

22 **Abstract**

23 Ascomycota, the largest and best-studied phylum of fungi, contains three subphyla:
24 Saccharomycotina (budding yeasts), Pezizomycotina (filamentous fungi), and
25 Taphrinomycotina (fission yeasts); organisms from all three subphyla have been invaluable as
26 models in diverse fields (e.g., biotechnology, cell biology, genetics, and medicine). Despite
27 its importance, we still lack a comprehensive genome-scale phylogeny or understanding of
28 the similarities and differences in the mode of genome evolution within this phylum. To
29 address these gaps, we examined 1,107 genomes from Saccharomycotina (332),
30 Pezizomycotina (761), and Taphrinomycotina (14) species to infer the Ascomycota
31 phylogeny, estimate its timetree, and examine the evolution of key genomic properties. We
32 inferred a robust genome-wide phylogeny that resolves several contentious relationships and
33 estimated that the Ascomycota last common ancestor likely originated in the Ediacaran (~563
34 ± 68 million years ago). Comparisons of genomic properties revealed that Saccharomycotina
35 and Pezizomycotina, the two taxon-rich subphyla, differed greatly in their genome properties.
36 Saccharomycotina typically have smaller genomes, lower GC contents, lower numbers of
37 genes, and higher rates of molecular sequence evolution compared to Pezizomycotina.
38 Ancestral state reconstruction showed that the genome properties of the Saccharomycotina
39 and Pezizomycotina last common ancestors were very similar, enabling inference of the
40 direction of evolutionary change. For example, we found that a lineage-specific acceleration
41 led to a 1.6-fold higher evolutionary rate in Saccharomycotina, whereas the 10% difference in
42 GC content between Saccharomycotina and Pezizomycotina genomes stems from a trend
43 toward AT bases within budding yeasts and toward GC bases within filamentous fungi. These

44 results provide a robust evolutionary framework for understanding the diversification of the
45 largest fungal phylum.

46 **Main**

47 The fungal phylum Ascomycota is one of most diverse phyla of eukaryotes with ~65,000
48 known species that represent approximately three quarters of all known species of fungi¹. The
49 Ascomycota is divided in three subphyla. The Saccharomycotina subphylum is a lineage of
50 more than 1,000 known species and 12 major clades²; commonly referred to as budding
51 yeasts. Species in this lineage include the model organism *Saccharomyces cerevisiae*³ and
52 several notable pathogens, such as the human commensal *Candida albicans*⁴ and the
53 multidrug-resistant emerging pathogen *Candida auris*⁵. The Pezizomycotina subphylum
54 contains more than 63,000 described species in 13 classes⁶; commonly referred to as
55 filamentous fungi. This subphylum contains several major plant and animal pathogens
56 belonging to diverse genera, such as *Fusarium*, *Aspergillus*, *Zymoseptoria*, and
57 *Magnaporthe*⁷⁻¹⁰. Finally, the Taphrinomycotina subphylum contains ~140 described species
58 in 5 classes⁶; commonly referred to as fission yeasts. This subphylum contains the model
59 organism *Schizosaccharomyces pombe* and the human pathogen *Pneumocystis jirovecii*^{11,12}.
60

61 To better understand the evolution of species diversity and ecological lifestyles in
62 Ascomycota fungi, a robust framework of phylogenetic relationships and divergence time
63 estimates is essential. In the last two decades, several studies have aimed to infer the
64 Ascomycota phylogeny, either using a handful of gene markers from hundreds of taxa¹³⁻¹⁷ or
65 using hundreds of gene markers from tens of taxa¹⁸⁻²¹. To date, the most comprehensive
66 “few-markers-from-many-taxa” phylogeny used a 6-gene, 420-taxon (8 Taphrinomycotina,
67 16 Saccharomycotina, and 396 Pezizomycotina) data matrix¹³, whereas the most

68 comprehensive genome-scale phylogeny used an 238-gene, 496-taxon (12 Taphrinomycotina,
69 76 Saccharomycotina, and 408 Pezizomycotina) data matrix²² but was inferred using
70 FastTree, a program that is faster but typically yields phylogenies that have much lower
71 likelihood scores than those obtained by IQ-TREE and RAxML/RAxML-NG²³. Key
72 relationships supported by these studies include the monophyly of each subphylum and class
73 and the sister group relationship of subphyla Saccharomycotina and Pezizomycotina. In
74 contrast, relationships among classes are contentious between studies, particularly with
75 respect to relationships between the 13 classes in Pezizomycotina⁶. For example, there is
76 disagreement whether the sister group to the rest of classes in the Pezizomycotina is class
77 Pezizomycetes¹⁴, class Orbiliomycetes¹⁷, or a clade comprised of both¹⁹.

78

79 Previous molecular clock-based estimates of divergence times for Ascomycota have all been
80 based on few-markers-from-many-taxa data matrices^{14,15,24-26}, resulting in age estimates for
81 key events in Ascomycota evolution that have wide intervals. For example, analysis of a 6-
82 gene, 121-taxon (1 Saccharomycotina, 118 Pezizomycotina, and 2 Taphrinomycotina) data
83 matrix inferred that the origin of the phylum Ascomycota took place 531 million years ago
84 (mya) (95% credibility interval (CI): 671-410 mya) (see their Scenario 4 in Table 3)¹⁵, while
85 analysis of a 4-gene, 145-taxon (12 Saccharomycotina, 129 Pezizomycotina, and 4
86 Taphrinomycotina) data matrix inferred that the phylum originated 588 mya (95% CI: 773-
87 487 mya)¹⁴. More importantly, the sparser taxon sampling of previous studies has prevented
88 estimation of divergence times of several key divergence events of higher taxonomic ranks²⁴⁻

89 ²⁶ and stymied our understanding of their evolutionary pace. While these studies have
90 significantly advanced our understanding of Ascomycota evolution, a comprehensive,
91 genome-scale phylogeny and timetree stemming from the sampling of hundreds of genes
92 from thousands of taxa from the phylum are still lacking.

93

94 A robust phylogenomic framework would also facilitate comparisons of genome evolution
95 across the subphyla of Ascomycota. For example, the three subphyla differ in their genome
96 sizes, with the genomes of Pezizomycotina species being notably larger (~42 Mb) than those
97 of Saccharomycotina (~13 Mb) and Taphrinomycotina (~14 Mb)²⁷. While several recent
98 studies have analyzed major lineages within the two taxon-rich subphyla,
99 Saccharomycotina^{2,20,28} and Pezizomycotina²⁹⁻³¹, comparisons of genome evolution across
100 the two subphyla are lacking. For example, a recent analysis of the tempo and mode of
101 genome evolution in 332 Saccharomycotina found evidence of high evolutionary rates and
102 reductive evolution across this subphylum², but whether budding yeasts are faster evolving
103 than filamentous fungi remains unknown. However, a recent analysis of 71 Ascomycota
104 genomes showed that Pezizomycotina have much higher levels of gene order divergence than
105 Saccharomycotina²¹. Similarly, genome-wide examinations of horizontal gene transfer events
106 in dozens to more than a hundred Ascomycota genomes have revealed that Pezizomycotina
107 acquired significantly higher numbers of genes from prokaryotic donors than
108 Saccharomycotina^{32,33}. Although these studies have contributed to our understanding of
109 certain evolutionary processes in the phylum, we still know relatively little about the

110 evolution of Ascomycota genomes and their properties.

