Quantifying the efficiency and biases of forest *Saccharomyces* sampling strategies

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Abstract

Saccharomyces yeasts are emerging as model organisms for ecology and evolution, and researchers need environmental *Saccharomyces* isolates to test ecological and evolutionary hypotheses. However, methods for isolating *Saccharomyces* from nature have not been standardized and isolation methods can influence the genotypes and phenotypes of studied strains. We developed a direct isolation method for forest floor *Saccharomyces* and compared its success and phenotypic biases to a previously published enrichment-based isolation method. In a European forest, direct isolation was more successful at isolating *S. paradoxus*, but also more labor intensive, than enrichment culturing. Average growth rates of *S. paradoxus* isolates collected using the two methods did not differ at the enrichment isolation temperature, but variances in growth rates did: direct isolation produced a collection of *S. paradoxus* isolates with less variation in growth rates than enrichment culturing. In other words, enrichment culturing sampled more phenotypic diversity than direct isolation. Enrichment culturing also sampled more *Saccharomyces* species diversity than direct isolation, including our only isolations of rare *S. cerevisiae*. Enrichment culturing may sample higher *Saccharomyces* phenotypic and species diversity because of variations in interactions between yeasts and the other microbes that were present in the soil and leaf litter samples. We recommend direct culturing for researchers interested in randomly sampling their study habitats and enrichment culturing for researchers interested in discovering new *Saccharomyces* phenotypes or rare *Saccharomyces* species from natural environments. We include step-by-step sampling protocols in the supplemental materials.

41 **Introduction**

42 Naturally-occurring *Saccharomyces* populations are models for ecology and
43 evolution (Boynton & Greig, 2014). Use of these models has led to exciting
44 discoveries about the ecology and evolution of microbial phenotypes; for example,
45 adaptation to climate can lead to speciation (Leducq et al., 2014), domesticated *S.*
46 *cerevisiae* is more phenotypically diverse than wild *S. paradoxus* (Warringer et al.,
47 2011), and interspecific hybrids can have high fitnesses in stressful environments
48 (Bernardes, Stelkens, & Greig, 2017; Stelkens, Brockhurst, Hurst, Miller, & Greig,
49 2014). These studies made inferences based on the phenotypes and genotypes of
50 isolates collected from wild and domesticated substrates. And *Saccharomyces*
51 substrates are diverse: wild substrates include tree bark, insect guts, leaf litter, soil,
52 fruits, and parasitic *Cyttaria* galls, (Kowallik & Greig, 2016; Libkind et al., 2011;
53 Mortimer & Polsinelli, 1999; Sampaio & Goncalves, 2008; Stefanini et al., 2012), and
54 domesticated substrates include wine, beer, bread, kimchi, kombucha, palm wine, and
55 pulque, among many other substrates (Boynton & Greig, 2016; Carbonetto,
56 Ramsayer, Nidelet, Legrand, & Sicard, 2018; Estrada-Godina et al., 2001; Ezeronye
57 & Okerentugba, 2001; Gallone et al., 2016; Greenwalt, Steinkraus, & Ledford, 2000;
58 Jeong, Jung, Lee, Jin, & Jeon, 2013). One challenge of environmental yeast sampling
59 is to minimize sampling biases so researchers can assure that differences among
60 source environments, not sampling techniques, are responsible for observed
61 phenotypic patterns.

62 Enrichment culturing is a reliable and frequently-used method for isolating
63 difficult-to-culture microbes, including *Saccharomyces*, from natural environments
64 (Schlegel & Jannasch, 1967; Sniegowski, Dombrowski, & Fingerman, 2002) (Figure
65 1A). Microbiologists have been relying on enrichment cultures for over a century

