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Review

Why mushrooms have evolved to be so promiscuous: Insights from evolutionary and ecological patterns

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ABSTRACT

Agaricomycetes, the mushrooms, are considered to have a promiscuous mating system, because most populations have a large number of mating types. This diversity of mating types ensures a high outcrossing efficiency, the probability of encountering a compatible mate when mating at random, because nearly every homokaryotic genotype is compatible with every other. Here I summarize the data from mating type surveys and genetic analysis of mating type loci and ask what evolutionary and ecological factors have promoted promiscuity. Outcrossing efficiency is equally high in both bipolar and tetrapolar species with a median value of 0.967 in Agaricomycetes. The sessile nature of the homokaryotic mycelium coupled with frequent long distance dispersal could account for selection favoring a high outcrossing efficiency as opportunities for choosing mates may be minimal. Consistent with a role of mating type in mediating cytoplasmic-nuclear genomic conflict, Agaricomycetes have evolved away from a haploid yeast phase towards hyphal fusions that display reciprocal nuclear migration after mating rather than cytoplasmic fusion. Importantly, the evolution of this mating behavior is precisely timed with the onset of diversification of mating type alleles at the pheromone/receptor mating type loci that are known to control reciprocal nuclear migration during mating. While reciprocal nuclear exchange may have released some of the restrictions on the evolution of multiple mating types, it may also have fostered a nuclear "arms race" that favored the evolution of selfish alleles with greater access to conspecific mycelia. Such selection could potentially increase mating type diversity through rapid diversification of novel, more fully compatible forms or may actually reduce outcrossing efficiency by skewing mating type allele frequencies towards more selfish alleles. Distinguishing among the possible mechanisms involved in promoting promiscuity will require extensive testing of correlates between allele number and cytological, ecological, and demographic factors across a broad range of species.

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1.   Introduction. Why are mushrooms considered promiscuous?

What is promiscuous about the mushroom mating system? Promiscuity can be defined as the practice of having sex with multiple partners or being indiscriminate in the choice of sexual partners. Both of these criteria are applicable to the mating system of the mushrooms (Agaricomycetes) which is highly sexual as well as promiscuous. At least three aspects of the mushroom mating system facilitate promiscuity. Firstly, unlike most other fungal groups, mushrooms by and large have an obligately sexual life cycle. Secondly, mated mushroom mycelium is heterokaryotic rather than diploid (Anderson and Kohn, 2007). This heterokaryotic nuclear condition facilitates subsequent unmating (return of the mated nuclei to a homokaryotic state) as well as remating with a different partner. In mushrooms, because the haploid nucleus can be considered a gamete, the heterokaryon is therefore, in botanical terms, both a gametophyte and sporophyte. Lastly, mushroom species have a very large number of mating types (Brown and Casselton, 2001). Mating types distinguish groups of individuals that are capable of mating outside but not within a group. Thus, if there are a large number of mating types in a given population, then any individual is capable of mating with the majority of other individuals if matings happen at random.

These three claims of promiscuity warrant further explanation. First the obligate role of sex in the mushroom life cycle appears to be greater than that of other groups, though this point has been seldom made. There are very few examples of completely clonal or asexual species within Agaricomycetes. Some known exceptions are *Moniliophthora roreri*, a pathogen of cacao (Evans, 1981), *Leucoagaricus gongylophorus*, the cultivated fungus of leaf cutter ants (Chapela *et al.*, 1994), though see (Mikheyev *et al.*, 2006). Other potential exceptions are *Chaetospermum*, a coelomycetous conidial fungus in the early-diverging Agaricomycetes order Sebacinales (Tangthirasunun *et al.*, 2014), and *Sporotrichum pruinosum*, a widespread conidial fungus that may have a teleomorph related to *Phanerochaete chrysosporium* (Burdsall, 1981). Most other fungal groups have many examples of solely asexual species, with anamorphic forms common throughout classes of Ascomycota, and the subphyla Pucciniomycotina and Ustilaginomycotina of the Basidiomycota. For example many rusts are considered imperfect and only make asexual spores (aeciospores or urediniospores) (Petersen, 1974).

On the other hand, the presence of asexual or anamorphic states as part of the complete mushroom life cycle has been often overlooked (Brodie, 1936; Mukhin and Votintseva, 2002; Walther *et al.*, 2005). Both homokaryons and heterokaryons are known to produce asexual (i.e., non-meiotic) spores in the form of oidia, conidia, and chlamydospores. The asexual spores may be limited to the homokaryotic or heterokaryotic stage or may be found in both stages (Kües *et al.*, in press; Nobre *et al.*, 2014; Walther and Weiss, 2008). The production of these asexual spores by heterokaryotic mycelia provides a means by which homokaryons can be reformed, because these asexual spores may be uninucleate and haploid (Cao *et al.*, 1999; James *et al.*, 2008). Thus asexual sporulation of

heterokaryons also facilitates promiscuity through the unmating and remating of nuclei. These extra copulations allow nuclei to reassort which may provide fodder for sexual selection. Asexual conidia could themselves be involved in fertilization of homokaryons, acting as spermatia when they land on compatible homokaryotic mycelium (Brodie, 1936).

Because of the avoidance of nuclear fusion after cell fusion, heterokaryosis interjects remating as a central part of the life cycle of an individual nucleus. Perhaps the best known and most common route in which a nucleus can remate is the Buller phenomenon, when a heterokaryon mates with a homokaryon (Raper, 1966). When this happens one of the two nuclei of the heterokaryon can migrate into a recipient homokaryon. This form of unmating and remating allows nuclei to reassort which, again, may provide fodder for sexual selection or selection on growth or mycelial robustness (Nieuwenhuis *et al.*, 2011). The Buller phenomenon is not a laboratory phenomenon; there is ample evidence from field studies that nuclei in close proximity undergo reassortment (Johannesson and Stenlid, 2004; Kay and Vilgalys, 1992; Nieuwenhuis *et al.*, 2013c; Ullrich, 1977), such that one nucleus has clearly mated with multiple other nuclei.

