Genotyping, gene genealogies and genomics bring fungal population genetics above ground

James B. Anderson and Linda M. Kohn

As ubiquitous decomposers, symbionts and parasites, fungi build populations not easily accommodated by population genetic theory. Identifying and delineating individuals and populations is often difficult, and recombination can occur in complex and variable ways. Genotyping and gene genealogies provide the framework for identifying and delineating individuals and for detecting recombination in natural populations. Expanding genomic databases now make fungi ideal subjects for tracking mutation and expression in genes of adaptive importance in experimental populations.

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Fungi have provided some of the best model systems in genetics[1], including the first eukaryotic genome to be completely sequenced[2]. Although the genetic capabilities of fungi are well known from laboratory experiments, actual patterns of genetic transmission in natural populations have been less well understood. In this review, we consider recent approaches to two key questions that cannot be answered by direct observation. What are the fundamental units of fungal populations, and how important are asexual and sexual reproduction in determining their genetic structure? As fungal population genetics has been amply reviewed[3–6], we focus on recent studies identifying variation in DNA, especially nucleotide sequence data and genealogical analysis.

Fungi do not fit the classical models

Most fungi do not fit the classical models of population genetics. They have novel methods of colonization, dispersal, perennation and their generations overlap. Sexual reproduction can be irregular or absent altogether in some recently derived lineages[7]. Also, most fungi grow indeterminately within opaque substrates making direct measure of fungal biomass or census of individuals difficult or impossible. Under these conditions, there is no single definition of the fungal individual that serves all purposes. For example, the basidiomycete *Armillaria gallica*, can legitimately be considered to be one of the largest[8] or one of the smallest organisms. This depends on whether the unit counted is the entire genet or the ramet, which may consist of as little as a single totipotent cell. In fungi, even the fundamental distinction between growth and reproduction is not always clear. For example, several discrete mushrooms can be produced by one continuous mycelium that is hidden from view and is difficult, if not impossible, to trace physically within its substrate. In this case, which is the appropriate unit to count, the mushroom or the mycelium?

Like fungal growth, methods of fungal reproduction are extremely varied. Fungi produce a multitude of different kinds of propagule associated with meiotic and mitotic nuclear division[9] (Fig. 1). After their production, spores and other propagules can lie dormant in their substrates for long periods, and then germinate and grow rapidly under favorable conditions. This means that not all fungi in a locality or a habitat are active players at all times. Dormant fungal propagules, like seedbanks, effectively shelter genotypes from selection for various periods. Because of these complexities, fungal populations are sometimes difficult to define. By necessity, fungal populations are usually defined as a group of conspecific individuals in the same locality at the same time.

Genetic exchange is not limited to the sexual cycle

In addition to their varied methods of growth and reproduction, fungi have novel mechanisms of genetic exchange and recombination, which are not accommodated by existing population genetic theory – genetic exchange and recombination are not necessarily limited to the sexual cycle. Unlike plants, animals and bacteria, the vegetative cells of many fungi fuse readily with one another – hyphal fusion is a constitutive feature of nearly all ascomycetes and basidiomycetes (32 267 and 13 857 species are recognized in each group, respectively[10]). Vegetative hyphal fusion can be followed by a process of genetic exchange known as parasexuality[1]. In parasexuality, genotypically different nuclei coexist within the same hyphal compartments in a heterokaryon formed after hyphal fusion. Nuclei of different genotypes occasionally fuse with one another, and new genotypes are then produced by two independent processes: mitotic crossing over and haploidization with segregation of whole chromosomes. Both of these processes of recombination occur much less frequently in parasexual crosses than in sexual ones with meiosis.

Although parasexuality has been well documented in laboratory experiments, its occurrence in natural populations has not been proven conclusively. This would be difficult for two reasons. First, parasexuality is limited by the widespread occurrence of the somatic incompatibility (or vegetative incompatibility) response. This response is a self–nonself recognition system[11,12] that usually involves cell death and therefore restricts heterokaryosis among nuclei carrying different alleles at the loci determining the specificity of somatic recognition. Second, parasexuality has genetic consequences that are very similar to those of sexual recombination; both result in the reshuffling of entire chromosomes and in the recombination of alleles on the same chromosome through crossing over. However, parasexuality could be a potent evolutionary