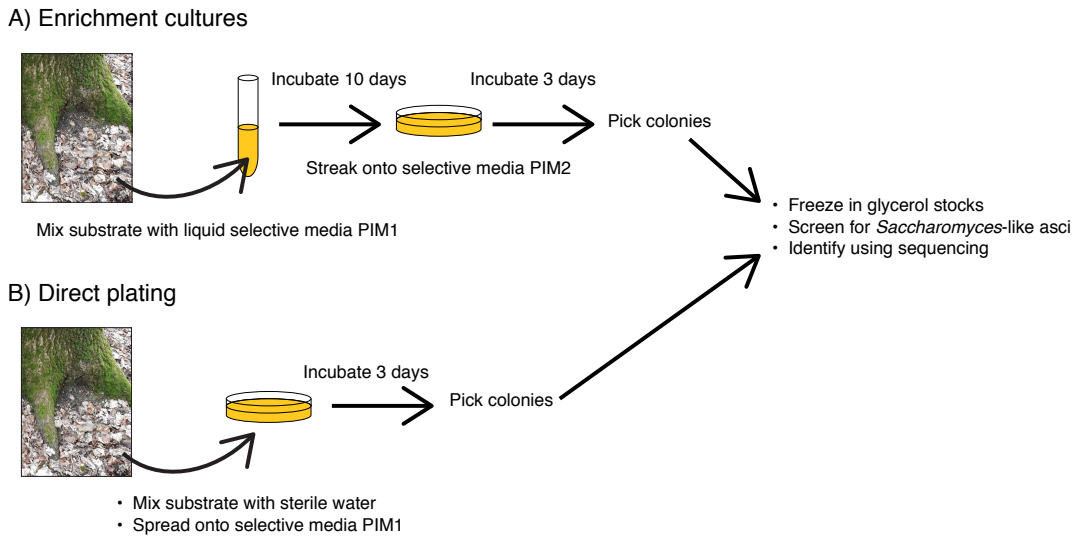

Figure 1: Schematic illustration of sampling strategies used to isolate *Saccharomyces* for this project. A) Enrichment culturing B) Direct plating. Photo: Doreen Landermann

(Beijernick, 1961), and have used enrichment culturing to isolate many of the model *Saccharomyces* strains commonly used in laboratory studies (Johnson et al., 2004; Liti et al., 2009; Sniegowski et al., 2002). To isolate a microbe using enrichment culturing, a researcher adds a small amount of natural material to a growth medium designed to be hospitable to the target microbe and inhospitable to other microbes (Liti, Warringer, & Blomberg, 2017; Schlegel & Jannasch, 1967). If the enrichment medium is well-designed, the target microbe is expected to grow in abundance, and after some incubation time, this enrichment culture can be streaked to solid media and colonies of the target microbe can be easily isolated. An alternative to enrichment culturing is to spread a microbial substrate directly onto a selective solid medium, with or without dilution, and to pick colonies which morphologically resemble the target microbe (Stefanini et al., 2012) (Figure 1B).

Because it can be difficult to isolate *Saccharomyces* from natural substrates, many investigations of wild *Saccharomyces* rely on enrichment culturing, usually in high-sugar, acidic media (Charron, Leducq, Bertin, Dube, & Landry, 2014; Robinson, Pinharanda, & Bensasson, 2016; Sniegowski et al., 2002; Sweeney, Kuehne, &

82 Sniegowski, 2004). Comparative studies of *Saccharomyces* genomes have been
83 carried out using *Saccharomyces* strains isolated using disparate strategies, including
84 both enrichment and direct culturing (Liti et al., 2009; Peter et al., 2018). However,
85 isolation strategy can influence the genotypes and phenotypes of isolated microbes
86 (Stefani et al., 2015), and we were concerned about the biases that might be
87 introduced during enrichment culturing of *Saccharomyces* yeasts. Specifically, we
88 were concerned that isolation method might bias results in studies of *Saccharomyces*
89 phenotypes. For example, enrichment culturing might select for individuals with high
90 relative fitness in the enrichment medium. Such potential biases in sampled yeast
91 phenotypes are likely to lead to biases in sampled genotypes because genetic
92 information is responsible for expressed phenotypes. Isolation biases have also been
93 suggested as potential explanations for differences between results of culture-
94 dependent and culture-independent studies of environmental *Saccharomyces*
95 (Alsammar et al., 2018).

96 This study's goals were to compare isolation success between enrichment
97 culturing and a direct culturing strategy, and to quantify biases in *Saccharomyces*
98 phenotypes (and therefore genotypes) that might be introduced when sampling a
99 forest environment. We tested the assumption that it is easier to sample
100 *Saccharomyces* from forest substrates using enrichment cultures than direct plating.
101 We also compared growth rates between *S. paradoxus* isolated using enrichment and
102 direct strategies. Enrichment culturing might decrease or increase sampled *S.*
103 *paradoxus* phenotypic diversity compared to direct plating, thereby decreasing or
104 increasing variance among *S. paradoxus* growth rates. For example, variance among
105 growth rates would be low (and average growth rates high) among *S. paradoxus*
106 isolated using enrichment cultures if the enrichment temperature and media select for

107 the fastest growing *S. paradoxus* present in every sample. Conversely, variance
108 among growth rates would be high for *S. paradoxus* isolated using enrichment
109 cultures if diversity in the non-*Saccharomyces* microbial communities present on
110 sampled substrates select for diverse *S. pardoxus* among samples.