Here I summarize the observed data for mating type allele diversity to demonstrate the magnitude of the promiscuity across the Agaricomycetes. Estimates of mating allele diversity within a fungal species are hard to obtain, because they require extensive crosses to find rare identical mating types (Table 1). Most estimates of mating type allele diversity in the Agaricomycetes are in the dozens to hundreds, with a few outliers, such as the bird's nest fungi for which three of four examined species have one or more loci with less than 10 alleles. Bipolar species have a single mating type locus, while tetrapolar species have two mating type loci, and one locus is known to encode for homeodomain transcription factors (*HD*), while a second locus encodes for pheromones and pheromone receptors (*P/R*). In the bipolar species, the estimates of diversity are similar to that observed at each of the loci of tetrapolar species. Estimates of mating type diversity can be used to calculate the outcrossing efficiency of a species, or the probability that an outcrossing event between randomly chosen gametes will be compatible, assuming an idealized population with the observed allele numbers. The expected outcrossing efficiency can be calculated assuming random mating and equal frequencies of all alleles using the equation: $((n_a-1)/n_a)((n_b-1)/n_b)$, where n_a is the number of estimated alleles at the *HD* mating type locus and n_b is the number of alleles at the *P/R* locus, using only the n_a term for bipolar species. This leads to values > 0.95 for most species and a median value for Agaricomycetes of 0.967 (Table 1). Where mating systems are tetrapolar, there is a frequent statement that there are tens of thousands of mating types. This might be true by the definition of mating type, but the overall outcrossing efficiency is limited by the number of alleles at the locus with the fewest alleles (Kües *et al.*, 2011), a point glossed over by many mating system enthusiasts. Therefore, even though some tetrapolar species may have tens of thousands of "mating types", no tetrapolar species has an outcrossing efficiency greater than a bipolar species with 100 + mating types (Table 1). There is still much to learn about the implications of

Table 1 – Summary of mating type numbers across species of Agaricomycetes. Values shown are reported estimates of total alleles, with observed values in parentheses. Where known, the *HD* mating type locus represents the locus encoding homeodomain transcription factors and the *PR* mating type locus the pheromone/receptor encoding locus. Where that information is not available, the locus with the most alleles is randomly assigned to *HD*. In bipolar species, the values are listed under *HD*. N = number of fruiting bodies investigated. When mating types, somatic incompatibility, or other loci suggested identical genotypes had been sampled, those data were considered to be only one sample. The values are not corrected for instances were nuclei may be shared across genotypes, and such scenarios may indicate mating through the Buller phenomenon (specifically estimates for *Heterobasidion annosum* and *Sistotrema brinkmannii* (Group II-2).
OE = maximum outcrossing efficiency is the probability of two randomly chosen homokaryons being compatible, assuming all alleles are found at equal frequency. A minimum number of 8 fruiting bodies were used for estimates shown.

Species	N	HD	P/R	OE	Ecology	Taxonomy	Reference
Sistotrema brinkmannii (Group II-2)	31	219 (55)		0.995	Wood	Corticiales	(Ullrich and Raper, 1974)
Coprinellus disseminatus	24	123 (40)		0.992	Wood	Agaricales	(James et al., 2006)
Ganoderma boninense	52	195 (81)[c]	218 (83)[c]	0.990	Wood	Polyporales	(Pilotti et al., 2003)
Ceriporiopsis carnegieae	18	90 (29)		0.989	Wood	Polyporales	(Lindsey and Gilbertson, 1977)
Pleurotus populinus	30	126 (49)	354 (56)	0.989	Wood	Agaricales	(Anderson et al., 1991)
Exidiopsis plumbescens	16	large (32)	86 (27)[c]	0.988	Wood	Auriculariales	(Wells and Wong, 1985)
Lentinula edodes	94[a]	121 (66)	151 (72)	0.985	wood	Agaricales	(Lin et al., 2003)
Schizophyllum commune	114	288 (96)	81 (56)	0.984	Wood	Agaricales	(Raper et al., 1958)
Heterobasidion irregulare	32	61 (40)[c]		0.983	Tree pathogen	Russulales	(Chase and Ullrich, 1983)
Spongipellis unicolor	24	112 (39)	112 (39)	0.982	Tree pathogen	Polyporales	(Eggertson, 1953)
Coprinopsis cinerea	33	164 (31)	79 (27)	0.981	Dung	Agaricales	(Day, 1963)
Trichaptum abietinum	14	200 (26)[e]	70 (23)	0.981	Wood	Polyporales	(Fries and Jonasson, 1941)
Pleurotus djamor	22[a]	58 (19)	231 (21)	0.979	wood	Agaricales	(James et al., 2004)
Gymnopus subnudus	10	45 (16)		0.978	Litter	Agaricales	(Murphy and Miller, 1997)
Fomitopsis cajanderi	18	33 (19)		0.970	Wood	Polyporales	(Neuhauser and Gilbertson, 1971)
Piptoporus betulinus	201[b]	30 (28)		0.967	Tree pathogen	Polyporales	(Burnett 1965 p. 108)
Sparassis crispa	18	23 (17)[c]		0.957	Tree pathogen	Polyporales	(Martin and Gilbertson, 1978)
Fomitopsis palustris	50[a]	20 (19)		0.950	Wood	Polyporales	(Raper, 1966 p. 107)
Agaricus bisporus var. *burnettii*	23	(18) 13		0.944	Soil	Agaricales	(Imbernon et al., 1995)
Armillaria luteobubalina	8	18 (11)[c]	14 (10)[c]	0.944	Tree pathogen	Agaricales	(Kile, 1983)
Laccaria bicolor	28	44 (32)[c]	27 (24)[c]	0.941	Ecto-mycorrhizae	Agaricales	(Raffle et al., 1995)
Cyathus stercoreus	12	39 (15)	24 (13)	0.934	Dung/wood chips	Agaricales	(Malloure and James, 2013)
Agrocybe aegerita	13	30 (18)	25 (16)	0.928	Wood	Agaricales	(Noël et al., 1991)
Auricularia polytricha	13	44 (20)[c]	18 (14)[c]	0.923	Wood	Auriculariales	(Wong and Wells, 1987)
Marasmiellus praeacutus	8	19 (11)	28 (12)	0.914	Wood	Agaricales	(Murphy and Miller, 1997)
Mycocalia denudata (Group II)	8	9 (7)		0.889	Wood/dung/litter	Agaricales	(Burnett and Boulter, 1963)
Laccaria longipes	17	17 (14)	18 (14)	0.889	Ecto-mycorrhizae	Agaricales	(Doudrick et al., 1990)
Marasmius oreades	16	8 (8)[c]		0.875	Soil	Agaricales	(Mallett and Harrison, 1988)
Crucibulum vulgare	15	3 (3)	15 (11)	0.622	Wood/wood chips	Agaricales	(Fries, 1942)
Cyathus striatus	9	4 (4)	5 (5)	0.600	Wood/wood chips	Agaricales	(Fries, 1940)
Serpula lacrymans var. *lacrymans*	10	4 (4)	5 (5)	0.600	Wood	Boletales	(Schmidt and Moreth-Kebernik, 1991)
Tremella mesenterica	46	146 (68)	2 (2)	0.497	Mycoparasite	Tremellales	(Wong and Wells, 1985)
Ustilago maydis[d]	62[a]	21 (18)	2 (2)	0.476	Plant pathogen	Ustilaginales	(Puhalla, 1970)

