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REVIEW

Longevity of Fungal Mycelia and Nuclear Quality Checks: a New Hypothesis for the Role of Clamp Connections in Dikaryons

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SUMMARY This paper addresses the stability of mycelial growth in fungi and differences between ascomycetes and basidiomycetes. Starting with general evolutionary theories of multicellularity and the role of sex, we then discuss individuality in fungi. Recent research has demonstrated the deleterious consequences of nucleus-level selection in fungal mycelia, favoring cheaters with a nucleus-level benefit during spore formation but a negative effect on mycelium-level fitness. Cheaters appear to generally be loss-of-fusion (LOF) mutants, with a higher propensity to form aerial hyphae developing into asexual spores. Since LOF mutants rely on heterokaryosis with wild-type nuclei, we argue that regular single-spore bottlenecks can efficiently select against such cheater mutants. We then zoom in on ecological differences between ascomycetes being typically fast-growing but short-lived with frequent asexual-spore bottlenecks and basidiomycetes being generally slow-growing but long-lived and usually without asexual-spore bottlenecks. We argue that these life history differences have coevolved with stricter nuclear quality checks in basidiomycetes. Specifically, we propose a new function for clamp connections, structures formed during the sexual stage in ascomycetes and basidiomycetes but during somatic growth only in basidiomycete dikaryons. During dikaryon cell division, the two haploid nuclei temporarily enter a monokaryotic phase, by alternatingly entering a retrograde-growing clamp cell, which subsequently fuses with the subapical cell to recover the dikaryotic cell. We hypothesize that clamp connections act as screening devices for nuclear quality, with both nuclei

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continuously testing each other for fusion ability, a test that LOF mutants will fail. By linking differences in longevity of the mycelial phase to ecology and stringency of nuclear quality checks, we propose that mycelia have a constant and low lifetime cheating risk, irrespective of their size and longevity.

KEYWORDS Peto's paradox, clamp connection, crozier, dikaryon, evolution, filamentous fungi, genetic conflicts, major transitions in evolution, multicellularity, mycology

INTRODUCTION

Lower-Level Selection Is Detrimental for Cooperation

In biology competitiveness generally trades off with collective performance. An example is the sex ratio. In most animal species with dimorphic sexes, there is an equal number of females and males. An equal sex ratio is suboptimal for the possible total number of offspring that a group can produce in a certain period of time. In these species, females are limiting for reproduction and thus a minimum number of males, just sufficient to mate with all females, would maximize the collective number of offspring. However, Fisher (1) showed that individual-level selection always favors the rare sex, so the evolutionarily stable sex ratio is 50/50. Since each individual has a father and a mother, the reason for this 50/50 sex ratio is that individuals of the rare sex on average have more offspring. At other levels in the biological hierarchy, often below that of the multicellular individual, competitiveness is also detrimental for collective performance. An extreme example is cancer, caused by cell-level selection of somatic mutations that provide a competitive benefit to the cell despite clearly harmful consequences for the individual. Even within cells, selection at the level of organelles can lead to "organelle cancers," detrimental for the cell. One of the best-characterized examples is the petite mutant found in *Saccharomyces cerevisiae* (2, 3). With a frequency of about 1% per cell division, yeast cells mutate to form miniature colonies containing far fewer cells than wild-type colonies. The petite phenotype is caused by mitochondrial DNA (mtDNA) deletions, leading to a loss of respiration of mutant cells. Although yeast can meet their energetic needs by fermentation alone, respiration yields more energy. Deleted mtDNA molecules have a replication benefit over wild-type mtDNA, so that the mutated mtDNA molecules increase in frequency within the cell. Therefore, since mutated mtDNA molecules impose a cost to cell fitness, petite strains are selected against in competition with wild-type cells.

But Nevertheless, Organisms Are Hierarchically Organized

Despite the scope for, and the detrimental effects of, selection at lower levels of biological organization, life has organized itself into subsequently higher hierarchical levels, characterizing the so-called major transitions in evolution (4). Superorganisms such as colonies of fungus-growing termites consist of individuals, individuals consist of cells, and cells consist of organelles. Which factors facilitated selection leading to adaptation at subsequently higher levels of organization despite the threat from selection at the lower level? This paper addresses this question for multicellular growth of filamentous fungi of the subkingdom Dikarya, the group that includes the phyla Ascomycota and Basidiomycota, both of which in general produce dikaryons, i.e., cells with two genetically different haploid nuclei. We first discuss general evolutionary hypotheses for how the multicellular individual could become a unit of evolution. Specifically, we address the potential effects of sex on the scope for competition and the measures that were required to repress competition. We then zoom in on the peculiarities of multicellular growth of filamentous fungi. We link differences between the two groups of Dikarya, ascomycetes and basidiomycetes, to differences in their ecology. We propose a new hypothesis for the function of the clamp connection, a unique characteristic of mushroom-forming basidiomycetes, linked to their predominantly dikaryotic condition. Finally, we propose an analogue to Peto's paradox for fungi. Peto explained the observation that species of mammals across orders of magnitude in body size and longevity have a relatively constant lifetime risk to develop cancer (5). Analogously, we propose that fungal mycelia have a constant lifetime risk to be hit by intramycelial somatically parasitic mutants, despite orders-of-magnitude differences in mycelial longevity.

HOW DID THE INDIVIDUAL BECOME AN IMPORTANT UNIT OF EVOLUTION?

Germline Sequestration or Single-Celled Bottleneck?

Population genetics textbooks generally consider individuals as the basic units of evolution. How is it that an individual can be a useful unit of evolution to consider, given that natural selection acts at multiple levels and given that lower-level selection generally is detrimental for the higher level? Leo Buss (6) argued that the early sequestration of the germ line, seen in most animal phyla, is an important mechanism to reduce the selective scope of *de novo* somatic mutants. Since somatic cells are excluded from the germ line, any selfish somatic mutant cell finds itself in an evolutionary dead end. However, the importance of early germ line sequestration as a means to repress cell-level competition has been challenged (4). Other major groups of multicellular organisms, such as plants and fungi, do not have an early germ line sequestration, with each cell retaining the potential to become a germ cell. Furthermore, it has been argued that clonal development of an individual from a single-celled zygote stage, found in the vast majority of multicellular life cycles (7, 8), is sufficient to stabilize multicellular cooperation (4, 7, 9, 10). A clonal developmental mode, with a single-celled bottleneck, maximizes between-cell relatedness in an organism, favoring altruistic strategies of cells to help clonally related germ line cells via kin selection (10, 11). Another way to see this is that high relatedness converts natural selection to evaluating selfish or cheater mutants—defined as mutants with an intraindividual benefit relative to a noncheater at the cost of individual fitness—based on the consequences of their selfishness for individual-level fitness as a criterion.

