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Defining individual size in the model filamentous fungus *Neurospora crassa*

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It is challenging to apply the tenets of individuality to filamentous fungi: a fungal mycelium can contain millions of genetically diverse but totipotent nuclei, each capable of founding new mycelia. Moreover, a single mycelium can potentially stretch over kilometres, and it is unlikely that its distant parts share resources or have the same fitness. Here, we directly measure how a single mycelium of the model ascomycete *Neurospora crassa* is patterned into reproductive units (RUs), meaning subpopulations of nuclei that propagate together as spores, and function as reproductive individuals. The density of RUs is sensitive to the geometry of growth; we detected 50-fold smaller RUs when mycelia had expanding frontiers than when they were constrained to grow in one direction only. RUs fragmented further when the mycelial network was perturbed. In mycelia with expanding frontiers, RU composition was strongly influenced by the distribution of genotypes early in development. Our results provide a concept of fungal individuality that is directly connected to reproductive potential, and therefore to theories of how fungal individuals adapt and evolve over time. Our data show that the size of reproductive individuals is a dynamic and environment-dependent property, even within apparently totally connected fungal mycelia.

1. Introduction

Although the individual-as-unit-of-selection is a key component of evolutionary models and experiments, there is no one-size-fits-all definition of what an individual is. In different contexts, individuals may be delimited based on their (i) genetic homogeneity, (ii) sharing of resources between the subunits, such as cells, that the organism is composed of, or (iii) possession of a separate, segregated germ-line [1]. Although paradigmatic individuals, including many animals with preformistic development, typically satisfy all three tenets, many organisms may violate one or more of them. For example, some species of plants form matrices of clones linked by rhizomes (horizontally growing stems), or unlinked clones produced by asexual seeds [2]. Levels of physiological integration between clones vary depending on species, age and plant environment: in some species, links between clones quickly break down, while in others they can persist for decades. Among physiologically linked clones, resources may be shared among all ramets or hoarded in the best clones [3,4]. Consequently, demographic studies of clonal plant communities differentiate between genetic individuals (genets), the ensemble of genetically identical clones derived from a single zygote, and physiological individuals (ramets), the clonal members that are capable of surviving or dying, independent of each other [2].

Similar to plants, fungi are capable of forming potentially enormous colonies; single mycelia have been identified stretching over kilometres [5,6], including an *Armillaria solidipes* mycelium that is thought to be the world's largest organism [7]. Where assayed, these mycelia seem to contain very little genetic diversity, and it is hypothesized that modes of division are organized to shelter a population of stem-cell-like nuclei from the effects of mutation or genetic drift, allowing the

entire mycelium to be populated with clonal nuclei [5,6]. But the amount of physiological integration, or even communication between disparate parts of these giant mycelia, is not known. Moreover, neither genet- nor ramet-based concepts of individuality can be directly mapped to fungal systems. In clonal plants, ramets are typically defined as single stems with attached root systems [2]; in a fungal mycelium, individual nuclei are totipotent [8], and any growing hypha may be capable of regenerating the entire mycelium. At the same time, mycelia can tolerate high levels of internal genetic diversity or heterokaryosis; single mycelia continuously accumulate mutations as nuclei divide, and new genomes can also be acquired, though much more rarely, by exchange of nuclei between two mycelia (reviewed in [9]). Internal genetic diversity may enhance growth on nutritionally complex substrates [10] or in variable environmental conditions [11]. Additionally, because proteins and mRNAs are pooled between nuclei [12], fungi can tolerate mutations that would be lethal at the level of single nuclei [13]. The basidiomycete fungus *Rhizoctonia solani* AG8, which harbours multiple nuclei in each cellular compartment, shows evidence of hypermutation in loci shared in common between its constituent nuclear populations [14], and it is speculated that hypermutation is associated with the ability of nuclei to compensate for deleterious mutations. Moreover, different adaptations are hypothesized to help preserve multi-genomic diversity: glomeromycete fungi create spores containing hundreds of nuclei [15], while in ascomycete fungi, multidirectional flows of nuclei [16] or cytoplasm [17] may physically mix genetically different nuclei through the entire mycelium.

The concept of the individual is a vital part of biology's modern synthesis—models of evolution posit the existence of units whose fitness can be computed and on which selective force can act. Pontecorvo proposed that in heterokaryotic fungi, nuclei themselves form the individuals, and the mycelium should be considered as a population of interacting individuals [18]. Indeed nuclear populations are capable of manifesting 'ecological' dynamics, including competitive exclusion and cyclical dominance [19]. However, atomizing mycelia into constituent nuclei is unwieldy, and misses the ability of these nuclear populations to be marshalled for mycelium-wide behaviours like directed growth and exploration [20]. The problem of identifying an individual within a genetically diverse mycelium is in many senses orthogonal to the deeply studied ramet/genet division of genetically homogeneous plant colonies into physiological individuals. However, plants also acquire somatic mutations as they grow, and genetic mosaicism has been proposed as one method by which long-lived trees may resist rapidly evolving pests [21]. Analysis of the functional effect of internal diversity in plants has been assisted by decomposition of single plants into ramet-like domains called integrated physiological units (IPUs), modules within which production and consumption of resources is sharply constrained [22,23]. IPUs are typically aligned with morphological features such as branches, or flowers and their surrounding leaves.

Much theoretical work has targeted the general question of under which general circumstances divisible entities, including fungi, mutualistic partners [24], colonial communities like ascidians [25] or social insects [26] can be regarded as single organisms. Queller & Strassmann [27] argue that the subunits function as a single organism when there is low conflict and high cooperation. Michod builds multi-level selection models to link the fitness of the high-level entity to the interaction-dependent fitnesses of their low-level units [28]. Both of these approaches require mechanistic understanding of the interactions between the low-level units. While it may be feasible to observe these interactions directly for social insects or even amoebae, nuclei within a fungal syncytium exist in a shared sea of mRNAs, and each mRNA may represent an unquantifiable interaction [12].

