Nuclear interactions in a heterokaryon: insight from the model Neurospora tetrasperma

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A heterokaryon is a tissue type composed of cells containing genetically different nuclei. Although heterokaryosis is commonly found in nature, an understanding of the evolutionary implications of this phenomenon is largely lacking. Here, we use the filamentous ascomycete Neurospora tetrasperma to study the interplay between nuclei in heterokaryons across vegetative and sexual developmental stages. This fungus harbours nuclei of two opposite mating types (mat A and mat a) in the same cell and is thereby self-fertile. We used pyrosequencing of mat-linked SNPs of three heterokaryons to demonstrate that the nuclear ratio is consistently biased for mat A-nuclei during mycelial growth (mean mat A/mat a ratio 87%), but evens out during sexual development (ratio ranging from 40 to 57%). Furthermore, we investigated the association between nuclear ratio and expression of alleles of mat-linked genes and found that expression is coregulated to obtain a tissue-specific bias in expression ratio: during mycelial extension, we found a strong bias in expression for mat A-linked genes, that was independent of nuclear ratio, whereas at the sexual stage we found an expression bias for genes of the mat a nuclei. Taken together, our data indicate that nuclei cooperate to optimize the fitness of the heterokaryon, via both altering their nuclear ratios and coregulation genes expressed in the different nuclei.

1. Introduction

Although Darwin [1] framed his theory of evolution by natural selection around individuals within species, the principle of natural selection implies that any entity in nature that exhibits variation, reproduction and heritability may evolve [2,3]. Examples of such entities could be genes, mitochondria, nuclei, cells and individuals. Thus, an interaction between natural selection acting at different levels may occur in an organism [3–5]. In this study, we focus on the interactions of two levels of biological hierarchy in filamentous fungi—the mycelium and the nuclei it harbours. There is a strong sense of individuality of the mycelium. It is able to discriminate between self and non-self during both vegetative growth and sexual reproduction [6,7], through a system analogous to the major histocompatibility complex in vertebrates [8] and self-incompatibility in plants [9]. Self/non-self recognition of fungal mycelia is widespread and expected to be maintained by natural selection [10,11], most likely in response to challenges such as spread of mycoviruses [12], selfish genetic elements [13] or parasitization by aggressive genotypes [14] between genetically different mycelia. This observation suggests that the components of the mycelium cooperate to form a single ‘unit of selection’ [15]. However, a mycelium has the potential to grow as a heterokaryon, i.e. to harbour genetically different nuclei, an observation expected to result in a challenge for the individualistic mycelium. A fungal heterokaryon may arise through spontaneous mutations of the nuclei during somatic growth, or through mating or vegetative fusion of individuals of different nuclear types [16,17]. Fungal heterokaryons are assumed to be functionally equivalent to diploids with respect to dominance and recessivity of traits [18–20], and there is empirical evidence of genetic complementation of mutations between nuclei cohabitating a heterokaryon of, for example, Neurospora crassa [21] and Schizophyllum commune [22]. These findings...
We used pyrosequencing of mating type (\textit{mat})-linked SNPs of three natural heterokaryons of \textit{N. tetrasperma} to demonstrate that nuclear ratio is consistently biased in favour of \textit{mat A} nuclei during mycelial development, but this ratio evens out during the course of sexual development. Furthermore, we investigated the association between the nuclear ratios and expression ratios of alleles of \textit{mat}-linked genes in different tissues and found evidence of tissue-specific congeulation to obtain a specific bias in expression ratio. Our data suggest that different expression ratios are optimized during different stages of the life cycle, and that nuclear ratio and expression ratio act in the same direction to achieve this goal. Taken together, our data show support for the ‘individualistic mycelium’ concept [15], i.e. that nuclei in the heterokaryon cooperate to optimize the fitness of the heterokaryon.

### 2. Material and methods

#### (a) Fungal material and growth conditions

Three heterokaryotic strains of \textit{N. tetrasperma} were selected for the study (Table 1). These heterokaryons were originally isolated from nature and belong to three distinct phylogenetic lineages of \textit{N. tetrasperma} [34]. The heterokaryotic strains and their homokaryotic, single mating-type, component strains were obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas City, MO, USA. In this study, the heterokaryotic strains are referred to by their lineage ID, L1, L6 and L7, and the homokaryotic strains are referred to by their lineage ID followed by mating type, L1A, L1a, L6A, L6a, L7A and L7a (Table 1).