Sex May Complicate Matters

However, the argument that clonal development from a single-celled stage stabilizes multicellular cooperation neglects the potential consequences of sexual reproduction. Following the work of Vreeburg et al. (12), we use a definition of sex that considers both the union and separation of haploid genomes ("the union of two haploid nuclei, each produced by meiosis, in due course followed by a reduction of the genome through meiosis"). Sexual reproduction, when outcrossed, brings two unrelated genomes together, which must cooperate during multicellular growth to form gametes. To ensure cooperation, in the vast majority of eukaryotic life, the two genomes unite in a single diploid nucleus, and the mitotic machinery operating during cell division guarantees that each cell of the multicellular individual receives a copy of that diploid genome. The two haploid gamete genomes thus enter "a lifetime monogamic relationship" (11). Lifetime commitment implies "unity of purpose" (13), i.e., a common interest of the genes, and their associated alleles, of an organism to maximize individual-level fitness. However, although lifetime commitment removes competition between unrelated genomes during somatic growth, lifetime commitment only delays competition until the formation of haploid gametes involving genome reduction at all loci. So now there is competition between the two alleles at a locus to enter the haploid gamete cells. Generally, both alleles at a locus have an equal probability to enter a gamete: Mendel's first law of equal segregation of the two alleles at a locus implies that meiosis is fair (14). Although selection on individual alleles will favor variants with a transmission advantage, an equal chance of transmission for the two alleles at a locus is in the common interest of the diploid genome since it prevents the possibility that a selfish copy that reduces total gamete production can increase in frequency (15). Therefore, the astonishing fairness of meiosis among autosomal loci has been attributed to reflect the interest of the majority of all genes of the genome other than a potential selfish genetic element, i.e., "the parliament of genes" (16, 17). Haig and Grafen (18) argue that recombination is a means to sustain the fairness of meiosis. Their argument is that recombination reduces the information available to genes that they could use to exploit segregation.

For cytoplasmic genetic elements, such as the mitochondrial (mtDNA) and chloroplast (cpDNA) genomes, the consequences of sex are different (19). Their numbers per cell and inheritance during cell division are not strictly regulated via a mitosis- or meiosis-like process. Therefore, potentially a more competitive mtDNA variant could obtain a benefit during gamete formation even if it reduced the total number of gametes (20). Uniparental transmission links the fate of a cytoplasmic genome with that of one of its parental lineages, and an associated

bottleneck restricts the selective scope for selfish variants. The almost universal observation of uniparental vertical transmission, usually via the mother in anisogamous organisms, is consistent with this argument, and a uniparental transmission mode is therefore believed to be an organismal adaptation against selfish cytoplasmic mutants (20–22).

Nevertheless, Secondary Conflicts May Arise

So, fair meiosis and uniparental vertical transmission of cytoplasmic genes eliminate nearly all conflict between selection at the level of genes and at the level of individuals, as these traits maximize the common interest of the diploid genome plus cytoplasmic genes. However, as in human society, "genetic outlaws" may still pursue their individual selfish interests against the common interest (23). An example is the phenomenon of segregation distortion, where one of the alleles of a heterozygous individual has a higher than 50% representation in the gametes, thus violating Mendel's first law of equal segregation. Sandler and Novitski first pointed out in 1957 (24) that chromosomes could selfishly exploit meiotic asymmetries to maximize their own transmission, in a process they termed "meiotic drive." Only recently, examples of such segregation distortion directly involving asymmetries during meiosis have been described in plants and animals (25). For example, in *Mimulus* crosses between outbreeding populations and inbreeding populations showed completely biased transmission of a centromere-linked locus from the outcrossing populations (26). The best-characterized examples of segregation distortion are based on postmeiotic processes of non-Mendelian inheritance, such as the *t* haplotype in mice (*Mus domesticus*), segregation distorters in *Drosophila melanogaster*, and spore killing in fungi (27–30). All those examples appear to consist of two components: a gene encoding a toxin and a linked second gene encoding an antidote to the toxin. A complex encoding both the toxin and the antidote can have the benefit of segregation distortion in a heterozygote with a sensitive allele at the antidote locus. In many fungi, segregation distortion can be directly seen since the products of one meiosis are contained in a closed sac, an ascus, but in animals and plants, demonstrating segregation distortion depends on an associated phenotype. For example, the *t* complex in mice has been observed due to the associated difference in tail length in heterozygotes. The fact that we are limited in seeing segregation distortion suggests that this phenomenon may be more common than currently appreciated. Current advances in sequencing methods may facilitate observations for loci without an associated phenotype (31). Furthermore, since, in the absence of an associated deleterious effect of segregation distortion, segregation distortion results in rapid fixation of the distorter allele, many genes may have an associated history of segregation distortion (32).

Another example of a secondary conflict is the phenomenon of cytoplasmic male sterility (CMS). CMS is the maternally inherited inability to produce functional male gametes in individuals from an otherwise hermaphroditic population of plants and perhaps basidiomycete fungi, where nuclear migration in a mating between two homokaryons can be unidirectional (33–36). CMS has generally been found to be caused by mitochondrial mutations. Since mitochondria are usually inherited only via the egg cell, a mitochondrial mutation reducing male fertility can be selected if it is associated with an increase in female fertility. However, since selection on nuclear genes favors an equal investment in male and female functions, selection on the nuclear genome favors resistance genes. Nevertheless, CMS is widespread in flowering plants, described in some 5% of species (23). CMS is observed particularly in crosses between different populations or species, as such crosses produce novel combinations of mitochondrial variants and noncoevolved nuclear genotypes (37).

