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# Measuring microbial fitness in a field reciprocal transplant experiment

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## Abstract

**Microbial fitness is easy to measure in the laboratory, but difficult to measure in the field. Laboratory fitness assays make use of controlled conditions and genetically modified organisms, neither of which are available in the field. Among other applications, fitness assays can help researchers detect adaptation to different habitats or locations. We designed a competitive fitness assay to detect adaptation of *Saccharomyces paradoxus* isolates to the habitat they were isolated from (oak or larch leaf litter). The assay accurately measures relative fitness by tracking genotype frequency changes in the field using digital droplet PCR (DDPCR). We expected locally adapted *S. paradoxus* strains to increase in frequency over time when growing on the leaf litter type from which they were isolated. The DDPCR assay successfully detected fitness differences among *S. paradoxus* strains, but did not find a tendency for strains to be adapted to the habitat they were isolated from. Instead, we found that the natural alleles of the hexose transport gene we used to distinguish *S. paradoxus* strains had significant effects on fitness. The origin of a strain also affected its fitness: strains isolated from oak litter were generally fitter than strains from larch litter. Our results suggest that dispersal limitation and genetic drift shape *S. paradoxus* populations in the forest more than local selection does, although further research is needed to confirm this. Tracking genotype frequency changes using DDPCR is a practical and accurate microbial fitness assay for natural environments.**

*Keywords*: dispersal, hexose transporter, local adaptation, population, relative fitness, yeast

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## Introduction

Evolutionary biologists use microbial fitness assays to describe evolutionary trajectories and make general predictions about evolution (Elena & Lenski 2003; Orr 2009; Kawecki *et al.* 2012). Unlike plants and animals, microbes reproduce quickly, and relative fitness can be measured directly by comparing two microbes' growth rates (Lenski *et al.* 1991). Unfortunately, existing protocols are ill-suited for field experiments, making it difficult to study natural selection pressures. Fitness assay protocols require controlled conditions and sterile media, and cannot be carried out on nonsterile substrates. Additionally, fitness assays are often performed on genetically modified microorganisms, which can be impractical, unethical or illegal to introduce to natural environments (Francescon 2001). Evolutionary biologists need methods that directly measure fitness differences *in situ* between nongenetically modified organisms.

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Detecting local adaptation is one application for microbial fitness assays. When a population is locally adapted, it has higher fitness than other populations in its native habitat and vice versa (Kawecki & Ebert 2004). Populations can be locally adapted over space or time; they can also be locally adapted to habitat types or host genotypes (Domínguez-Bello *et al.* 2008; Rengefors *et al.* 2015). In laboratory studies, microbial local adaptation has evolved under a variety of conditions (Bell & Reboud 1997; Travisano 1997; Kassen 2002). But microbes are cryptic, and it is difficult to directly observe local selection pressures in natural environments such as soils. In the absence of microbial fitness assays for the field, researchers have compromised by either using a proxy for fitness or conducting fitness assays in laboratory microcosms simulating field conditions (Belotte *et al.* 2003; Refardt & Ebert 2007; Kraemer *et al.* 2015). Both strategies have disadvantages: proxies may not faithfully describe fitness, just as microcosms may not faithfully replicate field environments. A method to directly measure fitness in the field can enable field studies of microbial local adaptation.

We designed a microbial fitness assay using digital droplet PCR (DDPCR) to detect individual clones' frequency changes in a natural fungal population. Like quantitative PCR (QPCR), DDPCR is a PCR-based method for quantifying DNA molecules. Unlike QPCR, which measures the number of cycles needed to reach a threshold concentration of PCR product, DDPCR measures the number of droplets containing amplified PCR product in an emulsion of PCR mix and oil (Heid *et al.* 1996; Hindson *et al.* 2011). To measure DNA concentration using DDPCR, template is first added to a PCR mix; then, the PCR mix is emulsified with oil to form thousands of droplets containing a Poisson distribution of template molecules. The emulsion is cycled on a thermal cycler, and individual drops are screened for fluorescently labelled PCR products after amplification. Both methods use similar fluorescent chemistry—dual-labelled probes or dyes that bind to double-stranded DNA—to detect PCR products. DDPCR is often easier to use and more precise than QPCR (Hindson *et al.* 2013), but see Hayden *et al.* (2013). For example, time-intensive standard curves are necessary for QPCR, but not for DDPCR. While both methods can measure natural genetic variation, avoiding the need for introduced genetic markers, to our knowledge neither has been used to measure intraspecific fitness outside the laboratory; however, QPCR has been used to quantitate individual microbe species in field studies of microbial species composition and competitive exclusion (Alkan *et al.* 2006; Kennedy *et al.* 2007; Andorrà *et al.* 2010).

An ideal field fitness assay compares relative genotype frequencies without the need for accurate individual counts. Traditional laboratory-based fitness assays count individuals before and after growth and compare growth rates (Lenski *et al.* 1991; Travisano & Lenski 1996). Under field conditions, genotype frequencies are often easier to accurately measure than absolute microbial cell numbers because PCR inhibitors co-extracted with organism DNA can compromise count accuracy. PCR inhibitors are common in a variety of natural microbial environments, including leaf litter, soil, stool and food (Opel *et al.* 2010; Schrader *et al.* 2012; Hedman & Rådström 2013). Instead of measuring absolute cell or gene copy numbers for each strain, we measured relative strain frequency changes over time in two-strain competitions (Goddard & Bradford 2003). A microbe that increases in relative frequency at the expense of a competitor has higher fitness than the competitor. We extended the strategy of modelling frequency changes over time to test for local adaptation: in pairs of microbes containing one local strain and one foreign strain, the local strain increases in frequency if it is adapted to the local habitat.