The nutrient medium used for the cultures was synthetic crossing medium [35], and when not stated differently, the cultures were grown at 25°C in darkness. For the cultures where we harvested tissues for analyses of nuclear and expression ratio, we used a layer of cellophane on top of the solid medium to facilitate tissue harvesting.

#### (b) Experimental design and tissue sampling for the nuclear and expression ratio analyses

First, we investigated the relative abundance of \textit{mat A} and \textit{mat a} nuclei in extending heterokaryotic mycelia. We inoculated the heterokaryotic strains at the centre of large Petri dishes (Ø140 mm), and when the mycelium had reached the edge of the plate (67 h for L1 and L7, and 74 h for L6), we performed concentric harvesting of mycelia in three zones along the radius.
of the developed mycelium. Three replicate plates were used for each heterokaryon, resulting in 27 tissue samples.

Second, we investigated the nuclear dynamics in the heterokaryons in different stages of the life cycle. We investigated both the mat A/mat a nuclear ratio and the relative expression of mating-type-linked genes in sexual and vegetative tissues. We harvested tissue rich in perithecia and tissue dominated by mycelia from Petri dishes (c90 mm; see the electronic supplementary material, figure S1, note that the inoculum plug was never included in the collected tissue samples). We refer to these two tissues as ‘perithecial’ and ‘mycelial’ throughout the paper. Tissue was harvested at two time-points post-inoculation. The first sampling of perithecial and mycelial tissue from the dishes was performed at 9 days post-inoculation. At this stage, the perithecia of L7 had matured and started to eject ascospores, whereas the perithecia of L1 and L6 were still immature and did not yet eject ascospores. The sampling procedure was repeated 7 days later (i.e. 16 days post-inoculation) when ascospore discharge was ongoing in all three strains. For each of these two time-points, five Petri dishes/strain were used, thus this experiment resulted in altogether 60 tissue samples.

The resulting 87 samples were immediately after harvesting frozen in liquid nitrogen and stored in −80 °C until DNA and RNA extraction.

(c) Nucleic acid extraction and cDNA synthesis

We extracted both RNA and DNA from each of the tissue samples. For this extraction, we used the TRI Reagent kit (Molecular Research Center, Inc., Cincinnati, OH, USA) for RNA and subsequent DNA extraction, following the manufacturer’s instructions. This protocol allowed us to analyse both RNA and DNA from the same nucleic acid extraction. All RNA samples were treated with DNase I (Fermentas, Sweden) before performed CDNA synthesis, which was performed on 1 μg total RNA/sample using iScript (Bio-Rad, CA, USA) following the manufacturer’s instructions with oligo(dT)20 primers. The random primer mix included in the iScript kit was also used in a subset of 96 samples to confirm that cDNA synthesis was not dependent on the use of primers (data not shown). The DNA and cDNA samples were dissolved in nuclease-free water for storage at −20 °C before pyrosequencing.

(d) Development of molecular markers for the analyses of mating-type-linked SNPs

Seven different coding genes, each located within the region of suppressed recombination on the mat chromosome [36] and thus genetically linked to mating type, were chosen for the study. The genes selected were: ad-9, arg-1, cse-9, erg-8, eth-1, hse-4 and upr-1. For a relative position of the genes along the chromosome, see the electronic supplementary material, figure S2. PCR primers were designed using the PSQ ASSAY DESIGN Software (Biotage) to amplify regions flanking mat-linked SNPs (identified in [36] and shown in the electronic supplementary material, figure S3). In addition, for each amplified region, a specific sequence primer was designed for the pyrosequencing reaction. All primers used in this study are listed in the electronic supplementary material, table S1.

(e) PCR, pyrosequencing and estimation of ratio of DNA and cDNA in tissue samples

All PCR reactions were based on the Expand High Fidelity PCR System, using the buffer with 15 mM MgCl2 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Each reaction contained 5 ng template of DNA or cDNA. The reactions were carried out on an Eppendorf epgradient S thermocycler with the following thermal profile: 94 °C for 5 min, then 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, followed by a final 7 min extension at 72 °C.