111

112 There are currently more than one thousand genomes from Ascomycota species that are
113 publicly available, which span the diversity of Saccharomycotina (332 genomes representing
114 all 12 major clades), Pezizomycotina (761 genomes representing 9 / 13 classes), and
115 Taphrinomycotina (14 genomes representing 4 / 5 classes) (1,107 genomes as of December
116 14, 2018). These 1,107 genomes represent a much larger and representative source of
117 genomic data across the entire Ascomycota phylum than previously available, providing a
118 unique opportunity to infer a genome-scale phylogeny and timetree for the entire subphylum
119 and compare the mode of genome evolution across its subphyla.

120

121 **Results and Discussion**

122 **A genome-scale phylogeny of the fungal phylum Ascomycota**

123 To infer a genome-scale phylogeny of Ascomycota fungi, we employed 1,107 publicly
124 available genomes from species belonging to Ascomycota (Saccharomycotina: 332;
125 Pezizomycotina: 761; Taphrinomycotina: 14) and six outgroups from the sister fungal phylum
126 Basidiomycota. All genomes were retrieved from the NCBI GenBank database, ensuring that
127 only one genome per species was included (Supplementary Tables 1 and 2). Analysis of
128 genome assembly completeness reveals that 1,021/1,113 (~92%) genomes have more than
129 90% of the 1,315 full-length BUSCO genes³⁴ (Supplementary Fig. 1).

130

131 1,315 BUSCO genes from 1,107 Ascomycota fungi and six outgroups were used to construct
132 a phylogenomic data matrix (see Methods). After constructing the multiple amino acid
133 sequence alignment and trimming ambiguous regions for each of these 1,315 BUSCO genes,
134 we kept only the 815 BUSCO genes that had taxon occupancy of $\geq 50\%$ for each subphylum
135 (i.e., ≥ 7 Taphrinomycotina, ≥ 166 Saccharomycotina, and ≥ 381 Pezizomycotina) and whose
136 amino acid sequence alignments were ≥ 300 sites in length. In the final set of 815 BUSCO
137 genes, alignment lengths range from 300 to 4,585 amino acid sites (average = 690) and
138 numbers of taxa range from 851 to 1,098 (average = 1,051) (Supplementary Table 3). The
139 final data matrix contains 1,107 taxa, 815 genes, and 562,376 amino acid sites.

140

141 Inference using concatenation- and coalescent-based approaches yielded a robust,
142 comprehensive phylogeny of the Ascomycota phylum (Fig. 1). The vast majority of
143 internodes in both the concatenation-based (1,103 / 1,110; 99%) and the coalescent-based
144 phylogeny (1,076 / 1,110; 97%) received strong ($\geq 95\%$) support and were congruent
145 between the phylogenies inferred using the two approaches; only 46 / 1,110 (4%) internodes
146 were incongruent between the two phylogenies (Supplementary Figs. 2 and 3).

147

148 Our higher-level phylogeny of Ascomycota is generally more congruent with previous
149 genome-scale phylogenies^{2,18,19,35} than with few-genes-from-many-taxa phylogenies¹³⁻¹⁶,
150 particularly with respect to relationships among the nine classes in the subphylum
151 Pezizomycotina. For example, genome-scale studies, including ours, consistently favor a

152 clade consisting of Pezizomycetes and Orbiliomycetes as the sister group to the rest of the
153 Pezizomycotina^{18,19}, while studies based on a few genes recovered either Orbiliomycetes^{15–17}
154 or Pezizomycetes¹⁴ as the sister class to the rest of the Pezizomycotina (Fig. 2a). Our
155 phylogeny also strongly supported the placement of the class Schizosaccharomycetes, which
156 includes the model organism *Schizosaccharomyces pombe*, as the sister group to the class
157 Pneumocystidomycetes, which contains the human pathogen *Pneumocystis jirovecii* (Fig.
158 2b). Interestingly, a recent genome-scale study of 84 fungal genomes showed that our result is
159 consistent with the phylogeny inferred using an alignment-free composition vector approach
160 but not with the phylogeny inferred using maximum likelihood, which instead recovered
161 Schizosaccharomycetes as the sister group to Taphrinomycetes¹⁸. Finally, both concatenation-
162 and coalescent-based approaches supported the placement of the subphylum
163 Saccharomycotina as the sister group to the subphylum Pezizomycotina (Figs. 1 and 2c). This
164 result is consistent with most previous studies that analyze multiple sequence alignment
165 data^{16,18,20,36–38}, but not with a recent study that analyzed genomic data with an alignment-free
166 method and placed the subphylum Saccharomycotina as the sister group to the subphylum
167 Taphrinomycotina³⁹.

168

169 To evaluate whether our genome-scale data matrix robustly resolved the three historically
170 contentious branches discussed in the previous paragraph, we quantified the distribution of
171 phylogenetic signal for alternative topologies of these three phylogenetic hypotheses at the
172 level of genes and sites using a maximum likelihood framework presented by Shen et al.⁴⁰.

173 First, we found that phylogenetic support for each of the three branches stemmed from many
174 genes, i.e., it was not dominated by a small number of genes with highly disproportionate
175 influence (Supplementary Table 4). Second, we found that the topology recovered by both
176 concatenation- and coalescent-based approaches in our study had significantly the highest
177 frequencies of supporting genes and supporting sites (G -test), ranging from 0.45 to 0.65, in
178 all three branches examined (Fig. 2a-c, Supplementary Tables 4 and 5). Importantly, none of
179 two alternative conflicting phylogenetic hypotheses for each of the three branches received
180 frequencies of supporting genes and supporting sites that were equal or greater than 1/3
181 (0.33), the value expected if the relationships among the taxa were represented by a
182 polytomy. The very small fraction of branches where concatenation- and coalescent-based
183 inference conflicted (<5%) and the robust support of individual genes and sites for specific
184 historically contentious branches (Fig. 2a-c) suggest that the coupling of genome-scale
185 amounts of data and comprehensive taxon sampling will provide robust resolution to major
186 lineages of the tree of life^{2,41}.