a Indicates number of homokaryotic strains tested.
b Ploidy of strains uncertain.
c Values were newly estimated using the method of (O'Donnell and Lawrence, 1984).
d This member of the smut fungi (Ustilaginomycetes) is shown for comparison.
e Indicates uncertainty regarding which mating locus was *HD* versus *PR*.

tetrapolar vs. bipolar mating systems in natural populations and why these transitions have occurred so frequently (Nieuwenhuis et al., 2013a).

This paper is written in memoriam to Dr. Lorna Casselton, a leading figure in mycology who helped usher in the era of molecular biology to the field of mushroom genetics. Her work on chimeric, self-activating mating type mutants of *Coprinopsis cinerea* laid the foundation for molecular analysis of mushroom mating type genes (Kües et al., 1994; Olesnicky et al., 1999), which provided great insights in understanding

how promiscuity is generated at the mating type loci. Her work with transformation also cleared many of the obstacles needed for robust tests of gene function (Binninger et al., 1987; Mellon and Casselton, 1988). The passion she showed for understanding how mating type genes of *Coprinopsis cinerea* promote promiscuity is the inspiration for this paper (Brown and Casselton, 2001; Casselton and Kües, 2007). The goal of the rest of this paper is to discuss the why part of why are mushrooms promiscuous. Why questions are hard in biology, because they seek to understand causation or at

least perseverance in an ever-changing world. As you will see if you persevere in reading this paper, the answer is not entirely clear. But the phenomenon is so interesting and fundamental to the life cycles of this most sexually diverse group of fungi, that a number of hypotheses can be put forward that hopefully could be tested in the future.

2.   Evolutionary hypotheses and patterns to explain promiscuity

First I will clarify what are fungal mating types and then discuss how mating types and mating systems promote outcrossing. Then I will review the phenomenon of multiple mating types or sexes across the eukaryotes and lastly discuss three hypotheses that might explain why Agaricomycetes have multiple mating types while other fungi do not.

Mating types vs. sexes

The term sexes should be restricted to organisms with compatibility groups defined by the presence of morphologically distinct parents and/or gametes during sex. Isogamous species, including many unicellular organisms, are considered to have mating types rather than sexes. Sex in fungi is roughly equally split along the anisogamous (most molds, cup fungi, lichens, rusts) and isogamous (zygomycetes, yeasts, smuts, jelly fungi, mushrooms) lineages. However, anisogamous fungi are all hermaphroditic, meaning individuals can produce both male and female gametes, and for this reason anisogamous fungi also have mating types rather than sexes. There is ample theory explaining why sexes and mating types mostly come in twos (Hurst, 1995, 1996; Iwasa and Sasaki, 1987; Power, 1976). Most of these theories involve either the stability of systems with large and small gametes controlled by sex or systems where sex controls cytoplasmic inheritance.