We designed our fitness assay to test for local adaptation to habitat type in the yeast *Saccharomyces paradoxus*. *Saccharomyces paradoxus* has been isolated from a variety of forest substrates and is the wild sister species of the model domesticated microbe *Saccharomyces cerevisiae* (Naumov *et al.* 1998; Glushakova *et al.* 2007; Vaughan-Martini & Martini 2011). We found *S. paradoxus* strains on the leaf litter of several tree species in a mixed forest in northern Germany. The two most frequent *S. paradoxus* leaf litter habitats in the forest are oak and larch litter (V. Kowallik & D. Greig, unpublished observations); we hypothesized that *S. paradoxus* strains are locally adapted to the litter type from which they were isolated.

To test for local adaptation, we developed a DDPCR assay targeting the *S. paradoxus HXT3* gene. *HXT3* codes for a hexose transport protein, and has at least two alleles in the European *S. paradoxus* population. After genotyping a collection of *S. paradoxus* strains isolated from larch and oak litter, we conducted a reciprocal transplant experiment. First, we randomly assigned strains to pairs; each strain pair included one strain each from larch and oak litter, each with a different *HXT3* genotype. Both source population/genotype combinations were represented. Then, strain pairs were mixed together and re-inoculated to oak and larch litter in the forest. *HXT3* relative frequencies were tracked over 20 days using DDPCR, and we inferred local adaptation when the *HXT3* genotype matching that of the locally sourced strain increased in frequency with time.

While *S. paradoxus* is widespread in local forests, it is generally not abundant enough to detect using PCR (Kowallik *et al.* 2015). To ensure detection of experimental *S. paradoxus* isolates, we inoculated them to leaf litter at higher abundances than previously observed. We expected *S. paradoxus* population sizes to decrease over the course of the experiment. When measuring changes in relative frequencies between strains, we therefore could only measure the survival component of fitness (i.e. fitness under negative growth conditions), and not differences in growth rates or reproductive output. Throughout this manuscript, we will use the terms 'fitness' and 'local adaptation' to refer primarily to differences in *S. paradoxus* survival in the presence of competitors.

## Materials and methods

### *Digital droplet PCR assay*

The DDPCR assay used naturally occurring genetic variation to count individuals with two *S. paradoxus* genotypes. We used probes targeting *HXT3* to distinguish strains because there are at least two *HXT3* alleles, differing from each other by multiple linked SNPs, in the

European *S. paradoxus* population. The gene was chosen by visually inspecting a five-genome *S. paradoxus* alignment for polymorphic regions using Integrative GENOMICS VIEWER v.2.3.34 (Liti *et al.* 2009; Robinson *et al.* 2011; Bergström *et al.* 2014). We designed primers and dual-labelled probes around a 165-bp target region containing three linked SNPs within 23 bp of one another (Table 1) Dual-labelled probes are reviewed in Smith & Osborn (2009). Primers and probes were designed with PRIMER3 (Koressaar & Remm 2007). Dual-labelled probes included either a FAM or HEX molecule at the 5′ end, and the quencher BHQ1 at the 3′ end. We identified the targeted region as homologous to *S. cerevisiae HXT3* using BLASTX (Gish & States 1993), and arbitrarily designated *S. paradoxus* genotypes matching the two probes as *'hxt3-1'* and *'hxt3-2'*.

We verified the accuracy of DDPCR primers by comparing *HXT3* copy and colony-forming unit (CFU) frequencies in test mixtures of two genetically labelled *S. paradoxus* strains. Strains were grown overnight in liquid media and resuspended in sterile water, then combined in nine different ratios. We diluted strain combinations 1:10 in water, and mixed 100 μL of each dilution with about 1 mL of each of larch and oak litter in a 15-ml centrifuge tube. Strain combinations were also further diluted and plated onto selective media; colonies were counted when they became visible.

Yeast DNA was then extracted from the cells rinsed from each leaf litter sample. We added 2 mL of PCR-grade water to every centrifuge tube, vigorously shook tubes and removed 1 mL of water for DNA extraction. Cells were pelleted from water samples by centrifuging for 10 min at 16 837 rcf, and DNA was extracted from pellets using a method modified from Hoffman (2001) and Sambrook & Russell (2001). We first resuspended each pellet in 200 μL of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA). Two hundred microlitres of 0.5-mm glass beads and 200 μL of chloroform:phenol:isoamyl alcohol (25:24:1) were added, and cells were lysed by vortexing for 2 min. Mixtures were then centrifuged for 5 min at 16 837 rcf. One hundred microlitres of the aqueous layer were removed, added to 2 μL 5 μg/μL RNase A and incubated for 30 min at 37 °C. Then, 10 μL of 3 M sodium acetate and 250 μL of 95% ethanol were mixed with each sample, and samples were incubated for 1 h at −20 °C. After incubation, samples were centrifuged for 15 min at 16 837 rcf, supernatants were removed, and each pellet was washed with 0.5 mL of 70% ethanol and centrifuged again for 10 min. Ethanol was removed and pellets were dried on the bench for 10 min before resuspension in 50 μL of TE buffer.

