Species and Speciation in Mushrooms

Development of a species concept poses difficulties

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> It is really laughable to see what different ideas are prominent in various naturalists' minds when they speak of species; in some, resemblance is everything and descent of little weight—in some, resemblance seems to go for nothing, and Creation the reigning idea—In some, descent is the key,—in some, sterility an unfailing test, with others it is not worth a farthing. It all comes, I believe, from trying to define the indefinable. (Letter from Darwin to Hooker, 24 December 1856; Darwin 1887, p. 88)

Speciation is the process by which a genetically cohesive group of interbreeding individuals diverges into two or more genetically distinct groups of individuals. A requisite in this process is a hiatus in gene exchange between the two emerging groups. This hiatus allows each group to accumulate genetic differences through the natural processes of evolution, including mutation, selection, and genetic drift. When two groups have become sufficiently different, they are recognized as species. In many cases, however, fruitbody phenotypic differences are subtle and there is disagreement as to whether two groups are different species or just locally adapted but interbreeding groups within a species (Figure 1). Because speciation is a process that occurs over time, there is uncertainty concerning the exact point in this process when a population becomes a species.

For these fungi, the species concept should be grounded in a demonstration of phenetic cohesiveness, common evolutionary descent, and reproductive isolation where possible

Fungi pose special problems in determining species status because little is known about what constitutes an individual; the extent of variation within populations is largely unexamined; fungi often have complex, varied life cycles, each stage of which may be subject to different evolutionary pressures; and reproduction (sexual and asexual) is extremely complex and may affect evolutionary patterns in ways that we do not yet understand. For many fungi, the sexual stage of their life cycle is unknown and, in fact, may not exist. Many species of *Penicillium*, from which penicillin is derived, are among such fungi.

Classification of the major groups of fungi is based on aspects of their sexual reproductive cycle. Thus, ascomycetes (including the highly edible truffles and morels as well as *Neurospora*, widely used in genetic studies) produce meiotic spores in a sac called an ascus. Basidiomycetes (the mushrooms and their relatives) produce meiotic products from a microscopic club-shaped structure called a basidium. The zygomycetes (black bread molds) form sexual spores as an elaborately ornamented zygosporangium. Fungi that do not reproduce sexually are placed in an artificial group, the Deuteromycetes ("imperfect fungi"). In discussing species concepts and speciation processes in this article, we emphasize the basidiomycete fungi.

Species concepts in fungi

"Species concepts" are the philosophical criteria through which investigators communicate their definition of the term "species." Conversely, species concepts are *not* the taxonomic characters used to distinguish a particular species from other species. Implicit in most species concepts is the idea that two species are genetically distinct from each other and are reproductively isolated, either by intrinsic reproductive barriers (i.e., genetic incompatibility) or by extrinsic factors, such as ecology of the organism or geographical separation. Each group of organisms poses special problems for investigators, and species concepts that have been developed for one group of or-

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ganisms may not be workable for another group, although numerous attempts have been made to find species concepts that are broadly applicable across kingdoms (Carson 1985, Mayden 1997). Numerous species concepts exist, all of which have their proponents and detractors (see Mayden 1997). The three species concepts that are most applicable to fungi are the "morphological" species concept (i.e., differences in morphology reflect underlying genetic differences and can thus imply a genetic hiatus between two groups), the "phylogenetic" species concept (i.e., populations may be grouped and ranked as species only if they share a common evolutionary lineage, usually expressed as a terminal node on a phylogenetic tree), and the "biological" species concept (i.e., the ability of individuals in two groups to interbreed).

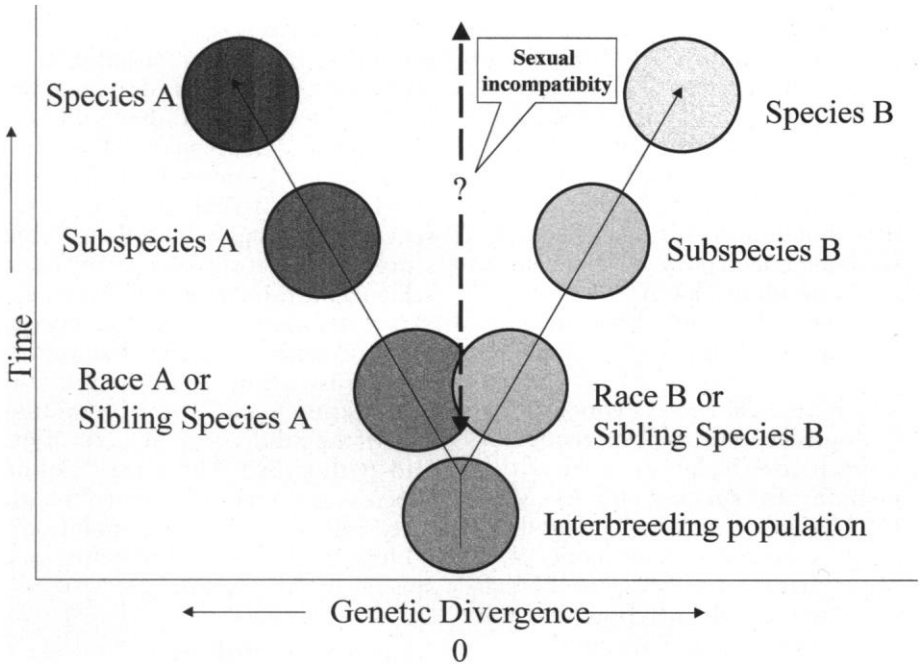

Figure 1. Interbreeding populations may diverge genetically over time until speciation occurs. Sexual incompatibility may occur quickly, leading to sibling species with little genetic divergence; may accompany the gradual genetic divergence; or may not develop, even between populations judged sufficiently divergent to be designated species.

The morphological species concept. In this concept, characters (phenotypes) of individual organisms are compared, and similar individuals are designated as a species. Inherent in this construction is an assumed genetic hiatus between dissimilar organisms. Decisions about similarity and dissimilarity of characters, of course, are left to the taxonomist. Traditionally, characters used to identify mushrooms and their relatives have been taken from the macro- and micromorphology of the basidioma (i.e., the fruiting body or mushroom). It is little wonder, then, that mushroom systematics has been informed by the morphological species concept (see Smith 1968, Clemençon 1977).

The use of morphology to determine species boundaries in fungi has, however, proven inadequate for several reasons: all characters are based on only one part of the life cycle, the fruitbody; the mushroom fruitbody is relatively simple, so limited characters are available for separating species; fungi of divergent evolutionary lineages may share similar morphological characters as a consequence of convergent evolution; and, finally, the breadth of infrataxon phenotypic plasticity in mushrooms is largely unknown. In part, this inadequacy arises because most fungal fruitbodies are ephemeral, forming only when environmental conditions are appropriate and then lasting only a short time. The result of these problems has been an intuitive classification, with each taxonomist grouping and ranking specimens in his or her own way.

