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THE EVOLUTION OF ASEXUAL FUNGI: Reproduction, Speciation and Classification

JW Taylor[1], DJ Jacobson[2] and MC Fisher[1]
[1]*Department of Plant and Microbial Biology, University of California at Berkeley, Berkeley, California 94720–3102;* [2]*Department of Biological Sciences, Stanford University, Stanford, California 94305-5020; e-mail: jtaylor@socrates.berkeley.edu, djjacob@leland.stanford.edu, mfisher@nature.berkeley.edu*

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■ **Abstract** Phylogenetic and population genetic methods that compare nucleic acid variation are being used to identify species and populations of pathogenic fungi and determine how they reproduce in nature. These studies show that asexual or sexual reproductive morphology does not necessarily correlate with clonal or recombining reproductive behavior, and that fungi with all types of reproductive morphologies and behaviors can be accommodated by a phylogenetic species concept. Although approximately one fifth of described fungi have been thought to be asexual and clonal, recent studies have shown that they are also recombining. Whether a particular pathogen reproduces clonally or by recombination depends on factors relating to its biology and its distribution in space and time. Knowing the identity of species and populations and their reproductive modes, while taking a broad view of pathogen behavior in space and time, should enhance the ability of pathologists to control pathogens and even predict their behavior.

INTRODUCTION

Mitosporic fungi represent more than half of the Ascomycota and are very important to plant pathology. These fungi generally fall in two groups: species that lack the morphology of sex altogether and make only mitospores or no spores at all, and those that can make sexual structures with meiospores, but only rarely. In nature, both groups are usually encountered only in their mitosporic states. Until very recently, these organisms have been segregated in the Fungi Imperfecti or Deuteromycota and generally assumed to be clonal with very widespread distributions. In the last half-decade, analysis of nucleic acid variation has shown that mitosporic fungi can be classified with their meiosporic relatives and, where examined, that they can recombine in nature and show genetic differentiation and

isolation as do meiosporic fungi. In this review, we discuss how to use nucleic acid variation to find genetically isolated populations in nature and with this information how to detect clonality and recombination in nature. We show how the same approach and data can be used to diagnose species, to classify mitosporic and meiosporic fungi together, and to identify them. Finally, in a review of examples from recent studies of plant pathogenic fungi, we show that neither the type of spore nor the life cycle observed is predictive of the genetic structure, and that the reproductive mode in the same fungus can vary with space and time. Treating the fungi in groups based on life cycle, we illustrate that the tests for recombination are very sensitive, and that nearly all of the fungi studied show recombining population structures in addition to clonality. However, the tests currently available cannot tell how recombination occurs, how often it occurs, or when it last occurred. Answering these questions is the next challenge in the population biology of mitosporic fungi.

Plant pathologists have led the way in fungal population genetics, making fungi in genera such as *Ophiostoma, Cryphonectria, Puccinia, Armillaria, Sclerotinia, Mycosphaerella, Blumeria*, and *Phytophthora* among the best understood in terms of how they reproduce in nature, the limits of their genetically differentiated or isolated groups, and their spread through space and time. Plant pathogenic fungi lacking the morphology of sexual reproduction, e.g. species of *Fusarium* or *Verticillium*, have not received as much attention, despite their importance as plant pathogens. Recent studies of asexual animal pathogenic fungi have shown that population genetic studies of morphologically asexual fungi can have surprising results. Morphological species may contain cryptic species that are genetically isolated, and asexual fungi have been shown to be recombining in nature. With this information in hand, mycologists know which taxa are worth identifying and the type and number of genetic markers needed to identify species, populations, and individuals.

Access to nucleic acid variation and its analysis by cladistics have revolutionized phylogenetics and classification of asexual species by allowing mitosporic and meiosporic fungi to be classified in one common system (138). Characterizing nucleic acid variation within species and applying phylogenetic theory to population genetic questions is having a similarly iconoclastic effect on our notions of the reproductive mode and genetic differentiation of these fungi (2, 155). Our challenge in this review is to convince practicing plant pathologists that seemingly academic questions about evolutionary biology have practical application.

As a first introductory example we offer *Fusarium oxysporum* f. sp. *cubense*. This banana pathogen has been the subject of three studies published in 1997 (93) and 1998 (13, 123) that show that (*a*) the morphological species concept for *F. oxysporum* is so broad as to be meaningless, (*b*) the forma specialis concept is phylogenetically misleading, and (*c*) one cryptic species in this mitosporic fungus is likely to be recombining in nature. Pathologists now know that there are several genetically different *F. o. cubense* "species" infecting banana and have defined which variable DNA sequences can be used to identify them. This information

should help advance research on control methods, at the very least by ensuring that representatives of each group of *F. o. cubense* are considered when testing new fungicides or host cultivars.

A second introductory example is *Blumeria graminis* (= *Erysiphe graminis*) f. sp. *hordei*, the agent of barley powdery mildew throughout Europe. A series of remarkably thorough studies for the entire continent over several decades document both clonal and recombining reproduction and one large long-lived European population (170). Shorter-term regional genetic variation caused by strong selection due to differences in host resistance is also demonstrated, as are the homogenizing effects of migration (genotype flow) and subsequent mixing (gene flow). One retrospective study of mildew migration from the former Czechoslovakia to Switzerland and the UK shows how such large-scale studies with precise data could be used to predict disease outbreaks (169). Similarly thorough studies of other pathogens could make phytopathology a predictive science.

REPRODUCTIVE MODES IN FUNGI

There are two fundamental means by which fungi and other organisms transmit genes to the next generation, via clonal reproduction or via mating and recombination. Under clonality, each progeny would have one parent, its genome would be an exact mitotic copy of the parental one, and all parts of the genome would have the same evolutionary history. At the other extreme are genetically novel progeny formed by the mating and meiotic recombination of genetically different parental nuclei, events that cause different regions of the genome to have different evolutionary histories. However, fungi do not fit neatly into these two categories. Recombination need not be meiotic or sexual because mitotic recombination via parasexuality can mix parental genomes. Clonality need not be mitotic and asexual because self-fertilizing or homothallic fungi make meiospores with identical parental and progeny genomes. Even mating and recombination between genetically different fungi may approximate clonality if the partners routinely develop from sibling meiospores, as in pseudohomothallism. In addition to the observation that reproductive mode, clonal or recombining, is uncoupled from reproductive morphology, meiosporic or mitosporic, there is the complication that the same fungus may display different reproductive modes in different localities at different times. As a result, to discuss asexual fungi we must consider all types of fungal life cycles and reproductive modes. One more caveat: The methods currently available for study of microbial reproduction in nature can distinguish recombination from its absence, but they cannot reveal the mechanism nor estimate the amount of recombination. Therefore, in this review, reproduction by recombination is defined as the production of progeny genomes that are mixtures of genetically different parental genomes, and reproduction by clonality is defined as the production of progeny genomes that are identical to the parental genome.

Distinguishing Between Recombination and Clonality

Direct observation of microbial reproduction in nature is often difficult, whereas indirect assessment via analysis of genetic variation is not. All genetic methods used to distinguish between these two modes of reproduction exploit the fact that different regions of the genome have different evolutionary histories due to recombination but are inherited as a unit under clonality (155, 158). Evidence for clonality can be as simple as demonstrating an overrepresentation of identical genotypes, provided that the loci have been shown to be polymorphic among some members of the population. If only one polymorphic locus is available, reproductive mode can be inferred for diploid organisms because the clonal association of alleles raises the proportion of homozygotes or heterozygotes above that expected under Hardy-Weinberg equilibrium. For haploids, with only one allele at a locus, the same logic can be used with pairs of polymorphic loci. Here, clonality should reduce the frequencies of two of the four possible combinations of alleles for the two biallelic loci [00, 01, 10, 11] due to their association. Increasing the number of loci from two to ten or more to create multilocus genotypes makes it possible to use two other methods of distinguishing between clonal and recombining reproduction, the index of association (I_A) (115) and the parsimony tree length permutation test (PTLPT) (28). As before, both the I_A and PTLPT exploit the fact that all regions of the genome are inherited similarly in clonal populations, but not in recombining ones. The I_A was developed to study recombination in barley using the logic that genetic distances among recombining organisms should be normally distributed, whereas those for clonal organisms will show an excess of very close and very large distances (23). The statistic used by the I_A to describe the distribution of genetic distances among all pairs of organisms in a population is the variance; it is low when most of the samples are near the mean, as seen in normal distributions, but rises as the distribution is skewed toward the extremes. To establish the significance of the test, the I_A is calculated for the observed multilocus genotypes, and then for 1000 or more data sets for which the alleles at each locus have been resampled without replacement to simulate the effects of recombination. The mean I_A for the artificially recombined data sets is defined as zero, and significance is determined from the position of the observed variance in the distribution of variances for the resampled data (115). The PTLPT is derived from methods developed to detect signal in phylogenetic analyses (6). Parsimony trees are built for the observed multilocus genotypes and for data sets that have been resampled to simulate recombination as described above. The expectation is that a clonal population will support one or a few short, well-resolved trees, whereas a recombining population will support many, longer, and poorly resolved trees. In the parlance of phylogenetics, clonal populations evolve cladistically, whereas the recombining ones do not owing to reticulation as a consequence of each mating. Again, the null hypothesis is recombination, and significance is determined by the fraction of tree lengths based on resampled data that are as long as or longer than those based on the observed data (28, 83).

If there is sufficient nucleotide variation among individuals in the population to build well-supported gene genealogies, the topologies of the genealogies can be compared directly with the expectation that gene genealogies will be congruent for clonal populations and incongruent for recombining ones. The first method of comparison is a likelihood ratio test comparing the sum of the highest likelihoods of the gene genealogies independently and with the stipulation that all genealogies must have the same topology. Under clonality, with all regions of the genome having the same evolutionary history, the sum of the likelihoods with and without the stipulation should be similar, but not under recombination (28). The second, the partition homogeneity test, calculates the sum of parsimony tree lengths for trees built for the several regions of the observed data and compares the sum to the distribution of sums after the variable sites in each region have been swapped via resampling without replacement (54, 82). The sums should be similar under clonality, but not under recombination. For both tests, the null hypothesis is clonality, and significance is determined for the first test by the difference in likelihoods and for the second by the position of the sum for the observed data in the distribution of sums for resampled data.

All of the tests mentioned above detect association of alleles, and they can be influenced by phenomena unrelated to clonality or recombination. If the test supports clonality, there is the possibility that the loci are associated because of (*a*) physical linkage, (*b*) epigenetic effects, or (*c*) selection for more than one locus, all of which lead to association of alleles. Or, the individuals may have been sampled from reproductively isolated groups, in which case fixed differences in alleles between the groups would cause association among alleles even if recombination were the norm within each group (Figure 1). In cases in which the tests support recombination, an alternative explanation is that the loci are hypervariable, causing alleles to be identical by convergence and not by descent. However, these alternative hypotheses can be tested. Loci can be genetically mapped to ensure independence, populations can be tested for genetic differentiation or isolation, and hypervariable nucleic acid loci such as microsatellites and other short tandem repeats (STRs) can be identified and compared to loci with slow mutation rates, such as single nucleotide polymorphisms (SNPs). The problem of including fungi from more than one genetically isolated group in a test of reproductive mode is particularly important when working with plant pathogenic fungi because agricultural practices move these fungi beyond their site of origin. Defining and detecting genetically isolated groups of individuals places one on the slippery slope leading to species concepts, a topic that seems always to be the subject of intense debate (9, 21, 41a, 114).

