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Classical Methods and Modern Analysis for Studying Fungal Diversity

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10.1   INTRODUCTION

In this chapter, we examine the use of classical methods to study fungal diversity. Classical methods rely on the direct observation of fungi, rather than sampling fungal DNA. We summarize a wide variety of classical methods, including direct sampling of fungal fruiting bodies, incubation of substrata in moist chambers, culturing of endophytes, and particle plating. We also cite and discuss study designs for documenting diversity and monitoring species, and analytical methods that have been used for data produced using classical methods. Selected examples of such mycological studies are cited so they may serve as models for future research.

The advantages and disadvantages of using classical methods and how these differ from molecular methods are discussed first. Next, we discuss differences in study goals

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as an important factor when selecting methods for sampling and analyzing fungal diversity. For example, detecting rare or endangered species generally requires different methods from general assessments of fungal diversity or monitoring changes in populations or communities. Selection of appropriate methods for assessing fungal diversity is also highly dependent on the ecological characteristics and distribution or dispersion patterns of the targeted fungal group in both time and space. The types of methods that have been used in classical fungal diversity studies are discussed in the context of the ecological groups for which they are appropriate, and we have cited selected examples in which each of these has been successfully employed. In the last section, we cover statistical and other analytical methods that can be applied for different goals or hypotheses regarding fungal diversity and conservation.

10.2   ADVANTAGES OF CLASSICAL METHODS

Despite recent advances in the use of molecular methods, there are still many advantages to classical methods for studying fungal diversity. Classical protocols have been developed for studying any substratum or group of fungi, and are described in detail in Mueller et al. (2004).

One of the most useful products of a classical study is a list of species found during the study. Due to the relatively small number of species that have been sequenced, it is often impossible for a molecular-based study to present a similar list. Assembling a species list enables researchers to compare data across sites and studies and among different taxonomic or ecological groups. By combining species lists from multiple studies, researchers can determine basic information about individual species, such as geographic range, host relationships, and ecological distribution. The fungal communities of different areas can be compared to determine patterns of species diversity. Additionally, the data from various studies can be combined in order to perform meta-analyses, which can be used to determine the biological and environmental factors that influence fungal community structure at large scales. Classical methods are also the only methods that can be used to demonstrate which fungi are reproducing in a particular environment or on a given substratum, as opposed to which fungi are present but cannot reproduce.

Classical methods are often used to inventory fungi over a clearly defined area or amount of substrate. Researchers often measure environmental variables, such as pH, soil nutrient content, weather-related variables, and biotic variables (e.g., plant community composition or biomass), on the same plots or substrates. Numerous statistical techniques are available to help investigators evaluate the impact that these factors have on fungal communities. Studies using molecular methods can also include environmental measurements, but the data for molecular studies and environmental measurements are often taken at vastly different scales. Combining molecular data from a few grams of substrata with environmental data taken over a much wider area is a major challenge for fungal ecology.

One final advantage of classical methods is that compared to molecular methods, they are generally less expensive and need less specialized equipment. These are important considerations for many investigators, especially those in developing nations.

10.3   DISADVANTAGES OF CLASSICAL METHODS

Despite their widespread use, classical methods have certain disadvantages when compared to sampling using molecular techniques. Some species may not grow or produce repro-

ductive structures in culture and may reproduce rarely in natural settings. These species will be missed by traditional sampling methods, even though they could be important members of the fungal community. The fact that some species will not be detected clearly has the potential to bias classical studies. Unfortunately, it is difficult to assess how many species are missed by classical techniques or to determine if this can bias the results of any particular study. While molecular-based studies of fungal diversity can provide an independent assessment of the fungal community, they are limited to sampling a small area, which can result in a different set of biases.

Compared to molecular techniques, classical sampling methods can be considerably more time consuming. Studies based on macrofungal fruiting bodies have shown that even in areas that have been repeatedly sampled for many years, new species can be found (e.g., Arnolds, 1988; Perini et al., 1989; Tofts and Orton, 1998, Straatsma et al., 2001). Additionally, more taxonomic expertise is required for classical methods than for molecular methods, as all of the species must be identified based on morphological characters. The relative scarcity of trained taxonomists can lengthen the time it takes to identify all of the collections, and thereby lengthen the time it takes to carry out a study.

10.4   STUDY GOALS

10.4.1   Documenting Diversity

One of the most common reasons to conduct a study of fungi is to document the diversity of species present in a particular area. Diversity studies are sometimes used to document the presence of rare or endangered species, but more commonly to demonstrate that they are indeed rare (e.g., Arnolds, 1998, 2001; Ing, 1996; Molina et al., 2001; Otto and Ohenoja, 1998; Courtecuisse, 2001). General survey and inventory methods are not as efficient as targeted surveys for detecting species that are known to be rare or endangered (see following section; Molina et al., 2001). Quantitative, plot-based diversity studies are also used to provide baseline community data in anticipation of future plant succession, disturbance, or stressors such as climate change and air pollutants (Arnolds, 2001; Ing, 1996). In addition, such studies are used to compare fungal communities in different areas, either to aid in prioritizing conservation decisions (Courtecuisse, 2001; Senn-Irlet, 1998) or to gain insight into what factors influence fungal community composition (Lodge, 1997; Heilmann-Clausen, 2001) and temporal variation in fruiting (Straatsma et al., 2001).

