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1 **Diversity and dynamics of fungi during spontaneous fermentations and association with**
2 **unique aroma profiles in wine**

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10

Abstract

Microbial activity is an integral part of an agricultural ecosystem and influences the quality of agricultural commodities. Microbial ecology influences grapevine health and crop production, conversion of sugar to ethanol during fermentation, thus wine aroma and flavour. There are regionally differentiated microbial patterns in grapevines and must but how microbial patterns contribute to wine regional distinctiveness (*terroir*) at small scale (<100 km) is not well defined. Here we characterise fungal communities, yeast populations, and *Saccharomyces cerevisiae* populations during spontaneous fermentation using metagenomics and population genetics to investigate microbial distribution and fungal contributions to the resultant wine. We found differentiation of fungi, yeasts, and *S. cerevisiae* between geographic origins (estate/vineyard), with influences from the grape variety. Growth and dominance of *S. cerevisiae* during fermentation reshaped the fungal community and showed geographic structure at the strain level. Associations between fungal microbiota diversity and wine chemicals suggest that *S. cerevisiae* plays a primary role in determining wine aroma profiles at a sub-regional scale. The geographic distribution at scales of less than 12 km supports that differential microbial communities, including the dominant fermentative yeast *S. cerevisiae* can be distinct in a local setting. These findings provide further evidence for microbial contributions to wine *terroir*, and perspectives for sustainable agricultural practices to maintain microbial diversity and optimise fermentation function to craft beverage quality.

Keywords: microbial biogeography, fungal microbiota, *Saccharomyces cerevisiae*, wine, spontaneous fermentation

32 ## 1. Introduction

33 Wine grapes (*Vitis vinifera*) are an economically and culturally important agricultural commodity for
34 which microbial activity plays key roles in grape and wine production and quality (Barata et al., 2012;
35 Swiegers et al., 2005). The grapevine harbours complex and diverse microbiota, such as bacteria,
36 filamentous fungi, and yeasts (Barata et al., 2012; Liu and Howell, 2020; Stefanini and Cavalieri,
37 2018), which substantially modulate vine health, growth, and crop productivity (Berg et al., 2014;
38 Gilbert et al., 2014; Müller et al., 2016). Grapevine-associated microbiota can be transferred to the
39 grape must/juice and have an influence on wine composition, aroma, flavour, and quality (Barata et
40 al., 2012; Ciani et al., 2010; Morrison-Whittle and Goddard, 2018). Wine fermentation is a complex
41 and multispecies process, involving numerous transformations by fungi and bacteria to sculpt
42 chemical and sensory properties of the resulting wines (Swiegers et al., 2005; Verginer et al., 2010).
43 While these consortia all contribute to wine flavour formation, the fermentation process is principally
44 driven by diverse populations of *Saccharomyces cerevisiae* (Fleet, 2003; Goddard, 2008; Howell et
45 al., 2006).
46
47 Microbial biogeography contributes to regional distinctiveness of agricultural products, known as
48 "*terroir*" in viticulture [reviewed by Liu et al. (2019)]. Biogeographical patterns in the microbiota
49 associated with the grape and must have been demonstrated for both fungi and bacteria at a regional
50 scale (Bokulich et al., 2014; Mezzasalma et al., 2018; Pinto et al., 2015; Taylor et al., 2014), which
51 are conditioned by multiple factors, such as cultivar, climate and vintage, topography, and soil
52 properties (Bokulich et al., 2014; Liu et al., 2019; Miura et al., 2017; Portillo et al., 2016;
53 Zarraonaindia et al., 2015). Bokulich et al. (2016) showed that the bacterial and fungal consortia
54 correlated with metabolites in finished wines, highlighting the importance of fermentative yeasts (for
55 example, *S. cerevisiae*, *Hanseniaspora uvarum*, *Pichia guilliermondii*) and lactic acid bacteria
56 (*Leuconostocaceae*) on the abundance of regional aromatic signatures. Our previous research revealed
57 that wine-related fungal communities structured and distinguished vineyard ecosystems by impacting
58 the flavour and quality of wine, and weather with a contribution by soil properties affected soil and

59 must fungal communities and thus the composition of wines across six winegrowing regions in
60 southern Australia (Liu et al., 2020). However, whether grape-associated microbiota exhibit distinct
61 patterns of distribution at smaller geographic scales (for example individual vineyards) and their
62 associations with wine aroma profiles are not well understood.

63 Geographic differentiation of *S. cerevisiae* populations is evident at global (Legras et al., 2007; Liti et
64 al., 2009) and regional scales (Gayevskiy and Goddard, 2012; Knight and Goddard, 2015), revealing a
65 picture of distinctive populations at large scales more than ~ 100 km. Drumonde-Neves et al. (2018)
66 showed higher genetic divergence among *S. cerevisiae* populations between rather than within
67 islands/regions (~ 1.5 – 260 km scale) and suggested a prevailing role of geography over ecology
68 (grape varieties and agricultural cultivation) in shaping diversification, as previously reported
69 (Goddard et al., 2010). At small scales, several studies characterised significant genetic differences
70 between *S. cerevisiae* populations residing in different vineyards within the same region [Börlin et al.
71 (2016); <10 km] and different sites within a vineyard [Schuller and Casal (2007); 10 – 400 m]. Knight
72 et al. (2015) experimentally demonstrated that regional strains of *S. cerevisiae* produce distinct wine
73 chemical compositions, suggesting a prominent route by which regional *S. cerevisiae* shape wine
74 *terroir*. While few studies have investigated how *S. cerevisiae* differentiation can affect wine aroma,
75 flavour, and characteristics, and none have considered *S. cerevisiae*, fermentative yeasts, and the
76 global fungal communities simultaneously to quantify their contributions to the resultant wine.
77

78 To investigate these questions, we sampled microbial communities associated with Pinot Noir and
79 Chardonnay grape must and juice from three wine estates with 8 - 12 km pairwise distances to include
80 grapes from 11 vineyards in the Mornington Peninsula wine region of Victoria, Australia. Using
81 culture-independent sequencing to characterise fungal communities, we disentangled the influences of
82 geographic origin (estate/vineyard), grape variety, and (spontaneous) fermentation stage on the
83 diversity, structure, and composition of the fungal communities. Yeast populations were isolated
84 during spontaneous wine fermentation and taxonomically identified, and the *S. cerevisiae* populations
85 differentiated using microsatellite analysis. To identify the volatiles that differentiated the wine estates

86 we used headspace solid-phase microextraction gas-chromatographic mass-spectrometric (HS-SPME–
87 GC-MS) for metabolite profiling of the resultant wines. Associations between fungal communities
88 and wine metabolites were elucidated with partial least squares regression (PLSR) and structural
89 equation model (SEM). We demonstrate that the grape/wine microbiota and metabolites are
90 geographically distinct, identify multiple layers of fungal microbiota that correlate with wine aroma
91 profiles, and demonstrate that distinctive *S. cerevisiae* exert the most powerful influences on wine
92 quality and style at small geographic scales.
93

94 2. Materials and methods

95 2.1 Sampling

96 Five *Vitis vinifera* cv. Pinot Noir and six *Vitis vinifera* cv. Chardonnay vineyards from three wine
97 estates (designated A, B, and C) in the Mornington Peninsula region were selected to conduct this
98 study in 2019 (Supplementary Fig. S1). The distance between wine estates A and B, A and C, B and C
99 is 8 km, 12 km, and 10 km, respectively. Within these estates, vineyards are within a 5 km radius of
100 one another. All vineyards were commercially managed using similar viticultural practices, for
101 example, grapevines were under vertical shoot positioning trellising systems and were applied with
102 the same sprays. Chemical constituents of harvested grapes (°Brix, pH, total acidity) were similar and
103 listed in Supplementary Table S1. Fermentations sampled in this study were conducted without
104 addition of commercial yeasts following similar fermentation protocols. Tanks were cleaned and
105 decontaminated before filling grapes. For Pinot Noir, crushed grapes were held for three days at a
106 cool temperature (known as cold-soaking) and followed by warming so fermentation could
107 commence. Fermentation samples were collected at three time points in duplicate: before fermentation
108 (BF; destemmed and crushed grape musts of Pinot Noir, Chardonnay juice following clarification), at
109 the middle of fermentation (MF, around 50% of sugar fermented), and at the end of fermentation (EF,
110 before pressing, 6-7 °Brix) (Supplementary Table S1). Samples (n = 66) were shipped on ice to the
111 laboratory. Each sample was divided into two subsamples, one was used immediately to isolate yeasts

112 and the other was stored at -20°C for DNA extraction, next-generation sequencing and wine volatile
113 analysis.
114