So, Sex Is a Janus Head

Sexual reproduction thus has two opposing effects on the stability of multicellularity, as it is associated with traits facilitating the transition to multicellular growth but also with the introduction of potential conflicts. On the one hand, in most organismal groups sex is associated with a genetic and cytoplasmic bottleneck, leading to a single-celled diploid zygote with a single clone of cytoplasmic genes, which develops in a multicellular organism by mitotic division. We saw that clonal development removes the evolutionary prospects of cellular and intracellular cheaters. Since cheaters per definition depend on association with noncheaters, clonal development from a single-celled stage implies that such cheaters can have

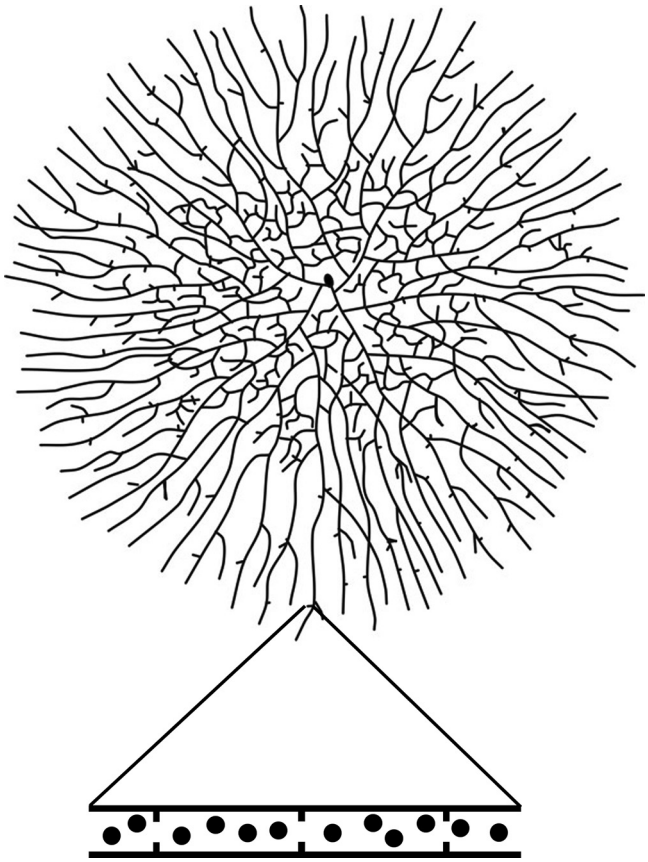

FIG 1 A fungal mycelium (86). The inset shows that cell compartments are leaky, to various degrees in different species, and that compartments can have various numbers of haploid nuclei. A mycelium with a single nuclear genotype is called a homokaryon, while a mycelium with different nuclear genotypes is called a heterokaryon. The main mycelial stage of basidiomycetes has two nuclear genotypes per cell and is called a dikaryon.

only a temporary benefit during somatic growth but not after the bottleneck. However, on the other hand, sex is a temporary "uneasy partnership" of two unrelated haploid genomes in a single diploid zygote that first cooperate for the common good of the total number of gametes but ultimately also compete when diploid germ cells go through meiosis to form new gametes. Several individual-level adaptations, such as fair meiosis, recombination, and uniparental cytoplasmic transmission, remove the scope for competition, thus "enforcing" selection for the common good of "the parliament of genes" (15, 38).

Sex in the best-studied multicellular life cycles—those of animals and plants—is characterized by, first, a lifetime relationship between the two haploid gamete genomes in a single diploid zygote and, second, the only single-celled bottleneck in the life cycle. However, the other major lineage of multicellular organisms, that of fungi, has completely different life cycles, including asexual single-cell bottlenecks and a less strict commitment between two haploid genomes (39, 40). It is then the question how general the theory is that has been developed to understand the major transition to multicellular growth that we have discussed so far. We now shift to fungi, asking the question of how they solved the tension between cooperation and competition during multicellular growth, including the consequences of sexual reproduction.

INDIVIDUALITY IN FUNGI

Fungal Individuals Are Haploid and Grow as Mycelial Networks

In contrast to animals and plants, fungal individuals generally are haploid, and many grow as a mycelium (Fig. 1). Although many groups of fungi do not have a mycelial body plan, for the purposes of this discussion we consider filamentous fungi as synonymous with fungi, as the described selective forces are dependent on a multicellular state. Mycelia

consist of hyphae that branch and fuse regularly to form a dense, radially growing network (41–43), although the frequency of hyphal fusion varies between species. Mycelia lack an early sequestration of the germ line, and each fragment can reproduce via the formation of sexual and—in some groups—also asexual spores. Spores are produced by specialized structures, which differentiate late in development from the mycelium and are supported by a substrate-bound somatic hyphal network that acquires the organic resources for growth. The cells in most fungal mycelia are incompletely compartmentalized so that the cytoplasm, and in some species also the nuclei, can move through most if not all parts of the fungal colony's mycelial network (40, 44). Therefore, the cooperating units in a fungal colony are the haploid nuclei and not the cells, as in most other multicellular organisms (42, 45). This hyphal organization means that the nuclei inside each hyphal tip to some extent evolve independently due to genetic drift and selection on genetic variation resulting from somatic mutations during mitosis. The formation of heterokaryons, i.e., mycelia with genetically different nuclei, by mutation or fusion of compatible homokaryons, has been demonstrated to facilitate adaptation (46, 47). Furthermore, in the parasexual cycle, mitotic recombination can occur between genetically different nuclei in a heterokaryon, which may result in new nuclear genotypes, further facilitating adaptation (48, 49). However, intramycelial competition among nuclei may also oppose mycelium-level fitness (see below). Mutation accumulation has been best demonstrated in long-lived basidiomycetes, where sampling of mycelia separated by decades to hundreds of years has shown accumulation of independent mutations (50–52). Somatic mutations are often visible in laboratory culture, as sectors of a colony that grow faster than the rest of the colony. Often overlooked, these sectors are the result of selection for higher mycelial growth rate—when the nuclei in one hyphal tip obtain a mutation that allows faster growth, they will outcompete their neighbors. The effects of this selection have been best visualized in mutant strains with artificially low growth rates, for example, due to the cost of fungicide resistance, where compensatory mutations can be selected due to a major effect in restoring growth rate (53).