187

188 **A genome-scale evolutionary timetree of the fungal phylum Ascomycota**

189 We next used the robust phylogeny, a relaxed molecular clock approach, and six widely
190 accepted time calibration nodes (see Methods), to infer the timescale of evolution of
191 Ascomycota. We inferred the origin of the phylum to have taken place 563 million years ago
192 (mya) (95% credibility interval (CI): 631–495 mya); the origin of the subphylum
193 Saccharomycotina 438.4 mya (CI: 590–304 mya); the origin of the subphylum

194 Pezizomycotina 407.7 mya (CI: 631–405 mya); and the origin of Taphrinomycotina crown
195 group 530.5 mya (CI: 620–417 mya). Notably, the taxonomic placement of all budding yeasts
196 into a single class, Saccharomycetes, whose origin coincides with the origin of the
197 subphylum Saccharomycotina, means that the last common ancestor of this sole class of
198 budding yeasts is much more ancient than those of any of the 9 classes (based on current
199 taxon sampling) in the subphylum Pezizomycotina (Supplementary Fig. 4 and Supplementary
200 Table 6). For example, the most ancient class in Pezizomycotina is Pezizomycetes, whose
201 origin is dated 247.7 mya (CI: 475-193 mya) (Supplementary Fig. 4 and Supplementary Table
202 6). The other outlier, albeit with much larger confidence intervals, is class Neolectomycetes
203 in Taphrinomycotina, which we estimate to have originated 480.4 mya (CI: 607-191 mya)
204 (Supplementary Fig. 4 and Supplementary Table 6).

205

206 Comparison of our inferred dates of divergence to those of a recent study using a 4-gene,
207 145-taxon data matrix¹⁴ shows that our estimates are younger (563 vs 588 mya for
208 Ascomycota and 408 vs 458 mya for Pezizomycotina; sparser taxon sampling in the previous
209 study prevents comparison of dates for Saccharomycotina and Taphrinomycotina). This result
210 is consistent with findings of previous studies^{42,43}, where inclusion of large numbers of genes
211 was found to also result in younger estimates of divergence times, perhaps because of the
212 influence of larger amounts of data in decreasing the stochastic error involved in date
213 estimation. In summary, generation of a genome-scale timetree for more than 1,000
214 ascomycete species spanning the diversity of the phylum provides a robust temporal

215 framework for understanding and exploring the origin and diversity of Ascomycota

216 lifestyles⁴⁴.

217

218 **Contrasting modes of genome evolution in fungal phylum Ascomycota**

219 To begin understanding the similarities and differences in the modes of genome evolution

220 between subphyla, we focused on examining the evolution of seven different genomic

221 properties between Saccharomycotina (332 taxa) and Pezizomycotina (761 taxa), the two

222 most taxon-rich subphyla in Ascomycota (Fig. 3). Specifically, we found that

223 Saccharomycotina exhibited a 1.6-fold higher evolutionary rate (on average, 1.80

224 substitutions per site in Saccharomycotina vs. 1.12 substitutions per site in Pezizomycotina),

225 1.24-fold lower GC content (40% vs. 50%), 3-fold smaller genome size (13 Mb vs. 39 Mb),

226 1.9-fold lower number of protein-coding genes (5,734 vs. 10,847), 1.3-fold lower number of

227 DNA repair genes (41 vs. 54), 1.2-fold higher number of tRNA genes (179 vs. 146), and 1.3-

228 fold smaller estimates of non-synonymous to synonymous substitution rate ratio (d_N/d_S)

229 (0.053 vs. 0.063), compared to Pezizomycotina (Fig. 3a, Table 1, and Supplementary Table

230 7).

231

232 Analysis of standard Pearson's correlations among the seven genomic properties revealed that

233 two pairs exhibited statistically significant contrasting patterns between Saccharomycotina

234 and Pezizomycotina. Specifically, evolutionary rate shows negative correlation with GC

235 content in Saccharomycotina but positive correlation in Pezizomycotina and GC content

236 shows negative correlation with number of DNA repair genes in Saccharomycotina but
237 positive correlation in Pezizomycotina (Fig. 3b). These correlations are largely consistent
238 before (i.e., standard Pearson's correlations) and after (i.e., phylogenetically independent
239 contrasts) accounting for correlations due to phylogeny (Supplementary Table 8).

240

241 For each of the seven properties, we used our genome-scale phylogeny (Fig. 1) to infer the
242 ancestral character states and reconstruct their evolution in the Saccharomycotina ancestor
243 and the Pezizomycotina ancestor. Comparison of ancestral states along branches on the
244 Saccharomycotina part of the phylogeny to those on the Pezizomycotina part of the
245 phylogeny shown that all genomic properties, except the number of tRNA genes, exhibited
246 different modes of evolution (Fig. 4 and Table 1). For example, most Saccharomycotina
247 branches exhibit evolutionary rates of at least 1.0 amino acid substitutions / site, whereas
248 those of Pezizomycotina exhibit evolutionary rates between 0.7 and 1.4 substitutions / site
249 (Fig. 4a). However, the inferred values for these properties in the Saccharomycotina last
250 common ancestor and in the Pezizomycotina last common ancestor nodes are quite similar.
251 For example, the inferred state values for the Saccharomycotina last common ancestor and
252 the Pezizomycotina last common ancestor are 1.1 and 0.9 substitutions / site for evolutionary
253 rate and 43% and 47% for GC content (Table 1), respectively. Interestingly, the same trends
254 are also observed across lineages, such as Lipomycetaceae, which is the sister group to the
255 rest of the Saccharomycotina, and the clade consisting of Pezizomycetes and Orbiliomycetes,
256 which is the sister group to the rest of the Pezizomycotina (Fig. 4a and b).

257

258 Comparison of the trait values for the seven genome properties between extant
259 Saccharomycotina and Pezizomycotina branches to those of the Saccharomycotina and
260 Pezizomycotina last common ancestors showed that evolutionary rate, GC content, genome
261 size, and number of protein-coding genes were the properties with the highest amounts of
262 evolutionary change (Figs. 3 and 4, Table 1). Ancestral state reconstruction also enabled
263 inference of the direction of evolutionary change for each of the evolutionary properties. For
264 example, the Saccharomycotina and Pezizomycotina last common ancestors, as well as
265 branches in Lipomycetaceae and branches across Pezizomycotina, exhibit similar
266 evolutionary rates, whereas the rest of the nodes and branches in the Saccharomycotina part
267 of the phylogeny exhibit much higher evolutionary rates. This pattern suggests that the higher
268 levels of genomic diversity in Saccharomycotina stem from an acceleration of evolutionary
269 rate that occurred within the subphylum, after the divergence of Lipomycetaceae from the
270 rest of the Saccharomycotina (Fig. 4a and b).

