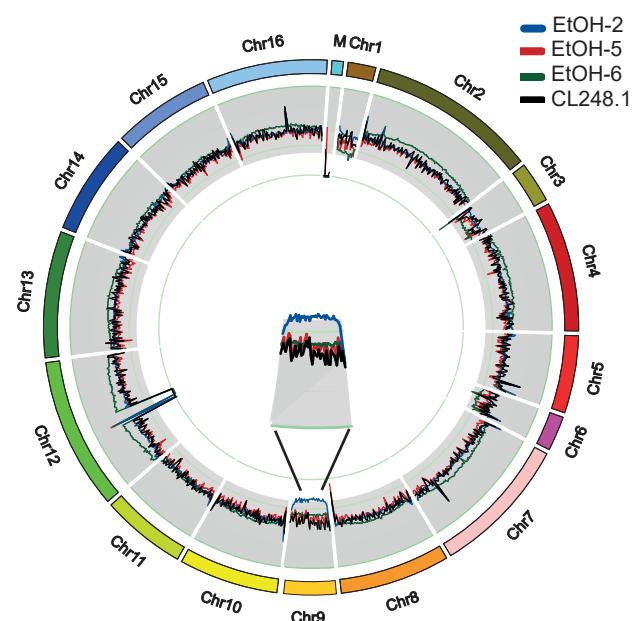
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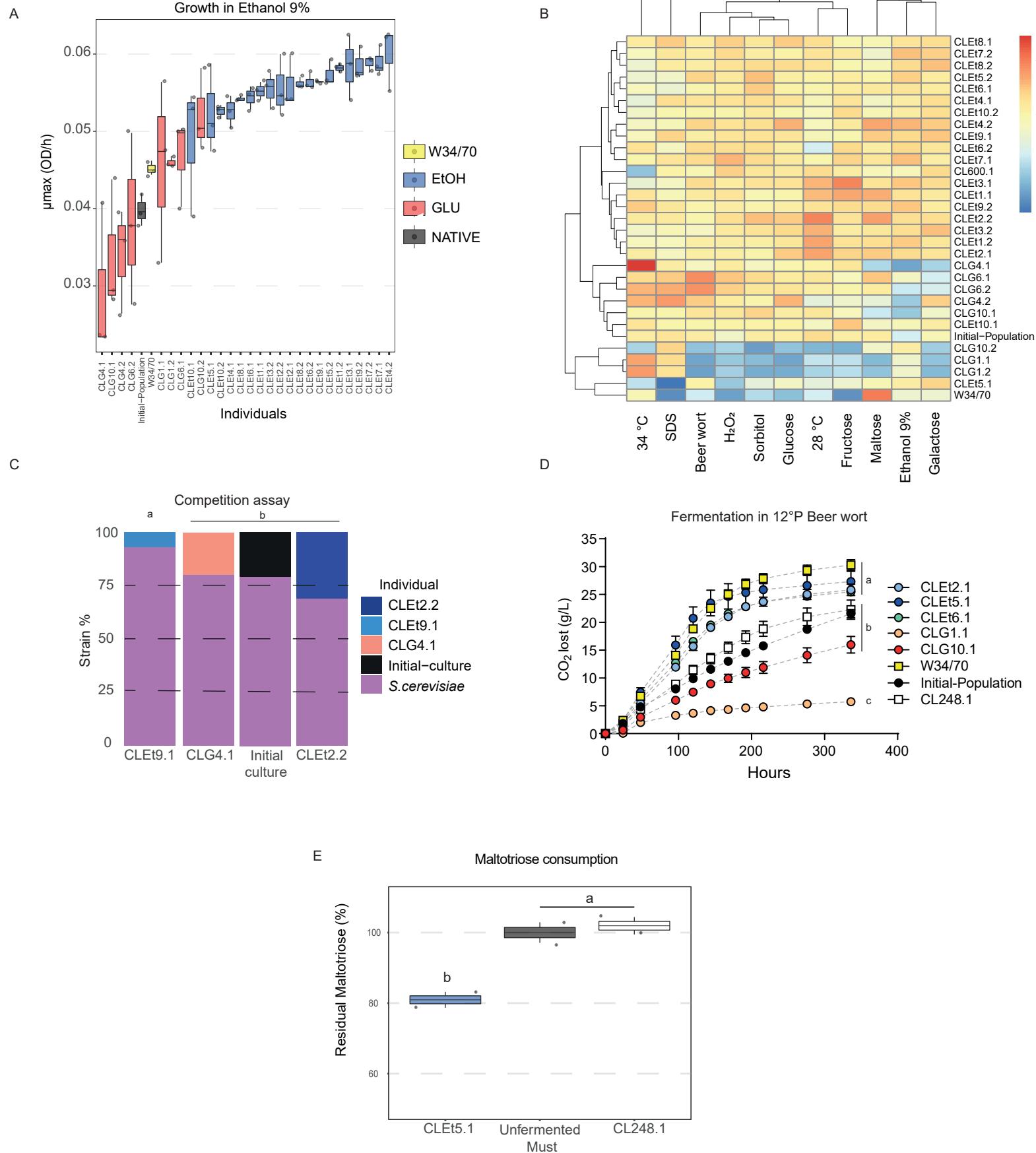