We counted copies of both *HXT3* alleles in DNA extracts simultaneously using DDPCR. PCR mixtures consisted of 1× DDPCR supermix for probes (Bio-Rad, CA, USA), 0.17 μg/μL of bovine serum albumin, 440 nM each primer, 240 nM each probe and 1 μL undilute template per 20 μL of PCR mix. Droplets were produced using 20 μL of PCR mixture and 70 μL of droplet generator oil for probes (Bio-Rad) in a X100 droplet generator (Bio-Rad), according to the manufacturer's instructions. Droplets were cycled on a FlexCycler thermalcycler (Analytik-Jena, Jena, Germany) using the following program: 10 min at 90 °C, 40 cycles of 30 s at 94 °C and 1 min at 57 °C, followed by one cycle of 10 min at 98 °C and a hold temperature of 10–12 °C. Droplets were read on a X100 droplet reader (Bio-Rad) and analysed using QUANTASOFT v.1.7 (Bio-Rad). We examined droplet reader outputs and manually adjusted thresholds between negative and positive droplets when needed.

### *Saccharomyces paradoxus strain isolation and genotyping*

All strains were collected from a mixed conifer-hardwood forest in Nehmten, Germany (54°06′18″N, 10°21′36″E). We isolated 117 *S. paradoxus* strains from leaf litter within 1 m of larch and oak trees throughout the forest in November, 2014, and June, 2015. To avoid collecting duplicate strains, we never collected more than one strain from beneath the same tree in the same month.

*Saccharomyces paradoxus* strains were isolated from litter using enrichment culturing, and identified using morphological characteristics and ribosomal DNA sequencing. About 2 mL of compressed leaf litter were collected and mixed with modified PIM 1 *Saccharomyces* enrichment media (0.3% yeast extract, 0.3% malt extract,

**Table 1** Primer and probe sequences for digital droplet PCR assay

| Name | Description | 5′ modification | Sequence | 3′ modification |
|---|---|---|---|---|
| HXT3.dd.F | Forward primer | | AGTCAACGATATGTACGCCG | |
| HXT3.dd.R | Reverse primer | | CACTACGGTTCAGCGAGAA | |
| HXT3-1.probe | Probe annealing to genotype *hxt3-1* | 6FAM | TGCT***T***CTTGGGTTCCAAC***T***TC***C***C | BHQ1 |
| HXT3-2.probe | Probe annealing to genotype *hxt3-2* | HEX | TGCT***G***CTTGGGTTCCAAC***A***TC***T***C | BHQ1 |

0.5% peptone, 1% sucrose, 8% ethanol, 0.001% chloramphenicol and 0.52% 1 M HCL) (Sniegowski *et al*. 2002). After enrichments had been incubated at 30 °C for 10 days, 25 μL was streaked onto modified PIM 2 media (2% methyl-α-D-glucopyranoside, 0.67% yeast nitrogen base without amino acids or ammonium sulphate, 2% agar, 0.005% antifoam A (Sigma-Aldrich, MO, USA), 0.4% 1 M HCl) (Sniegowski *et al*. 2002). We transferred colonies with *S. paradoxus*-like morphology to sporulation agar (2% potassium acetate, 0.22% yeast extract, 0.05% glucose, 0.087% complete amino acid mix, 2.5% agar). Colonies producing *Saccharomyces*-like asci after 3–5 days were sequenced using the primer pair ITS1/ITS4 (White *et al*. 1990) to confirm that they were *S. paradoxus*. Sporulated cultures were stored at −80 °C in 20% glycerol.

*Saccharomyces paradoxus* strains were genotyped using DDPCR. Frozen stocks were streaked onto solid YPD media (2% dextrose, 2% bactopeptone, 1% yeast extract, 2.5% agar), and DNA was extracted using the MasterPure™ Yeast DNA Purification Kit (Epicentre), according to the manufacturer's instructions. Each culture's *HXT3* genotype was determined using the DDPCR protocol described above. We assigned a strain to a genotype when 99% or more of the total copy concentration corresponded to the genotype. Other cultures were assumed to be heterozygous. We made all strains homozygous by dissecting asci to single haploid spores and allowing spores to germinate and self-fertilize.

### *Field experiment*

We set up a reciprocal transplant experiment in the Nehmten forest using 68 genotyped *S. paradoxus* strains. Strains were randomly matched into pairs containing one strain from each litter type and one of each *HXT3* genotype, and pairs were inoculated on the leaf litter beneath an oak tree and a larch tree. Each strain was used in exactly one pair, and each pair was inoculated under exactly one tree of each type. We had previously identified fourteen experimental trees (seven of each type) to approximately evenly cover an area of 0.09 $km^2$ in the Nehmten forest (Fig. S1, Supporting information). Each strain pair was inoculated in the laboratory onto unsterilized leaf litter collected from its assigned tree, and the inoculated litter was buried between the soil and litter horizons at the base of the tree. We used unsterilized leaf litter in order to expose *S. paradoxus* strains to the most realistic environments possible, including abiotic and biotic selection pressures. We collected samples of inoculated litter immediately after sample burial (time = 0 days), and every 5 days for the next 20 days, to determine strain frequencies with DDPCR. The experiment ran between 4 August 2015 and 24 August 2015. During the experiment, the average daily high temperature was 24 °C and the average daily low temperature was 13 °C. There were rain events on 8 days, and a total of 42 mm of rain fell (weather data are Deutscher Wetterdienst data from the Dörnick weather station, approximately 4 km from the Nehmten forest; http://www.dwd.de/EN/climate_environment/cdc/cdc_node.html, accessed 4 May 2016).