271

272 Why do Saccharomycotina exhibit higher evolutionary rates compared to Pezizomycotina?
273 Studies in other lineages, such as vertebrate⁴⁵ and invertebrate⁴⁶ animals, have previously
274 shown that evolutionary rate is positively associated with generation time. Assuming that
275 mutation rates are equal, species with shorter generation times will replicate their genomes
276 more frequently, accruing more mutations per unit time. While the generation times of most
277 fungi in our phylogeny are unknown, the generation times of model organisms in

278 Saccharomycotina are thought to be shorter than those in Pezizomycotina. For example, the
279 doubling time of the budding yeasts *S. cerevisiae* and *C. albicans* under optimal conditions is
280 90 min^{47,48}, while that of the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* is
281 between 2-3 hours^{49,50}. An alternative but not mutually exclusive explanation may be that
282 Saccharomycotina have, on average, 13 fewer DNA repair genes (41) than Pezizomycotina
283 (54) (Fig. 3 and Table 1), since it is well established that absence or loss of DNA repair genes
284 increase mutation rates⁵¹⁻⁵³. The lower numbers of DNA repair genes in budding yeasts, but
285 not their higher evolutionary rate, was also recently reported in a recent analysis of 328
286 ascomycete proteomes by Milo et al.⁵⁴. Finally, other life-history traits (e.g., smaller cell size,
287 faster metabolism, and larger population size) that have been associated with variation in the
288 rate of molecular evolution⁵⁵ might also contribute to higher evolutionary rates of the
289 Saccharomycotina.

290

291 Variation in genomic GC content has historically been of broad interest in biology⁵⁶. Average
292 GC content values of different genomic regions (e.g., intergenic regions, protein-coding
293 regions) in Saccharomycotina are consistently lower than those in Pezizomycotina
294 (Supplementary Fig. 5). Similarly, gene-wise average estimates of GC content showed that all
295 815 BUSCO genes in Saccharomycotina have lower GC content values than those in
296 Pezizomycotina (Supplementary Fig. 6). Moreover, we found that the frequencies of amino
297 acids encoded by GC-rich codons in Saccharomycotina are much lower than those of amino
298 acids encoded by GC-rich codons in Pezizomycotina (Supplementary Fig. 7). Ancestral state

299 reconstruction of genomic GC content along branches on the phylogeny shows that the
300 Saccharomycotina and Pezizomycotina last common ancestors, as well as branches in
301 Lipomycetaceae and branches in classes Pezizomycetes and Orbiliomycetes, exhibit
302 intermediate GC content around 45%. In contrast, GC content of most branches within the
303 rest of Saccharomycotina (i.e., all major clades of Saccharomycotina, including extant taxa,
304 except Lipomycetaceae) evolved toward 40%, while GC content within the rest of
305 Pezizomycotina (i.e., all classes, including extant taxa, except Pezizomycetes and
306 Orbiliomycetes) evolved toward 50%. This pattern suggests that the evolution of lower levels
307 of GC content in Saccharomycotina occurred after the divergence of Lipomycetaceae from
308 the rest of Saccharomycotina and that the evolution of higher levels of GC content in
309 Pezizomycotina occurred after the divergence of the clade consisting of Pezizomycetes and
310 Orbiliomycetes from the rest of Pezizomycotina (Fig. 4a and b).

311

312 Why are Pezizomycotina genomes more GC-rich compared to Saccharomycotina genomes?
313 There are two possible explanations. The first one is that mutational biases have skewed the
314 composition of Saccharomycotina genomes toward AT content⁵⁷. For example, Steenwyk et
315 al. showed that *Hanseniaspora* budding yeasts with higher AT content lost a greater number
316 of DNA repair genes than those with lower AT content⁵³, suggesting that the loss of DNA
317 repair genes is associated with AT richness. Consistent with these results, we found that
318 Pezizomycotina genomes contain a higher number of DNA repair genes than
319 Saccharomycotina (Fig. 3 and Table 1). The second potential, not necessarily mutually

320 exclusive, explanation is that mutational biases have skewed Pezizomycotina genomes
321 toward GC richness. It was recently shown that increasing GC-biased gene conversion
322 (gBGC), a process associated with recombination that favors the transmission of GC alleles
323 over AT alleles⁵⁸, can result in a systematic underestimate of d_N/d_S in birds⁵⁹. If this is true for
324 Ascomycota, due to the higher GC content of Pezizomycotina genomes, we would expect
325 that their d_N/d_S would be underestimated due to the higher levels of gBGC compared to
326 Saccharomycotina. Consistent with this expectation, by calculating differences in d_N/d_S
327 before and after accounting for gBGC across 815 codon-based BUSCO genes, we found that
328 the underestimate of d_N/d_S in Pezizomycotina is 2-fold higher than that in Saccharomycotina
329 (Pezizomycotina: average of differences in $d_N/d_S = 0.004$; Saccharomycotina: average of
330 differences in $d_N/d_S = 0.002$) (Supplementary Fig. 8).

331

332 **Concluding Remarks**

333 In this study, we took advantage of the recent availability of the genome sequences of 1,107
334 Ascomycota species from Saccharomycotina (332), Pezizomycotina (761), and
335 Taphrinomycotina (14) to infer a genome-scale phylogeny and timetree for the entire phylum
336 and compare the mode of genome evolution across its subphyla. Leveraging genome-scale
337 amounts of data from the most comprehensive taxon set to date enabled us to test the
338 robustness of our inference for several contentious branches, potentially resolving
339 controversies surrounding key higher-level relationships within the Ascomycota phylum. For
340 example, our study robustly supported Saccharomycotina as the sister group to

341 Pezizomycotina and a clade comprised of classes Pezizomycetes and Orbiliomycetes as the
342 sister group to the rest of the Pezizomycotina. Our first genome-scale timetree suggests the
343 last common ancestor of Ascomycota likely originated in the Ediacaran period. Examination
344 of mode of genome evolution revealed that Saccharomycotina, which contains the single
345 currently described class Saccharomycetes, and Pezizomycotina, which contains 13 classes,
346 exhibited greatly contrasting evolutionary processes for seven genomic properties, in
347 particular for evolutionary rate, GC content, and genome size. Our results provide a robust
348 evolutionary framework for understanding the diversification of the largest fungal phylum.
349