Here, we revisit an idea advanced by Lewontin [29] by turning the search for the individual into a search for units of selection: that is, for a group of nuclei that are transmitted intergenerationally at the same frequency, and that therefore, in principle, have a shared fitness. Although this idea forms the basis of general multi-level selection models [28], we show here that it is not necessary to model or measure the interactions between nuclei to be able to measure their tendency to reproduce together. Inspired partly by the concept of an IPU, we call these nuclear populations reproductive units (RUs). Unlike IPUs, resources need not be shared only within the nuclear members of an RU; indeed, resource translocation can occur over far longer scales than single RUs [30]. Also unlike IPUs, RUs are identified without needing to find functional or morphological substructures within the mycelium. Because nuclei within an RU propagate together as spores they have a shared fitness, making the RU a natural concept of individuality for studies of fungal evolution.

To measure the number of nuclei reproducing together, we grow heterokaryotic *Neurospora crassa* mycelia containing different fluorescently labelled (*hH1::gfp* and *hH1::DsRed*) nuclei [17]. Figure 1*a* produces a schematic of how we analyse the nuclear diversity of spores to identify RUs. Our method makes use of the statistical isotropy of the mycelium; that is, it assumes that points in the mycelium at the same age (i.e. the same distance from the points where the mycelium was inoculated) will contain a similar number of RUs. Atwood & Mukai [31] showed that multikaryotic spores have less genetic diversity than would be expected if the nuclei present within the mycelium were randomly assorted into spores, suggesting that the nuclear contents of spores are drawn from smaller populations than the entire mycelium. We use the diversity deficit between spores and the patch of mycelium from which they are taken to calculate the number of spore-producing populations in the patch of mycelium. Specifically, we identify the diversity of a population of nuclei by its heterozygosity (also called Gini-Simpson Index), viz. the probability that two randomly selected nuclei have different genotypes (i.e. one nucleus is *hH1::gfp* and the other is *hH1::DsRed*). For each sampled patch, we compute two different heterozygosity values: (1) the heterozygosity of a single spore; and (2) the heterozygosity of all nuclei isolated from the patch, including nuclei isolated from different spores. (1), which we call the spore heterozygosity, measures the diversity of the RU from which the spore originated, while (2), which we call the sample heterozygosity, measures the diversity of all nuclei present within the patch of mycelium. In §2c, we derive a method for estimating the number of RUs present based on the difference between (1) and (2).

Our data show that the patterning of the mycelium into RUs depends on whether the mycelium grows outwards in multiple directions or whether it is only allowed to grow in one direction. It also changes when the mycelium is exposed to chemical or environmental stress. At the same time, parts of the mycelium with different ages show essentially the same patterning.

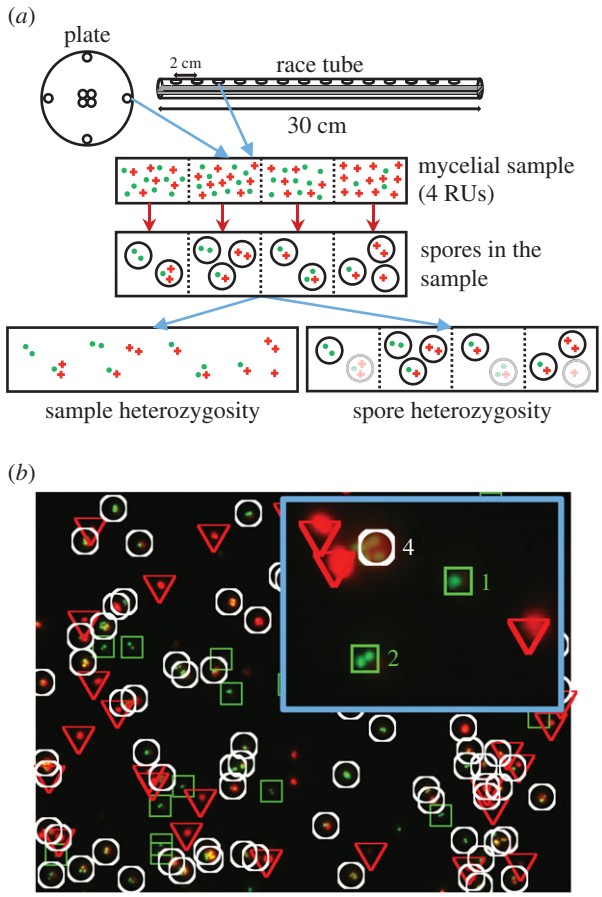

Figure 1. (*a*) Schematic of sampling method, and the biological interpretation of sample and spore heterozygosities for a mycelium containing *hH1::DsRed* nuclei (red pluses) and *hH1::gfp* nuclei (green dots). The sample heterozygosity *H* measures the diversity of nuclear genotypes in the sample, and the spore heterozygosity *h* the diversity of dikaryotic spores. (*b*) Classification of real spores by PerkinsCS. Spores are identified by template matching in transmitted light images then classified as either *hH1::DsRed* homokaryons (white circles), *hH1::gfp* homokaryons (green squares) or heterokaryons (white squares). In the magnified image, the number of nuclei is also shown. (Online version in colour.)

2. Material and methods

(a) Mycelial preparation and analysis

Heterokaryotic *N. crassa* mycelia were started from mixtures containing equal numbers of homokaryotic *hH1::gfp* and *hH1:DsRed* spores; spores from the two strains freely fused with each other to create a heterokaryotic mycelium. To prepare homokaryotic spore suspensions, 7–10-day-old cultures were washed and the resulting suspension was filtered to separate spores from hyphal fragments. To create mixtures with equal numbers of each spore type, we developed custom spore counting software to count thousands of spores (see §2b). The two homokaryotic strains were built by ectopic insertion of transformed DNA into the same background strain, as described in [17]. The two homokaryotic cultures had similar rates of growth: the radial growth rate is 0.199 ± 0.006 cm h^{-1} for *hH1::gfp* and 0.233 ± 0.037 cm h^{-1} for *hH1:DsRed* ($p > 32\%$ against different means by two-sample *t*-test).

Mycelia were grown in two different geometries: (i) *plates*, in which spores were inoculated in the centre of a Petri plate and mycelia grew radially outward; and (ii) *race tubes*, in which spores were inoculated at one end of a long polycarbonate tube and mycelia grew in one direction along the tube.