The amplicons for each sample were prepared, sequenced and analysed on a PSQ 96MA (Qiagen) using pyrosequencing technology following the manufacturer’s guidelines. The SNPs were identified and automatically edited by the PSQ ASSAY DESIGN Software (Qiagen, Solna, Sweden). Genotyping the SNPs in a sample with both alleles allowed us to quantify the relative abundance of mat A and mat a nuclei, as well as the relative abundance of the mRNA transcribed by the mat-linked genes, in each sample. We based our estimations of nuclear ratio in perithecial and mycelial tissues on the mean of combined output from the different markers (for details on which markers were used for which samples, see the electronic supplementary material, table S1). For the cDNA samples, the data from the markers were analysed separately, because different genes may be expressed at different levels in a sample. Samples that yielded insufficient signal in the pyrosequencer were repeated in order to obtain strong signal. A set of 25% of all samples were randomly chosen as a control and repeated to check for any inconsistencies in pyrosequencing. No inconsistencies were detected.

(f) Analyses of the interaction between nuclear and expression ratio

We investigated whether the relative abundance of mat A and mat a nuclei (i.e. the mat A/mat a ratio) corresponded to the relative abundance of their respective gene transcripts, by regressing the proportion of mat A in the DNA (estimated from all markers combined) against the proportions of mat A in the RNA sample of each of the seven genes. We compared the ratio of expression of the above-mentioned genes between tissues and time points among the three studied strains L1, L6 and L7 with an analysis of covariance (ANCOVA). Analyses were run separately for each gene and included four factors: strain (L1, L6 and L7), time (9, 16 days in culture), tissue (perithecial, mycelial) and the proportion of mat A in the DNA. We analysed all the strains together and separately. According to the DNA analysis, the proportion of mat A varied among time points and tissues. In order to compare expression among the different time points–tissue combinations, we calculated the expected expression at the same proportion of mat A. Expected expression means a ratio of 50% mat A were then compared by Tukey’s HSD (honest significant difference) test with the general linear model procedure of SAS/STAT v. 9.3. Also, at each time point we compared whether there were slope or intercept differences between tissues, and conversely for each tissue we compared whether there were slope and intercept differences between time points. For each time point and tissue, we adjusted a different regression.

(g) Growth rate estimations

We investigated growth rate as a proxy for fitness [37] for the strains on synthetic crossing medium. Growth rates were measured for the heterokaryons and their component single mating-type strains, as in [28]. Specifically, the growth rate was measured in Petri dishes (c90 mm) at four time-points, starting at 19 h post-inoculation and ending at 33 h, or when the strain had reached the edge of the plate. For each time point, the mycelial growth was determined by measuring the mycelial radius at the maximum and minimum point from the centre of inoculation, and a mean growth rate value was derived from the five biological replicates at each time point.

3. Results

(a) Nuclear ratio in heterokaryotic mycelia and sexual tissue

The data on the relative abundance of mat A and mat a nuclei in tissue of the three natural heterokaryons of N. tetrasperma