Finding Polymorphic Loci

Methods of finding polymorphic loci and the relative utility of the different types of loci have recently been reviewed for fungi (155). Polymorphic protein loci continue to provide useful data for determining reproductive mode or genetic

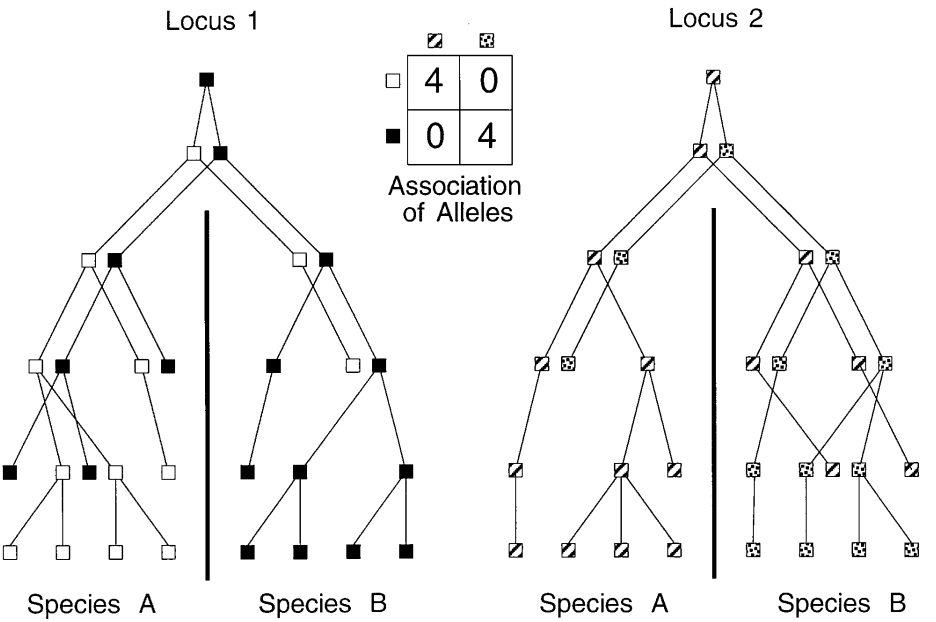

Figure 1 A demonstration with two neutral loci showing how genetic isolation leads to association among alleles of individuals from different species. For each locus, a barrier to genetic exchange (*vertical bar*) divides an ancestral population into two progeny populations. In each progeny population, ancestral variation is lost due to drift as described by Maddison (112a) and Avise (7a). If individuals from both species A and B are included in tests of association among alleles, there will be strong support for association and the inference of clonality. However, within each species, each locus is monomorphic and unsuitable for addressing association among alleles.

differentiation leading to isolation, but much nucleotide variation can go undetected when only the protein is studied. The best-characterized polymorphic DNA loci are those found by sequencing. They may be gene fragments or SNPs. An electrophoretic screening for variation in PCR amplified gene fragments or arbitrary DNA sequences via single strand conformation polymorphism electrophoresis [SSCP (29, 124)] prior to sequencing saves wasted effort. Testing for congruence of gene genealogies works only if substitution rates are high enough, say 1%, to provide sufficient variation to make well-supported genealogies. If the rates are low, 0.5 or lower, analysis of multilocus genotypes made of ten or more independent SNPs is the method of choice. Scoring SNPs can be simplified if the SNP lies in the footprint of a restriction enzyme, which happens about 75% of the time in our experience (30). A key advantage of both gene sequence and SNPs is that all alleles at a locus are observed so that diploid heterozygotes can be distinguished from homozygotes, i.e. the loci are codominant.

The many other methods of sampling nucleic acid variation at single loci, e.g. RFLPs, AFLPs, and RAPDs, can be problematic because there is more than one

way to lose a restriction site or a PCR priming site, leading to mistaken assumptions about allele identity (155). This ambiguity becomes more of a problem at greater genetic distances, and nucleotide sequencing should be used to confirm the alleles, if possible. Methods that sample many loci at once to make a fingerprint also suffer from the problem of unknowingly pooling different alleles, and the problem is compounded by the many loci being examined at once. Although fingerprint data may not be trustworthy for tests of reproductive mode, they can be useful for confirming clonal genomes or genetically isolated populations.

Loci may be misleading owing to hypervariability or selection. Loci that are suspected of being hypervariable, such as microsatellites and similar STRs, may be unsuitable for tests of reproductive mode. Fortunately, STRs are easy to identify from their nucleotide sequence. Loci under strong directional or balancing selection may also be misleading for tests of reproductive mode or genetic isolation. Selection can be detected by comparing alleles within and among closely related species (117), but the preponderance of neutral loci should make it unlikely that the odd selected one will skew results when ten or more loci constitute the multilocus genotype.

Defining Genetically Isolated Populations Using the Phylogenetic Species Concept

Fungal species concepts are overwhelmingly phenotypic, although there is general agreement that the Biological Species Concept (BSC) is well suited to fungi and should be applied where possible (132). Unfortunately, most fungi cannot be cultivated, and even fewer can be induced to mate in the laboratory. Fortunately, cladistic analysis and the nearly unlimited characters made available through direct sequencing of PCR amplified DNA have provided an alternative to the BSC in the form of a phylogenetic species concept [PSC, or rather PSCs because there are quite a few of them (114)]. Whereas the BSC is founded on the potential to interbreed in nature as determined from experimental matings, PSCs rely on cladistic analysis of variable characters, which can be the same polymorphic nucleotide positions used to analyze reproductive mode. The original PSC defined species as the smallest monophyletic clade of organisms that share a derived character state. This PSC certainly groups individuals, but deciding the limit of the species is arbitrary. For example, if the gene being used has two sequences, are they simply alleles in one population, or fixed differences in two populations? If several gene genealogies are available, their shared branches can separate genetically isolated groups, while the terminal branches that are incongruent reveal the conflicting evolutionary histories of individuals that are exchanging genes (7). The interface between gene genealogy congruence and incongruence appears to be an objective way to define the limits of species under the genealogy concordance PSC (Figure 2).

The *Gibberella fujikuroi* complex probably provides the best current test of compatability between species diagnosed using the BSC and the PSC. The BSC for *Fusarium* was begun over two decades ago by Snyder and colleagues (81) who used laboratory matings to identify three mating populations (= biological species). Since then, more mating populations have been characterized to give

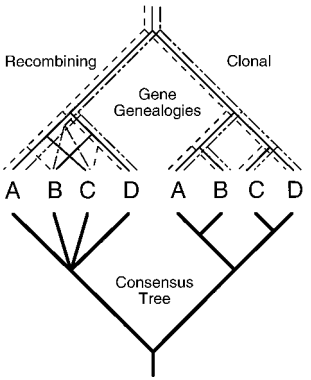

Fig. 2

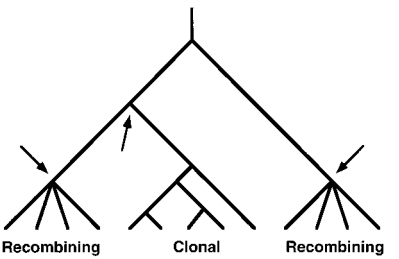

Fig. 3

Figure 2 Use of gene genealogies to distinguish between recombining and clonal reproduction. Within recombining species, gene genealogies are incongruent; within clonal species, they are congruent. Between genetically isolated species, gene genealogies are congruent. Consensus trees made from multiple gene genealogies show poor resolution within recombining species, but good resolution between genetically isolated species and within clonal species.

Figure 3 Diagnosing species with consensus trees made of multiple gene genealogies. For recombining species, the interface between incongruence (shown as a polytomy with its lack of resolution) and congruence of gene genealogies can be used to define the limit of the species. Clonal species would include all individuals subsequent to the last common ancestor of the clonal and recombining species.

a total of nine, seven of which are named (A–G) (105). O'Donnell et al (122) identified species in the *Gibberella fujikuroi* complex using a PSC. They studied what proved to be 45 species in the complex by first sequencing part of the β-tubulin gene for all isolates (as many as 50 or more for some morphological species, as few as one for others) and using the variation in this gene to sort the isolates into groups. The ITS and mt ssu rDNA were then sequenced for one representative of each β-tubulin group, and phylogenetic trees were constructed for the combined data. Among the 45 phylogenetic species are all 9 biological species, and 23 of the 45 species are new. The complete concordance of the PSC and BSC for the nine mating populations shows the compatibility of the BSC and PSC. Admittedly, the complete compatibility may be due to the conservative interpretation of a fertile cross in these species; perithecia must be large and ascospore production abundant before the cross is considered successful (105). The compatability may also be enhanced by the practice of lumping isolates based on one gene prior to examining other genes. Even if future studies where all isolates are characterized for all genes alter the PSC somewhat, it seems certain that the PSC and BSC will be very similar. In terms of mitosporic fungi, the two most important points are that 80%

of the *Fusarium* species were not diagnosed by the BSC and the ease with which mitosporic and meiosporic fungi are integrated in one tree using the PSC.

The lack of sexual morphology is no longer an impediment to understanding the evolutionary relationships of "imperfect" fungi [cf. (78, p. 282; 138)]. Nucleotide sequence has been used to integrate mitosporic and meiosporic species (109) and to place mitosporic species within a meiosporic genus [*Sporothrix schenckii* in *Ophiostoma* (14)] and a mitosporic individual within a meiosporic species [*Trichoderma reesei* in *Hypocrea jecroina* (101)]. There is the concern, however, that although the interface of gene genealogy congruence and incongruence can be used to define a recombining species, it would not exist for a clonal species because there would be no incongruence. This lack would be a severe problem if clonality proved to be very common among fungi, or if clades of exclusively clonal fungi were found. Fortunately, wholly clonal fungi appear to be rare, as will be seen below, and phylogenies combining mitosporic and meiosporic species, e.g. in the genera *Fusarium* and *Penicillium*, have not found exclusively mitosporic clades. Should an exclusively clonal fungus be found among recombining relatives, the entire clonal clade, up to but not including the presumed last common ancestor of the clonal and recombining sister groups, would be defined as a species (Figure 3).

The ease of phylogenetic integration of mitosporic and meiosporic taxa leaves the problem of naming taxa unresolved. Would it be worthwhile to rename all *Fusarium* species related to *Gibberella fujikuroi* as *Gibberella* species, or to rename all *Penicillium* species in subgenus *Biverticillium* as *Talaromyces* species? Given the nomenclatoral anarchy caused by the difficulty of adapting Linnean classification to phylogenetics and the impending creation of a code for rank-free classification (79), it is probably prudent to wait to rename large groups of mitosporic taxa until a consensus emerges. However, when mitosporic genera are found not to be monophyletic, renaming all but one of the clades would have the advantage of alerting researchers to the phylogenetic truth. For example, *Penicillium* subgenus *Penicillium* is not even the sister clade to *Penicillium* subgenus *Biverticillium* (15). Should someone decide to keep the species in the former as *Penicillium* and rename those in the latter as *Talaromyces*, confusion would be reduced.

FUNGAL REPRODUCTIVE STRATEGIES

The two examples given in the introduction showing how knowledge of reproductive mode and genetic isolation are germane to plant pathology do not do justice to the breadth of fungi or pathology. Therefore, we survey some of the better studied fungal systems to review our current state of knowledge of fungal reproduction. Our survey is organized using key aspects of the fungal life cycle and divided into four categories (Table 1): I. Heterothallic mating with a definite asexual phase; II. Heterothallic mating with little to no asexual reproduction; III. Homothallic (and

TABLE 1 Fungal reproductive modes inferred from population genetic data; species are grouped by sexual morphology and mating strategy

	Group	Species name	Population genetic structure*	Selfing?	References
Meiosporic taxa	I. Heterothallic with mitospores	*Blumeria graminis*	R & C	Yes	169, 170
		Botryotinia fuckeliana	R & C	Yes	43, 65
		Ceratocystis spp.	R	Yes	77a, 77b
		Cochliobolus heterostrophus	R		160
		Cryphonectria parasitica	R	Yes	113, 118b, 119
		Fusarium moniliforme (*Gibberella*)	R? & C?		81, 81a, 105
		Fusarium circinatum (*Gibberella*)	R & C		71, 164, 168a
		Histoplasma capsulatum	R	Yes	90, 152
		Magnaporthe grisea	R & Cr	Yes	107, 175, 176
		Mycosphaerella graminicola	R		38, 39, 178
		Ophiostoma novo-ulmi	R & C		19, 22, 118c
		Phytophthora infestans	R & C		49, 67, 68
		Puccinia graminis	R & C		25, 25a, 74, 97
		Rhizoctonia solani (*Thanatephorus*)	R & C		46, 112, 143, 162, 174
		Uromyces appendiculatus	R & C		73
	II. Heterothallic with no mitospores	*Armillaria gallica*	R		147, 148, 151
		Cronartium ribicola	R		66, 76
		Heterobasidion annosum	R		58, 59, 104
		Suillus pungens	R	Possibly	18
	III. Homothallic and pseudo-homothallic with or without mitospores	*Agaricus bisporus*	R	Yes, predominately	91, 172, 173
		Aspergillus nidulans	R	Yes, predominately	61, 62
		Sclerotinia sclerotiorum	R & Cr	Yes, predominately	31, 95, 96

TABLE 1 (*continued*)

	Group	Species name	Population genetic structure*	Selfing?	References
		Microbotryum violaceum	*	Probably	80, 89, 127
		Neurospora tetrasperma	R & C	Yes, predominately	85, 118, 133
Mitosporic taxa	IV. Mitosporic	*Aspergillus flavus*	Cr		10, 11, 63, 127a
		Coccidioides immitis	R or Cr		28, 56, 99
		Puccinia recondita	Cr		97, 128
		Rhynchosporium secalis	Cr?		26a, 116a
		Fusarium oxysporum f. sp. *cubense*	Cr		13, 93, 123
		Fusarium oxysporum f. sp. *melonis*	C?		5, 5a, 70
		Melampsora lini	R & C		24
		Peridermium harknessii	C		166, 167
		Verticillium dahliae	C?		48a, 89a, 118a, 145

*Key for genetic structure: R = Predominately recombinant; Cr = Predominantly clonal, but recombination occurs, either rare or cryptic; C = Clonal with no recombination demonstrated to date; * = Status undetermined at present, ? = Data suggestive, but not conclusive.

pseudohomothallic) mating with or without sexual reproduction; and IV. Asexual reproduction without known sexual mating. We realize that the lines between these categories are blurred at best, but use them to better illustrate the great variety inherent within fungi.