115 **2.2 Wine volatile analysis**

116 Volatile compounds of EF samples were determined using headspace solid-phase microextraction
117 gas-chromatographic mass-spectrometric (HS-SPME–GC-MS) method (Liu et al., 2016; Zhang et al.,
118 2015) with some modifications. Analyses were conducted with Agilent 6850 GC system and a 5973
119 mass detector (Agilent Technologies, Santa Clara, CA, USA), equipped with a PAL RSI 120
120 autosampler (CTC Analytics AG, Switzerland). In brief, 10 mL wine sample was added to a 20 ml
121 glass vial containing 2 g sodium chloride and 20 µL internal standard (4-Octanol, 100 mg/L), and then
122 equilibrated at 35 °C for 15 min. A polydimethylsiloxane/divinylbenzene (PDMS/DVB, Supelco) 65
123 µm SPME fibre was immersed in the headspace for 10 min at 35°C with agitation, and followed by
124 desorbing in the GC injector for 4 min at 220 °C. Volatile compounds were separated on an Agilent
125 J&W DB-Wax Ultra Inert capillary GC column (30 m × 0.25 mm × 0.25 µm), with helium carrier gas
126 at a flow rate of 0.7 mL/min. The column temperature program was as follows: holding 40 °C for 10
127 min, increasing at 3.0 °C/min to 220 °C and holding at this temperature for 10 min. The temperature
128 of the transfer line of GC and MS was set at 240 °C. The ion source temperature was 230 °C. The MS
129 was operated in positive electron ionization (EI) mode with scanning over a mass acquisition range of
130 35 to 350 m/z. Raw data were processed with Agilent ChemStation Software for qualification and
131 quantification. Volatile compounds (n = 79) were identified in wine samples according to retention
132 indices referencing standards and mass spectra matching with NIST11 library. 13 successive levels of
133 standards in model wine solutions (12% v/v ethanol saturated with potassium hydrogen tartrate and
134 adjusted to pH 3.5 using 40% w/v tartaric acid) were analysed by the same protocol as wine samples
135 to establish the calibration curves for quantification. Peak areas of compounds were integrated via
136 target ions model. The concentrations of compounds were calculated with the calibration curves and
137 used for downstream data analysis.

138

2.3 DNA extraction and sequencing

Samples were thawed, and biomass was recovered by centrifugation at 4,000 × g for 15 min, washed three times in ice-cold phosphate buffered saline (PBS) with 1% polyvinylpolypyrrolidone (PVPP) and centrifuged at 10,000 × g for 10 min (Bokulich et al., 2014). The obtained pellets were used for DNA extraction using PowerSoil™ DNA Isolation kits (QIAgen, CA, USA). DNA extracts were stored at -20 °C until further analysis.

Genomic DNA was submitted to Australian Genome Research Facility (AGRF) for amplicon sequencing. To analyse the fungal communities, partial fungal internal transcribed spacer (ITS) region was amplified using the universal primer pairs ITS1F/2 (Gardes and Bruns, 1993). The primary PCR reactions contained 10 ng DNA template, 2× AmpliTaq Gold® 360 Master Mix (Life Technologies, Australia), 5 pmol of each primer. A secondary PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Clontech). Amplification was conducted as follows: 95 °C for 7 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 60 s, and a final extension at 72 °C for 7 min. The resulting amplicons were cleaned again using magnetic beads, quantified by fluorometry (Promega Quantifluor), and normalised. The equimolar pool was cleaned a final time using magnetic beads to concentrate the pool and measured using a High-Sensitivity D1000 Tape on an Agilent 2200 TapeStation. The pool was diluted to 5nM and molarity was confirmed again with a High-Sensitivity D1000 Tape. This was followed by 300 bp paired-end sequencing on an Illumina MiSeq (San Diego, CA, USA).

Raw sequences were processed using QIIME v1.9.2 (Caporaso et al., 2010). Low quality regions ($Q < 20$) were trimmed from the 5′ end of the sequences, and the paired ends were joined using FLASH (Magoč and Salzberg, 2011). Primers were trimmed and a further round of quality control was conducted to discard full length duplicate sequences, short sequences (< 100 nt), and sequences with ambiguous bases. Sequences were clustered followed by chimera checking using UCHIME algorithm from USEARCH v7.1.1090 (Edgar et al., 2011). Operational taxonomic units (OTUs) were assigned

166 using UCLUST open-reference OTU-picking workflow with a threshold of 97% pairwise identity
167 (Edgar, 2010). Singletons or unique reads in the resultant data set were discarded. Taxonomy was
168 assigned to OTUs in QIIME using the UNITE fungal ITS database (v7.2) (Kõljalg et al., 2005). To
169 avoid/reduce biases generated by varying sequencing depth, sequences were rarefied to 10,000 per
170 sample prior to downstream analysis. Raw sequencing reads are publicly available in the National
171 Centre for Biotechnology Information (NCBI) Sequence Read Archive under the bioproject
172 PRJNA646865.

173 **2.4 Yeast isolation and identification**

174 Yeast populations were evaluated during spontaneous fermentations (BF, MF, EF). Aliquots of 0.1 ml
175 from serially diluted samples (from 10^{-2} to 10^{-5}) were spread on Wallerstein Laboratory Nutrient
176 (WLN) agar plates (Oxoid, Australia) that were supplemented with 34 mg/mL chloramphenicol and
177 25 mg/mL ampicillin to inhibit bacterial growth. After 5 days of incubation at 28°C, colonies were
178 counted (30-300 colonies) and colony morphology was recorded to differentiate yeast species
179 according to Pallmann et al. (2001) and Romancino et al. (2008). Characteristic yeast colonies were
180 isolated by subculturing on fresh WLN plates. DNA was extracted from pure colonies using the
181 MasterPure™ Yeast DNA Purification Kit (Epicentre, Madison, WI) following the manufacturer's
182 instructions. The 26S rRNA D1/D2 domain was amplified using primers NL1/4 (Kurtzman and
183 Robnett, 1998) for sequencing by AGRF. Species identity was determined using BLAST hosted by
184 the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), considering an identity threshold of at least 98%.
185 The colonies identified as *S. cerevisiae* were analysed by microsatellites as described in the following
186 paper. Sequence data was uploaded to Genbank with accession numbers MT821080-MT821098.
187

188 **2.5 Microsatellite characterisation**

189 A set of 94 *S. cerevisiae* strains, listed in Supplementary Table S1, was characterised for allelic
190 variation at 12 microsatellites as previously described (Legras et al., 2007). Briefly, two multiplex
191 assays of primers corresponding to loci C5, C3, C8, C11, SCYOR267c and C9, YKL172w, ScAAT1,
192 C4, SCAAT5, C6, YPL009c, were amplified using the QIAGEN Multiplex PCR Kit according to the

193 manufacturer's instructions. PCRs were run in a final volume of 12.5 µL containing 10 – 250 ng yeast
194 DNA, and used the following program: initial denaturation at 95°C for 15 min, followed by 34 cycles
195 of 94°C for 30 s, 57°C for 2 min, 72°C for 1 min, and a final extension at 60°C for 30 min. PCR
196 products were sized for 12 microsatellite loci on an ABI 310 DNA sequencer (Applied Biosystems)
197 using the size standards HD400ROX. Allele distribution into classes was carried out using Genious
198 software v9.1.6 (Biomatters) and the corresponding alleles classes were described in Legras et al.
199 2007.

200

201 **2.6 Data analysis**

202 Alpha diversities of fungal communities, yeast populations, and wine volatile compounds were
203 calculated using the Shannon index with the "vegan" package (Oksanen et al., 2007). One-way
204 analysis of variance (ANOVA) was used to determine whether sample classifications (e.g.,
205 fermentation stage, wine estate) contained statistically significant differences in the alpha-diversity.
206 Principal coordinate analysis (PCoA) was performed to evaluate the distribution patterns of fungal
207 communities, yeast populations, and wine aroma based on beta-diversity calculated by the Bray–
208 Curtis distance with the "labdsv" package (Roberts, 2007). Permutational multivariate analysis of
209 variance (PERMANOVA) using distance matrices with 999 permutations was conducted within each
210 sample class to determine the statistically significant differences with "adonis" function in "vegan"
211 (Oksanen et al., 2007). Significant taxonomic differences of fungi in the BF must between sample
212 categories (wine estate, grape variety) were tested using linear discriminant analysis (LDA) effect size
213 (LEfSe) analysis (Segata et al., 2011) (https://huttenhower.sph.harvard.edu/galaxy/). The OTU table
214 was filtered to include only OTUs > 0.01% relative abundance to reduce LEfSe complexity. The
215 factorial Kruskal–Wallis sum-rank test ($\alpha = 0.05$) was applied to identify taxa with significant
216 differential abundances between groups (all-against-all comparisons), followed by the logarithmic
217 LDA score (threshold = 2.0) to estimate the effect size of each discriminative feature. Significant taxa
218 were used to generate taxonomic cladograms illustrating differences between sample categories.
219 Dynamics and successions of the dominant species (relative abundance > 1.00%) demonstrating

220 significant differences among stages (ANOVA; FDR-corrected p value < 0.05) were illustrated by
221 alluvial diagrams using the "ggalluvial" package (Brunson, 2018) in "ggplot2".

222

223 Multilocus genotypes were calculated with the "poppr" package (Kamvar et al., 2014). The Bruvo's
224 distance (Bruvo et al., 2004) was calculated between each strain by the "ape" (Paradis et al., 2004)
225 and "poppr" packages. The tree was obtained from the distance matrices with "poppr", and drawn
226 using MEGA X (Kumar et al., 2018). The tree was rooted by the midpoint method. An alternative
227 method of genetic clustering- discriminant analysis of principal components (DAPC) was also applied
228 to infer the population structure with "adegenet" package (Jombart et al., 2010). Analysis of
229 molecular variance (AMOVA) was conducted to determine the degree of differentiation of variations
230 between designated partitions (estates, vineyards, varieties) with "poppr" (Excoffier et al., 1992). The
231 genetic distance F_{ST} (Reynolds et al., 1983) between estates were calculated by the "hierfstat" package
232 (Goudet, 2005). PCoA based on the Bruvo's distance was conducted to evaluate the geographic
233 distribution pattern of *S. cerevisiae* populations with "vegan" (Oksanen et al., 2007).