Other characters have recently been added to the repertoire of traits used to estimate species status of fungi. Fungi are often potent producers of secondary metabolites (indeed, many drugs and antibiotics are derived from fungi). Analysis of secondary metabolites has identified relationships between morphologically divergent groups of fungi (Gill and Steglich 1987, Whalley and Edwards 1987). In addition, electrophoretic analysis of isozymes is now being used to evaluate population structure, as are some DNA-based techniques, such as restriction fragment length polymorphism (RFLP) analysis. When such additional characters are treated as indicators of genetic differences (in much the same way that morphological characters have been used), the species concept is termed the "phenetic species concept." In this concept, overall phenotypic similarity—including morphology and any other characters used—is the primary criterion defining a species (Mayden 1997). In practice, however, these groups may or may not reflect the evolutionary history of the species.

The phylogenetic species concept. The phylogenetic species concept (Hennig 1966), by contrast, requires that species represent monophyletic groups (i.e., the product of a single evolutionary lineage or the terminal taxa on a cladogram estimating an evolutionary lineage). Although cladograms are often DNA based, they can be developed from any available data, including morphological data. Most modern phylogenetic species concepts support the idea that species lie on the boundary between interbreeding populations and reproductively isolated, genetically divergent taxa (Davis 1996). In this respect phylogenetic and biological species concepts agree, but in the phylogenetic species concept the terminal taxa may or may not retain the ability to interbreed. The phylogenetic approach to conceptualizing "species" has been criticized because it may rank well-defined populations as species and because it fails to

recognize that taxa can be paraphyletic (Brummitt 1997). Nevertheless, in basidiomycete systematics, some investigators argue for a phylogenetic species concept. Hibbett et al. (1995), for example, argued for recognition of morphologically distinct but interfertile lineages of the shiitake mushroom as a means of understanding character evolution, biogeography, and distribution of genetic variation.

The biological species concept. The biological species concept recognizes reproductive isolation as the critical element in speciation. As Mayr (1942), Dobzhansky (1951), and others presented this species concept, all other character suites were subordinated to the ability (or potential ability) to interbreed. Thus, in this species concept, if two populations are able to interbreed, then, regardless of other variation (i.e., morphological, physiological, ecological, molecular), they constitute a shared gene pool and belong to one (biological) species.

The biological species concept has met with criticisms that are aimed mainly at the primacy of the interbreeding requirement (Donoghue 1985, Bock 1986, Templeton 1989). This concept is perhaps more applicable to animals than to plants, for which good morphological species exist in nature that still retain an ability to interbreed. The genetic hiatus between two such plant species may be due to a variety of factors, including geographic separation, ecology, pollinator preferences, and post-reproductive barriers, such as seed inviability.

In determining species boundaries in mushrooms, all available data ought to be considered, but as we show in this article, mating studies have proven among the most enlightening (e.g., Boidin 1986, Stenlid and Karlsson 1991, Vilgalys 1991, Petersen and Hughes 1993, 1998, Petersen 1995a, 1995b, Hallenberg et al. 1996). Mushrooms have mating systems that are designed to encourage outbreeding, and strong reproductive barriers often accompany differentiation into species. Thus, the biological species concept (Mayr 1942) provides a good starting point for a philosophical species concept in mushrooms.

Species terminology. Existing terminology for fungal infraspecific ranks is complex because it is derived from fungal genetics and studies with fungal pathogens. Brasier and Rayner (1987) have examined terminology and concepts associated with infraspecific groupings and have suggested simplified terminology. In their scheme, the primary hierarchical subdivisions are races and subspecies, with or without associated reproductive isolation. Additional qualifying terms would describe the nature of the subdivision (e.g., serotype and pathotype). The term "sibling species" is reserved for morphologically similar or identical populations. Sibling species are ranked below true species in this hierarchical system.

Mushroom biology

Fungal life forms are unique in many ways, and comprehending their biology is important to understanding speciation patterns and processes. Among the important features of fungi are the identification of a fungal individual, the complexity of the mushroom life cycle, the importance of clonal reproduction, and the structure of fungal populations.

The mushroom individual. Just as species comprise populations, so populations comprise individuals. Species concepts in mushrooms rest, in part, on identifying individuals within a "group." This identification is the first step in evaluating whether a genetic hiatus exists between two groups and, therefore, whether these groups can be classified as populations, races, or species.

There is usually little doubt over what is meant by an individual mite, redwood tree, or bacterium, even when considering clonal reproduction, but fungal individuals are less discrete (Anderson and Kohn 1995). With the filamentous vegetative organism existing within an opaque substrate (e.g., wood or soil) and thus hidden from view, there can only be conjecture about whether all the mushroom fruitbodies of one species within a few feet of one another are being produced by a single mycelium or by more than one. For example, nuclear and mitochondrial markers showed that an individual of the mushroom *Armillaria bulbosa* covered several hectares, with all fruitbodies belonging to the same individual (Smith et al. 1992). In contrast, we were able to identify five different individuals of the coral fungus *Clavicorona pyxidata* within a 10 m^2 plot, some represented by more than one fruitbody. The largest individual of *Amanita francheti* in a 300 m^2 plot spanned 25 m^2, but for *Suillus pungens*, the largest individual spanned 300 m^2 (Bruns et al. 1998). Kay and Vilgalys (1992) found individuals of *Pleurotus ostreatus* to be quite small, often with more than one individual occupying a single piece of rotten wood. For experimental purposes, however, it is likely that collections (or mycelia) separated by several miles represent different individuals; many studies showing that widely separated individuals have different mating-type alleles confirm this assumption.

The mushroom life cycle. As traditionally presented, the mushroom life cycle begins at the sexual spore stage (Figure 2A). These spores, borne on specialized organs termed basidia, are called basidiospores and are normally the result of meiosis, either directly or with one of several variations in pre- or post-meiotic nuclear behavior. Whereas basidiospores of saprophytic mushrooms (i.e., mushrooms that obtain nutrition by degrading nonliving organic material) are usually easily germinated on undefined agar media, basidiospores of ectomycorrhizal mushrooms (i.e., mushrooms in a symbiotic relationship with plant roots) are notoriously more difficult to germinate, although some progress has been made (Fries 1978). Under appropriate conditions, basidiospores imbibe water, autolyse their spore wall, and extrude a "germ tube" (Figure 2B) that soon begins to produce exogenous enzymes to digest the surrounding substrate and elongates into a hypha (i.e., a single thallus filament). The hypha in turn grows and branches into an aggregate of hyphae called a mycelium (Figure 2C). The mycelium that results from the germination of a single basidiospore perpetuates the nuclear number and mating type (i.e., equating to "sex," for the purposes of this article) of the