To fully describe the breadth of fungal reproduction and genetic differentiation we should include all fungal studies. However, the obvious constraints on the authors, publisher, and reader force us to focus on selected, recent studies to illustrate our points about the relative contribution of clonality and recombination, and the need to define reproductively isolated groups before attempting to assess reproductive mode in natural isolates.

Group I: Heterothallic Fungi with Mitospores

We start by considering the most common fungal life cycle, that which demonstrates heterothallic as well as mitosporic reproduction. Although it may seem odd

to begin a review of asexual fungi with those that are known to have heterothallic sexual reproduction, and to devote so much space to this topic, this is done to facilitate our understanding of the relative effects of these two reproductive modes on population genetic structure. Moreover, there is a direct connection because these are the fungi from which all "asexual fungi" appear to be derived [e.g. (109)]. In addition, the perception of what is a sexual or asexual fungus is controversial (157, 163) because fungi may display different behavior in different places and at different times. These differences are dependent on features that can be sorted into three categories: biology, space, and time.

Biology, space, and time have profound effects on amounts of recombination in nature, and examining studies of fungi in light of these three features may show how and why truly asexual taxa may evolve. With biology, the key factors are variation within the mating system of a fungus and its interactions with the host or hosts. For space, scale is a key aspect, and whether one is studying one wheat field or a whole continent has implications for the perception of reproductively isolated units and reproductive mode. Time is also a matter of scale, and both population structure and reproduction can show dramatic differences on recent, historical, and evolutionary time scales. To complicate matters, biology, space, and time are interrelated and all of these factors interact to influence each species. In focusing on population structure and reproductive mode in light of the three factors, we see that some species serve as examples of more than one factor.

Differences in Reproduction Based on Biotic Factors

Self-Fertilization Whether a fungus can self-fertilize and the amount of selfing that occurs in any given population have a basic effect on the rate of recombination. The line between strict outcrossing (heterothallism) and selfing (homothallism) may be illusory, and these terms no longer describe categories but only indicate the boundaries of a continuum from predominantly homothallic to predominantly heterothallic. A growing list of fungi reportedly have mixed mating systems, at least in the laboratory, where varying degrees of selfing are combined with predominantly heterothallic outcrossing [reviewed in (43, 131, 160)]. Many of these fungi appear to switch mating type to create sexually compatible individuals from a single haploid nucleus, although not necessarily by the mechanism described for yeast. Of particular note here is *Cryphonectria parasitica*, where the outcrossing rate has been estimated in natural populations by directly sampling ascospore progeny from perithecia in the field (113, 119). Based on RFLP multilocus genotypes and a DNA fingerprint marker, outcrossing rates were estimated to be between 0.68 and 0.79. Interestingly, selfing appears much higher in the field than the laboratory (RE Marra & MG Milgroom, personal communication), another indication of the need to base conclusions of mating systems on direct assay of field populations. Viruses complicate mating in *C. parasitica* because they can severely debilitate the fungus and cause hypovirulence. A significant symptom of viral infection is almost total loss of female fertility as well as reduced mitospore reproduction (also see the discussion of *Ophiostoma novo-ulmi*, below).

Requirement for Two Hosts Rusts provide the classical plant pathological examples of alternating meiosporic and mitosporic reproduction related to host interactions. Kolmer (97) recently provided a comparative review of virulence in North American populations of wheat stem rust (*Puccinia graminis* f. sp. *tritici*), wheat leaf rust (*P. recondida* f. sp. *tritici*), and oat crown rust (*P. coronata*). The heteroecious rust *P. graminis* f. sp. *tritici* requires barberry, as well as wheat, to complete its sexual cycle. In North America east of the Rocky Mountains, barberry has been eradicated and *P. graminis* f. sp. *tritici* has become a collection of persistent asexual clones. This host distribution has effectively switched this pathogen from being heterothallic with mitospores (group I) to being solely mitosporic (group IV) in this geographic area. *P. recondita* f. sp. *tritici* is also asexual throughout North America owing to lack of a susceptible alternate host. *P. coronata*, on the other hand, freely recombines throughout its range due to abundant sexual reproduction.

Recent characterizations of white pine blister rust, caused by *Cronartium ribicola*, have used Hardy-Weinberg equilibrium to support panmixis within spatially limited pine plantations (66, 76). This result is biologically comforting because pines are infected by only the meiotic basidiospores. However, geographically localized studies of this rust might show extensive clonal spread by mitospores on the alternate host, *Ribes* spp. The population structure needs to be placed in the context of the entire life cycle so as not to ignore the interacting organisms in larger spatial and temporal scales.

Uromyces appendiculatus, the bean rust pathogen, also has both sexual and asexual forms, but the separation, while often appearing in geographically different populations, may be based more on host preference. Asexual forms that have lost the ability to produce teliospores are found mostly on snap bean varieties in the tropics and subtropics where live host tissue is always available. Such asexuals are regularly transported to northern temperate regions, although they presumably cannot overwinter. A recent study comparing small sexual and asexual sympatric populations from Minnesota showed no difference in isozyme or virulence diversity between sexual and asexual populations (73). The high diversity in the asexual population was unexpected based on the wheat stem rust model. One of the two asexual populations assayed could have been the result of rare sexual recombination, but the other appeared stable in its asexuality. This result suggests that the migration of at least one asexual form north to Minnesota must have been both large in spore numbers and from a highly diverse population.

Genetic Isolation Due to Host Preference: Formae Speciales Commonly, the classification of groups of pathogens that have a common morphology but attack-different host plants is based on host specificity as exemplified by pathotypes and formae speciales. In some cases, the ability to reproduce sexually correlates with host preference and is used to classify "sibling species." This is well illustrated by the anamorphic genus *Colletotrichum* and its associated *Glomerella* teleomorph (156). For example, *C. graminicola* commonly occurs on maize, sorghum, and johnsongrass. Vaillancourt & Hanau (161) have induced both maize and sorghum

isolates to reproduce sexually in the laboratory, although the teleomorph has not been seen in the field. Isolates from the different hosts are not interfertile and they conclude that the two groups are genetically isolated sibling species. However, two studies (75, 144) present virulence and molecular data demonstrating that johnsongrass (*Sorghum halepense*) and sorghum isolates, at least, have a predominately clonal population structure. In this case, sexual compatibility may only correlate with genetically isolated clonal groups in nature, rather than having an important role in structuring populations.

Host species is not always an isolating factor and sibling species may occur on the same host. *Botryotinia fuckeliana* is the meiosporic state of *Botrytis cinerea* and causal agent of gray mold on many hosts. Giraud et al (65) characterized four RFLPs and two retrotransposon-like elements in 356 isolates of *B. fuckeliana* to show that there are two genetically isolated groups of this fungus on *Vitis vinifera*. One group, *Transposa*, had both repeated elements, whereas the other, *Vacuma*, had neither. Most pairs of loci were in equilibrium, and 30% of those that were not came into equilibrium the following year. *B. fuckeliana* mates in the laboratory, and the two mating types were in equal frequency in the field collection. All of these observations support two recombining populations. How two sympatric species with what must be very similar life histories can exist on the same host poses a puzzle. Giraud et al (65) suggest that *Transposa* is a resident pathogen on berries, whereas *Vacuma* is migratory and affects leaves. Here the host-isolating behavior would have to exist at the tissue level rather than species level, and it appears that isolation must be complete since the two sibling species show some sexual compatibility in the laboratory (T Giraud, personal communication).

Differences in Disease Cycles The heterothallic ascomycete *Histoplasma capsulatum* provides an example of how the same fungus can have a very different mode of reproduction depending on the biology of the disease that it causes. *H. capsulatum* also produces mitospores and causes infections in mammals, including humans. Three varieties are described (103): *capsulatum* is centered in the New World and affects humans, *farciminosum* is in the Old World and affects horses and other equidae, and *duboisii* is African and affects humans. The interesting biological difference between *H. c. farciminosum* and the other varieties is that it is a superficial skin infection passed from host to host by epidermal contact, whereas the other two cause pulmonary infections started by spores acquired from the environment, and host-to-host transmission is unknown. Nucleic acid variation had been used to define two classes of isolates in North American *H. c. capsulatum* (152) and to demonstrate recombination in the more common class (33). Kasuga et al (90) then used congruence of gene genealogies to discover at least six genetically isolated groups in *H. capsulatum*: two North American clades, which were similar to the classes defined earlier (152); one Central American clade; and two South American clades, one of which was quite variable and recombining. All isolates of *H. c. farciminosum*, however, had identical genotypes and fit solidly in one of the South American clades, despite their existence throughout

the Old World. Kasuga et al (90) explained this surprising finding about *H. c. farciminosum* in terms of the biology of infection: Whereas *H. c. capsulatum* is always acquired from the environment and samples the population of recombined pathogen genotypes, *H. c. farciminosum* is passed from host to host as a single clonal lineage.

Differences in Reproduction Based on Spatial Distribution

Biological variation in fungal life cycles is often expressed in spatial distribution; specifically for our purposes, the division and persistence of clonal and recombinant phases is often directly due to the biological properties of different spores stages. Mode of distribution (e.g. wind-borne, rain-splash, or insect-vectored), survival potential, and sheer numbers are often significantly different between meiospores and mitospores of the same species. The results can be profound, as alluded to above, affecting population structure at both very large scales (continental and subcontinental) down to the local scale of single fields.

Large-Scale Differences in Population Structure Plant pathology has provided many examples of how moving a fungus from its normal biogeographic range to a new environment across or between continents can change its reproductive mode and population structure. The change may be due to a new environment not being conducive to meiospore development or to a genetic bottleneck that reduces an individual's chance of finding a mating partner. This phenomenon is well illustrated by the movement of *Phytophthora infestans* out of Mexico to the United States and Europe. For *P. infestans*, founder effects have severely curtailed opportunities for recombination because the worldwide population appears to have been founded from a single genotype of the A1 mating type, rendering reproduction exclusively mitotic for 120 years (67). Widespread transmission of strains of the A2 mating type in the 1970s demonstrated that recombinant genotypes can occur rapidly (49) or may take longer to emerge (68, 69), depending on the physical proximity of individuals with the two mating types.

Similar geographical patterns of genetic diversity are seen in the rice pathogen *Magnaporthe grisea* [recently reviewed in (175)], where populations found in Europe and North and South America are composed of low numbers of lineages (typically 5 to 17), with populations often dominated by a single lineage (107, 171). In this system, lineages are defined by the authors as isolates sharing $>80\%$ similarity in RFLP fingerprints of the multilocus marker MGR586. Here, lineages are generally correlated with limited pathotypic variation and show strong specificity to certain rice cultivars (146); these data have been interpreted as showing that *M. grisea* is evolving clonally in these populations (175). In contrast, levels of genetic variation seen within presumably native Asian populations of *M. grisea* are high, with up to 46 lineages identified within small geographical areas (175). Although perithecia have never been observed in nature, they can be demonstrated in the laboratory in vitro and in planta (150), which shows that recombination in nature is theoretically possible. That rice isolates of *M. grisea* are typically female-sterile

compared to fertile isolates derived from wild grasses and millets (175) has led to the viewpoint that *M. grisea* is asexual on rice and sexual on other grasses, and may be composed of host-limited lineages. Studies of *M. grisea* on rice in the Indian Himalayas have shown that all four allelic combinations are observed for the most common alleles of pairs of loci defined by single-locus probes, and that loci for clone-corrected data sets are in linkage equilibrium (175). This result suggests that recombination is contributing to genetic structure here, although whether recombination occurs on rice or another host is not known. The geographic transposition of rice pathotypes out of Asia appears to result in new populations that have low sexual competence and a predominately clonal population structure, in contrast to the population structure seen within the endemic range of the pathogen. Although the relative contributions of meiotic and mitotic recombination are as yet unknown, there is evidence that mitotic recombination may have occurred between isolates in the field (176).

As mentioned above, absence of the alternate host has created large-scale geographically isolated asexual rust populations. Classic studies of stem rust isozyme and virulence diversity in *P. graminis* (25, 74) have shown (*a*) that there is significantly more virulence and isozyme diversity in the sexual versus asexual populations; (*b*) that there is a strong association between isozyme and virulence phenotypes only in the asexual population; and (*c*) that the sexual population approximates the structure expected from panmixis. It is postulated that the majority of the asexual population is composed of direct descendants of the genotypes present at the time of barberry eradication when the population ceased to recombine. The cereal rusts also provide good examples of the relationship between sexual reproduction in the pathogen and durability of host resistance (97). The effectiveness of resistance to the asexual population of *P. g. tritici* stem rust is high, whereas host resistance to sexual populations of oat crown rust (*P. coronata*) is much more short-lived. Leaf rust (*P. r. tritici*) provides a more complicated and apparently contradictory example. Like stem rust, it relies entirely on mitospores, but, compared with stem rust, it has considerably more diversity of pathogenic phenotypes and a smaller-scale regional structure of these pathogenic races. The durability of host resistance to *P. r. tritici* is more variable due to the apparent ability of the fungus to "respond quickly to the selective effects of host resistance."