111 To test these predictions, we compared *Saccharomyces* sampling success and
112 isolates' growth rates among soil and leaf litter samples from a well-studied northern
113 German forest (Kowallik & Greig, 2016; Kowallik, Miller, & Greig, 2015). A
114 previous study showed that *S. paradoxus*, the wild sister species of the lab model *S.*
115 *cerevisiae*, is readily isolated using enrichment cultures from oak leaf litter in this
116 forest (Kowallik & Greig, 2016). We were also previously able to isolate *S.*
117 *paradoxus* directly from these forest substrates without enrichment (Kowallik, 2015).
118

119 **Methods**

120 Field sampling and yeast isolation

121 All isolates were sampled from a mixed hardwood and conifer forest in
122 Nehmten, Schleswig-Holstein, northern Germany (Nehmtener Forst). We sampled
123 leaf litter and soil material from close to the bases of ten oak trees at four sampling
124 dates (Table 1), although not all trees were sampled at every date. Trees were between
125 12 and 744 m from one another. At each date, samples were collected from leaf litter
126 and the top organic layer of soil within one meter of the base of each tree. Paired leaf
127 litter and soil samples were collected on the north, south, east, and west side of each
128 tree at all collection days except 7 April, when samples were collected at an arbitrary
129 two of the four cardinal directions.

130 Material was collected simultaneously for the **direct plating** and **enrichment**
131 collections at each sampling point (Figure 1). First, leaf litter was collected by

Table 1: Number of sampling points from each tree at each timepoint (each sampling point includes one enrichment and one direct plating sample)

	21 March 2017		7 April 2017		12 June 2017		10 July 2017	
	soil	litter	soil	litter	soil	litter	soil	litter
Tree 1	4	4	2	2	4	4	4	4
Tree 2	4	4	2	2	4	4	4	4
Tree 3	4	4	2	2	4	4	4	4
Tree 4	4	4	2	2	4	4	4	4
Tree 5	4	4					4	4
Tree 6			2	2	4	4	4	4
Tree 7			2	2	4	4	4	4
Tree 8			2	2	4	4	4	4
Tree 9					4	4	4	4
Tree 10							4	4

aseptically transferring litter into sterile collection tubes: approximately 5 ml of compressed leaf litter was collected for the direct plating method and approximately 2 ml for the enrichment method. Then, the remaining leaf litter was removed from the soil surface and the top approximate 2 cm of soil (mostly composed of soil organic layer) were aseptically transferred into sterile collection tubes. As for leaf litter, approximately 5 ml of compressed soil was collected for the direct plating method and approximately 2 ml for the enrichment method. Instruments were sterilized between samples using 70% ethanol. Samples were transported between the field and lab at ambient temperature and processed within four hours of collection.

For **direct plating** (Figure 1A), material was mixed with 20 ml sterile water in a sterile 50 ml tube, the mixture was vigorously mixed for at least 10 seconds with a vortex mixer on its highest setting, and 0.2 ml of the resulting dirty liquid was pipetted on each of two plates containing solid modified selective media PIM1 (3 g yeast extract, 5 g peptone, 10 g sucrose, 3 g malt extract, 1 mg chloramphenicol, 80 ml ethanol, 5.2 ml 1 M HCl, and 20 g agar per liter) (Kowallik & Greig, 2016; Sniegowski et al., 2002). Liquid was spread on plates using sterile glass beads, and

151 plates were left open in a laminar flow hood until dry. Plates were incubated for three
152 days at 30 ºC before colonies were picked.

153 For **enrichments** (Figure 1B), material was mixed with 10 ml liquid selective
154 media PIM1 (composition as for solid PIM1 but without agar) in a 15-ml sterile tube,
155 mixtures were inverted, and tubes were incubated, slightly open and without shaking,
156 at 30 ºC. After 10 days, a sterile wooden stick was inserted into each enrichment tube
157 and a small amount of liquid was streaked onto a single plate with solid selective
158 media PIM2 (20 g Methyl-(alpha)-D-glucopyranoside, 1 ml 5% Antifoam Y-30
159 emulsion, 6.7 g Yeast Nitrogen Base without amino acids, 4 ml 1M HCl, and 20 g
160 agar per liter) (Kowallik & Greig, 2016; Sniegowski et al., 2002), and plates were
161 incubated 4 days at 30ºC before colonies were picked.

162 We include these procedures as step-by-step protocols for the convenience of
163 future researchers in the supplementary materials (Supplemental File 1).

164

165 Yeast identification

166 After incubation, we streaked colonies with yeast-like morphology to fresh
167 YPD media (10 g yeast extract, 20 g peptone, 20 g dextrose, and 25 g agar per liter).
168 For each method, up to 6 (March and April sampling days) or 12 (June and July
169 sampling days) colonies per sample were selected. After one day of growth on YPD at
170 30 ºC, cultures were frozen at -80 ºC in 20% glycerol and a small amount of each
171 culture was transferred to sporulation media (20 g potassium acetate, 2.2g yeast
172 extract, 0.5 g dextrose, 870 mg complete amino acid mixture, and 25 g agar per liter).
173 Any cultures with bacteria-like morphology on YPD media (slimy culture and/or cells
174 smaller than 1 micron across) were not frozen and were discarded. Sporulation