To make the 34 strain pairs used for the experiment, we randomly matched 68 of the 117 genotyped *S. paradoxus* strains. Each pair included one homozygous strain isolated from each litter type (oak and larch). In 20 pairs, the oak strain's genotype was *hxt3-1* while the larch strain's genotype was *hxt3-2*; in 14 pairs, the larch strain was *hxt3-1* while the oak strain was *hxt3-2*. Strains were mixed into pairs and grown together overnight in 10 mL of liquid YPD medium. Each 10 mL culture was centrifuged for 1 min at 16 837 rcf, washed twice with 1 mL sterile water, and resuspended in 1 mL sterile water. Resuspended cultures were mixed with about 25 mL of uncompressed leaf litter from the strain pair's assigned tree in a plastic bag. Inoculated leaf litter was then distributed among five sterile 175-mL tea filters. We avoided processing collected leaf litter in any way (aside from inoculating *S. paradoxus* strains and mixing) in an effort to alter the field litter environment as little as possible.

Filters containing strain pairs were buried beneath the litter layer of seven oak and seven larch trees in total. Each strain pair was buried under one randomly chosen larch and one randomly chosen oak tree, and each tree housed four to five strain pairs. Five samples of each strain pair were buried under each tree type (20–25 bags per tree total), and strain pairs were mixed with leaf litter previously collected from the tree under which they were buried. Each strain pair was buried within 1 m of the base of each tree chosen.

We harvested one sample from each pair under each tree type after 0, 5, 10, 15 and 20 days. We also collected one uninoculated leaf litter sample from beneath each tree in April, 2016, to estimate the influence of background *S. paradoxus* DNA on our DDPCR assay. Immediately after harvesting, approximately 1 mL of leaf litter was aseptically transferred to a 15-mL centrifuge tube and stored at −20 or −80 °C until DNA could be extracted. DNA was extracted as described above for test mixtures, and all DNA extracts were diluted 1:10 with TE buffer and analysed using DDPCR as described above. PCR mixes included 2 μL dilute template per 25 μL PCR mix; 20 μL of this mixture was used to make droplets. We reran samples with <100 total detected droplets at higher concentrations (up to 1:1). A few samples did not produce 100 drops, even

when run without dilution; we combined data from several runs for these samples. We also reran samples with unclear separation between positive and negative drop clusters at dilutions up to 1:100. Unclear separation was generally due to droplets with weak signal lying between positive and negative clusters. We suspect that the weak droplets were a result of contaminating PCR inhibitors from the leaf litter; further dilution to 1:100 improved separation.

### *Statistical analyses*

We correlated test strain CFU and genotype frequencies using Pearson's correlation, and modelled local adaptation in the field experiment using a mixed-effects linear model. A strain is locally adapted when its frequency relative to a foreign strain increases in its home environment. If there is local adaptation, we expect local strain frequencies to increase with time; that is, time point sampled would significantly predict local strain frequency. If one strain source (oak or larch) houses more fit strains than the other, we expect an interaction between time point and strain source to influence local strain frequencies, regardless of the tree identity. And if *HXT3* genotype is important for fitness, we expect an interaction between time point and genotype (*hxt3-1* or *hxt3-2*) to influence local strain frequencies, regardless of the tree identity.

When testing for local adaptation and other fitness effects, the response variable ('local ratio') was ln($L$/$F$), where $L$ = concentration of DNA from the locally originated strain and $F$ = concentration of DNA from the foreign-originated strain. The response variable is high when the frequency of the locally originated strain is high. We predicted local ratio as a function of the fixed predictors *time point*, *local strain source*, *local strain genotype* and all interactions. We included the crossed random predictors *tree identity* and *strain pair*; both random intercepts and random slopes with respect to *time point* were modelled. We selected the most appropriate model by defining the most complex possible fixed and random structure, then removing explanatory factors stepwise and comparing log-likelihoods. We retained a predictor only when a model with the predictor had a significantly lower AIC than a model without it. Random effects were removed before fixed effects.

We described change in total DDPCR signal with time using a similar mixed-effects linear model. We modelled the response variable $\log_{10}$(DNA concentration), the fixed predictor *time point* and the same random predictors as above. Model selection was as described above.

All statistics were performed using R version 3.1.1 and the car and LME4 packages (Fox & Weisberg 2011; R Core Team 2014; Bates *et al.* 2015).

## Results

The DDPCR primers amplify a 165-bp region covering 145 bp of the C-terminal end of *HXT3*, plus 20 flanking base pairs. This region includes six SNPs among the genomes used to design the DDPCR assay (genome Q32.3 had low coverage in this region and may include further variation), one of which is nonsynonymous. The DDPCR probes match three of these SNPs (Table 1). In test samples, *hxt3-1* genotype frequencies measured using DDPCR and CFU counts were highly correlated, indicating that DDPCR measures individual frequencies as accurately as colony counts do (Pearson's $r = 0.98$, $t = 18.76$, d.f. = 16, $P < 0.001$, Fig. 1).