Within-Mycelium Selection Can Oppose Mycelium-Level Fitness

Since fungi lack an early germ line sequestration, the question of how individuals can persist in the face of selection within the mycelia is more urgent than in animals since parasitic mutants with a benefit within the mycelium can make it to the offspring. Experimental lab studies have demonstrated that intramycelial selection has detrimental effects for mycelium-level fitness (54–56). One class of selfish variants with an intramycelial benefit is mitochondrial mutants. In some fungal species, mitochondrial plasmids are found that have a deleterious effect on mycelial fitness and induce a so-called senescence phenotype, first leading to reduced growth and sporulation and ultimately to death (55). The best-studied examples are found in the ascomycete genera *Neurospora* and *Podospora*. Senescence starts with insertion of the plasmid in the mitochondrial genome, and the plasmid causes the loss of normal mitochondrial function associated with oxidative phosphorylation, leading to increased reactive oxygen species (ROS). ROS damage the mitochondria and eventually the cells and cause cell death through necrosis (57). For yet unknown reasons, mtDNA molecules carrying an integrated plasmid copy are "suppressive", i.e., they accumulate during growth, gradually replacing the wild-type molecules (58). Some populations are polymorphic for the presence of senescence plasmids, as a consequence of a balance between processes increasing the frequency of the senescence plasmid, i.e., horizontal and vertical transmission, and processes decreasing its frequency, i.e., somatic incompatibility (59) and selection at the level of the mycelium (60). Species showing senescence typically are found on ephemeral substrates such as dung or have other external limits to the mycelial life stage such as regular fires (61). The presence of senescence plasmids in such species can thus be attributed to a "selection shadow", i.e., the absence of selection to sustain mycelial growth beyond the expected life span (62). The process of senescence in a particular strain carrying a senescence plasmid is influenced by the frequency of single-cell bottlenecks, the frequency of hyphal fusion, and the presence of somatically incompatible strains, processes that can delay or prevent the accumulation of suppressive mitochondria, and thereby the senescence phenotype (60). Single-cell bottlenecks enable selection at the level

of the mycelium and thus selection against suppressive mitochondrial variants. This requires that there is variation in the frequency of suppressive mitochondria between spores. It has been found that the senescence phenotype of the cytoplasmic donor to sexual spores affects the average phenotype of the sexual offspring in *Neurospora intermedia* but that there is nevertheless selectable variation among sexual spores in the senescence phenotype (63).

The other category of mutants with an intramycelium growth benefit at the cost of mycelial fitness is nuclear. It has been known for a long time that selection can operate between genetically different nuclei within the mycelium (e.g., reference 56). Davis (54) demonstrated that intramycelial selection can be detrimental for mycelial fitness, since a pantothenate-requiring variant had a competitive benefit in heterokaryons with a wild-type nucleus on a medium where the mutant could not grow as a homokaryon, and the mutant could increase in frequency within the heterokaryon up to the point that growth was arrested. In 2016, experimental evidence was provided for selection on *de novo* nuclear cheater mutants in *Neurospora crassa* (64). Starting with a single-spore culture, parallel experimental evolution with transfers of asexual spores at high density resulted in a repeatable significant decrease in mycelial fitness, as measured by asexual spore production. This decline was caused by so-called cheater mutants with reduced investment in somatic functions and increased competitive success during asexual reproduction relative to noncheaters. This advantage existed only at low frequencies—when common, the wild-type nuclei had a competitive benefit, and this negative-frequency-dependent selection resulted in a stable polymorphism of cheaters and wild type in evolved lines. Recently, the causal mutations of cheating were identified and found to be loss-of-function mutations in mycelial fusion genes (here referred to as loss-of-fusion [LOF] mutations), most often at the *so* locus (65, 66). Experiments showed that while these mutants cannot initiate fusion, they can be involved in fusion with wild-type mycelia with the ability to fuse. When the frequency of LOF mutants in the population is low, the LOF mutants are surrounded by wild-type mycelia that form an altruistic network, facilitating resource uptake and distribution, supporting the formation of spores in the aerial hyphae. The chances that at least one or a few wild-type hyphae will fuse with a LOF mutant are high, giving the LOF mutant access to the network and thus the resources. LOF mutants are cheaters in that they do not contribute to this supportive network and have a higher probability to become overrepresented in the aerial hyphae that later form spores. At higher frequencies in the population, the LOF mutant will be increasingly surrounded by its own genotype, providing a relative benefit to now-relatively isolated wild-type mycelial patches (66). There are indications that LOF mutants not only occur in *N. crassa* but are a general problem for the stability of mycelial growth. First, experimental evolution showed analogous emergence of LOF mutations that induce similar reductions of spore production in *Aspergillus nidulans* (K. Nandimath, B. Auxier, J. van den Heuvel, E. Bastiaans, M. Klatter, M. Slakhorst, S. E. Schoustra, A. J. M. Debets, V. Nanjundiah, B. J. Zwaan, D. K. Aanen, and J. A. G. M. de Visser, unpublished data). Further, the scientific literature suggests that so-called "strain degradation" during mycelial growth may be very common in molds, although it usually has not been ultimately attributed to nucleus-level selection within the mycelium (67, 68).

In Fungi Single-Celled Bottlenecks Can Also Occur after Asexual Reproduction

Although cheaters emerge easily under artificial conditions, their occurrence under natural conditions may be limited (but see reference 69). First, consistent with the general significance of clonal development from a single-celled zygote phase, fungi regularly pass through a spore phase. In many ascomycetes, this bottleneck can also be an asexual spore, which contains at most a few nuclei, usually mitotically derived from a recent common ancestral nucleus (70). These spores are dispersed and subsequently grow in isolation at least initially. Since cheaters per definition depend on coexistence with noncheaters, the passage through a bottleneck tests their effect on mycelium-level fitness. Second, related to asexual spore dispersal, many fungal species, particularly ascomycetes, are pioneer species found in early stages of succession and so have short-lived mycelial stages limiting the time for emergence and selection of selfish mutants (71). Third, fungi have highly sensitive nonself-recognition mechanisms (72), so that cheaters are limited in their ability to find "victims," especially after sexual reproduction (see below). Meunier et al. (69) found selfish

nuclei in one strain of the secondarily homothallic ascomycete *Neurospora tetrasperma*, enjoying better replication and transmission than sister nuclei while at the same time being detrimental to the heterokaryon. However, this case could be attributed to a recent introgression event, thus potentially disrupting previous coevolution between nuclear types in this strain.

We note here that other phylogenetic groups of mycelial fungi, like species of the Glomeromycota or Mucoromycota, often have multinucleate spores and would seem like targets of similar selection. However, in the Mucoromycota hyphal fusion is exceedingly rare (73), while in the Glomeromycota hyphal fusions are somewhat more frequent (74).