Modification of Spatial Differences by Migration of Sexual Progeny Changes in the genetic diversity of isolated asexual populations have been well documented (26). The mechanisms behind these changes can vary from mutation to somatic recombination to immigration of new genotypes from separate populations. Often the influence of immigration from more diverse sexually recombining populations is dependent on geographic barriers and the proximity of sexual and asexual populations. For *P. graminis* stem rust in North America, the Pacific Northwest sexual and Great Plains asexual populations are separated by the Rocky Mountains, and consequently migration of genotypes from the sexual to asexual populations appears to be rare. Recently, however, a new race appeared in the Canadian plains

that has numerous virulence determinants and rDNA different from the predominate asexual clusters [reviewed in (97)]. This race appears to have migrated from the sexual population to the asexual one.

The rust *Melampsora lini* on native *Linum marginale* in Australia provides a different example of migration between geographically separated sexual and asexual populations on a subcontinental scale. This system has been used as a prime example of metapopulation dynamics in plant-host systems where small discrete populations are linked by migration events, but undergo extinction and colonization events independently of each other. A series of studies in Mt. Kosciusko National Park used race along with isozymes and RFLPs to make the case for clonal spread and migration among several small, infected host populations [reviewed in (24)]. In this limited geographic area, host-resistance and pathogen-virulence genes were randomly dispersed, which suggests that host-pathogen genetic interactions were less important than genetic drift in shaping the rust populations. Pathogen race frequencies changed markedly from year to year, and all possible combinations of pairs of race and RFLP markers were not found, providing no evidence for recombination within the national park. In the absence of recombination, the effects of local extinction and recolonization were very important in determining virulence structure of the pathogen population. However, this fungus is known to reproduce sexually further south in Australia. This separation of reproductive styles is due to climatic factors, not presence or absence of alternate host species. Moreover, the presence of very different RFLP types at Mt. Kosciusko provides some support for immigration of new pathogenic races (24). Such studies highlight the potential of fungi to reproduce by different modes in different locations and to exchange genes among distant locations by migration. The working pathologist is often interested in a small geographic area, but unraveling the behavior of the pathogen may also require studying distant populations.

The effect of migration on population structure is also clearly seen in *P. infestans*, as mentioned above. Drenth et al report a rapid breakdown of the clonal population structure of isolates recovered from potato fields in Europe after introduction of the A2 mating type into the region (49). In the United States, however, new, recombined strains have been observed much less frequently. That the A2 strains tend to have high virulence and resistance to the fungicide metalaxyl perhaps explains why they tend to displace the A1 mating strains (e.g. the US1 genotype) rather than mating with them (68) (also see below).

The Unrealized Potential for Recombination in Geographically Separated Populations One species of the *Fusarium/Gibberella fujikuroi* complex, *F. circinatum* (=*F. subglutinans* f. sp. *pini*) deserves further mention because its reproductive mode appears to vary with geography. *F. circinatum* is currently causing outbreaks of pitch canker of pine in California and South Africa and has long been known on pine in the southeastern United States. Viljoen et al (164) used vegetative compatibility to show that South African isolates reproduce clonally [69 isolates fell into 23 vegetative compatibility groups (VCGs)], but the relatively high number

of VCGs and the demonstration that South African isolates will mate in culture led them to conclude that recombination was also a possibility in nature. With isolates from Florida, 45 VCGs had been found in a sample of 117 individuals, which provides direct evidence for clonality and indirect support for recombination as well. In California, where the pathogen was recently introduced, only 8 VCGs were found in a survey of 152 testable isolates (71), which indicates that clonal reproduction was occurring, and suggests that recombination was absent. However, Wikler & Gordon (K Wikler & TR Gordon, unpublished data) have just shown that California isolates will mate in many combinations to produce recombined progeny, so the possibility that recombination is occurring in nature in California exists and perhaps the low number of VCGs simply reflects the low number of polymorphic *het* or *vic* loci in this population.

An extreme example of the potential for sexual reproduction, which is probably unrealized, is seen in the *Colletotricum gloeosporioides/Glomerella cingulata* complex. Isolates from different hosts (pecan and jointvetch) are sexually compatible and are able to cross on jointvetch stems under controlled conditions, but there is no conclusive demonstration of hybridization in the field (40, 41). The similar finding with *Botryotinia fuckeliana* mentioned above (65) was explained by finding two "cryptic species," and one wonders if this is a possibility with *C. gloeosporioides*. Dogma has it that fungal hybrids are rare in nature, as suggested by these examples, but they are not unknown and one case is germane to asexual fungi. Schardl and colleagues have documented natural hybrids between an asexual grass endophyte (*Acremonium lolii*) and its closely related meiosporic pathogen (*Epichloë typhina*) (149, 159).

Local to Regional Scale Differences in Population Structure Clonal reproduction via mitospores makes it possible for a local collection of clones to predominate within a much larger recombining population. Again, the biological features of these spore stages, especially the mode of dissemination, will influence the scale of clonal spread.

Blumeria graminis f. sp. *hordei* causes powdery mildew of barley and has been studied intensively in Europe since the early 1980s in response to the ability of the pathogen to develop virulence to a series of host-resistance genes, the first of which was introduced in the 1930s (170). Evidence from multilocus phenotypes (combinations of virulence loci) and nucleic acid variation indicates that one population is present in all of Europe, but that strong selection for virulence alleles by host resistance makes for local variation over periods of several years. Migration of pathogens that are virulent on newly planted host genotypes provides clear evidence of clonal spread via wind-borne mitospores, but these invaders rapidly mix with resident populations to provide new genotypes that show recombination of virulence loci and DNA markers. Maps of Europe showing the change in pathogen genotype over space and time offer the possibility of predicting outbreaks due to migration (169), and of preventing them by planting hosts with different resistance genes or ameliorating the problem by planting mixtures of different host genotypes

(121). Other formae speciales of *B. graminis* have not been studied as closely, and given the confusion regarding formae speciales in genera such as *Fusarium*, it would be worthwhile to do so.

The contribution of sexual and asexual reproduction in the wheat pathogen *Mycosphaerella graminicola* has been well studied (38, 39, 116, 178). Through hierarchical sampling of lesions on wheat and genetic characterization by means of RFLP loci and DNA fingerprints, geographically distinct populations all showed high levels of genotypic diversity and loci under gametic equilibrium, which suggests that genetic structure was determined primarily by sexual reproduction and that epidemics were established by ascospore rain. The role of asexual reproduction, the morphologically predominant reproductive mode of these pathogens, was limited to spreading clones on very small spatial scales ($<$10 m). These studies are noteworthy because they demonstrate the power of molecular methods to indirectly demonstrate sexual reproduction in the apparent rarity or absence of a teleomorph.

Rhizoctonia solani, a complex of genetically isolated species with common morphology, is associated with a *Thanatephorus* meiosporic state (162). Hyphal recognition involved in self-nonself recognition has been used to establish anastomosis groups (AG) in *R. solani* that correlate with phylogenetic species defined by analysis of nuclear large subunit rDNA and ITS variation (102, 162). Sexual reproduction has been demonstrated for about half of the AGs (46) and clonal reproduction is by sclerotia. Some AGs have been subdivided into several phylogenetic species (162), e.g. AG1 comprises AG1-IA, -IB, and -IC. Pettway et al (132a) characterized seven codominant RFLP loci for 182 AG1-IA isolates in Texas rice fields, and found 36 different multilocus genotypes. There was no evidence for genetically isolated groups, and the authors consider all of these Texas isolates to be one population. There is clear evidence of clonal reproduction in finding repeated genotypes, and in finding the same genotype in well-separated fields. However, just over half of the loci were in Hardy-Weinberg equilibrium and only one of all possible pairs of loci was in linkage disequilibrium, which indicates that recombination is also occurring. The authors suggest that the loci not in Hardy-Weinberg equilibrium can be explained by a recent bottleneck in population size, a likely explanation given that this rice pathogen was almost certainly introduced from Asia in the recent past (112, 132a). Macnish et al (112) have used careful examination of anastomosis and isozymes to study AG8 in Australia. Here they find an association of anastomosis reaction type with isozyme phenotype, and an overrepresentation of certain phenotypes, which they take as evidence for clonality. Yang et al found a similar lack of variation with RAPDs, which again argues for clonal spread (174). Given the importance of space and time in the evolution of pathogens, studies of *R. solani* AGs in their native locations (e.g. Japan for AG1-IA) might be very useful in explaining the behavior of this fungus in newly colonized areas such as Texas or Australia.

A different type and scale of spatial division of reproductive strategy has been apparent in the recent epidemics of Dutch elm disease in Europe caused by *Ophiostoma novo-ulmi* (19). Here the progressing front of the epidemic (pathogenic

phase) was characterized as asexually reproducing collections of limited VCGs all of a single mating type. Behind this front, when *O. novo-ulmi* entered the saprophytic phase in elm bark, the teleomorph was common and the population then consisted of many apparently recombining progeny. Also associated with the saprophytic phase was a cytoplasmic mycovirus, d-factor, which had a detrimental effect on the host fungus fitness by reducing growth rate and mitospore germination [reviewed in (22, 168)]. It is postulated that d-factor is readily lost by the fungus both during growth in the pathogenic phase and after sexual reproduction in the saprophytic phase. Therefore, its effect would be limited to growth while in elm bark and perhaps during dissemination by beetle vectors. The spatial division between the clonal and recombining phases of *O. novo-ulmi* may be mediated by d-factors that prevent infected, debilitated strains from colonizing xylem in the pathogenic phase (168).

Differences in Reproduction Over Time

Obviously, each of the examples already mentioned has a temporal component; all change takes place over time. For example, the metapopulation approach to describing host-pathogen interactions combines spatial and temporal aspects of local extinction, colonization, and migration events. Epidemiology, moreover, describes disease spread by combining spatial and temporal aspects of pathogen reproduction. One of our main concerns in this review is how agriculturally relevant time periods and long historical time periods both affect the contribution of clonal and recombining reproduction to structuring populations.

Changes in Reproduction in the Short Term The short term for most pathogens can be considered as both within and between epidemics, that is usually within and between years or growing seasons. A classical epidemic cycle, fitted to our Group I fungi, would involve primary inoculum, most likely meiospores, establishing the pathogen among the host, and then subsequent secondary spread of the pathogen, usually through mitospores. A seasonal transition to sexual reproduction of the pathogen would provide survival until the next cycle begins. This model fits most of our examples covered so far, if allowances are made for variation in the frequency of reproductive events.

Of particular note here are those examples that deviate significantly from this model cycle. Recent studies of *M. graminicola* sampled over three years in a single naturally infected wheat field found that the pathogen's genetic diversity and population structure conform to a hypothesis of random mating both within and between epidemics, with little evidence for clonal spread (38). A very low frequency of clones was found among separate leaves in the same field (maximum of 4 isolates from the same clone in 444 isolates sampled), and no clones were "conserved" between years. Furthermore, a maximum of only 7% of 617 genotypes were found to have reproduced clonally during a single year (39), contrary to the expectation that the clonal proportion of the pathogen population would increase during an epidemic. Immigration and recombination during an epidemic were tested for in a controlled experiment whereby a field was artificially inoculated and sampled

three times over the season (178). By the third sampling, the authors estimate that 10% of the isolates sampled originated from immigration and 24% originated from recombination within the field, with the remainder the result of asexual reproduction of the original inoculated strains. These studies together suggest that asexual spread is of minimal importance to the epidemiology of this disease. This is particularly surprising given that the teleomorph is not commonly noticed on wheat during an epidemic.

Medically important fungi may behave similarly. For example, *H. c. capsulatum* makes meiospores and mitospores, and the small microspores (microcondida) are the infective propagule. In spite of the necessity of mitospores in the disease cycle, clinical isolates show great variation and their multilocus genotypes support recombination (33).

Changes in Reproduction in Historical Time It is easiest to think of historical time in terms of generations, human not fungal. Human intervention in transporting pathogens to new areas, changing host genotypes through introducing resistance, and changing environments by reducing biodiversity and establishing large-scale monoculture has had profound effects on fungal reproduction in historical time. However, time has not obscured the footprint of sex in some predominately clonal fungi, as has been alluded to above. The footprint may be so old that the clones are now the genetically isolated units, which raises a most pressing question: how to determine the length of time since recombination last made a footprint?