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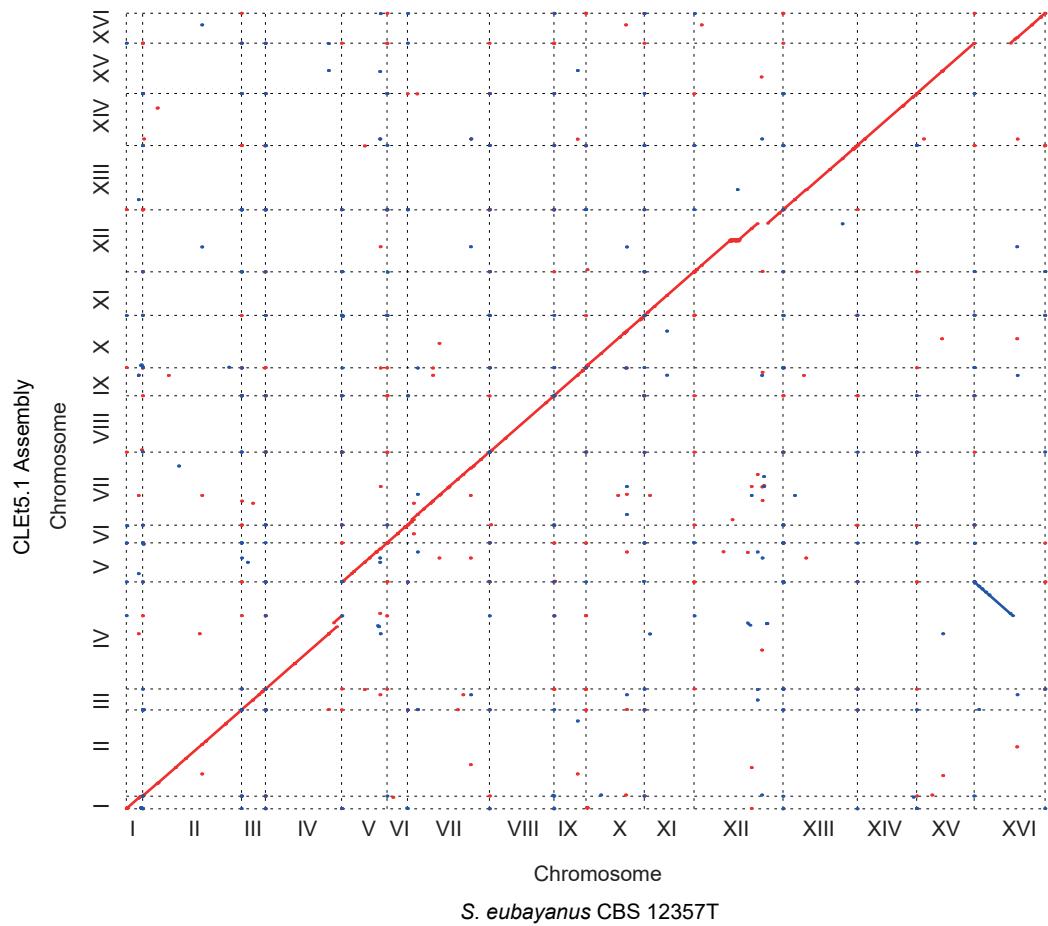