350 **Methods**

351 **Data collection**

352 To collect the greatest possible set of genome representatives of the phylum Ascomycota as
353 of 14 December, 2018, we first retrieved the 332 publicly available Saccharomycotina yeast
354 genomes (<https://doi.org/10.6084/m9.figshare.5854692>) from a recent comprehensive
355 genomic study of the Saccharomycotina yeasts². We then used “Pezizomycotina” and
356 “Taphrinomycotina” as search terms in NCBI’s Genome Browser
357 (<https://www.ncbi.nlm.nih.gov/genome/browse#!/eukaryotes/Ascomycota>) to obtain the basic
358 information of strain name, assembly accession number, assembly release date, assembly
359 level (e.g., contig, scaffold, etc.), and GenBank FTP access number for draft genomes from
360 the subphyla Pezizomycotina and Taphrinomycotina, respectively. For species with multiple
361 isolates sequenced, we only included the genome of the isolate with the highest assembly
362 level and the latest release date. We next downloaded genome assemblies from GenBank data
363 via FTP access number (<ftp://ftp.ncbi.nlm.nih.gov/genomes/>). Collectively, we included 332
364 species representing all 12 major clades of the subphylum Saccharomycotina², 761 species
365 representing 9 / 13 classes of the subphylum Pezizomycotina^{1,6}, and 14 species representing 4
366 / 5 classes of the subphylum Taphrinomycotina^{1,6}. Finally, we used the genomes of six
367 representatives of the phylum Basidiomycota as outgroups. Detailed information of
368 taxonomy and source of the 1,113 genomes in our study is provided in Supplementary Tables
369 1 and 2.

370

371 **Assessment of genome assemblies and phylogenomic data matrix construction**

372 To assess the quality of each of the 1,113 genome assemblies, we used the Benchmarking
373 Universal Single-Copy Orthologs (BUSCO), version 3.0.2³⁴. Each assembly's completeness
374 was assessed based on the presence / absence of a set of 1,315 predefined orthologs (referred
375 to as BUSCO genes) from 75 genomes in the OrthoDB Version 9 database⁶⁰ from the
376 Ascomycota database, as described previously^{28,61}. In brief, for each BUSCO gene, its
377 consensus orthologous protein sequence among the 75 reference genomes was used as query
378 in a tBLASTn search against each genome to identify up to three putative genomic regions,
379 and the gene structure of each putative genomic region was predicted by AUGUSTUS v
380 3.2.2⁶². Next, the sequences of these predicted genes were aligned to the HMM-profile of the
381 BUSCO gene. BUSCO genes in a given genome assembly were considered as single-copy,
382 "full-length" if there was only one complete predicted gene present in the genome,
383 duplicated, "full-length" if there were two or more complete predicted genes present in the
384 genome, "fragmented" if the predicted gene was shorter than 95% of the aligned sequence
385 lengths from the 75 reference species, and "missing" if there was no predicted gene present in
386 the genome.

387

388 To construct the phylogenomic data matrix, we started with the set of 1,315 single-copy, full-
389 length BUSCO genes from 1,107 representatives of the phylum Ascomycota and six
390 outgroups. For each BUSCO gene, we first translated nucleotide sequences into amino acid
391 sequences, taking into account the different usage of the CUG codon in Saccharomycotina^{2,63}.

392 Next, we aligned the amino acid sequences using MAFFT v7.299b⁶⁴ with the options “--
393 thread 4 --auto --maxiterate 1000” and trimmed amino acid alignments using the trimAl
394 v1.4.rev15⁶⁵ with the options “-gappyout -colnumbering”. We mapped the nucleotide
395 sequences on the trimmed amino acid alignment based on the column numbers in the original
396 alignment and to generate the trimmed codon-based nucleotide alignment. Finally, we
397 removed BUSCO gene alignments whose taxon occupancy (i.e., percentage of taxa whose
398 sequences were present in the trimmed amino acid alignment) was < 50% for each
399 subphylum (i.e., < 7 Taphrinomycotina, < 166 Saccharomycotina, and < 381 Pezizomycotina)
400 or whose trimmed alignment length was < 300 amino acid sites. These filters resulted in the
401 retention of 815 BUSCO gene alignments, each of which had $\geq 50\%$ taxon occupancy for
402 each subphylum and alignment length ≥ 300 amino acid sites.

403

404 **Phylogenetic analysis**

405 For each of 815 BUSCO genes, we first inferred its best-fitting amino acid substitution model
406 using IQ-TREE multi-thread version 1.6.8⁶⁶ with options “-m TEST -mrate G4” with the
407 Bayesian information criterion (BIC). We then inferred best-scoring maximum likelihood
408 (ML) gene tree under 10 independent tree searches using IQ-TREE. The detailed parameters
409 for running each gene were kept in log files (see the Figshare repository). We inferred the
410 concatenation-based ML tree using IQ-TREE on a single node with 32 logical cores under a
411 single “LG +G4” model with the options “-seed 668688 -nt 32 -mem 220G -m LG+G4 -bb
412 1000”, as 404 out of 815 genes favored “LG +G4”^{67,68} as best-fitting model (see
413 Supplementary Table 3). We also inferred the coalescent-based species phylogeny with

414 ASTRAL-III version 4.10.2^{69,70} using the set of 815 individual ML gene trees. The reliability
415 of each internal branch was evaluated using 1,000 ultrafast bootstrap replicates⁷¹ and local
416 posterior probability⁷², in the concatenation- and coalescence-based species trees,
417 respectively. We visualized phylogenetic trees using the R package *ggtree* v1.10.5⁷³.
418
419 We used the non-Bayesian RelTime method, as implemented in the command line version of
420 MEGA7⁷⁴ to estimate divergence times. The very large size of our data matrix, both in terms
421 of genes as well as in terms of taxa, prohibited the use of computationally much more
422 demanding methods, such as the Bayesian MCMCTree method^{75,76}. The concatenation-based
423 ML tree with branch lengths was used as the input tree. Six time calibration nodes, which
424 were retrieved from the TimeTree database⁷⁷, were used for molecular dating analyses: the
425 *Saccharomyces cerevisiae* – *Saccharomyces uvarum* split (14.3 mya – 17.94 mya), the
426 *Saccharomyces cerevisiae* - *Kluyveromyces lactis* split (103 mya – 126 mya), the
427 *Saccharomyces cerevisiae* - *Candida albicans* split (161 mya – 447 mya), the origin of the
428 subphylum Saccharomycotina (304 mya – 590 mya), the *Saccharomyces cerevisiae* –
429 *Saitoella complicata* split (444 mya – 631 mya), and the origin of the subphylum
430 Pezizomycotina (at least 400 mya) based on the *Paleopyrenomycites devonicus* fossil⁷⁸.