SEX PROVIDES OPPORTUNITIES FOR NUCLEAR QUALITY CHECKS

Sex Is Associated with Bottlenecks

The benefit of sex is often considered mainly one of genetics—increased genetic variation in offspring favors both adaptation to new environments and more efficient elimination of deleterious alleles (75). However, genetic consequences are but one benefit. In fungi, a major benefit of sexual reproduction is the restriction to a single-cell stage. A single-cell stage allows for a reset of the competition between haploid mitotic lineages, through this bottleneck. This bottleneck occurs due to the fusion between just two haploid gametes, producing a single diploid cell which then undergoes reduction to a haploid in most fungal lineages. This bottleneck means that a single genotype now needs to stand on its own merits. Additionally, in most organisms, cytoplasmic genomes such as the mtDNA are uniparentally transmitted in association with a bottleneck, again facilitating selection against selfish mtDNA variants and other deleterious cytoplasmic elements such as mycoviruses (23). As fungi do not have differentiated sexes, either parent can in theory donate the mitochondria and other cytoplasmic elements (33, 76). However, in filamentous fungi there is usually very limited cytoplasmic mixing between sexual partners, and female and male roles can be distinguished in a mating based on the parent that donates the cytoplasmic genes to a spore. Usually, these roles are played simultaneously in a mating, meaning that fungi mate in a hermaphroditic way and that mtDNA transmission is "doubly uniparental" (33, 76–78). In yeast-forming fungi either parent can be the cytoplasmic donor, and while cytoplasmic mixing does occur, the two mitochondrial types are usually quickly segregated. However, for some other yeast fungi, most notably the basidiomycete yeasts *Cryptococcus* and *Microbotryum*, the cytoplasm is predictably inherited from one parent only, apparently determined by the mating locus (79, 80).

Thus, even in homothallic fungi, where increasing genetic diversity through outcrossing is rare, the bottleneck occurring during meiosis is beneficial even in the absence of genetic recombination. This meiotic bottleneck is particularly useful for species that do not have asexual spore formation, such as most basidiomycetes, or species where asexual spores are multinucleate and where there are no mitotic bottlenecks, like the well-studied *Neurospora*, where conidia have between 2 and 5 nuclei (70).

Sex May Also Be an Opportunity for Additional Genetic Quality Checks

The sexual stage is often imagined as a random union of two genotypes, with the resulting offspring being selected by the environment based on their fitness. However, there is some evidence that the sexual stage in fungi may also include significant intraorganismal quality checks. Particularly, the elaborate machinery of sexual reproduction may suggest that some form of intraorganismal selection occurs, testing for genetic quality (81). In the subkingdom Dikarya consisting of ascomycetes and basidiomycetes, sexual reproduction starts with the formation of a dikaryon (Fig. 2 and 3). The cells of the dikaryon have two separate haploid nuclei, resulting from the fusion of two haploid homokaryotic individuals (77). In ascomycetes, the dikaryon is the result of "courtship behavior," involving fusion between receptive female hyphae and migration of male nuclei through these hyphae (81). The dikaryon stage in ascomycetes is very short, just preceding the formation of a diploid ascogonial cell, which undergoes meiosis to form four haploid ascospores. The functional female grows a long structure called a trichogyne that fuses with an enlarged cell in the compatible filament. The result is the emergence of a filament that remains haploid with two distinct nuclei (dikaryotic). As it divides, the terminal end makes a hook cell (called a crozier [Fig. 2])

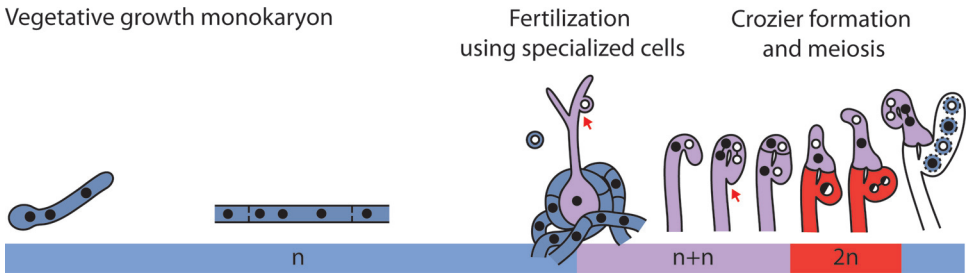

FIG 2 Generalized life cycle of ascomycete fungi. The dominant life stage is homokaryotic and haploid (n), and during sexual reproduction there is a brief dikaryotic phase (n+n) followed by a brief diploid stage immediately followed by meiosis. During the brief dikaryotic stage, croziers are formed, with a single haploid cell that fuses with a cell with the other nucleus. The fused cell becomes diploid, immediately followed by meiosis. The red arrows indicate the moments where somatic quality checks may occur.

that sequesters one of the nuclei to ensure that each daughter cell has the full complement of haploid nuclei. This dikaryon is short-lived and after a few cell divisions leads to the development of the ascus, within which the haploid nuclei fuse and then undergo meiosis to form the ascospores (81). We propose that crozier formation and fusion provide a test selecting against LOF mutants, since ascus formation depends on fusion between two haploid cells. Consistent with this hypothesis, LOF mutants in *Neurospora crassa* are female sterile (65). Although this female sterility does not seem to be caused by impaired communication or fusion failure between mating partners, it remains to be tested if LOF mutants in this species with female sterility are impaired in crozier formation.

In *Aspergillus nidulans*, it has been shown that there is selection after this fertilization stage within a mycelium, presumably between zygotes (cleistothecia), for progeny with fewer deleterious mutations, indicating that the sexual stage forms a "selection arena" (82, 83). Similarly, earlier work also in *A. nidulans* showed that when offered a choice, the sexual dikaryons that produced spores were preferentially those with genetically different parents, showing that fungi are capable of mate choice as seen in other organisms (84).

Thus, in the typical ascomycete life cycle, with both sexual and asexual reproduction, both stages present a genomic bottleneck. The regular occurrence of either sexual or asexual reproduction can then allow a genotype to be tested in isolation, removing cheating genotypes from a population. For those species that produce multinucleate conidia, the sexual stage may provide a more effective bottleneck. Additionally, during sexual reproduction in ascomycetes quality checks may occur at three moments: (i) during mating, (ii) after mating between zygotes, and (iii) also during the brief dikaryotic stage prior to ascus formation by crozier formation.