The role of mating types in controlling outbreeding/inbreeding ratios

In heterothallic fungal species, mating types enforce outcrossing and therefore prevent haploid-selfing which would lead to genome-wide homozygosity. Selfing at the diploid level, i.e., among meiotic progeny of a diploid, however, is not prevented by mating types, and is a form of inbreeding that reduces heterozygosity and may lead to inbreeding depression. Tetrapolar species are hypothesized to experience less inbreeding than bipolar species because the intercompatibility of sibling monokaryons is 50 % lower (Nieuwenhuis et al., 2013a). Despite the free-living homokaryotic stage of mushroom fungi that should purge deleterious recessive alleles, there appears to be evidence that they suffer from inbreeding depression when examined in the lab (Malloure and James, 2013; Xu, 1995). If these limited studies on inbreeding depression are applicable to the broader group of mushrooms, then there may be strong selection to avoid inbreeding which fits in well with data that show populations of mushroom fungi are often highly outcrossing, with a panmictic population structure (Kauserud and Schumacher, 2003). Given, then, that many mushrooms are highly outcrossing, the greater

outcrossing efficiency provided by increased mating type allele diversity should be naturally selected so as to reduce the incompatibility genetic load (dead-end matings), or the amount of wasted mating opportunities suffered due to mating between incompatible partners (Crow and Morton, 1960).

What organisms have multiple mating types?

In order to understand why systems with multiple mating types or sexes are so rare, it is worth investigating the phylogenetic distribution and life cycles of the multi-sex anomalies. Withstanding a few poorly known exceptions, only Agaricomycetes, ciliates, and slime molds (Dictyosteliomycetes and Myxomycetes) have multiple mating types. All species are isogamous. Estimated mating type numbers range from 3 to 354 per locus (Table 1) in Agaricomycetes, 2–100 in ciliates (Phadke and Zufall, 2009), 2–18 in Myxomycetes (Clark and Haskins, 2010), and 2–7 in Dictyosteliomycetes (Bloomfield, 2011; Mehdiabadi et al., 2010). There are no commonalities to the mating systems of these anomalies, but one similarity between Agaricomycetes and ciliate mating systems is that in both, conjugating parents exchange nuclei but not cytoplasm and organelles such as mitochondria (Casselton and Condit, 1972; Roberts and Orias, 1973), whereas in slime molds, amoeboid gametic stages undergo complete plasmogamy. Despite this one similarity, these three groups of organisms have numerous differences in their life cycles. For example, ciliates are highly motile, whereas mushrooms and slime molds rely on spores for long distance dispersal. Another difference is that cell conjugation in ciliates (Kuhlmann et al., 1997) and slime molds (Youngman et al., 1981) only, or preferentially, occurs between different mating types as opposed to mushrooms in which fusions are not mating type controlled (Raudaskoski, 2015). An additional difference between ciliates and slime molds versus the mushrooms is that mating types often are as few as two within a species (Phadke and Zufall, 2009), whereas a diversity of less than three mating types is essentially unobserved in mushrooms (Table 1).

Exceptions to the taxonomic patterns are few and poorly documented. Three mating types are reported in the dinoflagellate Alexandrium excavatum (Destombe and Cembella, 1990). In the Ascomycota, there is only one report of multiple mating types; the species Glomerella cingulata is reported to have three (Cisar and TeBeest, 1999), and there is only one in the non-Agaricomycete basidiomycetes, the smut fungus Sporisorium reilianum (Schirawski et al., 2005), which has three mating types at one locus and five at a second locus. Low diversity of mating types (specifically two) extends throughout most Pucciniomycotina, Ustilaginomycotina, and even the Tremellomycetes of the Agaricomycotina where these life cycles have what is known as a modified tetrapolar system with two alleles at the mating type locus encoding pheromone receptors yet often dozens at an unlinked HD mating type locus (Wong and Wells, 1985) (Fig. 1). In contrast to the Agaricomycetes, in the modified tetrapolar species fusions are dictated by the alleles at the pheromone/pheromone receptor locus, and the second locus (the homeodomain encoding) functions to determine compatibility after cytoplasms are mixed. As I argued above, I think it is inappropriate to consider these two systems to have a high number of mating types as
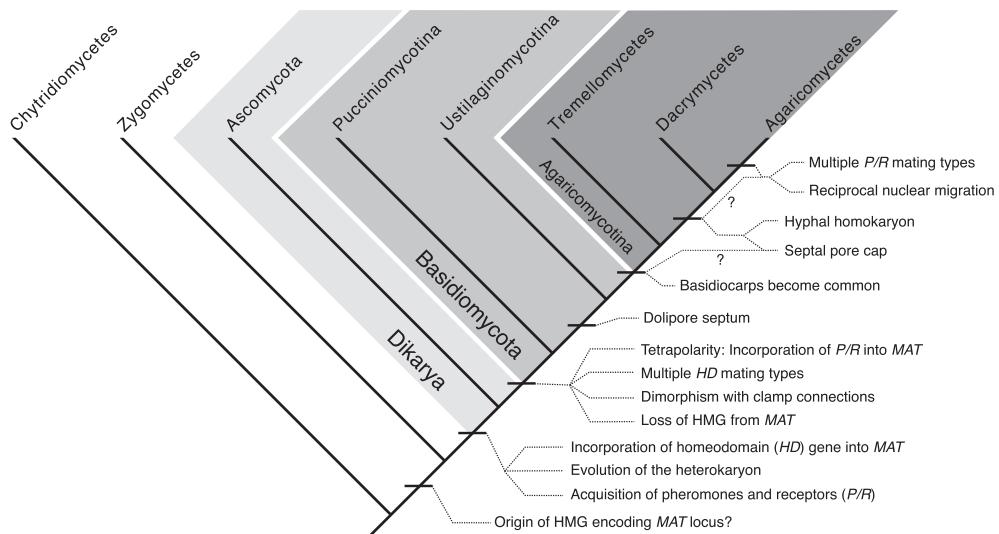
Fig. 1 – **Major evolutionary trends in the mating genetics and cell biology leading to the Agaricomycetes. Phylogeny based on (Hibbett *et al.*, 2014). Character evolution mapped on tree based on several publications (Ingold, 1983; Kües *et al.*, 2011; Lee *et al.*, 2010; Van Driel *et al.*, 2009).**

outcrossing efficiency (and fusion) is solely determined by the locus with fewer alleles.