431

432 **Examination of seven genome properties**

433 As the subphylum Taphrinomycotina (No. species = 14) has a much smaller number of
434 species than the subphylum Saccharomycotina (No. species = 332) and the subphylum
435 Pezizomycotina (No. species = 761) in our dataset, we focused our analyses on the

436 comparisons of seven genome properties (evolutionary rate, GC content, genome size,
437 number of genes, number of DNA repair genes, number of tRNA genes, and d_N/d_S) between
438 Saccharomycotina and Pezizomycotina. Specifically, for a given taxon, 1) evolutionary rate is
439 a sum of path distances from the most common ancestor of the subphyla Saccharomycotina
440 and Pezizomycotina to its tip on the concatenation-based ML tree (Fig. 1); 2) GC content is
441 the percentage of guanine-cytosine nucleotides in genome; 3) genome size is the total number
442 of base pairs in genome in megabases (Mb); 4) number of genes is the number of protein-
443 coding genes in genome. The gene structure was predicted with AUGUSTUS v3.3.1⁷⁹ on
444 *Aspergillus fumigatus* and *Saccharomyces cerevisiae* S288C trained models for
445 Pezizomycotina and Saccharomycotina, respectively; 5) number of DNA repair genes was
446 estimated by counting the number of unique protein-coding genes with GO terms related to
447 DNA repair using InterProScan version 5⁸⁰; 6) number of tRNA genes is the number of tRNA
448 genes inferred to be present using the tRNAscan-SE 2.0 program⁸¹; and 7) d_N/d_S was
449 estimated by calculating the average of the ratio of the expected numbers of non-synonymous
450 (d_N) and synonymous substitutions (d_S) across 815 trimmed codon-based BUSCO gene
451 alignments under the YN98 (F3X4)⁸² codon model and the free ratio model using bppml and
452 MapNH in the bio++ libraries⁸³, following the study by Bolívar et al.⁵⁹.

453

454 **Statistical analyses**

455 All statistical analyses were performed in R v. 3.4.2 (R core team 2017). Pearson's correlation
456 coefficient was used to test for correlations among seven variables. To account for phylogenetic
457 relationships of species in correlation analysis, we used the R package ape v5.1⁸⁴ in order to

458 compute phylogenetically independent contrasts following the method described by

459 Felsenstein⁸⁵.

460

461 **Ancestral state reconstruction**

462 To reconstruct ancestral character states for each of seven continuous properties, we used the

463 R package phytools v0.6.44 function *contMap*⁸⁶ to infer ancestral character states across

464 internal nodes using the maximum likelihood method with the function *fastAnc* and to

465 interpolate the states along each edge using equation [2] of Felsenstein⁸⁵. The input tree was

466 derived from the concatenation-based ML with branch lengths, which was then pruned to

467 keep the 1,093 taxa from the subphyla Pezizomycotina and Saccharomycotina.

468

469 **Data availability**

470 All genome assemblies and proteomes are publicly available in the Zenodo repository:

471 <https://doi.org/10.5281/zenodo.3783970>. Multiple sequence alignments, phylogenetic trees,

472 trait ancestral character state reconstructions, log files, R codes, and custom Perl scripts are

473 available on the figshare repository (<https://doi.org/10.6084/m9.figshare.12196149>;

474 <https://figshare.com/articles/>

475 [Phylogenomics_and_contrasting_modes_of_genome_evolution_in_Ascomycota/12196149](https://doi.org/10.6084/m9.figshare.12196149) –

476 please note that this link will become active upon publication).

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704 Study conception and design: X.X.S., C.T.H., A.R.; Acquisition of data: X.X.S.; Analysis

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707

708 **Competing interests**

709 The authors declare no competing financial interests.

710 **Figure Legends**

711 **Fig. 1 Maximum likelihood (ML) phylogeny of 1,107 taxa in the fungal phylum**

712 **Ascomycota.** The concatenation-based ML phylogeny ($\ln L = -269043834.145$) was inferred
713 from a set of 815 BUSCO amino acid genes (total 56, 2376 sites) under a single LG + G4
714 substitution model using IQ-TREE multicore version 1.5.1. The number of species sampled
715 in each subphylum is given in parentheses. Internal branch labels are acronyms for 12 major
716 clades in the subphylum Saccharomycotina and 9 classes in the subphylum Pezizomycotina.
717 The bar next to each species indicates the guanine-cytosine (GC) content. On average,
718 lineages in the subphylum Saccharomycotina have significantly lower GC content (49.6% vs.
719 40.6%; Wilcoxon rank-sum test; P -value = 3.07×10^{-103}) but higher evolutionary rate (1.80
720 substitutions per site vs. 1.12 substitutions per site; Wilcoxon rank-sum test; P -value = $6.57 \times$
721 10^{-126}) compared to lineages in the subphylum Pezizomycotina. The complete phylogenetic
722 relationships of 1,107 taxa are given in Supplementary Fig. 2 and in the Figshare repository.
723 For easy determination of the relationships among any subset of taxa, the phylogeny is also
724 available through Treehouse⁸⁷.

725

726 **Fig. 2 Distribution of phylogenetic signal for three historically contentious relationships**

727 **within Ascomycota.** For each relationship / internal branch (**a**: which class(es) is the sister
728 group to the rest of the Pezizomycotina?; **b**: what is the relationship among three classes
729 Schizosaccharomycetes, Pneumocystidomycetes, and Taphrinomycetes in the subphylum
730 Taphrinomycotina?; **c**: what is the relationship among three subphyla Pezizomycotina,
731 Saccharomycotina, and Taphrinomycotina in the phylum Ascomycota?), we applied the

732 framework presented by Shen et al.⁴⁰ to examine proportions of genes (left panel) and sites
733 (right panel) supporting each of three competing hypotheses (topology 1 or T1 in red,
734 topology 2 or T2 in green, and topology 3 or T3 in yellow). Note that both concatenation- and
735 coalescent-based approaches supported T1 in our study. Dashed horizontal lines on 1/3 y-axis
736 value denote expectation of proportion of genes / sites under a polytomy scenario. The *G*-test
737 was used to test if the sets of three values are significantly different (***: P -value ≤ 0.001).
738 All values are given in Supplementary Tables 4 and 5. Input and output files associated with
739 phylogenetic signal estimation are also deposited in the Figshare repository.

740

741 **Fig. 3 Contrasting patterns for seven genomic properties between Pezizomycotina and**

742 **Saccharomycotina. a**, For each species in Pezizomycotina (colored in red, $n=761$) and
743 Saccharomycotina (colored in green, $n=332$), we calculated evolutionary rate, GC content,
744 genome size, number of protein-coding genes, number of DNA repair genes, number of tRNA
745 genes, and d_N/d_S (see the Methods section for details). The Wilcoxon rank-sum test was used
746 to test if the sets of values in two subphyla are significantly different. **b**, Pairwise standard
747 Pearson's correlation coefficient among pairs of the seven genomic properties were
748 conducted using R 3.4.2 for Pezizomycotina (lower diagonal) and Saccharomycotina (upper
749 diagonal), respectively. For each cell, the top value corresponds to P -value (NS: P -
750 value >0.05 ; *: P -value ≤ 0.05 ; **: P -value ≤ 0.01 ; ***: P -value ≤ 0.001), whereas the
751 bottom value corresponds to Pearson's coefficient value. Orange cells denote instances where
752 correlation trends in Pezizomycotina and Saccharomycotina are in opposite directions,

753 whereas blue cells denote instances where the trends are in the same direction. The detailed
754 values of all seven properties in Pezizomycotina and Saccharomycotina are given in
755 Supplementary Table 7. The correlations among these seven properties are largely consistent
756 before (i.e., standard Pearson's correlations) and after (i.e., phylogenetically independent
757 contrasts) accounting for correlations due to phylogeny (see Supplementary Table 8).

