Temporal and spatial dynamics within the fungal microbiome of grape fermentation

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Abstract

Wine fermentation is a highly complex and competitive environment, imposing harsh selective pressures on fungal community ecology and diversity. The composition of fungal communities inhabiting the surface of grapes will directly impact fermentation progression, wine quality, and contribute to the distinctiveness between wines from different geographical regions. Despite this, the extent of microbial community diversity between geographies, termed 'microbial terroir', remains highly debated. We amassed a large survey of grape spontaneous ferments over six years, encompassing 3105 fungal microbiomes across 14 geographically separated grape-growing regions, and nine grape cultivars. Investigation into the biodiversity of these ferments identified that few high abundance genera form the core of the initial grape microbiome and can be partitioned into three clear community types that do not correlate with geographic region. Analysing ferments across temporal and spatial scales revealed significant differences in species richness and compositional heterogeneity between wineries and grape growing regions. However, microbial communities were transient between years in the same winery, regularly transitioning between the three broad community types. We then investigated microbial community composition throughout the fermentative process and observed that initial microbial community composition is predictive of the diversity during the early stages of fermentation, with *Hanseniaspora uvarum* detected as the main non-*Saccharomyces* species within this large cohort of samples. Our results help to formulate a clear understanding of the spatial and temporal characteristics of the grape spontaneous ferment niche and suggest that geography does not play a major role in community composition on a larger ecological scale.

Introduction

The surfaces of vines and grapes harbor a naturally occurring, intricate consortium of fungal and bacterial species, which exert a direct influence on vine growth and overall health [1-3]. These communities can vary significantly in their composition and may be affected by numerous factors, including grape variety, geographical location, climate and viticultural practices [4-6].

These microorganisms are transferred into the grape must during harvesting and processing, where they can play a pivotal role in shaping fermentation dynamics and contributing to the sensory attributes of the resulting wine. This influence is particularly significant during traditional uninoculated ("wild") fermentation, where the absence of commercial yeast strains allows specific members of the microbial community to directly impact the fermentation process. Under this production framework, a complex microbial succession of yeasts and yeast-like fungi is generally observed as the grape must ferments. Initially, a diverse mixture of aerobic and apiculate yeasts,

which normally reside on the surface of intact grape berries, dominate the fresh grape must. However, most of these species succumb to the decreasing oxygen levels and increasing ethanol concentrations, allowing mildly fermentative species to transiently increase in abundance [7-11]. Ultimately however, due to a higher fermentative ability, growth rate and tolerance to ethanol, most wild wine ferments converge to near monocultures of the major wine yeast, *Saccharomyces cerevisiae*. This is despite almost undetectable levels of this species being present on intact grape berries [12, 13].

While traditional microbiological techniques have provided important insights into the microbial succession that occurs in spontaneous ferments, both the breadth of ferments investigated and the depth at which individual species contributions could be resolved has been limited. Recent advances in culture-independent methods for species analysis, such as shotgun metagenomics and amplicon-based ITS phylotyping provides a high-throughput means to analyse large numbers of microbiological samples in great detail [14, 15].

Phylotyping techniques have been employed to investigate grape and fermentation microbiomes, aiming to uncover whether regional variations in wines can be partially attributed to the distinctiveness of microbial communities, supporting the notion of a "microbiome terroir". While studies have revealed geographic patterns and considerable variation in microbial community composition among specific locations [4, 5, 16, 17], the extent to which these biogeographic patterns persist on a broader ecological scale remains uncertain. To address this, an extensive survey of grape juice samples was carried out to assess the microbial diversity inherent across a large cohort of uninoculated fermentations within Australia, that extended across nine grape varieties and 14 wine regions over the six successive vintages from 2016 to 2021. This comprehensive dataset enabled a thorough exploration of the core microbial communities in grape must and fermentation at a large ecological scale, thereby enhancing our understanding of the fungal species associated with grapes and the fermentation process.

Materials and Methods

Sample collection

A total of 36 wineries were sampled during six years across 14 different wine growing regions covering six states of Australia (Fig. 1a, Table S1). Wineries were supplied with pre-labelled, 50 mL falcon tubes. Approximately 40 mL of grape must was collected at four timepoints per fermentation tank, based upon refractometry levels (T1, freshly pressed grape juice; T2, after a 1 Bé drop in sugar; T3, 50% sugar remaining; T4, 10% sugar remaining) (Fig. 1b). Samples were stored frozen until collection and processing. All samples were obtained from strictly un-inoculated spontaneous ferments.

Sample processing and ITS sequencing

Samples were thawed, homogenized, and 1.9 mL of sample was centrifuged and washed twice with 1 mL of 1X PBS. DNA was extracted from pellets using the DNeasy PowerFood Microbial DNA Isolation Kit (Qiagen) following the manufacturer's instructions. Bead-beating was carried out using a combination of 0.1 mm and 0.5 mm zirconia/silica beads (BioSpec Products) in a Precellys Evolution homogenizer (Bertin Instruments) at 8,000 RPM for 4 x 60 sec.

A two-step PCR was performed using 1 ng of DNA with primer sequences designed to amplify the fungal ITS region, while adding experiment-specific inline barcodes and appropriate adaptors for the

Illumina sequencing platform as previously described by Sternes, Lee [14]. The first-round of amplification (20–30 cycles, 55°C annealing, 30-second extension, KAPA 2G Robust polymerase) targeting the ITS region was performed using primers BITS (ACCTGCGGARGGATCA) and B58S3 (GAGATCCRTTGYTRAAAGTT) [18]. 2 uL of first-round PCR product was used in the second amplification (15 cycles, 55°C annealing, 30-second extension, KAPA 2G Robust polymerase). Sequencing was performed using 2 x 300 bp paired-end chemistry in an Illumina instrument at the Ramaciotti Centre for Genomics (UNSW Sydney, Australia).