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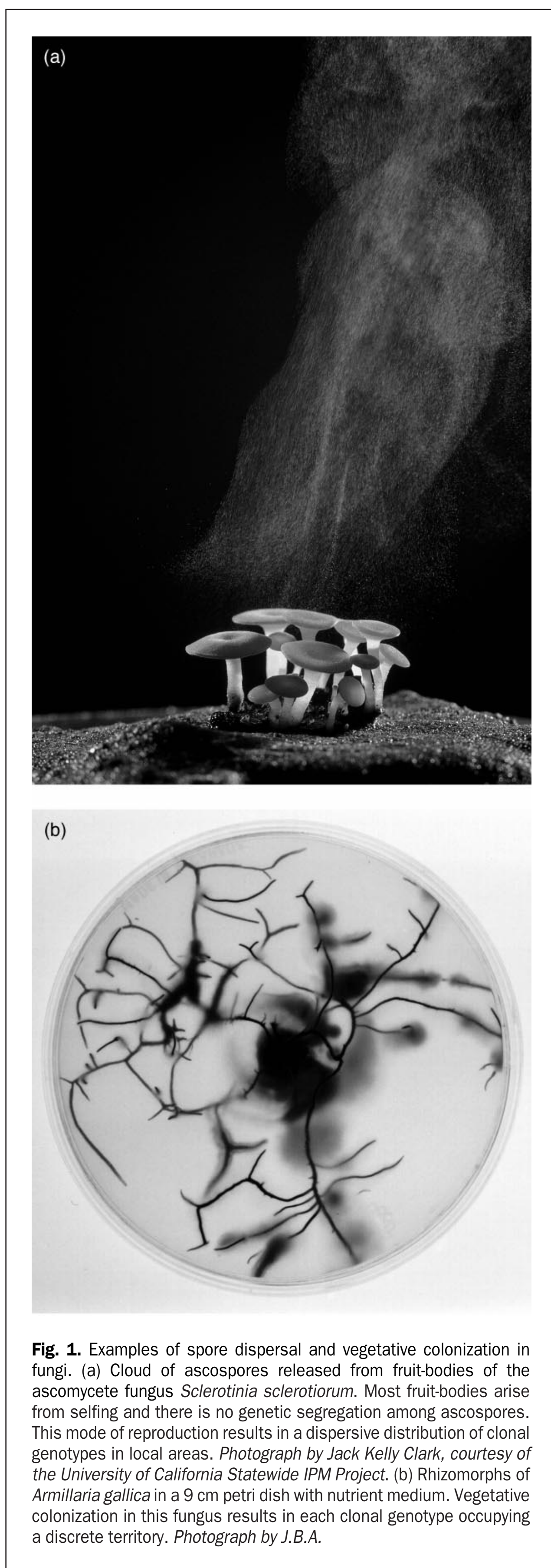


Fig. 1. Examples of spore dispersal and vegetative colonization in fungi. (a) Cloud of ascospores released from fruit-bodies of the ascomycete fungus *Sclerotinia sclerotiorum.* Most fruit-bodies arise from selfing and there is no genetic segregation among ascospores. This mode of reproduction results in a dispersive distribution of clonal genotypes in local areas. *Photograph by Jack Kelly Clark, courtesy of the University of California Statewide IPM Project.* (b) Rhizomorphs of *Armillaria gallica* in a 9 cm petri dish with nutrient medium. Vegetative colonization in this fungus results in each clonal genotype occupying a discrete territory. *Photograph by J.B.A.*

force in many fungi lacking a sexual cycle, because relatively few recombination events can have a disproportionately large impact on population structure[13].

Genetic clones are units of fungal populations

Given the diverse life histories of fungi, what are the appropriate units to count when characterizing fungal populations? One obvious approach is to identify clones resulting from asexual reproduction. Clones are identified as repeatedly sampled, multilocus genotypes that are unlikely to arise by chance in sexual reproduction[14]. Although most clones are of recent origin, some appear to be extremely ancient. For example, certain fungal clones cultivated by highly derived species of leaf-cutting ants could represent exceptional lineages that have managed, as the bdelloid rotifers have, to survive without a sexual cycle for tens of millions of years[15].

The patterns of clonal propagation detected in fungal populations are highly varied. In many fungi, clones are disconnected from their origins by dispersal, which can be local[16], continental[17] or even global[18]. In local areas, the spatial distribution of clones can be random[19] or nonrandom[20], and clone frequencies can change with time[19].