The three phylogenetic instances of multiple mating types in mushrooms, slime molds, and ciliates are certainly not the limits to the phenomenon but likely reflect the difficulty of demonstrating it. Many more are predicted to exist by general theory (Iwasa and Sasaki, 1987). To test for multiple sexes a large number of single genotype cultures must be obtained and they must be amenable to experimental crossing. Predictions are that organisms that reciprocally exchange nuclei during the mating process are more likely to evolve multiple mating types.

Major hypotheses on the origin and maintenance of promiscuity and their predictions

The origin of mating types has been recently reviewed (Billiard *et al.*, 2011), and I will merely attempt here to present the major hypotheses that explain why there should or should not be more than two mating types and then discuss how they conform with the data and biology of Agaricomycetes. As there is probably a single origin of mating types in *Dikarya*, an inference based on the shared presence of transcriptional regulation of pheromone control (Kronstad and Staben, 1997), this question reduces to what are the factors that keep allele numbers from increasing or have facilitated their increase.

The mating kinetics model
Iwasa and Sasaki (1987) modeled the predicted number of mating types under varying types of "mating kinetics". These mating kinetics reflect how quickly mating partners are found and the number of opportunities to find a mate. When there is no cost for waiting to mate, i.e., being choosy, then there are no stable equilibria with more than two mating types. On the other hand, if there is only a single opportunity to mate and mating occurs in proportion to mating type abundance,

this imposes a high cost to those who do not find partners and provides a rare advantage. This leads to an ever-increasing number of mating types at equal frequency, limited only by their ability to be generated by mutation and to be maintained by balancing selection versus genetic drift. This model could support the observations that only mushrooms among fungi have large numbers of mating types. Specifically, yeasts signal and communicate with pheromones and are less sessile than mushrooms, giving them more opportunities to find partners. Likewise, filamentous ascomycetes and rusts produce aerial gametes (spermatia) that make them more vagile (Hiscock and Kües, 1999). Furthermore, the cost of not finding a partner in mushrooms is potentially larger than other fungi, because most species are obligately sexual and outcrossing. Mushroom basidiospores have evolved for long distance dispersal; billions are made per generation, and therefore the odds of any one surviving to reproductive maturity are vanishing, suggesting most spores contribute nothing to the next generation. Despite the fact that most spores only travel a minimal distance from the fruiting body of origin (Dam, 2013; Galante *et al.*, 2011), the low dispersal cohort is likely composed of numerous interacting conspecific and sibling mycelia, whereas the rare spores that disperse a long distance to arrive in prime habitat are strongly selected to be intercompatible, facilitating the evolution of a system with very high outcrossing efficiency. Lastly, because it is believed that hyphal fusion in the Agaricomycetes happens without regard to mating compatibility, the cost of incompatibility is even higher. Among the predictions of the mating kinetics model are that allele number scales with effective population size and is influenced by substrate because this dictates mean parent-offspring spatial distance.

The origins models
There are a number models on the origin of mating types from zero that are rather mechanistically explicit and cause

implicit constraints on the likelihood of a third or more mating type invading the population (Billiard et al., 2011). Thus, origins models, as considered here, provide rules to explain why multiple mating types seldom evolve. Where Agaricomycetes violate these model assumptions, therein reveals the explanation for why they can evolve multiple mating types.

Probably the most widely championed is the organelle inheritance model (Hurst and Hamilton, 1992). In this model, mating types evolved as a way to neutralize the negative impacts of selfish organellar genomes. The model hypothesizes that an ultracompetitive cytoplasmic genotype evolves that eliminates or out-replicates that contributed by the other parent during cytoplasmic fusion of gametes. Unchecked, such a selfish element is expected to go to fixation. A nuclear suppressor may arise that can lead to suppression of this competition by preventing its own cytoplasmic genome from killing. Both suppression and presence of ultracompetitive cytoplasmic elements are envisaged as having a cost, and therefore matings between suppressor + non-suppressor phenotypes which have enforced uniparental inheritance have the maximal fitness. This scenario selects for the evolution of a choosy allele that favors matings between cells of different suppression types. In this manner a mating type locus can be established that controls uniparental cytoplasmic inheritance. This model has great appeal in that it fits with the known systems by which mating type loci control the inheritance of organellar DNA in isogamous organisms like the alga *Chlamydomonas reinhardtii* (Gillham, 1969), the slime mold *Physarum polycephalum* (Moriyama and Kawano, 2003), basidiomycete yeasts and the zygomycete *Phycomyces* (Xu and Wang, 2015). The cytoplasmic fusion of gametes is predicted to lead to constant selection for ultracompetitive cytoplasmic genomes, and if the mating type locus controls cytoplasmic inheritance, it becomes difficult to maintain more than two mating types (Hurst, 1995, 1996). Therefore the model also explains the presence of multiple mating types in mushrooms and ciliates as a result of the fact that these organisms do not undergo cytoplasmic fusion during mating but rather have reciprocal nuclear exchange. Nuclear migration in Agaricomycete mating is controlled by the *P/R* mating type locus encoding pheromones and their receptors (Brown and Casselton, 2001). Nuclear migration, which can occur at rates as high as 4 cm/hr (Ross, 1976) and involves the dissolution of the septal pore complex occluding the passages between cells (Raper, 1966), is phylogenetically timed with the origin of hyper-polymorphic pheromones and receptors at the *P/R* locus, the switch to a filamentous homokaryon from a haploid yeast stage, and increasingly complex septal pore ultrastructure (Fig. 1). Whether this occurred as a series of events or concomitantly is unclear due to the paucity of mating studies on Dacrymycetes and other early diverging Agaricomycotina.