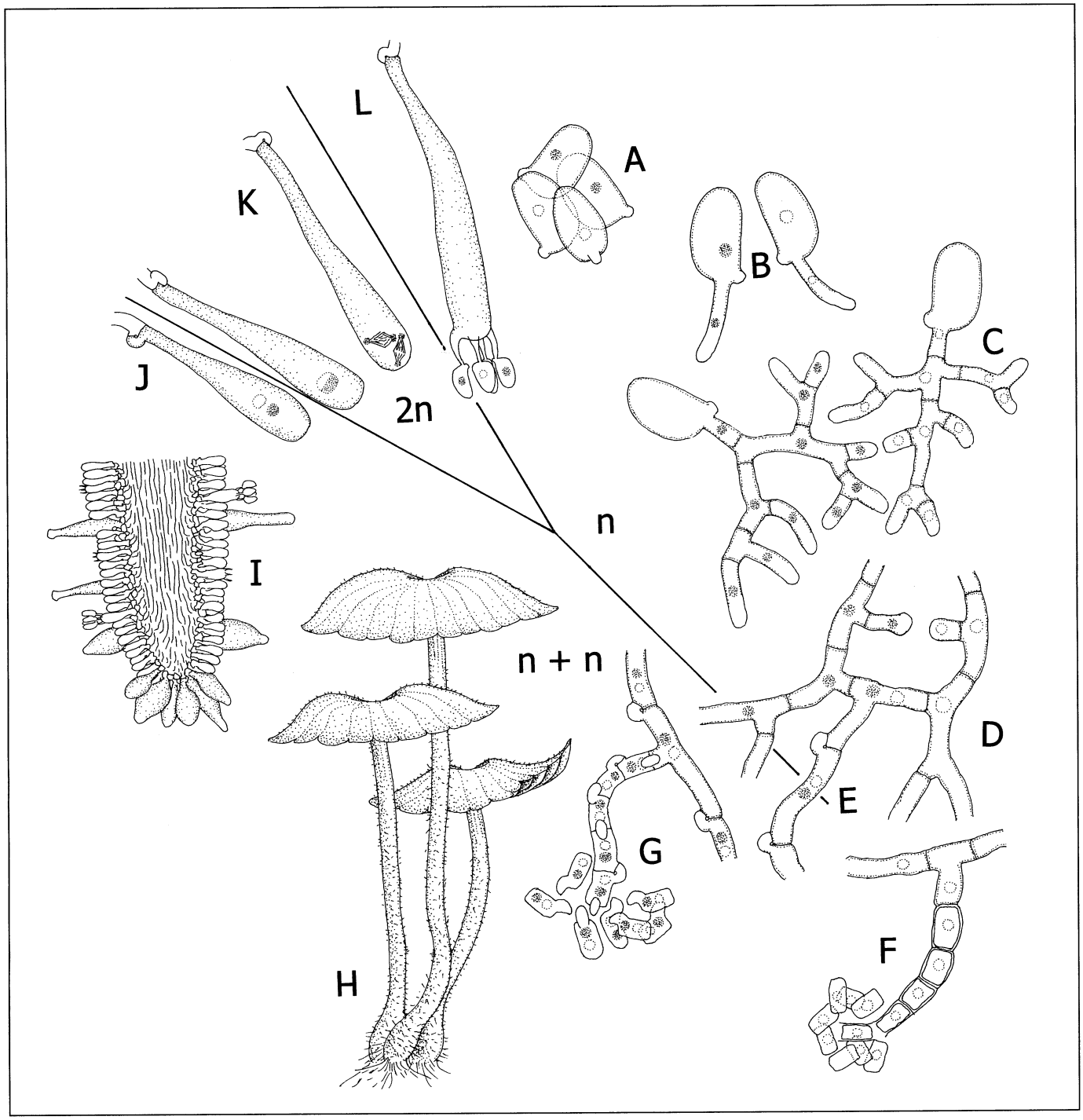

Figure 2. Mushroom life cycle. n, haploid monokaryon state ("haplont"); n + n, dikaryon state; 2n, diplont state. Basidiospores (A) undergo germination (B); germ tubes branch into germling mycelium (C). Monokaryon hyphae anastomose (D) to produce dikaryon hyphae (E) with clamp connections. Monokaryon hyphae can produce asexual spores (F); dikaryon hyphae can produce asexual spores with clamp connections (G). Under appropriate conditions, the dikaryotic mycelium produces mushroom basidiomata (H; the individual on the right has upturned pileus, revealing lamellae). A cross-section of the lamella (I) shows sterile cells (cystidia) and basidia. Nuclear fusion occurs within the young basidia (J; the lower basidium has two nuclei, and in the upper basidium the nuclei have fused). Meiosis takes place in the basidial initial (K), ultimately resulting in a mature basidium with sterigmata and four haploid monokaryon basidiospores (L).

basidiospore.

The most common sexual system in mushrooms is governed by two unlinked mating-type genes, each with multiple allelic forms. Such a system is termed "tetrapolar" (or "bifactorial," for the two mating-type genes). A minority of mushrooms, by contrast, have only one mating-type gene (a system called "bipolar" or "unifactorial"). (For a summary of the molecular basis for mating-type genes, see Casselton and Kües 1994.) In bifactorial mating systems, individuals with different

alleles at both loci will mate. The presence of multiple alleles for mating type within a species encourages outcrossing at the expense of selfcrossing between individuals in a population and thus acts in opposition to evolutionary forces that might fragment a population.

The mycelium from a typical basidiospore is often termed a "monokaryon," which reflects the single nucleus per hyphal compartment (usually called a cell, although narrow holes in the transverse septa of the hypha allow cytoplasmic continuity and even nuclear migration). The mycelium at this stage is typically haploid (as was its meiotic basidiospore; Figure 2C). This monokaryon (haploid) mycelium, if isolated at this time, acts as a gametic generation and can be used in mating experiments of various types: among sibling isolates from a single fruitbody, among individuals from different populations of a given species, or between individuals classified as different morphological species within a genus.

Although the mechanistic details of mating systems in mushrooms continue to be elucidated, it is clear that two monokaryon hyphae of sexually compatible mating types can anastomose and proceed through plasmogamy (Figure 2D). In occasional groups (e.g., *Armillaria*), karyogamy soon follows to produce a diploid mycelium, but, typically, donor nuclei pair but do not fuse. Nuclear division, followed by nuclear migration, may take place; the result will be a proliferating "dikaryon" (from the two nuclei per cell; Figures 2E and 3a). The dikaryotic condition is accomplished by the formation of "clamp connections" at the transverse septa in the majority of mushroom taxa (Figure 3a). These hyphal connections allow nuclei to migrate between two cells, maintaining the dikaryotic condition. Dikaryotic mycelia are assumed to be the major vegetative unit of the individual mushroom fungus.

The growth of dikaryon mycelia appears to be limited only by a variety of micro- and macro-ecological factors, such as appropriate substrate or host availability; substrate moisture; substrate preparation by other organisms, from bacteria to annelids; and symbiont availability. Conventional wisdom, based presumably on in vitro experience, says that monokaryon mycelia are more fragile or ephemeral than dikaryotic mycelia of the same "species" and, thus, that dikaryotic tissue has a selective advantage in a given substrate.