234 Partial least squares regression (PLSR) analysis with cross-validation was used to model associations
235 between normalised mean values for fungal taxa (species with relative abundance > 0.10%) and wine
236 volatile compounds using the "pls" package (Wehrens and Mevik, 2007). The structural equation
237 model (SEM) (Grace, 2006) was used to evaluate the direct and indirect relationships between must
238 fungal communities and yeast populations, *S. cerevisiae* populations, and resulting wine aroma (the
239 first axis values of PCoA analysis). SEM is an a priori approach partitioning the influence of multiple
240 drivers in a system to help characterise and comprehend complex networks of ecological interactions
241 (Eisenhauer et al., 2015). An *a priori* model was established based on the known effects and
242 relationships among drivers of distribution patterns of wine aroma to manipulate the data before
243 modelling (including must aroma, °Brix, pH; data not shown). A path coefficient described the
244 strength and sign of the relationship between two variables (Grace, 2006). The good fit of the model
245 was validated by the $\chi 2$-test ($p > 0.05$), using the goodness of fit index (GFI > 0.90) and the root MSE
246 of approximation (RMSEA < 0.05) (Schermelleh-Engel et al., 2003). Standardised total effects of

247 each factor on the wine aroma distribution pattern were calculated by summing all direct and indirect
248 pathways between two variables (Grace, 2006). All these analyses were conducted using AMOS
249 v25.0 (AMOS IBM, NY, USA).

250 **3. Results**

251 **3.1 Fungal microbiota vary by geographical origin and grape variety**

252 To elucidate the influences of geographic locations, grape variety, and fermentation process on the
253 wine microbiota, 66 duplicate samples covering three wine estates, Pinot noir and Chardonnay, from
254 the beginning, middle and end of fermentation were collected to analyse fungal communities. A total
255 of 1,566,576 ITS high-quality sequences were generated from all samples, which were clustered into
256 277 fungal OTUs with a threshold of 97% pairwise identity. *Ascomycota* was the most abundant
257 phylum in the grape must/juice before fermentation (BF) comprising 92.00% of all sequences,
258 followed by *Basidiomycota* (7.98%) and *Mortierellomycota* (< 0.01%) (Data not shown). Fungal
259 profiles were dominated by filamentous fungi, mostly of the genera *Cladosporium*, *Aureobasidium*,
260 and *Epicoccum*, with notable populations of fermentative yeasts including *Saccharomyces* and
261 *Hanseniaspora*, as well as the basidiomycetous yeasts *Vishniacozyma*, *Rhodotorula*, and
262 *Sporobolomyces* (Fig. 1A).

263

264 Individual vineyards and wine estates were distinguished based on the microbial community present
265 in the grape must/juice before fermentation (Fig. 1B; Supplementary Table S2). Permutational
266 multivariate analysis of variance (PERMANOVA) based on the Bray–Curtis distance confirmed that
267 fungal composition was significantly different between estates ($R^2 = 0.310$, $p < 0.001$) and at least two
268 vineyards ($R^2 = 0.573$, $p < 0.001$) regardless of grape variety. Pinot Noir demonstrated stronger
269 geographical differentiation (PERMANOVA, $R^2_{Estate} = 0.683$, $p < 0.001$; $R^2_{Vineyard} = 0.779$, $p < 0.001$)
270 than Chardonnay (PERMANOVA, $R^2_{Estate} = 0.470$, $p < 0.001$; $R^2_{Vineyard} = 0.566$, $p < 0.001$). Grape
271 variety weakly but significantly impacted fungal composition (PERMANOVA, $R^2 = 0.103$, $p =$
272 0.021). This impact was more distinct with an improved coefficient of determination (R^2) within a
273 certain wine estate (Fig. 1B; Supplementary Table S2). Principal coordinate analysis (PCoA) showed

274 the geographic patterns (95% confidence interval based on estates) of fungal communities across both
275 varieties, with 65.0% of total variance explained by the first two principal coordinate (PC) axes (Fig.
276 1B). Linear discriminant analysis (LDA) effect size (LEfSe) analysis further confirmed that these
277 patterns related to significant associations (Kruskal–Wallis sum-rank test, $\alpha < 0.05$) between fungal
278 taxa and wine estates (Fig. 1C), and grape varieties (Fig. 1D), respectively. *Microbotryomycetes*,
279 notably *Rhodotorula babjevae* and *Sporobolomyces roseus*, were observed with higher abundances in
280 the grape must/juice from wine estate A, with *Saccharomycetaceae* (notably fermentative yeast *S.*
281 *cerevisiae*), *Tremellomycetes* (including *Filobasidium* spp.), *Cladosporium grevilleae*,
282 *Mycosphaerellaceae*, *Stemphylium*, *Didymella* from wine estate B, and *Capnodiales* (notably
283 *Cladosporium* spp.), *Pleosporales* (including *Didymellaceae*, *Alternaria*, *Epicoccum*,
284 *Sporormiaceae*), *Exobasidiomycetes*, *Leotiomycetes* (including *Botrytis*), *Sordariomycetes* (including
285 *Cystofilobasidiales*, *Trichosphaeriales*, *Diaporthales*) from wine estate C. For varietal influences,
286 *Phaeomoniellales* including *Phaeomoniellaceae* and *Entylomataceae*, *Dothiora*, *Epicoccum*
287 *brasiliense*, *Sporobolomyces phaffii*, *Blastobotrys*, and *Ilyonectria* were significantly abundant in
288 Chardonnay juice, while *Botryosphaeriales*, *Capnodiales* (in particular *Cladosporium ramotenellum*),
289 *Dothideales* (in particular *Aureobasidium pullulans*), *Eurotiales* (including *Penicillium*),
290 *Saccharomycetes* (in particular *Hanseniaspora* spp.), *Microbotryomycetes_ord_Incertae_sedis*,
291 *Boeremia*, *Diaporthaceae*, *Sarocladium*, and *Amphisphaeriaceae* in Pinot Noir musts.
292
293 **3.2 Microbiota dynamics during spontaneous wine fermentation**
294 Significant decreases in the fungal diversity (ANOVA, $p < 0.001$) were recorded between
295 fermentation stages regardless of grape variety (Fig. 2A, Supplementary Fig. S2A). PCoA showed
296 that ferments were grouped according to their fermentative stage, where PC1 explained 66.0% and
297 57.3% of the total variance for Pinot Noir (Fig. 2B; PERMANOVA, $R^2 = 0.648$, $p < 0.001$) and
298 Chardonnay (Supplementary Fig. S2B; PERMANOVA, $R^2 = 0.608$, $p < 0.001$), respectively. BF
299 samples were clearly separated from MF and EF, while the latter groups were partially overlapped.
300

301 Within grape varieties, tracking major species across vineyards and estates (relative abundance >
302 1.00%) revealed fungal dynamics and succession during fermentation. All these species shown in the
303 alluvial diagrams presented significantly different relative abundances as the fermentation progressed
304 (ANOVA; FDR-corrected p value < 0.05) (Fig. 2C, Supplementary Fig. S2C). BF musts/juice
305 displayed diverse and variable collections of fungi, of which *A. pullulans* and *C. ramotenellum* were
306 the most abundant species for Pinot Noir and Chardonnay, respectively. In the beginning of Pinot
307 Noir fermentation, filamentous fungi and non-*Saccharomyces* yeasts dominated 89.9% of the
308 community, with 5.45% relative abundance for *S. cerevisiae*. After fermentation started, *S. cerevisiae*
309 grew and gradually dominated the community, occupying 66.5% of the MF community and 80.9% of
310 the EF community, while other species underwent drastic decreases in relative abundances during the
311 fermentation (Fig. 2C). Likewise, this succession pattern was also observed in Chardonnay ferments,
312 except that few taxa (*A. pullulans*, *R. babjevae*, *Hanseniaspora lachancei*) showed slight recoveries in
313 relative abundances between MF and EF (Supplementary Fig. S2C). Correspondingly, geographical
314 differences in fungal communities were not significant based on estates in both MF (PERMANOVA,
315 $R^2 = 0.162$, $p = 0.104$) and EF (PERMANOVA, $R^2 = 0.145$, $p = 0.162$) ferments, although fungi
316 differentiated vineyard origin of some vineyards in both stages ($p < 0.001$) (Supplementary Table S2).
317

318 **3.3 Yeast population dynamics during spontaneous wine fermentation**

319 To estimate yeast population dynamics during spontaneous fermentations, we isolated colonies based
320 on morphology on WLN medium and identified yeasts using the taxonomically distinctive 26S rRNA
321 D1/D2 region. A total of 359 yeast isolates were obtained (Supplementary Table S1), corresponding
322 to 14 species: *Hanseniaspora uvarum*, *Hanseniaspora opuntiae*, *Torulaspora delbrueckii*,
323 *Metschnikowia andauensis*, *Meyerozyma guilliermondii*, *Candida africana*, *Candida intermedia*,
324 *Candida oleophila*, *Candida ishiwadae*, *Rhodotorula mucilaginosa*, *Pichia membranifaciens*,
325 *Wickerhamomyces anomalus*, *Saccharomyces bayanus*, and *S. cerevisiae* (Supplementary Table S3).
326 All of these species had also been identified with ITS amplicon sequencing (give figure/table
327 number). *S. cerevisiae* and the non-*Saccharomyces* species of *H. uvarum*, *H. opuntiae*, *T. delbrueckii*,
328 *M. andauensis* dominated the isolates, whereas other species appeared sporadically. At the beginning