Of the 117 collected and genotyped leaf litter *S. paradoxus*, 33 were *hxt3-1*, 83 were *hxt3-2*, and one was heterozygous. There was no significant difference in genotype frequency between oak and larch litter habitats ($\chi^2 = 0.14$, d.f. = 1, $P = 0.70$). We inoculated and buried 340 total leaf litter samples, made from 34 *S. paradoxus* strain pairs. Of the 340 samples, 15 went missing in the field and 12 lost their labels. Deer or other mammals probably damaged these samples. We did not include these missing samples in the models. Of the 27 missing samples, 25 were from larch trees and two were from oak trees, and more samples were from later than earlier collection times (two samples were missing at 5 days, three at 10 days, nine at 15 days and 13 at 20 days). Five more experimental samples did not produce any DDPCR signal and were discarded. We also collected 14 uninoculated litter samples, one beneath each experimental tree, several months after the experiment. We detected DDPCR signal from both genotypes in three uninoculated samples (litter from Oak 1, Oak 2 and Oak 7). All

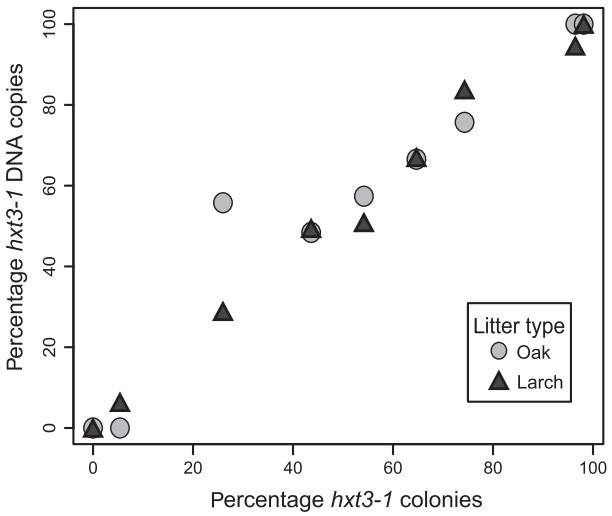


**Fig. 1** Correlation between *HXT3* copy read frequency and colonies counted in test samples.

experimental data, including a list of all collected and missing samples, metadata, and DDPCR output, are available in Dryad (doi: 10.5061/dryad.mb780).

*Saccharomyces paradoxus* strains were not locally adapted in our field experiment, although strain source and *HXT3* genotype both influenced local strain fitness. Local strains did not consistently increase in frequency in local environments (Fig. 2a, Table 2). Instead, oak-originated strains increased in frequency over time (significant interaction between *time point* and *strain source*, $\chi^2 = 9.73$, d.f. = 1, $P = 0.002$, Fig. S2, Supporting information), and strains with the *hxt3-2* genotype increased in frequency with respect to *hxt3-1* strains over time (significant interaction between *time point* and *genotype*, $\chi^2 = 138.22$, d.f. = 2, $P < 0.001$, Fig. 2b). The most parsimonious model also includes a random effect of *strain pair*, indicating that strain relative frequencies were dependent on the two strains' genetic backgrounds. DDPCR signal decreased over time in samples, but was always higher than DDPCR signal in uninoculated leaf litter (Fig. 3, Table S1, Supporting information).

## Discussion

### *Changes in leaf litter Saccharomyces paradoxus frequencies*

The DDPCR assay successfully detected frequency changes between *S. paradoxus* strains in the field. DDPCR is as accurate as colony counting at determining strain relative frequencies (Fig. 1). We detected survival differences between strains, as indicated by changes in strain frequencies, over the 20 days of the experiment (Fig. 2). Specifically, we found that strains from oak litter survived better than strains from larch, and strains with the *hxt3-2* allele survived better than strains with the *hxt3-1* allele (Fig. 2b, Fig. S2, Supporting information).

The DDPCR assay detected consistent frequency differences between strain pairs even when absolute cell numbers were difficult to accurately count. We expected to encounter difficulties measuring absolute cell numbers because leaf litter contains PCR inhibitors, and because leaf litter is a heterogeneous substrate. PCR inhibitors in the leaf litter probably influenced total DDPCR signal (Opel *et al.* 2010). Also, some litter samples may have adhered more closely than others to surface microbes, including *S. paradoxus* cells, when we harvested cells for DNA extraction (Dang *et al.* 2007). We did not expect either PCR inhibitors or litter heterogeneity to bias the detected strain relative frequencies, and we found no such biases in test samples (Fig. 1). However, the DDPCR assay did not accurately measure total cell number in test samples: total DDPCR signal and colony count did not correlate based on incidental variation in cell numbers, although we did not explicitly manipulate absolute cell numbers in the test samples (Pearson's $r = 0.01$, $t = 0.04$, d.f. = 16, $P = 0.98$). In general, we expect all PCR-based methods to give coarse estimates of