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are found in the electronic supplementary material, table S2. In the investigation of the relative abundance of \textit{mat A} and \textit{mat a} nuclei in young and extending heterokaryotic mycelial networks of the three heterokaryons, we found that nuclear ratio was stable over mycelial development, and that all samples were strongly dominated by \textit{mat A} nuclei (mean of 87\%, range: 67–99\% among strains and samples) (figure 1). Furthermore, as in young extending mycelia, a strong \textit{mat A} bias was found in older mycelia (i.e. sampled at day 9 and 16 post-inoculation) and in the premature perithecia (i.e. the sexual fruiting bodies, of day 9 for L1 and L6), while at the later stage of perithecial development (perithecia of day 9 in L7 and of day 16 in all heterokaryons) there was a more even nuclear distribution (figure 2). Specifically, the mean proportion of \textit{mat A} nuclei in L1 mycelial samples showed a slight decrease of \textit{mat A} relative frequency from 76 to 73\% from day 9 to day 16, while the mean relative abundance of \textit{mat A} nuclei in the perithecial sample showed a decrease from 75 to 57\% between day 9 and 16. Using ANOVA tests on differences between time points, tissue and their interactive effects, we found that the proportion of \textit{mat A} in the 16 days old perithelial sample of L1 was significantly lower than in the three other samples (figure 2). Similarly, for L6, the mean relative frequency of \textit{mat A} to \textit{mat a} nuclei in the mycelial samples showed a decrease from 70 to 63\%, while the mean ratio \textit{mat A} nuclei in samples rich in perithecia showed a decrease from 71 to 40\% from 9 to 16 days: also for this lineage, ANOVA-analyses revealed that the 16 days old perithelial sample was significantly lower than the all three other samples (figure 2). As for L1 and L6, in the mycelial samples of L7 \textit{mat A} DNA was more abundant than \textit{mat a} DNA, but the ratios were even in both perithelial samples of this lineage (51\% and 55\% \textit{mat A} nuclei of day 9 and 16, respectively) and no significant changes were found between the four samples according to ANOVA (figure 2). The discrepancy in the patterns observed for the different strains can be explained by different speed of sexual maturation between them: as mentioned in the Material and methods section, L7 was forming mature perithecia and already ejecting ascospores at day 9, which was not the case for L1 and L6.

(b) The correlation between nuclear and expression ratios in mycelia

By correlating the nuclear ratio with the expression ratio of alleles of \textit{mat}-linked genes (data shown in the electronic supplementary material, table S2), we found significant differences between strains when comparing the expression ratio with its relative proportion of DNA in the tissue (electronic supplementary material, table S3). Strain L6 was the only strain showing a consistent pattern across markers, according to which (i) the amount of RNA would change depending on the cell density and the type of studied tissue, and (ii) the association between the RNA-ratio and the DNA-ratio would change with time (electronic supplementary material, table S3). Thus, from here on, we focus on presenting detailed data from L6.

In heterokaryon L6, the association between the relative frequencies of \textit{mat A} nuclei and relative expression of \textit{mat A}-linked genes varied between mycelia sampled at the two different time points. In mycelia tissues sampled at 9 days post-inoculation, we found indications of nuclear interaction in that, independently of the nuclear ratio, the \textit{mat A}/\textit{mat a} expression ratio was high (figures 3 and 4). Specifically, in all markers but one, we found that the observed expression ratio of \textit{mat A} at an even nuclear ratio would be significantly higher than 50\% (figure 3). Furthermore, as expected under coregulation of gene expression, we revealed a high intercept and a flattened slope when regressing the relationship between RNA and DNA proportion of \textit{mat A} for all six markers studied (figure 4). The average slope was 0.29, indicating that every 10\% decrease of \textit{mat A} nuclei would be compensated by \approx 70\% (1–0.29) increase in expression of \textit{mat A}. Remarkably, in four out of six markers, the slope was not significantly different from 0, indicating that the \textit{mat A} expression ratio...
was independent of the amount of *mat A* nuclei in the tissue (figure 4).

In mycelia at 16 days post-inoculation, the relative amount of *mat A* DNA remained higher than 50% (figures 2 and 4). However, in contrast to the results from the mycelium sampled at day 9, the expression ratio was proportional to, and thus dependent on, the relative amount of *mat A* DNA (figure 4): the average slope of all markers was 1.13 and for none of the markers studied the slope departed significantly from 1. Note-worthy, the observed expression remained significantly below 50% in five out of six markers (figure 3), indicating a consistent *mat a* bias in expression. A consistent *mat a* bias was also supported by the fact that almost all points were below the 1:1 line in figure 4, although in only two out of six markers the intercept values significantly differed from 0.

(c) The correlation between nuclear and expression ratios in perithecia

In perithecial samples of both day 9 and 16, we found indications of coregulation between nuclei, independently of nuclear ratio, to obtain a tissue-specific expression ratio. Specifically, in premature perithecia of L6, in which a strong *mat A* nuclear bias was found (figure 2), we found support for coregulation between nuclei to obtain a slight *mat a* bias in expression: by comparing the expression at perithecia-rich tissues where *mat A* represented 50% of the nuclei, we found that the gene expression ratios of *mat A*-linked genes were significantly below 50% in three out of six cases (figure 3), and the regressed interactions between gene expression and DNA shown in figure 5 suggest that gene expression is independent of nuclear ratio: all of the slopes are significantly lower than 1, although none of them are completely flattened (i.e. they are all significantly different from 0).