Sequencing data processing

Raw sequencing reads were quality trimmed using Trimmomatic v0.38 [19], adaptor trimmed using Cutadapt v.1.16 [20] and merged using FLASH v2.2.00 [21]. Merged reads were hashed, de-replicated and counted as previously described by Sternes, Lee [14]. Operational taxonomic unit (OTU) clustering was performed using Swarm v2.2.2 [22]. Taxonomic classification was performed with the assign_taxonomy.py module of QIIME v1.9.1 [23] with a 98 % similarity cut off against the UNITE database (qiime_ver8_dynamic_10.05.2021). The taxonomic classification of all OTUs was also manually checked and curated against the BLAST database.

Diversity analysis

All diversity analyses were performed in R v4.1.1 [24] and plots produced using a combination of ggplot2 [25] and the Ampvis2 package v2.8.3 [26]. Samples containing less than 2000 total read counts were removed from the dataset prior to analysis. Alpha (Shannon index) and beta diversity (Bray-Curtis dissimilarities) were calculated on rarefied OTU counts using Vegan v2.6 [27]. Differences of alpha diversity between categorical variables were investigated using Dunn's multiple comparison test and Holm adjusted P-values. Investigation of differences in species diversity between categorical variables was tested using a permutational multivariate analysis of variance (MANOVA) [28] implemented in the adonis function with 999 permutations. Multivariate homogeneity of groups dispersions between categories was investigated using the betadisper function of Vegan and statistical differences between groups within categories were tested using a permutational ANOVA with 999 permutations. Pair-wise comparison between samples was performed with the permutest function implemented in Vegan. PERMANOVA is known to behave unreliably in unbalanced designs with heterogenous dispersion [29]. To address this issue the F_2 statistic was [30] also used to test categories that showed heterogeneity in group dispersion (https://github.com/AWRI/permanovaModF). Investigation of specific taxonomic differences between categories was tested using the LDA effect size [31] on normalised OTU tables either at the OTU or Genus level. Community partitioning of T1 samples was performed using Dirichlet multinomial mixture (DMM) modelling [32] on a rarefied OTU abundance level matrix. The number of groups was obtained using the lowest Laplace estimation score.

Data availability

The sequencing data included in this study is available in NCBI under BioProject PRJNA1053885.

Results

The core fungal community of grapes is represented by few highly abundant species

126 To determine the core community of fungi associated with grapes and fermentation a large-scale
127 survey of grape juice was conducted that covered 14 wine grape-growing regions of Australia (Fig.
128 1a). Samples were obtained from uninoculated grape juice samples across four timepoints during
129 fermentation (Fig. 1b) from a total of 36 wineries throughout a period of six years (Fig. 1c),
130 representing the largest microbial survey of grape juice to date. The fungal microbial community
131 composition was characterized using amplicon sequencing targeting the ITS region. After quality
132 filtering and OTU clustering, a total of 3105 samples were obtained containing 4309 OTUs.

133 T1 samples, which were taken as soon as fermentation tanks were filled with freshly crushed juice,
134 largely reflected the composition of the fungal communities that inhabited the surface and flesh of
135 grapes and were selected to estimate the grape-derived core fungal microbiome. While the T1
136 timepoint was shown to contain the highest diversity across the 772 samples, a small number of very
137 abundant OTUs dominated the overall read abundance within most samples with the top 10 and 20
138 most abundant OTUs represented 70% (± 3.4%) and 80% (± 2.7%) of the total reads on average,
139 respectively (Fig. 2a). OTUs that made up at least 80% of the reads in at least one sample were
140 defined as abundant (Fig. 2b). To be considered a core OTU, we defined an abundance-occurrence
141 threshold of 50% of the samples. While no single OTU was observed as abundant in all the samples
142 investigated (Fig. 2b), two OTUs, one belonging to the genus *Aureobasidium* (observed in all samples,
143 abundant in 659) and the other *Cladosporium* (observed in 770 samples, abundant in 424), passed
144 the applied threshold for comprising core members of grape fungal microbiome (Fig. 2b). Species
145 within the genus *Aureobasidium* are recognized for their ubiquitous presence in the phyllosphere of
146 various plants, including grapevines [33] and these results confirm *Aureobasidium* as the
147 predominant fungal genus across this large cohort of grape must samples (Fig. 2b and c). OTUs
148 taxonomically assigned to *Cladosporium ramotenellum* (757 samples, abundant in 281),
149 *Saccharomyces cerevisiae* (690 samples, abundant in 204) and *Hanseniaspora uvarum* (689 samples,
150 abundant in 190) were also abundant across a large number of samples (Fig. 2b). When considering
151 only the occurrence of individual OTUs, 54 OTUs were observed in at least 50% of the samples, 25
152 OTUs in 80% (Fig. 2c) and only a single OTU (genus *Aureobasidium*) in 100% of the samples analysed.

153 A global overview of all samples clustered by genus showed similar profiles to the OTU analysis and is
154 consistent with previous studies [4, 6, 14, 34, 35]. The most abundant genera corresponded to
155 *Aureobasidium*, *Cladosporium*, *Saccharomyces*, *Hanseniaspora* and *Metschnikowia* (Fig. 2c). As
156 opposed to *Aureobasidium*, which comprised only four OTUs and with a single OTU accounting for
157 most of the abundance (Fig. 2b), the genera *Cladosporium* (13 OTUs), *Saccharomyces* (67 OTUs),
158 *Hanseniaspora* (46 OTUs) and *Metschnikowia* (53 OTUs) were comprised of high numbers of
159 individual OTUs and suggests a higher species diversity within these genera.