It is the dikaryotic mycelium that, under appropriate physiological and biochemical conditions, produces hyphal ganglia and subsequent basidiome primordia and mushrooms (Figure 2H). The mushroom is normally composed of dikaryotic hyphae that differentiate into cap (pileus), gills (lamellae), and stem (stipe) tissues. In mushrooms and their relatives, any one or a combination of these tissues can be reduced or missing, but ordinarily the basidiospore-producing tissue, the "hymenium," covers the surface of the lamellae (Figure 2I) and is composed of microscopic hyphal tips in a palisade. These hyphal tips often comprise sterile elements, called cystidia, and "fertile," club-shaped basidia. Within the basidia, karyogamy, or nuclear fusion, occurs (i.e., the paired haploid nuclei of the dikaryon fuse into a diploid (Figure 2J); fusion is then followed by meiosis (Figure 2K). During late meiosis, the basidia produce narrowly curved conical outgrowths called sterigmata. Four sterigmata typically form per basidium, reflecting the four products of meiosis. The tips of the sterigmata are "blown out" into spores (Figure 2L) into which the post-meiotic nuclei migrate; the spores are subsequently ejected forcibly from the sterigmata. The basidiospores (Figure 2A), which are also technically known as "meiospores" because they are the result of meiosis, complete the sexual life cycle. In rare cases, segregation of nuclei into spores may vary within a single morphospecies. For example, *Mycena* and *Trogia* species occur in two- and four-spored forms, with two nuclei (n + n) or one nucleus (n), respectively, per basidiospore (Kühner and Lamoure 1958, Corner 1991). This nuclear variation would presumably be reflected in the nuclear condition of the single-basidiospore mycelium.

For mating studies, single basidiospores are collected and germinated to form individual monokaryotic mycelial colonies. Monokaryon inocula of the same putative morphospecies are paired (i.e., placed near each other on nutrient agar) and allowed to grow together. The contact zone is then examined for the presence of viable dikaryotic hyphae, which are indicative of mating (Figure 3b).

Clonal reproduction. Clonal reproduction occurs commonly in fungi. Asexual spores give the mushroom mycelium a means of dissemination separate from the sexual life cycle with its meiotic basidiospores (Anderson and Kohn 1995). In fact, mushroom production is unnecessary for dissemination (although beneficial for gene recombination) if a clonal state is adequate for perpetuation of the organism. Clonal reproduction has important evolutionary implications. If the monokaryon hypha forms asexual spores, the mycelium has a means of extending and widening its availability to an appropriate mate. This reproductive shortcut might be an advantage when a fungus is colonizing new territory. If the dikaryon hypha forms asexual spores, then the dikaryon genotype is perpetuated unchanged (Anderson and Kohn 1995). This means of dissemination could be an advantage in perpetuating rare gene combinations that allow colonization of new ecological niches; it also has implications for speciation processes. It is no surprise, therefore, that many fungi produce asexual spores through a variety of unusual and unique processes. Moreover, the mechanisms of their production provide phenetic information that can be used to group fungi into taxonomic units.

Asexual propagules are termed "conidia," with additional technical terms used for particular modes of production. In mushrooms, conidia are typically the result of fragmentation of a hypha; therefore, they are called "arthroconidia" or "arthrospores." Such asexual spores are rarely produced as part of the mushroom itself but, when produced, are almost always formed by the vegetative mycelium, whether monokaryon (Figure 2F) or dikaryon (Figure 2G). Because the vegetative mycelium is not normally seen in nature (being

immersed in the woody, leafy, or soil substrate, and microscopic as well), asexual spore production is usually discovered only when the mushroom is cultured in vitro. As more and more mushroom species are cultured, the number of species in which asexual reproduction is known to occur continues to grow. Certain mushroom groups (e.g., *Flammulina*, *Collybia* sensu stricto, *Melanotus*, *Psathyrella*, and the related genus *Polyporus*) are well known for the production of asexual spores, but others are only now being discovered.

In some mushroom groups, much of the fruitbody (basidiome) is converted into asexual propagules. The most famous is the genus *Asterophora*, whose basidiomata form on basidiomata of other mushrooms and undergo an almost complete conversion to thick-walled conidia (often termed "chlamydospores"; McMeekin 1991). Species of the parasitic genus *Squamanita* cause distorted growth of their mushroom host and produce asexual spores on the surface of the host carcass and on their own stipes (Redhead et al. 1994). In *Flammulina stratosa*, a newly identified species from New Zealand, abortive fruitbodies also convert into small balls of brown, thick-walled chlamydospores (Redhead et al. 1998). DNA sequence data show that *F. stratosa* is highly divergent from all other *Flammulina* species that we have examined. Its restricted island habitat and genetic divergence suggest that chlamydospore production may have contributed to invasion of the New Zealand island habitat and rapid adaptive evolution, a possibility that we are currently investigating.

Clonal reproduction can be accomplished in at least one other way. Certain strains of *Volvariella bombycina* (Chiu 1993) and *Xerula radicata* (Petersen and Methven 1994) form fruitbodies composed of monokaryon hyphae. Basidia do not undergo meiosis and therefore produce spores of only a single mating type.

Asexually reproducing stages are termed the "anamorph"; the meiotic, sexual stage is termed the "teleomorph"; and the entire life cycle, with all possible stages, is termed the "holomorph."

In fungal groups in which teleomorphs form ascospores (ascomycetes), anamorphic morphology is extremely varied, and the anamorph is often found (i.e., isolated in culture) without the teleomorph. Many workers have spent many years using conventional means (e.g., light and electron microscopy or growth media enhancement) to determine which anamorphs and which teleomorphs represent alternate stages of a single species. Newer techniques (e.g., DNA sequencing and fingerprinting) hold the probability of confirming or elucidating these anamorph–teleomorph relationships. Conversely, in other fungi—mushrooms and their relatives—asexual reproduction has been overlooked for taxonomic character suites. Most of the known anamorphic stages have not even received "form taxon" status or rank.

Needless to say, an understanding of the entire life cycle of each mushroom taxon is invaluable before one can be confident about its taxonomy and phylogenetic placement. Conidia can be used in pairing experiments, just like single-basidiospore isolates can. Thus, in addition to perpetuating the organism asexually, appropriate single-conidial mycelia can form dikaryons and reinitiate the sexual life cycle.

The mushroom population. Locally adapted populations are the first stage in divergence leading to speciation. Although several studies have examined population structure in plant pathogenic fungi (Anderson and Kohn 1995 and references therein), only a few have attempted to characterize the relationships between individual mushrooms and their populations in any detail (Kay and Vilgalys 1992, Smith et al. 1994, Anderson and Kohn 1995, Kerrigan et al. 1995, Gardes and Bruns 1996). The paucity of such studies is due in part to the difficulty of collecting a sufficient sample size in an organism that produces fruitbodies ephemerally and in part to the complexity of the mushroom individual.

Gene flow, or the movement of genes between populations, determines if and how fast populations can evolve into separate species (Carlile 1987). High gene flow favors population coherence, whereas low gene flow favors population divergence. There are two methods by which gene flow can occur between populations: through asexual hyphal fusion and through spore production. In many species, hyphal fusion is prevented by a genetic system of vegetative incompatibility, which acts to protect an individual genotype (Carlile 1987, Worrall 1997); thus, the primary mechanism of gene flow between populations is sexual and asexual spore production. There has been considerable debate about long-distance spore dispersal in fungi (Vilgalys and Sun 1994b), but isozyme and molecular evidence from many mushroom disjunct populations suggests that long-distance spore dispersal does not occur at a rate sufficient to prevent population divergence (Gordon and Petersen 1997, Methven et al. 1997). When gene flow cannot be measured directly, divergence is inferred by finding reproductively isolated subgroups (i.e., biological species) within a morphological species or well-differentiated morphological subgroups within a biological species.

