

Multilevel Selection in the Filamentous Ascomycete *Neurospora tetrasperma*

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ABSTRACT: The history of life has been driven by evolutionary transitions in individuality, that is, the aggregation of autonomous individuals to form a new, higher-level individual. The fungus *Neurospora tetrasperma* has recently undergone an evolutionary transition in individuality from homokaryosis (one single type of nuclei in the same cytoplasm) to heterokaryosis (two genetically divergent and free-ranging nuclear types). In this species, selection can act at different levels: while nuclei can compete in their replication and transmission into short-lived asexual spores, at the level of the heterokaryotic individual, cooperation between nuclear types is required to produce the long-lived sexual spores. Conflicts can arise between these two levels of selection if the coevolution between nuclear types is disrupted. Here, we investigated the extent of multilevel selection in three strains of *N. tetrasperma*. We assessed the ratio between nuclear types under different conditions and measured fitness traits of homo- and heterokaryotic mycelia with varying nuclear ratios. We show that the two nuclei have complementary traits, consistent with division of labor and cooperation. In one strain, for which a recent chromosomal introgression was detected, we observed the occurrence of selfish nuclei, enjoying better replication and transmission than sister nuclei at the same time as being detrimental to the heterokaryon. We hypothesize that introgression has disrupted the coevolution between nuclear types in this strain.

Keywords: levels of selection, transitions in individuality, heterokaryosis, conflict, cooperation, cheating.

Introduction

The history of life is characterized by evolutionary transitions in individuality. Such transitions involve independently replicating individuals that aggregate to form new, higher-level individuals (Buss 1987; Maynard-Smith and Szathmari 1995; West et al. 2015). For example, genes cooperated to form genomes, cells cooperated to form mul-

ticellular organisms, and solitary insects formed colonies (Maynard-Smith and Szathmari 1995; West et al. 2015). During evolutionary transitions in individuality, two major steps are identified: the formation of the cooperating group and the transformation of the group to an integrated organism. The latter involves the emergence of new, higher-level traits, such as division of labor and mediation of within-group conflicts (Michod 1996, 2005; Clarke 2014). Conflicts result from discordant multilevel selection—for example, selection of selfish variants at the lower level that disrupts the higher-level organization (Buss 1987; Michod 1997; Frank 2003). Indeed, several empirical studies have shown the emergence of “cheaters” when unrelated cells group to cooperate (Velicer et al. 2000; Foster et al. 2002; Rainey and Rainey 2003; Bastiaans et al. 2016), and selection of mutant cells in cancers also exemplifies discordant multilevel selection (Aktipis et al. 2015). Hence, the evolution of mechanisms that ensure genetic homogeneity within the organism has been considered crucial during transitions in individuality (Maynard-Smith and Szathmari 1995; Queller 2000; Roze and Michod 2001). Yet, naturally occurring within-organism variation has been documented from all major branches of the tree of life (Gill et al. 1995; Santelices 1999; Pineda-Krch and Lehtila 2004), suggesting that this phenomenon is more common than previously believed. In essence, these findings challenge the concept of the individual as a single unit of selection (Santelices 1999), and arguments have been raised that within-organism variation even benefits the organism, either by providing phenotypic flexibility to a changing environment (Jinks 1952; Pineda-Krch and Lehtila 2004) or by allowing purging of deleterious cell lineages (Otto and Orive 1995).

Because within-organism variation is common in fungi, there has been a long-lasting debate about the concept of individuality in this group (Pontecorvo 1946; Buss 1987; Johannesson and Stenlid 2004; Booth 2014). Here, we use the filamentous ascomycete *Neurospora tetrasperma* as a model for the study of multilevel selection. This species has recently (approximately 1 mya) evolved a mating system in which mating-type heterokaryosis is dominant throughout the life cycle

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(fig. 1; Corcoran et al. 2014). Heterokaryosis is the coexistence of genetically different nuclei in the same cytoplasm. In *N. tetrasperma*, haploid nuclei of opposite mating types, *mat A* and *mat a*, coexist throughout the life cycle. They are packaged into the ascospores (sexual spores) in a 1:1 proportion, enabling the individual to complete the life cycle by selfing (Raju and Perkins 1994; fig. 1). In addition, homokaryotic spores that contain a single nuclear type may also occasionally be produced (Corcoran et al. 2012; fig. 1). Evolving from a homokaryotic ancestor, the mating system transition in *N. tetrasperma* has implied a substantial increase in within-organism variation. Extensive suppression of recombination around the mating-type (*mat*) locus in *N. tetrasperma* results in over 1,500 genes being linked to *mat* and thus to the nuclear type (Merino et al. 1996; Ellison et al. 2011; Corcoran et al. 2016). The sequence divergence of nuclei of the two alternative mating types is up to 3.2% (Merino et al. 1996; Ellison et al. 2011; Corcoran et al. 2016) as a result of both mutation accumulation and, in certain lineages, introgression from closely related species (Sun et al. 2012; Corcoran et al. 2016).

We hypothesize that the evolution of the novel genetic system in *N. tetrasperma* has led to a shift in individuality from the unit of the homokaryotic (i.e., the nucleus) to the heterokaryotic (fig. 1) individual. The nuclei are thought to freely move across cells of the mycelium (Roper et al. 2013), and hence, nuclei represent the units among which cooperation (but also competition) can occur. Cooperation between nuclear types is required to complete the life cycle of a heterokaryon in *N. tetrasperma*, in particular, to produce the ascospores, which are long-lived and an efficient vector of both spatial and temporal dispersion. Yet, nuclear types may also compete, for example, in their replication rates and/or in their transmission to the short-lived asexual spores (conidia; fig. 1). We predict that selection at the nuclear level occurs during the vegetative phase, enhances the production of homokaryons, and conflicts with the interests of the heterokaryon. Furthermore, we expect introgression from other species to disrupt the coevolution of nuclei in a lineage, thereby shifting the selection level from the heterokaryotic individual to the nucleus.

We studied the extent of multilevel selection in three lineages of *N. tetrasperma*, with varying levels of nuclear divergence and introgression (Corcoran et al. 2016). As nuclear ratios can depart from 1:1 in the mycelium (Samils et al. 2014), we studied how the proportion of the nuclear types varies under different conditions in mycelia and asexual spores. Furthermore, we measured fitness traits of the homokaryotic and heterokaryotic mycelia, contrasting performances during the vegetative or the sexual phase of the life cycle. Our findings are consistent with emergent transition in individuality in *N. tetrasperma*, with evidence for both cooperation and cheating among nuclei.

Material and Methods

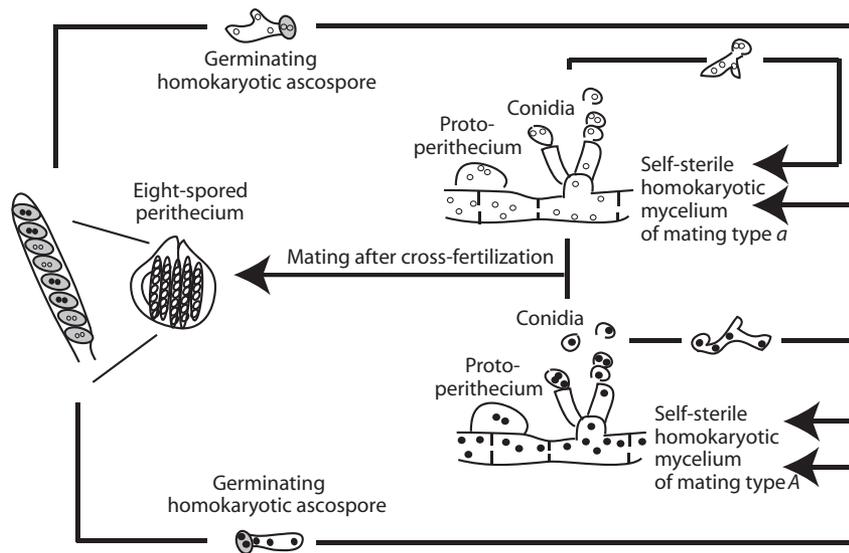
Strains Used in the Study

We used three heterokaryotic strains of *Neurospora tetrasperma* and their component single mating-type homokaryons (app. A; apps. A–I are available online). Homokaryotic strains were obtained in previous studies by isolating mycelia grown from asexual spores (conidia) of single mating type that the heterokaryon occasionally produces (Raju 1992; Corcoran et al. 2012; fig. 1). The investigated strains were obtained from the Fungal Genetics Stock Center (FGSC; University of Missouri, Kansas City) and selected to represent the phylogenetic diversity of the 10 phylogenetically and reproductively isolated lineages that have been identified in this morphologically determined species (Menkis et al. 2009; Corcoran et al. 2014, 2016): one heterokaryon was selected from each of three different lineages (L1, L6, and L10). The investigated strains have been used frequently in previous *N. tetrasperma* studies (Ellison et al. 2011; Corcoran et al. 2012, 2016; Samils et al. 2013, 2014) and span the range of divergence between nuclei in heterokaryons of *N. tetrasperma*. The divergence of the nonrecombining *mat* chromosomes of nuclei in these heterokaryons amounts to an average of 1.4% in L1, 2% in L10, and 3.2% in L6 (Corcoran et al. 2016). The other chromosomes (autosomes) are strikingly similar in L1 and L6, while in L10, extensive differences are observed (e.g., up to 2% on the chromosome corresponding to linkage group IV in *Neurospora crassa*; Corcoran et al. 2016). In L1, weak or no support for introgression into the genomes is found, whereas in L6, introgression from a yet-undetermined source is confined to the *mat A* chromosome. In L10, a recent introgression of both the *mat A* chromosome and autosomes has been confirmed from the closely related species *Neurospora hispaniola* (Sun et al. 2012; Corcoran et al. 2016).