(i) Plates

Ten-centimetre Petri dishes were prepared containing Vogel's minimal media (1.5% sucrose, 1.5% agar wt/vol) [32], and inoculated with 3 µl of mixed spore suspension at the plate centre. They were then grown at 25°C under conditions of constant, weak illumination. After 5–7 days of growth, we sampled spore composition both around the edge of the plate and at the plate centre by punching out 4–8 spots of agar with a standard 0.5 cm diameter drinking straw (figure 1*a*). Agar spots were vortexed with 250 µl of de-ionized (DI) water to remove spores and passed through a filter tip to remove agar and mycelial fragments. To increase spore concentration the suspension was spun down in a microcentrifuge for 5 min, clear fluid was removed and the spore pellet was then resuspended. We imaged spore suspensions at 260× magnification using a Zeiss AxioZoom microscope with DsRed and E-GFP filter-sets, as well using transmitted light, photographing at least 1000 spores per sampling spot. We analysed the images to compute the spore and sample diversities of each sampling spot using a custom image analysis algorithm, PerkinsCS (see §2b and the electronic supplementary material).

(ii) Race tubes

We constructed race tubes [33] by pre-drilling 1.5 × 30 cm clear polycarbonate tubes with 5 mm diameter sampling ports spaced 2 cm apart (figure 1*a*). After autoclaving, the sampling ports were sealed with sterile cotton wool, and 25 ml of Vogel's minimal media was poured into each race tube. Mycelia took approximately 10 days to grow and conidiate along the entire 30 cm sampling length. We prepared race tubes with three different experimental conditions: minimal media, desiccation stress and using 1% L-sorbose instead of sucrose as a carbon source. The desiccation stress race tubes were grown for 17 rather than 10 days, to dry the agar. Because of their slower growth rate, sorbose race tubes were 6 cm long rather than 30 cm long, and sampling ports were spaced 1 cm apart.

(b) Image analysis

Each spore field was imaged using transmitted light, DsRed and GFP fluorescence channels. We used template matching in the transmitted light image to identify spores, and in the GFP image to identify any GFP-containing nuclei within the spores. We thresholded the DsRed image to identify DsRed-containing spores. Because fluorescently tagged histones are translated within the cytoplasm, they are freely exchanged between nuclei [12]. Accordingly, in heterokaryotic spores, nuclei contain both types of labelled histones, independent of their genotype. The first step in our image analysis algorithm is to classify spores as GFP (homokaryotic), DsRed (homokaryotic) or heterokaryotic. For the GFP and heterokaryotic spores in which nuclei could be clearly counted, we further divided spores by the number of nuclei that they contain. We found DsRed label typically did not remain localized in nuclei and diffused through the entire of the spore, making it impossible to count nuclei within a homokaryotic DsRed spore. The software that we wrote to analyse images, which we call PerkinsCS, is described in detail in the electronic supplementary material.

To calculate spore and sample heterozygosities, we use maximum-likelihood estimation to compute the following parameters: $\{p_K\}$, the probability that a randomly chosen spore contains K nuclei for $K = 1$, 2 or 3, and p, χ and λ, the probabilities that a spore is homokaryotic DsRed given that it has a total of 1, 2 or 3 nuclei, respectively. We do not fit data for spores with four or more nuclei. These spores make up fewer than 8% of the spores that were imaged. In general, we can count the number of nuclei in any spore that contains at least one *hH1::gfp* nucleus; spores containing four or more nuclei and at least one *hH1::gfp* nucleus can be filtered from the dataset on which fitting is performed. However,

homokaryotic *hH1::DsRed* spores with four or more nuclei could not be filtered out of our fitted data. However, as most RUs have close to equal proportions of each genotype, the fraction of spores wrongly left in the fitted data can be estimated *a priori* to be less than $8\% \times (1/2)^4 = 0.5\%$, creating a negligible source of error for our parameter estimates. The parameter fitting method is described in more detail in the electronic supplementary material.

(c) Population model

The key idea for measuring the number of RUs present in a sample is shown in figure 1*a*. Although single RUs may have very different proportions of *hH1::DsRed* nuclei, if a sampled patch of mycelium contains multiple RUs, the fluctuations in proportions of *hH1::DsRed* nuclei will tend to average out between different RUs; so fluctuations in diversity between different sampled patches will tend to be smaller than fluctuations between different spores. Here, we develop a mathematical framework for calculating the number of RUs in each sample by comparing the sizes of the two fluctuations.

Suppose that the sample contains an unknown number, N, of RUs. Assume that a proportion p_i of the nuclei in the ith RU have the *hH1::DsRed* genotype. We assume that the random variables p_i are independent and identically distributed (i.i.d.) but no assumption is made about their distribution, except to define the first two moments of the distribution $\mathbb{E}[p_i] = \bar{p}$ and $\mathbb{E}[p_i^2] = \overline{p^2}$.

Suppose that our sample includes M mononucleate spores. Let X_i be the number of mononucleate spores from the ith RU that are *hH1::DsRed* nuclei: To simplify computations, assume that each of the N RUs are equally sampled. Relaxing the assumption of equal sampling has a provably small effect on the estimate for N (see appendix A).

We can calculate the expectation and variance of our estimator for the proportion of *hH1::DsRed* nuclei:

$$\mathbb{E}[\hat{p}] = \frac{1}{M}\sum_{i=1}^{N}\mathbb{E}[X_i] = \frac{1}{M}\sum_{i=1}^{N}\mathbb{E}[\mathbb{E}[X_i|\,p_i]] = \frac{1}{M}\sum_{i=1}^{N}\mathbb{E}\left[\frac{Mp_i}{N}\right] = \bar{p}, \tag{2.1}$$

where we have used the law of total expectation to expand $\mathbb{E}[X_i]$ in terms of conditional expectations. For example, the random variable $\mathbb{E}[X_i|\,p_i]$ is the conditional expectation of X_i given the value of p_i. Similarly, by the law of total variance,

$$\begin{aligned}\mathrm{Var}[\hat{p}] &= \frac{1}{M^2}\sum_{i=1}^{N}\mathrm{Var}[X_i] = \frac{1}{M^2}\sum_{i=1}^{N}(\mathbb{E}[\mathrm{Var}[X_i|\,p_i]] + \mathrm{Var}[\mathbb{E}[X_i|\,p_i]])\\ &= \frac{1}{M^2}\sum_{i=1}^{N}\left(\mathbb{E}\left[\frac{M}{N}p_i(1-p_i)\right] + \mathrm{Var}\left[\frac{M}{N}p_i\right]\right)\\ &= \frac{1}{N^2}\sum_{i=1}^{N}\mathrm{Var}[p_i] = \frac{1}{N}(\overline{p^2} - \bar{p}^2).\end{aligned} \tag{2.2}$$