329 of fermentation, the grape must/juice harboured high species diversity (Shannon index) of yeasts, with
330 non-*Saccharomyces* yeasts dominating the populations (Supplementary Table S3). The amount and
331 distribution of yeast species differed among vineyards, estates, and varieties (Supplementary Table
332 S3), with significant geographical differences observed in population compositions (PERMANOVA
333 based on Bray–Curtis distance, $R^2_{Estate} = 0.287$, $p = 0.019$; $R^2_{Vineyard} = 0.426$, $p = 0.002$). As
334 fermentation proceeded, the viable population of yeast in the must increased from initial values of
335 10^4–10^6 to 10^7–10^8 CFU/mL in the middle fermentation (MF) and declined coinciding with decreased
336 species diversity during fermentation (Supplementary Table S3). Non-*Saccharomyces H. uvarum* and
337 *H. opuntiae* were isolated throughout some fermentations, with *T. delbrueckii* isolated at the
338 beginning and middle fermentation points. In spite of low initial abundance, *S. cerevisiae* dominated
339 the ferments at middle and end fermentation points and occupied 100% of some yeast populations
340 from wine estate B (Supplementary Table S3).
341
342 From the isolated yeasts, 94 *S. cerevisiae* strains were further analysed through 12 microsatellite loci,
343 resulting in 80 multilocus genotypes, with 14 strains showing genotypes identical to others in this
344 study. The 12 microsatellite loci recorded from 4 to 23 different alleles per locus, of which C5 and
345 SCAAT1 displayed the highest diversity with 24 and 21 alleles, respectively. Two strains were shared
346 between vineyards and varieties, while other identical strains were isolated from within a single
347 vineyard and are likely the same strain. The neighbour-joining tree built from the Bruvo's distance
348 showed clustering linked to the geographica l origin of strains (Fig. 3A). Some branches clustered
349 isolates from one wine estate with very close genetic relationships as group I (A), group II (B), and
350 group III (C). Some strains from estate A and B gathered in clusters, with only few strains from estate
351 C (group IV, V). Group VI was composed of clusters originating from all three estates. The
352 relationships among *S. cerevisiae* strains inferred using DAPC were consistent with the genetic tree
353 (Fig. 3B). The total amount of genetic variation explained by the first 35 principal components was
354 93.8%, of which 24.12% was conserved by the first two axes. Most populations were clearly
355 separated into estate A, B, and C, with overlaps observed among groups (Fig. 3B). Strains V1.2.1,
356 V9.1.2, and V11.2.4 among three estates were coinciding with group VI in the phylogenetic tree, as

357 well as strains V4.3.4 and V7.2.6 between estate A and B clusters with group IV (Fig.3). AMOVA
358 confirmed significant influences of geographic origins (estate/vineyard, $p = 0.001$) and the grape
359 variety ($p = 0.023$) on the *S. cerevisiae* population structure based on, in particular based on estates
360 (Supplementary Table S4). Within estates, the difference between vineyards or varieties was not
361 significant (Supplementary Table S4). Higher differentiation between was observed between wine
362 estates A and C (12 km; $F_{ST} = 0.143$, $p < 0.001$), B and C (10 km; $F_{ST} = 0.133$, $p < 0.001$), than
363 between A and B (8 km; $F_{ST} = 0.076$, $p < 0.001$) (Supplementary Table S4).
364

365 **3.4 Aroma profiles are distinctive for wines of each geographical origin**

366 Using GC-MS, we analysed the volatile compounds of Pinot Noir and Chardonnay wine samples (at
367 the end of fermentation) in triplicate to represent wine metabolite profiles. In all, 79 volatile
368 compounds were identified in these wines. Pinot Noir wines contained 37 geographically differential
369 compounds based on wine estates, and Chardonnay wines contained 46 (Supplementary Table S5).
370 Within grape varieties, wine complexity (as determined by Shannon index) was not significantly
371 different amongst wine estates (ANOVA; $F_{\text{Pinot Noir } (2, 12)} = 2.393$, $p = 0.161$; $F_{\text{Chardonnay } (2, 15)} = 2.313$, $p =$
372 0.284). Wine aroma profiles are clearly separated with PCoA based on Bray–Curtis dissimilarity
373 according to estates, where PC1 explained 65.8% and 62.7% of the total variance for Pinot Noir wines
374 (PERMANOVA, $R^2 = 0.722$, $p = 0.011$) and Chardonnay wines (PERMANOVA, $R^2 = 0.773$, $p =$
375 0.002), respectively (Fig. 4).
376

377 **3.5 Fungal microbiota correlate to wine aroma profiles**

378 To elucidate the relationship between geographically differential fungal microbiota and wine
379 metabolites, partial least squares regression (PLSR) was used to model covariance between fungal
380 species and volatile compounds. PSLR projections were made with dominant species in the grape
381 must (relative abundance > 0.01% across samples; Pinot Noir, 20 species; Chardonnay, 23 species)
382 and volatile compounds in the resulting wines. Compounds shown in the plots explained > 20% of the
383 variance in the first two components (Fig. 5), of which many were the same compounds identified by
384 ANOVA (Supplementary Table S5, S6). PLSR showed highly covariable relationships between

385 fermentative yeasts and volatile compounds. In Pinot Noir wines, some interesting correlations were
386 observed; for example *S. cerevisiae* and *T. delbrueckii* correlated strongly with several esters (C33,
387 Ethyl octoate; C26, Ethyl lactate; C55, 3-Methylbutyl octanoate; C38, Ethyl 6-heptenoate); *M.*
388 *guilliermondii* with monoterpenes (C43, linalool; C48, terpinen-4-ol; C68, nerol; C59, *α*-terpineol), *β*-
389 damascenone (C70), nonanals (C41, 2-nonanol; C56, 1-nonanol) and the related ester (C42, ethyl
390 nonanoate); and *H. uvarum* and *H. lachancei* with alcohols (C46, 2,3-butanediol; C63, 1-decanol) and
391 esters (C62, methyl salicylate; C76, ethyl tetradecanoate). In Chardonnay wines, *S. cerevisiae* was
392 associated with some esters (for example, C33; C55; C16, ethyl hexanoate; C61, benzyl acetate; C20,
393 hexyl acetate), *T. delbrueckii* and *M. guilliermondii* with some alcohols (C63; C44, 1-octanol) and
394 esters (C26; C38; C42; C76), and *H. uvarum* and *H. lachancei* with C46, C62, and fatty acids (C45, 2-
395 methyl-propanoic acid; C72, hexanoic acid; C77, octanoic acid).
396
397 To disentangle the role of microbial communities on wine metabolites, we used structural equation
398 modelling (SEM) (Grace, 2006) to testify fungal community compositions (the first axis of PCoA) at
399 multiple levels simultaneously: fungal communities, yeasts (*S. cerevisiae* and non-*Saccharomyces*
400 yeasts), and *S. cerevisiae* populations. The SEM explained 84.9% of the variance found in the
401 geographical pattern of wine aroma (Fig. 6). Fungal communities in the must drove wine aroma
402 profiles directly (path coefficient = 0.286***) and indirectly by effects on yeasts and *S. cerevisiae*
403 populations, in particular strong influences on yeast populations (path coefficient = 0.562). *S.*
404 *cerevisiae* populations had the highest direct positive effects on the resulting wine aroma
405 characteristics, while yeast populations had the lowest but significant effects (Fig. 6A). Overall, *S.*
406 *cerevisiae* populations were the most important driver of wine characteristics (Fig. 6B).
407
408 **4. Discussion**
409 There is mounting evidence for geographical differentiation of wine-related microbial communities at
410 regional scales (Bokulich et al., 2014; Gayevskiy and Goddard, 2012; Jara et al., 2016; Pinto et al.,
411 2015; Taylor et al., 2014). Our previous work revealed that different wine-producing regions in
412 southern Australia possess distinct, distinguishable microbial patterns (especially fungal microbiota)

413 at the scale of 400 km, correlated with local weather conditions and soil properties (Liu et al., 2020).
414 In the current study, within a single sub-region spanning 12 km, we demonstrate geographical
415 differentiation of grape must/juice fungal communities, yeast populations, and *S. cerevisiae*
416 populations, with influences from the grape variety.