Growth Conditions

Strains were grown at 25°C, with 12L:12D conditions, either on 90-mm petri dishes or in glass tubes with 1.5 mL of slanted medium. As media, we used Vogel's medium N (Vogel 1956), which promotes vegetative growth and is hereafter referred to as "medium N," or media with low nitrogen promoting sexual reproduction and hereafter referred to as "medium low N." As medium low N, we used modified Vogel's medium N (Russo et al. 1985) except in a few cases indicated in appendix B, where synthetic cross medium (SC; Westergaard and Mitchell 1947) was used. Sucrose (1%) was used as a carbon source in these media. We also grew conidia on "sorbose medium" (i.e., medium N with 2% sorbose), 0.05% glucose, and 0.05% fructose as carbon sources. Sorbose is toxic to the mycelium and prevents extensive hyphal growth, allowing the separation of colonies

Heterothallic life cycle in *Neurospora*



Mating-system transition

Pseudohomothallic life cycle in *Neurospora tetrasperma*

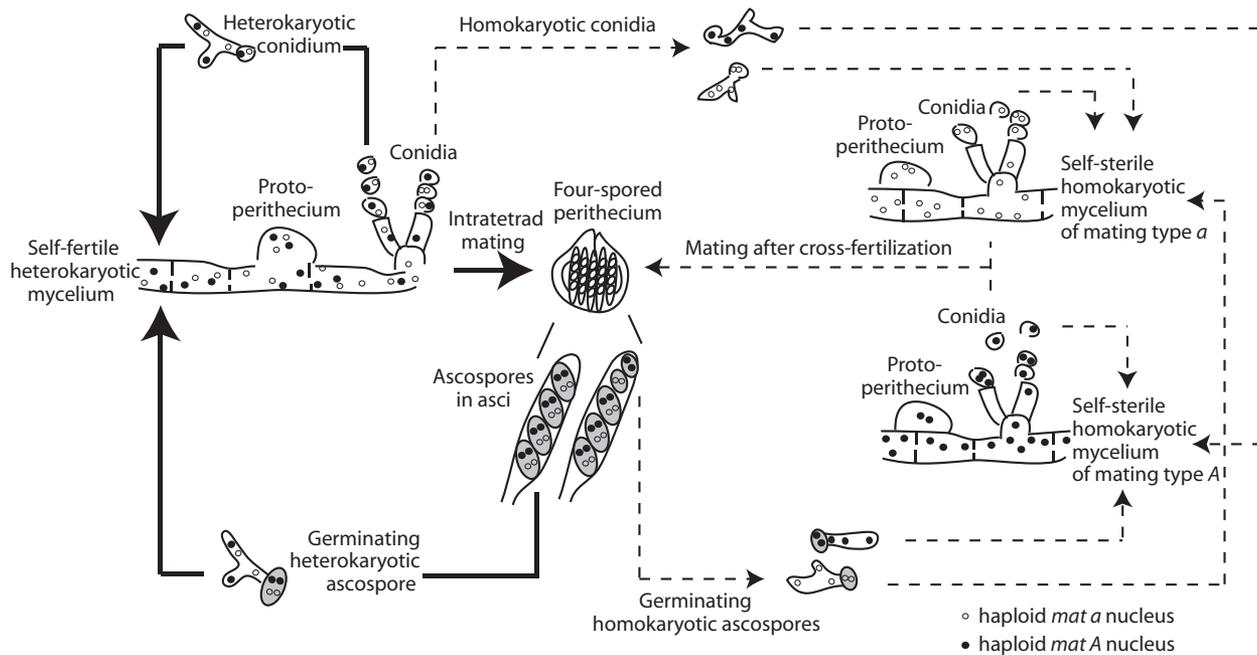


Figure 1: Schematic drawing of the *Neurospora* life cycles. The life cycle of the terminal clade of *Neurospora* includes a sexual and an asexual path, and both ascospores (sexual spores) and conidia (asexual spores) are produced. In heterothallic species, both ascospores and conidia give rise to self-sterile, homokaryotic mycelia of single mating type, which need to cross-fertilize to go through the sexual cycle. One million years ago, a shift in mating system evolved pseudohomothallism in *Neurospora tetrasperma*. In this species, ascospores and conidia are heterokaryotic for mating type. The germinating heterokaryotic mycelium is self-fertile and can go through intratetrad mating to produce heterokaryotic ascospores in the mature perithecium (fruiting structure) as well as bud off heterokaryotic conidia; both of these spores form new heterokaryotic mycelia on germination and allow completion of the life cycle without separating nuclei of the two mating types. These self-fertile and heterokaryotic routes of the life cycle are shown by solid lines in the figure. In addition, ascospores and conidia of *N. tetrasperma* that are homokaryotic for mating type may be produced. The resulting homokaryons can grow as mycelia forming protoperithecia (immature fruiting structures) but are self-sterile, and mating requires the encounter of an individual with the opposite mating type to cross-fertilize. Mating may take place following hyphal fusion of heterokaryon-compatible homokaryons or through fertilization. This alternative, heterothallic route of the life cycle of *N. tetrasperma* is indicated with dashed lines. Structures are not drawn to scale.

originating from single conidia after plating. For all media, we used agar (1.5%) for solidification, and when necessary, the agar surface was covered with cellophane to facilitate harvesting.

Estimation of Nuclear Ratios

Nuclear ratios were estimated using a quantitative polymerase chain reaction (qPCR). DNA was extracted from conidia or harvested tissue by using the ZR Fungal/Bacterial DNA MiniPrep kit, and an allele-specific qPCR was used to determine the quantities of *mat A* or *mat a* DNA in the samples. The primers used in the qPCR were designed to target either of the *mat A* or *mat a* idiomorphs (i.e., the highly dissimilar allelic variants at the *mat* locus; Glass et al. 1990) or genes tightly linked to these idiomorphs on the mating-type chromosome. When targeting genes outside of the idiomorphs, primers were designed to span several single-nucleotide polymorphisms or indels, allowing a clear discrimination between the alleles linked to *mat A* or *mat a*. The sequence and annealing temperature of primers for each mating type, lineage, and targeted gene are given in appendix C. Each 10- μ L PCR reaction contained 5 μ L SsoAdvanced Universal SYBR Green Supermix kit (Bio-Rad, Hercules, CA), 200 nM of each primer, and 1 μ L DNA. Samples were amplified in triplicates. The following PCR protocol was used: 98°C for 5 min, 39 cycles of 95°C for 15 s, annealing for 30 s, and 72°C for 15 s. Amplifications were conducted on a Bio-Rad CFX384 machine. Primer amplification efficiency was deduced from amplification of standard curves using genomic DNA. The built-in method in the Bio-Rad software was used to calculate DNA starting quantities of *mat A* and *mat a* in the samples (N_A and N_a , respectively). Nuclear ratio, p_A , was then calculated as the proportion of *mat A* to the total as follows: $p_A = (N_A/N_a)/(1 + N_A/N_a)$. The accuracy of this method for estimation of nuclear ratios was verified as described in appendix D.

Preparation of Starting Inocula Varying in Nuclear Ratio

As inocula for our experiments, we used either a single heterokaryotic ascospore or a mix of conidia varying in the proportion of *mat A/mat a* nuclei. Heterokaryotic ascospores pass through a stage where they contain *mat A* and *mat a* nuclei in the proportion 1:1 (fig. 1; Raju 1992). By contrast, the use of conidia of different mating type, which fuse after germination to form a heterokaryotic mycelium (see below and app. E), allowed the building of heterokaryotic inocula with initial *mat A/mat a* ratios deviating from 1:1.

Ascospores for inoculation were harvested from fruiting heterokaryotic mycelia of each lineage after 10–15 days of growth in glass tubes with medium low N. We used a single ascospore as inoculum for each replicate. Before inoculation,

each ascospore was induced to germinate by heat shocking at 60°C for 30 min.