Morphological species versus biological species: Examples

A number of recent studies allow morphological species concepts in fungi to be compared with biological species concepts. The use of a biological species concept must be tempered by the large number of mushroom species that have not yet been established in culture, much less in monokaryon culture. Ectomycorrhizal mushroom taxa, including such large genera as *Cortinarius*, *Lactarius*, and *Russula*, are notorious for resisting domestication. Furthermore, even if two fungi are able to mate in culture, there may be barriers in nature to mating or to the survival of hybrid offspring (Brasier 1987). Mating studies, however, provide some "ground truth" in that if two taxa can mate, they are genetically related. If they cannot mate, they are genetically separate species.

Biological species within morphological species. The "classic" case of using crossing studies to identify species can be found in studies of *Armillaria*, the honey mushroom. For many years, fastidious taxonomists used small differences in basidiome

characters to describe "species" of *Armillaria*. As might be expected, opinions about such practices differed with the philosophy and taxonomic experience of the user—in short, whether the user was a "lumper" or a "splitter." There was, of course, no overarching authority to render binding judgments. Over time, however, most mushroom taxonomists agreed that there was only one species of *Armillaria*, *A. mellea*, but that it varied morphologically, depending on substrate, geographic location, and other factors.

In the 1970s, however, the mating system of *A. "mellea"* was elucidated, paving the way for inter-collection crossing experiments—and, therefore, for other crossing experiments, such as inter-substrate and inter-geographic (Hintikka 1973, Ullrich and Anderson 1978). The first revelation was that there were at least five "biological species" of *Armillaria* in Scandinavia (Korhonen 1978); intercollection crossing experiments revealed at least 10 such "intersterility groups" in northeastern North America (Korhonen 1978, Anderson and Ullrich 1979). With discovery of this genetic complexity, mycologists re-examined *Armillaria* morphology and ecology. In the following years, each "biological species" was matched to its basidiomata and substrate (Guillaumin et al. 1991). The majority of intersterility groups were found to represent morphological species that had previously been named but were historically overlooked. Current studies involve the molecular characterization of these genetic–taxonomic units and their placement on a phylogeny (Anderson and Stasovski 1992).

Much the same story applies to *Pleurotus*, the genus of "oyster mushrooms" now popular in supermarkets. For many years, most oyster mushrooms were identified as *Pleurotus ostreatus* (usually without reference to keys or descriptions), which was assumed to fruit throughout the North Temperate Zone. Vilgalys and coworkers (1993), however, identified three "intersterility groups" (i.e., biological species) in North America by mating haploid monokaryon isolates. Two of these already bore names: *P. ostreatus* and *Pleurotus pulmonarius*. A previously undescribed species, found mostly west of the Mississippi and usually fruiting on aspen (*Populus* spp.) was named *Pleurotus populinus*. Furthermore, the biological species *P. ostreatus* exhibits geographical morphological variation. Whereas European *P. ostreatus* basidiomata usually have gray, charcoal gray, blue gray, or dark gray–black caps (Figure 4e), those from eastern North America are off-white to tan to brown. So far, the only American collections of *P. ostreatus* exhibiting European colors have been made from woody *Lupinus* shrubs on the northern California coast.

In both the *Armillaria* and *Pleurotus* examples, once mating studies pointed toward enlarged species numbers, delineation of the appropriate taxa was relatively straightforward. Mating studies can also reveal the presence of "sibling species" (i.e., populations whose basidiome morphology appears identical but that sort into two or more "biological species"). One such situation has been discovered in *Xerompha-lina campanella* (Johnson 1997). Over 80 cultured collections of *X. "campanella"* from all over the North Temperate Zone were found to fall into two intersterility groups. Mushrooms of the two groups differed morphologically only in the pigmentation of individual sterile cells on the stipe. A similar situation occurs in *Lentinula*, the genus of the popular shiitake mushrooms. Until recently, the common species of subtropical America was known as *Lentinula boryana*, but recent studies (Petersen et al. 1998) show that another species with almost identical basidiomata fruits along the Gulf Coast from east Texas to central Florida and in Puerto Rico and Cuba in the Caribbean. This species (now called *Lentinula raphanica*) is sexually incompatible with collections of *L. boryana* from Costa Rica and tropical Mexico. The most striking difference between the two species is the shape of the sterile cells that form on the edge of the lamellae. Finally, mating tests with *Marasmius androsaceus* clearly reveal two taxa, one in Europe and northeastern North America, the other from the southern Appalachian Mountains. From voucher herbarium specimens, the southern Appalachian taxon could be linked to *Quercus* (oak) litter, whereas the more widespread northern collections seem to prefer conifer debris as a fruiting substrate (Gordon and Petersen 1997).

The relationship between *Omphalotus illudens* and *Omphalotus subilludens* is another example of diverging biological species. *O. illudens* (called the "Jack-O-Lantern mushroom" because its basidiomata are brilliant orange and bioluminescent; Figure 4c) is distributed in the eastern United States from Georgia northward and is commonly collected on hardwood stumps in the southern Appalachians. *O. subilludens*, which fruits on dead palmetto in Florida, is only doubtfully separable from *O. illudens* based on morphology. Analysis of the internal transcribed spacer (ITS) region of the rRNA gene by restriction analysis gave an estimated sequence difference of 7.35% (Hughes and Petersen 1998). This level of divergence is high—higher than that observed between the geographically separated morphological species *Omphalotus olearius* from Europe and *Omphalotus olivascens* from California (for comparison, European and eastern North American *P. pulmonarius* collections differ by 0.58% and collections of *P. ostreatus* by 1.41%; data from Vilgalys 1994a). Crossing studies between *O. illudens* and *O. subilludens* showed a reduced ability to interbreed (only 4 of 24 crosses produced hybrid mycelia in vitro; Petersen and Hughes 1998). The combination of reduced ability to intercross in vitro, different substrate preference, and sequence divergence strongly suggests that these species do not exchange genes in nature despite the potential to do so. In this case, therefore, both mating studies and molecular analysis were keys to understanding that *O. illudens* and *O. subilludens* are different species, despite morphological similarities.

Numerous other examples of biological species within morphospecies are presented by Brasier (1987) for all fungal groups, not just mushrooms. Brasier classified the processes leading to divergence as geographical (i.e., allopatric), as sympatric speciation in response to ecological influence, and as sympat-

ric speciation with unknown cause. For some groups, two causes could be identified. Of the examples given by Brasier (1987), only 5 were geographical, 33 were ecological, and 11 were probable sympatric speciation with unknown cause.