160 **Temporal stability and regional variations in fungal communities**

161 To investigate changes in fungal diversity over the course of the fermentation process, three
162 sequential samples were collected after the T1 sampling point, as delineated by sugar consumption
163 (Fig. 1b). The fermenting grape juice ecosystem exhibits a pronounced selective bias, favouring
164 species with fermentative capabilities and increased tolerance to ethanol [36, 37]. Alpha and beta-
165 diversity assessments across fermentation time points revealed clear reduction in both diversity and
166 beta dispersion (Fig. 3a and Fig. S1a), accompanied with rapid shifts in microbial community
167 composition (Fig. 3b) towards Saccharomycetes yeast species (Fig. S1b). In alignment with findings
168 from many previous studies, 94% of T4 samples were predominantly dominated (> 50% relative
169 abundance) by OTUs classified as *Saccharomyces cerevisiae*. Additionally, *Hanseniaspora uvarum*,
170 *Schizozaccharomyces japonicus* and *Metschnikowia* were observed as the most abundant non-
171 *Saccharomyces* at T4.

172 Aside from *S. cerevisiae*, *H. uvarum* was observed as the most abundant yeast during the initiation of
173 fermentation (1 Be drop), with 333 of 781 T2 samples containing greater than 10% relative
174 abundance of *H. uvarum*. Additional genera of non-*Saccharomyces* species were also observed in
175 high relative abundance (>10%) at the initiation of fermentation, albeit in a lower number of samples
176 (*Aureobasidium*: 159, *Metschnikowia*: 77, *Cladosporium*: 50, *Pichia*: 29, *Issatchenkia*: 24, *Candida*:
177 18, *Starmerella*: 16, *Penicillium*: 15, *Torulaspora*: 14, *Schizosaccharomyces*: 7, *Botrytis*: 4 and
178 *Lachancea*: 3 of 783 samples).

179 Significant differences in species richness (Dunn's, Holm-adjusted $p<0.05$) and compositional
180 heterogeneity (Permutest of beta dispersion $p<0.05$) were observed within T1 samples across various
181 wineries and grape-growing regions (Fig. S2-S3, Tables S2-S5). Additionally, species richness and
182 diversity exhibited substantial dispersion, indicating instability of the microbial communities (Fig. S2).
183 To assess the temporal microbial stability, a comparative analysis of beta diversity was conducted
184 across a panel of 16 wineries that were repeatedly sampled over three successive vintages (Fig. 3e).
185 While a small number of wineries displayed a consistent microbial composition over time, the
186 majority exhibited compositional alterations either after one or two years (Fig. 3e).

187 Previous studies have suggested that viticultural regions harbour regionally distinct microbial
188 communities [4, 5, 16, 17]. To investigate this effect, a Permutational MANOVA of the Bray-Curtis
189 dissimilarities between categories was utilized to assess fungal community structure between
190 wineries, grape-growing regions, states, grape varieties, and years across the dataset (Fig. 3c). All
191 tests were significant (Table S6), however a meaningful effect-size was only observed when
192 comparing between wineries (Adonis R^2 of wineries = 0.29) (Fig. 3c). To account for the observed
193 heterogenous dispersion between categories, a modified F_2 statistic was also calculated [30],
194 producing similar results (Table S6). Principal Coordinates Analysis (PCoA) revealed no discernible
195 clustering across the various categories examined (Fig. S4). Furthermore, we observed no significant
196 associations (Log LDA score > 2) between categories and specific taxa or OTUs (Table S7 and S8).
197 Subsequent pairwise comparisons across individual wineries identified that the most pronounced
198 dissimilarities were primarily associated with three specific wineries, namely K, E, and L (Table S9),
199 situated within the Margaret River, Langhorne Creek, and Canberra District wine regions. Principal
200 Component Analysis (PCA) analysis between these wineries indicated that they largely differ in the
201 abundance of three OTUs corresponding to the genera *Aureobasidium*, *Cladosporium* and
202 *Saccharomyces* (Fig. 3d).

203 **Three community types partition the grape fungal microbiome**

204 Considering the absence of meaningful associations between the taxa and regional categories, the
205 fungal diversity between samples was further investigated by partitioning samples into community
206 types using a DMM-based approach [32]. The 772 T1 samples were partitioned into three distinct
207 community types (defined as DMM1-3) (Fig. 4a and b) based on the lowest Laplace approximation (*n*
208 DMM1 = 430, *n* DMM2 = 250 and *n* DMM3 = 92). Inspection of the 30 most abundant genera
209 revealed clear differences between communities. DMM1 exhibited a high abundance of
210 *Aureobasidium*, *Cladosporium*, *Metschnikowia* and several lower abundance genera (Fig. 4a). DMM2
211 displayed the highest abundance of *Aureobasidium* and the highest species richness (Fig. 4c),
212 characterized by the presence of several rare genera (Fig. 4a). DMM3 had the lowest species richness
213 (Fig. 4c) and was mainly characterized by a high abundance of *Saccharomyces* and *Hanseniaspora*
214 (Fig. 4a). Assignment of winery samples to community types indicated that many of the wineries
215 were composed of mixed fungal community types (Fig. 4d), further supporting the lack of significant
216 associations between specific taxa and individual wineries when investigating the whole dataset
217 (Table S7 and S8).

To evaluate the impact of initial community type on the microbial dynamics of fermentation, results from T2 through T4 were partitioned based on the community type assignment of their corresponding T1 sample. DMM1 and DMM2 retained an overall higher species richness at T2 (Fig. S1c) compared to DMM3. However, in all community types, fermentative species such as *Saccharomyces* and *Hanseniaspora* were already abundant at T2 (Fig. 4e). Non-fermentative taxa, namely *Aureobasidium* and *Cladosporium*, appeared to be more persistent in community types that initially contained a higher abundance of these genera in T1 (Fig. 3a). While our data suggests that the initial community type does influence the microbial dynamics of fermentation, it is evident that other external factors, such as grape juice composition and fermentation conditions, likely exert a substantial influence on microbial community dynamics.