In some fungi, however, clones are spatially connected to their origins and are highly territorial[21]. In many basidiomycetes, each individual begins with a discrete mating event and then grows vegetatively to occupy a continuous territory. The territoriality of these individuals is evident at strikingly different spatial scales (Fig. 2). Some smaller individuals grow in discrete patches with visible borders that are the result of somatic incompatibility reactions between neighbors, whereas larger individuals grow vegetatively over long periods to occupy many adjacent root systems. In these fungi, somatic incompatibility maintains the integrity of individuals and prevents cytoplasmic continuity among genotypically distinct mycelia. Spatial dispersal by asexual propagules or mycelial fragments in these fungi appears to be rare. Although the dynamics of long-term competition and turnover among adjacent individuals is not known, the best information on the short-term dynamics of colonization and competition among individuals comes from cut spruce stumps and the subsequent colonization of root systems by *Heterobasidion annosum*[22]. Here, many individuals colonize the cut stump surfaces, but usually only one individual predominates in the lower portions of the root system. Whether the outcome of this process is random or determined by fitness differences among genotypes is not known.

Both sexual and asexual reproduction shape population structure

Once genotypes are identified, what are their patterns of descent? Fungal population structures range from almost completely clonal to panmictic[3]. The types of population structure described for various fungi could not have been predicted from what was initially known about their life histories. For example, in *Sclerotinia sclerotiorum*, a pathogen of a wide range of plants, sexual fruiting and meiosis occur every season, but the population structure is highly clonal[15]. The reasons for this are prolific asexual reproduction plus sexual reproduction by selfing of haploid mycelia, which results in meioses that are completely homozygous, with no genetic segregation. In *Mycosphaerella graminicola*, a pathogen of wheat, the sexual stage is not always observed, but the population structure is highly recombined[23]. Even for populations in which some individuals are extremely long lived, such as in the basidiomycete *A. gallica*, there is ample evidence of recombination, and no evidence of geographic subdivision, over thousands of kilometers within a continent[24].

