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Ploidy tug-of-war: evolutionary and genetic environments influence the rate of ploidy drive in a human fungal pathogen

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Running title: Evolutionary and genetic environments impact ploidy drive

Abstract

Variation in baseline ploidy is seen throughout the tree of life, yet the factors that determine why one ploidy level is selected over another remain poorly understood. Experimental evolution studies using asexual fungal microbes with manipulated initial genome sizes intriguingly reveals a propensity to return to the historical baseline ploidy level, a phenomenon that we term here as 'ploidy drive'. To assess whether evolution under nutrient limitation, an environmental condition hypothesized to select for low ploidy levels, could counteract ploidy drive, we evolved haploid, diploid and polyploid strains of the human fungal pathogen *Candida albicans*. We found that strains indeed tended to maintain or acquire smaller genome sizes in a minimal medium and under phosphorus depletion compared to in a complete medium, yet tended to maintain or acquire increased genome sizes under nitrogen depletion. Genetic background also contributed to ploidy drive dynamics, as one diploid and one polyploid genotype were markedly less stable than others. Combined, this work demonstrates a role for both the environment and genotype in determination of the rate of ploidy drive.

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Introduction

Baseline ploidy levels vary among closely related extant species and whole genome sequencing has revealed widespread paleopolyploidy throughout the eukaryotic tree of life (Gregory TR and Mable 2008; Albertin and Marullo 2012; Wendel 2015, and references within). Ploidy variation has the potential to affect many aspects of evolutionary dynamics (Thompson and Lumaret 1992; Orr and Otto 1994; Otto and Whitton 2000; Gerstein and Otto 2009, and references within). There are theoretical advantages to both high and low ploidy levels, primarily related to differences in the mutation rate and the efficiency of selection. In brief, more mutations arise in higher ploidy backgrounds, thus if new beneficial mutations are the rate-limiting step in adaptation, higher ploidy populations should be advantageous. However, if the adaptive mutations are not completely dominant or have low penetrance, they will take longer to reach high frequency in higher ploidy populations and may be lost due to chance. Additional complexities regarding the influence of ploidy level on the characteristics of beneficial mutations have been revealed in recent experimental studies with yeast. Different ploidy levels may have different suites of mutations available to them (Orr and Otto 1994; Gerstein and Otto 2009; Selmecki et al. 2015). Furthermore, the same mutations can have different effect sizes in different ploidy backgrounds, independent of dominance (Gerstein 2012; Selmecki et al. 2015), implying that ploidy itself has important consequences on the mutational pathways available to evolution. Therefore, the theoretical "optimal" ploidy level may depend heavily on the selective environment, genetic background, and characteristics of the specific set of available beneficial mutations.

Experimental evolution studies *in vitro* using ploidy variants of fungal microbes revealed a puzzling ploidy phenomenon: convergence towards the species historical, baseline ploidy within tens to hundreds of generations under diverse environmental conditions. This phenomenon has been repeatedly observed in the limited number of batch culture experiments tracking ploidy and across evolutionarily divergent fungal taxa. These studies include ascomycete and basidiomycete fungal species whose historical baseline ploidy is diploid, including *S. cerevisiae* (Gerstein et al. 2006; Gresham et al. 2008; Voordeckers et al. 2015) and *Candida* species (*C. albicans*: Hickman et al. 2015)**;**

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C. tropicalis: Seervai et al. 2013), and in species whose historical baseline ploidy is haploid (*C. neoformans*: Gerstein et al. 2015; *Schizosaccharomyces pombe*: V. Perrot et al., pers comm; *A. nidulans*: Schoustra et al. 2007). Here, we term the force acting on ploidy to push it towards its baseline level as 'ploidy drive'.

Intriguingly, a clear selective advantage to diploid cells over haploids has only been identified in two of the recent experiments where ploidy drive was identified in *S. cerevisiae* (under carbon limitation, Venkataram et al. 2016; and under ethanol stress, Voordeckers et al. 2015). In contrast, in the majority of identified cases of ploidy drive there was no clear fitness advantage attributable to ploidy *per se* (e.g., Gerstein and Otto 2011; Hickman et al. 2015), and thus the mechanism that enabled ploidy variant cells to be maintained and selected within populations remains extremely cryptic. The evolutionary environment clearly plays a significant role in this process, however, as the rate at which ploidy drive operates (i.e., how quickly a population of atypical ploidy returns to the baseline ploidy) is significantly influenced by the evolutionary environment. For example, *S. cerevisiae* haploids more rapidly became diploids and tetraploids maintained high ploidy states under salt stress compared to rich medium, (Gerstein et al. 2006). Similarly, *C. albicans* tetraploids more frequently reduce in genome size at high temperatures and when sorbose is the carbon source compared to glucose (Bennett and Johnson 2003).

In single-celled eukaryotes, ploidy directly correlates with physiological characteristics such as cell volume (Schmoller et al. 2015). The nutrient limitation hypothesis, first posed by Lewis (1985), suggests that since haploid cells have a higher surface area:volume ratio (SA:V) relative to higher ploidy cells, nutrient scarcity serves as a selective force promoting haploidy because of their increased capacity for passive nutrient uptake from the extracellular environment. An increase in energy and material costs is also predicted to select against higher ploidy under nutrient scarcity, particularly when there is selective pressure on growth rate due to the high demand for P for ribosomes and N for proteins (Hessen et al. 2010). Thus, in the context of ploidy drive, we predict that nutrient limitation might be able to counteract ploidy drive, such that haploid populations will maintain haploidy and polyploid populations will reduce in ploidy more rapidly under nutrient limitation compared to a complete or rich medium.

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In this study, we investigated how selection environment and strain background influence the dynamics of ploidy drive by tracking ploidy transitions in *C. albicans* during experimental evolution under nutrient limitation. C. *albicans* is an opportunistic fungal pathogen of humans and other warm-blooded animals. It can proliferate in a large diversity of sites including kidneys, skin, nails, and mucosal surfaces (Odds 1988). Although *C. albicans* is predominantly diploid, recent studies uncovered both haploid and polyploid strains of *C. albicans* of clinical and laboratory origin (Suzuki et al. 1982; Marr et al. 1997; Hickman et al. 2013; Abbey et al. 2014). The isolation of ploidy variants, particularly of clinical origin, suggests there that genetic background and/or environmental factors influence the maintenance of atypical ploidy states. In the laboratory, a variety of stress conditions result in karyotypic changes (Janbon et al. 1998; Bennett and Johnson 2003; Wellington and Rustchenko 2005; Chang et al. 2014; Harrison et al. 2014). Here, we evolved strains with different genetic backgrounds that were initially haploid, diploid, and polyploid under four environmental conditions, and identified that specific evolutionary environment as well as strain background as significant factors that influenced the rate of ploidy drive.

Methods

Strains & environments

We utilized nine *C. albicans* strains for this study that vary in their relationship to each other. (Table 1). The first set are homozygous lab strains, consisting of two related haploid strains (1N1 & 1N2, that are ~91% similar) and a diploid strain (2N1) that is isogenic to strain 1N1 (note that clinical homozygous strains have not yet been recovered). The second set are strains of clinical origin, and contain a heterozygous diploid strain (2N2), a polyploid strain with a complex karyotype isolated from the same patient (4N1) and a euploid tetraploid strain isolated from a different patient (4N2). The remaining strains include the diploid laboratory reference strain SC5314 , 2N3), and two related polyploids (4N3 & 4N4, Table 1).