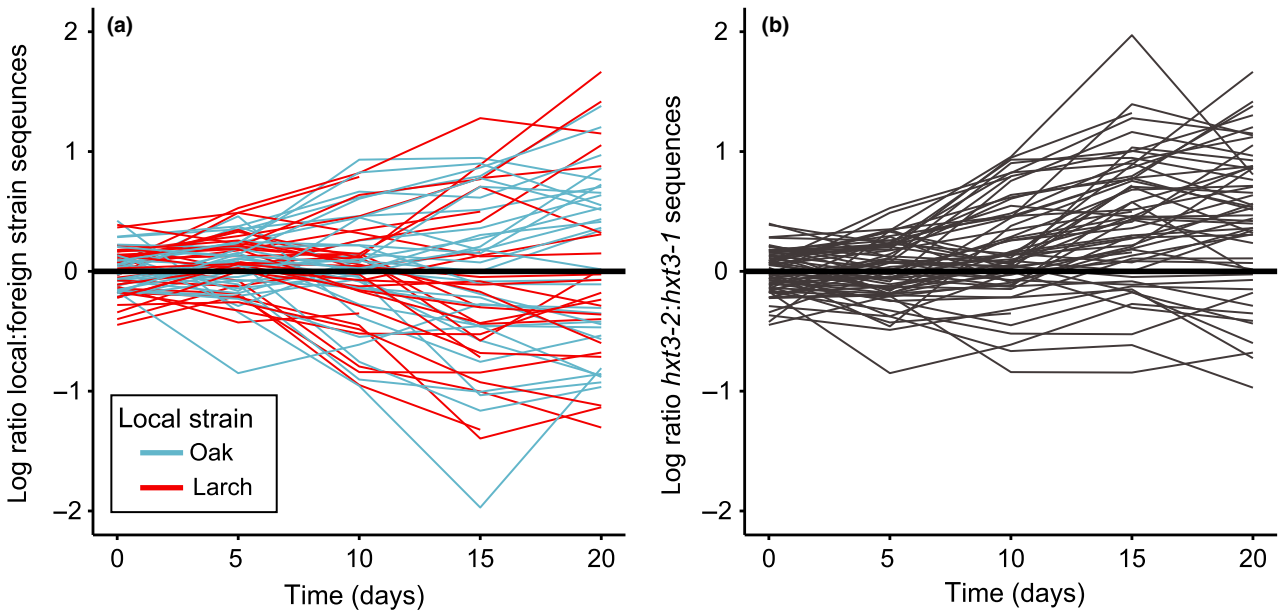


**Fig. 2** Change in gene count frequencies over time. Each trace is a single strain pair under a single tree. Heavy black lines indicate equal numbers of both strains. (a) Change in the ratio of local:foreign individuals over time. The *y*-axis is ln(local strain *HXT3* gene copies/foreign strain *HXT3* gene copies). (b) Change in the ratio of individuals with the two *HXT3* genotypes over time. The *y*-axis is ln(*hxt3-2* copies/*hxt3-1* copies). [Colour figure can be viewed at wileyonlinelibrary.com]

**Table 2** Fixed and random factors predicting the ratio of sequences originating from oak and larch strains

| Model | Fixed effects | Random effects | AIC | Compared to | $\chi^2$ | d.f. | *P* | Better model |
|---|---|---|---|---|---|---|---|---|
| 1 | G + Ls + Tp + G:Ls + G:Tp + Ls:Tp + G:Ls:Tp | Tp\|P + Tp\|Tr | 349.94 | | | | | |
| 2 | G + Ls + Tp + G:Ls + G:Tp + Ls:Tp + G:Ls:Tp | 1\|P + Tp\|Tr | 345.94 | Model 1 | 0 | 2 | 1 | 2 |
| 3 | G + Ls + Tp + G:Ls + G:Tp + Ls:Tp + G:Ls:Tp | Tp\|Tr | 350.28 | Model 2 | 6.34 | 1 | 0.012 | 2 |
| 4 | G + Ls + Tp + G:Ls + G:Tp + Ls:Tp + G:Ls:Tp | 1\|P + 1\|Tr | 342.24 | Model 2 | 0.30 | 2 | 0.86 | 4 |
| 5 | G + Ls + Tp + G:Ls + G:Tp + Ls:Tp + G:Ls:Tp | 1\|P | 340.78 | Model 4 | 0.54 | 1 | 0.46 | 5 |
| 6 | G + Ls + Tp + G:Ls + G:Tp + Ls:Tp | 1\|P | 338.84 | Model 5 | 0.06 | 1 | 0.81 | 6 |
| 7 | G + Ls + Tp + G:Tp + Ls:Tp | 1\|P | 336.92 | Model 6 | 0.08 | 1 | 0.78 | 7 |
| 8 | G + Ls + Tp + G:Tp | 1\|P | 339.48 | Model 7 | 4.56 | 1 | 0.033 | 7 |
| 9 | G + Ls + Tp + Ls:Tp | 1\|P | 394.77 | Model 7 | 59.85 | 1 | <0.001 | 7 |
| 10 | G + Tp + Ls:Tp + G:Tp | 1\|P | 335.04 | Model 7 | 0.12 | 1 | 0.73 | 10 |
| 11 | Tp + Ls:Tp + G:Tp | 1\|P | 335.07 | Model 10 | 2.02 | 1 | 0.15 | 11 |
| 12 | Ls:Tp + G:Tp | 1\|P | 335.07 | Model 11 | 0 | 0 | 1 | 12 |

G, genotype of the local strain; Ls, local strain source (oak or larch litter); Tp, time point; P, pair of strains; Tr, tree identity.

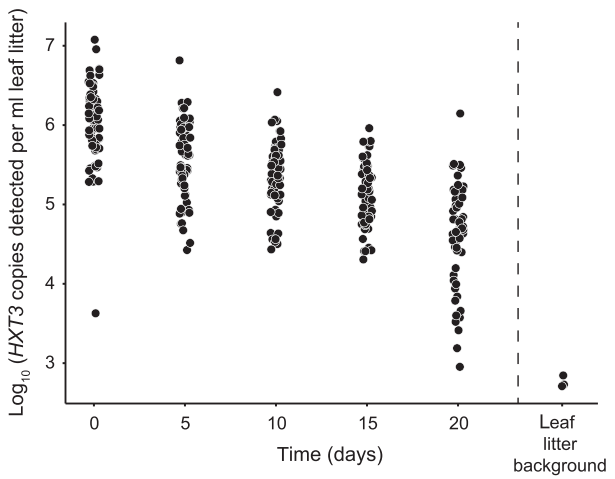


**Fig. 3** Decay in *Saccharomyces paradoxus* digital droplet PCR (DDPCR) signal over time and background DDPCR signal from uninoculated leaf litter. Points are slightly offset on the *x*-axis for clarity. Samples with no DDPCR signal are not shown (five of 313 experimental and 11 of 14 leaf litter background samples).

total microbial cells when cells are rinsed from heterogeneous surfaces containing soil particles.