For all basidiomycetes that have cytoplasmic fusion rather than nuclear migration, the number of mating types is low (at maximum three). Many of the early diverging basidiomycetes have evolved morphological adaptation during mating in which there is minimal cytoplasmic mixing of gametes, such as the long bridge formed by fusion of conjugation tubes between gametes from which the dikaryon often, but not always, emerges, e.g., *Ustilago* and *Tremella* (Bandoni, 1963; Snetselaar and Mims, 1992). However, this gametic fusion

mechanism may actually increase competition amongst cytoplasmic organelles as they race to enter the developing dikaryon. Long conjugation bridges coupled with unidirectional nuclear migration across the bridge as reported in *Cryptococcus neoformans* (McClelland et al., 2004), may be the means by which uniparental cytoplasmic inheritance is generated and in which the negative impact of cytoplasmic warfare is reduced. Ultimately, if the cytoplasmic inheritance model accurately describes why mating type numbers remain low in most groups of basidiomycetes, it would predict that if species of basidiomycete yeasts are discovered with bidirectional migration across a conjugation bridge into fusing gametes, then these would evolve a multitude of mating types at the *P/R* locus and a higher outcrossing efficiency. The cereal smuts are an excellent system to test some of these predictions, as the group has 2–3 mating types at the *a* MAT locus which is known to control uniparental mitochondrial inheritance in *Ustilago maydis* through the action of two genes (*lga2* and *rga2*) (Fedler et al., 2009). It has been recently shown that the origin of the third mating type is ancient, occurring at least 90 mya (Kellner et al., 2011), however the majority of species have only a two alleles, showing the partial instability of the three mating type system. Understanding how cytoplasmic inheritance has evolved in concert with mating type over the phylogeny of cereal smuts would be an excellent test of the organelle inheritance theory for limiting mating type diversity.

Two other origins models both explain the origin of mating types and provide a constraint on the evolution of more than two mating types. In the "by-product" model a pan-sexual gamete type with a pheromone and a receptor evolves into two mating types by the loss of the pheromone in one mating type and the loss of the receptor in the other (Billiard et al., 2011; Hoekstra, 1982). After establishment of such a system, a third mating type is unable to evolve because it cannot be compatible with both of the existing mating types. In the "anisogamy consequence" model, following the establishment of anisogamous gamete types, a mating type locus evolves to enforce fusion between large and small gamete types to achieve maximal fitness (Billiard et al., 2011; Parker, 1978). As it stands, neither the by-product nor anisogamy consequence models are clearly related to observed constraints on mating type number in fungi. Anisogamy in fungi, where known, is always linked to hermaphroditism and has never evolved to be mating type specific. Pheromones and their receptors, likewise, always come in full complements, where both mating types have both receptor and pheromone genes in their genomes (except perhaps in zygomycetes) (Lee et al., 2010). Moreover, essentially all Ascomycetes have the same set of pheromones (**a** and α factor) and receptor genes and use differential gene expression to produce and secrete a mating type-specific signal that is used for mating (Hiscock and Kües, 1999). This is fundamentally different in Basidiomycetes where the **a**-factor pheromones and receptors have become mating type-specific by their genes being incorporated into a separate *MAT* locus (Fig. 1).

Although the organelle inheritance model has the best fit to the observed patterns of release from the origins imposed constraints in Agaricomycetes, it suffers from being a single phylogenetic event, concomitant with other cellular features,

and moreover not linked to the origin of mating types in fungi as this long predated the evolution of the Dikarya (Fig. 1). Moreover, there have been theoretical developments that suggest mating types may not have arisen to control cytoplasmic inheritance (Hadjivasiliou et al., 2013). On the other hand, the early diverging groups of Basidiomycota mate through cytoplasmic fusion and have uniparental inheritance controlled by mating type, which fits well with the model's prediction that more than two mating types are unlikely to be stably maintained. As a group, the organelle inheritance and all other origins models fail to explain the lack of multiple mating types in other branches of the tree where mating type is neither controlling organellar inheritance, gamete size, nor receptor-ligand presence.

The selfish nucleus model
A hypothesis that has yet to considered is that the long-lived heterokaryotic phase that so characterizes Agaricomycete mycelia, creates an arena that selects for high numbers of mating types. Mushroom heterokaryons have been likened to superorganisms (Rayner and Franks, 1987), because they may be comprised of multiple genetically different nuclei that together cooperate within a single mycelium, but only a portion (in space and genotype) actually contributes to reproduction. Studies in the field reveal that Agaricomycete mycelia quite often consist of more than two nuclear components (Anderson and Catona, 2014; Johannesson and Stenlid, 2004; Murata et al., 2005), and in the lab, trikaryons with three nuclear genotypes have been synthesized and readily maintained (James et al., 2009). Thus, although the concept of the individualistic mycelium showing somatic incompatibility between dikaryons has received much credibility through field and lab studies (Rayner, 1991), it appears that chimeric individuals are more common than appreciated. While these chimeras can facilitate the superorganism behavior of the mycelium, they could also facilitate the evolution of selfish genotypes, i.e., nuclei which have increased competitive abilities with respect to access to conspecific migration and partitioning into asexual spores (Rayner and Franks, 1987). Genes involved in generating these selfish behaviors are unknown, but leading candidates are mating type genes, because the mating type loci override the expression of somatic incompatibility observed between genetically distinct mycelia (Rayner, 1991). I hypothesize that multiple mating type alleles are required for creating higher order heterokaryons, because mating type genes control nuclear migration and communication (Brown and Casselton, 2001). Thus, a rare mating type would be advantageous for exploiting and reassorting with the largest number of other nuclei during mycelial encounters, and new or rare mating types should rapidly invade a population. Selfish nuclei may be able to use the shared mycelial connections to extract resources from other genotypes and use them for growth and reproduction, e.g., somatic cell parasitism (Buss, 1982; Coates and Rayner, 1985).