Discussion

The fungal communities of grapes have a direct effect on both the fermentation dynamics and the sensory attributes of spontaneously fermented wines [16, 38, 39]. To elucidate if the regional variation of wines could be partially explained by the distinctiveness of microbial communities, several studies have investigated the grape microbiota across different geographical scales using high-throughput amplicon sequencing, supporting the notion of a "microbiome terroir" [4, 5, 16, 17]. While it is clear from these studies that there is an extensive variation in microbial communities between particular geographical locations, they lack the sampling depth to obtain a global overview of the fungal communities of grape must. To address this, we carried out an extensive survey of 3105 grape juice samples across 14 grape-growing regions throughout a period of six years (Fig. 1), representing the most comprehensive microbial survey of grapes to date. Rather than concentrating on small scale regional or year-specific differences we analysed the dataset as a whole to elucidate general trends in community composition.

The core fungal community of grapes was surprisingly small, comprising only two OTUs and in addition, fewer than 20 OTUs accounted for the majority of the sequencing reads across the 772 T1 samples analysed. Relative to other open ecosystems, where the assembly of the communities is initiated by a multitude of species, grapes appear to display a very low number of core OTUs [40-44] and resemble the fungal microbiome of other cultivated fruit species [45]. Grapes therefore represent a highly selective ecosystem that differs significantly from other regions of the grapevine, such as the rhizosphere or bark, where a higher fungal taxonomic diversity have been observed [35, 46]. *Aureobasidium* appears to be particularly well adapted to the grape environment, with a single OTU dominating the abundance associated to this genus. While a single species could not be confidently assigned to this OTU, it is highly probable that it corresponds to *Aureobasidium pullulans*, given its more frequent isolation from grapes [47-50]. It is also interesting to note that community types featuring a higher abundance of this genus exhibit an overall greater species richness, suggesting that *Aureobasidium* species may not engage in broad antagonism but rather exhibit targeted antagonism against specific species, as reported in several studies [51, 52]. *Cladosporium*, known for causing post-harvest rot diseases in various fruits [53-55], is also part of the core microbiome of grapes, implying that its abundance in grapes is not necessarily linked to disease development.

The fungal dynamics of fermentation progressed as expected [7, 8] with *S. cerevisiae* dominating by T2 in nearly all the samples analysed, yet many samples exhibited a prolonged period of high abundance of *H. uvarum*. Although prior research noted the prevalence of this species in early fermentation stages [56], these results highlight its role as the primary non-*Saccharomyces* yeast in grape juice fermentation. While other *Hanseniaspora* and non-*Saccharomyces* yeast were also

263 detected at T1, their frequency was marginal by T2. From an evolutionary viewpoint, *H. uvarum*
264 seems less genetically equipped among *Hanseniaspora* species for fermentation due to a notable
265 loss of genes related to cell-cycle and DNA repair [57]. Yet recent studies suggest that losing specific
266 genes, including key cell-cycle regulators, might be an adaptive strategy, enabling a quicker cell cycle
267 [58]. This discovery prompts the need for more research on *H. uvarum* to fully uncover its
268 competitive advantage during early fermentation, especially compared to the various non-
269 *Saccharomyces* yeast species found in grape must.

270 Wineries displayed distinct and temporally unstable fungal communities, and this is consistent with
271 the observations reported in similar studies [4, 17, 35, 59, 60]. Despite this, the effect size of this
272 associations was considerably lower than previously reported, likely due to differences in sampling
273 size, geographic coverage, number of grape varieties and years of sampling. Large-scale geographic
274 denominations exhibited a weak association with community composition. For instance, samples
275 collected from Tasmania, despite its geographical isolation from mainland Australia, displayed
276 remarkably similar fungal microbial communities to those in mainland states, all falling under a single
277 community type. Similarly, samples obtained from Western Australia, positioned more than 2000 km
278 away from the nearest wine region, exhibited a fungal microbial composition highly similar to
279 samples obtained from South Australia, also falling within the same community type. We observed
280 no strong association between grape variety and community structure, even when focusing on the
281 two primary varieties sampled, Shiraz and Chardonnay (n = 231 and 353, respectively). These
282 findings were further supported by the lack of associations between specific taxa/OTUs and any of
283 the categories tested, providing contrast to previous work suggesting correlations between
284 geographic locations and diversity [4, 5, 16, 17]. Rather than containing distinctive microbial
285 communities, the data suggests that the disparities among geographic locations can be largely
286 attributed to the prevalence of a few select genera, including *Aureobasidium*, *Cladosporium* and
287 *Saccharomyces* and suggests that regions transition between three broad community types
288 characterised by differences in diversity. Although environmental data was not collected during this
289 study, it is probable that environmental conditions (e.g., climate and anthropogenic factors) play a
290 pivotal role in driving the observed geographical shifts in community composition, as previously
291 suggested [4]. These community types exhibited differences in several important genera known to
292 impact wine composition (e.g., *Saccharomyces*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Torulaspora*,
293 *Starmerella* etc.) [61] and likely show functional heterogeneity with regards to fermentation. Indeed,
294 more diverse communities retained a higher fungal diversity during the initial stages of fermentation,
295 likely influencing fermentation performance and the chemical composition of wine. Divergence from
296 past studies may be due to the large number of sites we utilized, allowing for a greater
297 understanding of the spectrum of microbial communities within regions that is absent when fewer
298 sites are sampled. However, due to the utilization of ITS sequences we were unable to test for strain
299 specific population structure between categories, which has been suggested to occur [17]. Recent
300 work characterizing 411 *S. cerevisiae* genomes isolated from spontaneous ferments from a subset of
301 the wineries utilized in this study over the 2014-2019 period identified limited strain level population
302 structure across multiple grape varieties, locations, and years [62] suggesting this may not be
303 observed in *S. cerevisiae*.