175 cultures were incubated for at least three days at room temperature before being
176 screened under a compound microscope for *Saccharomyces*-like asci (tetrads).
177 All cultures producing tetrads were identified using sequencing of the internal
178 transcribed sequence (ITS), a region neighboring rRNA-coding DNA (Schoch et al.,
179 2012). We sequenced every strain using the ITS1/ITS4 primer pair (White, Bruns,
180 Lee, & Taylor, 1990). PCR mixes were 7-15 µl in volume and contained one yeast
181 colony, 0.5 µM each primer, and either 50% Phusion® High-Fidelity PCR master mix
182 with HF buffer or 1x HF-buffer, 100 µM dNTP mix, 3% DMSO, and 1 U/50 µl
183 Phusion DNA polymerase. PCR reactions were cycled at 98 ºC for 30 s, then 35
184 cycles of 98 ºC for 5 s, 62 ºC for 20 s, and 72 ºC for 30 s, plus a 10 min terminal
185 extension at 72 ºC. PCR products were cleaned using illustra™ ExoProStar™
186 according to the manufacturer's instructions, and sequenced on an ABI 3130xl
187 sequencer.
188 ITS sequences were compared to sequences from the type or neotype strains of
189 *S. paradoxus*, *S. cerevisiae*, *S. kudriavzevii*, and *S. mikatae* (Genbank accession
190 numbers NR_138272.1, NR_111007.1, KY105195.1, and KY105198.1). If a
191 sequence did not align with *Saccharomyces* sequences, we compared the sequence
192 with all sequences in the NCBI database from type strains using BLAST (Zhang,
193 Schwartz, Wagner, & Miller, 2000). If the sequence aligned with *Saccharomyces*
194 sequences but had more than one base pair different from its closest match, we
195 supplemented ITS sequences with sequences from the gene for translation elongation
196 factor 1 using primers EF1-983F and EF1-2212R (Rehner & Buckley, 2005) using the
197 protocols above, but with a PCR annealing temperature of 57 ºC. In some cases,
198 cultures originating from apparent single colonies were in fact mixtures of two yeast

199 species. We counted these colonies as *Saccharomyces* if sequences from one of the
200 species was *Saccharomyces*.
201
202 Growth rates
203 We compared the distributions of maximum growth rates between two groups
204 of *S. paradoxus* strains: strains collected using enrichment culturing and strains
205 collected using direct plating. To avoid confounding effects of environmental source
206 (*i.e.*, combination of substrate, date collected, and tree), we compared growth rates for
207 pairs of *S. paradoxus* strains originating from the same environmental source. In other
208 words, we collected a dataset of *S. paradoxus* growth rates from two groups of strains
209 with equal representations of combinations of substrate, date collected, and tree, and
210 differing only in the method used to isolate the strains. To ensure that all isolates were
211 pure *S. paradoxus* cultures, we streaked all isolates used for growth rate
212 measurements to single-colony cultures a second time and reidentified these cultures
213 by mating them with a *S. paradoxus* tester strain (NCYC 3708, α, ura3::KANMX,
214 ho::HYGMX). In total, 110 isolates (55 from each sampling method) were measured.
215 Growth rates were measured using an Epoch 2 microplate reader (Biotek
216 Instrument, Inc., Winooski, VT, USA) and calculated using the included Gen5
217 software version 3.03.14 (Biotek Instrument, Inc., Winooski, VT, USA). We first
218 inoculated strains in 0.2 ml liquid YPD media (composition as for solid YPD, but
219 without agar) in a 96-well microplate and incubated cultures without shaking or
220 measurement in the microplate reader at 30 ºC for 24 hours to condition strains to
221 microplate reader conditions. We then transferred 2 µl from each culture to 198 µl
222 fresh liquid YEPD in a new microplate and incubated the new microplate under the
223 same conditions for 20-24 hours. OD_{660} was measured during the second incubation

224 every ten minutes, and maximum growth rate (mOD_{660}/min) was calculated from the
225 maximum slope of each growth curve over four points (30 min total) using Gen5
226 software. Reported growth rates for each isolate are means of three replicates.
227
228 Statistical analyses
229 We compared sampling success across substrates (leaf litter or soil) and
230 methods (direct plating or enrichment) using a generalized linear mixed-effects model
231 with probability of isolating *Saccharomyces* (including *S. paradoxus* and *S.*
232 *cerevisiae*) as the response variable, substrate and method as fixed effects, and tree
233 and date as random effects. We selected the best model using a top-down strategy,
234 comparing Akaike's Information Criteria (AIC) after removing predictors from a full
235 model one by one.
236 We compared growth rate distributions by first comparing variances using
237 Levene's test (Levene, 1960) for homogeneity of variance, and then comparing
238 medians using a paired Wilcoxon signed rank test. Statistics were computed using R
239 version 3.3.1 (R Development Core Team, 2016) and the car and lme4 packages
240 (Bates, Machler, Bolker, & Walker, 2015; R Development Core Team, 2016).
241 Graphics were produced using the ggplot2 package (Wickham, 2016).
242
243 **Results**
244 Influence of sampling method on success isolating *Saccharomyces*
245 Direct plating was more successful than enrichment culturing for isolating
246 *Saccharomyces* from natural substrates ($z = 6.1$, $p < .001$) (Tables 2, 3, Figure 2). We
247 found *Saccharomyces* isolates in 45% of direct plating cultures and 19% of
248 enrichment cultures. However, enrichment culturing produced the only *S. cerevisiae*