LONG-LIVED BASIDIOMYCETES NEED MYCELIAL QUALITY CHECKS

Basidiomycete Fungi Can Be Extremely Long-Lived and Have Low Mutation Rates

Basidiomycetes, specifically those producing mushroom fruiting bodies, are particularly

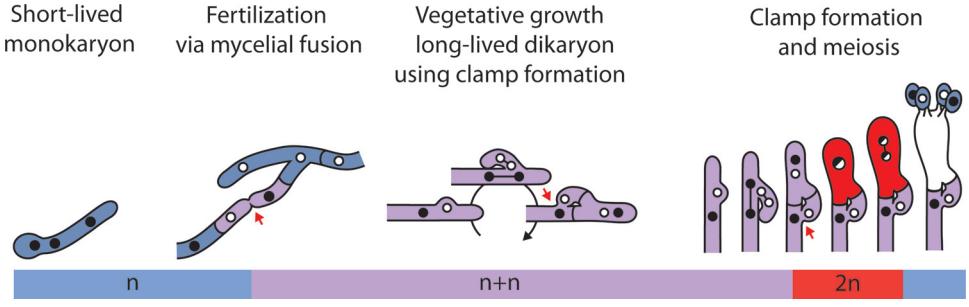

FIG 3 Generalized life cycle of basidiomycetes. Uniquely, the main life stage is the dikaryon with two haploid nuclei (n+n) derived from two compatible homokaryons (n). Most basidiomycete species form clamp connections during cell division of the dikaryon and also prior to the formation of basidia, where a brief diploid phase is immediately followed by meiosis and basidiospore formation (n). The clamp cell grows backward, receives a single nucleus, and splits off as a homokaryotic cell, just before it fuses with the subapical cell. The red arrows indicate the moments where somatic quality checks may occur.

susceptible to lower-level selection. First, many basidiomycetes have extremely long-lived mycelia, with life spans often in the tens to hundreds of years (52, 85). Additionally, the two haploid genomes of the dikaryon, the main somatic stage in basidiomycete fungi, retain the option to fertilize subsequent monokaryons encountered in so-called di-mon matings (86, 87). The two nuclear genotypes of the dikaryon compete for di-mon matings, potentially with detrimental consequences for mycelium-level fitness (88–90). Finally, as most species do not produce asexual spores (91), unlike for ascomycetes, presumably the sexual stage presents the only single-cell bottleneck. This extended hyphal growth increases the tension of lower-level selection, in the absence of any compensatory factors. One factor reducing the tension of lower-level selection may be a lower mutation rate. In animals, somatic and germ line mutation rates per year are higher for short-lived than for long-lived species, resulting in similar per-generation mutation rates (92, 93). Similarly, long-lived basidiomycetes have also been found to have an exceptionally low mutation rate (50–52). A decreased mutation rate will limit the chance that a mycelium develops a selfish mutation during its life span. Supporting the idea that long-lived organisms have reduced mutation rates, the comparatively short-lived *Schizophyllum commune* has a mutation rate much higher than that of longer-lived basidiomycetes (94). It has been hypothesized that a mechanism to achieve a low mutation rate is the preferential retention of the template DNA strands in the tip cells of the mycelial growth front (95, 96). By keeping the template strands at the front, the growing "germ line" of the mycelium will have a reduced mutational load.

Did Basidiomycetes Adopt Sexual Quality Checks during Mycelial Growth?

However, adaptation of the somatic mutation rate is perhaps not the only way that an organism can limit the effects of lower-level selection. As described above in sexual reproduction, a direct test of organismal fitness can allow for the limitation of growth of selfish mutants. Based on findings in ascomycetes, the act of fusion seems to be the primary trait under lower-level selection, and thus a test of this process may suffice to limit its devastating effects. Interestingly, in basidiomycetes the clamp connection, the morphological connection between adjacent cell compartments, lacks a sufficient explanation (Fig. 3). An interesting factor of the clamp connection is that it requires fusion of a hyphal tip with a single nucleus inside. While not a single-cell bottleneck, it comes tantalizingly close.

Clamp connections in basidiomycetes and croziers in ascomycetes are probably homologous structures, as they are shared only between these two sister phyla (97–99). In basidiomycetes clamp connections are present in the vegetative mycelia as well as at the base of the basidia where diploid formation occurs. In ascomycetes, the croziers are limited to the ascogenous hyphae, immediately prior to meiosis (100). In general, then, both basidiomycetes and ascomycetes produce these morphological structures immediately prior to meiosis, usually interpreted to function for the precise regulation of nuclear number. In basidiomycetes, the presence of clamp connections during mycelial growth has long fascinated mycologists. It is often hypothesized that the clamp connections on mycelia primarily function to regulate the nuclear ratio between the two genotypes of a dikaryon (cf. references 6 and 96). However, many species of basidiomycetes lack clamp connections on the mycelia and yet maintain both nuclear types, even though their cells generally contain more than two nuclei (101, 102). So, clamp connections are not strictly necessary for this role, and yet their formation on mycelia persists in most extant basidiomycetes. It has been shown in *Coprinopsis cinerea* that the identity of the nucleus in the clamp alternates between the two genotypes (96, 103). This means that, at least in *C. cinerea*, clamp cell formation would provide an opportunity to equally test the two genotypes in isolation.

This isolated clamp cell needs to grow only a few micrometers and does not have any competitors, so it may seem unclear what can be tested during this growth. But a critical factor of the clamp cell is the fusion of the retrograde growth. While clamp connections screen only a portion of the phenotype of a nucleus, as we describe above, in ascomycetes the loss of fusion seems to be a common trait under nucleus-level selection within mycelia. Given the central role of fusion for lower-level selection, the simple test may be sufficient to eliminate much of the detrimental effects of nucleus-level selection (Fig. 4). Assumptions and predictions

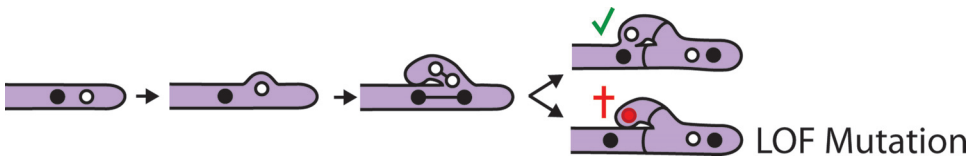

FIG 4 There is no satisfactory adaptive explanation for the function of clamp connections. We propose that clamp-cell fusion is a test of somatic quality of a haploid nucleus. A mutant that fails to fuse will be stuck in a false clamp, which is a dead end in mycelial growth or the spontaneous formation of homokaryotic mycelium or a di-mon mating (indicated with a red cross), while a variant that fuses passes the test and can grow further in the dikaryotic form (indicated with a green checkmark).