417 **4.1 Fungal ecology at multiple layers during spontaneous wine fermentation**

418 In the freshly crushed grape must/juice, fungal communities were highly diverse and characterised by
419 ubiquitous genera such as *Aureobasidium*, *Cladosporium*, *Saccharomyces*, and *Rhodotorula*, deriving
420 from the vineyard ecosystem. Geographical origin had a greater impact on the fungal community
421 (Supplementary Table S2) and yeast populations (results 3.2) than grape variety, and this is in line
422 with other studies in grapevine-associated microbiota (Bokulich et al., 2014; Mezzasalma et al., 2018;
423 Wang et al., 2015). In particular, *S. cerevisiae* yeasts was one of geographical features (Fig. 1C).
424 Given the small scale of the vineyards in this study (< 12 km), macroclimate does not differentiate the
425 sites, and so local conditions appear to modulate communities. Geographical features (for example
426 vineyard orientation), microclimate, and soil properties (for example nutrient availability) could
427 explain some variation among vineyard sites, but these measures were beyond the scope of this study.
428 Within a single estate, grape variety played a significant role in shaping fungal communities (Fig. 1B;
429 Supplementary Table S2), suggesting a genetic component to plant-microbial interactions (Bokulich
430 et al., 2014; Mezzasalma et al., 2018). Cultivar variation in the microhabitat and environmental stress
431 responses may explain how grapevines recruit their associated microbiota, including both the normal
432 microbiota and cultivar-specific susceptibilities to disease pressures (Fung et al., 2008). Our results
433 show that differential taxa between varieties are ubiquitous fungi in agricultural ecosystems, and some
434 of these taxa are considered to be grapevine pathogens, for example *C. ramotenellum* and
435 *Mycosphaerella tassiana* (Bensch et al., 2012), were more abundant in Pinot Noir musts (Fig. 1D).
436

437 Geographical signatures diminished during spontaneous wine fermentation as growth of fermentative
438 yeasts reshaped the community diversity and composition, in particular *S.cerevisiae* (Fig. 2, S2;
439 Supplementary Table S2, S3). Regardless of grape variety, fungal and yeast species diversity

440 collapsed as alcoholic fermentation progressed, with a loss of the environmental fungi (Fig. 2A, S2A;
441 Supplementary Table S3), indicating evolution through selection associated with wine fermentation.
442 Non-*Saccharomyces* yeasts start the fermentation process (especially genera *Hanseniaspora*,
443 *Candida*, *Pichia* and *Metschnikowia*), but they are quickly replaced by *S.cerevisiae* that lead
444 fermentation until the end (Capozzi et al., 2015; Fleet, 2003). The inability of non-*Saccharomyces*
445 yeasts to sustain their presence in ferments has been attributed to their oxidative and weakly
446 fermentative metabolism, their sensitivity to increasing fermentation rate, ethanol and heat production
447 induced by *S.cerevisiae* growth, and being less tolerant to low oxygen availability (Bokulich et al.,
448 2016; Combina et al., 2005; Goddard, 2008). Thus, fermentation conditions, such as the chemical
449 environment and interactions within the community drive the microbial pattern into a population
450 dominated by *S. cerevisiae* (Fig. 2C, S2C; Supplementary Table S3) (Liu et al., 2017).
451
452 Spontaneous wine fermentation is characterised not only by significant intraspecific biodiversity
453 (Cocolin et al., 2000), but also by high genetic polymorphism in the *S.cerevisiae* population present
454 during the fermentation. Biogeography of *S.cerevisiae* has been previously reported from regional
455 (Gayevskiy and Goddard, 2012; Knight and Goddard, 2015) and global (Legras et al., 2007; Liti et al.,
456 2009) scales to small scales between vineyards within a single region (Börlin et al., 2016; Schuller
457 and Casal, 2007), while Knight et al. (2019) demonstrated no vineyard demarcation between *S.*
458 *cerevisiae* that could be ascribed to gene flow. Here, we show that distinct geographical
459 differentiation between wine estates, with increased genetic divergence with distance (Schuller and
460 Casal, 2007). A certain degree of mixed strains from various vineyard sites indicates gene flow among
461 populations at small scales. Within the wine estates, the vineyards are within a 5 km radius from one
462 another, and thus insect vectors like honeybees, wasps, and fruit flies, as well as birds, may
463 homogenise the yeast populations (Francesca et al., 2012; Goddard et al., 2010; Lam and Howell,
464 2015; Stefanini et al., 2012). Shared staff and agricultural implements may also facilitate the
465 movement and exchanges of *S.cerevisiae* populations between vineyards managed by the same estate
466 (Goddard et al., 2010). Harvested grapes from various vineyards within the estate were processed at
467 the same winery, where equipment surfaces may harbour large populations of *S.cerevisiae* and other

468 yeasts under normal cleaning conditions, acting as potential reservoir for wine microbiota during
469 spontaneous fermentation (Bokulich et al., 2013; Ciani et al., 2004; Sabate et al., 2002). All these
470 factors contribute to the estate-specific pattern of *S.cerevisiae* distribution.
471
472 **4.2 Association between fungal microbiota and composition of the wine metabolome**
473 Previous research suggested that fungal microbiota structured and distinguished vineyard ecosystems
474 impacting the aroma and quality of wine at the regional scale (Liu et al., 2020). Here we show that the
475 geographical diversification observed in fungal communities, yeasts, and *S.cerevisiae* could translate
476 to aromatic differences in wines within a single region. Wine aroma profiles highly associated with
477 fermentative yeasts, in particular, *S.cerevisiae* displayed the most important effects on wine
478 characteristics at this scale (Fig. 5, 6). Other fungal species, although non-fermentative (or not known
479 to be associated) with wine fermentation, could produce some sensory-active compounds associated
480 with wine aroma formation (Verginer et al., 2010), or substantially modulate vine health, growth, and
481 fruit quality in the vineyard (Berg et al., 2014; Gilbert et al., 2014). The most widespread fungi *A.*
482 *pullulans* are known to have an antagonistic effect on mould development, like *Botrytis cinerea*,
483 causing grey rot, and *Aspergillus* spp., producing ochratoxin (Barata et al., 2012). Further, host-
484 microbe interactions could promote plant resistance to environmental/abiotic stress thus benefiting
485 crop production (Berg, 2009; Lugtenberg and Kamilova, 2009).
486
487 In the resultant wines, most volatile compounds were esters, alcohols, acids and aldehydes, many of
488 which are fermentation-derived products. Some compounds were grape-derived, for example
489 monoterpenes, and potentially modified by microbial metabolism during fermentation (Swiegers et
490 al., 2005). Non-*Saccharomyces* yeasts dominating the initial spontaneous fermentation can contribute
491 to the overall wine aroma profiles by producing flavour-active compounds, which depends on yeast
492 species and strains (Capozzi et al., 2015; Jolly et al., 2014). Here, our work shows the presence of *T.*
493 *delbrueckii*, *M. guilliermondii*, *and Hanseniaspora spp.* correlated with some volatile compounds,
494 such as higher alcohols, ethyl esters, and acids (Fig. 5), thus potentially affecting wine characteristics.
495 *M. guilliermondii*, which is known to produce *β*-glucosidases to release bound terpenoids (Silva et al.,

496 2005), associated with monoterpenes including linalool, terpinen-4-ol, nerol, and α-terpineol, thus
497 enhancing varietal aroma in wine and local *terroir* expression. Beyond fermentative contributions, the
498 presence of non-*Saccharomyces* yeasts and the species diversity indirectly affect wine ecosystem
499 function by altering the ecological dominance of *S. cerevisiae* through antagonistic interactions in
500 early succession (Bagheri et al., 2017; Boynton and Greig, 2016). *S. cerevisiae* eventually dominate
501 the fermentation, where they are naturally initially rare, by modifying the environment through
502 fermentation, and drive wine ecosystem function (Boynton and Greig, 2016; Goddard, 2008). *S.*
503 *cerevisiae* show genetic diversity at strain level and it is well documented that different genotypes
504 produce variable amounts of volatile compounds as fermentation by-products, with desirable or
505 undesirable impacts on wine aroma and flavour (Capece et al., 2012; Howell et al., 2004; Pretorius,
506 2000; Romano et al., 2003). Knight et al. (2015)found that *S. cerevisiae* genotypes and wine
507 phenotypes were correlated with geographic dispersion, and regional populations produced distinct
508 aroma profiles in Sauvignon Blanc wine fermentation. Our results further suggest that within a single
509 region, wine aroma profiles were affected by the geographical origin and genomics of *S. cerevisiae*
510 natural strains. As the keystone driver of the wine ecosystem, *S. cerevisiae* was associated with many
511 ethyl esters and acetate esters, exerting the most powerful influences of multiple layers of fungal
512 microbiota on wine quality and style (Fig. 5, 6). A causative relationship between the geographically
513 differentiated fungi, yeasts, and *S. cerevisiae* should be established to show the impact on wine
514 compounds for regional differentiation based on sensory characteristics.
515

516 ## 5. Conclusions

517 Our study describes the diversity of fungal communities during spontaneous wine fermentation in
518 carefully selected vineyards comprising two cultivars. As predicted, we observed ecological
519 dominance *Saccharomyces* spp., but showed that geographical diversification is evident in the initial
520 fungal community composition and the strain level diversity of *S. cerevisiae*. Fungal species
521 correlated with wine volatile compounds, of which *S. cerevisiae* is likely the primary driver of wine
522 aroma and characteristics within the sub-region (less than 12 km). A better understanding of how

523 multiple layers of fungal microbiota vary at different scales, and the effects of these communities on
524 agricultural ecosystems, provides perspectives for sustainable management practices maintaining the
525 biodiversity and functioning thus optimising plant food and beverage production.
526

527 Declaration of competing interest

528 We declare that this research was conducted in the absence of any commercial or financial
529 relationships that could be constructed as a potential conflict of interest.
530