Again, the separation of *P. graminis* stem rust into sexual and asexual populations provides an excellent example. This system may also provide a good test of the sensitivity of the gene genealogy methods proposed here. The loss of sexual reproduction east of the Rocky Mountains is known to have occurred with the eradication of barberry in the 1920s. This loss allows us to ask whether historical evidence of recombination remains in these populations, as judged by congruent genealogies. If so, we can then address the question of how long the footprint of recombination remains evident under very severe selection pressure for host virulence. Similar tests with *P. recondita* leaf rust may reveal any differences in the loss of congruence under less severe selection pressures, but still in asexually reproducing populations. These rusts may provide a benchmark on which to judge the timing of cryptic recombinational events evident in other morphologically asexual fungi (see below).

Reproduction in *P. infestans* is currently changing in the opposite way. As mentioned, reproduction has been clonal due to the presence of a single mating type, and the other mating type has been migrating throughout Europe and North America, which has introduced the potential for sexual recombination. Here again, we have the opportunity to correlate timing of the clonal expansion and then subsequent recombination. Although the appearance of recombinant genotypes has been slow in the United States and Canada (68), European populations show the breakdown of clonal population structures in a relatively short span of time (49, 153). Why these differences are seen is not known, although measures of mating-type diversity show that, in the United States and Canada, fields containing both mating types

are still rare and opportunities for sexual recombination may be limited relative to the diversity seen in Europe [(68) and references therein]. This example illustrates the importance of considering the small-scale distribution of fungi, even with global pathogens, when determining population structure.

Group II: Heterothallic Fungi with No Mitospores

Departures from the Group I life cycle, which encompasses both outcrossing and clonal spread, involve losing either the mitosporic stage, the ability to outcross, or sexual reproduction altogether. Although these losses would appear to be mostly modifications in the biological factors, we point out areas where spatial and temporal issues should be considered when addressing our general questions: "What are the reproductively isolated groups and how clonal and recombining reproduction affect population structure?"

The homobasidiomycete pathogens, *e. g. Armillaria* species (1), appear to be good examples of heterothallic fungi that lack mitospores. However, comparing recent work on mating systems and population structure in *Heterobasidion annosum* presents a more complex view of these organisms. Haploid basidiospores (meiospores) provide long-distance spread to infect freshly cut tree stumps, and mating (dikaryotization) between haploid mycelia produces dikaryotic (N + N) individuals. Subsequent and persistent growth of dikaryotic mycelia from tree to tree via grafted or closely associated roots gives rise to mortality centers, i.e. contiguous clusters of infected trees within forest stands. Here it becomes convenient to consider the ramet as equivalent to the genet, with asexual reproduction solely due to growth of the mycelium because there is no discontinuous dissemination of mitospores. The key to such an association is the genetic stability of the ramet over its entire life span.

H. annosum can be divided into reproductively well-differentiated, genetic intersterility groups (ISG), or biological species, which correlate with host specificity on fir (F-ISG), spruce (S-ISG), and pine (P-ISG). Phylogenetic and geographic analyses of the ISGs from Europe and North America have given some indication of the origin and evolution of ISGs. For example, S- and F-ISGs in Europe are very closely related and probably originated from sympatric speciation related to host specificity (58, 104). The ISGs, however, are not completely reproductively isolated in North America where their host ranges overlap, which suggests that here they originated allopatrically and have recently come together. In California, a P/S hybrid was characterized on pine and western juniper (59). The long-term persistence, the contribution to intergroup gene flow, and the evolutionary importance of such hybrids are unknown.

Two notable laboratory studies (135, 136) indirectly tested genetic stability of hybrids by mating haploid individuals derived from basidiospores in both sympatric and allopatric combinations. After mating, conidia were isolated at various times and assayed for nuclear components. Although it appears unlikely that such mitospores have any role in the field or contribute to the population genetic structure, they were used to compare the stability of dikaryons as indicators of

mating success. As might be expected, dikaryons made up of allopatric and inter-ISG combinations were less stable than were sympatric, intra-ISG dikaryons. Unfortunately, these matings were not fruited to show stability through meiosis, or compare fitness of the resulting progeny. Still, they show how the genetic isolation of ISGs can be maintained.

Garbelotto et al (60) found that 37–92% of genets collected from seven natural populations were haploid, unmated monokaryons. The high percentage of haploids was surprising as it is usually thought that the monokaryotic phase of the basidiomycete life cycle is short and only lasts until the mycelium encounters a compatible mate. However, *H. annosum* monokaryons were able to persist and spread to nearby trees. From these and other data, Garbelotto et al conclude that mortality centers are mosaics of mono- and dikaryons due to the high proportion of basidiospore infections, possible de-dikaryotization events, and persistence of parental monokaryotic hyphae among mated dikaryons. The connection between mosaic mortality centers and the dikaryon instability shown by Ramsdale & Rayner (135, 136) is consistent with the conclusions of Garbelotto et al.

Recent work on the population structure of *Armillaria gallica* reinforces the notion of each individual being a genet arising from unique mating events (148). However, the expansive and long-lived growth of a single genet of *A. gallica* presents an even more dramatic example of clonal spread by growth in a sexual species, while maintaining the equality of ramet and genet [reviewed in (1)]. The now-famous study of Smith et al (151) characterized a single individual of this species as covering several ha, weighing 10,000 kg, and being 1500 years old, which makes it one of the largest and oldest organisms on earth. The concept of temporal genetic stability stretches to $>10^7$ mitotic generations with the tests of mtDNA sequence variation within three very large genets (147). This longevity is interesting in itself, given that fungi are usually considered ephemeral organisms.

The genetic stability seen in *Armillaria* may be related to its having moved from being dikaryotic to diploid, as shown by laboratory studies demonstrating the potential of this species (and other related *Armillaria* spp.) to participate in haploid-diploid matings, which result in recombination by nonmeiotic means (34). Although somatic recombination, aneuploidy, and triploidy are possible, the most common outcome of haploid-diploid interactions is nuclear replacement of the haploid in its mycelium by the invading diploid. Moreover, such nuclear replacement may contribute to genetic stability of an established diploid in the wild by eliminating each generation of haploid basidiospore progeny within and at the margins of its territory. Haploid-diploid matings have also been studied in a related species, *A. ostoyae*, with similar outcomes, but without direct evidence of somatic recombination (140, 141).

Group III: Homothallic Fungi With or Without Mitospores

Many fungi reproduce sexually by meiospores resulting from self-fertilization without genetically different individuals coming together to mate. These taxa fall into two general classes: (*a*) homothallic, such as *Aspergillus nidulans* and

Sclerotinia sclerotiorum, where meiosis proceeds from a single haploid nucleus and (*b*) pseudohomothallic, such as *Agaricus bisporus* and *Neurospora tetrasperma*, where two haploid nuclei, each of a different mating type, are required for meiosis, and remain intimately associated throughout the life cycle by being packaged together in the meiospore and remaining as a heterokaryon in the subsequent vegetative mycelium.

In homothallic species, the selfed sexual elements, along with any mitosporic reproduction, intuitively point to predominantly clonal populations with a principally clonal mode of evolution. The best-studied examples include *S. sclerotiorum* (96) and *A. nidulans* (44). Geiser et al (61) tested this prediction in *A. nidulans* with a sample of >100 British isolates that had been sorted into 20 vegetative compatibility groups (VCGs). Markers that were polymorphic between single representative isolates from these 20 groups were developed by probing genomic digests with cosmids of known chromosomal origin. For 11 physically unlinked markers, linkage disequilibrium was low (only 9.1% pairwise associations were nonrandom) and I_A was not significantly greater than zero. This lack of association between genetic markers and VCG was interpreted as showing that these lineages arose from a process of meiotic outcrossing and that recombination was an important determinant of population genetic structure in this species. As mentioned above, what is unknown is the amount and frequency of recombination occurring in this population.

In *S. sclerotiorum*, clonal reproduction has had a considerable impact on the genetic structure of populations on monocultured crops, as evidenced by the repeated sampling of identical multilocus genotypes (45, 94) over wide geographical areas and from year to year. Kohli & Kohn (95) recently showed by fingerprinting analyses that of 2747 isolates of *S. sclerotinium* from Canadian canola, 594 genotypes were unique, and that isolates sharing a genotype also shared VCG. Analyses of pairwise linkage disequilibrium demonstrated that more than a quarter of loci showed significant association within the sample of 594 unique genotypes and the multilocus index of association was significantly greater than zero (95), which demonstrated a significant clonal component to the data set. However, that all four genotypic classes were represented in 75% of the pairwise comparisons between loci was taken to indicate that a proportion of the genotypes sampled originated from outcrossing events. A second study on North Carolina cabbage found less evidence for linkage between VCG and multilocus genotypes (45). Here, partial compatibility between VCGs was found and a lack of association between fingerprint pattern and VCG was observed, which suggests that in this population recombination may have occurred between compatibility loci. That putative heterokaryons were detected in the Canadian population is suggestive that somatic recombination may play a role in the population structure of this fungus (95).

Carbone et al (31) argued that the fingerprint probe used to genotype *S. sclerotiorum* in these studies relied on a retrotransposable-like element and that parallel gains and losses of fingerprint fragments in clonal *S. sclerotiorum* lineages could

provide an alternative explanation to recombination as the cause of homoplasy. Further investigation was undertaken by comparing gene genealogies of four unlinked loci with genealogies derived using fingerprint characters. Comparison of the combined gene genealogies of the four loci with genealogies inferred from fingerprint characters demonstrated significantly more homoplasy in the fingerprint data, consistent with the hypothesis of hypervariable fingerprint loci (31). Tests of congruency between trees from each of the four sequenced *S. sclerotiorum* loci by the partition homogeneity test demonstrated significantly different topologies for each of the data partitions, a result consistent with recombination. *S. sclerotiorum* is one of the few fungi for which there are data sufficient to analyze reproductive mode. Because the data were published, we could reanalyze them by grouping characters into monophyletic biallelic families for each locus and then comparing these groupings between loci. This analysis demonstrated that all four combinations of characters were found for five of the six pairwise-locus combinations and that the I_A was nonsignificant for the clone-corrected data (Figure 4), which supports the conclusions made by Carbone et al about recombination due to gene genealogy incongruity. Therefore, outcrossing appears to occur in agricultural populations of *S. sclerotiorum* at a rate that is important in generating new

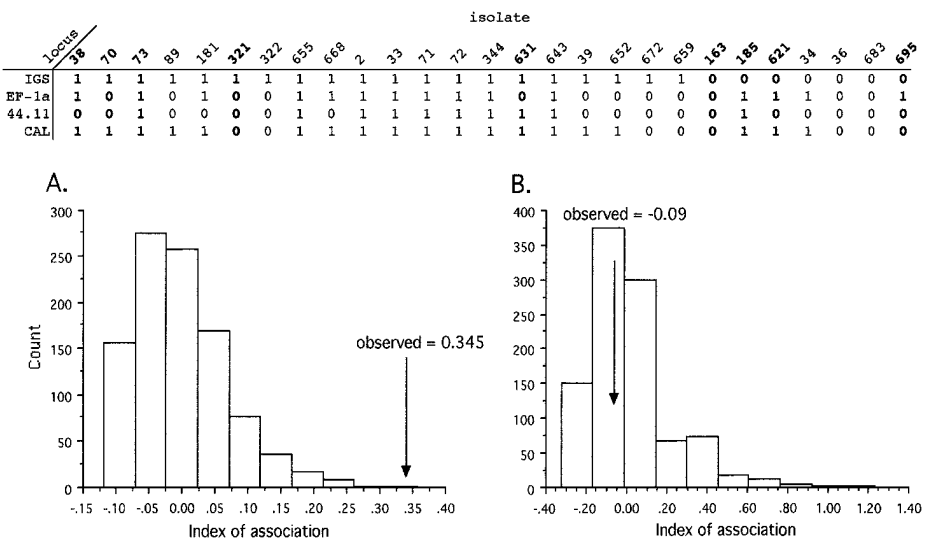

Figure 4 Reanalysis of the *Sclerotinia sclerotiorum* data from Carbone et al (31). The data set was constructed by grouping characters into monophyletic biallelic families for each of four loci, IGS, EF-1a, 44.11, and CAL (table insert). *A.* Analysis of the index of association (for method, see text) the entire data set shows that the observed I_A is significantly different from that of the recombined data. *B.* For the clone-corrected data set (bold characters in table), the observed I_A value is not significantly different from that expected from a recombining population.

genotypes, which make it likely that long-term evolution of asexual lineages does not contribute significantly to population genetic structure.

Pseudohomothallism

Pseudohomothallism is a reproductive strategy unique to fungi and may belong midway in the continuum from inbreeding to outbreeding. Although highly inbred pseudohomothallic populations have the potential to persist as such, most species maintain mechanisms that allow some outcrossing (92, 133, 134). Conflicting data exist on the influence of the pseudohomothallic life cycle on population genetic structure. In *Agaricus bisporus*, Xu (172) suggested that homokaryon progeny backcrossed to the parents were less fit than outcrossed progeny of the same parents: a case of inbreeding depression. That inbreeding depression appears to occur suggests a role for outcrossing in the wild. Analysis of natural populations of *A. bisporus* in California has demonstrated that exotic European cultivars are now commonly encountered within native habitats (91, 173). The occurrence of isolates with the European-type mitochondrial haplotype, but the Californian type nuclear background, is cited as evidence that there is introgression of European alleles into natural Californian populations.