As noted in the Introduction, we compute the measures of diversity. First, we define the sample heterozygosity, H, to be the probability that two nuclei, selected randomly and independently from the sampled area of mycelium (including from different RUs), have different genotypes: $\mathbb{E}[H] = 2\bar{p}(1-\bar{p})$. Second, we define the spore heterozygosity, h, to be the probability that a randomly chosen dikaryotic spore is heterokaryotic. As the nuclei that populate a single spore are drawn from the same RU, h measures the diversity within individual RUs: $\mathbb{E}[h] = 2(\bar{p} - \overline{p^2})$. A schematic of the two different concepts of heterozygosity is shown in figure 1*a*. $\mathbb{E}[H]$, $\mathbb{E}[h]$ and $\mathrm{Var}[\hat{p}]$ can all be measured experimentally: $\mathbb{E}[h]$ is computed from the fluctuations in diversity of spores measured from a single sampled area of mycelium, and $\mathrm{Var}[p]$ and $\mathbb{E}[H]$ are computed from the fluctuations of diversity between different sampled patches. We can combine equations (2.1) and (2.2) to estimate the number of RUs, N:

$$N = \frac{\overline{p^2} - \bar{p}^2}{\mathrm{Var}[\hat{p}]} = \frac{\mathbb{E}[H] - \mathbb{E}[h]}{2\mathrm{Var}[\hat{p}]}. \tag{2.3}$$

The expression on the right-hand side of the above equation is proportional to the fixation index (F_{ST}) used to detect subpopulation structure in populations of sexual organisms [34]. When cast in terms of spore and sample heterozygosities, the F_{ST} is defined to be $F_{ST} = (\mathbb{E}[H] - \mathbb{E}[h])/\mathbb{E}[H]$. Thus, a large number of RUs will typically be associated with a high F_{ST} value, which concords with how F_{ST} is interpreted as an indicator of whether a sexual population contains inbreeding subpopulations (analogous to the existence of RUs in the pool of asexually dividing nuclei).

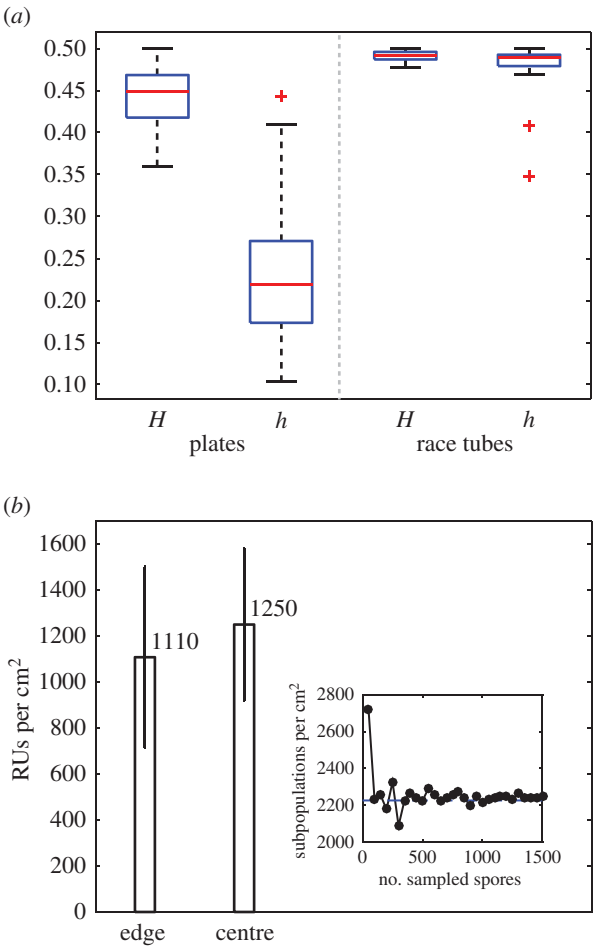

Figure 2. (*a*) In mycelia grown in plates, sample heterozygosity (H) is systematically larger than spore heterozygosity (h), allowing us to measure the number of RUs present. H and h are indistinguishable in mycelia with one-dimensional growth. (*b*) Densities of RUs in the plates (no. RUs per cm^2) do not depend on sampling location. (Inset) RU density estimates as a function of number of spores counted—convergence occurs before 1000 spores (our experimental sample size). (Online version in colour.)

3. Results

Consistent with previous experiments [17,35], sample heterozygosities varied little between replicate mycelia, and matched the heterozygosity of the initial inoculum (figure 2*a*). *hH1::DsRed* nuclei are therefore present in the same overall proportions in every 0.2 cm^2 patch of the mycelium. However, in Petri dishes spore heterozygosity h was consistently less

than the sample heterozygosity. In fact, dikaryotic spores were approximately 2 times more likely to contain nuclei of the same genotype than would be expected by chance, though there was no significant difference between the two heterozygosities for mycelia grown in race tubes (figure 2*a*).

Differences between spore and sample diversity are consistent with the presence of subpopulations of nuclei that reproduce together (see §2c). We identify these subpopulations as RUs. We can compute the number of such subpopulations using equation (2.3). From the formula, we find approximately 1250 RUs per cm^2 for mycelia grown in Petri plates (figure 2*b*). To check that we have sufficient spores in our sample to estimate the number of RUs, we increased the number (M) of spores sampled, and found that the estimate for the number of subpopulations converged long before we reach the minimum sample size, $M = 1000$ (figure 2, inset panel).