We tentatively interpret the decrease in DDPCR signal over time in the field experiment as evidence for a decrease in total cell numbers (Fig. 3, Table S1, Supporting information). Variation in DDPCR signal was much higher in the field experiment than in test samples, and the decrease in field sample DDPCR signal occurred on a log scale. Sample cell numbers could have decreased either due to death or washing out of the litter. Both processes can be components of survival fitness in leaf litter if washing carries *S. paradoxus* cells to soil layers where they cannot survive. Unfortunately, we do not understand *S. paradoxus* natural history in soil well enough to know the fitness consequences of washing from leaf litter.

We speculate that viable *S. paradoxus* cell number decreased over the course of the field experiment because we inoculated leaf litter with yeast cultures containing large numbers of cells grown in YPD medium, and we expect leaf litter to maintain smaller *S. paradoxus* populations than YPD. The predominant component of leaf litter is lignocellulose, which *Saccharomyces* yeasts cannot use (Gupta & Malik 1999; Lynd *et al.* 2002). Unfortunately, we cannot detect ecologically relevant *S. paradoxus* population sizes using PCR; we were limited to observing decreases in large inoculated populations (the survival component of fitness) instead of measuring differences in total reproductive output among strains (both survival and growth components of fitness, Orr 2009). Local adaptation is a function of total fitness integrated over all local conditions an organism encounters. Survival is crucial for fitness: individuals that do not survive have zero reproductive output. Eventually, we would expect cells in our experiment to reach population sizes comparable with those naturally occurring on leaf litter, at which point frequent genotypes would contribute more to the next generation than infrequent genotypes. Selection on survival may be particularly important for *Saccharomyces* fitness in forest habitats, especially if yeasts use leaf litter as reservoirs for long periods of time when high-sugar environments are not available (Knight & Goddard 2016). Our experiment measured *S. paradoxus* relative fitness, but only under conditions in which growth rates were negative.

While observed differences in *S. paradoxus* strain frequencies are most likely due to differences in survival between the two inoculated strains, other factors may have influenced strain frequencies in the field. If isolates from one strain source had more or less genetic variation overall than the other strain source, we would expect our results to be driven by the behaviour of common genotypes. We attempted to avoid isolating exact clones from

litter by only using isolates from different trees or different isolation times, but did not measure genetic variation beyond *HXT3* genotypes. However, the even distribution of *HXT3* genotypes across strain sources suggests that the two strain sources had similar levels of genetic variation. DNA from dead cells could also have influenced our results because PCR can detect intact DNA from nonviable cells (Josephson *et al.* 1993). Unfortunately, we do not know enough about *S. paradoxus* mortality in the field to evaluate the influence of dead cells on our results, although the observed decrease in DDPCR signal (Fig. 3) suggests that large numbers of dead cells were not detected. We could not directly test the influence of dead cells on our results because we expect the cause of cell death to influence DNA availability for PCR; for example, DNA from cells killed by exposure to ultraviolet light probably persists on leaf litter, while DNA from cells eaten by invertebrates is probably digested. If detected, we expect persisting DNA to make it more difficult to detect changes in allele frequencies in leaf litter, contributing to potential false negatives and making our results more conservative.

While *S. paradoxus* source populations and genotypes showed consistent differences in persistence (Fig. 2b, Fig. S2, Supporting information), we found no evidence of local adaptation. Local strains did not consistently increase in frequency over time (Fig. 2a). However, this negative result does not rule out local adaptation: strains may be locally adapted over space but not between litter habitats; we may have investigated local adaptation at an inappropriate scale; or local adaptation may be temporal. Temporal local adaptation has previously been suggested as a mechanism for coexistence of different *Saccharomyces* species (Sweeney *et al.* 2004; Sampaio & Gonçalves 2008). Strains may also be locally adapted with respect to the growth component of strain fitness instead of the survival component.

Alternatively, high or low dispersal may have prevented local adaptation in the Nehmten forest. It is unclear how *S. paradoxus* disperses in nature, but high or low dispersal can prevent populations from locally adapting (Kawecki & Ebert 2004). If dispersal is high, gene flow prevents local populations from specializing. For example, high dispersal can lead to source–sink dynamics. Source–sink dynamics occur when a population in a low quality habitat is maintained, not by local reproduction, but by immigrants from a higher quality habitat (Pulliam 1988). Migrants from source habitats can prevent adaptation to sink habitats, and even lead to local maladaptation (Dias 1996; Dias & Blondel 1996; Holt & Gomulkiewicz 1997). Larch litter is a potential sink habitat because *S. paradoxus* population sizes are larger on oak litter than larch litter (V. Kowallik & D. Greig, unpublished observations). If oak litter is a *S. paradoxus* source and larch litter a sink, we would expect no effect of strain source on relative fitness because all larch *S. paradoxus* strains would be migrants from the oak litter habitat.