The mixes of conidia used for inoculation were prepared as follows: conidia less than 7 days old were harvested from each respective single mating-type homokaryons (app. A) grown on glass tubes with medium N. Conidia were washed off the surface of the mycelium, suspended in 0.01% tween ddH₂O, and counted using a Casy cell counter (Innovatis). Suspensions of *mat A* and *mat a* conidia were adjusted to the same concentration, after which conidia from opposite mating type were mixed into three different proportions of *mat A* nuclei (90%, 50%, or 10%). The same number of conidia were inoculated to start growth for each replicate in an experiment, as conidia densities can affect growth rates of the developing mycelium (Richard et al. 2012; Bastiaans et al. 2015).

We verified the initial nuclear ratios in the conidia inoculum with qPCR, estimated the relative germination rate of conidia of the two mating types, and verified that fusion takes place between germinating conidia. These tests are outlined in detail in appendix E.

Investigation of Variation of Nuclear Ratios in Sectors of Mycelia

To investigate the variation of nuclear ratios among sectors of the radiating mycelium, we inoculated cellophaned petri dishes with medium N with conidia of known proportions of the *mat* nuclei (either 90%, 50%, or 10% *mat A*). We inoculated the center of each plate with 10⁵ conidia and used three replicate plates for each initial inoculum ratio and lineage (app. F). After 48 h of growth, the mycelium was divided into eight sectors. Nuclear ratio was estimated in four noncontiguous sectors out of eight for each plate, initial inoculum ratio, and lineage (36 samples per lineage and 108 samples in total; app. F). To assess whether there was a difference in nuclear ratio among sectors of a plate, we compared the variance among sectors in each plate with the technical variance due to qPCR estimation. To estimate technical variance, we randomly selected one plate for each ratio and lineage and performed five qPCRs for the DNA isolated from each sector of these plates (app. F; 60 qPCRs per lineage and 180 in total). Confidence intervals (CIs) on the mean sectors' variance and the mean technical variance were computed using bootstrapping in R. We tested for the difference between the variances using a Kruskal-Wallis test, due to nonnormal distributions and high heterogeneity of variances.

Factors Determining Nuclear Ratio in Heterokaryons: Experimental Setup

We assessed the effects of (i) strain identity/lineage, (ii) starting inoculum, (iii) medium, and (iv) duration of growth on nuclear ratios in individual mycelium by using the following

experimental setup: ascospores, or mixes of conidia varying in the proportion of *mat A* nuclei (90%, 50%, or 10%), were inoculated onto the center of cellophane petri dishes. Tissue was grown on either medium N or medium low N and harvested at two different time points, T1 and T2. Duration of growth was 48 h (T1) and 7 days (T2) when starting from conidia and 72 h (T1) and 8 days (T2) when starting from ascospores, since we observed differences in the rate of colony development when using different inocula. Time point T1, whatever the starting material, corresponds to the stage where the mycelium covered the plate and only vegetative structures were produced (mycelia and conidia; fig. 1). At T2, the mycelium had also produced maturing sexual structures (protoperithecia in homokaryons, perithecia in heterokaryons; fig. 1). For this time point, harvesting took place before discharge of mature ascospores from perithecia produced by heterokaryons. As expected (see Growth Conditions) at T1, the mycelia grown on medium N produced more conidia than mycelia grown on medium low N, while at T2, more perithecia were formed on medium low N (data not shown). The number of replicates for each combination of treatment is given in appendix B. After harvesting, DNA was extracted and the nuclear ratio was estimated for each sample with qPCR, as outlined above.

Factors Determining Nuclear Ratio in Heterokaryons: Statistical Analyses

The results of the experiment on factors determining nuclear ratio were separately analyzed depending on whether ascospore or conidia were used as inoculum; for the latter, we divided the results from the different lineages. We refer to the data sets below as the ascospore or conidia data sets. All results were analyzed using three-way ANOVA in R, with the nuclear ratio as the response variable; the factors were time point, medium, and lineage for the ascospores data set and time point, medium, and initial ratio for the conidia data set.

We visually verified a normal distribution of residuals. Homoscedasticity of error among factors was tested using a Levene test. When heteroscedasticity was present, we used White-adjusted, heteroscedasticity-corrected standard errors to correct for it, as transformation of the variable improved neither normality nor heteroscedasticity. We used sum of squares type III to remediate for unequal sample sizes in groups and to be able to interpret main effects in the presence of interactions. We also computed 95% CIs on the median of nuclear ratios for each treatment using bootstrapping methods in R.

Investigation of Nuclear Bias in Transmission into Conidia

We compared the nuclear ratio in mycelia and conidia from the same culture. For this experiment, three replicate petri

dishes per lineage were covered with cellophane and inoculated with one heterokaryotic ascospore on medium N. After 24 h of mycelial growth, the mycelia were covered with another cellophane sheet with a diameter smaller than the plate (75 mm) and allowed to grow for another 48 h. The periphery of the plate then acquired more O₂, required for the aerial hyphae to produce the conidia. This experimental setup allowed separating mycelium from the conidia, since the mycelium grows between the two cellophane sheets, while the conidia are produced on the periphery and above the second cellophane cover. The study by Samils et al. (2014) showed that the nuclear ratio is homogeneous in the central part and periphery of an emerging mycelial colony of *N. tetrasperma*, and hence, we assumed that any difference in nuclear ratio between the mycelia and conidia in this experiment is due to a bias in transmission from mycelia into conidia.

For each plate, we harvested four samples of conidia-free mycelium (36 samples in total) and five samples of conidia (45 samples in total). Conidia were dry-harvested from the plate with a scalpel, microwaved for 5 min to improve extraction efficiency (Ferreira and Glass 1996), and then suspended in 0.01% tween ddH₂O. The conidial suspension was filtered using Easy strainers (40 μm; Greiner Bio-One) to remove any mycelial contaminants, after which the conidial solution was concentrated by centrifugation and removal of supernatant. DNA was extracted and the nuclear ratio was estimated for all samples.

The results were analyzed plate by plate using a one-factor ANOVA with conidia/mycelium as the factor and observed nuclear ratios as the response variable. In addition, we used nonparametric tests to study differences in ratio between mycelia and conidia.

Estimation of Fitness Components in Homokaryons and Heterokaryons Varying in Nuclear Ratio

The production of conidia (fig. 1) was used as a proxy for fitness during the vegetative phase. Note that in this assay we were not able to distinguish between heterokaryotic and homokaryotic conidia produced by heterokaryons. For each lineage, we inoculated glass tubes containing medium N with 10⁴ conidia, either homokaryotic for *mat A* or *mat a* or mixed into three different ratios (90%, 50%, and 10% *mat A*, respectively). Twenty replicate tubes were inoculated per treatment (100 tubes per lineage, 300 total). After 2 days of mycelial growth from the inoculum, next-generation conidia were harvested with 1 mL 0.01% tween ddH₂O and counted using a Casy cell counter (Innovatis).

Components of fitness expressed during the sexual phase were measured in both the self-sterile homokaryons and the self-fertile heterokaryons (fig. 1), using different fitness proxies: for the homokaryons, we measured the investment

in protoperithecia production (fig. 1), while for the heterokaryons, we measured the sexual reproductive output in ascospore production (rate and total amount) after intratetrad mating (fig. 1). The size of the protoperithecia was measured in unfertilized homokaryons of mating type *A* and *a* at a time when all heterokaryons of the same lineage had sporulated: these protoperithecia were thus mature and ready to be fertilized. The largest protoperithecia were chosen in 10 random areas in five tubes of homokaryons of each lineage and measured under a dissecting microscope. For the heterokaryons, 20 replicate tubes containing medium low N were inoculated with 10^5 conidia of each lineage, mixed in different proportions (90%, 50%, 10% *mat A*; hence, a total number of 60 tubes were used per lineage). For each lineage, we measured the total number of ascospores produced in a specific time interval, determined by the time between the sporulation of the first and last replicate of that lineage (day 6–7 for L1, day 7–8 for L6, and day 8–13 for L10). Ascospores were harvested by washing the inside of the tubes with 500 μ L of 0.01% tween ddH₂O and counted as follows: after dilutions to adjust spore number to a 0–100 range for each 5- μ L droplet, photos from five replicate droplets per sample were taken under the dissecting microscope. Images were treated using the Fiji software (Schindelin et al. 2012; Schneider et al. 2012) with an in-house script to contrast the image and count the ascospores (B. Nieuwenhuis, personal communication).

Statistical Analyses of Fitness Data

A one-way ANOVA model was applied to the data on conidia total counts, per-tube mean ascospore concentration, and protoperithecia size. We separated the data sets by lineage, measuring the effect of initial inoculum. The response variable was log-transformed, as it improved normality of residuals and homogeneity of variance. We used White-adjusted corrected standard errors to deal with the remaining heteroscedasticity. We also used bootstrapping methods to compute 95% CIs on the median of conidia and

ascospore production as a function of lineage and initial nuclear ratio.