304 Understanding the environmental drivers behind shifts in community types could serve as a valuable
305 tool to modulate grape health and fermentation. For instance, DMM1 exhibited a higher abundance
306 of *Botrytis*, which is considered highly detrimental to wine quality [63], while DMM3 was dominated
307 by *Saccharomyces*, suggesting faster fermentation kinetics.

308

Conclusion

Taken together our results help to formulate a broader understanding of the community dynamics within grape fermentations across varietal, spatial and temporal scales utilizing the largest survey of grape ferment microbiomes to date. A holistic approach was taken with respect to the data analysis, investigating the role that geography, grape variety and year has on microbiome composition and diversity. When considering all scales, little structure was identified with wineries displaying highly unstable fungal communities that transitioned within three broad community types. In contrast to previous work suggesting that geography might play a large role in microbiome composition, it was observed that both regional and state level geographies showed a small effect size on the overall diversity and composition of ferments. These analyses suggest that sampling number within locations and the geographic scale investigated are key factors when considering the "microbial terroir" hypothesis. In general, it was observed that initial community diversity was transient between fermentation timepoints while playing an important, but imperfect, role in predicting the diversity of the fungal microbiome throughout fermentation. This suggests that initial microbial community is not deterministic and highlights the role abiotic factors have in determining microbiome composition throughout the fermentation process.

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Figure Legends

Fig. 1: Sampling design of the study. **a** Geographical location of the Australian wineries sampled throughout a six-year period. Each point represents a winery and labels show the closest town/city. **b** Sampling point for each grape juice sample: T1= freshly pressed juice, T2= 1 Bé drop in sugar, T3= 50% sugar remaining; T4= 10% sugar remaining. **c** Number of samples collected each year for each timepoint.

Fig. 2: The core fungal community of grape juice. **a** Cumulative read abundance for 772 samples and 3594 OTUs. Red line represents the mean read abundance ± SD in red shading. OTUs were ranked in order of abundance for all samples. The 10 most abundant OTUs represent 70 % (± 3.4 %) of the total reads on average. **b** Frequency and observation as high abundant (OTUs making up the top 80% of the reads as in plot **a**) of OTUs across all samples. Points are coloured based on number of OTUs and Genus classification is shown for the top 5 OTUs observed as abundant. **c** Box plot showing the relative abundance of the top 50 genera sorted by mean read abundance. Genera in red represent taxa observed in at least 80% of all samples. Line inside the boxplot depicts the median and upper and lower ends of each box represent the 25th and 75th percentiles. Outer lines show the minimum and maximum value and dots represent potential outliers.

Fig. 3: The fungal diversity of grape juice. **a** Violin-plot showing the alpha-diversity (Shannon index) of 3113 samples separated by sampling timepoint. Multiple-comparison test was performed between samples. $^{**}P < 0.005$ (Dunn's, Holm-adjusted). **b** Non-metric multidimensional scaling (NMDS) of all samples based on Bray-Curtis (BC) dissimilarities. **c** Bar-plot showing the effect size (R^2) of each metadata category calculated by PERMANOVA (Adonis). **d** Principal component analysis (PCA) of the Hellinger transformed OTU abundances of the three wineries displaying the highest F2 values after pairwise comparison (Table S9). Individual OTUs are shown in grey with the three most extreme OTUs labelled to the genus level. **e** Temporal stability of the fungal communities. Baseline represents the average within BC distances for each winery in the year 2018. 1 and 2 years represent the average BC distances between the baseline and 2019 and 2021, respectively.

Fig. 4: Dirichlet Multinomial Mixtures (DMM) partitioning of grape juice fungal communities. **a** Relative abundance for the top 30 Genera observed in each DMM partition. **b** Principal coordinate analysis (PCoA) of T1 samples based on Bray-Curtis (BC) dissimilarities. Samples are coloured by DMM as in panel (c) **c** Violin-plot showing the alpha-diversity (Shannon index) of samples belonging to each DMM partition. Multiple-comparison test was performed between samples. $^{*}P < 0.05$, $^{**}P < 0.005$ (Dunn's, Holm-adjusted). **d** Total number of samples allocated to each DMM partition for each winery with more than 10 samples. Samples are coloured by DMM as in panel (c). **e** Relative abundance for the top 5 genera presented in panel (a) for timepoints T1 to T4 separated by DMM partition. Only DMM partition samples containing timepoints T2 to T4 were used to produce the plot.