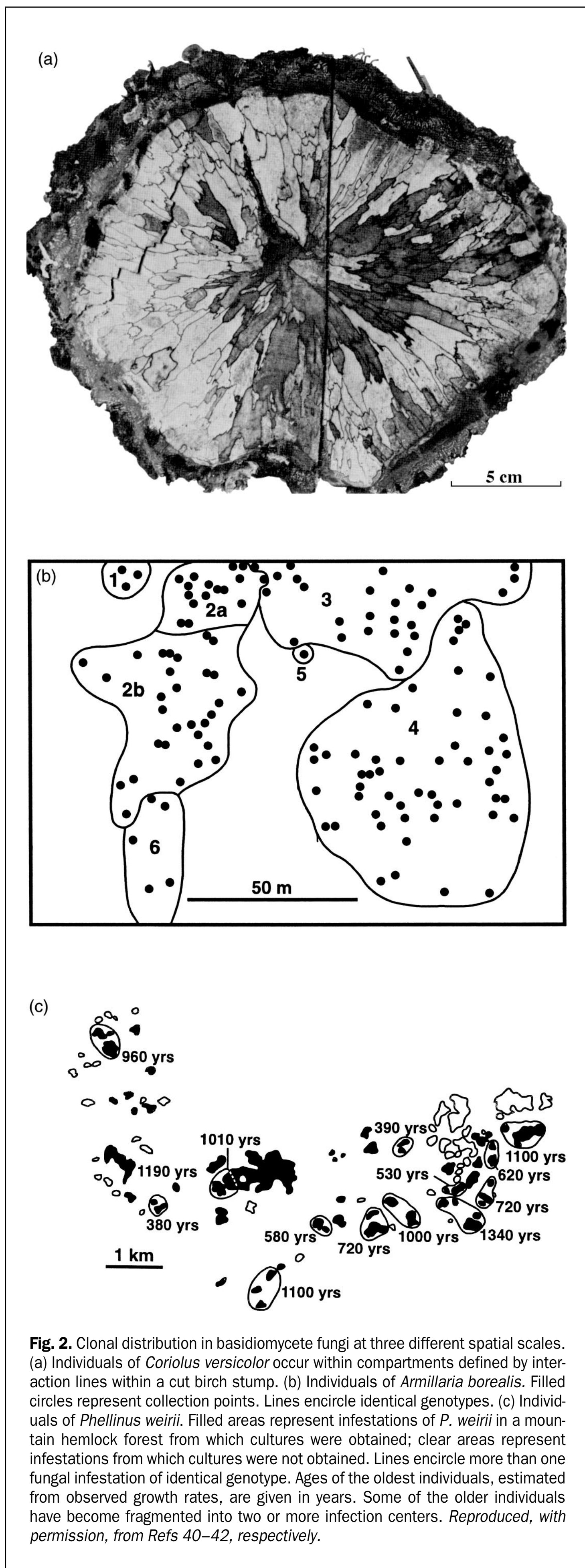


Fig. 2. Clonal distribution in basidiomycete fungi at three different spatial scales. (a) Individuals of *Coriolus versicolor* occur within compartments defined by interaction lines within a cut birch stump. (b) Individuals of *Armillaria borealis.* Filled circles represent collection points. Lines encircle identical genotypes. (c) Individuals of *Phellinus weirii.* Filled areas represent infestations of *P. weirii* in a mountain hemlock forest from which cultures were obtained; clear areas represent infestations from which cultures were not obtained. Lines encircle more than one fungal infestation of identical genotype. Ages of the oldest individuals, estimated from observed growth rates, are given in years. Some of the older individuals have become fragmented into two or more infection centers. *Reproduced, with permission, from Refs 40–42, respectively.*

Recombined populations that present no direct evidence of sex

The discovery of recombined population structures in species that present no morphological evidence of mating and meiosis[13] was unexpected. For these fungi, nucleotide sequence data and genealogical analysis allow a test of whether the observed genotypic diversity in a sample of isolates from a population is caused by the accumulation of mutations under a strictly clonal mode of reproduction or by the shuffling of mutations through recombination. The best criterion for detecting whether any recombination has occurred is the same as that used for bacteria[25]: the signature of recombination is incongruency in the genealogies of different genomic regions of DNA (Box 1).

Coccidioides immitis, the causal agent of valley fever in humans, is an example of a fungus with no known sexual stage, but with a recombined population genetic structure[13]. A recent study included 30 isolates of *C. immitis* from 25 patients and found 17 polymorphic sites in 14 anonymous regions of nuclear DNA. Twelve of these sites were phylogenetically informative in that each of two alleles was found in two or more isolates in the sample. Two different kinds of analysis of genotypic variation were consistent with genetic exchange and recombination and were irreconcilable with strict clonality. The first test was based on an overall measure of linkage disequilibrium, the index of association (I_A) among alleles at different loci. The I_A calculated from the observed genotypic data fell within the range expected under random mating. Although a high mutation rate alone might result in the absence of allelic association, this is difficult to reconcile with the low nucleotide diversity in *C. immitis*.

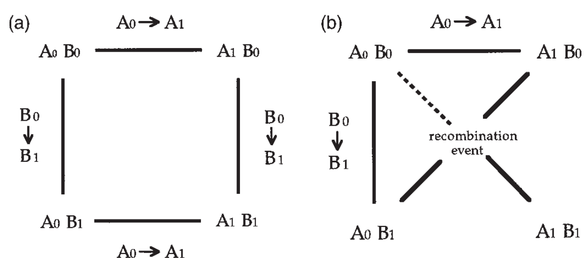
The more conclusive test of recombination in *C. immitis* is phylogenetic. If evolution is strictly clonal, then it should be possible to use nucleotide positions as characters and the nucleotides as states, and to infer a bifurcating tree of multilocus haplotypes of high internal consistency, but this was not the case. Instead, the shortest trees in *C. immitis* have high levels of homoplasy distributed throughout and are only marginally shorter than the average of those expected under random mating (Fig. 3). The consensus of the best trees from the observed genotypes has essentially no resolution. The homoplasy cannot be attributed to parallel mutation because the proportion of variable sites is low in all DNAs sequenced and only two of the four bases were present at each of these sites.

The failure of parsimony analysis to resolve relationships among haplotypes of *C. immitis* in the conventional phylogenetic sense leaves only one viable explanation: this fungal population is recombining (or at least has recombined in the past) and evolution is reticulate. The different nucleotide sites within an individual do not share one common history of descent, because alleles at different loci are shuffled by genetic exchange and recombination to create new genotypes. Recombination is also the more parsimonious explanation for the observed genotypes. When reticulation is allowed, and haplotypes are not forced to fit the tips of a bifurcating tree, substantially fewer mutational steps are required to explain the observed genotypes (Fig. 3). The recombination could be caused by sexual crossing, which has escaped direct observation, or by parasexual exchange.