Such examples are satisfying in that they reveal the underlying population structure and genetics of speciation processes. However, descriptions of "new taxa" that are based on mating behavior (with or without small differences in morphology) do not make the tasks of identifying species and estimating biodiversity easier for the "clients" of taxonomy (e.g., foresters, plant pathologists, and ecologists). It may well be that, for the purposes of assessing biodiversity, species concepts based on distinct morphological characters will be preferred.

Morphological variability within biological species. Crossing studies can, as already described, lead to more accurate taxonomic conclusions by increasing the number of recognized species. However, in other situations, crossing studies have led to *decreased* numbers of putative taxa.

For example, *Pleurotus djamor* fruits along the Gulf Coast into Florida and extends somewhat up the Atlantic Coastal Plain. It also fruits throughout eastern Asia and Southeast Asia and in parts of New Zealand, southern Australia, and subtropical North, Central, and South America. It has also been reported from super- and sub-Saharan Africa. Basidiomata occur in several rather distinct morphological statures and colors. Caps vary from bright pink (Figure 5a) to off-white (Figure 5b) to sallow olive to tan (Figure 5c) and from smooth to rather hairy. These color variants and textures were previously given separate species names. Names such as "*Pleurotus opuntiae*" refer to the New Zealand grey fibrillose form, "*Pleurotus salmoneostramineus*" to the pink northeast Asian basidiomata, and "*Plerotus ostreatoroseus*" to the rosy fruitbodies from tropical America.

By fruiting cultures of upward of 60 collections from all the locations where *P. djamor* fruits except Africa, Nicholl (1996) found that the color and texture variations noted in nature were damped when fruiting took place under more or less standard conditions. This result indicates that some substrate or edaphic factors may maintain the natural variants. Crossing experiments showed that the natural color and texture forms result from nongenetic phenotypic plasticity and are not indicative of discrete genetic units. McCleneghan and Hughes (1998) further confirmed the high genetic similarity of the color variants by RFLP mapping of the rRNA ITS region. As might be expected from the *Armillaria* experience, at least one taxonomist (Corner 1981) had correctly described most of the variants as infra-specific units within *P. djamor* as it fruited in Southeast Asia.

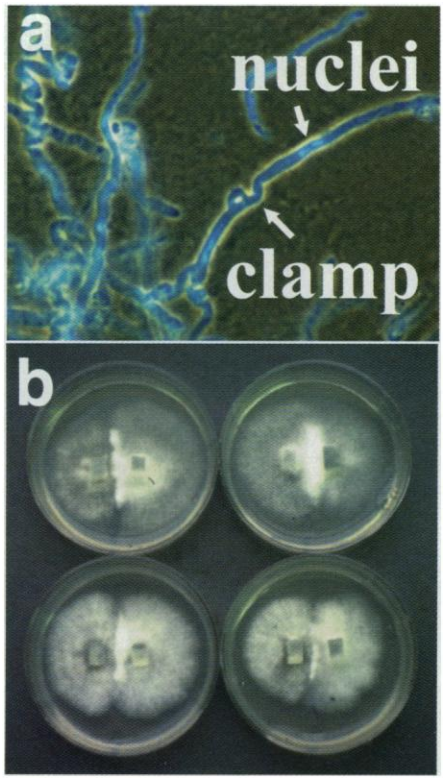

Figure 3. Fungal hyphae and typical pairing experiment. (**a**) Basidiomycete hypha showing clamp connection and dikaryon condition. 1000× magnification. (**b**) Pairing experiments with monokaryon mycelium. Contact zone mycelium is more congested than donor mycelium.

Another example of decreased taxon number comes from studies of *Collybia* by Vilgalys (1991), who showed that even though European and eastern North American basidiomata of *C. dryophila* exhibit different morphologies, they are significantly sexually intercompatible. Similarly, using morphology, isozyme analyses, and mating compatibility, McCleneghan (1996) was able to justify only a single species within the *Pholiota alnicola* complex (Figure 4d), in which seven taxa had originally been described (Smith and Hesler 1968). Similar studies with the *Pholiota spumosa* complex reduced the number of species from 11 to 2. Chang and Mills (1992) showed that several morphotaxa in the *Psilocybe subaeruginosa* complex could be subsumed under significantly fewer biological taxa, and that enzyme electrophoretic patterns were consistent and confirmatory with this conclusion.

Allopatric speciation: Physical barriers to interbreeding

For many mushroom species, populations on different continents or separated by other geographical barriers may be completely interfertile. John Raper, one of the early promoters of the biological species concept in mushrooms, showed that worldwide collections of *Schizophyllum commune* were intercompatible in spite of morphological variation (Raper and Flexer 1971). Collections of *Auriscalpium vulgare* (tooth fungus) from Asia, North and Central America, and Scandinavia were all intercompatible, with minimal morphological variation of the fruitbody and spores (Petersen 1994). Worldwide collections of *Panellus stypticus* (Figure 4b) were found to be compatible (Macrae 1937, Petersen and Bermudes 1992a, 1992b), as were collections of *Clavicorona pyxidata* (coral fungus; Figure 4f) from China, Scandinavia, and eastern North America (Wu et al. 1995) and worldwide collections of *P. pulmonarius* (Petersen and Hughes 1993, Petersen 1995b). In *Omphalotus*, as discussed above, the morphological species *O. olearius* from Europe, *O. subilludens* from Florida, and *O. olivascens* from California were completely interfertile. In all of these cases, morphological differentiation based on geographically disjunct separation could be concluded.

When fungi from different continents are able to interbreed in vitro, three explanations are possible: long-distance distribution of viable spores