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740 ## Figures and figure legends

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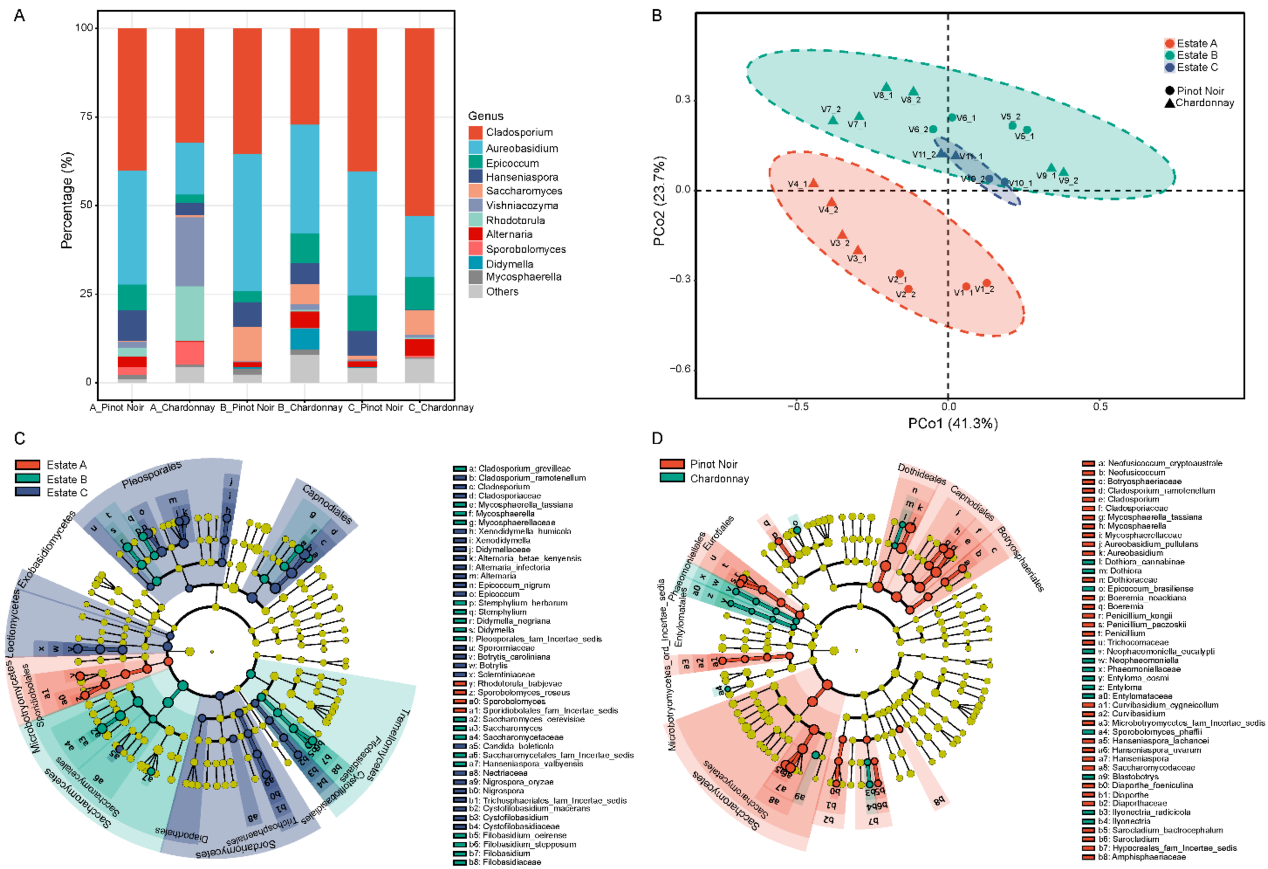

756 **Fig. 1** Musts before fermentation (BF) have differential fungal communities depending geographical origins and grape varieties. (A) Microbial community
757 composition characterised to the genus level (11 genera, relative abundance > 1.00% shown); (B) Principal coordinate analysis (PCoA) based on Bray-Curtis
758 distances among wine estates across both varieties; (C) Linear discriminant analysis (LDA) effect size (LEfSe) taxonomic cladogram identifying significantly
759 discriminant (Kruskal–Wallis sum-rank test $\alpha < 0.05$; LDA score > 2.00) taxa associated with wine estates; (D) LEfSe taxonomic cladogram identifying
760 significantly discriminant taxa associated with grape varieties.

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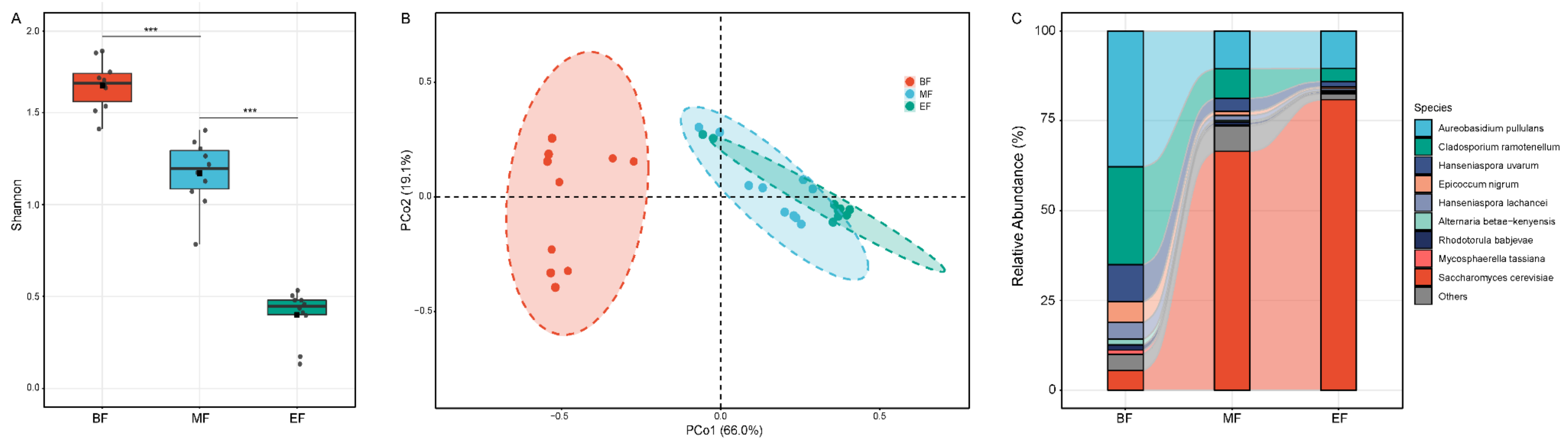

772 **Fig. 2** Stage of fermentation influences microbial diversity and composition of Pinot Noir. (A) α-diversity (Shannon index) significantly decreases ($p <$
773 0.001***) during the fermentation; (B) Bray-Curtis distance PCoA of fungal communities according to the fermentation stage (BF, before fermentation; MF,
774 at the middle of fermentation; EF, at the end of fermentation); (C) Relative abundance changes of major fungal species (9 species; relative abundance >
775 1.00%) that significantly differed by fermentation stage (ANOVA; FDR-corrected p value < 0.05).