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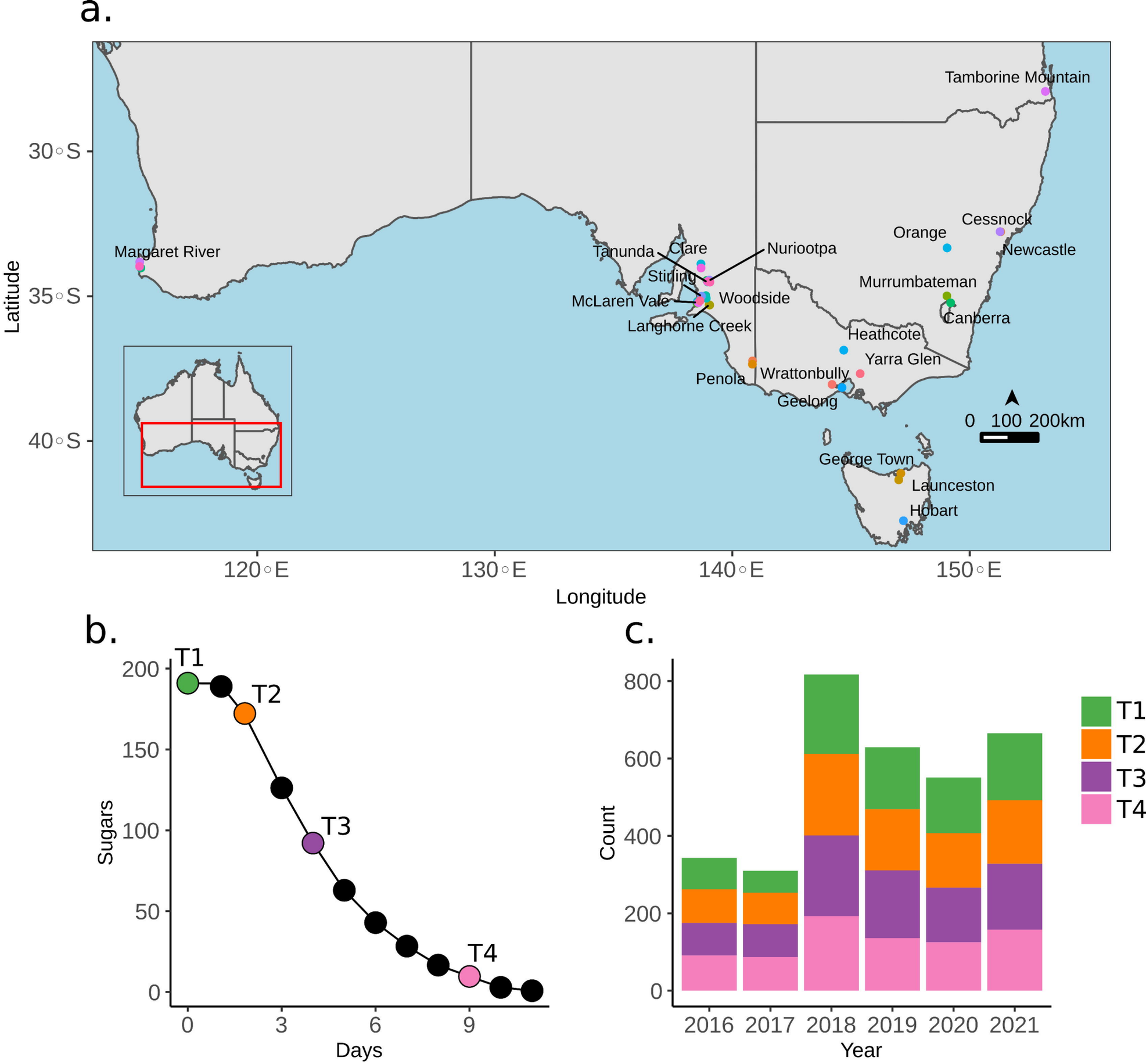