To determine whether nuclei segregated before or during the production of aerial hyphae, we compared spore heterozygosity between mycelia grown in race tubes and in plates. We found no significant difference between aerial hyphae length or density in plates and in race tubes (length of aerial hyphae: 6.2 ± 0.9 mm versus 7.1 ± 1.0 mm, nuclei density: $2.39 \times 10^8 \pm 0.56 \times 10^8$ nuclei per cm^2 versus $1.67 \times 10^8 \pm 0.53 \times 10^8$ per cm^2, $p > 40\%$ against different means by two-sample t-test). Thus, if the mycelia below the aerial hyphae were well mixed and nuclei only segregated into RUs during their passage through aerial hyphae and into spores, we would expect to detect as many RUs for mycelia grown in race tubes as in plates, because nuclei travel the same distance and through the same number of aerial hyphae in both cases. However, we detected a much higher density of RUs for mycelia grown in plates ($1250\ cm^{-2}$) than for mycelia grown in race tubes ($25\ cm^{-2}$). It follows that RUs exist within the growing mycelia and are not created during sporulation.

In plates, RUs do not extend more than 0.5 cm. We increased the area of the sample by using the punch to remove touching circles. We found that the number, N, of RUs in the sample increased in direct proportion to the sample area (i.e. that the estimate for the number of RUs per unit area was very similar whether one punch or two was removed: $333\ cm^{-2}$ versus $316\ cm^{-2}$). If the region of mycelium occupied by an RU extended beyond the linear dimensions of the punch, we would expect the single-punch samples and double-punch samples to have RUs in common (i.e. the detected number of RUs would not increase in proportion to sample area). Our data show that each RU is completely contained within the punch area. By measuring the number of nuclei in single-punch samples, we estimated that an RU contains $14.2 \times 10^4 \pm 7.5 \times 10^4$ (mean $\pm$ s.d.) nuclei, corresponding to the linear length of 0.93 ± 0.49 m of hyphae (based on 1.5 nuclei per 10 μm of hyphae [17]).

The densities of RUs in the centre and at the edge of the plates were statistically indistinguishable (figure 2*b*): $1250 \pm 390\ cm^{-2}$ for samples taken from the centre of the plate, $1110 \pm 330\ cm^{-2}$ for samples taken along the plate edge ($p > 80\%$ against different means by two-sample t-test). Taken together with the data above, that each RU extends over no more than 0.5 cm of mycelium, these data suggest that the RU structure of the mycelium does not represent the breakdown of the mycelium into genetically homogeneous sectors, because the width of sectors would increase (i.e. density of sectors would decrease) with distance grown [36].

Micropatterning of mycelium into RUs depends on the geometry of mycelial growth. As already noted, when the mycelium was constrained to grow in only one direction, along a race tube, we found a much lower density of RUs than when the mycelia had an expanding periphery (i.e. was grown in a plate). In race tubes, we detected between 5 and 36 RUs per cm^2, and the density of RUs was not dependent on the distance the mycelium had grown along the race tubes (see the electronic supplementary material). Physical mixing of nuclei is known in *Neurospora* to maintain genetic diversity down to the scale of individual hyphae [17]. However, data from colonies of unicellular microbes show that the physical mixing (for microbial colonies, this mixing takes the form of local rearrangements between cells) is stronger in colonies that grew in one dimension than in colonies that grew radially in two dimensions, because on the expanding frontier of a radially growing colony patches of different cells become further apart as the colony expands [37].

We tested whether RUs maintained a memory of the initial distribution of genotypes by starting mycelia from mixed and unmixed spores. Specifically, rather than inoculate with a single well-mixed suspension of *hH1::gfp* and *hH1::DsRed* spores, we started the mycelium from two spots containing homokaryotic suspensions of *hH1::gfp* and *hH1::DsRed* spores, respectively. Although spores could freely fuse, even when the spots were placed directly on top of each other, spores of the same genotype were more likely to be grouped together in the inoculum. In mycelia with radial growth, these patches of genetically homogeneous nuclei persisted as RUs throughout growth: the RUs detected at the edge of the plate were typically much less genetically diverse than in mycelia founded by well-mixed spores (figure 3*b*). In mycelia with one-dimensional growth unmixed spores still produced genetically diverse RUs (figure 3*b* and inset).

Hypothesizing that physical mixing of nuclei affected the fragmentation of the mycelium into RUs, we perturbed the hyphal network to alter the amount of physical mixing. Specifically, for a mycelium with one-dimensional growth, we (i) replaced sucrose with sorbose as a carbon source or (ii) desiccated the substrate by growing the mycelium for an extra 7 days (see Material and methods). Previous experiments have shown that sorbose alters hyphal network geometry, producing hyphal networks with denser branching and fewer fusion points [38]. We observed that hyphal death on highly desiccated agar produces sparsely connected hyphal networks. Because nuclear mixing is the strongest in highly connected networks [17], we hypothesized that reducing network connectivity would create smaller RUs within mycelia with one-dimensional growth. Indeed, we detected 40% more RUs in desiccated agar and five times more on sorbose-supplemented media (figure 3*a*).

4. Discussion

We have shown that the nuclei within the growing filamentous fungus *N. crassa* reproduce together as reproduction units (RUs), with potentially more than 1000 RUs in a single square centimetre of fungus. Physical mixing of nuclei, already known to be a key component of the mycelium's ability to maintain genetic diversity at the scale of single hyphae, controls the density of RUs. In particular, when mycelia are grown in one dimension, RUs are much larger than when the