Results

Nuclear Ratio Is Homogeneous in Sectors of Heterokaryotic Mycelium of Neurospora tetrasperma

When comparing the variance of nuclear ratios among sectors of a given mycelium with the technical variance obtained in qPCR estimations (app. F), we found that the mean sector variance did not differ significantly from the mean technical variance (overlapping CIs among mean technical and sector variances; app. F). This result was supported by Kruskal-Wallis tests where we compared the mean standard deviation, as well as the range of variation in nuclear ratio, between sectors within a mycelium and technical replicates (table 1). We conclude that nuclear ratios are homogeneous within the individual mycelium.

Ascospore Inoculum: Effect of Strain Identity and Growth Conditions on Nuclear Ratios

The use of heterokaryotic ascospores as inoculum allowed for investigation of the effect of strain and growth conditions on nuclear ratio in developing heterokaryotic mycelia with 1:1 initial nuclear ratios. Figure 2 shows the median and 95% CI of the nuclear ratio for each treatment (strain, medium, and duration of growth). First, we observed that the mycelium growing out from the ascospore showed strain-specific differences in nuclear ratio. When merging data for all conditions (i.e., media and time points), the proportion of *mat A* nuclei was larger than 50% in L10 (median = 0.72; 95% CI: 0.66–0.76). By contrast, for the strains from L1 and L6, the CIs on the median of the nuclear ratio spanned 50% (L1 overall: median proportion of *mat A* nuclei = 0.52; 95% CI: 0.48–0.55; L6 overall: median = 0.55; 95% CI: 0.50–0.58). The discrepancy among strains was confirmed by the ANOVA: the effect of strain on nuclear ratio was sig-

Table 1: Comparison of the range and mean standard deviation of the nuclear ratio between technical replicates and between sectors within a mycelium

Lineage	Technical divergence		Sector divergence		χ^2	<i>P</i> ^c
	Range ^a (%)	Mean SD ^b (%)	Range ^a (%)	Mean SD ^b (%)		
L1	2–7	1.4	2–9	2.2	3.98	.05
L6	4–10	2.5	3–12	3.2	.61	.43
L10	2–9	2.9	1–9	2.0	2.02	.16

Note: Data underlying this figure are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.5t8pq> (Meunier et al. 2017).

^a Range of divergence in nuclear ratios among technical replicates and among sectors of a plate.

^b Mean standard deviation in nuclear ratios among technical replicates and among sectors of a plate.

^c *P* is the result of the Kruskal-Wallis test on the difference between the mean variance of technical and sector samples.

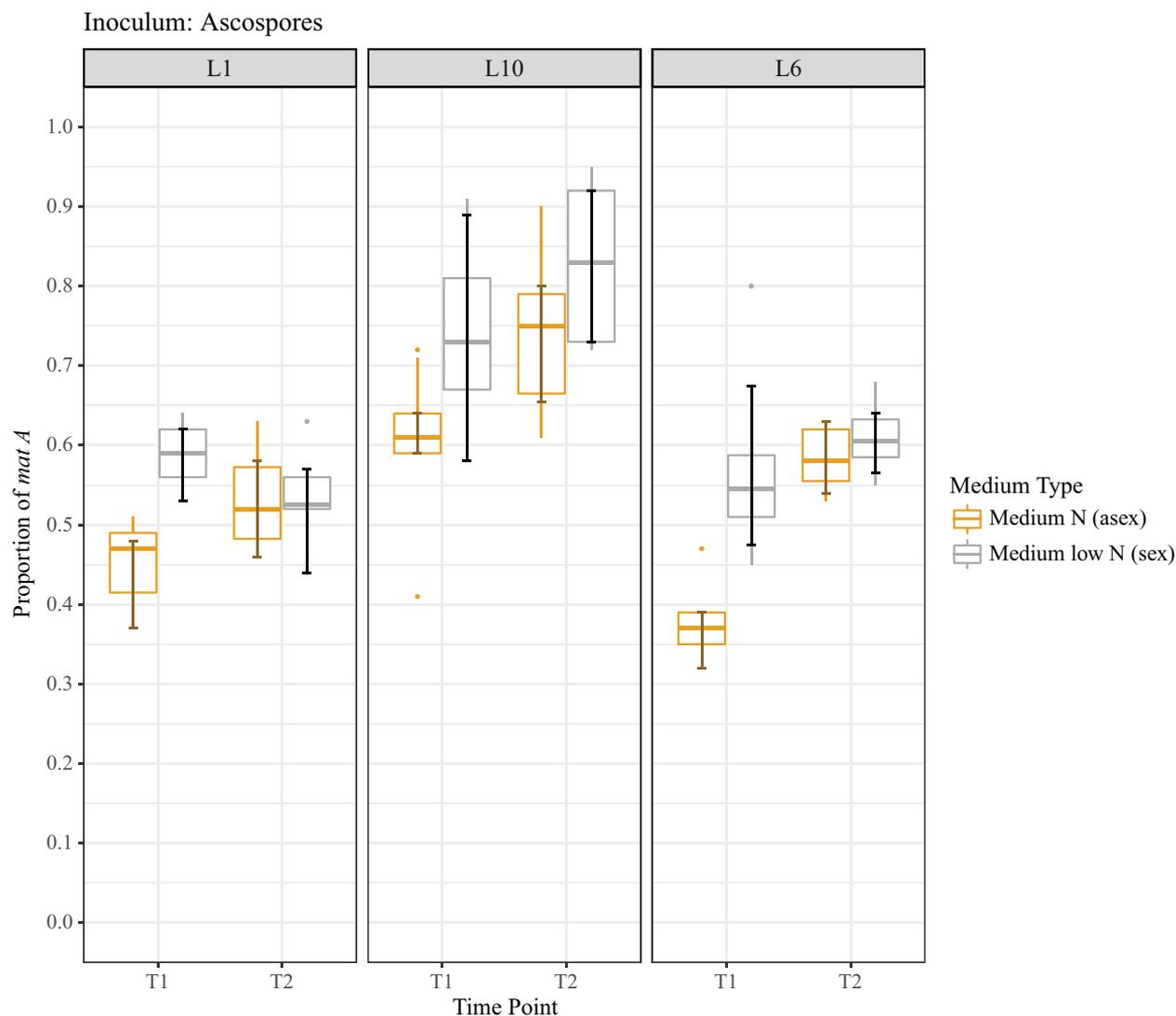


Figure 2: Proportion of *mat A* nuclei in mycelia emerging from a single ascospore. Boxplot of observed nuclear ratios in mycelia, shown by lineage, medium, and duration of growth. L1, L10, L6 = lineage 1, 10, and 6, respectively. T1 is 3 days after inoculation, and T2 is 8 days after inoculation. Bars represent the 95% confidence intervals on the median of the nuclear ratios for each treatment, and dots represents outliers. Data underlying this figure are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.5t8pq> (Meunier et al. 2017).

nificant (ANOVA SSIII: $P < 10^{-6}$), with the proportion of *mat A* nuclei being significantly higher in L10 compared to L1 and L6 (contrast corrected value, L10-L1 and L10-L6: $P < 10^{-6}$), whereas no difference was detected between L1 and L6.

Growth medium also had a significant effect on the nuclear ratio. Specifically, when merging for all conditions, a higher proportion of *mat A* nuclei was found in mycelia grown on medium low N than on medium N (ANOVA SSIII: $P < 10^{-5}$). The proportion of *mat A* was generally higher at T2 than at T1 (fig. 2). However, this effect was dependent

on medium (interaction significant, ANOVA $P = .01$), with an effect of time being stronger on medium N (i.e., the nuclear ratio increased between T1 and T2 on medium N and was more stable on medium low N; see L6 and L10 on fig. 2).

To summarize, the nuclear ratios depended on strain identity and growth conditions and could, in spite of an equal ratio in the inoculum, become significantly biased away from 1:1 in the developing mycelium. This was especially the case for L10, where nuclear ratios were systematically biased toward *mat A*.

*Conidia Inoculum: Effects of Variable
Inoculum Nuclear Ratios*

Statistical analyses were conducted separately for each lineage. In the strain from L1, the nuclear ratio in the established mycelium stayed very close to the inoculum ratio, even after 1 week (fig. 3A). Accordingly, the ANOVA analyses showed a very strong effect of initial inoculum ratio on the nuclear ratio in the outgrown mycelium ($P < 2.10^{-16}$) but no effect of medium and duration of growth. However, the effect of duration of growth depended on the initial ratio, with a tendency for a balancing of the nuclear ratio by time (fig. 3A).