found in this study: we found six *S. cerevisiae* isolates from a single enrichment culture from tree 3 in March of 2017. All other *Saccharomyces* isolates found in this study were *S. paradoxus*. Other detected yeast species included *Saccharomycodes ludwigii*, *Torulaspora delbrueckii*, *Pichia membranifaciens*, and *Hanseniaspora osmophila*, all of which have previously been found alongside *Saccharomyces* yeasts in beverage fermentations (Domizio et al., 2011; Gschaedler, 2017).

Table 2: Model selection (Mixed-effects generalized linear model)

model	fixed effects	random effects	AIC	compared to	better model
1	method + substrate + method:substrate	1|tree + 1|month	447.20		
2	method + substrate + method:substrate	1|month	470.94	model 1	1
3	method + substrate + method:substrate	1|tree	451.99	model 1	1
4	**method + substrate**	**1|tree + 1|month**	**446.76**	**model 1**	**4**
5	method	1|tree + 1|month	480.34	model 4	4
6	substrate	1|tree + 1|month	485.71	model 4	4

Table 3: Model summary table (model 4)

	Estimate	Std. Error	z	p
intercept	-2.5324	0.4599	-5.506	< .001
method (plating)	1.5494	0.2559	6.054	< .001
substrate (soil)	1.4445	0.2542	5.683	< .001

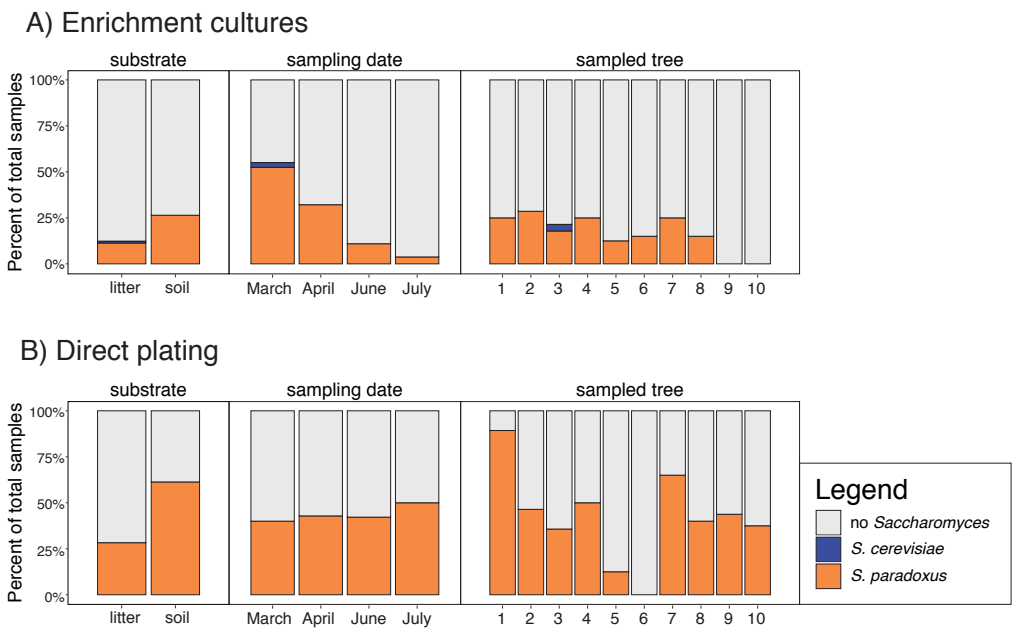

Figure 2: Percentages of samples in which *Saccharomyces* could be detected using A) enrichment cultures or B) direct plating. Bars represent all samples for each category of sampling, and colors represent *Saccharomyces* species. *S. paradoxus* and *S. cerevisiae* were the only detected *Saccharomyces* species.