Another pathogen of humans, *Candida albicans*, appears to have elements of both clonality and recombination[26,27]. Because *C. albicans* is diploid, the analyses are more complicated than for haploid fungi such as *C. immitis*. The gametic phases of alleles in *C. albicans* at different loci cannot

Box 1. The phylogenetic criterion for recombination

Incompatibility, the existence of all four possible combinations of two alleles at two loci, can be explained in either of two ways (a) by mutation alone or (b) by mutation with recombination. In the examples illustrated below, A and B are loci with two alleles, 0 and 1. A locus can be defined either as a segment of DNA with alternate nucleotide sequences as alleles, or as a nucleotide site with alternate nucleotides as alleles.



Mutation alone: a minimum of three mutational steps are required, including one parallel mutation or reversal.
Mutation with recombination: only two mutational steps are required, with no parallel mutation or reversal. Recombination can be detected only after the mutation at A and B have occurred[43]. The dotted line from the recombination event leads to the ancestral genotype and is therefore not detectable. Similarly, recombination between individuals of the same genotype, or between individuals having different alleles at only one of the two loci, is not detectable.

Incompatibility among loci can be interpreted as evidence for recombination if the overall mutation rate is low, and each allelic state reflects common descent for that locus. Multiple examples of incompatibility between loci provide the basis for arguments for recombination[12,28,32,33]. Detecting recombination in this way, however, neither shows how, nor how often, it has occurred.