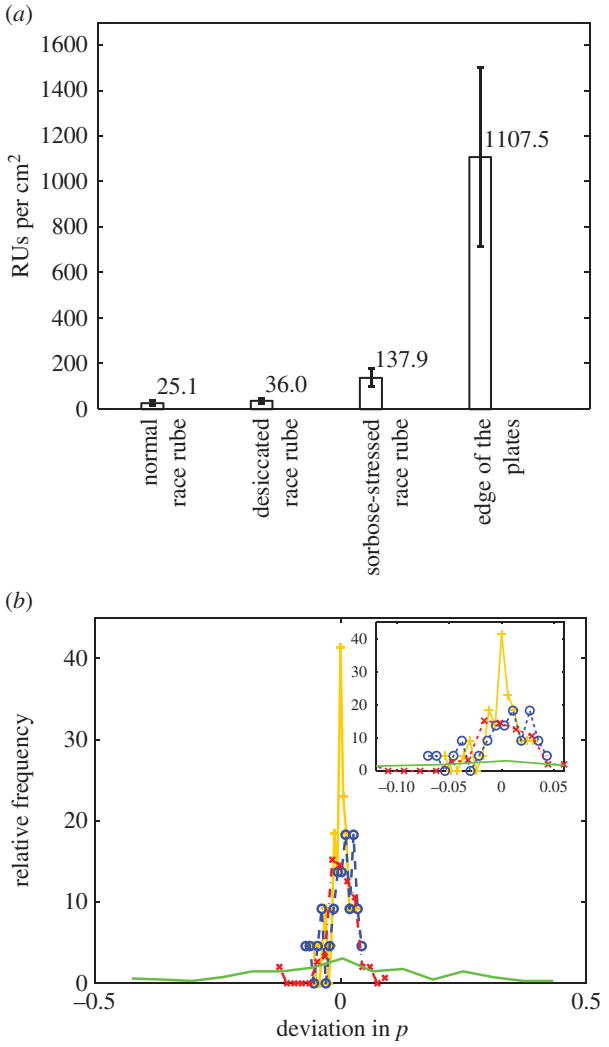

Figure 3. (*a*) RU formation depends on physical mixing within the mycelium: mycelia with a growing frontier had more RUs than mycelia constrained to grow in one dimension. Perturbing network connectivity using sorbose or dessication stress also increased the number of RUs. (*b*) Starting mycelia with unmixed spores tests for whether RUs are constituted early in mycelial development. In plates, unmixed spores produce unmixed RUs (green solid line, unmixed spores; red dash-dot line with × symbols, mixed spores) but RUs in race tubes remained uniformly diverse (orange solid line with + symbols, unmixed spores; blue dashed line with O symbols, mixed spores). Here we measure RU diversity by plotting fluctuations in p (fraction of *hH1::DsRed* nuclei) across RUs. Inset shows the same data over a smaller interval of p fluctuations. (Online version in colour.)

mycelium is grown radially (i.e. has an expanding frontier), in line with previous data showing that mixing of cells in colonies is strongest during one-dimensional growth [37]. Similarly, when mixing was disrupted by stressing the mycelium in ways known to alter network connectivity, RUs become smaller. Both modes of growth are seen in nature; filamentous fungi may be constrained to grow in one dimension as they grow through the roots or stems of a host, or form linked two- or three-dimensional networks as they forage across the forest floor. In particular, the largest fungal mycelia probably form essentially two-dimensional hyphal networks.

We have found no evidence, so far, of competitive dynamics between different RUs; indeed there is likely to be extensive resource sharing as well as nuclear exchange between RUs. Different RUs contain exactly the same two types of nuclei, albeit in different proportions, and so would not be found by their genetic signatures alone—in other words, the RU structure exists independently of genetic differences between nuclei. Moreover, as a single nucleus reproduces only with the nuclei in the same RU, different RUs could potentially have different fitness. On this basis, it is natural to identify these RUs as different individuals.

Are RUs constituted early in mycelial development, or at each stage of mycelial growth? We found that starting mycelia from unmixed spores produced less diverse RUs in mycelia with radial growth, but RUs remained genetically diverse in mycelia with one-dimensional growth. We hypothesize, based on these data, that RUs are constituted when spores first germinated and began to fuse. Mixing within the hyphal network can alter RU populations as the mycelia grows, but only effectively in the well-connected networks associated with one-dimensional growth.

Nucleotypes in this study differed only by a fluorescent protein, and have the same rate of growth when grown independently as homokaryotic mycelia, thus nuclei associate into RUs even in the absence of fitness differences between genotypes. However, in nature, mycelia can harbour nuclei with functionally different genomes and different intrinsic division rates [35], and these nuclei can form complex ecosystems [19]. Previous work has shown that even when nuclei have different division rates when grown separately from each other, they can be maintained in stable (though not equal) proportions in heterokaryotic mycelium, when large (approx. 1 cm) patches of mycelium are measured [35]. Our data reveal an important length scale on which interactions must be considered—within RUs of around 10^5 nuclei—and we plan to revisit classic studies of inter-genomic interactions [19] to see how RUs are constituted in the presence of fitness differences or antagonistic interactions between nuclei.

Grosberg & Strathmann [39] have hypothesized that colonial organisms capable of harbouring internal genetic variation must incorporate a unicellular or spore stage in their life history. The syncytial nature of the fungal mycelium means that nuclei carrying deleterious mutations are protected from these mutations because of the availability of wild-type proteins created by the other nuclei within the syncytium [9,12,19]. By contrast, spores will be able to found new mycelia only if they contain a full complement of proteins, creating a bottleneck that prevents deleterious mutations from being transmitted to new mycelia. In *N. crassa*, most spores contain more than one nucleus. In heterokaryotic mycelia containing two different nucleotypes and grown with an expanding frontier, fewer spores than would be expected by chance are genetically diverse, so that about 80% of the spores produced by mycelia grown on plates are homokaryotic and would function in the way proposed by Grosberg & Strathmann [39]. However, fewer than 50% of the spores produced by mycelia grown in one dimension were homokaryotic. Previous studies on glomeromycete fungi have shown that fungal spores can carry genetic diversity [40,41], but have tended to focus on this property in absolute terms—that a single species may always create diverse spores or otherwise. Heterokaryosis may carry adaptive benefits for fungi [9,10], but there are also trade-offs from the potential for inter-genomic conflict and from the ability of deleterious mutations to persist in a mycelium. Our data show that spore diversity can be controlled by altering RU density. Dynamic control of RU density may allow mycelia to reweight the importance of

producing heterokaryotic spores against the trade-offs of doing so, depending on the environment in which the mycelium grows.

Data accessibility. Data are available in the electronic supplementary material.

Authors' contributions. L.M., B.S., T.C., E.D. and M.R. designed the research. L.M., B.S., T.C., N.P. and M.R. performed the research. L.M., B.S. and M.R. wrote the paper.