In the strain from L10, by contrast, we observed *mat A*-biased nuclear ratios regardless of the initial inoculum ratio, and this bias was increasing with time (fig. 3B). Even when starting from only 10% of *mat A* conidia, the nuclear ratio eventually reached a high proportion (80%–90%) of *mat A* at T2 (fig. 3B). This bias for *mat A* was consistent with the result obtained when using ascospores as inoculum (fig. 2) and seemed to be a lineage-specific trait: we replicated this experiment using one additional heterokaryon of the same lineage (FGSC 10715/10716), and the observed ratios here were also highly skewed toward nuclei of mating type *A* (app. G). The ANOVA *P* value gave all main effects of medium, time, and ratio and interactions of these, significant except for the three-way interaction. The nuclear ratios were significantly more *mat A* biased on medium low N than on medium N (contrasts: corrected $P < 2.10^{-16}$) and were significantly more biased at T2 than at T1 ($P < 2.10^{-16}$; fig. 3B). The effect of time depended on medium: no difference in nuclear ratios could be found at T2 between the two media (fig. 3B).

The pattern of the nuclear ratio dynamics showed yet a different pattern in L6 than in L1 and L10 (fig. 3C). The nuclear ratio in this strain significantly depended on the initial ratio (ANOVA SSIII: $P < 2.10^{-16}$), but in contrast to L10, nuclear ratios were biased toward nuclei of mating type *a*: at T1, the observed ratio changed from the initial *mat A*-biased inoculum to about equal ratio in the mycelium, from the initial equal ratio to about 30% *mat A* nuclei, but did not change when the inoculum was *mat a* biased (fig. 3C). An effect of time, dependent on the initial ratio, was also present (interaction time point \times initial ratio: $P < .0001$; fig. 3C).

Comparison of the nuclear ratio in hyphae germinating from ascospore inoculum and from conidia mixed in equal nuclear ratios, where both inocula had a 1:1 nuclear ratio, showed consistent results for L1 (figs. 2, 3A) and L10 (figs. 2, 3B). By contrast, in L6, the nuclear ratios of hyphae were more *mat a* biased when inoculating with conidia as compared to ascospores (figs. 2, 3C). We found a germination bias for *mat a* conidia in L6, but this bias was not sufficient

to explain this inconsistency (table E1; tables A1, B1, C1, E1, H1 are available online). We suggest that recombination events occurring during ascospore production may represent one contributing factor. Furthermore, the effects of medium on nuclear ratio were not consistent between ascospore and conidia inocula in L6 and L1 (figs. 2, 3A, 3C). In L10, however, the effect of medium was in agreement for both experiments (figs. 2, 3B).

To summarize, in L1, the nuclear ratio changed slowly over time. By contrast, in L10 and L6, the nuclear ratio was more dynamic. In L10, the bias toward *mat A* was consistent across all conditions, held whatever the initial conditions, and held for different strains in the same lineage.

Bias in Transmission of Nuclei into Conidia

When investigating for biased transmission of *mat A* and *mat a* nuclei from heterokaryotic mycelium into the conidia, we found that the strains from L1 and L6 did not show any deviation of the nuclear ratio between mycelium and conidia. However, in L10, the nuclear ratio was significantly more *mat A* biased in conidia compared to the nuclear ratio in the mycelium in all three plates tested (fig. 4). Appendix H gives detailed ANOVA results for L10 plates. Nonparametric tests gave the same results. The mean deviation in nuclear ratio between conidia and mycelium reached 14%. We conclude that *mat A* nuclei are better transmitted into conidia than *mat a* nuclei in L10.

*The Effect of Nuclear Ratio on Fitness:
The Vegetative Phase*

We measured conidia production as a proxy for fitness in the vegetative phase in heterokaryons with different initial nuclear ratios and in the corresponding homokaryons. Performance in conidia production differed significantly among mycelia with different nuclear content (one-way ANOVA, White-adjusted correction, $P < 10^{-9}$ for the effect of nuclear content for each strain; fig. 5). The direction of the advantage was not conserved among lineages. In the strains from L1, homokaryons of mating type *a* had the highest conidia production, whereas homokaryons of *mat A* showed the highest conidia production in L6 and L10 (fig. 5). This analysis showed that the performances of heterokaryons changed in a roughly additive way with respect to mating-type dosage: heterokaryons formed by inocula containing most of the more productive mating type also performed better (fig. 5). Furthermore, no heterokaryon for any lineage produced fewer conidia than the homokaryon producing the fewest, and no one produced more than the best homokaryon (see CIs on medians in fig. 5).

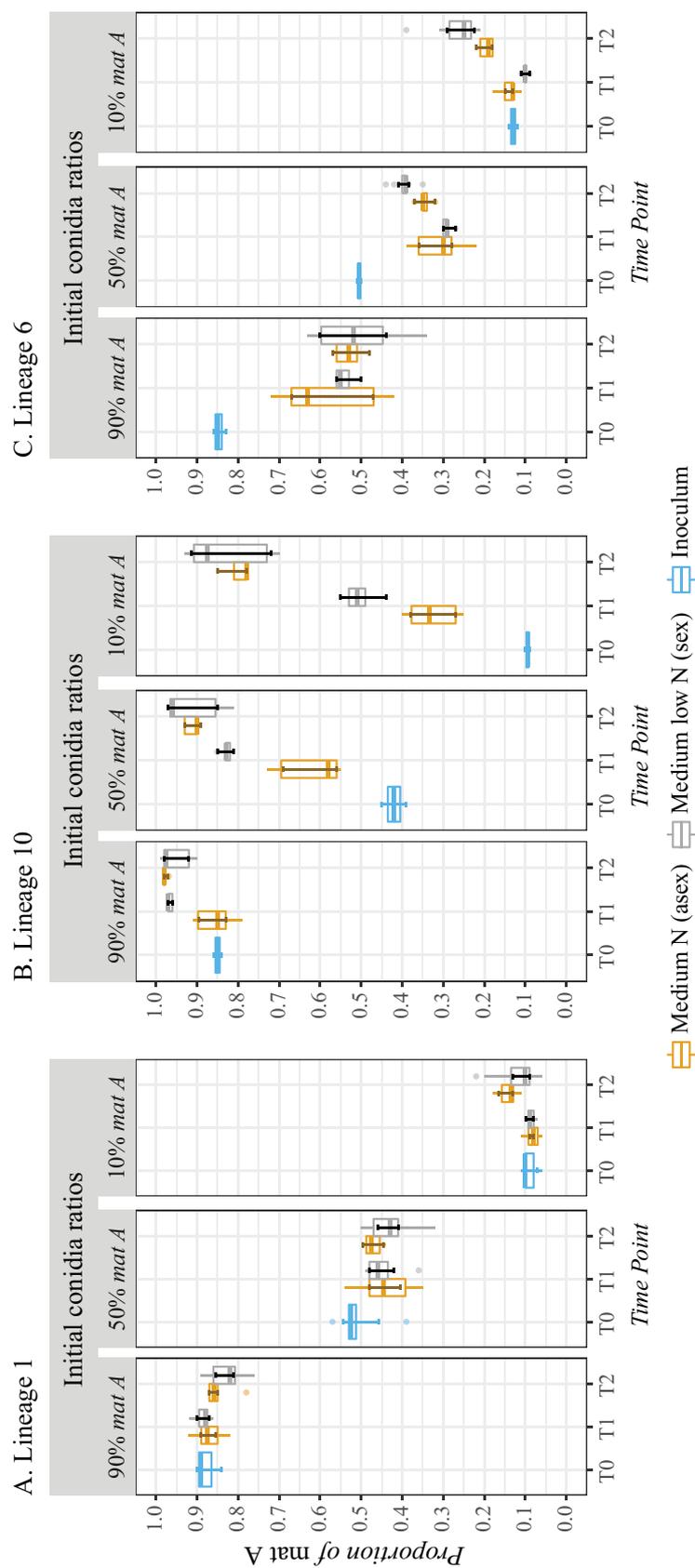


Figure 3: Proportion of *mat A* nuclei in mycelia from conidial inocula. Boxplots of observed nuclear ratios in inoculum and outgrown mycelia are shown for each lineage (lineage 1 [A], lineage 10 [B], lineage 6 [C]). Data is shown by aimed proportion of *mat A* in the mixes of conidia (90%, 50%, and 10% *mat A* nuclei) medium and duration of growth. T1 is 2 days after inoculation, and T2 is 7 days after inoculation. Bars represent the 95% confidence intervals on the median of the nuclear ratios for each treatment, and dots represent outliers. Data underlying this figure are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.5t8pq> (Meunier et al. 2017).