of this hypothesis need to be tested. First, a critical assumption is that the nucleus trapped in a clamp cell is at a dead end in a growing mycelium. The retrograde growth of a clamp cell is consistent with this. Second, our hypothesis assumes that most *de novo* mutations will end up in the clamp cell and will not be retained in the tip cell. If one of the nuclei in the tip cell is itself a LOF mutant, this hyphal tip could continue growing, even if the subapical cells would then be partially homokaryotic since clamp-cell fusion would be interrupted. One way to achieve mutations mostly ending in the clamp cell is that the template DNA strands preferentially are retained by the tip cell as has been proposed for stem cells in mammalian tissues but recently also for microorganisms (95, 96, 104, 105). Although direct evidence for such fusion-related selection in basidiomycetes is currently lacking, there are some strong clues that this selective force acts in basidiomycetes as well. Already in the 1950s, it was noticed that spontaneous mutants could be observed in *Schizophyllum commune* homokaryons that had a flat morphology, from reduced aerial hyphae, so-called *thin* mutants (106). These mutants could be reliably isolated by using a blender to fragment mycelia, or by visual inspection for sectors with increased aerial hyphae, which upon subculturing produced reduced aerial hyphae (107). At least one cause of this phenotype was subsequently shown to be from transposon insertions, although we stress here that the transposons are only the mechanisms and are not the selective force itself (108). The first gene shown to cause this phenotype was designated *thn1*, encoding a regulator of G-protein-coupled receptors, involved in cell signaling, consistent with a role in hyphal fusion (109). In line with our hypothesis, *thn1* mutants have a very low frequency of clamp-cell fusion and are also partially defective in mating (108). *Thin* mutants have increased formation of aerial hyphae, when connected to a wild-type mycelium, but reduced aerial hyphae when grown as a monoculture (107), exactly analogous to the phenotype of *so* mutants in *Neurospora crassa* (64, 66). However, in the latter species, this has been found to be associated with a competitive benefit in asexual sporulation relative to wild-type nuclei at low frequency. For basidiomycetes, the competitive success of *thin* mutants within an otherwise wild-type mycelium remains to be established, as well as the hypothesized role of clamp connections in preventing this.

THE LIFETIME CHEATING RISK HYPOTHESIS AND PETO'S PARADOX

In Animals, the Lifetime Cancer Risk Is Constant across Species

In animals, only germ line mutations can be transmitted to future generations, so they are the ultimate source of variation for evolutionary processes. Yet, since somatic mutations can affect survival and reproduction of an individual, they indirectly do affect the fitness of the germ line (Fig. 5). For example, cancer is caused by somatic mutations that provide a replication benefit to a cell lineage but are detrimental to the individual. With every cell division there is a risk of a cancer-causing mutation, and so a naive expectation is that large individuals have a higher probability to develop cancer due to the increased number of mitoses. However, cancer rates across animals vary by only approximately 2-fold, even though the size difference among mammals alone can be on the order of a millionfold (110, 111). The absence of a correlation between body size, longevity, and cancer is referred to as Peto's paradox (5). The explanation of this paradox comes from natural selection interacting with the life history of a species and favoring adaptations that suppress cancer through the expected period of fertility of an organism. Therefore, Peto's paradox is consistent with life-

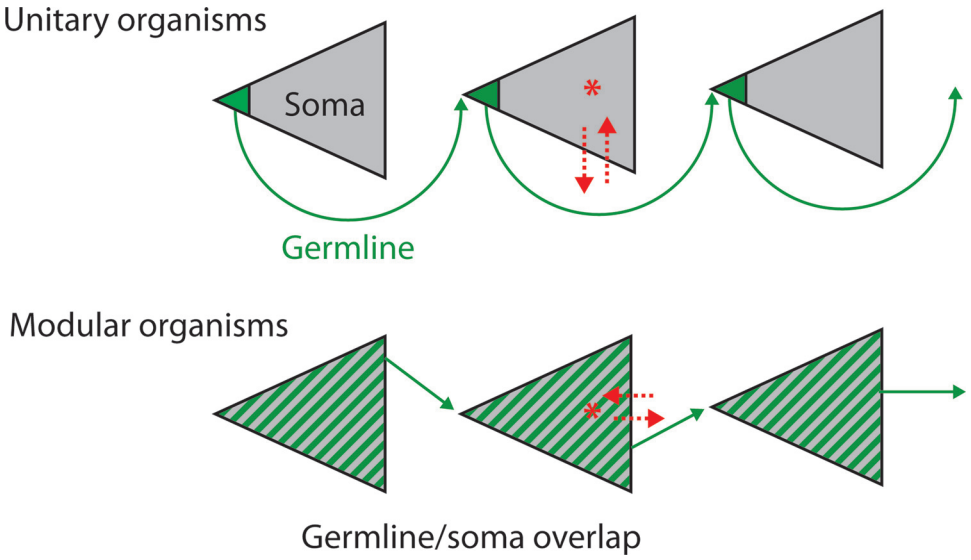

FIG 5 The consequences of mutations in unitary (top) and modular (bottom) organisms. In unitary organisms, only germ line mutations are transmitted, but somatic mutations (indicated with an asterisk) indirectly matter as well: they influence the survival and reproduction of the soma (downward broken arrow). So, natural selection between individuals has favored adaptations to reduce the risk of selfish mutants during an organism's lifetime, such as policing mechanisms (broken arrows). The power of this selective force is influenced by ecology such as external mortality and explains that mammals of very different size and longevity have remarkably similar lifetime cancer risks (Peto's paradox [5, 107, 108]). Mycelium-forming fungi do not have early germ line differentiation, meaning that selfish mutants can enter the germ line. However, in species with long-lived mycelia selfish nuclear mutants may threaten mycelial fitness (broken arrow), thus indirectly favoring adaptations that reduce the scope for such deleterious mutations (leftward broken arrow). We hypothesize that the force of this selective pressure depends on external limitations to mycelial life span and historical constraints depending on phylogenetic history. These factors determine the opportunities for within-mycelium selection, which influences selection for mycelial adaptations against deleterious mutations. This logic is analogous to Peto's paradox, but for modular organisms.

history theory, since one expects cancer rates to be similar across species after correcting for reproductive life spans (110).