259 While the direct plating method was more successful than the enrichment
260 method, it was also more labor-intensive (Table 4). We screened 3.4 times as many
261 colonies for tetrads when using the direct plating method than we did using the
262 enrichment method. Only 32% of the processed direct plating colonies were *S.*
263 *paradoxus*, compared to 74% of enrichment colonies.
264
265 Table 4: Colonies processed and sampling success for each method

method	**total colonies picked**	**sequenced colonies with *Saccharomyces*-like ascus morphology**	***Saccharomyces* isolates**
enrichment	284	246 (87%)	211 (74%)
plating	969	344 (35%)	307 (32%)

266
267 Both methods isolated *Saccharomyces* colonies from both substrates, most
268 trees, and all timepoints (Figure 2). We had significantly more sampling success on
269 soil than leaf litter substrates ($z = 5.7$, $p < .001$, Table 3), but other relationships
270 among sampling success, sampling method, and sampling environments were
271 idiosyncratic. For example, direct plating did not produce any *Saccharomyces*
272 isolates from tree 6, while three enrichment samples from this tree isolated *S.*
273 *paradoxus*, and enrichments produced more *Saccharomyces* isolates in March than
274 direct plating did (Figure 2). Because our sampling effort was not the same for all
275 trees at all months, we did not model tree habitat or sampling month as fixed effects;
276 instead, we modeled these parameters as random effects, and found that models
277 including tree and month fit the data better than models without tree and month (Table
278 2). A list of sequenced yeasts from each sample is included in the supplemental
279 materials (Supplemental File 2).
280
281 Phenotypes of sampled *S. paradoxus*

282 Variances in growth rate, but not median growth rates, were different between
283 strains isolated using the two methods (Figure 3). Growth rates of *S. paradoxus*
284 isolated using enrichment culturing had a larger variance than growth rates of *S.*
285 *paradoxus* isolated using direct plating (Levine's test $F_{1,108} = 5.42$, p = .02). Median
286 growth rates for the two groups of *S. paradoxus* strains did not differ (Wilcoxon
287 signed rank test V = 6320, p = .39). Measured growth rates of each strain are included
288 in the supplemental materials (Supplemental File 2).

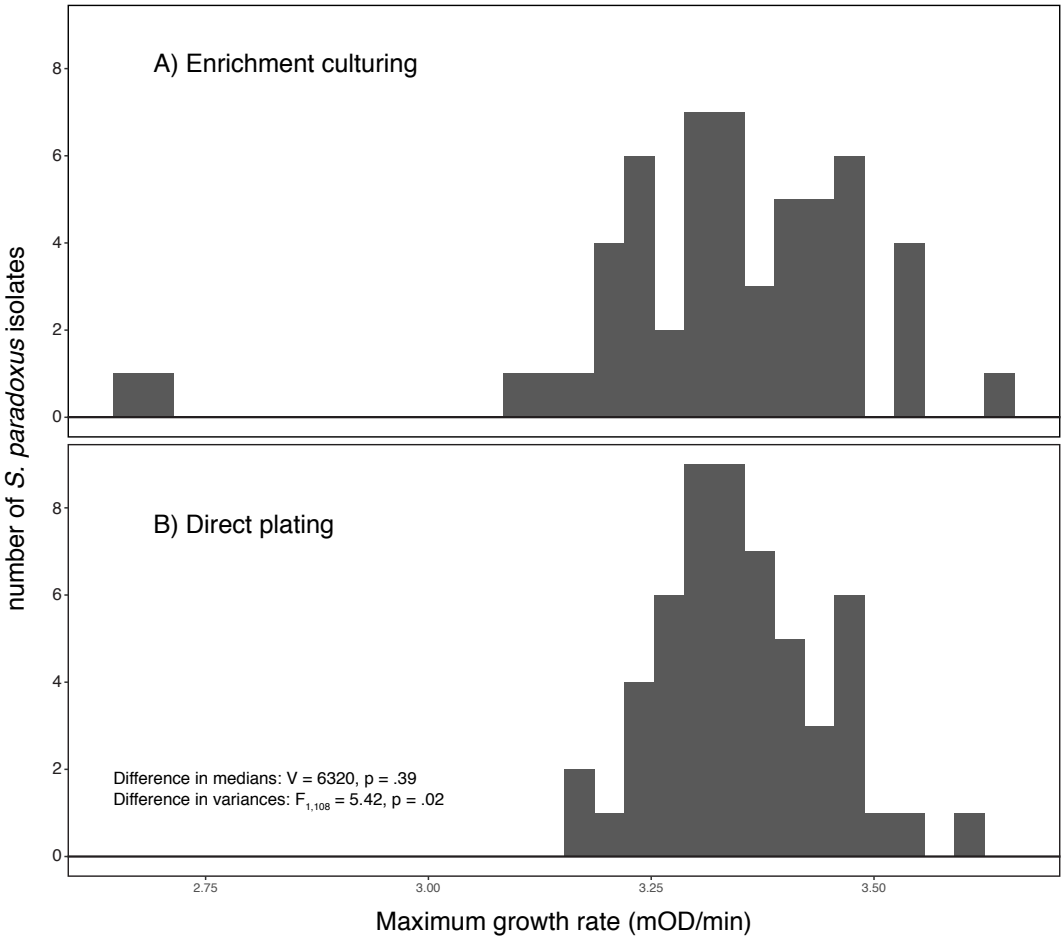

Figure 3: Histograms representing distributions of growth rates for *S. paradoxus* clones isolated using A) enrichment culturing or B) direct plating.