In contrast, outcrossing in the pseudohomothallic species *Neurospora tetrasperma* results in significant sexual dysfunction (85). The fact that dysfunction occurs between isolates from a small geographic area (<5 ha) suggests that *N. tetrasperma* may exhibit outbreeding depression (DJ Jacobson, unpublished data). A recent study, however, has indirectly shown that some outcrossing must be taking place within populations of *N. tetrasperma* to maintain allelic polymorphism at the *het-c* locus, one of many genes controlling heterokaryon incompatibility (AJ Powell & DO Natvig, unpublished data). Pseudohomothallism in *N. tetrasperma* is achieved by suppressing crossing over on the mating-type chromosome so that mating-type genes segregate at the first meiotic division. This suppression of recombination on the mating-type chromosome has the effect of preserving heterozygotes at the many other loci on the chromosome, even though loci on other chromosomes are homozygous, presumably due to selfing (118; A Gallegos, DJ Jacobson & DO Natvig, unpublished data).

Other fungi, including plant pathogens, may also have a pseudohomothallic life cycle, although the evidence is preliminary. Ota et al (125) describe Japanese isolates of *Armillaria mellea* that are homothallic and may represent a variant of pseudohomothallism. Single basidiospore cultures (2N) were fruited and cytological studies of basidium development and meiosis revealed putative 4N and haploid stages limited to the basidium. Whether this is homothallism or pseudohomothallism remains in question; however, it places both heterothallic and homothallic life cycles in the same morphospecies, albeit with considerable geographic separation.

The cause of anther smut of Caryophyllaceae, *Microbotryum violaceum* (formerly *Ustilago violacea*), also has some features of pseudohomothallism. Promcelium (basidium) development and meiosis recently have been studied cytologically (80). Infection of the plant is caused only by a dikaryon, which is

formed by conjugation of haploid meiotic products of opposite mating type. Although haploid sporidia can be cultured from promycelia, it is likely that under natural environmental conditions, conjugation takes place between cells of the promycelium or within one or two mitotic generations of sporidia (80). Recently, two groups (89, 127) reported that natural populations of teliospores when cultured in the lab do not produce sporidial cultures with equal proportions of the two mating types. Genetic data suggest a model involving a lethal genetic element linked to the mating type that cannot be recovered. Antonovics et al (4) tested this model mathematically to show that such haplo-lethals linked to mating type could form a stable polymorphism or spread to fixation in a population if there was an advantage to the dikaryon, or if there was a high degree of intratetrad selfing, or both.

Such studies argue for the predominately intratetrad selfing life cycle of this organism. Segregation of mating type at first meiotic division provides a mechanism for maintained heterozygosity of mating type and is reminiscent of pseudohomothallism in *N. tetrasperma* (above). On the other hand, the ability of this pathogen to undergo nonmeiotic forms of recombination, again deduced from laboratory studies, may mitigate effects of selfing [reviewed in (48)]. At this point, baseline measures of genetic diversity in natural populations of *M. violaceum* do not exist, but would be valuable if they could demonstrate the reproductive mode in nature.

That clonal genotypes occur and persist due to selfing in homothallic and pseudohomothallic fungi is apparent. Although counterintuitive, the possibility for inbreeding depression, or other similar selective pressure, in a selfing organism must be examined further. Together, these examples illustrate the need and importance of classical genetic and cytological studies to accompany measures of genetic structure of populations when evaluating the reproductive strategy of any particular fungus.

Group IV: Fungi Lacking Sexual Morphology and Thought to Reproduce Clonally

Within this group are some of the most important plant pathogens and many fungi that have been used to illustrate the evolutionary enigma of persistent clonality. We begin with a mitosporic human pathogen that provides an example of recombination, and then move to a toxigenic fungus and a rust before reviewing mitosporic *Fusarium* species.

Coccidioides immitis is the agent of coccidioidomycosis in humans and has an exclusively mitosporic life cycle. Comparisons of five gene genealogies showed that isolates taken from throughout the New World distribution of the fungus fell into two genetically isolated groups (99, 100). These groups represent phylogenetic species and correlate with geography in that one species is in the San Joaquin Valley of California and the other is outside of it. There was insufficient sequence variation within each species to distinguish between clonality or recombination, but PTLPT and I_A analysis of 14-locus SNP genotypes for 25 Arizona isolates detected recombination in the species outside the San Joaquin Valley (28).

MC Fisher et al (personal communication) showed in a recent study of 37 isolates and 14 locus SNP genotypes that recombination was also occurring in the San Joaquin Valley species (56). Estimates of gene flow between isolates from the San Joaquin Valley and Arizona or Texas using SNPs also supported genetic isolation of the two species (30).

A second example employing congruence of gene genealogies is that of *Aspergillus flavus*, a common soil saprophyte that can infect seeds and, most important, produce aflatoxin. Geiser et al (63) showed, through comparison of five gene genealogies and also through phylogenetic analysis of multilocus genotypes, two distinct cryptic species in a collection from Australian agricultural soils. Analyzing the larger group for concordance of gene genealogies showed convincing evidence for recombination. However, if these two groups were not separated and the complete data set was analyzed as one species, it would give the mistaken impression of a population with clonal structure. As with *C. immitis*, it is essential to determine all reproductively isolated groups within a collection prior to asking whether population structure is clonal or recombining.

In separate studies, populations of *A. flavus* from Arizona were characterized by VCG, morphology, DNA polymorphism (RAPDs), and aflatoxin production (10, 11). Sampling a single field over three years revealed 13 VCGs from 61 isolates and an additional 44 isolates that could not be assigned to a VCG. This situation is typical of the overall genetic diversity that we have now come to expect even in asexual fungi. Distribution and frequency of VCGs changed significantly over the three years sampled with the most frequent VCG disappearing in year 3, whereas new VCGs were detected in years 2 and 3. Here, as with most such studies of morphologically asexual fungi, absence of recombination was assumed. Unfortunately, the Australia and Arizona populations of *A. flavus* cannot be directly compared, because of the different methodologies used. Combining morphology, VCG analysis, and molecular characterization would provide extremely useful information. The two sclerotial size types (groups S and L) collected in Arizona correlate with VCG and DNA polymorphisms (11); however, whether these groups correspond with the Australian *A. flavus* groups I and II is unknown. In addition, VCG structure of the *A. flavus* group I populations may provide clues to the mechanism of recombination. Heterokaryon formation is controlled by VCG, itself a mutilocus trait, and is a prerequisite for parasexuality. Therefore, VCG diversity should be low within *A. flavus* group I if parasexuality is influential. However, if sexual outcrossing is the predominant mechanism, the expectation is that VCG diversity would be higher due to recombination among VCG loci. The frequency of the different VCGs could change over time in small sample sizes due to genetic drift or selection at other loci, again, similar to what Bayman & Cotty (10) characterized.

Not all studies of reproductive mode employ nucleic acid variation, as shown by studies of protein variation in gall rust of pine caused by *Peridermium harknessii*. This fungus has been the subject of a controversy over possible basidium production, with the most recent data suggesting that meiosis does not occur and that no basidia are produced (166). In a study by Vogler and colleagues (167),

six polymorphic isoenzymes were characterized for 341 presumably dikaryotic aeciospore isolates from coastal California, Oregon, and Washington; the Sierra Nevada and Cascade mountain ranges; and Idaho. Strong evidence was found for two genetically isolated groups (zymodemes I and II), which were sympatric in parts of their ranges. Within each group the isozyme genotypes were monomorphic. In the smaller group, zymodeme II, each of the polymorphic loci was heterozygous, and this fixed heterozygosity indicated that this taxon was reproducing clonally. In the larger and more widespread group, zymodeme I, all loci were apparently homozygous and, without any polymorphisms within the zymodeme, the question of reproductive mode remains open. In subsequent work (166), it was found that zymodeme I is dikaryotic and zymodeme II is uninucleate but diploid, which could explain the fixed heterozygosity in zymodeme II provided that meiosis is not occurring. A complicating factor is that phylogenetic analysis of isozyme variation shows zymodeme I to be a closer relative to the sexual rust *Cronartium quercuum* than either is to zymodeme II (165). Nucleotide variation in the ITS also showed *P. harknessii* to be the closest relative of *C. quercuum*, but did not address the distinction between zymodemes I and II. Because *P. harknessii* zymodeme II is often cited as the best example of a strictly clonal fungus, it would be worthwhile to challenge the hypothesis of clonality by studying polymorphic nucleic acid markers.

Finally, we arrive at the exclusively mitosporic, plant pathogenic fungi that are central to our review, members of the *Fusarium oxysporum* group. In a recent review of the evolutionary biology of the species, Gordon & Martyn (70) stated that there was no evidence for sex in the taxon, whereas there was plenty of evidence for clonal spread, principally from many cases of overrepresented genotypes in natural collections. On the other hand, they noted the presence of high genetic variation in the species and pointed to cases where virulence, VCG, and nucleic acid variation were found in many combinations, acknowledging that these occurrences might be explained by sex. However, in the conclusion, they considered sex to be unlikely, citing an RFLP study of *F. o. cubense* that supported clonality (93).

The study of reproductive mode in *F. o. cubense* is one of the most thorough in plant pathology because Koenig et al characterized 9 polymorphic RFLP loci and then looked for association between pairs of loci in 72 unique genotypes found among 165 isolates. Their test showed association between almost all pairs of alleles, as expected for clonal populations, but this test is compromised if the isolates are taken from two or more genetically isolated groups. The parsimony analysis of multilocus genotypes in this report [Figure 2 in (93)] provided evidence for several strongly supported clades that could be as genetically isolated as are species. In one of the largest clades (labeled FOC I + FOC VIII), there was very little resolution represented by only one internal branch, a condition consistent with recombination. A subsequent study of *F. o. cubense* by Bentley et al (13) used PCR fingerprints and VCG to characterize a very similar collection of isolates. They used the fingerprint data to make a phenogram by distance analysis and noted a good correspondence between VCG and fingerprint pattern, except for isolates in one

clade, which they labeled DFG IV. Although these authors consider *F. o. cubense* to be exclusively clonal, their observation of a lack of association between fingerprint and VCG is evidence for recombination, at least in DGF IV. Interestingly, the VCG types in DFG IV are nearly identical to those in FOC I + VIII described by Koenig et al and must represent the same genetically isolated unit.

Most recently, O'Donnell et al (123) used gene genealogies for elongation factor alpha and mt ssu rDNA to investigate the phylogenetic relationships of 33 isolates of *F. oxysporum* representing multiple individuals of formae speciales *cubense*, *lycopersici*, *melonis*, and *radicis-lycopersici*. None of the formae speciales proved to be monophyletic, and *F. o. cubense* formed five clades, each of which may represent a cryptic species. Clade 1, representing one of the earliest diverging *F. o. cubense* clades, is equivalent to FOC I + VIII and DFG IV. It seems probable that at least one of the several cryptic species in the *F. o. cubense* group exhibits recombination in addition to clonal reproduction.

To test the hypothesis that recombination could be occurring among isolates in FOCs I + VIII, the data set of Koenig et al (93) was obtained and trimmed to the 25 unique genotypes in FOCs I + VIII and the restriction fragments that varied among them (Table 2). For the restriction fragments that were variable and informative, only one fragment was analyzed for each combination of hybridization probe and restriction enzyme to avoid the possible association of alleles by physical proximity. With the reduced number of polymorphic restriction fragments, ten of the genotypes were no longer unique and were excluded from the PTLPT and I_A tests. With the PTLPT (PTP in PAUP* 4.0b2) using a heuristic search, tree-bisection-reconnection branch swapping and steepest descent, the 14-step tree length of the observed data was not significantly different from the distribution of tree lengths for 10,000 data sets that had been resampled to simulate recombination ($p = 0.85$, Figure 5). When the analysis was repeated without steepest descent,

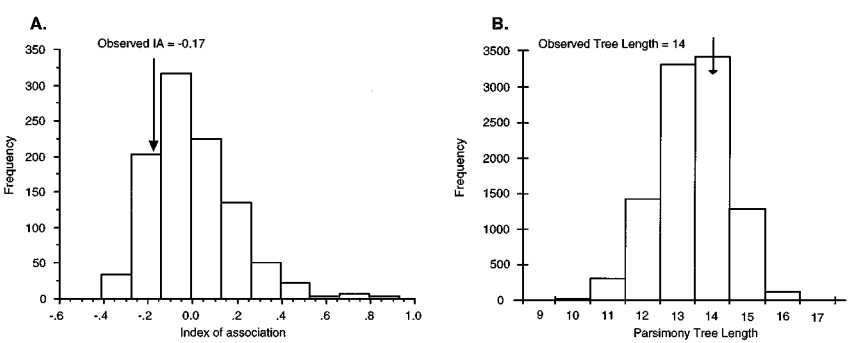

Figure 5 Reanalysis of the *Fusarium oxysporum* f. sp. *cubense* data from Koenig et al. (93) as described in the text. *A*. Index of Association. The observed I_A is not significantly different from that expected under recombination ($p = 0.83$). *B*. PTLPT. Neither is the observed tree length significantly different from that expected under recombination ($p = 0.85$).