This hypothesis is partially supported by data showing that mating type controls nuclear identity, migration, and sexual selection (Brown and Casselton, 2001; Crowe, 1963; Nieuwenhuis et al., 2013b). This model predicts a more rapid turnover of mating types than other models through the consistent evolution of more "sexy" alleles that allow greater access to mycelia. This selection for alleles of higher male fertility, be they more compatible or promote greater access to other mycelia through nuclear migration, could promote diversification of both pheromone and receptor alleles through sexually antagonistic selection as seen in other reproductive proteins, such as sperm accessory proteins (Clark et al., 2009; Wong and Wolfner, 2012). This would arise because for example, the traits that promote greater male function, nuclear invasion/migration, have a direct negative impact on female function, through increased competition and resource depletion on the female side (accepting mycelia). On the other hand, although explaining the origin and maintenance of multiple mating types, the model is expected to lead to less promiscuity than other models because of the rise of selfish alleles that skew allele frequencies and cause the loss of rare alleles. One prediction of the model is that species that have multiple nuclei per heterokaryotic cell rather than the two observed in dikaryotic species would should have lower numbers of mating types and higher allele skew due to the increased competition, sexual selection, and selfish behavior promoted by this nuclear arrangement (Coates and Rayner, 1985; James et al., 2008).

Synopsis and prognosis

No theory fully explains the evolution of more than two mating types across the eukaryotes as it seems to be intrinsically different in ciliates, slime molds and mushrooms. Agaricomycetes are unique among the fungi by having nuclear exchange rather than cytoplasmic fusion during mating, frequent but costly sex (fusion without regard to incompatibility), coupled with sessile growth and low chances of finding a partner. Distinguishing among these theories could rely on the fact that the selfish nucleus hypothesis predicts that there would be greater skew in allele frequencies as some alleles are more sexy while the mating kinetics hypothesis argues that all alleles should be equivalent. The organelle inheritance model predicts that nuclear migration proceeded the evolution of multiple mating types of P/R. This could be tested by looking at mating in more of the earlier Agaricomycotina, especially Dacrymycetes. In the next Section I discuss the additional ecological and cytological correlates of mating allele diversity that may be used to test these three models. As mentioned, surveys of mating allele diversity are hard to conduct, but as population genomics with large sample sizes becomes more routine, these data will soon be readily accessible. Future emphasis will also need to be placed on cytological studies of nuclear migration, septal ultrastructure, and molecular and genetic studies of pheromone production and function.

3. Ecological and morphological patterns that are expected to correlate with mating type number

Regardless of why mating types evolved in the first place, what determines their numbers are likely multiple factors including mating kinetics, population biology, cell biology, and spore dispersal. If this assertion is true then there are several ecological and morphological traits that are expected to correlate with mating type numbers for each species. Below

I highlight some of these factors. According to the mating kinetics model the central driver of allele diversity is the optimization of outcrossing efficiency relative to the cost of not finding a mate. In cases where the odds of finding a mate are low, selection should promote mating type diversity. On the other hand, under the selfish nucleus model, selection on nuclear types for the purposes of exploiting other mycelia is expected to lead to skews in mating allele frequencies, as not all alleles have the same competitive abilities, and thus in species where traits facilitate selfish nuclear behaviors fewer mating type alleles are expected.

Habitat preference

Some correlations between mating system and substrate have already been noted for Agaricomycetes (Bayman and Collins, 1989; Gilbertson, 1980). These correlations relate the selective pressure for fast reproduction with the need for inbreeding avoidance mechanisms. Substrates should be variably selective for outcrossing efficiencies. For example, given that dung is an ephemeral and patchy resource, there is a high cost of not finding a partner quickly, hence the selection for dung inhabiting species to be homothallic. Likewise, the smaller the resource colonized, the less a homokaryotic mycelium will have to be choosy about a partner. In general, dung-inhabiting mushrooms should be selected for high mating type numbers when they are heterothallic, as observed for *Coprinopsis cinerea* (Table 1). Long-lived soil inhabiting species, such as the fairy ring mushroom *Marasmius oreades* and *Armillaria* spp., should have fewer mating types under the mating kinetics model.

Effective population size

One constraint on maximum allele number is likely to be effective population size, a population genetic concept relating the genetic diversity of the population in question to that maintained by a given number of individuals in a randomly mating, idealized population. This value often differs from the census population size because of unequal fecundity among parents or historical bottlenecks. Under the mating kinetics model, rare mating types are always favored by negative frequency dependent selection, and the limit of how many alleles can be maintained is an equilibrium between loss by genetic drift and creation by mutation (or recombination). In this scenario, species like *Schizophyllum commune* which have huge effective population sizes as measured by genetic diversity (Baranova *et al.*, 2015) also have high mating type diversity (Table 1). In contrast, largely inbreeding species like bird's nest fungi (Malloure and James, 2013) with smaller effective population sizes are shown to have lower mating type diversity (Table 1).