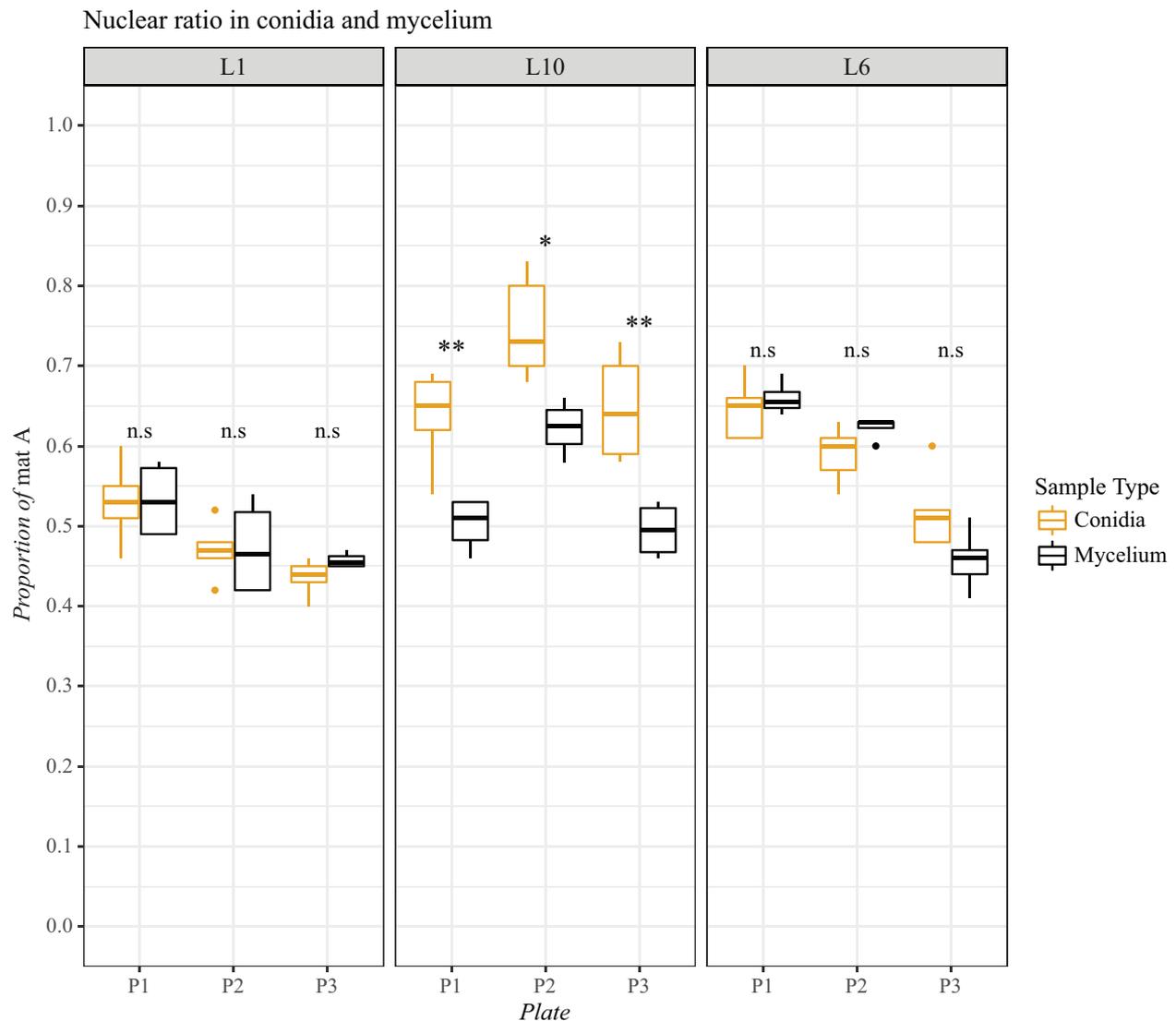


Figure 4: Nuclear ratios in individual mycelia and their produced conidia. Boxplot of nuclear ratios by lineage (L1, L10, L6 = lineage 1, 10, and 6, respectively) and individual replicate (P1, P2, P3 = one inoculum each of a single ascospore). Bars represent the 95% confidence intervals on the median of the nuclear ratios for each treatment, and dots represent outliers. Asterisks refer to the result from the statistical test of the difference of means between conidia and mycelium for each replicate (one asterisk: $P < .05$; two asterisks: $P < .01$; n.s. = nonsignificant). Data underlying this figure are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.5t8pq> (Meunier et al. 2017).

The Effect of Nuclear Ratio on Fitness: The Sexual Phase

We found that protoperithecia size, used as a proxy for reproductive investment of the homokaryons into the sexual cycle, differed significantly among single mating-type homokaryons for each lineage (L1 and L10: $P < 10^{-4}$; L6: $P = .03$). The investment of nuclei of the two mating types depended on the lineage; homokaryons of mating type *A* invested more in the protoperithecia size in the strain from L1, whereas in L6 and L10 it was homokaryons of mating type *a* that invested more (fig. 6A).

When using ascospore production in heterokaryons as a proxy for heterokaryotic fitness in the sexual phase, we found that the initial nuclear ratio significantly impacted the performances of the heterokaryons (one-way ANOVA on heterokaryons; $P = .002$ for L1, $P = .003$ for L6, and $P < 10^{-16}$ for L10; fig. 6B). Ascospore production in heterokaryons correlated with the investment in the sexual reproduction in homokaryons; the heterokaryons having the highest ascospore production were the ones biased toward the mating type investing most in the sexual pathway (fig. 6). Strikingly, the trend was reversed compared to conidia production (cf.

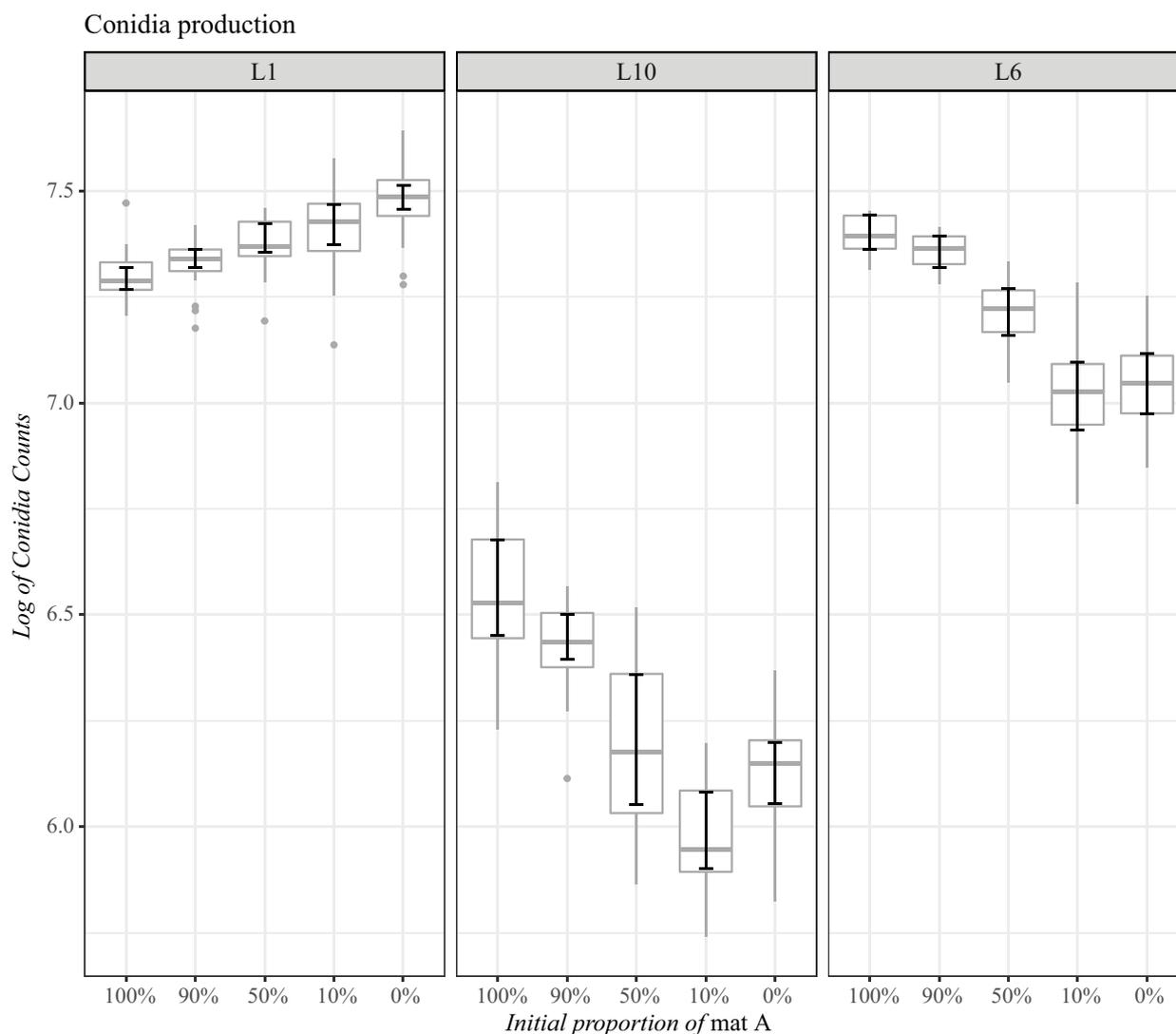


Figure 5: Vegetative proxy of fitness: conidia production as a function of nuclear ratio. Boxplots of the logarithm of conidia counts by lineage (L1, L10, L6 = lineage 1, 10, and 6, respectively) as a function of nuclear ratios. Bars are 95% confidence intervals on the median of the conidia counts, and dots are outliers. Note that the X-axis shows the initial ratio and not the attained ratio by the time of conidia production. Data underlying this figure are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.5t8pq> (Meunier et al. 2017).

fig. 5, fig. 6B; the heterokaryons that produced most conidia produced least ascospores and vice versa). Ascospore production changed in an additive way with respect to the mating-type dosage in both L1 and L6. However, in L10, we observed a dramatic fall in ascospore production in the heterokaryons with the most highly *mat A*-biased initial nuclear ratios (50% and 90%; fig. 6B). Notably, our results revealed a long-lasting effect of initial conditions on ascospore production. For example, in L10, by the time of ascospore production (8–13 days), nuclear ratios among the three treatments should have been very similar (highly *mat A*-biased ratios; see fig. 3B at T2), yet ascospore yield was very different (fig. 6B).