TABLE 2 Polymorphic restriction fragments in *Fusarium oxysporum* f. sp. *cubense* FOC 1 + VIII (92a, 93)

	Polymorphic Loci[a]	1	2	**3**	4	5	**6**	7	**8**	9	10	**11**	**12**	13	**14**	15	16	17	18	19	**20**	21	22	23
	Probe number	7	7	**7**	7	7	**120**	204	**204**	225	225	**225**	**260**	260	**260**	260	260	260	260	261	**261**	261	261	261
	Enzyme	*Dra*	*Dra*	***Hae***	*Hae*	*Hae*	***Hae***	*Eco*	***Eco***	*Eco*	*Eco*	***Eco***	***Dra***	*Dra*	***Eco***	*Eco*	*Eco*	*Eco*	*Eco*	*Eco*	***Eco***	*Eco*	*Eco*	*Eco*
	Fragment	2	5	**1**	2	3	**5**	4	**6**	2	3	**4**	**2**	4	**2**	4	6	8	9	1	**2**	3	4	5
Isolates[b]																								
1	**VCG0124-A36**	1[c]	0	**0**[d]	1	1	**1**	0	**0**	1	1	**0**	**1**	0	**0**	1	1	0	0	0	**0**	1	0	0
2	**VCG0124-GMB**	1	0	**0**	1	1	**1**	0	**1**	1	1	**1**	**1**	0	**0**	1	1	0	0	0	**0**	1	0	0
3	**VCG0124-Maca**	1	0	**0**	1	1	**1**	0	**1**	1	1	**0**	**1**	0	**0**	1	1	0	0	0	**0**	1	0	0
4	**VCG0124-STJ2**	1	0	**0**	1	1	**1**	0	**0**	1	1	**1**	**1**	0	**0**	1	1	0	0	0	**0**	1	0	0
5	**VCG0124-MW43**	1	0	**0**	1	1	**1**	0	**0**	1	1	**0**	**1**	0	**0**	1	1	0	0	0	**1**	0	0	0
6	VCG0124-MW47	1	0	0	1	1	1	0	0	?	?	?	1	0	0	1	1	0	0	0	1	0	0	0
7	**VCG0124-JLTH15**	1	0	**0**	1	1	**1**	0	**0**	1	1	**1**	**1**	0	**0**	1	1	0	0	0	**1**	0	0	1
8	VCG0124-MW64	1	0	0	1	1	1	0	0	1	1	0	1	0	0	1	1	0	0	0	1	0	1	0
9	VCG0124-STN5	1	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	0	0	0	0	1	0	0
10	VCG0124-STN7	1	0	0	1	1	1	0	0	1	0	1	1	0	0	1	1	0	0	0	1	0	0	0
11	**VCG0124/25-MW9**	1	0	**0**	1	1	**0**	0	**0**	1	1	**1**	**1**	0	**0**	1	1	0	0	0	**1**	0	0	0
12	**VCG0124/25-MW11**	1	0	**0**	1	1	**0**	0	**0**	1	1	**0**	**1**	0	**0**	1	1	0	0	0	**1**	0	0	0
13	**VCG0124/25-MW53**	1	0	**0**	1	1	**0**	0	**0**	1	1	**0**	**?**	?	**0**	1	1	0	0	0	**1**	0	0	0
14	VCG0124/25-MW63	1	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	0
15	**VCG0124/25-MW86**	1	0	**0**	1	1	**1**	0	**1**	1	1	**1**	**1**	0	**0**	1	1	0	0	0	**1**	0	0	1
16	VCG0124/25-JLTH16	0	1	0	1	1	1	0	0	1	1	0	1	0	0	1	1	0	0	0	1	0	0	0
17	VCG0124/25-JLTH17	1	0	0	1	1	1	0	0	1	1	0	1	0	0	1	1	0	0	1	0	0	0	0
18	**VCG0124/25-JLTH18**	1	0	**0**	1	1	**1**	1	**0**	1	1	**1**	**0**	1	**0**	1	1	0	0	0	**1**	0	0	1
19	**VCG0124/25-JLTH19**	1	0	**0**	1	1	**1**	0	**0**	1	1	**0**	**0**	1	**0**	1	1	0	0	0	**1**	0	0	0
20	**VCG0125-8606**	1	0	**0**	1	1	**1**	0	**1**	1	1	**0**	**1**	0	**0**	1	1	0	0	0	**1**	0	1	0
21	VCG0125-22479	1	0	0	1	1	1	0	0	1	1	0	1	0	0	0	1	1	1	0	1	0	1	0
22	VCG0125-22541	1	0	0	1	1	1	0	0	1	1	1	1	0	0	1	1	0	0	0	1	0	0	0
23	VCG0128-/A47	1	0	0	1	1	1	0	1	0	1	0	1	0	0	1	1	0	0	?	?	?	?	?
24	**VCG01212-STNP1**	1	0	**1**	0	0	**1**	0	**1**	0	1	**0**	**1**	0	**1**	0	0	0	0	0	**1**	0	0	0
25	**VCG01212-STNP4**	1	0	**1**	0	0	**1**	0	**0**	0	1	**0**	**1**	0	**1**	0	0	0	0	0	**1**	0	0	0

[a]Loci in bold are informative and apparently unlinked, [b]Isolates in bold have unique genotypes when the data set is limited to informative and apparently unlinked loci,

[c]Key to alleles, 1 = presence, 0 = absence, ? unknown, [d]Alleles in bold were used in the analyses shown in Figure 5.

but using nearest neighbor branch swapping, the same result was obtained ($p = 0.93$, not shown). With the I_A test, the I_A for the observed data was -0.17, again not significantly different from the mean of the distribution of I_As calculated for 1000 artificially recombined data sets ($p = 0.83$, Figure 5). Therefore, by neither test can we reject the null hypothesis that individuals in FOC I and VIII are recombining in nature. Certainly, this question should be reinvestigated with other approaches, such as congruity of gene genealogies, but the fact remains that our analysis does not support the claim that *F. o.* f. sp. *cubense* is exclusively clonal in nature.

F. o. cubense is not the only forma specialis that has been carefully studied for reproductive mode. In *F. oxypsporum* f. sp. *melonis*, Appel & Gordon (5) found that associations between VCG, pathogen race, and mtDNA variation were not simple and could be explained by convergence, parallelisms, or recombination. With these data, pathogenic and nonpathogenic isolates appeared to be closely related, and it was impossible to define a clade made exclusively of pathogenic isolates. Subsequent phylogenetic analysis of IGS sequence contradicted the previous studies by forming an exclusively pathogenic clade, but support for the clade was not strong and the IGS tree was not congruent with a mtDNA tree. Three of the isolates, one of them pathogenic, had two IGS sequences per individual, suggesting that IGS may not be the most reliable molecule for studies of *F. o. melonis*. This forma specialis was another of the taxa shown to be polyphyletic by parsimony analysis (123). This result was predicted by Appel & Gordon (5) from their IGS phylogeny, which placed an isolate of *F. o. cubense* among a collection of isolates of *F. o. melonis*. Until the genetically isolated groups of *F. o. melonis* are fully delimited [only two isolates were included in the elongation factor α/mt ssu rDNA analysis (123)], it will not be possible to make careful tests of reproductive mode in this fungus. In the absence of better data, the lack of association between pathogenicity, VCG, and mtDNA genotype is suggestive of recombination.

Genetic differentiation has also been demonstrated in *F. o. radicis-lycopersici*. Rosewich et al (143) used four multitarget RFLP probes (which produce simple fingerprints) to characterize 387 isolates collected from Florida and Europe. They showed that different localities in Florida showed significant genetic differentiation from each other, but that isolates from Palm Beach, Florida, and Europe were not significantly different from each other. In the course of their study, they found that some isolates collected as *F. o. lycopersici* had the same VCG as *F. o. radicis-lycopersici*. Knowing that neither forma specialis is monophyletic (123), it would be prudent to include representatives of both formae speciales in future population genetic studies to see if the two formae speciales are truly genetically isolated.

F. o. albedinis on date palm provides an example of the difficulty in assessing reproductive mode in closely related individuals (154). All isolates from Morocco have the same VCG, mtDNA, and RAPD profiles, but there is variation in RFLPs based on hybridization to the repeated DNA element *Fot 1*. Because some *Fot 1* patterns are shared by many isolates, there is good evidence of clonal spread. However, *Fot 1* may be an active transposon and too variable to be used to assess reproductive mode [reminiscent of DNA fingerprinting in *S. sclerotiorum* (31)].

Because the other markers are monomorphic, it is not possible to address the question of recombination with them either, a conundrum noted by the authors (154).

The mechanism of cryptic recombination in a mitosporic and asexual fungus has been demonstrated in at least one case. In Australia, *P. recondida* f. sp. *tritici* is asexual, again because the alternate host is not present. New virulence genotypes, (races or pathotypes) do arise periodically. Park et al (128) have shown, using virulence, isozymes, and RAPDs, that one pathotype that appeared in 1990 could not have arisen by simple mutation, but must be a recombinant between two existing races as the result of somatic hybridization or a parasexual event. They suggest that a similar mechanism operates in *P. graminis*, where both laboratory and field data have shown somatic hybridization [reviewed in (26)].

THE EVOLUTION AND MAINTENANCE OF SEXUAL RECOMBINATION

Why Is It Hard to Demonstrate That Truly Asexual Fungi Exist?

This survey has shown that most, if not all, mitosporic taxa examined exhibit the footprint of recombination in their population genetic structure and that truly asexual lineages, where they exist, do not appear to persist for evolutionarily significant lengths of time. Again, a distinction needs to be made between homothallic, predominately selfing species and fungi without a meiosporic state. Within the kingdom Fungi, a survey of 10,596 ascomycetes showed that 55% are meiosporic, of which 90% lack mitospores and are presumably obligately sexual (138), although the proportion that are homothallic is unknown. Although phylogenetic evidence has been used to support the argument that sexuality is an ancestral character and that asexual taxa are an evolutionary endpoint (62, 109), here our hypothesis extends one step further and suggests that even these putatively asexual taxa are recombining to some degree and that genuinely asexual taxa are, at best, extremely rare. The test of this hypothesis will come when we are able to determine the length of time between rare recombinational events (the strides between footprints) and the mechanism of recombination, sexual or somatic.

Attempts to demonstrate that a taxon lacks sexual reproduction suffer from the general impossibility of demonstrating a negative result. Failure to find sex can be criticized by challenging assumptions that the appropriate strains (mating types) and the correct environmental conditions to induce fruiting were used. On the other hand, the genetics of reproduction is more complicated than simple mating type. Studies to induce anamorphic *Cochliobolus* relatives to mate have shown that supplying missing mating-type genes is not enough to restore sexuality (160).

Truly asexual populations can probably arise within a species capable of meiosporic as well as mitosporic propagation and having a significant metapopulation structure. Leslie & Klein (106) use theory to show that in a heterothallic haploid ascomycete capable of sexual and asexual reproduction, female-sterile

(FS) mutants will arise each generation as loss-of-meiosis mutations accumulate during vegetative reproduction. In taxa where sexual reproduction is rare, these mutations will be effectively neutral and drift will take these polymorphisms to fixation in approximately 2log(N) generations [where N is the effective population size (47)]. As discussed earlier, founder effects, mating-type biases, and selection by host-resistance genes found in specific cultivars may lead to highly structured metapopulations of small N in fungi found on monocultures of agricultural hosts. These fungal populations should show an increased rate of loss of sexuality relative to their wild conspecific relatives. Comparisons between mating populations (biological species) of the *G. fujikuroi* complex show that there is considerable variation in the frequency of FS strains. It was noted that mating populations found on single, widely dispersed host plants have high frequencies of FS strains, which suggests that sexuality is being lost more rapidly than in biological species of the *G. fujikuroi* complex that are found with wide host distributions (106). Thus, an appropriate, and testable, population genetic model for asexual fungi is that mitosporic taxa are mosaics of recombining and asexual populations. Here, sexual populations found on wild, genetically heterogeneous hosts act as a source of recombined genotypes to sink asexual populations of pathogens on genetically uniform agricultural hosts (Figure 6).

Viewing the issue from an opposite perspective raises the question: Why is sexual recombination so prevalent? It is well demonstrated that significant costs exist to sexual reproduction (see below), so it remains a dilemma why asexual populations do not outcompete their sexual relatives. If persistent asexuality is as rare as we suggest and inbreeding depression is prevalent in homothallic species, these factors are consistent with wider debate on the advantages of maintaining sexual recombination as a life-history strategy. There has been much recent debate on the mechanisms maintaining sex within natural populations, given the significant costs associated with recombining genomes (8, 84, 126); however, there has not been a satisfactory resolution of the debate. Modern attempts to answer the question fall into two broad groups. The first is that recombination acts to create advantageous genotypes in changing environments (environmental deterministic hypotheses, EDH); the second is that recombination is maintained as a method of protecting genomes against accumulation of a mutational load in stable environments (mutational hypotheses, MH).