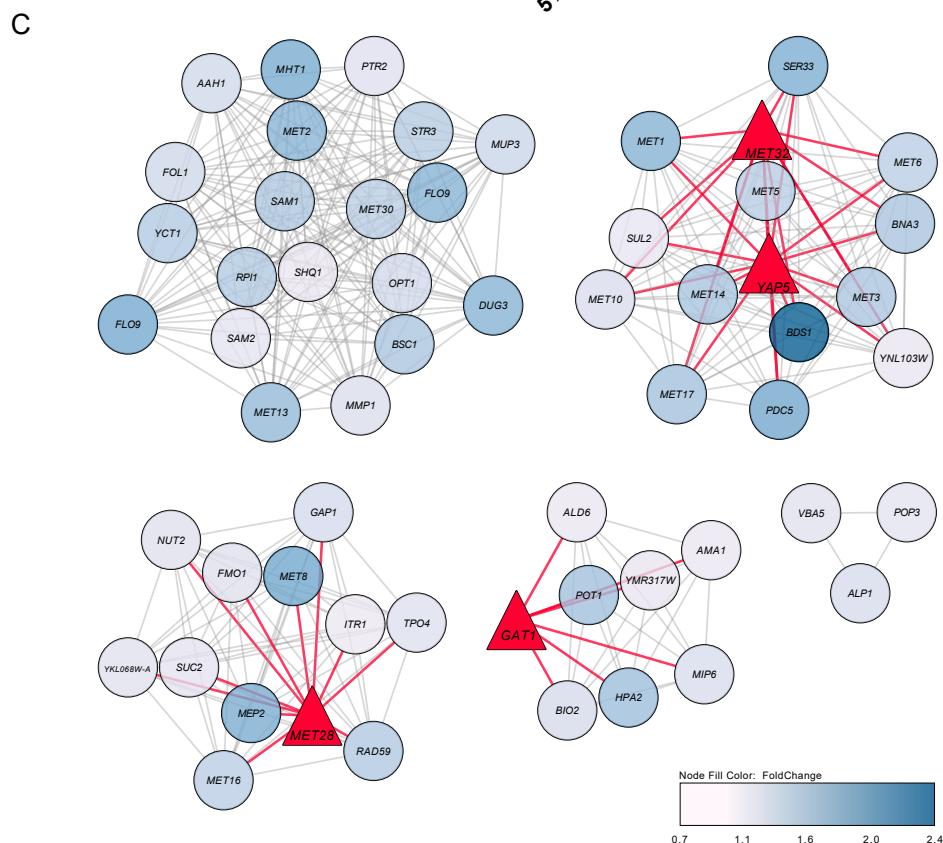
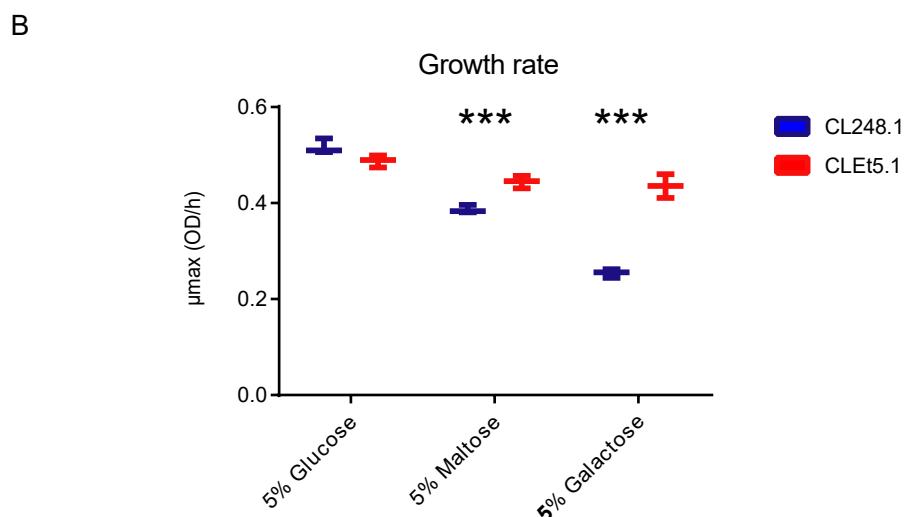
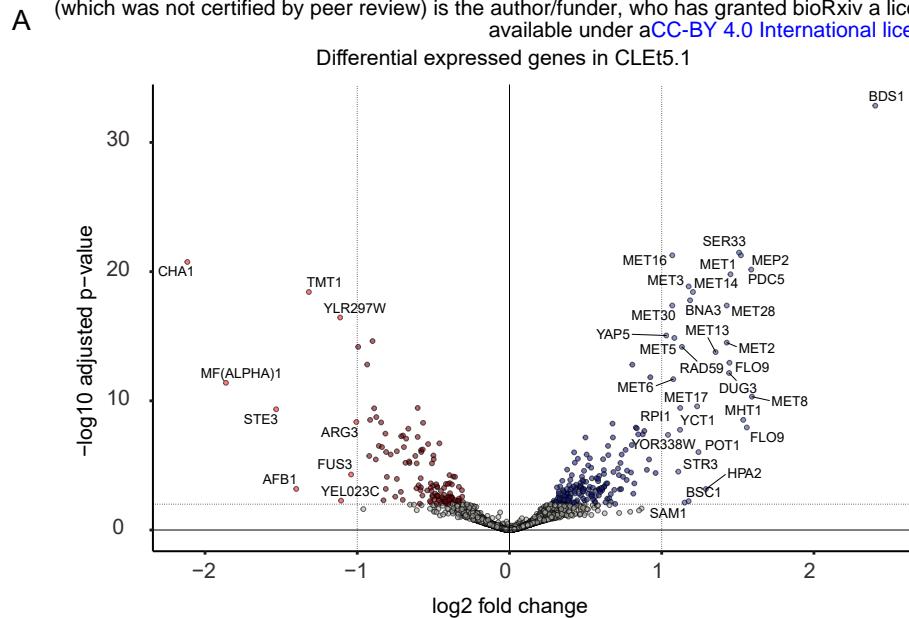