758

759

760 **Fig. 4 Contrasting modes of genome evolution in Pezizomycotina and**

761 **Saccharomycotina. a**, For each of the seven genomic properties examined (see the Methods
762 section for details) , we reconstructed them as continuous traits on the species phylogeny
763 (Fig. 1) and visualized their ancestral states with the R package phytools v0.6.44⁸⁶. Heatmap
764 bars denote ancestral state values from small (blue) to large (red). Three ancestral state values
765 next to three red dots are shown for the ancestor of the subphyla Pezizomycotina and
766 Saccharomycotina, the ancestor of the subphylum Pezizomycotina, and the ancestor of the
767 subphylum Saccharomycotina, respectively. **b**, Phylogeny key showing the placement of the
768 21 nodes representing the last common ancestors of the 12 major clades in the subphylum
769 Saccharomycotina and of the 9 classes in the subphylum Pezizomycotina; the 21 nodes are
770 indicated by the red dots. The orders of branches in **a** are identical to those in **b**.

771

Table 1. Summary of values for seven genomic properties in extant Saccharomycotina and Pezizomycotina and in the last common ancestors of Saccharomycotina and Pezizomycotina.

Property	Extant Saccharomycotina* (n=332)	Extant Pezizomycotina* (n=761)	Saccharomycotina ancestor	Pezizomycotina ancestor	Difference between two extant lineages	Difference between two ancestors
Evolutionary rate (amino acid substitutions/ site)	1.80	1.12	1.1	0.9	0.68	0.2
GC content (%)	40	50	43	47	10	4
Genome size (Mb)	13	39	23	42	26	19
No. of genes	5,734	10,847	7,000	9,400	5,113	2,400
No. of DNA repair genes	41	54	44	52	13	8
No. of tRNA genes	179	146	160	170	33	10
d_N/d_S	0.053	0.063	0.052	0.058	0.01	0.006

* denote average values.