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Table 1. Stains used in this study

Strain	Alias	Ploidy	Background	Reference
1N1	YJB12804	haploid	SC5314-derived haploid after fluconazole exposure (*his-*)	Hickman et al 2013
1N2	YJB12801	haploid	SC5314-derived haploid selected for multiple LOH events (*his-*)	Hickman et al 2013
2N1	YJB12908	diploid	YJB12804-derived homozygous diploid	Hickman et al 2013
2N2	FH1	diploid	Clinical isolate from marrow transplant patient	Marr et al 1997
2N3	SC5314	diploid	Laboratory reference strain	Gillum et al 1984
4N1	FH6	polyploid	Clinical isolate from same marrow transplant patient 4 × ChrR, Chr1, Chr2, Chr3, Chr7, 3 × Chr4, Chr5, Chr6), 2 × [i(5L)]	Marr et al 1997
4N2	PN1	polyploid	Clinical strain recovered from vaginal infection Euploid 4N	this study
4N3	10154	polyploid	Result of mitotic failure during chemical transformation (*ade-*) 4 × ChrR, Chr1, Chr2, Chr4, Chr5 and Chr7, 3 × Chr3, Chr6	this study
4N4	RBY18	polyploid	Mating product between two laboratory strains Euploid 4N	Bennett and Johnson 2003

Each strain was initially streaked from glycerol stocks stored at -80°C onto an SDC (synthetic defined complete medium) plate. After 48 h at 30°C, a single colony was arbitrarily chosen and transferred to a microcentrifuge tube in 15% glycerol. We refer to these single-genotype stocks as the "ancestral strains."

We assessed ploidy drive in four different environments. Standard complete yeast medium ("SDC") contains 1.7g/L yeast nitrogen base (which includes the phosphorus source, 1.0g/L KH_2PO_4), 5.0g/L ammonium sulfate (the nitrogen source), 0.08g/L uridine, 0.04g/L adenine, and standard amino acids (Rose et al. 1990). The three nutrient reduction environments were SD (complete medium without amino acids, i.e., a general minimal medium), nitrogen-depleted ("Ndep"), and phosphorus-depleted ("Pdep"). We chose nitrogen and phosphorus due to their biological relevance at both the ecological and genomic levels (Lewis 1985; Elser et al. 1996; Sterner et al. 2002; Elser et al. 2007) and theory posits an advantage to genome streamlining (and lower ploidy) under phosphorus and nitrogen limitation (Hessen et al. 2010). Furthermore, as a commensal organism in the human body *C. albicans* experiences general nutrient limitation through

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competition with co-commensal microbial organisms in the human gut, a primary site of *C. albicans* colonization.

Preliminary experiments were conducted to determine the maximal amount of nitrogen and phosphorus that could be removed while still achieving a growth plateau by 48 h (results not shown). Ndep was thus constructed with the same base medium as SD without adding any ammonium sulfate (i.e., 100% reduction of the nitrogen source). For Pdep we found that we could not completely eliminate the primary phosphorus source (KH_2PO_4) as strains begun to go extinct after three transfers. We thus constructed Pdep medium with YNB without KH_2PO_4 (purchased from Sunrise Scientific powders) and added back 10% of the typical amount of KH_2PO_4 (0.1 g/L; i.e. 90% reduction of the phosphorus source). We also added 0.493g/L KCl to Pdep to compensate for the potassium lost from reduction of KH_2PO_4 (following Homann et al. 2009). Due to auxotrophic strains in the strain set (Table 1), we were required to supplement SD, Pdep, and Ndep with 20mg/L adenine and 20mg/L histidine, thereby adding a secondary, albeit minor, nitrogen source beyond ammonium sulfate. The amount of carbon (glucose) was 2% in all environments.

Initial growth ability in Ndep and Pdep

Initial growth rates were measured in all environments. The ancestral strains were streaked onto SDC and incubated for 48 h at 30°C. A single colony was randomly chosen and inoculated into 4 mL SDC and incubated while shaking at 250 rpm for 24 h at 30°C. Optical density (OD) at 595 nm was measured using a NanoDrop (Thermo Scientific) and sterile dH_2O was added as necessary to standardize all overnight cultures to the same (minimum) OD. Standardized cultures were then diluted 1:100 into 1 mL of the appropriate medium. 150 µl of diluted culture was transferred to a clear round bottom 96 well plate and placed on a Tecan microplate reader, with four replicates per strain. OD was automatically read every 15 min for 48 h at 30°C with on the highest shaking setting.

Batch culture evolution

We conducted batch culture evolution experiments in SDC, SD, Pdep and Ndep. To initiate each evolution experiment, the ten ancestral strains were streaked from frozen

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onto SDC plates. After 24 h at 30°C, a single colony from each was transferred to 4 mL SDC and incubated shaking at 250rpm at 30°C for 24 h. Overnight culture OD was standardized to the lowest OD, and 10 µL was inoculated into 1 mL of the appropriate medium in deep well culture blocks. Twelve replicate wells were inoculated for each strain.

We transferred all lines for 21 transfers in each environment; for SDC, where all ancestral strains grew quickly, we conducted 1:101 transfers every 24 ± 2 h; for SD, Ndep, and Pdep we conducted 1:101 transfers every 48 ± 2 h to allow lines to reach stationary phase. SD and Ndep transfers were conducted from the same starting culture. Deep (3 mL) 96 well culture blocks were incubated at 30°C with continuous shaking at 250 rpm. Ancestral and evolved samples were frozen after transfers 1 and 21 in 50% glycerol maintained at -80°C. We thus evolved a total of 480 lines (12 evolved lines x 10 ancestral strains x 4 growth conditions) for ~140 generations (21 transfers x 6.67 generations between each transfer).

Growth rates of ancestral strains and evolved lines

From the initial growth experiments used to determine the appropriate levels of Ndep and Pdep we measured four replicates from all ancestral strains in each of the four environments. We also conducted a growth rate experiment on the evolved lines. Frozen ancestral strains and evolved lines were thawed and 10 µL of culture was transferred into 500 µL SDC and incubated at 30°C for 24 h, shaking at 250 rpm. Due to space limitations we measured only ten of the twelve evolved lines (always the first ten replicate wells) from each strain and 2 replicate measurements from each ancestral strain. Overnight culture ODs were measured on the Tecan plate reader and standardized with sterile dH_2O to the lowest OD. Standardized culture was diluted 1:100 into 1 mL of the appropriate medium and two replicates of 150 µl were transferred to a clear round bottom 96 well plate and placed on a Tecan microplate reader. Optical density at 595 nm was recorded every 15 min for 48 h, with growth at 30°C with high shaking. Growth rate was determined using custom R scripts that calculate the maximal growth rate in each well as the spline with the highest slope from a loess fit through log-transformed optical density data that reflects the rate of cell doubling (as in Gerstein and Otto 2011). We

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limited the analysis of direct comparisons between ancestral and evolved growth rates to minimize the influence of differences in replicate numbers and potential variation from day effects, which resulted from a consequence of machine space limitations. For each ancestral strain we calculated the mean ancestral growth rate across all measured replicates for our analyses (i.e., three biological replicates, each with two replicates), which focused primarily on the mean differences between evolved and ancestral growth rates.

Ploidy analysis with flow cytometry

Flow cytometry was used to determine the genome size of populations from all evolved lines. Sample preparation was similar to previously published methods in *C. albicans* (Hickman *et al.,* 2013) with the following modification: SybrGreen (a dye that stains DNA and thus enables genome size quantification by measurement of fluorescence intensity) was diluted 1:100 in 50:50 TE and incubated in the dark overnight at room temperature (the previously published protocol used 1:85 diluted SybrGreen solution and incubated overnight at 4°C). All ancestral and evolved lines from all environments from the same initial ploidy level were always prepped simultaneously and run on the same day on an LSRII flow cytometer (BD Biosciences); this enables us to accurately compared ancestral and evolved genome sizes. We collected FITC-A fluorescence data from 10 000 cells from each population. As we purposefully did not bottleneck populations prior to analysis, in some cases multiple peaks representing subpopulation of differing genome sizes were present.