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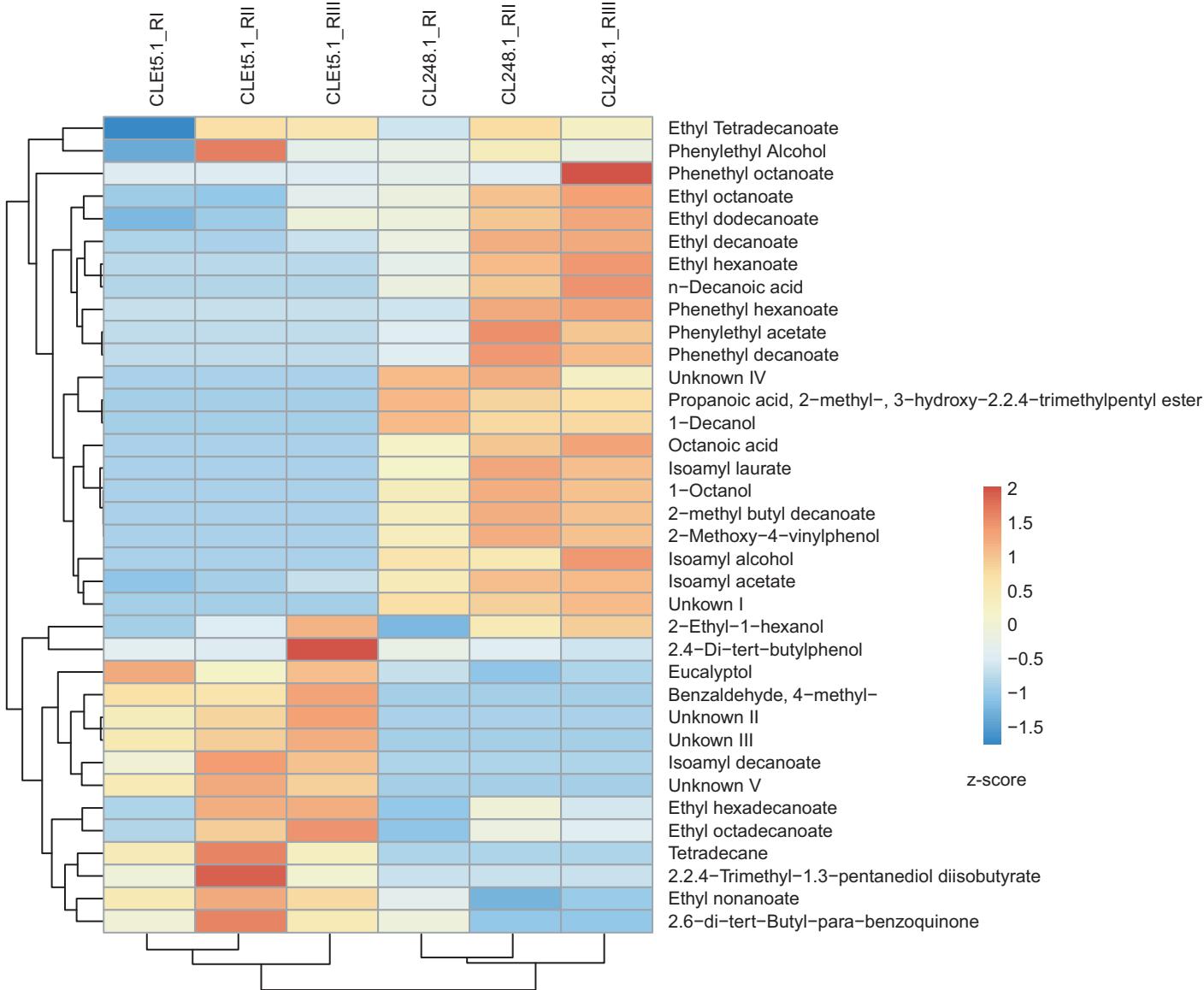


Figure 6