Genetic Diversity and the Red Queen Hypothesis

The EDH maintains that recombination is selectively advantageous when different gene combinations are favored in different generations. A well-adapted genotype should persist as long as the environment is static. However, the environment never is static, especially the biological part and parasites in particular. The image of host and parasite both recombining in an effort to keep pace with one another reminded evolutionary biologists (86) of Lewis Carroll's Red Queen, who admonished Alice, "Now, here, you see, it takes all the running you can do, to remain in

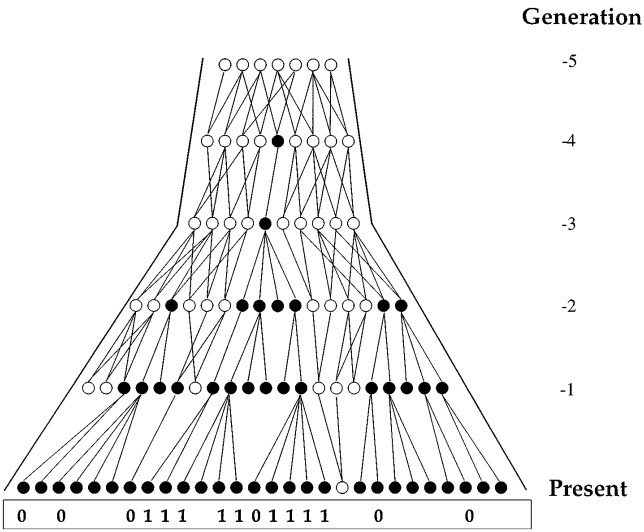

Figure 6 Figure demonstrating how a long-term sexual footprint may be seen in gene genealogies of presently asexual populations. Here, an historically recombining fungus (*unfilled circles*) capable of both mitosporic and meiosporic reproduction experiences loss-of-sex mutations over time (*filled circles*). Sampling the '1' individuals and analysis of multiple gene-genealogies would demonstrate a clonal population structure. However, sampling and analysis of the '0' individuals would demonstrate an apparently recombining population structure, a consequence of multiple origins of asexuality preserving homoplasies in the gene genealogies. Eventually, as time increases, the effect of mutation and genetic drift will eradicate the signature of recombination.

the same place" (32). A key assumption of this model is that pathogens adapt to particular host genotypes, and that recombination of the host genotype renders the parasites less fit in the subsequent generation. The main support for the Red Queen hypothesis comes from theoretical models of antagonistic coevolution demonstrating the stable advantages of sex in systems where resistance is determined by a number of loci (77). However, these models remain controversial owing to their assumption that pathogens may only be optimally adapted to a single host genotype. It has been argued that the nature of gene-for-gene interactions commonly seen between plants and fungi (57) results in the evolution of generalist pathogens that do not support this condition unless extremely high costs to virulence and resistance are postulated (42, 129).

The experimental evidence, where it exists, is broadly compatible with the existence of antagonistic coevolution. Facultatively asexual parasitic nematodes recombine when attacked by specific host responses (64), and, conversely, generation time in hosts is positively correlated with recombination rates (27). A

series of studies by Lively et al have demonstrated that common clones of the parthenogenetic snail species *Potamopyrgus antipodarum* are often disproportionately infected over their rare electromorphs (50) and that oscillations in the most common clone vary temporally (51). An analogous study of the apomictic plant *Chondrilla juncea* and the rust fungus *Puccinia chondrillina* found that in 13 of 16 plant populations, the only clone to carry disease was also the commonest, further support for the idea that pathogens adapt to the most common host genotype (35). However, no study has yet demonstrated the frequency-dependent cycling of homologous pairs of host-parasite alleles necessary to satisfy the conditions implicit in theoretical treatments showing selection for recombination under Red Queen hypothetical conditions (8).

Interestingly, support for the EDH is found in situations where resistance of the fungus itself to disease may be important in the maintenance of sexual reproduction [reviewed in (87)]. The spread of cytoplasmic d-factors in *Ophiostoma novo-ulmi* appears to be only between members of a single vc type, and are more common in epidemic fronts where reproduction of the fungus is clonal and vc diversity limited (19). Here, it is postulated that sexual reproduction is a response to increase genetic diversity in *O. novo-ulmi* and thus acts to limit the spread of d-factors (20). The converse situation is seen in the infection of *Cryphonectria parasitica* by dsRNA viruses, where symptoms of infection include an almost total reduction of sexual reproduction in the fungus (52). Taken together, these examples suggest that arms race–type scenarios may be occurring commonly between fungi and their parasites. Although this evidence is circumstantial, further investigation is certainly warranted.

Mutations and Genomic Fitness

Theory predicts that in small asexual populations, there will be a decrease in mean fitness as genetic drift causes the stochastic loss of the least mutated class of lineages, a process called Müller's ratchet (120). If the mean fitness of asexual populations decreases before they outcompete sexual populations, then recombination will be maintained. Although the ratchet has been shown to occur in laboratory populations of prokaryotes (3), it is not certain to what extent it actually occurs in nature. If populations are large, then the probability of losing the least mutated class of individual approaches zero and the ratchet stops. However, a related model [the mutational deterministic model (98)] predicts an advantage to recombination in populations of infinitely large size as deleterious mutations are concentrated in certain individuals, selection against whom removes these mutations from the gene pool. Here, asexual populations are unable to purge deleterious mutations in this manner, and the loss of fitness results in a large increase in the probability of extinction of a lineage (111).

Evidence on the instability of asexual genomes is strong. Recent estimates on the numbers of deleterious mutations arising in hominid populations are as high as 1.6 per individual per generation (53), well above the threshold value of

1.0 that allows a theoretical advantage of sex over asex. The evolutionary degeneration of nonrecombining genetic material, such as sex chromosomes and perhaps mitochondria, demonstrates the necessity of recombination in maintenance of long-term genetic integrity (110, 139). Recent experiments on the maintenance of sex in evolving populations involving competition between sexual and asexual strains of *Saccharomyces cerevisiae* show a consistent advantage of sexuals over the long term (16, 72, 177); however, the studies reach different conclusions as to the cause of this advantage. Zeyl & Bell (177) showed that sexual yeasts increased in mean fitness relative to isogenic asexuals in a stable environment as opposed to an environment where directional selection occurred. This was interpreted as support for the mutational deterministic hypothesis, that recombination was purging deleterious mutations from the population. On the other hand, Greig et al (72) demonstrated that sexuality was advantageous under conditions of directional selection only if the yeast strains were heterozygous. Here, it appears that the advantage to recombination accrues from the creation of novel adapted genotypes, a situation that supports the EDH. The discovery of fungi with clones that have persisted for $>10^7$ mitotic generations (1) opens the opportunity for experimental evolution to test theory on the rates of accumulation of deleterious mutation in asexual genomes.

Despite the polarization of the debate, it may be that the most plausible explanation for why sex is maintained within populations will involve a pluralistic view. It is quite possible that the mechanisms described above all operate within populations and may act synergistically (126, 130). However, despite ambiguity as to the exact mechanism maintaining recombination within natural populations, it is theoretically clear that the loss of recombination within a population is normally fatal over evolutionary time.

Ancient Asexual Organisms: Do They Exist?

A number of putative asexual taxa have been proposed that have dispensed with sex for several million years (88). If real, it is important to understand what they do that enables them to escape the costs to asexuality that appear to be so prevalent within the majority of taxa.

That putatively obligately asexual taxa tend to be rare and show limited evolutionary divergence from sexual relatives (12, 109) argues that they undergo rapid extinction. Although a number of asexual taxa have been claimed to be ancient based on fossil data and the observed lack of males, this evidence is too weak to conclude that they actually exist (88, 108). Proof of ancient asexuality requires the demonstration that loss of sex was monophyletic, that gene genealogies are congruent, and that alleles show considerable mutational diversification (17). However, such genetic data have yet to be published on that paradigm of ancient asexuality, the bdelloid rotifers.

Recent data on another taxon of putative ancient asexuals, the unisexual Ostracod crustaceans, found high levels of interclone genetic diversity and strong

correlations in the extent of divergence between clones for mitochondrial and nuclear markers. While these data are consistent with the loss of sexuality for up to 5 million years, they are also consistent with the polyphyletic occurrence of asexuality as a result of an ongoing process of hybridization between several genetically diverse lineages of sexual ancestors (37). Here, utilizing the population genetic tests discussed in this review would be useful in discriminating between these two hypotheses.

Within the fungi, demonstration of the coevolution between attine ants and their symbiotic fungi (*Lepiota* spp.) suggests that some lineages have been ant-propagated for up to 23 million years (36). That these lineages produce occasional basidiocarps, typically as the ant nest is dying off, shows that recombination is theoretically possible. It is possible that within this group, genetic fidelity is maintained by rare outcrossing events with wild conspecifics, followed by long periods of clonal propagation. The appropriate genetic analyses to show whether clones persist for evolutionarily significant lengths of time in this group are ongoing (IH Chapela, personal communication) Other fungi, such as the arbuscular mycorrhizal genus *Glomus*, are thought to be asexual, and the fossil record shows that they have existed for the past 400 million years (137). That the genomes of VA mycorrhizal fungi show extreme levels of polyploidy may provide a shelter for the organism from the accumulation of mutations (142). However, at this time of writing, the uniting factor of all these putative asexuals is that the appropriate genetic analyses to demonstrate long-term asexuality have not been performed (see above); until these have been done, support for the loss of recombination and long-term evolutionary success within these groups remains tenuous.

CONCLUSIONS

Phylogenetic and population genetic methods using comparisons of nucleic acid variation are making it possible for plant pathologists and mycologists to identify fungal species and populations and the way in which they reproduce in nature. This knowledge is essential for such basic reasons as standardized nomenclature for species and subspecies, and predicting the pace of pathogen evolution.

Diagnosing the biologically important specific and subspecific groups of fungi, which are the focus for pathologists, requires knowledge of the reproductively isolated groups and their reproductive modes (20).

Recent studies of fungal population genetics have made it clear that reproductive mode and reproductive morphology are uncoupled. As a result, terms such as sexual and asexual are ambiguous; it is preferable, therefore, to use more precise terms such as mitosporic, meiosporic, clonal, and recombining. Reproductive mode in nature can be assessed by phylogenetics through the use of the congruence of gene genealogies or population genetics by testing for association of loci. However, recombination cannot be detected if isolates are sampled from more than one genetically differentiated group. Therefore, it is essential to discover the

genetically differentiated or isolated populations before attempting to determine reproductive mode. Reproductive isolation can be detected by the shift from congruence to incongruence of gene genealogies, and reproductively isolated populations identified in this way can be usefully described as phylogenetic species. All fungal species can be diagnosed by a phylogenetic species concept based on comparisons of gene genealogies because there is no requirement for cultivation or mating.

The same fungus can display different modes of reproduction at different geographic locations and at different times. Often the clonal component can be geographically restricted due to agricultural practices. However, there are numerous examples of migration and genetic exchange between agriculturally clonal fungi and recombining members of the same species in other geographical locations. Knowledge of pathogen reproductive behavior on the largest possible geographic and temporal scales will allow pathologists a better chance of controlling it on the local scale. With enough information, pathologists should be able to predict pathogen evolution.

A question that has driven much of the recent work in fungal population genetics has been, "Is the fungus clonal or recombining in nature?" Almost always the answer has been, "The fungus is both clonal and recombining in nature." The next big question for the field is the relative contribution of both modes of reproduction to each fungus, in different locations, and at different times. We hope that methods being developed to assess the amount of recombination (114a) will be applied to fungal pathogens as part of larger integrated studies of their epidemiology, evolution, and population biology.

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GLOSSARY

AFLP, Amplified Length Polymorphism;
AG, Anastomosis Groups;
BSC, Biological Species Concept;

EDH, Enviromental Deterministic Hypothesis;
FS, Female-Sterile;
I_A, Index of Association;
ISG, Intersterility Groups;
ITS, rDNA Internal Transcribed Spacer;
mtDNA, Mitochondrial DNA;
MH, Mutational Hypothesis;
PCR, Polymerase Chain Reaction;
PSC, Phylogenetic Species Concept;
PTLPT, Parsimony Tree Length Permutation Test;
RAPD, Randomly Amplified Polymorphic DNA;
rDNA, Nuclear Ribosomal DNA;
RFLP, Restriction Fragment Length Polymorphism;
SNP, Single Nucleotide Polymorphism;
SSCP, Single Strand Conformational Polymorphism;
STR, Short Tandem Repeats;
VCG, Vegetative Compatibility Groups.

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