The mean G1 peak of the predominant ("major") ploidy population (i.e., a histogram of FITC-A values) was recorded for each replicate in Flowjo (Tree Star) using the cell cycle platform to fit the Watson pragmatic model. There is some unavoidable ambiguity in ploidy determination through flow cytometry. Ancestral haploid cells always contained a large G2 peak (which is indistinguishable from a diploid G1 peak), thus when a visible haploid peak was retained after evolution we recorded the major peak for that population as haploid (Figure S1A). Conversely, ancestral diploid and tetraploid cells are predominantly in G1 phase after an overnight in YPD, thus for initially-diploid and initially-polyploid evolved populations we recorded the major ploidy population as

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the highest peak (Supplementary Figure 1B). All samples were prepared and measured twice by flow cytometry with extremely consistent results. We based the majority of our analyses on the major peak observed but also recorded additional ("minor") peaks when they were present (Figure S1). Genome size change was calculated by comparing the major evolved peak to the median ancestral genome size.

Statistical analyses

All analyses were conducted in the R programming language (R Core Team 2014). The influences of strain background and the evolutionary environment on the prevalence of genome size change (i.e., ploidy drive) was analyzed separately for each initial ploidy level, as different mechanisms are involved in the increase or decrease of genome size. For each initial ploidy level we ran a two way ANOVA with strain background and evolutionary environment (and their interaction) as the predictor variables and change in genome size as the response. When the interaction was not significant we re-ran the model without this term. To determine the relationship among environments we ran the HSD.test function (i.e., Tukey test with multiple comparisons) from the agricolae package (de Mendiburu 2015).

We tested whether initial ploidy and evolutionary environment (or their interaction) significantly influenced growth rate change across the entire dataset with a linear mixed-effect model with strain background as a random effect with the lmer function from the lme4 package (Bates et al. 2015) (degrees of freedom and p-values calculated using the Satterthwaite approximation implemented in the lmerTest package, (Kuznetsova et al. 2016). For each initial ploidy level we then individually assessed whether the observed changes in genome size could predict the improvement in growth rate by running similar linear mixed-effect models with both strain background and environment as random effects.

Results

We assessed the genome size stability of 432 lines evolved from nine different strain backgrounds and three initial ploidy states (haploid, diploid and polyploid) after ~140 generations of evolution in complete medium (SDC), minimal medium (SD),

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phosphorus-depleted medium (Pdep) and nitrogen-depletion medium (Ndep) with the aim to determine whether evolution under nutrient limitation could select against ploidy drive, the force acting on genome size to push it towards its baseline level (diploid in *C. albicans*). If the nutrient limitation hypothesis, which predicts that lower ploidy levels will be beneficial under nutrient-limiting conditions, governs ploidy dynamics, we would expect (1) when there is no nutrient restriction (SDC) there should be no selection against ploidy drive, and we would observe convergence towards diploidy, and (2) when nutrients are limiting (i.e. SD, Pdep and Ndep), ploidy drive should be mitigated by selection towards haploidy (or reduced genome sizes), manifested by maintenance of initially haploid strains and ploidy reduction in diploid and polyploid strains.

We observed that ploidy dynamics were largely influenced by initial ploidy and strain background (Figure 1, Figure S2). Heterozygous diploidy (purple symbols), was by far the most stable ploidy state, regardless of the growth environment. Only 4% of these lines (6/144: 3 strains x 12 evolved lines x 4 growth conditions) evolved non-diploid populations. All six ploidy-variant lines arose under Pdep from the same genetic background, 2N1, the only homozygous diploid strain (Figure S2), and included five lines with a major subpopulation and one line with a minor subpopulation of increased genome size. The increase in genome size under Pdep evolution of diploid lines is surprising and inconsistent with the nutrient limitation hypothesis.

In contrast to the high degree of ploidy stability observed in diploid lines, only ~50% (49/98) of initially-haploid, and ~74% (142/192) of initially-polyploid lines maintained even a minor haploid or polyploid cell population (respectively) after evolution (Figure S2), a result that is consistent with ploidy drive pushing both haploid and polyploid strains towards diploidy. These results indicate that a more-nuanced explanation for ploidy dynamics is required than simply the presence or absence of nutrient limitation.

The frequency of ploidy drive was influenced by both the evolutionary environment and strain background (Figure 1). We ran two way ANOVAs with the change in genome size as the response variable separately for each initial ploidy level (see methods section and Figure S1 for details). We found that the environment was a significant predictor of ploidy drive rate in haploids, while in both diploids and

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tetraploids there was an interaction between strain background and the environment (haploids—enviro: $F_{3,91}= 69.4$, $P < 0.0001$, strain: $F_{1,91}= 0.02$, $P = 0.89$; diploids—enviro: $F_{3,132}= 7.22$, $P < 0.0001$, strain: $F_{2,132}= 9.16$, $P < 0.0001$, strain*enviro: $F_{6,132}= 4.04$, $P = 0.001$; polyploids —enviro: $F_{3,175}= 10.8$, $P < 0.0001$, strain: $F_{3,1756}= 63.4$, $P < 0.001$, strain*enviro: $F_{9,1765}= 7.7$, $P < 0.001$). The precise relationship between environment and evolved genome size was influenced by initial ploidy (assessed by post-hoc tukey tests, Table S1). The two haploid strains responded very similarly: the lines from both increased in genome size in SDC (complete medium), generally maintained haploidy in SD and Pdep, and had variable ploidy (both haploid and diploid) in Ndep. As described above, the only change in genome sizes observed in initially-diploid strains occurred in lines of the homozygous genotype evolved under Pdep (Table 1). In initially polyploid lines, ploidy drive towards diploidy was least frequent in complete medium (SDC), most frequent in minimal media (SD) and intermediate in frequency in Pdep and Ndep. The rate of ploidy drive varied significantly among different initially-polyploid strains, driven by strain 4N1 lines that more frequently evolved towards diploidy compared to the other strains (Table S1, Figure S2).

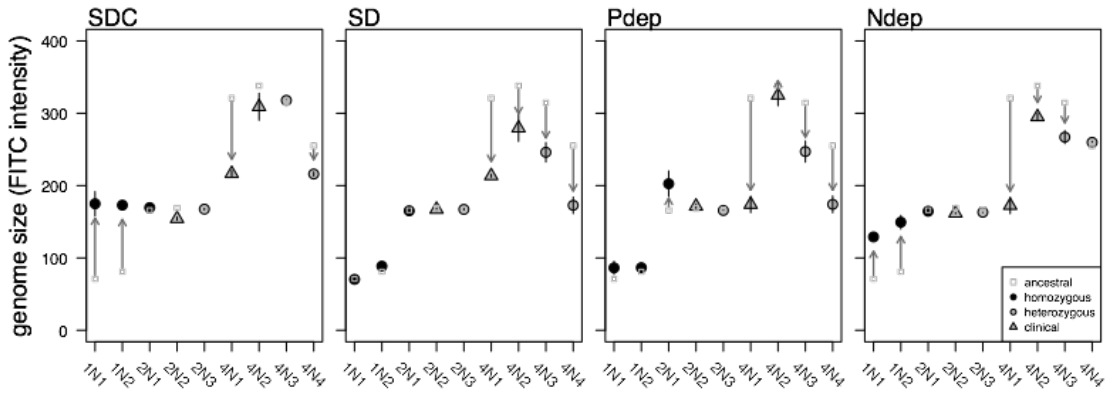

Figure 1. The frequency of ploidy drive was influenced by evolutionary environment and strain background and. Haploid, diploid and polyploid lines were evolved in complete (SDC), minimal (SD), phosphorus-depleted (Pdep) and nitrogen-depleted (Ndep) medium. Shown is the mean (+/- SE) major-population genome size of 12 replicate lines evolved for ~140 generations. Grey open squares indicate ancestral genome sizes measured as fluorescence intensity (i.e., FITC intensity of ~90 corresponds to haploidy, ~190 corresponds to diploidy, while ~ 310 corresponds to tetraploidy; note that fluorescence intensity does not scale linearly with genome size). Evolved ploidy levels intermediate to euploid are due to the presence of aneuploidy as well as a result of averaging among 12 lines (see Figure S2).