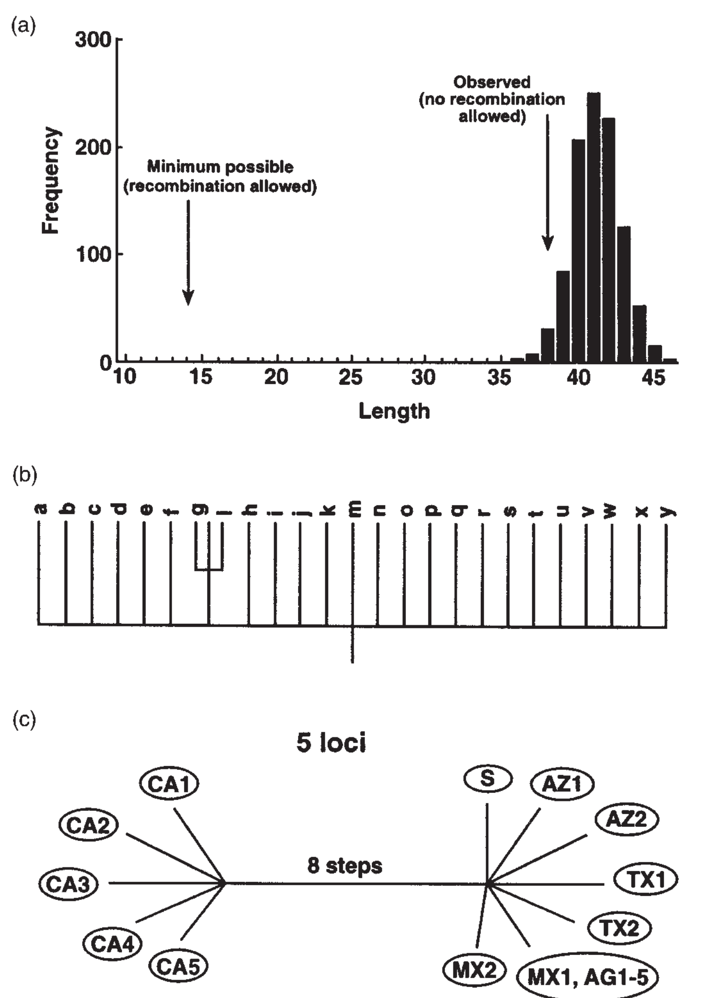


Fig. 3. Tests of recombination in *Coccidiodes immitis*. (a) Randomization test[13]: if no recombination is allowed, the minimum tree length required to explain the observed haplotypes of *C. immitis* is 38 steps. This value falls within the range of minimum tree lengths found for data sets in which alleles were randomly shuffled, and the difference from random expectation is only marginally significant (P=0.04; note, however, that in most phylogenetic data sets without recombination, the minimum tree length from the observed data is usually much less than those for randomly shuffled data sets). If recombination is allowed, the minimum number of mutational steps needed to explain the existing haplotypes is only 14. Given the low nucleotide diversity in *C. immitis*, recombination is therefore the more parsimonious explanation. (b) Lack of phylogenetic resolution[13]: strict consensus of the 62 most parsimonious trees showing only one internal branch. (c) Cryptic species of *C. immitis*[32]: polymorphic nucleotides in five different regions (loci) of DNA were considered jointly. The genealogy of 17 haplotypes has only a single internal branch of eight steps, which separates two reproductively isolated groups. These groups are found in different geographical locations. *Figures reproduced, with permission, from Refs 13 and 32.*

be determined. Also, gene conversion and mitotic crossing over in *C. albicans* lead to the loss of heterozygosity. The resulting genetic segregation occurs in the absence of any exchange of genes between individuals. We expect that gene genealogies for multiple nuclear loci will be necessary to resolve completely the question of whether genetic exchange and recombination occurs among individuals of *C. albicans*.

Whether the population structure of pathogenic fungi is recombining or clonal is important. Identification of fungal genotypes based on neutral markers would only be predictive of their other medically important characteristics, such as pathogenic capabilities and drug resistance, if the population were highly clonal, and this is not always the case. In a recombining population, any association between neutral markers and other traits would decay with time. Another important implication of recombination is that the fungi are expected to evolve more rapidly with regular genetic exchange and recombination than with strict clonality, because rare mutations to drug resistance or new pathogenic capabilities can be brought together more readily by recombination into new, more threatening genotypes. In a strictly clonal population, the same genotypes could only arise by sequential accumulation of rare mutations within a lineage[28].

Mitochondrial genomes also recombine

Although mitochondrial DNA (mtDNA) evolves clonally in many organisms, in a strictly clonal pattern of evolution, the observed mtDNA genotypes in natural populations of *A. gallica* are best explained by recombination, and not by mutation alone[29]. Recombination in mtDNA is well known from laboratory studies of fungi[29]. How can fungal mtDNAs of different descent come together and recombine? In basidiomycete fungi, there are no morphological sexes, and matings originate with the fusion of vegetatively growing haploid hyphae. Successful mating produces a dikaryon or diploid. At the same time, cytoplasmic mixing in the areas of hyphal fusion between paired mates carries the possibility of fusion of mitochondria and mixing of mtDNAs, with subsequent recombination. Because hyphal fusion is common in many fungi as part of the sexual cycle, mtDNA recombination, as described recently in *A. gallica*, could well be common in other fungi. This has two main implications. First, there will be no equivalent of the 'mitochondrial Eve' hypothesis[30] for fungi whose mtDNAs recombine, because there is no one genealogical history for the entire molecule. Second, mtDNA

recombination could be one way in which the accumulation of deleterious alleles is avoided, especially in populations of small effective size. In nonrecombining animal-mtDNA, the reduced stability of mitochondrial tRNAs relative to their nuclear counterparts is consistent with the operation of Muller's ratchet[31] (i.e. the inexorable accumulation of deleterious mutations in asexual populations of finite size).

Gene genealogies reveal barriers to gene flow

In fungi with genetic exchange and recombination, genealogical analysis can identify genetic barriers between groups of individuals[32]. In *C. immitis*, a second, sample of isolates showed the existence of two groups that are found in different geographical regions but are morphologically indistinguishable[33]. When nucleotide variation in five different regions of DNA is considered jointly, the genealogy of 17 isolates has only a single internal branch, which separates these two groups (Fig. 3). Within each group, gene genealogies are highly incongruent, indicating the prevalence of genetic exchange and recombination. Between groups, gene flow is absent. Consequently, these 'cryptic species' within *C. immitis* are highly differentiated with many fixed differences and no shared polymorphisms. A very similar situation occurs in *Aspergillus flavus*, except that one of the groups is clonal and the other recombining[34]. Complete genetic isolation carries the epidemiological implication that the fixed genetic differences might predict differences in pathogenicity or drug resistance.