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Appendix A. Population model for unequally sampled reproductive units

In general, our spore sample will not include identical numbers of spores from each RU. Variance in sampling between the different populations alters, but only slightly, estimates of the number N of RUs contained within the sample. Specifically, if we again let M be the total number of spores in this sample, and each of the N RUs is sampled with equal probability $1/N$, then the number of spores taken from the ith RU will have a multinomial distribution $M_i \sim \text{Multinomial}(M, 1/N)$. Following the derivation in §2c, we assume that the number of DsRed monokaryotic spores from the ith RU is still binomially distributed: $X_i \sim \text{Bin}(M_i, p_i)$, except that now the number of spores from this RU is itself a random variable that must be conditioned on. We can compute the conditional expectation of X_i given M_i and p_i by $\mathbb{E}[X_i|M_i, p_i] = M_i p_i$. Our estimator for the proportion of *hH1::DsRed* nuclei within the sample remains as: $\hat{p} = (1/M)\sum_{i=1}^{N} X_i$. To compute the mean and variance of $\hat{p}$, we apply the law of total expectation:

$$\mathbb{E}[\hat{p}] = \frac{1}{M}\sum_{i=1}^{N}\mathbb{E}[X_i] = \frac{1}{M}\sum_{i=1}^{N}\mathbb{E}[\mathbb{E}[X_i|M_i, p_i]] = \frac{1}{M}\sum_{i=1}^{N}\mathbb{E}[M_i p_i] = \frac{1}{M}\sum_{i=1}^{N}\frac{M}{N}\bar{p} = \bar{p} \tag{A 1}$$

and

$$\mathbb{E}[\hat{p}^2] = \frac{1}{M^2}\sum_{\text{i,j}}^{N}\mathbb{E}[X_i X_j] = \frac{1}{M}\left(\sum_{i=1}^{N}\mathbb{E}[X_i^2] + \sum_{i\neq j}^{N}\mathbb{E}[X_i X_j]\right). \tag{A 2}$$

We compute the summands separately. Since $\mathbb{P}(X_i|M_i, p_i) = \binom{M_i}{X_i} p_i^{X_i}(1-p_i)^{M_i - X_i}$,

$$\mathbb{E}[X_i^2|M_i, p_i] = \text{Var}(X_i|M_i, p_i) + (\mathbb{E}[X_i|M_i, p_i])^2 = M_i p_i(1-p_i) + M_i^2 p_i^2.$$

Thus by the law of total expectation, the first summand may be written as follows:

$$\mathbb{E}[X_i^2] = \mathbb{E}[\mathbb{E}[X_i|M_i, p_i]] = \mathbb{E}[M_i p_i(1-p_i) + M_i^2 p_i^2] = \frac{M}{N}(\bar{p} - \overline{p^2}) + \mathbb{E}[M_i^2]\overline{p^2} = \frac{M}{N}(\bar{p} - \overline{p^2}) + \left[\frac{M}{N}\left(1 - \frac{1}{N}\right) + \frac{M^2}{N^2}\right]\overline{p^2}. \tag{A 3}$$

Similarly, as $\mathbb{E}[X_i X_j|\{M_k, p_k\}] = M_i p_i M_j p_j$ and M_i and p_j are independent, the law of total expectation gives for $i \neq j$

$$\mathbb{E}[X_i X_j] = \mathbb{E}[\mathbb{E}[X_i X_j|\{M_k, p_k\}]] = \mathbb{E}[M_i M_j]\mathbb{E}[p_i]\mathbb{E}[p_j] = \mathbb{E}[M_i M_j]\bar{p}^2 = \left(\frac{-M}{N^2} + \frac{M^2}{N^2}\right)\bar{p}^2. \tag{A 4}$$

Substituting equations (A 3) and (A 4) into (A 2), we get

$$\mathbb{E}[\hat{p}^2] = \frac{1}{M}\bar{p} + \frac{M-1}{MN}\overline{p^2} + \frac{(N-1)(M-1)}{MN}\bar{p}^2. \tag{A 5}$$

Therefore,

$$\text{Var}(\hat{p}) = \mathbb{E}[\hat{p}^2] - \mathbb{E}[\hat{p}]^2 = \frac{1}{M}\bar{p} + \frac{M-1}{MN}\overline{p^2} + \frac{(N-1)(M-1) - MN}{MN}\bar{p}^2 = \frac{1}{N}\text{Var}(p_i) + \frac{1}{M}\left\{\bar{p} + \left(1 - \frac{1}{N}\right)\text{Var}(p_i)\right\}. \tag{A 6}$$

The above equation shows that the error we introduce by assuming that all the RUs are equally sampled is of the order of $1/M$, and is therefore negligible for the samples ($M > 1000$) in our experiments.

References

1. Buss LW. 1987 *The origin of individuality*. Princeton, NJ: Princeton University Press.
2. Cook RE. 1983 Clonal plant populations: a knowledge of clonal structure can affect the interpretation of data in a broad range of ecological and evolutionary studies. *Am. Sci.* **71**, 244–253.
3. Alpert P, Mooney H. 1986 Resource sharing among ramets in the clonal herb, *Fragaria chiloensis*. *Oecologia* **70**, 227–233. (doi:10.1007/BF00379244)
4. Alpert P. 1999 Clonal integration in *Fragaria chiloensis* differs between populations: ramets from grassland are selfish. *Oecologia* **120**, 69–76. (doi:10.1007/s004420050834)
5. Aanen DK. 2014 How a long-lived fungus keeps mutations in check. *Science* **346**, 922–923. (doi:10.1126/science.1261401)
6. Anderson JB, Catona S. 2014 Genomewide mutation dynamic within a long-lived individual of *Armillaria gallica*. *Mycologia* **106**, 642–648. (doi:10.3852/13-367)