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There must be a fitness benefit to ploidy-variant cells that arise within large populations in order for these mutants to arise to high enough frequency to be observed by flow cytometry. We tested whether the observed genome size changes could be explained by variation in initial growth rate or biomass production (final optical density). We found a strong and significant correlation between growth rate and biomass production in all environments (Pearson correlation tests; $p < 0.003$ and $r > 0.86$ in all cases; Table S2 & Figure S3), and thus focused on the growth rate data. We observed that initial fitness was primarily influenced by strain background, rather than initial ploidy (Figure 2). Specifically, the homozygous strains (1N1, 1N2 and 2N1) were always amongst the slowest growing for all growth environments, even in SD and Pdep, where haploidy was maintained. Furthermore, although the heterozygous diploids were always among the fastest growing strains, there were no clear or consistent differences in growth between heterozygous diploid and tetraploid strains (nor between laboratory or clinical strains) that could predict ploidy reduction. Taken together, these results indicate that there was no indication that differences in initial fitness could explain the observed variation among environments for ploidy drive.

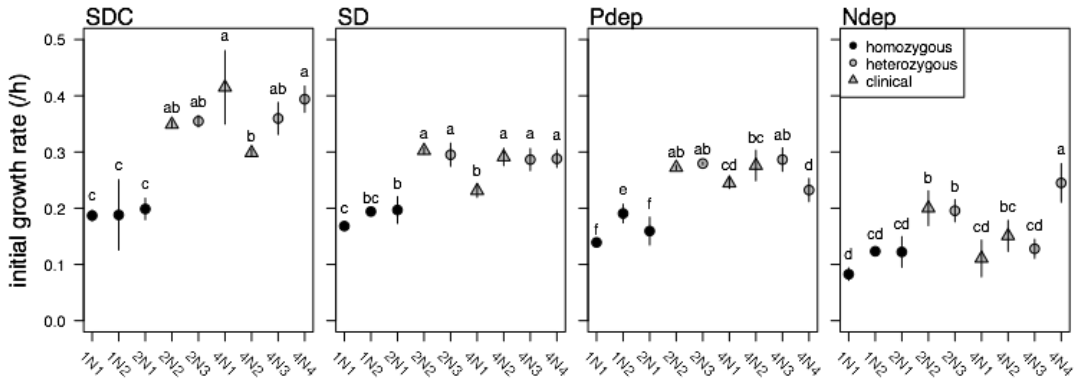

Figure 2. Initial ploidy does not predict initial growth rate. Mean (+/- SE) growth rate of ancestral strains in complete (SDC), minimal (SD), phosphorus-depletion (Pdep) and nitrogen-depletion (Ndep) medium. Letters above the symbols indicate statistical differences among strains for each environment from a post-hoc Tukey Test; when strains do not share a letter they are significantly different from each other.

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After ~140 generations of evolution, the majority of evolved lines did, however, increase in growth rate relative to the ancestral strains (Figure S4). Across the entire dataset, growth rate change was significantly influenced by an interaction between initial ploidy and evolutionary environment (analyzed by a linear mixed-effect model with strain background as a random effect; ploidy: $F_{2,6} = 0.01$, $P = 0.99$, environment: $F_{3,342} = 14.51$, $P < 0.0001$, ploidy*environment, $F_{6,342} = 37.4$, $P < 0.0001$; Table 2, Table S3). That we see an interaction between ploidy and evolutionary environment indicates that there is a role for the specifics of the environment to influence evolutionary dynamics in a ploidy-specific manner.

Interestingly, the improvement in growth rate after evolution was negatively correlated with initial fitness in SDC (Pearson correlation test; $t_7 = -8.99$, $p < 0.0001$, $r = -0.96$; Figure 3A), consistent with Fisher's geometric theory of adaptation (Orr 2005). In contrast, there was no significant relationship between improvement of growth rate and initial growth rate in any of the nutrient depletion environments (SD: $t_7 = 1.58$, $P = 0.16$; Pdep: $t_7 = -1.19$, $P = 0.27$; Ndep: $t_7 = -0.08$, $P = 0.94$, Figure 3A), suggesting that additional factors must play the predominant role in determining the mutation rate and/or effect size of beneficial mutations that arise in different genomic backgrounds. Despite finding significant changes in both genome size and growth rate, however, the observed changes in genome size across all lines did not predict the improvement of growth rate when strain background and growth environment were taken into account (linear-mixed effect models with strain and environment as random effects, p-value is calculated using the Satterthwaite approximation for degrees of freedom implemented in the lmerTest package in R (Kuznetsova *et al.* 2015); haploids: $F_{1,77} = 0.98$, $p = 0.09$, diploids: $F_{1,116} = 0.92$, $p = 0.34$, polyploids: $F_{1,155} = 0.22$, $p = 0.64$; Figure 3B). Taken together, changes in growth rate, a proxy for fitness, did not explain the significant observed differences in the rate of ploidy drive among strains and across environments observed throughout the evolution experiments.

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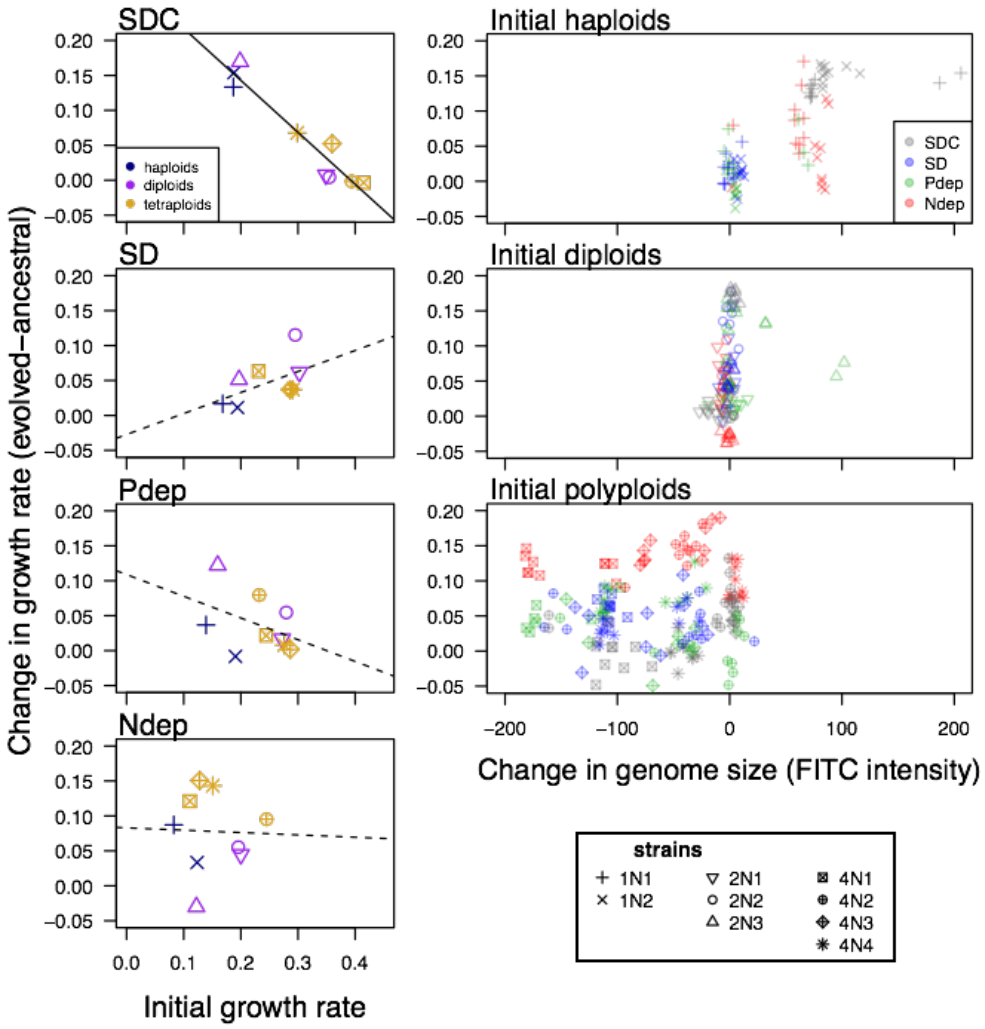

Figure 3. Change growth rate does not tend to correlate with either initial growth rate or change in genome size. A) Initial growth rate is negatively correlated with change in growth rate in SDC but not in other environments; a solid line indicates a significant ($p < 0.05$) correlation. B) Change in genome size does not correlate with change in growth rate.