289

290 **Discussion**

291 Direct plating detects *S. paradoxus* more frequently than enrichment culturing

292 Enrichment culturing did not increase *Saccharomyces* sampling success from
293 forest leaf litter and soil over direct plating, in spite of researchers' long history of
294 using enrichment culturing to isolate *Saccharomyces* from forest environments
295 (Kowallik & Greig, 2016; Naumov, Naumova, & Sniegowski, 1998; Sniegowski et
296 al., 2002). We expect reliable *Saccharomyces* isolation from this forest using direct
297 plating to be a result of high *S. paradoxus* abundance on forest floor substrates.
298 Indeed, it is common to find hundreds to tens of thousands of *S. paradoxus* cells per
299 gram of leaf litter near the bases of oak trees in this forest (Kowallik & Greig, 2016).
300 We expect direct plating to be less successful in environments in which
301 *Saccharomyces* are rarer, and note that enrichment culturing is frequently used to
302 isolate *Saccharomyces* from tree bark, which may be a habitat with lower
303 *Saccharomyces* density than the forest floor habitats we sampled (Kowallik et al.,
304 2015; Sniegowski et al., 2002).
305
306 <u>Isolation using enrichment culturing samples more phenotypic diversity than direct</u>
307 <u>plating</u>
308 Conditions in enrichment cultures resulted in isolating a different (albeit
309 higher) phenotypic diversity than random colony selection on plate cultures did
310 (Figure 3). There are several potential methodological and ecological explanations for
311 the high phenotypic diversity in enrichment isolates. But we expect that interactions
312 with microbes *during the enrichment culturing* are most likely to be responsible for
313 the increased phenotypic diversity among enrichment-sourced *S. paradoxus* isolates.
314 Microbes that potentially interact with *S. paradoxus* were doubtless present in
315 our enrichment cultures, and it is realistic to expect these microbes to influence the
316 phenotypes of the *S. paradoxus* that were ultimately recovered. Our enrichment

317 cultures contained all of the microbes that were present in the soil and leaf litter
318 samples, and soil and leaf litter include a wide diversity of bacterial and fungal taxa
319 that can interact with fungi (Curd, Martiny, Li, & Smith, 2018; Glassman et al., 2018;
320 Santonja et al., 2018). For example, some agricultural soils contain *Bacillus* and
321 *Pseudomonas* species that secrete compounds toxic to phytopathogenic fungi (Islam,
322 Jeong, Lee, & Song, 2012; Petatán-Sagahón et al., 2011). Conversely, some soil
323 bacteria promote mycorrhizal fungal growth and host plant colonization (Xie et al.,
324 2018).

325 We expect a variety of similar inhibition and facilitation interactions to
326 determine the identities of the *Saccharomyces* strains that ultimately reached high
327 frequencies in enrichment cultures. Bacteria and fungi co-occurring with *S. paradoxus*
328 on oak bark can both inhibit and facilitate *S. paradoxus* growth (Kowallik et al.,
329 2015). These effects are dependent on temperature and interacting microbe identities,
330 and likely also depend on other environmental conditions and *S. paradoxus* genotype.
331 The *Saccharomyces* strains recovered at the end of our enrichment cultures were
332 probably the fittest *Saccharomyces* strains present in the cultures, but this high
333 relative fitness was as likely to be a result of interactions with co-occurring microbes
334 that occur by chance in the same enrichment cultures as it was to be the result of
335 intrinsic growth rate. Microbial diversity during enrichment may similarly explain our
336 idiosyncratic sampling success across months and trees (Figure 2). For example, it is
337 possible that a bacterium that facilitates rare *S. paradoxus* growth in the enrichment
338 medium was more common in spring than summer months, resulting in higher
339 enrichment sampling success in spring than summer. Unfortunately, we did not
340 measure the microbial diversity of the enrichment cultures and do not know which
341 microbes may have interacted with *Saccharomyces*.

342 It is also possible, although in our opinion less likely, that methodological
343 biases resulted in relatively low phenotypic diversity among directly plated *S.*
344 *paradoxus* compared to enrichment *S. paradoxus*. While we aimed to randomly select
345 colonies with *Saccharomyces*-like morphologies from direct culture plates, biases in
346 colony picking could have selected for low variance in *S. paradoxus* phenotypes. For
347 example, if a *S. paradoxus* colony had an unusual morphology, it might have been
348 mistaken for a bacterial colony and not isolated. However, direct culturing isolated
349 more non-*Saccharomyces* yeast isolates than enrichment culturing did, and we expect
350 interspecific morphological variation to be higher than intraspecific morphological
351 variation. We therefore consider it unlikely that biases in colony picking decreased
352 phenotypic variance among *S. paradoxus* isolates.