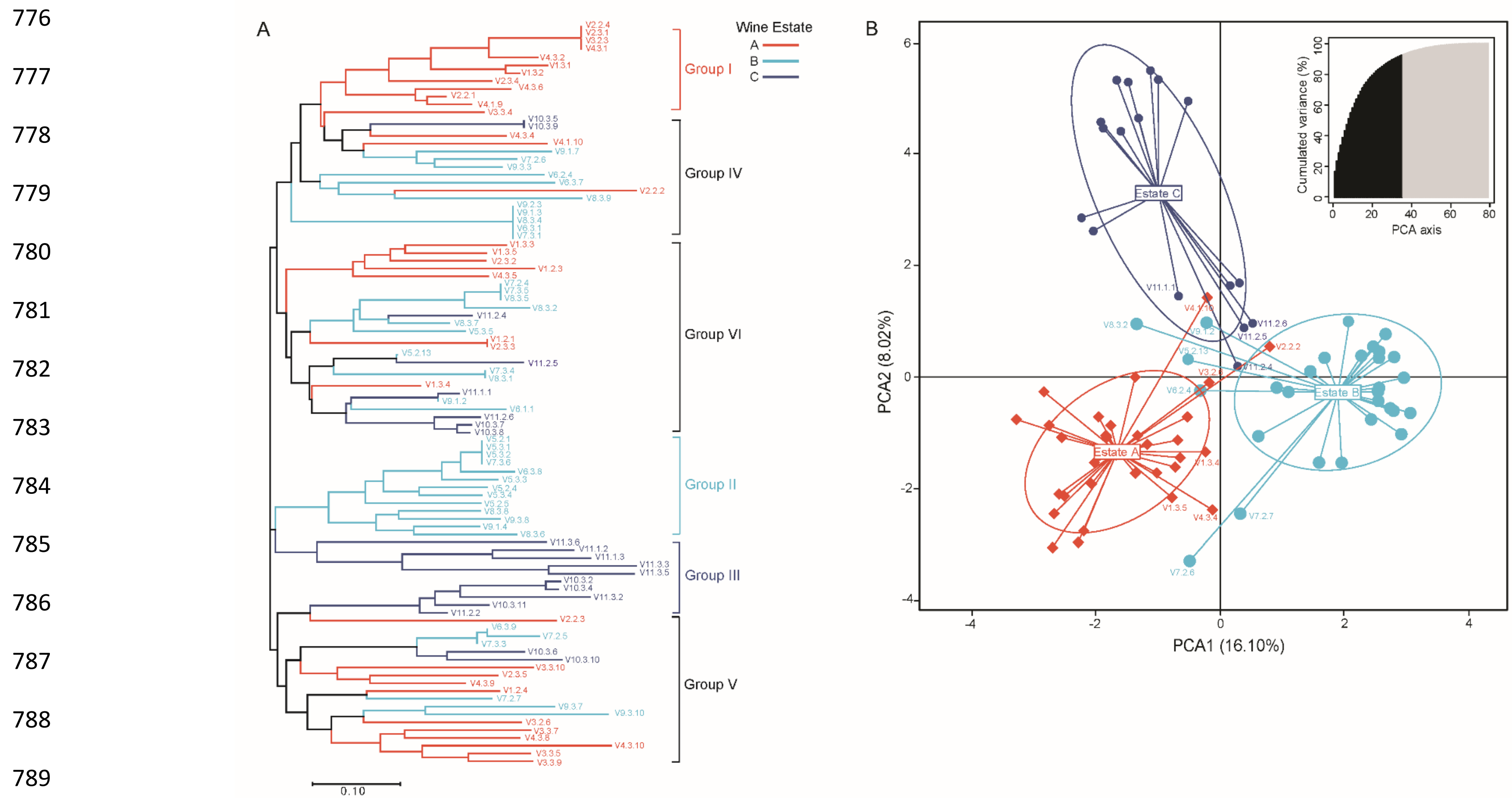

Fig. 3 *Saccharomyces cerevisiae* populations show geographic clustering. (A) Neighbour-joining tree showing the clustering of 94 *S. cerevisiae* strains isolated from three wine estates. The tree was constructed from the Bruvo's distance between strains based on the polymorphism at 12 loci and is rooted according to the midpoint method. Branches are coloured according to the wine estate from which strains have been isolated. (B) Scatterplot from discriminant analysis of principal components (DAPC) of the first two principal components discriminating *S. cerevisiae* populations by estates. Points/diamonds represent individual observations, and lines represent population memberships. Inertia ellipses represent an analog of a 67% confidence interval based on a bivariate normal distribution. Number of principal components (n = 35) at which the maximal reassignment of samples occurred are depicted as black lines the PCA graph on the topright corner, with subsequent components in grey line.

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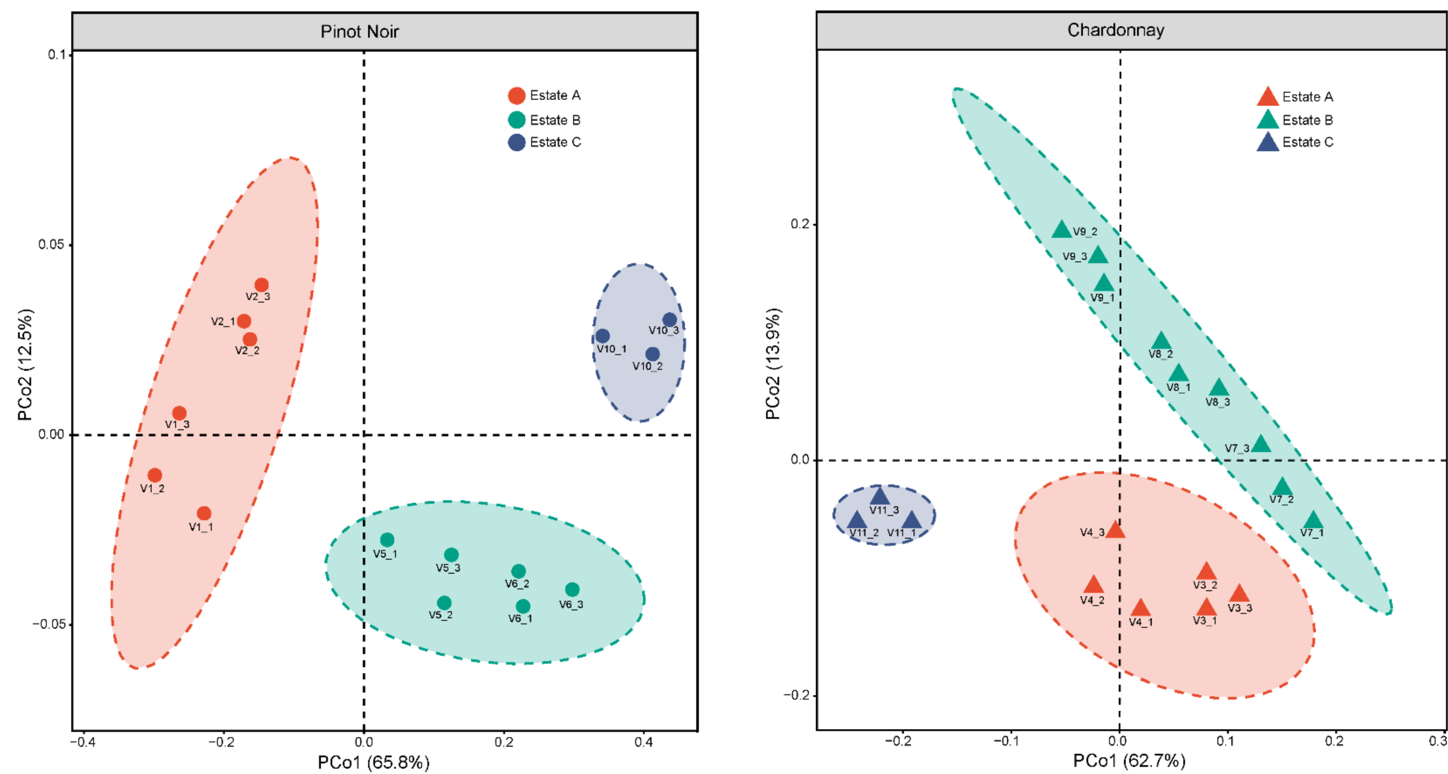

810 **Fig. 4** Wine metabolites exhibit geographical variation. PCoA based on Bray-Curtis dissimilarity obtained from comparing volatile profiles of Pinot Noir and

811 Chardonnay wines.

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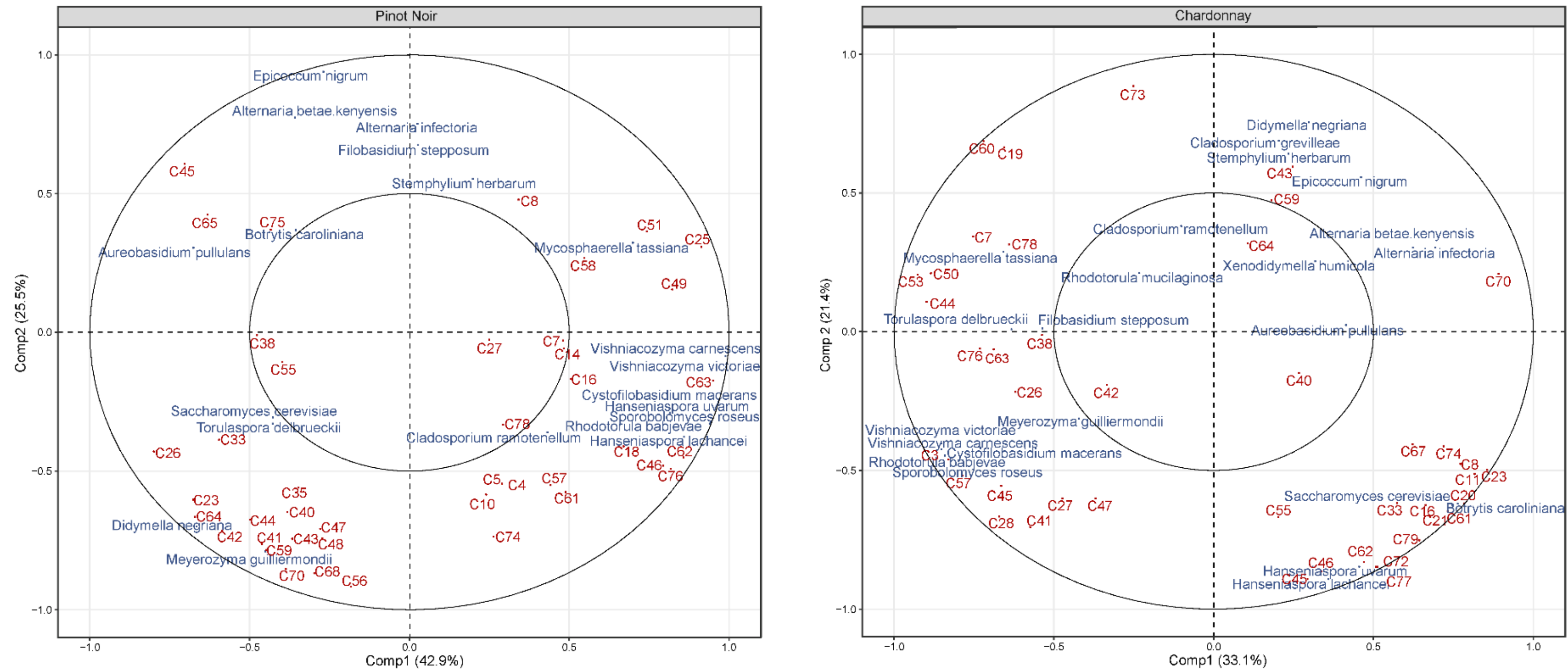

825 **Fig. 5** Partial least squares regression (PLSR) demonstrates microbial influence on volatile compounds of Pinot Noir and Chardonnay wines.