Fungi as ideal subjects for experimental population genetics

Most previous studies of fungal population genetics have focused on selectively neutral variation in DNA (Ref. 35). The emergence of the field of genomics facilitates a significant new avenue of research in fungal population genetics. Genes of adaptive importance can now be more easily tracked in both natural and experimental populations. In almost any fungus, homologs of the thousands of sequenced fungal genes, whose function is often known, can be located. Several fungal genes are timely candidates for study in natural fungal populations. These include genes for toxins, antibiotics, drug resistance, mating type, somatic incompatibility and virulence.

A shortcoming of many studies of fungal population genetics is that they represent a snapshot in time, with limited ability to infer history. The next wave of research will be experimental. Because fungi can be maintained as sexual or asexual populations whose trajectory can be followed over time, definitive experimental tests of hypotheses about the costs and benefits of sex in large or small populations[36] can be designed. For example, in comparing large sexual and asexual populations of *Saccharomyces*, Zeyl and Bell[37] conclude that sex is more important for eliminating deleterious mutations in stable environments, than for producing new combinations of genes and speeding adaptation in new environments. Such experimental tests might now be extended further to link changes in fitness with changes in specific genes. Genomics technology[38,39] now offers the opportunity to test hypotheses of sex and adaptation in eukaryotes while simultaneously monitoring mutation and expression in every gene in the genome.

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Recent advances in South American mammalian paleontology

John J. Flynn and André R. Wyss

Recently discovered deposits containing terrestrial mammal fossils, together with multidisciplinary studies of classical sequences, have yielded dramatic insights into the biotic and environmental history of South America. Notable advances include several new fossil primate taxa, an improved chronology of two major immigration events (caviomorph rodents and new world monkeys), documentation of the oldest mammalian faunas dominated by grazing taxa (which suggests that grasslands appeared at least 15 million years earlier than on other continents), evidence of early biogeographical provinciality within South America, and improved sampling of the best known Cenozoic tropical South American paleofauna.

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South America was an island continent for most of the past 80 million years, from its separation from most other Pangaean landmasses in the Late Cretaceous 65–80 million years ago (Mya) until the beginning of its full reconnection with North America via the Isthmus of Panama about 3–3.5 Mya. This long-term isolation produced a highly peculiar terrestrial biota, of which the paleontologically best known component is a diverse array of endemic mammals (marsupials, edentates, primates, rodents, and numerous 'ungulate' groups; Box 1). Much has changed since Patterson and Pascual's[1] landmark paper in 1968 summarizing knowledge of the South American mammalian fossil record. At that time, there existed only one Mesozoic mammal specimen, a single age-calibrating radiometric date and scant information from outside the high temperate latitudes (Argentine Patagonia). Recent efforts have resulted in vast improvements, including better age control, enhanced geographical, temporal, paleoecological and paleoenvironmental sampling and a greater understanding of the phylogeny and diversity of South American mammals.

Showing remarkably few signs of its age, Simpson's[2] 'three-phase' or '3-stratum' concept remains a serviceable summary of South American mammal evolution (Fig. 1). Simpson's Stratum 1 corresponds to the continent's early phase of isolation, and the establishment of basal clades within many of its indigenous, 'archaic' lineages (e.g. edentates, notoungulate and litoptern ungulates, and various marsupials). This Late Cretaceous to Early Cenozoic (~80–40 Mya) interval was dominated by warm and humid, tropical–temperate forest environments throughout South America[3]. The arrival of immigrant taxa (rodents and primates, most likely from Africa), and 'modernization' of other faunal aspects during the mid-Cenozoic (reflecting adaptation to major environmental changes, including increased aridity and cooling) marks the base of Stratum 2. Stratum 3 corresponds to development of the Recent (Holocene) fauna, beginning with a few North American immigrants ('waif dispersers' or 'heralds' arriving sporadically, well before the main influx of 'legions' of diverse other taxa) around 10 Mya, culminating in the Great American Biotic Interchange (~3.5 Mya to the Present)[4,5]. Isotopic data from paleosols[6] and mammalian dental enamel[6,7] support the idea that climate and habitat change [including major changes in plant communities (from C_3 to C_4 dominated) beginning ~8 Mya] continued during Stratum 3. Recent work somewhat complicates this simple picture, providing important new fossil material (Fig. 2) and welcome details about the timing, patterns and process explanations for evolutionary changes embodying the 3-stratum phases.

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