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Discussion

The evolutionary environment, initial ploidy, and genetic background all significantly influenced the rate of ploidy change in *C. albicans*. Consistent with previous experiments in both *C. albicans* (Hickman et al. 2013; 2015) and other diverse fungal microbes (Gerstein et al. 2006; Gresham 2006; Schoustra et al. 2007; Seervai et al. 2013; Voordeckers et al. 2015), we found that the ploidy changes observed over ~140 generations of batch culture evolution were nearly always towards the baseline ploidy (diploidy for *C. albicans*), a phenomenon that we term 'ploidy drive'. By tracking ploidy changes during evolution under nutrient limitation we tested whether the predicted costs of higher genome sizes (because of the extra P and N demands for DNA/RNA and proteins) can counteract ploidy drive. We found that the evolutionary environment did significantly influence the frequency of ploidy change (Figure 1). Evolution in phosphorus-depletion (Pdep) and minimal medium (SD) generally selected for lower ploidy levels, while the same strains evolved under complete medium (SDC) or nitrogen-depletion (Ndep), maintained or promoted higher ploidy levels.

We thus found contrasting results between Pdep and Ndep, inconsistent with a singular response to nutrient limitation. Rather, these results are consistent with phosphorus deprivation exerting a larger selective force counteracting ploidy drive compared to nitrogen deprivation in haploids, i.e., the ecological the cost of increasing genome size under Pdep could be higher than under Ndep, so that selection against higher genome sizes would be greater in Pdep. Selection for lower ploidy levels under Pdep (and SD) is consistent with the growth rate hypothesis, which posits that since nucleic acids contain ~9% phosphorus per unit dry mass and constitute a large fraction of organismal dry mass, there may be an increased sensitivity under phosphorus-limited conditions for higher ploidy levels (Elser et al. 1996; Sterner et al. 2002; Elser et al. 2003; Neiman et al. 2013a). Support for this hypothesis has also been found in freshwater snails, which naturally differ in ploidy level (Neiman et al. 2013b), as well as in zooplankton (Jeyasingh et al. 2015).

It is also possible that differences in the mutation rate exists among environments, and thus ploidy variant cells might arise more frequently under some conditions

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compared to others. Gains in ploidy occur through endoreplication, i.e. when a cell replicates its DNA yet fails to segregate during mitotic cell division (Otto and Whitton 2000; Storchová and Pellman 2004, and references within). The mutation from haploidy to diploidy might occur more frequently under Ndep compared to SD or Pdep, so that there are simply more diploids at each mitosis and these diploids are more likely to (by chance) acquire a beneficial mutation than haploids. Similarly, the rate of ploidy reduction may be less frequent under Ndep compared to SD or Pdep. Ploidy reductions can either occur though unequal segregation of chromosomes into daughter cells (Harrison et al. 2014; Schoenfelder et al. 2014; Vitale et al. 2015) or over several cell divisions due to consecutive chromosome non-disjunction events (Bennett and Johnson 2003; Hickman et al. 2015). Ploidy-variant causing mutations have indeed been previously shown to be more frequent in some environments compared to others (Harrison et al. 2014). Future studies that track single-cell dynamics will help distinguish between the contributions of mutation, selection, or both on the difference in ploidy drive in among environments.

Although the majority of strain backgrounds behaved quite similarly across the environments, the homozygous diploid strain (2N1) and one polyploid strain (4N1) emerged as outliers. Strain background has previously been shown to influence growth under nutrient depletion (Magasanik and Kaiser 2002; Zörgö et al. 2013), however, the influence of genetic background on genome size stability had not been explicitly tested. Of the initially-diploid strains, only lines from the homozygous diploid exhibited ploidy instability (in all cases an increase in genome size, Figure S2), and only in Pdep. It may be that the lines that changed in ploidy utilized a different set of adaptive mutations that are beneficial in altered copy number than the other strains used to evolve under Pdep. Interestingly, the replicates that increased in ploidy tended to have a reduced growth rate relative to replicates that stayed diploid (mean growth of the five measured replicates that increased in ploidy: 0.26 +/- 0.02; mean growth rate of the five replicates that remained diploid: 0.31 +/- 0.01), a further indication that ploidy variation is independent of evolved growth rates.

Of the initially-polyploid strains, all evolved lines from 4N1 showed ploidy reduction (Figure 1), while many evolved lines from the other three initially-polyploid

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strains retained sub-population of cells with their initial genome size (Figure S2). 4N1 has a unique karyotype that contains both tetrasomies and trisomies as well as two copies of isochromosome 5L (Table 1**,** Abbey et al. 2014)). Differences in the rate of ploidy drive may be due to differences in either the specific aneuploid chromosomes in 4N1 (i.e., compared to 4N4, also an aneuploid strain) or due to allelic variation in a gene (or genes) involved in mutation repair or cell cycle fidelity in these strains. To this end, recent analysis of heterozygous diploids revealed many genes that harbour one or more SNPs leading to allele-specific expression differences, including *POL3, RAD9, RRD1, CCN1, HAT1* and *RAD32* (Muzzey et al. 2014). Thus, future studies are needed to shed light on particular genomic features important in promoting (or counteracting) genome stability.

Initial growth rates were more influenced by genetic background than initial ploidy in all environments. The three strains that contain a single set of alleles (1N1, 1N2 and 2N1) were consistently among the slowest growing strains regardless of environment (Figure 2). Homozygosity is known to reduce competitive fitness in *C. albicans in vitro* and *in vivo* (Hickman et al. 2013), and homozygous strains have yet to be identified clinically. In contrast, *C. albicans* polyploids often grew at the same rate as diploids (Figure 2), despite having larger cell size and volume (Hickman et al. 2013). Thus the instability of polyploid lines cannot be due to differences in initial growth rate.

While the majority of lines had an increased growth rate in all environments (Figure S4), neither initial ploidy nor final ploidy correlated with the observed changes. This is consistent with prior empirical experiments that compared haploid and diploid growth rates in *S. cerevisiae* under nutrient limitation, where no consistent growth advantage to haploid strains has been identified (Adams and Hansche 1974; Glazunov et al. 1989; Mable 2001; Zörgö et al. 2013). Selection driving ploidy dynamics is thus independent of growth rate in these microbial taxa, which have streamlined genomes and low GC content, and are thus optimized to require minimal nutrient resources for replication (Giovannoni et al. 2014 and references within).

Why ploidy drive is such a frequent and potent force across broad taxa and environmental challenges remains a mystery. An *a priori* explanation for ploidy drive is that there is an immediate fitness advantage to a cell that is closer to the baseline ploidy

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(so that once such cells are present within the non-baseline ploidy population they rise to high frequency). We did not however observe an improvement in growth rate or biomass production in ploidy-variant cells in our experiments (Figure 3, S3), nor has such a fitness advantage been observed in previous batch culture evolution studies. The sole exception is evolution under carbon limitation, where diploidly alone was shown to have an advantage over ancestral haploids (Venkataram et al. 2016). Further perplexing from a theoretical standpoint, short-term fitness patterns do not predict long-term ploidy change: *S. cerevisiae* haploid strains adapted faster than diploids in both rich medium and salt-stressed medium (Gerstein et al. 2010), yet all haploid strains converged to diploidy within 1800 generations of batch culture (Gerstein et al. 2006). Thus, although ploidy drive was partially mitigated in some genetic background and environments in these experiments, we predict that had the experiments continued for longer and ever-increasing number of lines would have converged on diploidy. The overarching picture is that while both the growth environment and genetic background play a role in the evolutionary dynamics of ploidy drive, the major factor(s) that drive populations towards baseline ploidy in diverse fungal microbial taxa remains an evolutionary puzzle to be solved.