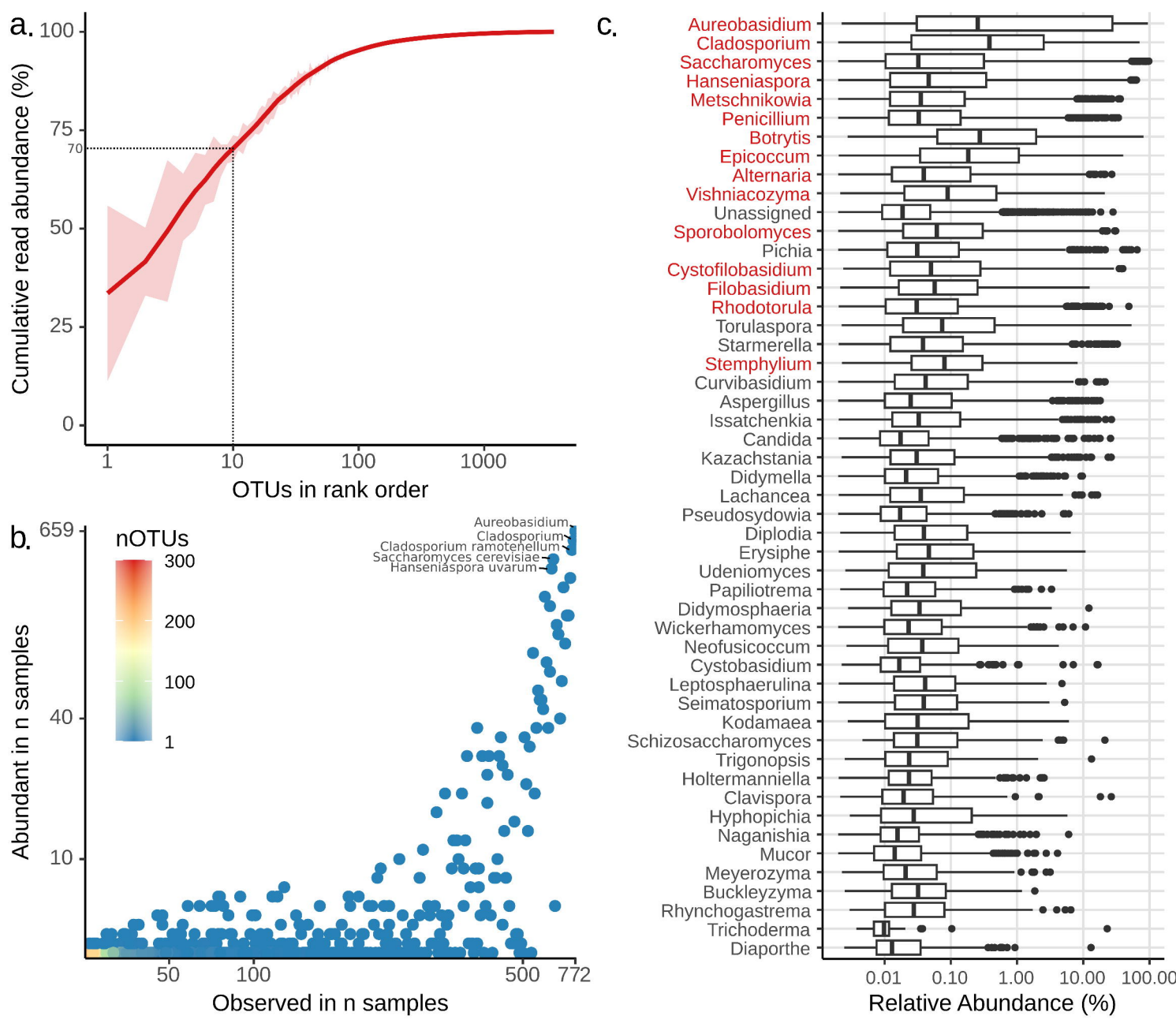
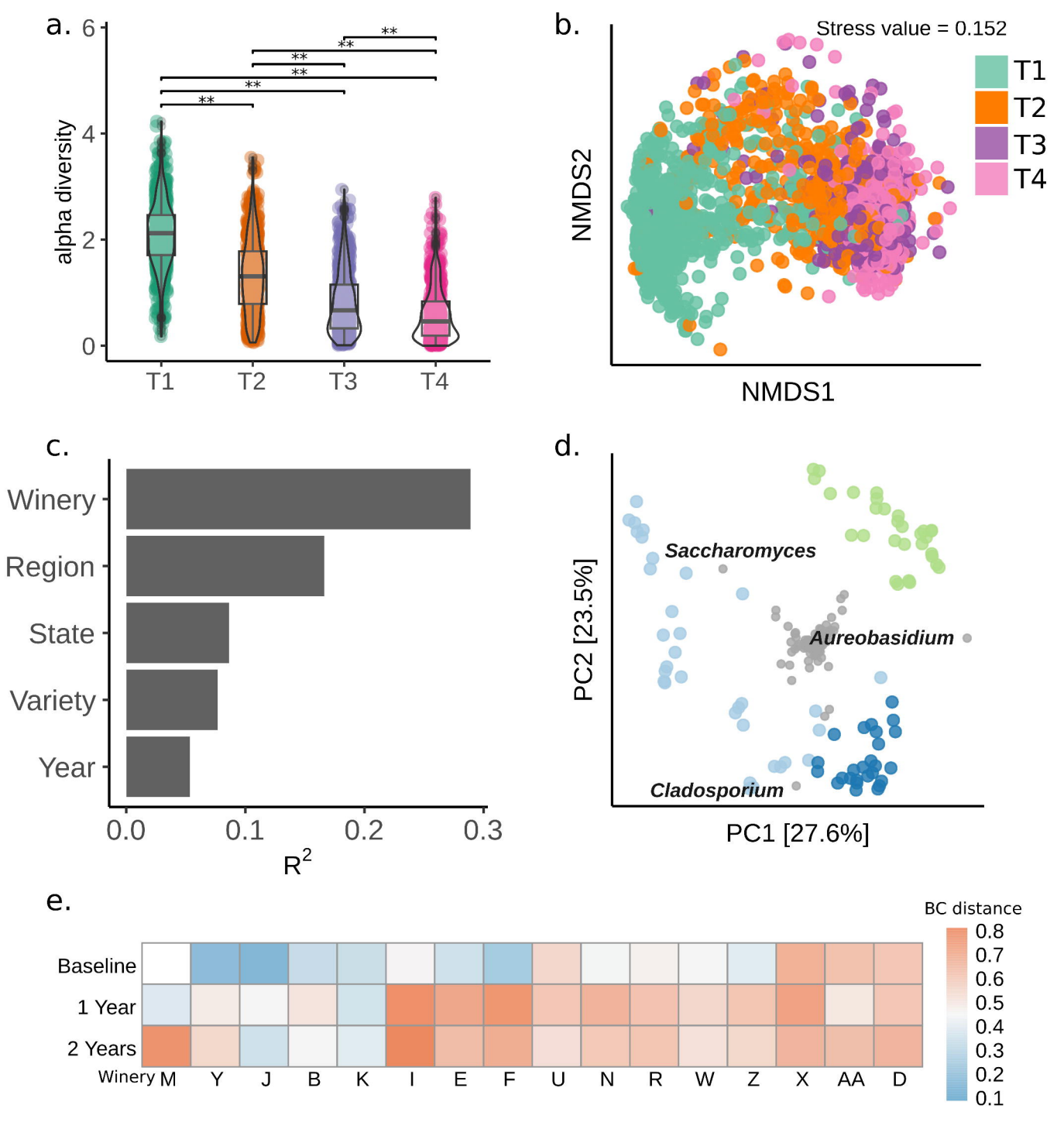