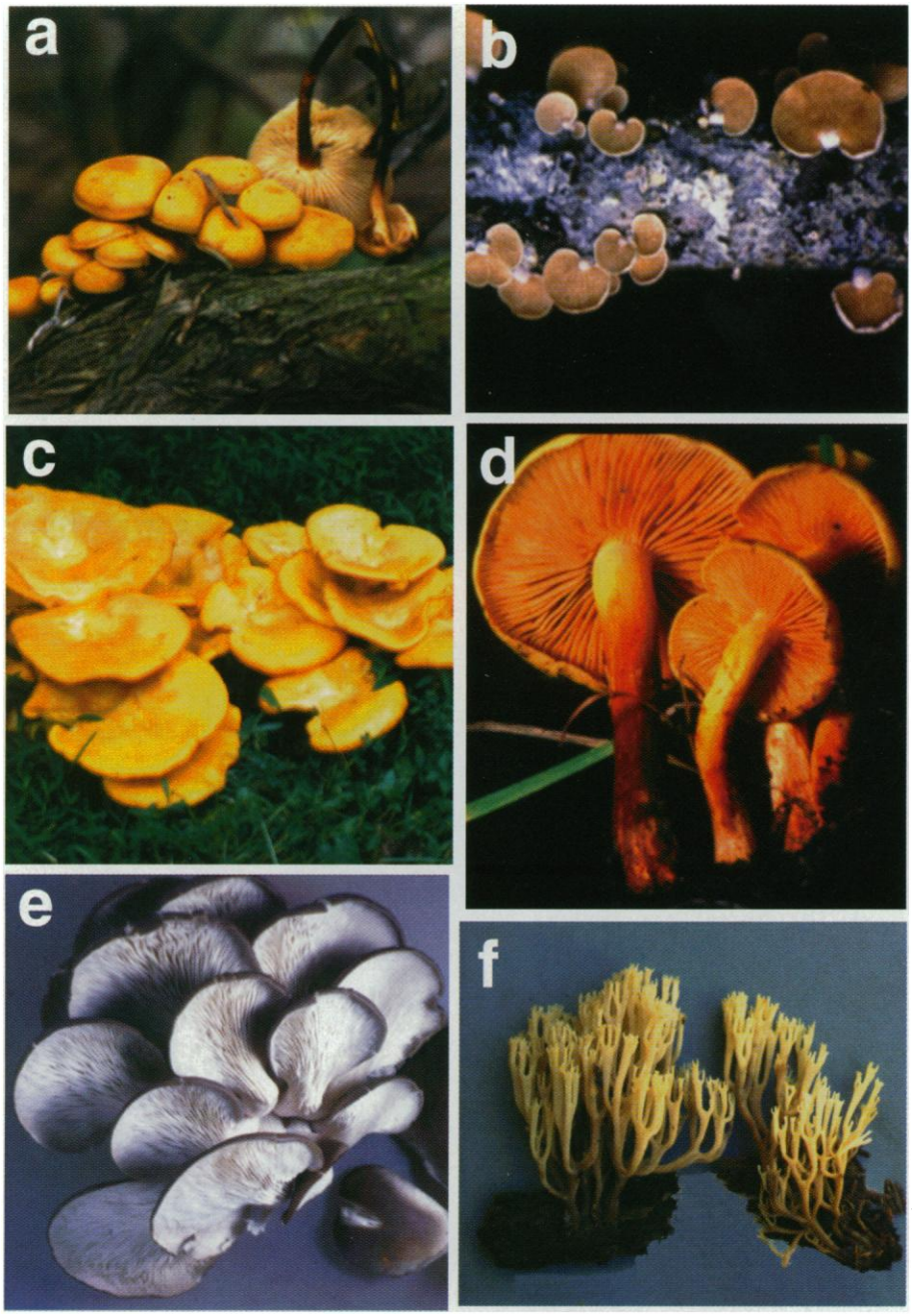

Figure 4. Basidiomata of mushrooms and related fungi. (**a**) *Flammulina velutipes*, 0.5× magnification. (**b**) *Panellus stypticus*, 0.8× magnification. (**c**) *Omphalotus illudens*, 0.3× magnification. (**d**) *Pholiota alnicola*, 0.5× magnification. (**e**) *Pleurotus ostreatus* from Europe, 0.2× magnification. (f) *Clavicorona pyxidata*, 0.5× magnification.

allows ongoing gene flow between the continents, vectors mediate transfer of fungi from one geographical area to another, or no current intercontinental gene flow occurs but relic intercompatibility persists. In the last case, populations on different continents may represent ancient distributions (i.e., distributions that were established before the continents separated) and may be in the process of allopatric speciation.

For a number of intercompatible taxa, there is good evidence that allopatric speciation is in progress. Unique alleles are not shared between intercontinental populations, and even within continents barriers such as mountain ranges can prevent gene flow. For these taxa, therefore, neither long-distance spore dispersal nor vector-mediated dispersal appears to be an effective means of sharing genes between populations. For example, *Flammulina velutipes* (Figure 4a) has a pan–North Temperate distribution, and collections from all geographical areas are interfertile (Ron Petersen and Karen Hughes, unpublished data). Part of a rRNA gene repeat was sequenced, and sequence differences were identified. One set of sequence differences was uniquely European, whereas another set was uniquely North American. Clearly, these two populations have not exchanged genes for a long time (Methven et al. 1997).

P. pulmonarius and *P. ostreatus* also show continental-specific sequence differences (Vilgalys and Sun 1994a, Petersen 1995b). And Chinese and North American populations of *Clavicorona pyxidata* differ in rRNA gene sequences (Ed Lickey, University of Tennessee, personal communication) and in laccase (a lignin-degrading enzyme) electrophoretic patterns (Wu et al. 1995). *Panellus stypticus* from New Zealand shows significant sequence divergence from European and North American *P. stypticus* populations (Jin et al. 1998) but easily mates with them (Petersen and Bermudes 1992a, 1992b). A further example of divergence: fruitbodies of *P. stypticus* are bioluminescent only in eastern North America and nowhere else in its worldwide distribution (Petersen and Bermudes 1992a).

An interesting question concerns how long fungi on different continents have been separated without evolving barriers to gene exchange. Some information can be derived from biogeographical patterns in higher plants. Plant taxa with widely disjunct distributions in temperate regions of the Northern Hemisphere have long been noted (Gray 1846). These distributions are thought to have been derived from a widespread mixed deciduous forest in the early to mid-Tertiary Period (Graham 1993 and references therein). During that time, a much warmer climate allowed plants to spread northward, and plants migrated between Europe and North America via the now sundered landmass of Euramerica. Following the Tertiary Period, the continents separated farther and colder climates pushed plant species' ranges farther south. If fungi followed the same distribution pattern, then ex-

tant members of one biological species on two continents may have been separated since the mid-Tertiary Period but may not have yet evolved crossing barriers, even in the face of time and diverging morphology.

Even within continents, there appear to be barriers to gene exchange leading to speciation. For example, the wet West Coast of the United States is geographically diverse and isolated from the eastern United States by the Cascade Mountains and the Rocky Mountains. *P. pulmonarius* isolates from the West Coast have a series of unique alleles that are not shared with isolates from east of the Rocky Mountains (Petersen 1995b). McCleneghan (1996) found a genetically isolated population of *P. spumosa* just south of Puget Sound, not far from the southern limit of the glacial ice shield. Likewise, there are more species of *Flammulina* from northern California to British Columbia than in any other part of the world (Ron Petersen and Karen Hughes, unpublished data). Two rare species of *Xeromphalina* are also found in the area (Johnson 1997). One is *Xeromphalina orickiana*, which fruits on redwood. The other, unnamed species has been found in only two locations: the Olympic Mountains and southern coastal British Columbia.

In the face of physical barriers over long time periods, and even morphological differentiation, why is it that reproductive barriers may not have evolved between intercontinental populations? According to Mayr's (1942) model of allopatric speciation, two things must happen for speciation to occur: an isolated population must be restructured (through mutation, selection, or genetic rearrangement) so that it survives in its new environment (see also Carson 1985), and reproductive isolating mechanisms must evolve that protect the reorganized gene pool. In Mayr's view, reproductive isolating mechanisms "arise as an incidental byproduct of genetic divergence in isolated populations" (Mayr 1970). Fungi, however, have unique mating systems with strong selection for outcrossing, and it may be that within geographical regions the gene pool is large enough and selection for outcrossing strong enough that the mating system is retained unchanged, even when morphological (and other) differentiation occurs. In essentially all cases we have studied involving genetically divergent populations of a single biological species, the populations were isolated from each other by some vicariance event (e.g., intercontinental or intermountain separation). When strong reproductive barriers have been observed, they have, as discussed above, occurred under conditions of relative sympatry (e.g., sibling species of *Xeromphalina*) or between genetically divergent species.