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Table S1. Tukey test results following ANOVA tests to examine the influence of environment and strain on the rate of genome size change.

Haploids

Environment

Comparison	diff	P
Pdep-Ndep	-52.8	*<0.0001*
SD-Ndep	-59.7	*<0.0001*
SDC-Ndep	34.7	*0.0009*
SD-Pdep	-6.9	0.8
SDC-Pdep	87.5	*<0.0001*
SDC-SD	94.4	*<0.0001*

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Diploids

Environment

Comparison	diff	P
Pdep-Ndep	14	*0.0002*
SD-Ndep	3.4	0.73
SDC-Ndep	-0.4	1
SD-Pdep	-10.6	*0.007*
SDC-Pdep	-14.4	*<0.0001*
SDC-SD	-3.8	0.64

Line

Comparison	diff	P
2N2-2N1	-4.2	0.56
2N3-2N1	-1.6	0.15
2N4-2N1	6.4	*<0.0001*
2N3-2N2	-5.8	0.85
2N4-2N2	2.2	*0.007*
2N4-2N3	-0.4	0.07

Tetraploids

Environment

Comparison	diff	P
Pdep-Ndep	-18.6	0.07
SD-Ndep	-20.9	*0.03*
SDC-Ndep	16.3	0.13
SD-Pdep	-2.3	0.99
SDC-Pdep	34.9	*<0.0001*
SDC-SD	37.2	*<0.0001*

Line

Comparison	diff	P
4N2-4N1	77.6	*<0.0001*
4N3-4N1	82.1	*<0.0001*
4N4-4N1	91	*<0.0001*
4N3-4N2	4.5	0.93
4N4-N42	13.5	0.28
4N4-4N3	8.9	0.63

Table S2. Ancestral growth rate and biomass production are well correlated in all environments.

Environment	Statistic	Correlation (r^2)
SDC	$t_8 = 6.76$, $p = 0.0001$	0.85
SD	$t_8 = 5.30$, $p = 0.0007$	0.77
Pdep	$t_8 = 4.44$, $p = 0.0022$	0.71
Ndep	$t_8 = 5.48$, $p = 0.0006$	0.79

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Table S3. Least square means pairwise posthoc tests to determine the influence of evolutionary environment on change in growth rate. Degree of freedom was 381 in all tests.

Haploids

Contrast	Statistic
Pdep-Ndep	t.ratio = 3.21, $P = 0.008$
SD-Ndep	t.ratio = 3.22, $P = 0.008$
SDC-Ndep	t.ratio = -5.81, $P < 0.0001$
SD-Pdep	t.ratio = 0.014, $P = 1$
SDC-Pdep	t.ratio = -9.02, $P < 0.0001$
SDC-SD	t.ratio = -9.03, $P < 0.0001$

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Diploids

Contrast	diff
Pdep-Ndep	t.ratio = -0.95, $P = 0.78$
SD-Ndep	t.ratio = -2.17, $P = 0.13$
SDC-Ndep	t.ratio = -1.24, $P = 0.60$
SD-Pdep	t.ratio = -1.22, $P = 0.61$
SDC-Pdep	t.ratio = -0.29, $P = 0.99$
SDC-SD	t.ratio = 0.93, $P = 0.79$

Tetraploids

Contrast	diff
Pdep-Ndep	t.ratio = 9.83, $P < 0.0001$
SD-Ndep	t.ratio = 8.25, $P < 0.0001$
SDC-Ndep	t.ratio = 9.74, $P < 0.001$
SD-Pdep	t.ratio = -1.58, $P = 0.39$
SDC-Pdep	t.ratio = 0.094, $P = 1.0$
SDC-SD	t.ratio = 1.48, $P = 0.45$

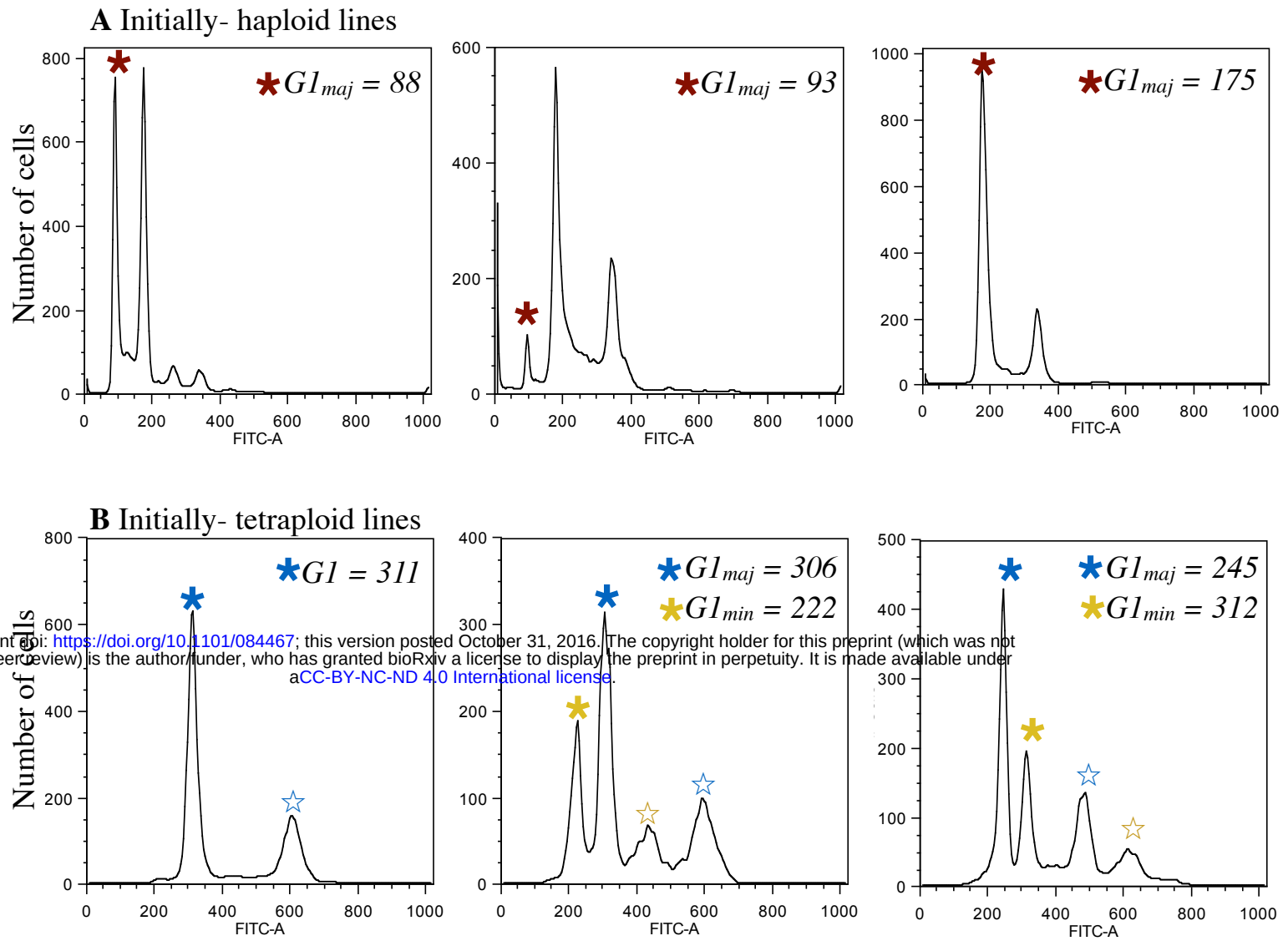

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Figure S1. Ploidy analysis from plots of FITC intensity. A. When a haploid peak was retained in an initially-haploid evolved line (red star) we recorded the G1 mean of this peak as the major ploidy peak ($G1_{maj}$), due to ambiguity between the haploid G2 and diploid G1 peak. B. For initially-diploid and initially-tetraploid replicates we recorded the major ploidy subpopulation as the G1 mean of the highest peak (blue star), with the minor population ($G1_{min}$) as the G1 mean of the next highest peak (yellow star). Note that since we assay cells at all stages of the cell cycle, we did not record the mean of peaks that are consistent with the G2 phase (open stars).