7. Ferguson B, Dreisbach T, Parks C, Filip G, Schmitt C. 2003 Coarse-scale population structure of pathogenic *Armillaria species* in a mixed-conifer forest in the Blue Mountains of northeast Oregon. *Can. J. Forest Res*. **33**, 612–623. (doi:10.1139/x03-065)
8. Money N. 2002 Mushroom stem cells. *Bioessays* **24**, 949–952. (doi:10.1002/bies.10160)
9. Roper M, Ellison C, Taylor JW, Glass NL. 2011 Nuclear and genome dynamics in multinucleate ascomycete fungi. *Curr. Biol*. **21**, R786–R793. (doi:10.1016/j.cub.2011.06.042)
10. Jinks JL. 1952 Heterokaryosis: a system of adaptation in wild fungi. *Proc. R. Soc. Lond. B* **140**, 83–99. (doi:10.1098/rspb.1952.0046)
11. Schoustra SE, Debets AJ, Slakhorst M, Hoekstra RF. 2007 Mitotic recombination accelerates adaptation in the fungus *Aspergillus nidulans*. *PLoS Genet*. **3**, e68. (doi:10.1371/journal.pgen.0030068)
12. Roper M, Lee C, Hickey PC, Gladfelter AS. 2015 Life as a moving fluid: fate of cytoplasmic macromolecules in dynamic fungal syncytia. *Curr. Opin. Microbiol*. **26**, 116–122.
13. Maheshwari R, Navaraj A. 2008 Senescence in fungi: the view from neurospora. *FEMS Microbiol. Lett*. **280**, 135–143. (doi:10.1111/j.1574-6968.2007.01027.x)
14. Hane JK, Anderson JP, Williams AH, Sperschneider J, Singh KB. 2014 Genome sequencing and comparative genomics of the broad host-range pathogen *Rhizoctonia solani* AG8. *PLOS Genet*. **10**, e1004281. (doi:10.1371/journal.pgen.1004281)
15. Jany J-L, Pawlowska TE. 2010 Multinucleate spores contribute to evolutionary longevity of asexual glomeromycota. *Am. Nat*. **175**, 424–435. (doi:10.1086/650725)
16. Gladfelter A, Berman J. 2009 Dancing genomes: fungal nuclear positioning. *Nat. Rev. Microbiol*. **7**, 875–886. (doi:10.1038/nrmicro2249)
17. Roper M, Simonin A, Hickey P, Leeder A, Glass N. 2013 Nuclear dynamics in a fungal chimera. *Proc. Natl Acad. Sci. USA* **110**, 12 875–12 880. (doi:10.1073/pnas.1220842110)
18. Pontecorvo G. 1946 Genetic systems based on heterocaryosis. *Cold Spring Harb. Sym*. **11**, 193–201. (doi:10.1101/SQB.1946.011.01.021)
19. Maheshwari R. 2005 Nuclear behavior in fungal hyphae. *FEMS Microbiol. Lett*. **249**, 7–14. (doi:10.1016/j.femsle.2005.06.031)
20. Rayner AD. 1991 The challenge of the individualistic mycelium. *Mycologia* **83**, 48–71. (doi:10.2307/3759832)
21. Gill DE, Chao L, Perkins SL, Wolf JB. 1995 Genetic mosaicism in plants and clonal animals. *Annu. Rev. Ecol. Syst*. **26**, 423–444. (doi:10.1146/annurev.es.26.110195.002231)
22. Watson MA. 1986 Integrated physiological units in plants. *Trends Ecol. Evol*. **1**, 119–123. (doi:10.1016/0169-5347(86)90005-4)
23. Hardwick R. 1986 Physiological consequences of modular growth in plants. *Phil. Trans. R. Soc. B* **313**, 161–173. (doi:10.1098/rstb.1986.0031)
24. Wilson D, Sober E. 1989 Reviving the superorganism. *J. Theor. Biol* **136**, 337–356. (doi:10.1016/S0022-5193(89)80169-9)
25. Zeng L, Jacobs M, Swalla B. 2006 Coloniality has evolved once in stolidobranch ascidians. *Integr. Comp. Biol*. **46**, 255–268. (doi:10.1093/icb/icj035)
26. Hölldobler B, Wilson E. 2009 *The superorganism: the beauty, elegance, and strangeness of insect societies*. New York, NY: W.W. Norton and Company.
27. Queller DC, Strassmann JE. 2009 Beyond society: the evolution of organismality. *Phil. Trans. R. Soc. B* **364**, 3143–3155. (doi:10.1098/rstb.2009.0095)
28. Michod RE. 1997 Evolution of the individual. *Am. Nat*. **150**, S5–S21. (doi:10.1086/286047)
29. Lewontin RC. 1970 The units of selection. *Annu. Rev. Ecol. Syst*. **1**, 1–18. (doi:10.1146/annurev.es.01.110170.000245)
30. Simonin A, Palma-Guerrero J, Fricker M, Glass N. 2012 Physiological significance of network organization in fungi. *Eukaryot. Cell* **11**, 1345–1352. (doi:10.1128/EC.00213-12)
31. Atwood K, Mukai F. 1955 Nuclear distribution in conidia of *Neurospora* heterokaryons. *Genetics* **40**, 438.
32. Vogel H. 1956 A convenient growth medium for *Neurospora* (medium N). *Microb. Genet. Bull* **13**, 2–43.
33. Ryan FJ, Beadle G, Tatum E. 1943 The tube method of measuring the growth rate of *Neurospora*. *Am. J. Bot*. **30**, 784–799. (doi:10.2307/2437554)
34. Hartl DL. 1988 *A primer of population genetics*. Sunderland, MA: Sinauer Associates, Inc.
35. Samils N, Oliva J, Johannesson H. 2014 Nuclear interactions in a heterokaryon: insight from the model *Neurospora tetrasperma*. *Proc. R. Soc. B* **281**, 20140084. (doi:10.1098/rspb.2014.0084)
36. Pontecorvo G, Gemmell A. 1944 Colonies of *Penicilium notatum* and other moulds as models for the study of population genetics. *Nature* **154**, 532–534. (doi:10.1038/154532a0)
37. Korolev K, Müller M, Karahan N, Murray A, Hallatschek O, Nelson D. 2012 Selective sweeps in growing microbial colonies. *Phys. Biol*. **9**, 026008. (doi:10.1088/1478-3975/9/2/026008)
38. Trinci A, Collinge A. 1973 Influence of L-sorbose on the growth and morphology of *Neurospora crassa*. *J. Gen. Microbiol*. **78**, 179–192. (doi:10.1099/00221287-78-1-179)
39. Grosberg RK, Strathmann RR. 2007 The evolution of multicellularity: a minor major transition? *Annu. Rev. Ecol. Evol. Sci*. **38**, 621–654. (doi:10.1146/annurev.ecolsys.36.102403.114735)
40. Kuhn G, Hijri M, Sanders IR. 2001 Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. *Nature* **414**, 745–748. (doi:10.1038/414745a)
41. Pawlowska TE, Taylor JW. 2004 Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. *Nature* **427**, 733–737. (doi:10.1038/nature02290)