Figure 1

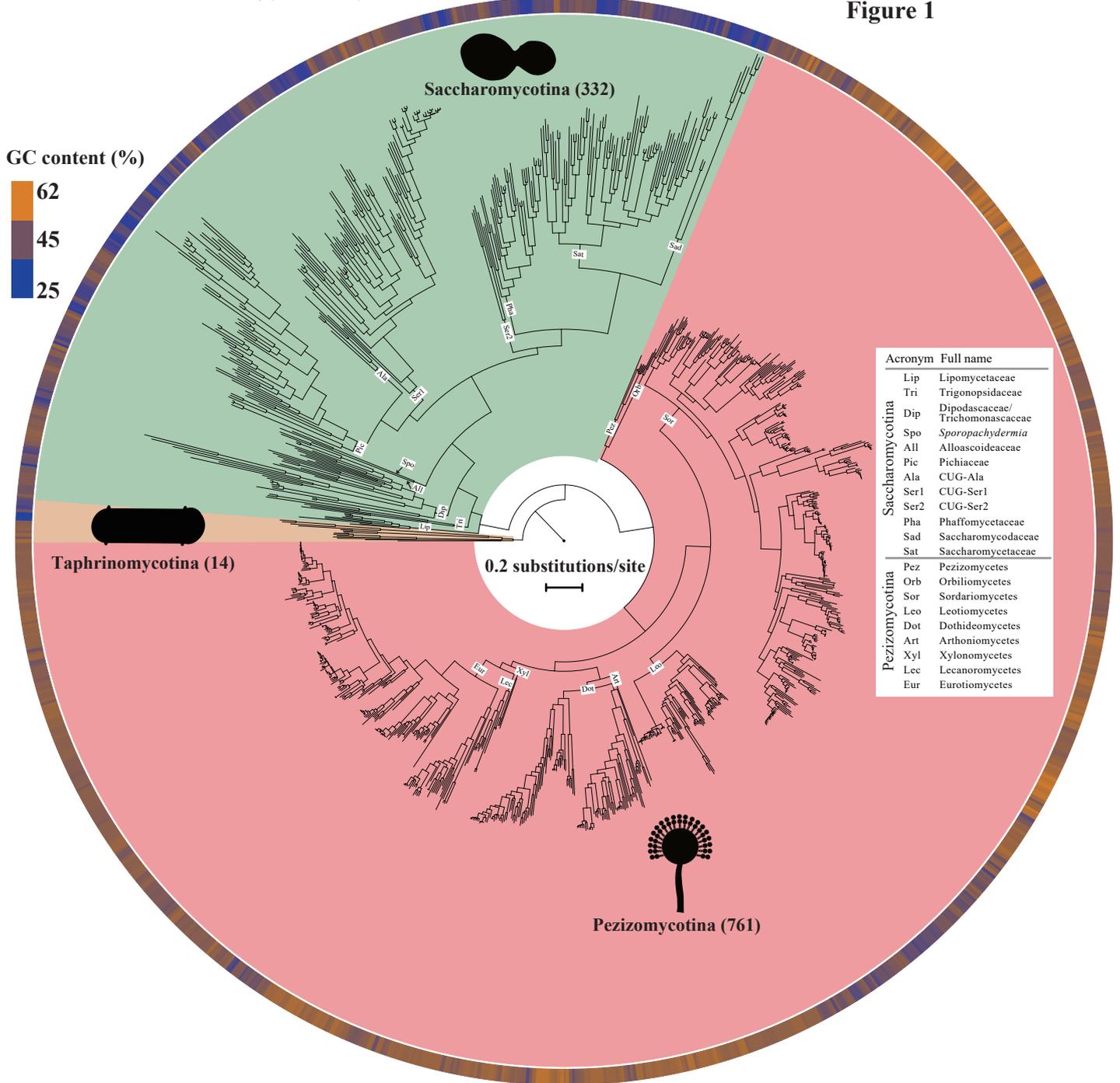


Figure 2

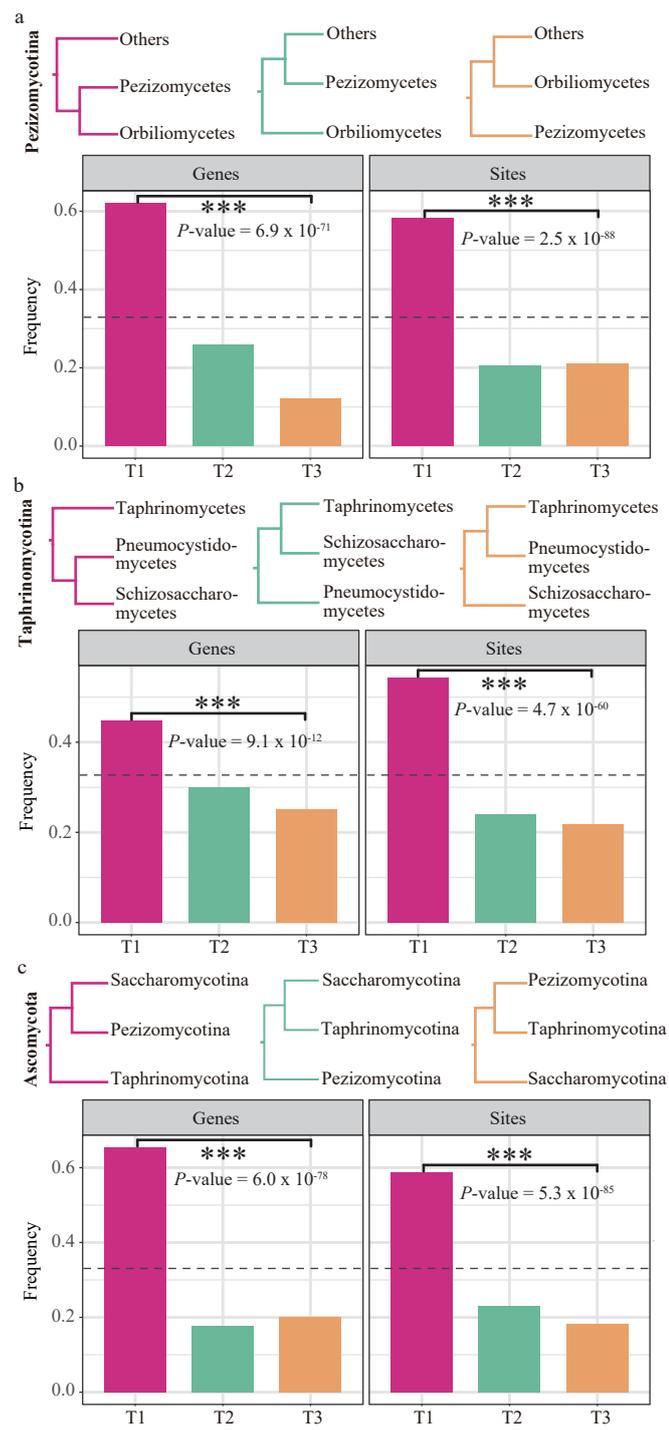


Figure 3

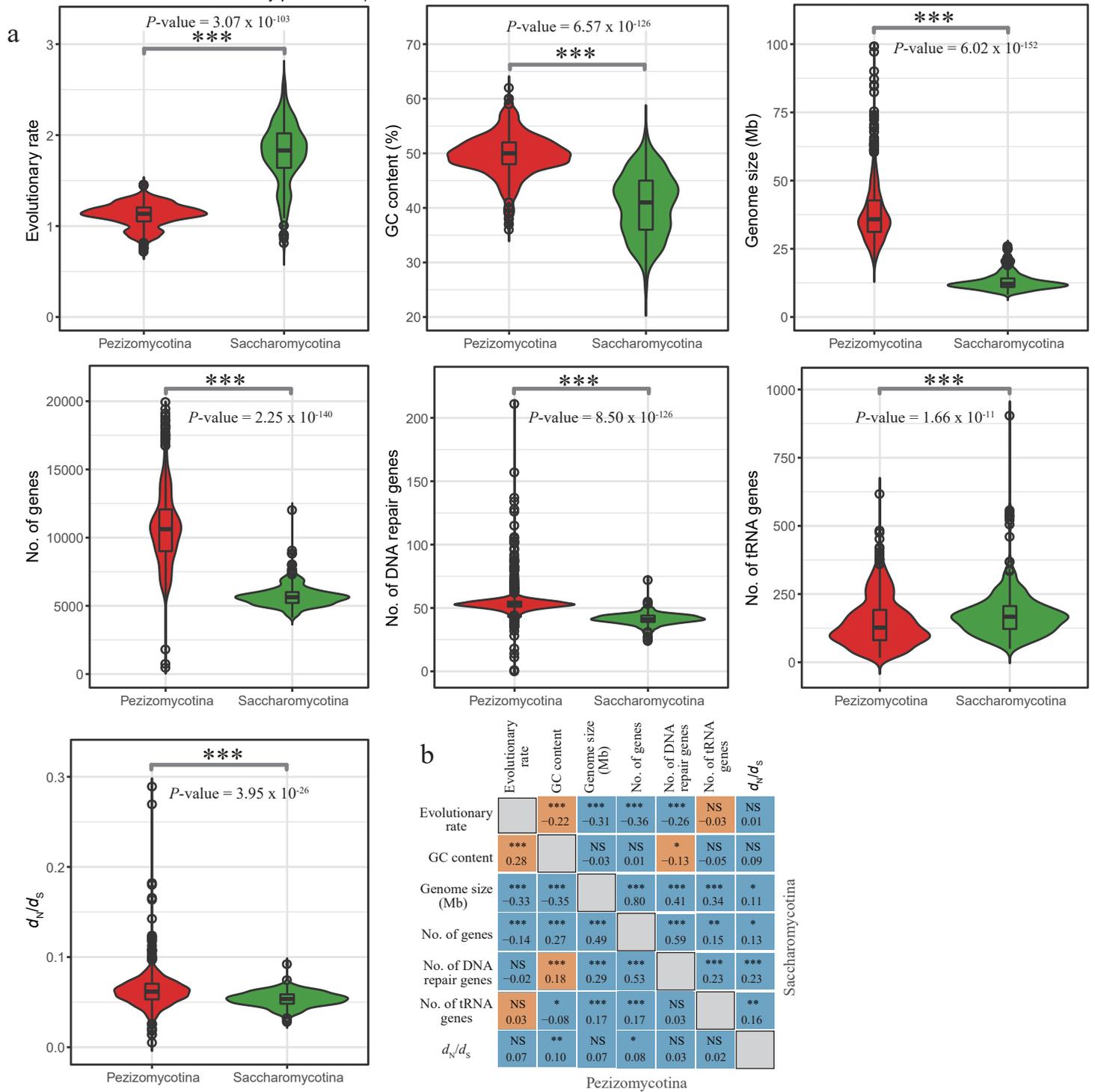


Figure 4