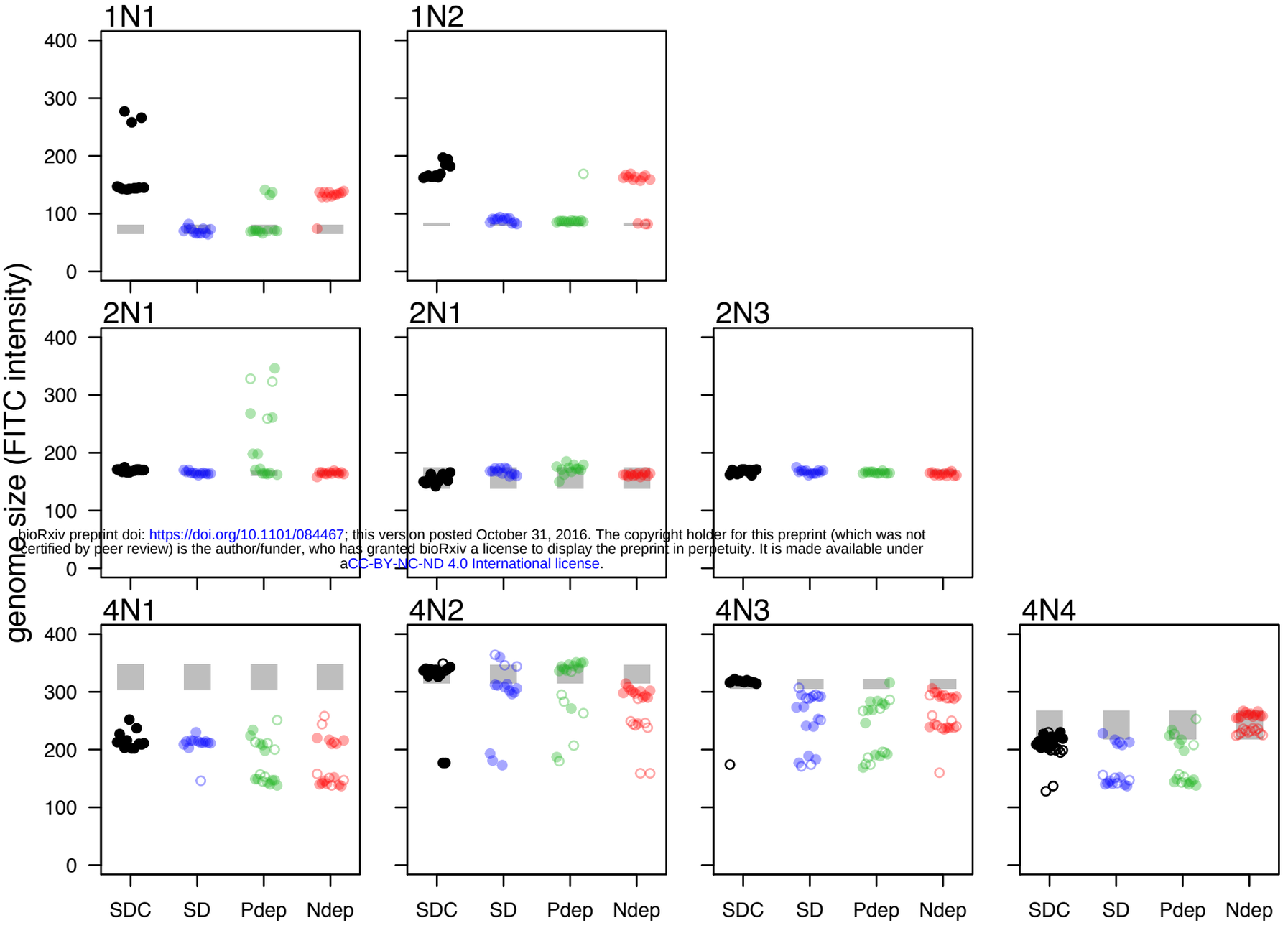

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Figure S2. Strain background and environment influence genome stability. Twelve lines of each ancestral strain were evolved in complete (SDC, black points), minimal (SD, blue), phosphorus-deprivation (green) and nitrogen-deprivation (red) medium. Filled-in points represents the major peak in FITC intensity from each population after ~140 generations of evolution. Hollow points indicate the minor peaks. Top row = initially haploid lines; middle row = initially diploid lines; bottom row = initially polyploid lines. Grey boxes indicate the range of genome sizes measured from twelve replicates of the ancestral culture from each strain.