353 We continue to expect plate culturing to be a more random sampling of
354 *Saccharomyces* diversity than enrichment culturing. Colonies are physically isolated
355 on a plate and unlikely to influence each other's growth, and plated colonies come
356 from a well-mixed mixture of environmental substrate and sterile water. We therefore
357 expect *Saccharomyces* colonies on plates to accurately reflect the diversity of
358 *Saccharomyces* strains present in nature, as long as all strains are able to grow on the
359 selective media chosen. Biases in sample *success* can still be introduced to direct
360 plating samples by the presence of morphologically similar yeast species. On petri
361 dishes, a high density of non-*Saccharomyces* yeasts with morphologies similar to
362 *Saccharomyces* could have prevented us from detecting *Saccharomyces*. This issue
363 can be mitigated by designing a more selective isolation medium, picking more
364 colonies per plate (we picked up to 6 or 12 colonies across two plates), or replica
365 plating selective plates to media containing a color indicator for the target yeast (*e.g.,*
366 Wallerstein media) (Hall, 1971).

367

368 Recommendations for future yeast sampling

369 Researchers should consider both resources available for sampling and study
370 goals when choosing a *Saccharomyces* field sampling strategy. Our results identified
371 a tradeoff between resources spent on sampling and resources spent on sequencing:
372 enrichment culturing was less successful than direct plating at finding *Saccharomyces*
373 per sample collected, but more successful per ITS region sequenced (Figure 2, Table
374 4). Researchers with a few precious samples are therefore better off isolating
375 *Saccharomyces* using direct plating than enrichments, especially if *Saccharomyces* is
376 common on their substrates. Conversely, if samples are easy to get but funds available
377 for sequencing are limited, researchers may prefer to use enrichment culturing.
378 Researchers with limited time or freezer space who would like assurances that most
379 picked colonies are *Saccharomyces* may also prefer enrichment culturing.

380 However, the scientific question to be answered by environmental
381 *Saccharomyces* samples may be more important than sampling and sequencing
382 resource limitations, especially as sequencing becomes cheaper. Direct plating
383 samples a more random collection of *Saccharomyces* cells from environmental
384 samples than enrichment culturing, and we recommend that researchers who need
385 random environmental samples to answer ecological and evolutionary questions rely
386 on direct plating. Researchers targeting phenotypic diversity, especially for applied
387 yeast biology (*e.g.,* food microbiology, drug discovery) may uncover more diversity
388 by isolating environmental *Saccharomyces* using enrichment cultures. Researchers
389 interested in detecting rare *Saccharomyces* species in an environment (*e.g., S.*
390 *cerevisiae* from our study forest, *S. mikatae* and *S. eubayanus* from European forests)

(Alsammar et al., 2018) may also have more success using enrichment culturing or a combination of enrichment and direct plating strategies.

Conclusions

Isolation protocols do indeed influence characteristics of isolated microbes. As researchers continue to develop *Saccharomyces* yeasts as model organisms for ecology and evolution, they must also consider how isolation history of environmental strains can influence the ecological stories the strains can tell. The results of this study highlight the need for consistent sampling within studies, and are a warning to researchers comparing phenotypes (and perhaps also genotypes) among *Saccharomyces* strains from different sources. Much of what we know about *Saccharomyces* evolution has come from culture collections (Strope et al., 2015; Warringer et al., 2011)—these collections are invaluable, as are the data and conclusions they enable, but sampling information about deposited strains is not always available. As technology improves and both genome sequencing and phenotype assays become cheaper and more accessible (Porter & Hajibabaei, 2018; Stewart et al., 2018; van Dijk, Auger, Jaszczyszyn, & Thermes, 2014; Zackrisson et al., 2016), environmental sampling may emerge as a limiting step to studying *Saccharomyces* ecology and evolution. Researchers should carefully consider the consequences of their chosen sampling strategies as they conduct this exciting microbiological research.

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Data Accessibility

All data for this project are included in Supplementary File 2.

Figure Legends

Figure 1: Schematic illustration of sampling strategies used to isolate *Saccharomyces* for this project. A) Enrichment culturing B) Direct plating. Photo: Doreen Landermann.

Figure 2: Percentages of samples in which *Saccharomyces* could be detected using A) enrichment cultures or B) direct plating. Bars represent all samples for each category of sampling, and colors represent *Saccharomyces* species. *S. paradoxus* and *S. cerevisiae* were the only detected *Saccharomyces* species.

Figure 3: Histograms representing distributions of growth rates for *S. paradoxus* clones isolated using A) enrichment culturing or B) direct plating.

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