a.
Tamborine Mountain
Cessnock
Orange
Newcastle
Margaret River
Tanunda
Clare
Nuriootpa
Stirling
Murrumbateman
McLaren Vale
Woodside
Canberra
Langhorne Creek
Heathcote
Yarra Glen
Penola
Wrattonbully
Geelong
0 100 200km
George Town
Launceston
Hobart
30◦S
35◦S
40◦S
Latitude
120◦E
130◦E
140◦E
150◦E
Longitude
b.
T1
T2
T3
T4
Sugars
Days
c.
Count
Year
2016
2017
2018
2019
2020
2021
T1
T2
T3
T4

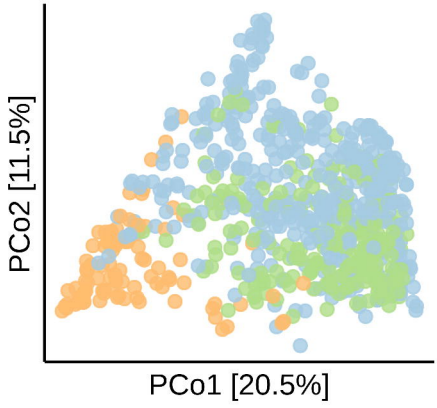

a.
Cumulative read abundance (%)
100
75
70
50
25
0
1
10
100
1000
OTUs in rank order
b.
nOTUs
300
200
100
1
659
40
10
Abundant in n samples
Aureobasidium
Cladosporium
Cladosporium ramotenellum
Saccharomyces cerevisiae
Hanseniaspora uvarum
50
100
500
772
Observed in n samples
c.
Aureobasidium
Cladosporium
Saccharomyces
Hanseniaspora
Metschnikowia
Penicillium
Botrytis
Epicoccum
Alternaria
Vishniacozyma
Unassigned
Sporobolomyces
Pichia
Cystofilobasidium
Filobasidium
Rhodotorula
Torulaspora
Starmerella
Stemphylium
Curvibasidium
Aspergillus
Issatchenkia
Candida
Kazachstania
Didymella
Lachancea
Pseudosydowia
Diplodia
Erysiphe
Udeniomyces
Papiliotrema
Didymosphaeria
Wickerhamomyces
Neofusicoccum
Cystobasidium
Leptosphaerulina
Seimatosporium
Kodamaea
Schizosaccharomyces
Trigonopsis
Holtermanniella
Clavispora
Hyphopichia
Naganishia
Mucor
Meyerozyma
Buckleyzyma
Rhynchogastrema
Trichoderma
Diaporthe
0.01
0.10
1.00
10.00
100.00
Relative Abundance (%)


a.
alpha diversity
0
2
4
6
T1
T2
T3
T4
**
b.
Stress value = 0.152
NMDS2
NMDS1
T1
T2
T3
T4
c.
Winery
Region
State
Variety
Year
0.0
0.1
0.2
0.3
R2
d.
PC2 [23.5%]
PC1 [27.6%]
Saccharomyces
Aureobasidium
Cladosporium
e.
Baseline
1 Year
2 Years
Winery
M
Y
J
B
K
I
E
F
U
N
R
W
Z
X
AA
D
BC distance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

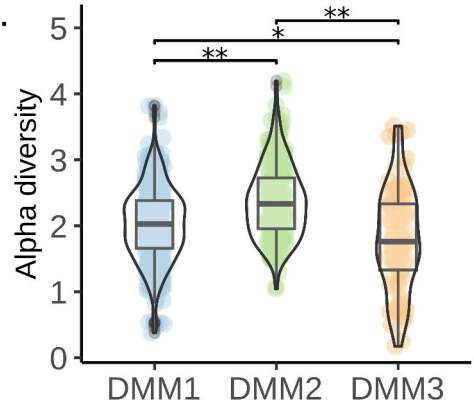
a.

	DMM1	DMM2	DMM3
Aureobasidium	33.8	43	9.2
Cladosporium	21.9	14.1	5.4
Saccharomyces	2.4	3.3	48.7
Hanseniaspora	7.8	5.1	15.1
Metschnikowia	4.5	2.5	2.2
Penicillium	2.9	3.3	1
Botrytis	4.5	0.8	0.2
Epicoccum	2.1	4.3	0.6
Alternaria	1.4	4.2	0.5
Vishniacozyma	2.7	1.4	1.1
Unassigned	1	2.1	1.2
Sporobolomyces	1.8	0.7	0.9
Pichia	1.4	0.6	2.8
Cystofilobasidium	1.7	0.9	0.4
Filobasidium	0.8	2.3	0.5
Rhodotorula	1.7	0.2	0.3
Torulaspora	0.6	0.9	1.8
Starmerella	1.2	0.1	1.3
Stemphylium	0.4	1.2	0.2
Curvibasidium	1.1	0.1	0.2
Aspergillus	0.2	1.2	0.4
Issatchenkia	0.5	0.5	0.4
Candida	0.4	0.3	0.7
Kazachstania	0.3	0.5	0.5
Didymella	0.2	0.5	0.1
Lachancea	0.2	0.2	0.8
Pseudosydowia	0.1	0.3	0
Diplodia	0.1	0.3	0
Erysiphe	0.1	0.3	0
Udeniomyces	0	0.3	0

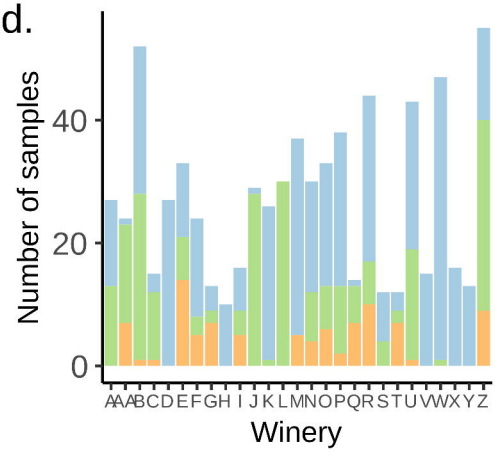
b.



c.



d.



e.

	DMM1				DMM2				DMM3			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
Aureobasidium	33.2	6.8	1.9	1.4	45.2	10.8	1.5	0.7	9.8	2.3	1.7	0.4
Cladosporium	22.7	3.9	1.7	0.8	14.5	2.3	0.5	0.2	5.8	1.2	0.5	0.2
Saccharomyces	2.3	58.7	79.9	88.1	3.1	52	79	84.3	47.4	73.9	84	89.7
Hanseniaspora	7.8	16.6	8.5	4.7	3.8	21.5	7.9	3.6	14.6	13.5	6.8	4.2
Metschnikowia	4.4	4.1	2.6	1.1	2.3	3.5	1.5	0.6	2.7	1.5	0.6	1.2