Instead, we observe a significant, but weak, effect of strain source on relative frequency, suggesting that *S. paradoxus* dispersal is low in the Nehmten forest. We would expect fitter oak strains to invade larch habitats if dispersal is high. If dispersal is low, we would expect drift to influence *S. paradoxus* evolution in small larch *S. paradoxus* populations more than local selection. We would also expect selection from intraspecific competition to be high on oak litter if oak *S. paradoxus* individuals directly compete more in larger populations. However, previous studies found evidence of extensive recombination among European *S. paradoxus* strains, suggesting that *S. paradoxus* is not dispersal limited in Europe (Koufopanou *et al.* 2006; Liti *et al.* 2009; Boynton & Greig 2014). Most strains from these population studies were isolated from oak surfaces, and future work is needed to confirm population patterns for larch *S. paradoxus*. Our observed difference between oak and larch strain relative fitnesses is slight; further direct observations coupled with studies of genetic diversity are needed to understand the extent of dispersal limitation, if any, in Nehmten *S. paradoxus*.

The field experiment identified strong effects of *HXT3* genotypes on persistence (Fig. 2b). *Hxt3-2* strains were consistently fitter in leaf litter, regardless of habitat or strain identity. *HXT3* probably plays a similar role in *S. paradoxus* as it does in *S. cerevisiae*: *S. cerevisiae HXT3* variants have differing rates of glucose and fructose uptake, and some genotypes prefer either fructose or glucose (Guillaume *et al.* 2007; Zuchowska *et al.* 2015). In the light of high *hxt3-2* relative fitness, there are several possible explanations for the polymorphism we observed when sampling *S. paradoxus* strains. *Hxt3-2* may be a fitter genotype under the conditions of our experiment (many cells inoculated onto a nutrient-poor substrate), but less fit when a cell encounters high nutrient conditions, pulses of nutrients or few conspecific competitors. Local spatial or temporal environments may also select *HXT3* genotypes on scales that we did not explicitly observe.

### *Recommendations for future researchers*

Researchers adapting this assay to their own systems should take particular care with experimental design and statistical analyses. For example, careful experimental design can avoid problems when, as in our study, the marker used to assay strain frequencies has fitness effects. An ideal experimental design would use a neutral marker, but marker fitness effects are not always

known. Researchers can avoid confounding marker fitness with experimental treatments by distributing markers evenly among treatments and accounting for marker fitness in statistical analyses. We also recommend carefully selecting a model's random effects to account for variation in strain genotypes and environmental conditions. Our model accounted for variation among strain pairs, although our experimental design did not allow for identification of particularly fit strains because each strain was paired with only one other strain. Researchers interested in identifying individual fit or unfit strains should replicate strains among several pairs. We also accounted for variation in litter environments among trees in our statistical model instead of experimentally. An alternative would have been to reduce environmental variation by mixing all collected leaf litter into a single batch, and then partitioning the litter among strain pairs. We hope researchers will modify our experimental design to suit the needs of their research questions.

We used DDPCR instead of QPCR to detect *in situ* changes in microbial frequencies, but the choice of a molecular method depends on the details of the study. DDPCR is advantageous on substrates containing PCR inhibitors because it is less sensitive to inhibitors than QPCR (Yang *et al.* 2014). DDPCR is also advantageous in assays of unculturable organisms because it does not require a standard curve of DNA quantity to cycle threshold. On the other hand, QPCR is advantageous when comparing more than two organisms because the currently available BioRad DDPCR system can only multiplex two fluorescent dyes (Koch *et al.* 2016). DDPCR is often, but not always, both more sensitive and more expensive per sample than QPCR (Kim *et al.* 2014; Nathan *et al.* 2014; Yang *et al.* 2014). The choice of a molecular method ultimately depends on the experimental needs of the researcher and the costs and availability of each method.

## Conclusion

Digital droplet PCR effectively measures microbial fitness in nature by tracking changes in genotype frequencies. Our protocol successfully found fitness effects of strain source and *HXT3* genotype in *S. paradoxus* strains in their natural habitat. These positive results allowed us to formulate hypotheses about *S. paradoxus* dispersal limitation and sugar utilization under realistic field conditions.

Researchers can measure microbial fitness using PCR to monitor genotype frequencies over time in any natural habitat desired. PCR-based methods target existing genetic variation, eliminating the need for transgenic markers in fitness assays. And researchers can overcome PCR sensitivity to inhibitors in natural substrates by measuring changes in relative frequencies instead of absolute fitness. Experimental protocols similar to ours will be particularly useful when studying interactions among soil microorganisms, but a variety of systems have limitations similar to those of soil and leaf litter. For example, PCR inhibitors are common in animal blood and tissues (Hedman & Rådström 2013). DDPCR or QPCR can be used to measure infectious microbial fitness *in situ* with minimal information about pathogen genotypes. In experimental systems where it is ethical to introduce pathogens to hosts, researchers can compare microbial competitive fitness with common fitness proxies, including infection persistence and virulence (Refardt & Ebert 2007). Finally, as in our study, this method can be used to detect local adaptation in a variety of microbial systems because it easily measures relative fitness between local and foreign individuals.

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P.J.B., R.S., V.K. and D.G. designed the field study. V.K. isolated *Saccharomyces paradoxus* strains, and P.J.B., R.S. and D.G. conducted the field experiment. P.J.B. designed the DDPCR assay and collected DDPCR data. P.J.B. and R.S. analysed the data. P.J.B. wrote the manuscript, with help from R.S., V.K., and D.G.

## Data accessibility

DNA sequences of *HXT3* alleles *hxt3-1* and *hxt3-2* are available in GenBank (accession nos. KX266864-KX266865). DDPCR outputs and sample metadata are available as .csv files in the Dryad Repository (doi: 10.5061/dryad.mb780).

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1** Locations of fourteen trees used for the field experiment.

**Fig. S2** Change in local strain frequency relative to foreign strain over time.

**Table S1** Random and fixed factors predicting DNA concentration over time