Discussion

Heterokaryosis in Neurospora tetrasperma

Heterokaryosis is known in several groups of fungi. The life cycle of most basidiomycetes, certain ascomycetes, and certain glomeromycetes includes a long-lived heterokaryotic mycelium, containing two haploid nuclear types of different mating type (Raper 1966; Raju and Perkins 1994; Ropars et al. 2016). In basidiomycetes, heterokaryosis is usually controlled by clamp connections to be in 1 : 1 ratio (Raper 1966), making it equivalent to diploidy in having an equal number of genotypes in cells and tissue. In this study, we verified that heterokaryosis in *N. tetrasperma* is not equivalent to dip-

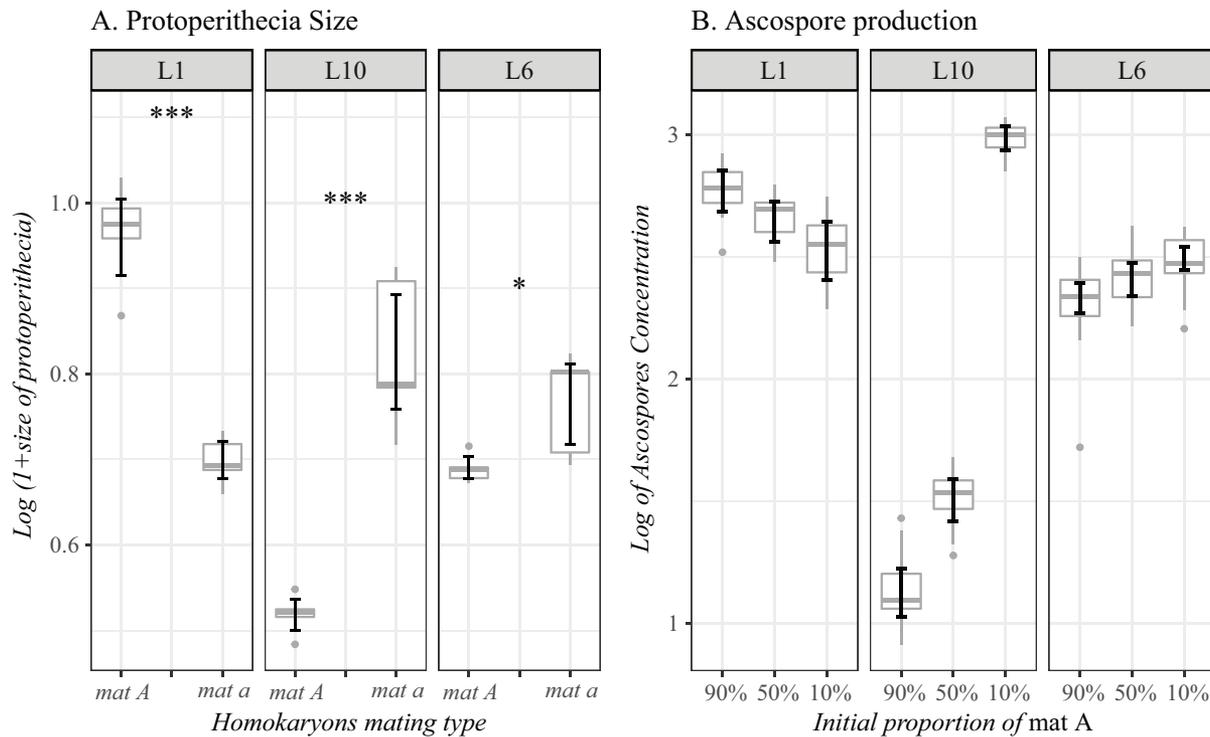


Figure 6: Sexual proxies of fitness. *A*, Protoperithecia size as a function of mating type for each lineage. Boxplot of the logarithm of (1 + size of protoperithecia) of the single mating type component strains of the different lineages (L1, L10, L6 = lineages 1, 10, and 6, respectively). Asterisks refer to the result from the statistical test of the difference of means between protoperithecia size of the mating type component homokaryons of each lineage (one asterisk: $P < .05$; two asterisks: $P < .01$; n.s = nonsignificant). Bars are 95% confidence intervals on the mean of the logarithm of protoperithecia size and dots are outliers. *B*, Ascospore production as a function of initial nuclear ratio in each lineage. Boxplots of the logarithm of ascospore production as a function of initial nuclear ratios in each lineage. Bars are 95% confidence intervals on the median of the log (ascospore concentration), and dots are outliers. Note that the X-axis shows the initial ratio and not the attained ratio by the time of ascospore production. Data underlying this figure are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.5t8pq> (Meunier et al. 2017).

loidy in this respect, since nuclear ratios can depart from 1 : 1. This result is in accordance with previous reports on biased nuclear ratios in *N. tetrasperma* (Samils et al. 2014) and in two basidiomycete species for which mating type nuclei are not constrained to propagate together, *Heterobasidion parviporum* (James et al. 2008) and *Termitomyces* sp. (Nobre et al. 2014). Here we further show that in *N. tetrasperma*, nuclear ratios can depart from evenness in the mycelium even when controlling the inoculum to be 1 : 1 (figs. 2, 3B, 3C), indicating that nuclei replicate at different rates in a radiating mycelium. Furthermore, we verified homogeneity of nuclear ratios among sectors of a radiating mycelium (table 1; app. F). Apart from previous reports on homogenous nuclear ratios between growth front and more aged parts of the mycelium (Samils et al. 2014), sectoring had not been explored in *N. tetrasperma*. This is a significant finding for the study of intraorganismal genetic variation in fungi, since sectoring is common in other ascomycetes (Jinks 1952; Roper 1971; Wang et al. 2005; Zheng et al. 2013) and allows the physical separation of genotypes within an individual.

Hence, our data suggest that some mechanism limits drift inside of the mycelium in *N. tetrasperma*, allowing nuclear diversity to be maintained throughout the mycelium. In the closely related species *Neurospora crassa*, nuclei have been demonstrated to move across cells under a permanent flow, maintaining genetic diversity in the mycelium (Roper et al. 2011, 2013). It is likely that the same active and probably costly mixing may also maintain homogeneous nuclear ratios in *N. tetrasperma*.

Nuclear Ratios Are Determined by Growth Conditions in a Strain-Specific Manner

Our data show that different sets of controlled conditions determine nuclear ratio in mycelia in different individuals or experiments (figs. 2, 3; app. G), suggesting that nuclear ratio is deterministic and not random. An important new finding in this study is the effect of initial nuclear ratio in the inoculum on latter nuclear ratio in the developing mycelium. The strain from L1 shows a nuclear ratio that, through the

course of this experiment, is well correlated with the inoculum and thus may be considered stable over time (fig. 3A). By contrast, in L6 and L10, ratios change toward different equilibria; in L6, we see a tendency for changing toward a slightly *mat a*-biased ratio (fig. 3C), whereas in L10, nuclear ratios consistently show a strong *mat A* bias that also increases over time (figs. 2, 3B; app. G). Taken together, these results indicate differential constraints on mitosis rates of the nuclei within a heterokaryon: in L1, both *mat A* and *mat a* nuclei might be constrained to have similar mitosis rates, whereas rates seem to differ in L6 and L10, with *mat A* nuclei in L10 enjoying a replication advantage.

Furthermore, we showed that nuclear ratios are sensitive to medium: under a range of conditions, nitrogen starvation triggered nuclear ratios that were more *mat A* biased than on standard medium (figs. 2, 3B). This finding is in accordance with previous studies showing an effect of medium on nuclear ratios (Jinks 1952; James et al. 2008). Based on these results, we hypothesize that nuclear ratio itself is determined by different mechanisms in the three lineages and/or that selection might favor different ratios in the different lineages.

The Advantages of Heterokaryosis in N. tetrasperma

The commonality of heterokaryosis in fungi suggests that there is an advantage of this genetic system over homokaryosis or diploidy. In this study, we approached the fitness effects of heterokaryosis by comparing hetero- and homokaryons in their conidia production, size of protoperithecia, and ascospore production. We are aware of limitations in analyzing and interpreting proxies for fitness and the fact that these characters may not completely represent the individual's reproductive success. Nevertheless, interpreted with caution, we argue that they provide insights into the pros and cons of fungal heterokaryosis.