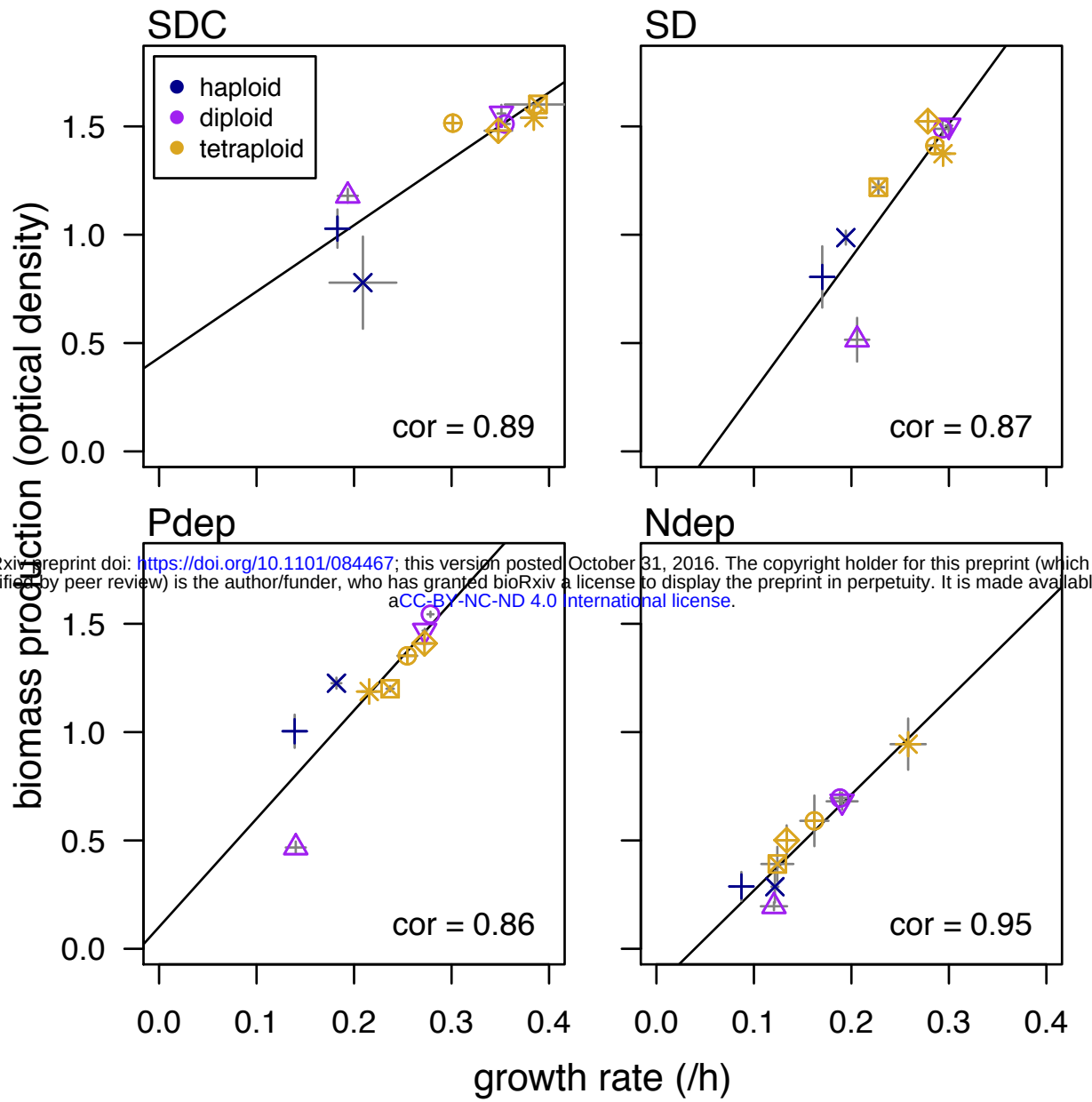

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Figure S3. Ancestral growth rate and biomass production are highly correlated in all environments.

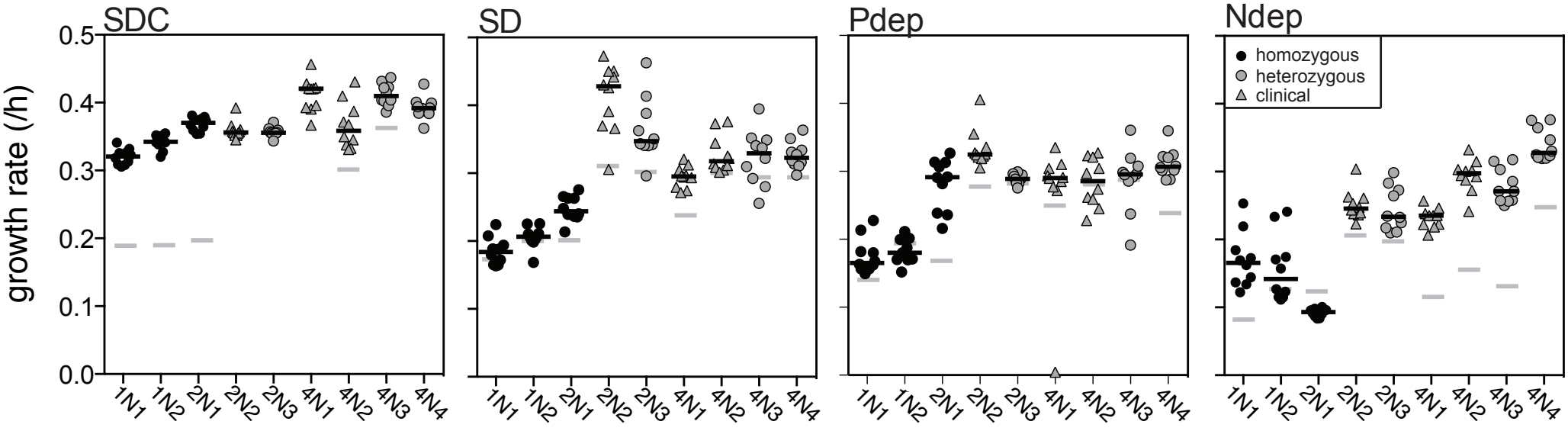

Figure S4. The majority of lines increased in growth rate after evolution. Each point is the evolved growth rate of one of 10 replicate lines evolved for each ancestral strain, The black bars indicate the median of evolved growth rates. Grey bars indicate the mean of ancestral growth rates (presented in Figure 2).

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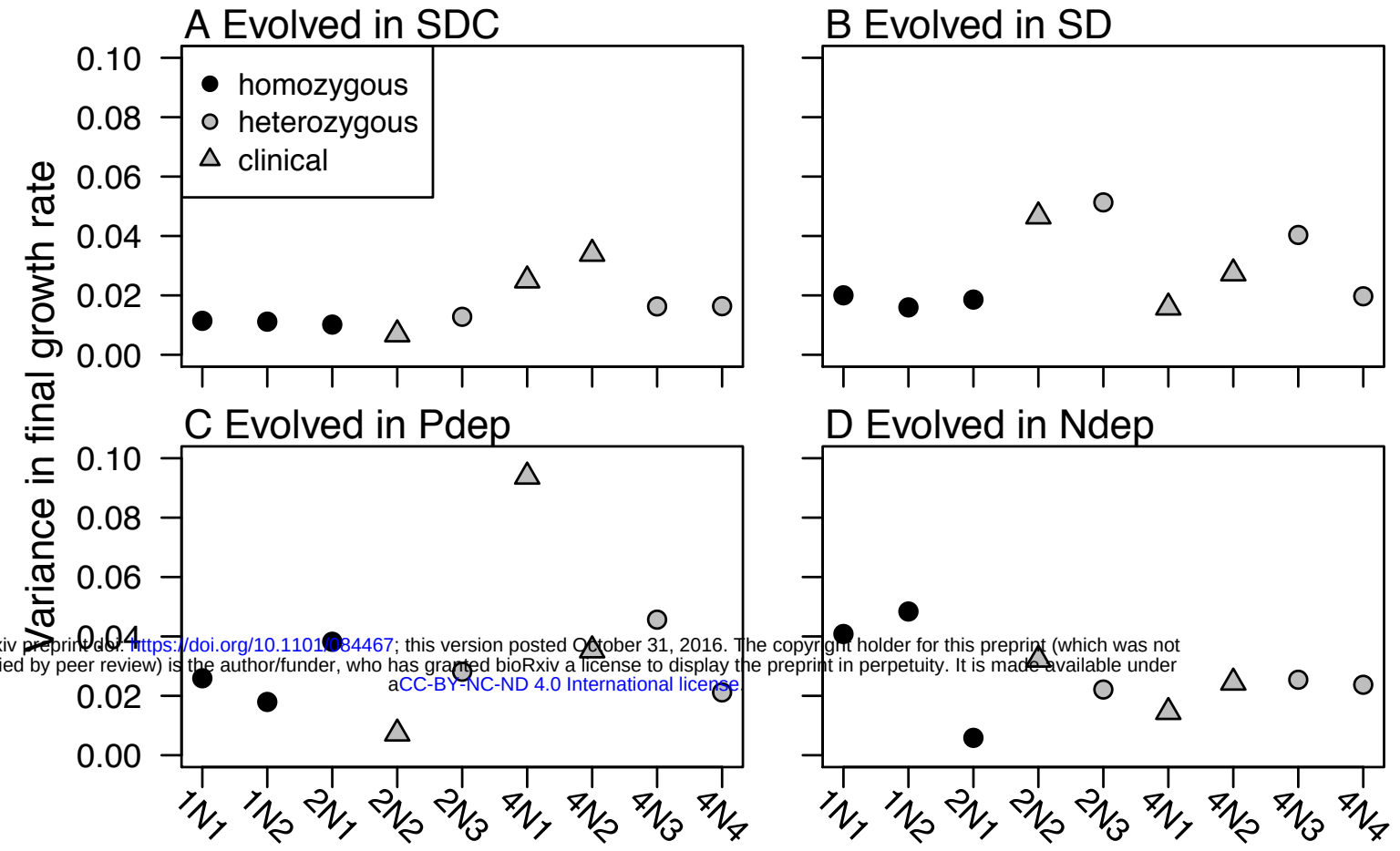

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Figure S5. Variation in final growth rate was not influenced by environment or initial ploidy.