At 16 days post-inoculation, nuclear ratio was more even than in any other tissue, and not significantly different from 50:50 (figure 2), and gene expression ratios of *mat A*-linked genes were found to be below 50% in all six genes (figures 3 and 5). The low slope found in the regressed interactions between gene expression and DNA (the average slope was 0.68) (figure 5), suggest that in this tissue coregulation of nuclei takes place to obtain a bias in expression of *mat a*-linked genes that is stronger than in premature perithecia.

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**Figure 2.** (a–c) Proportion of the DNA belonging to the *mat A* nuclei at different time points and in different tissues in the strains L1, L6 and L7. An ANOVA test on differences between time points, tissue and their interactive effects is also shown in the charts. The same letter above each average indicates non-significant mean differences according to Tukey-HSD test at *p* < 0.05. Bars indicate confidence interval of the mean at 95%. Bar intervals containing the 50% line (short dash) are not significantly (*p* < 0.05) different from 50%. (d–f) The radial growth rates of the heterokaryotic mycelia and their corresponding single mating-type component mycelia. Bars indicate standard error.
Radial growth rate of heterokaryons and their constituent homokaryotic components

In growth rate measurements of the heterokaryotic and homokaryotic strains (Table 1), we found that the mat A homokaryotic component strains of all three heterokaryons displayed the highest growth rates (Figure 2). In L1 and L6, the mat A strains were followed by the mat a strains, and the heterokaryon exhibited the slowest growth, whereas in L6 the mat a strain and the heterokaryon exhibited equal growth rate.

### 4. Discussion

Filamentous fungi, in which genetically distinct, free-ranging and totipotent nuclei populate the mycelium, may be viewed as an arena for multilevel selection. In this study, we approached the heterokaryotic *N. tetrasperma* for the study of the interplay between the nuclei of the mycelium. We show data in support of a non-random bias in nuclear ratio in the heterokaryotic *N. tetrasperma*, and a tissue-specific interaction between nuclei at the expression level. Our findings are consistent over loci, and thus, unlikely to be influenced by amplification and sequencing biases associated with the different SNPs. Our data suggest an optimization of the heterokaryosis in *N. tetrasperma* is associated with a fitness cost at the stage of mycelial growth. However, the benefits of carrying both nuclear types, and thereby being able to alter the nuclear ratio and also complete the life cycle on its own, are likely to overcome any negative effects at the organism level.

#### (a) The connection between nuclear ratio and life cycle stages

Our data indicate that nuclear ratio is strongly correlated with the stage of the life cycle, ranging from strongly biased during vegetative growth, to a more even ratio during the mature fruiting stage. The largely imbalanced nuclear ratios found in mycelial tissue (Figures 1 and 2) indicate that constrained and coordinated movement of nuclei, as found in many basidiomycetes with clamp connections [19], is not in action in *N. tetrasperma*. Instead, it is consistent with findings of the heterokaryotic mycelium of *Heterobasidion annosum* [28], a basidiomycete without clamp connections, and of the unbalanced nuclear ratio found in asexual spores (conidia) budding off the mycelium of *N. tetrasperma*, reported by Corcoran et al. [26]. However, this study is to our knowledge the first direct report on the nuclear ratio being imbalanced within the actual mycelium of a filamentous ascomycete, and on the finding of an imbalanced nuclear ratio in a heterokaryon being strongly connected to the stage of the life cycle.