We did not detect any difference in conidia production between heterokaryons and homokaryons of *N. tetrasperma* (fig. 5). Thus, potential advantages of heterokaryosis over homokaryosis remain speculative, as we outline below. First, heterokaryosis in *N. tetrasperma* leads to self-fertility (fig. 1), and a short-term advantage of heterokaryosis is reproductive assurance (Baker 1955). Second, heterokaryons might benefit from a size effect when germinating from sexual ascospores, since the heterokaryotic ascospores are larger than the occasionally produced homokaryotic ones (Raju 1992). Size-related effects of inoculum have been suggested to increase fitness (Roze and Michod 2001; Grosberg and Strathmann 2007; Clarke 2014) and have been proven experimentally in *Dictyostelium* chimeras (Foster et al. 2002) and *Neurospora* (Richard et al. 2012; Bastiaans et al. 2015). Finally, heterokaryons may take advantage of having two genomes, enabling them to adapt faster (Orr and Otto 1994; Clark and Anderson 2004; Otto and Gerstein 2008; Gerstein

et al. 2011), as has been verified experimentally for heterokaryons in the basidiomycete *Schizophyllum commune* (Clark and Anderson 2004).

We also investigated advantages of heterokaryosis over diploidy. Specifically, we showed an effect of a varying nuclear ratio on fitness, as estimated by both conidia and ascospore production (figs. 5, 6B), with a range of performances likely to reflect the allele dosage. To our knowledge, this is the first time such a fitness response has been shown in heterokaryons. Our results suggest that heterokaryosis in itself provides the mycelium with greater flexibility than diploidy, in which nuclear ratios are locked into a 1:1 ratio. The question remains whether the variation in nuclear ratio can be proved to be adaptive for the heterokaryon. This has been an idea long defended (Pontecorvo 1946; Jinks 1952), but to our knowledge, experimental data supporting or refuting it are still lacking (Jinks 1952; James et al. 2008). We have shown here that medium triggered, under certain conditions, shifts in nuclear ratios and that manipulating nuclear ratios had an effect on heterokaryon fitness (figs. 5, 6B). Further experiments of fitness effects of hetero- and homokaryosis are needed to show whether natural shifts of nuclear ratios within heterokaryons, in the absence of manipulation, can be directly linked to shifts in heterokaryon fitness.

Potential Conflicts among Nuclei in N. tetrasperma Heterokaryons

Bearing intraorganismal genetic heterogeneity might be a threat to the organism, as theoretical and experimental studies have shown (Michod 1997; Velicer et al. 2000; Kuzdzal-Fick et al. 2011; Bastiaans et al. 2016). In a heterokaryon, selection at the nuclear level may occur, in which a nuclear type enjoys an evolutionary advantage over the other at the same time as decreasing heterokaryon fitness. In *N. tetrasperma*, ascospore production is a relevant proxy for heterokaryon-level fitness, since in ascospores, nuclei are packed 1:1 and are observed to restore the heterokaryons with a frequency varying between 90% and 98% (Raju 1992). In this study, we showed that in L10, *mat A* nuclei enjoy a higher fitness via (i) a higher replication rate in the mycelia and (ii) a bias in their transmission to asexual progeny. In addition, *mat A*-biased heterokaryons in L10 perform very poorly in ascospore production (fig. 6B). Thus, *mat A* nuclei are transmitted with a higher probability via conidia but decrease the contribution of the heterokaryon to the next generation. Furthermore, due to the conidia being short-lived compared to ascospores (fig. 1), the dispersal as a homokaryon likely gives only a short-term benefit. Even if *mat A* nuclei get better access to conidia and a higher dispersal rate, homokaryons establishing from *mat A* conidia would no longer be self-fertile and would undergo mating partner restriction (Bistis 1996) or cost of outcrossing (Powell et al. 2001). Thus, recreating

heterokaryons from homokaryons seems compromised, and we interpret this finding as a result of conflict between levels of selection.

Notably, a former study showed that in L10, the *mat A* chromosome is introgressed from a homokaryotic close relative of *N. tetrasperma* and shows less molecular degeneration than the nonintrogressed *mat a* chromosome (Corcoran et al. 2016). Introgression in this study was hypothesized as a mechanism of rejuvenation of chromosomes in *N. tetrasperma*, which otherwise degenerate due to lack of recombination (Corcoran et al. 2016). Our results show a higher fitness for *mat A* nuclei/homokaryons in L10 that corroborate this rejuvenation hypothesis at the nuclear level. By contrast, *mat a* nuclei, which are not introgressed, are likely to have accumulated recessive deleterious alleles that get exposed at the homokaryon level. Alternatively, they may have evolved toward cooperation in heterokaryons, as they also fruit very poorly when homokaryotic (app. I), and we speculate that introgression on *mat A* in L10 has interrupted the coevolution between the nuclear types and disrupted selection at the heterokaryon level.

The Individualistic Mycelium: Mediation of Conflicts and Promoters of Cooperation

The evolution of mechanisms alleviating conflicts among lower-level parts of an organism is considered necessary for its stability (Michod and Roze 2001; Michod and Herron 2006; Grosberg and Strathmann 2007). One possible mechanism to avoid conflict in the first place is to limit within-organism genetic variation. In fungi, vegetative incompatibility is a potent mechanism limiting within-organism variation and has been viewed as proof that mycelia behave as individuals (Debets and Griffiths 1998; Czaran et al. 2014). *Neurospora* sp., just as other fungi, possess an allor-cognition system, limiting vegetative fusion between non-related strains through recognition of nonself at incompatibility loci (Glass and Kaneko 2003). Furthermore, additional mechanisms found in this study could also be considered as promoting the stability of heterokaryons. First, the absence of sectoring, that is, homogeneity of the genetic composition in the mycelium (table 1; app. F), is an argument for the individual mycelium behaving as a unit of selection. While mixing can provide opportunities to cheaters, preventing their isolation and exposure to selection at the higher level, mixing also results in fitness alignment of the nuclear types, since both nuclei experience the same environment (Clarke 2014). The active mixing of the cytoplasm in *Neurospora* may be seen as an analog to clamp connections in basidiomycetes: both mechanisms align fitness of the nuclei and prevent the complete takeover of harmful nuclear types. Second, constraints on mitotic rates might also reduce conflicts among nuclei. Nuclear ratio is quasi-constant in the strain of

L1 studied. In L6, a trend toward equalizing ratios also seems present. Regulation of mitosis rates can be a potential mechanism ensuring the limitation of cheating, even if the mechanism by which mitosis rates might be constrained to be constant is unclear in *Neurospora*. In *N. crassa*, mitosis has been shown to be asynchronous (Serna and Stadler 1978; Minke et al. 1999); mitosis clocks among asynchronous nuclei have been investigated only in *Ashbya gossypii* and were shown to be different (Roberts and Gladfelter 2015). Further studies are thus needed to understand how the mitosis rate might be constrained to constancy despite asynchrony.

A second hallmark of complex organisms is cooperation and division of labor. In this study, we showed that heterokaryons biased for one or the other mating types have different and opposite fitness performances depending on the life-cycle phase considered. In the three lineages tested here, we found trade-offs between performances of the two nuclear genotypes in the vegetative versus the sexual phase (figs. 5, 6B). Interestingly, this result corroborates Samils et al. (2013) study of the mating-type homokaryons of L1, which showed that a biased expression of genes on the mating-type chromosome has evolved. Other and similar phenotypic differences between *mat A* and *mat a* homokaryons had been described in another strain from L1 (Howe 1964). Mating-type phenotypic divergence thus seems lineage specific and consistent over time, which points toward genes being responsible for these divergent traits being linked to the mating-type chromosome. This specialization of function might have evolved in association with the transition toward heterokaryosis, since the two nuclear types in the ancestor differ in only one locus (Samils 2013; fig. 1). Specialization of function is considered as a hallmark of complex multicellularity, one of the traits emerging when fitness transfer to the higher level has occurred (Michod 2005; Clarke 2014). This last observation thus reinforces the concept of individual mycelium in our model species.

Conclusion

In this study, we investigated the switch in main unit of selection in *Neurospora* from the level of the homokaryon/nuclei to heterokaryotic mycelium and the effects of within-organism genetic variation. We identified that the heterokaryon functions as an individual in the form of gained additional phenotypic flexibility and division of labor, but we have also verified selection and selfish behavior at the nuclear level. The selfish behavior was detected in a nucleus reported to show recent chromosomal introgression from a sister species, suggesting that coevolution between the nuclei was interrupted in this lineage. Heterokaryosis in *N. tetrasperma* thus exemplifies a genetic system where transfer of fitness to the higher level of the organism seems at the same time advantageous and incomplete.

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