The Lifetime Cheating Risk Hypothesis as an Analogue of Peto's Paradox

We hypothesize that an analogue to Peto's paradox can be formulated for fungi and other modular organisms (Fig. 6). Since fungi, as modular organisms, do not have an early

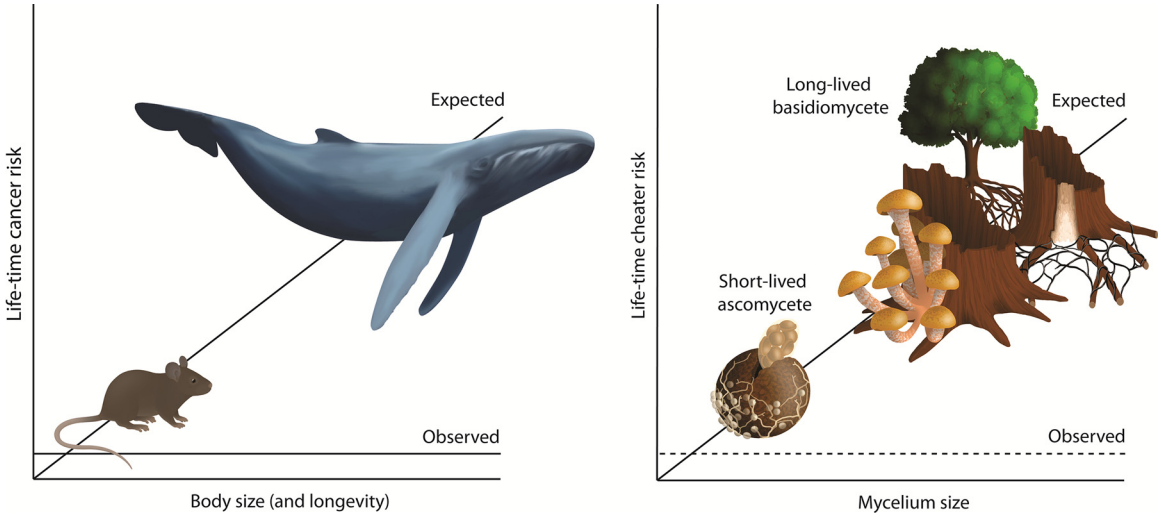

FIG 6 The lifetime cheating risk hypothesis and Peto's paradox. Peto observed that mammals have a more or less constant, low, lifetime cancer risk, independent of their body size and natural longevity. Analogously, we propose that fungi and other modular organisms have a constant, low, lifetime cheating risk, irrespective of their mycelium size and the natural longevity of their mycelial phase.

germ line sequestration, selfish mutations can affect the genetic quality of both the soma and offspring. However, fungi do differ in their longevity due to external and internal constraints such as external mortality risk and historical constraints (Fig. 5). We propose that basidiomycete mycelia generally are long-lived and that clamp connections are screening devices against selfish mutants during mycelial growth. Consistent with our idea, the longest-lived basidiomycete known, *Armillaria*, has diploid mycelia (85, 112) and likely has a reduced risk of haploid-nucleus-level selection. Also, the reversion to a temporary dikaryotic state and the occurrence of clamp-cell connections in fruiting bodies of the otherwise diploid phase in *Armillaria* are consistent with the hypothesis that clamp-cell fusion serves to check the genetic quality of the two haploid genomes (113–115). In contrast, molds generally are short-lived and do not have screens during mycelial growth but usually have regular asexual bottlenecks. As outlined above, within-mycelium selection can oppose mycelium-level fitness (64, 66), highlighting the need for long-lived mycelia to screen against selfish mutants that have a within-mycelium benefit. We thus predict an association between mycelial longevity and the presence of screening devices against selfish mutants, particularly loss-of-fusion mutants, and hypothesize that the lifetime risks of cheating mutants under natural circumstances will be similar for molds and mushrooms (the Lifetime Cheating Risk [LCR] hypothesis). The LCR hypothesis provides an analogue of Peto's paradox for mycelium-forming fungi.

The LCR hypothesis makes two testable predictions:

1. Species of mushrooms with clamp connections have higher mycelial stability, i.e., a lower rate with which cheaters occur during mycelial growth, than species without clamp connections. The level of cheating should negatively correlate with the frequency of clamp connections.
2. Species with higher mycelial stability typically have longer mycelial phases under natural conditions. For only a few species have estimates of mycelial longevity in nature been made (50–52, 85), so the natural longevity of mycelia deserves wider study.

OUTLOOK

An important question is what explains the observed difference between basidiomycetes and ascomycetes in the occurrence of asexual sporulation and the stringency of nuclear quality checks, as we argued above. Why might mushrooms generally require more stringent nuclear quality checks during somatic growth, and molds only during sexual reproduction? If the shorter mycelial phase of molds under natural conditions limits the accumulation of somatic deficiencies and particularly fusion mutants, the long mycelial phase of mushrooms should allow for intramycelial selection of such fusion mutants, thus requiring more stringent nuclear quality checks.

At first glance, mycelial longevity seems strongly linked to ecological strategies. While mycelial growth can initially be fast, space and resources quickly become limiting. Broadly speaking, fungi then follow two different growth strategies. Molds typically form asexual spores to disperse and start a new round of fast mycelial growth, which fits with a general ecological role as pioneer species. In contrast, mushroom mycelia typically are longer-lived and continue to grow longer without dispersal via asexual spores (91). While some data on the natural life span of fungi exist, more such data are needed to link potential adaptations to life cycle and ecology.

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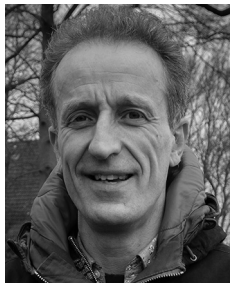

Duur K. Aanen is professor in Evolutionary Biology at Wageningen University, The Netherlands. He teaches courses in genetics, population genetics, and evolution and studies the origin and evolution of cooperation and conflict. He addresses levels of selection ranging from interspecific cooperation between fungus-growing termites and symbiotic fungi to cooperation between genomes within cells and cheating within fungal mycelia. After finishing his Ph.D. on speciation of fungi at Wageningen University in 1999, he became a postdoc and later assistant professor at the University of Copenhagen and returned to Wageningen in 2006. Duur Aanen uses fungi as his main experimental model system. Fungal "individuals" can fuse and form chimeras, can be used in evolution experiments, and are important mutualistic partners of other species, characteristics that make them excellent model systems for the evolution of conflict and cooperation. His empirical work is both guided by and feeds back into theoretical work.

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