The growth rate measurements of the homokaryotic and heterokaryotic strains of each component revealed that the mat
A component strains of all three heterokaryons displayed the highest growth rates (figure 2), suggesting the mat A nuclei encode for alleles in favour of fast hyphal growth. However, the absence of correlation between distance from inoculation point and an increase or decrease of the mat A/mat a ratio in the heterokaryons (figure 1) suggest that the uneven distribution of nuclei is neither controlled by factors related to uneven nuclear migration nor by the presence of homokaryotic components of the heterokaryotic mycelium. Rather, our result is suggestive of a replication (i.e. mitotic) bias in favour of mat A nuclei, in connection with fast and free migration of nuclei in the mycelium. This interpretation is supported by the finding of nuclei of many filamentous fungi, including species of Neurospora, being capable of dividing and moving freely through the interconnected mycelium with a rate that can reach several micrometres per second [16]. Furthermore, N. crassa, a close relative of N. tetrasperma, was recently found to exhibit a high level of cytoplasmic mixing [38], a phenomenon suggested by the authors to be adaptive for maintaining stable nuclear ratios in the mycelium. Accordingly, one may speculate that selection for both nuclei being present in the mycelium of N. tetrasperma, in spite of an uneven ratio, takes place to avoid the loss of the mat a nuclei by chance in homokaryotic sectors, and that a high level of cytoplasmic mixing is the mechanism involved.

The fact that mat A is the dominating nuclear type in all three investigated heterokaryons, which originate from different phylogenetic lineages of N. tetrasperma, suggests a generality of this finding across N. tetrasperma. However, in the study of Corcoran et al. [26], we see that nuclear ratio in conidia, emerging from mature hyphae, may be biased for nuclei of either of the two mating types. Although we cannot exclude the possibility that certain nuclei of N. tetrasperma have a stronger ability to be separated from a heterokaryon, and thereby be overrepresented in the conidia, the study of Corcoran et al. [26] suggests that the nuclear bias in a mycelium may also be in favour of mat a nuclei. In support for this expectation is the recent study of Roper et al. [38], in which it was reported that the nuclear ratio in the conidia of N. crassa accurately represented the genotype of the nuclei present within the mycelium, suggesting that nuclear ratio is connected in these tissues also for N. tetrasperma. Data connecting nuclei in the mycelia with the emerging conidia in N. tetrasperma are needed to resolve this issue.

During sexual development, our data suggest that nuclei of both mating types are needed in equal amounts, which is expected for an individual to undergo meiosis [39]. It is likely that the tissue containing the DNA from mature perithecia is maturing spores, and they are inherited in a Mendelian fashion: each spore contains nuclei of both mating-types [30].

Figure 4. Association between expression of the mat A-linked genes in mycelia-rich tissue and the corresponding proportion of mat A nuclei in the mycelia (DNA proportion) at 9 and 16 days. A line with slope b and intercept a has been adjusted for each marker and time point. The superscript above the intercept values displays the level of significance of the intercept being equal to 0, following, n.s., non-significant (p > 0.05), *p < 0.05, **p < 0.01 and ***p < 0.001. The superscript above the slope parameter shows the level of significance of the test of the slope parameter being equal to 0 and being equal to 1, respectively. The probability of the test of equal slopes at 9 and 16 days (b9 and b16), and equal intercepts (a9 and a16), are shown, respectively, with a superscript following the same system as for the previous test. Probabilities lower than 0.05 indicate a significant change of slope or intercept between 9 and 16 days for each marker. For the sake of comparison, a line representing a level of expression equal to a nuclear proportion of mat A in the tissue is shown (dotted line).
Our data suggest that different expression ratios are optimized during different stages of the life cycle of *N. tetrasperma*, and that both a bias in nuclear ratio and expression ratio are involved. This model is schematically depicted in figure 6. First, we suggest that the young extending hyphae (represented herein by extending mycelia, and mycelia sampled at day 9) is optimized for alleles present on *mat A* nuclei; the *mat A* bias is strong for DNA (figures 1 and 2), but even stronger for transcripts (figures 3 and 4), and our data suggest that coregulation is in play to keep the bias of *mat A*-linked genes high at the expression level. As the mycelium matures and ages, and is no longer extending by hyphal growth (i.e. sampled at day 16), the *mat A* nuclei remains dominant (figure 2) while the expression level is unbiased, or in certain cases even *mat a* biased (figures 3 and 4). These results suggest that the alleles present on *mat A* nuclei, possibly in favour of fast hyphal growth, are no longer highly beneficial at the stage of mature hyphae. From the premature to mature fruiting bodies, we see a significant change from a highly *mat A*-biased to an even nuclear ratio (figure 2), while the expression ratio is changing from unbiased to *mat a* biased (figures 3 and 5), suggesting transacting coregulation to keep genes on the *mat a* nuclei at a higher expression level.