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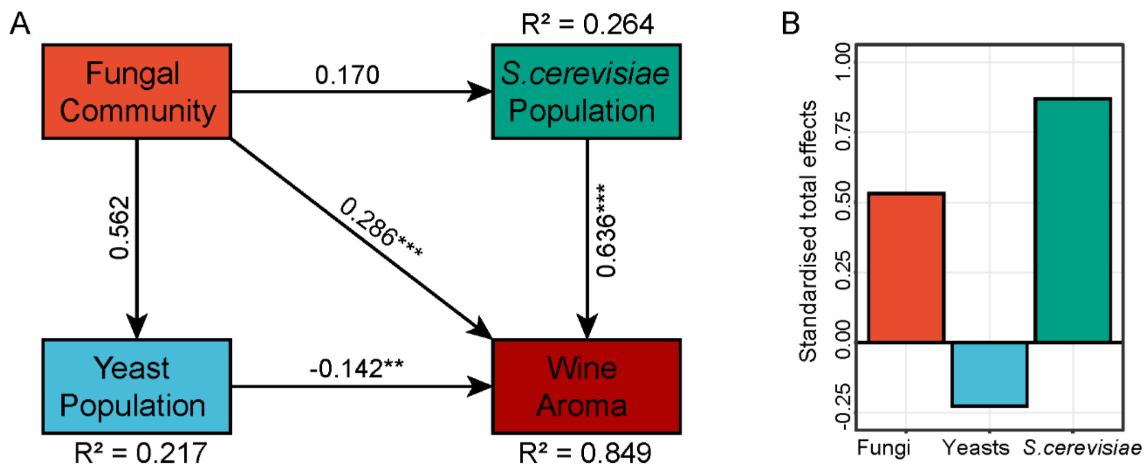

835 **Fig. 6** Direct and indirect effects of fungal community composition (the first axis of PCoA) at
836 multiple levels on wine aroma profiles. Structural equation model (SEM) fitted to wine aroma
837 composition (A) and standardised total effects (direct plus indirect effects) derived from the model
838 (B).

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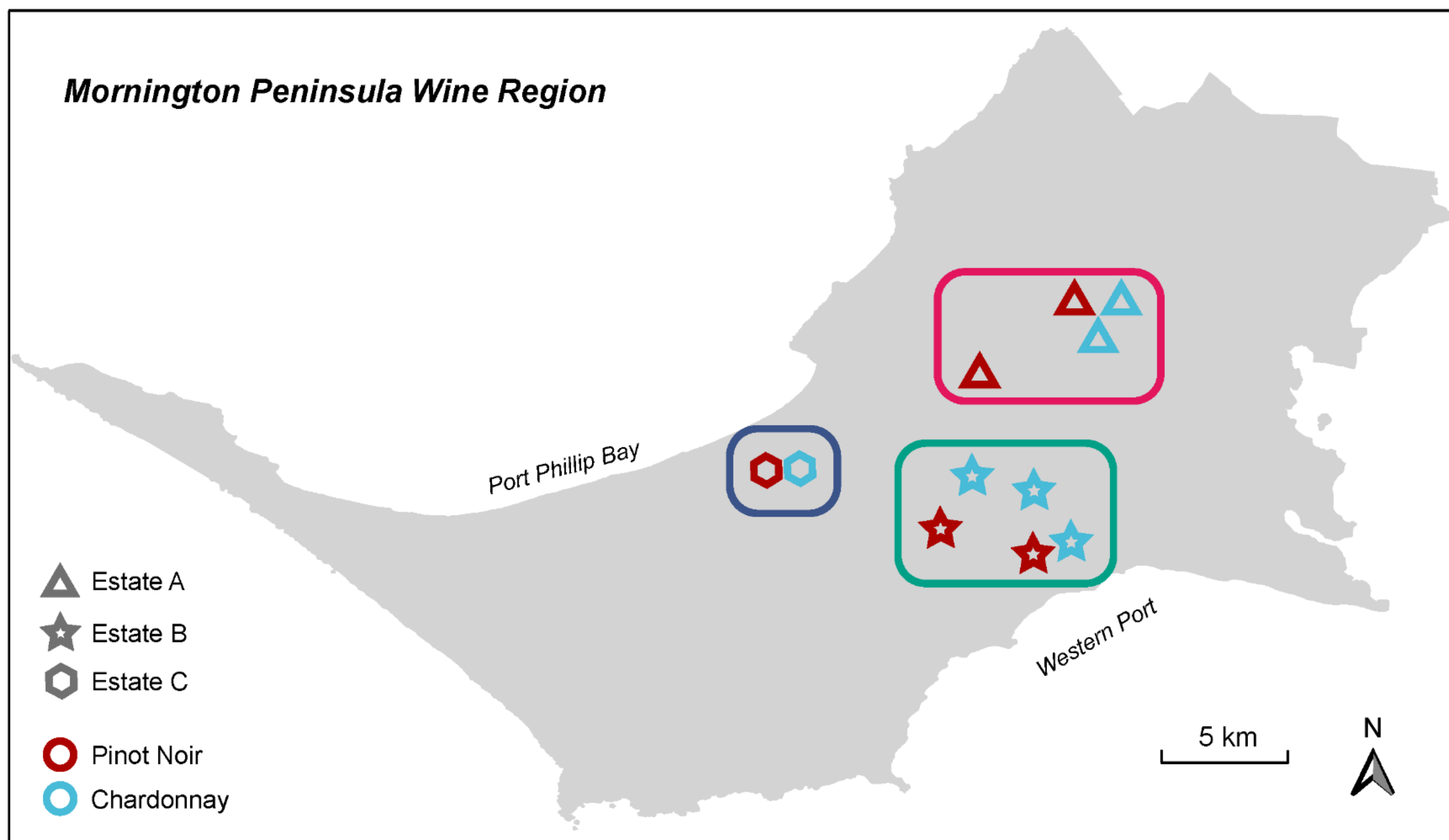

853 **Fig. S1** Sampling map of 11 vineyards from three wine estates in Mornington Peninsula wine region, Australia.

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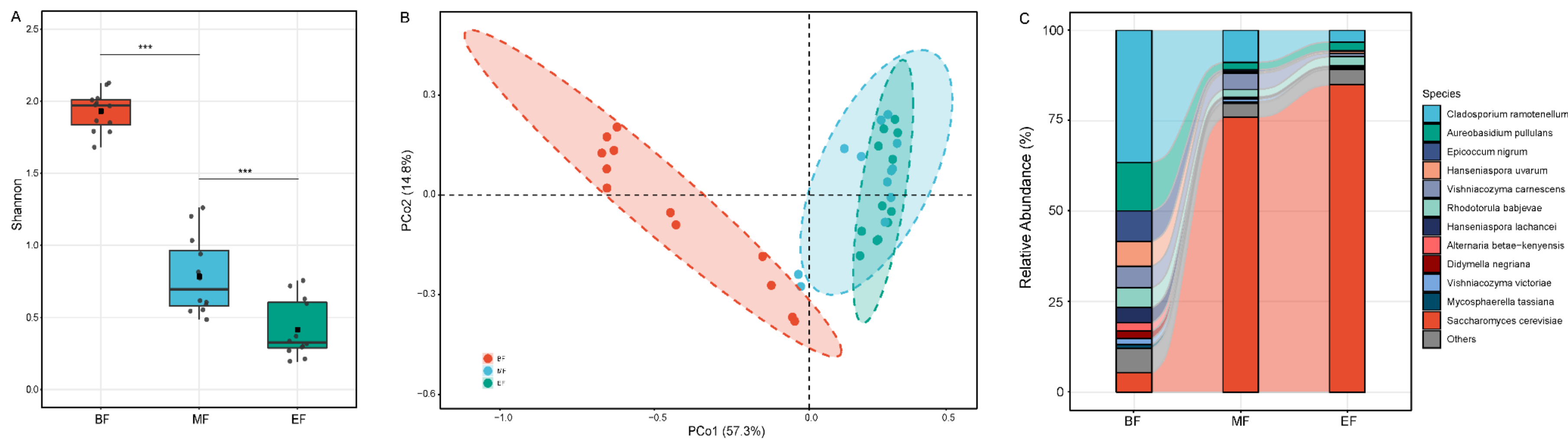

864 **Fig. S2** Stage of fermentation influences microbial diversity and composition of Chardonnay. (A) α-diversity (Shannon index) significantly decreases ($p <$
865 0.001***) during the fermentation; (B) Bray-Curtis distance PCoA of fungal communities according to the fermentation stage (BF, before fermentation; MF,
866 at the middle of fermentation; EF, at the end of fermentation); (C) Relative abundance changes of major fungal species (12 species; relative abundance >
867 1.00%) that significantly differed by fermentation stage (ANOVA; FDR-corrected p value < 0.05).