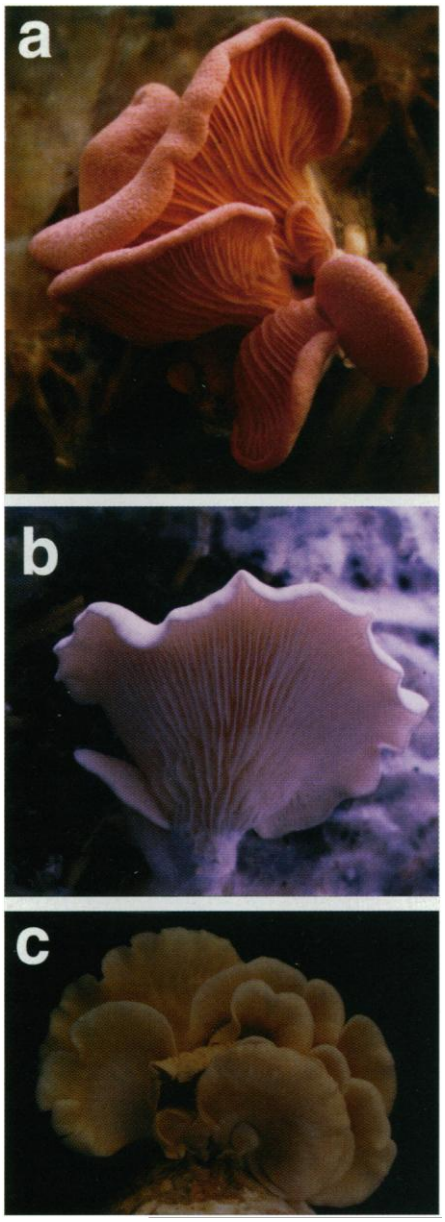

Figure 5. Basidiomata of *Pleurotus djamor*. (**a**) Pink form from Asia. (**b**) Off-white pan-tropical form. (**c**) Olive-tan form from New Zealand. Approximately 0.5× magnification. Photographs: David Nicholl.

Distributions that suggest recent dispersal. For many of the fungal groups that we have studied, there is no evidence of either long-distance spore dispersal or vector-mediated dispersal. There are some examples, however, of recent human-mediated introduction of fungi into new geographical areas. Because of these examples, the occurrence of a mushroom species far from its first reported range raises questions, not only about its accurate identification, but also about long-distance dispersal versus vectored introduction.

The "button mushroom" of supermarkets, *Agaricus bisporus*, is one such human-mediated introduction. This species has been domesticated for over a century, first in Europe (primarily France), then in eastern North America, and, most recently, in western North America. In California, Kerrigan et al. (1995) were able to distinguish two strains based on RFLP patterns. One strain, usually found fruiting in lawns, was similar to the commercial strains originally domesticated in Europe, whereas the other strain, fruiting at the margins of native woods, was unique and probably endemic to California. Other populations of *A. bisporus* endemic to the United States and Canada have now been identified, but there is strong genetic evidence that isolates of *A. bisporus* with the European RFLP patterns have probably escaped from commercial production sources and from home compost piles (with current dispersal by 18-wheelers).

Similarly, collections of *F. velutipes* from Chile and Argentina, all sexually compatible with their North Temperate Zone counterparts, have RFLP patterns matching the RFLP patterns of European isolates of *F. velutipes* but not those of North American isolates (Methven et al. 1997). Presumably, when European immigration and commercial intercourse with these Patagonian areas developed, along with the people and introduced trees came associated fungi.

Recently, *Pleurotopsis longinqua* was shown to be sexually inter-

compatible throughout its disjunctional range from Australia–New Zealand and Chilean and Argentine Patagonia to the Pacific Northwest of North America. The Australian–New Zealand populations showed significantly more basidiome morphological variation than those from other locations, but RFLP patterns and rRNA gene ITS sequences differed little, suggesting that the species may have been recently introduced into one or more of its locations (Petersen 1992, Petersen and McCleneghan 1995, Hughes et al. 1998).

Conclusions

Considerable time and effort has been spent trying to codify species concepts that are "universal" (i.e., broadly applicable to all forms of life), without resolution (Hull 1997). Suggesting a universal species concept for mushrooms is a particularly difficult exercise. Most investigators would agree that a species should be the result of an evolutionary process and should represent a cohesive interbreeding group that is reproductively isolated from other cohesive interbreeding groups. The use of a morphological species concept may be hampered by an inadequate suite of morphological characters and a considerable degree of convergent evolution for mushroom shape and spore dispersal mechanisms. The use of characters other than morphology (e.g., isozymes, DNA sequences, mating system analyses, and secondary-product chemistry) has been helpful in defining cohesive groups. Although a biological species concept also has problems if applied strictly, the use of mating studies to reveal genetic barriers to gene exchange has been valuable in uncovering cryptic species, identifying unexpected evolutionary relationships, and elucidating members of a population.

If a universal species concept is desirable for fungi, then that concept must be phenetic (i.e., based on overall similarity of organisms, whether morphological or other characters), simply because many known fungi lack sexual reproduction, a problem that has proven intractable for many species concepts (Hull 1997). For fungi, therefore, developing a universal species concept may be unwise because it limits definition of species to phenetic analysis, even when additional data are available. We suggest that for the fungi, the species concept should be based on a demonstration of phenetic cohesiveness, common evolutionary descent, and reproductive isolation where possible. The concept of using a variety of species concepts within a group is not new (Mishler and Donoghue 1992, Claridge et al. 1997), and it may be necessary to capture the complexity of speciation patterns in nature.

In this article, we have argued that mating studies have been especially helpful in elucidating speciation patterns in the mushroom fungi, a point others have also noted (Brasier 1997 and references therein). It is now a "given" that various character suites (e.g., morphology, ecological preference, physiology, biochemistry, and molecular biology) change and diverge at different rates with little predictability. Through processes of selection, it may be that sexual recognition, compatibility, and interfertility are among the *last* suites to diverge in allopatric situations, and that speciation is far from abrupt. Thus, in our studies, when strong reproductive barriers are present among allopatric groups, they are accompanied by phenotypic and genotypic differences that provide ample evidence for speciation. However, the converse—that the ability to interbreed implies conspecificity—is not necessarily true (Brasier 1997). When two allopatric groups are fully interfertile but represent different evolutionary lineages (e.g., *Pleurotus* intersterility group I; Vilgalys and Sun 1994a), determination of speciation status is not clear. That is, have these populations progressed through sufficient divergence to call them species (Figure 1)?

Sibling species, which are reproductively isolated but genetically nearly identical, present a special challenge, with some investigators choosing to group them within a single species (Brasier and Rayner 1987, Hallenberg et al. 1994). Yet these are reproductively isolated and already on the irreversible road to genetic divergence. As such, they may just as well be given species rank. This is a question about which even the authors of this article disagree. Ultimately, the determination of species status depends on the best judgment of the experienced investigator based on all available data.

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