**Figure 5.** Association between expression of the *mat A*-linked genes in perithecia-rich tissue and the corresponding proportion of *mat A* nuclei in the perithecia-rich tissues (DNA proportion) at 9 and 16 days. A line with slope $b$ and intercept $a$ has been adjusted for each marker and time point. The superscript above the intercept values displays the level of significance of the intercept being equal to 0, following, n.s., non-significant ($p > 0.05$), *p* < 0.05, **p < 0.01 and ***p < 0.001. The superscript above the slope parameter shows the level of significance of the test of the slope parameter being equal to 0 and being equal to 1, respectively. The probability of the test of equal slopes at 9 and 16 days ($b_9$ and $b_16$), and equal intercepts ($a_9$ and $a_16$), are shown, respectively, with a superscript following the same system as for the previous test. Probabilities lower than 0.05 indicate a significant change of slope or intercept between 9 and 16 days for each marker. For the sake of comparison, a line representing a level of expression equal to a nuclear proportion of *mat A* in the tissue is shown (dotted line).

**(b) Optimization of nuclear and expression ratios across the life cycle**

The mechanism of coregulation of gene expression is unknown, but our data suggest that this phenomenon is taking place at the nuclear level, as all of our genes show similar trends (figures 4 and 5). However, differences between genes do
occur, and we have not studied genes located on the autosomes: hence, the data used herein are not enough to disentangle gene-specific patterns from chromosomal or nuclear patterns.

(c) Costs and benefits of heterokaryosis in *Neurospora tetrasperma*

Taken together, our data support the hypothesis that heterokaryons of *N. tetrasperma* possess an additional level of adaptive flexibility to a changing environment than diploidy, and that the phenotype could be altered by changing nuclear ratios, but also by transacting gene regulation. Early work on the ascomycete moulds *Penicillium cyclopium* and *N. crassa* has indicated that nuclear ratios of heterokaryons changed depending on environmental conditions in a manner that reflected the underlying relative fitness of the constituent homokaryons grown in isolation [40,41]; however, here we show this more precisely in a natural heterokaryon.

Furthermore, our data suggest cooperation and altruistic behaviour of the nuclei of *N. tetrasperma* to optimize the life of the heterokaryon. This is particularly evident for *mat A* nuclei, which seem to perform a large part of the somatic function, but are equally represented in the offspring. Cooperation among nuclei of *N. tetrasperma* may be expected, due to long-term coevolution of the different components of the heterokaryon; the benefits of maintaining heterokaryosis for mating type, due to the resulting self-fertility, may be expected to be large in this species. Hence, the benefits of carrying both nuclear types, and thereby being able to alter nuclear ratio and also complete the life cycle on its own, is likely to overcome any negative effects of heterokaryosis at certain stages of the life cycle.

Nevertheless, the possibility of nuclei to separate from the heterokaryon via conidia or monokaryotic sexual spores leaves room for conflict. Such conflict is to a large extent limited to the mating-type chromosome, because the autosomes are largely homoallelic due to the selfing history [34,36,42]. Our study shows that the homokaryons displayed a higher growth rate than their corresponding heterokaryons (figure 2), suggesting a cost for the *mat A*, and in *L1* and *L7* also for *mat a*, nuclei to grow as a heterokaryon. The finding contradicts previous reports on a reduced vigour of homokaryotic strains of *N. tetrasperma*, relative to heterokaryons [25,43], and also from expectations of separated coadapted nuclei of a dikaryon to show reduced fitness [22]. One possible explanation to our results is that comparing radial growth rate, as herein, may not show differences in vigour, but rather reflect differences in developmental programmes between heterokaryons and homokaryons. This explanation is supported by the finding of a higher growth rate of both homokaryons as compared with the heterokaryon. Further studies invoking more characters and more heterokaryotic strains from a broad range of species, growing on their natural substrates, are needed to fully explore the costs and benefits of heterokaryosis in nature.

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