Spore dispersal mechanism

Spore dispersal is intimately linked with mating kinetics. Agaricomycete species that are eaten by herbivores, such as bird's nest fungi, stink horns, truffles, and other hypogeous fungi must have evolved under higher inbreeding because multiple spores from the same fruiting body are deposited into a substrate by animals. These species are expected to have lower mating type allele diversity as the costs of not finding a mate are less likely to be realized and inbreeding is likely. For epigeous species, if height is any indication of how far a species is likely to be dispersed, then costs to not finding a mate are higher, leading to greater selection for outcrossing efficiency. Predictions are that genera such as *Pleurotus*, *Fomes*, and *Ganoderma* that fruit high above the ground on standing wood should have species with high numbers of *MAT* alleles (Table 1).

Cytology

In many mushrooms the number of nuclei per cell in a heterokaryon is strictly limited to two, and dividing dikaryotic cells are usually distinguished by the presence of characteristic clamp connections involved in partitioning nuclei faithfully into cells (Kües, 2000). These cellular constraints limit the ability of higher order heterokaryons to form, confine interactions between nuclei to pairs, and thus limit the capacity for nuclei to behave selfishly. However, for >30 % of Agaricomycete species, the number of nuclei per heterokaryotic cell is greater than two and the presence of clamp connections irregular or absent (Boidin, 1971). Under the selfish nucleus hypothesis such species that have cellular pre-dispositions to forming higher order heterokaryons or unequal ratios of component nuclei would be subject to invasion by selfish mating types that drive out rare mating types. The rate of nuclear migration during mating is observed to vary over at least two orders of magnitude (Ross, 1976). The selfish nucleus hypothesis also predicts that species with high rates of nuclear migration may foster nuclear competition and mating allele skews.

Presence of asexual spores

The production of conidia or oidia on a heterokaryotic mycelium facilitates reassortment of nuclei and would increase opportunities for selfish nuclei to exploit mating partners. For example, in many species, the biased recovery of mating types among asexual and homokaryotic spores of heterokaryotic mycelium has been observed several times (James *et al.*, 2008; Kitamoto *et al.*, 2000). Under the selfish nucleus hypothesis, we would expect to see fewer mating types in species that make these spores. However, conidia could also function as a means to overcome the high cost of not finding a partner. Specifically, asexual sporulation of non-mated homokaryotic mycelia may be a means of dispersing from substrates lacking a compatible mate for colonization of additional substrates and continued search for compatible mates. This form of dispersal would reduce the cost of not finding a mate quickly and may reduce the pressure on outcrossing efficiency. The mating kinetics model predicts lower mating type diversity in species that make asexual spores in the homokaryotic state, while the selfish nucleus model predicts lower mating type diversity in species that make asexual spores in the heterokaryotic state.

Mating system

Tetrapolarity predominates among the two forms of heterothallic mating systems, and bipolarity seems to be irreversibly

evolved from tetrapolar (Nieuwenhuis *et al.*, 2013a). In every case in which bipolarity has evolved in Agaricomycetes, it involved the loss of mating types at the *P/R* mating type locus that encodes pheromones and their receptors (Heitman *et al.*, 2013). Because the pheromones and receptors are involved in nuclear migration and positioning, the switch to mating type determination being regulated solely by the *HD* mating type locus predicts that bipolar species may have different allele numbers because of different internuclear dynamics or mating kinetics. Bipolar species, in general, have greater potential for inbreeding than tetrapolar, especially through heterokaryon-homokaryon encounters. It has been speculated that bipolar species have lower numbers of mating type alleles per locus than tetrapolar (Raper, 1966), though the more recent appraisal of the data do not support this claim (Table 1).

In order to test whether the above correlates fit the observed patterns of diversity many more species need to be examined, and both allele number and ideally frequency would be estimated. Stringent tests of the above correlations should use phylogenetically corrected data to factor out shared evolutionary history (Felsenstein, 1985). Despite the temptation to analyze the existing data, it is probably too early to adequately test correlations between morphological and ecological traits and mating type numbers as those traits are highly labile within the lower taxonomic levels (families and even genera). Additional surveys of mating type number in more ecologically and morphologically diverse mushroom groups are needed.

4. Conclusions

Mushrooms are distinct among all eukaryotes in having both a long-lived heterokaryotic phase and high numbers of mating types. However, it should be clarified that the statement that they have tens of thousands of mating types is in direct conflict with their function. Instead emphasis needs to be placed on the minimum number of mating types per locus which will determine the overall outcrossing efficiency. If there is a deeper explanation for why only three major groups of eukaryotes have regularly maintained more than two mating types, it is unclear as mushrooms, ciliates, and slime molds have dramatically different mechanisms for finding a partner and creating a zygote. On the other hand, reciprocal nuclear exchange without cytoplasmic fusion is a common thread that unites the two examples of truly high polymorphism of mating types in ciliates and mushrooms; and this seems too much to be coincidence. Cytoplasmic fusion in early diverging basidiomycetes may have limited them to low numbers of mating types (usually two), and thus one answer to why mushrooms are promiscuous may be the evolution of reciprocal nuclear migration during mating. This innovation in basidiomycetes is coincident with the explosive allelic diversification of pheromone receptors of the *P/R* mating type locus and with the evolution of filamentous haploid stages. Within mushrooms, it seems mating kinetics as well as population genetic diversity must also play a role in determining how many mating types are in each species. While most models to explain the vast number of mating types in

mushrooms argue that outcrossing efficiency is the primary goal, it may be that the unique long-lived heterokaryotic stage of mushrooms provided the training grounds upon which the semi-autonomous nuclei could evolve selfish behavior as determined by mating type genes.

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