10.4.2   Detecting Rare Species

If certain species are known to be rare, or are restricted to limited or endangered habitats such as old-growth forests or unfertilized grasslands, it is often most efficient to use targeted surveys to locate populations of these species or their habitats (Molina et al., 2001; Parmasto, 2001). This generally begins with a survey of previously known localities based on herbarium records and reports (Jalink and Nauta, 2001; Molina et al., 2001). If a species is known or suspected to occur only in a restricted habitat, a search for areas with the same or similar habitats that are then searched for the rare species usually follows (Jalink and Nauta, 2001; Parmasto, 2001; Molina et al., 2001; Rotheroe, 2001). This approach has been referred to as habitat modeling by Molina et al. (2001) and gap analysis in conservation literature; it is useful for locating additional populations. Systems for ranking and prioritizing rare and endangered species for conservation purposes, such as Red Data Lists, generally use a combination of criteria that include the number of populations of a species in addition to rarity of occurrence and occurrence in a limited range or in threatened habitats (see Kotiranta, 2001; Molina et al., 2001). Rarity of occurrence

and range limits are often established using large-scale mapping and recording programs (Courtecuisse, 1993; Fraiture, 1993; Nauta and Vellinga, 2002). If new sites are discovered to be rich in rare and endangered species, these sites are sometimes inventoried to assess overall fungal diversity.

10.4.3   Monitoring

Recently, there has been interest in monitoring populations of individual fungal species. This can be due to the economic importance of a species, or because it is believed to be in danger of extinction in all or part of its range (Moore et al., 2001). Unfortunately, there is relatively little information on the population dynamics of fungi growing in the wild, which makes it challenging to design a monitoring program. Molina et al. (2001) discuss many of the issues involved in monitoring individual species. They point out that several factors need to be considered in a monitoring program, including establishing clear goals. In the case of rare or poorly known species, populations need to be located before they can be monitored. Due to variations in fruiting, several surveys may be needed each year. For commercially harvested species, monitoring needs to include the amount harvested, length of fruiting season, etc., as well as information on harvesting techniques and land use that may have an impact on fruit body production (Molina et al., 2001). At least two studies (Egli et al., 1990; Norvelle, 1995) have shown that mushroom picking alone does not have an apparent effect on fungal fruiting.

10.5   TYPES OF CLASSICAL METHODS

10.5.1   Opportunistic

Mycologists have traditionally used an opportunistic approach to collecting fruiting bodies of macromycetes, and it is often the most efficient way to record new species in a study area. Typically, this entails collecting fruit bodies that are in good condition and are visible along trails. There are several disadvantages of this method. Data from opportunistic collecting are not easily quantifiable, thus limiting comparisons among areas. This method also requires a highly trained collector who can recognize taxa in the field, but there is also a danger of collector bias affecting the results. Some collectors only detect large or brightly colored fruit bodies, or favor particular groups of fungi because they have developed a search image for them. Furthermore, inconspicuous species and those that are easily confused with more common species are often overlooked (Lodge et al., 2004). Some mycologists and other field biologists have adapted this approach to make the search area quantifiable by using a band-transect method in combination with existing trails. In order to quantify the area searched, the length of the trails must be measured, and only fungi that are found fruiting within a set distance from the trail (e.g., 1 m on either side) are collected or recorded.

10.5.2   Substrate Based

The importance of substratum type cannot be overemphasized in relation to selection of a sampling scheme for quantification of macrofungi. Basidiomycetes and ascomycetes have been found to fruit differentially on different types of substrata or diameter classes of wood, and species rarely fruit on dissimilar substrata (Lodge, 1996; Huhndorf and Lodge, 1997). While some fungi fruit rather dependably, others fruit only sporadically, often requiring surveys lasting a decade or more to be recorded from a particular area (Straatsma et al., 2001). Most surveys therefore employ one or several methods (discussed below) to estimate total species richness in their study area based on a finite number of samples. Many years

of surveying will be required if several hyperdiverse communities are combined in a single survey, such as orb weaving and hunting spiders (Colwell and Coddington, 1994) or fungi occurring on different types of substrata (Lodge et al., 2004). Furthermore, fruiting patterns of fungi differ among substratum types, and the abundance and dispersion of the substrata differ, so the methods used for large woody debris are unlikely to be efficient for fungi on leaf litter or soil and visa versa. If a complete inventory is desired, it is better to use different methods for fungi fruiting on large woody debris vs. small substrata or soil, and to treat the data as belonging to separate data sets (Lodge et al., 2004).

Substrate-based sampling methods are used for fungi that occur only on discrete, discontinuous, or patchy resources or are restricted to a particular host. If many resource patches or units occur within a conveniently sized measurement plot, then plot methods may be used. If the patches or substrata of interest are widely dispersed, however, it is more efficient to use a substrate-based method. In addition, relative frequencies of fungal species per substrate unit can be used to directly compare areas that differ in substrate frequency, making substrate-based methods advantageous in such cases. Substrate-based methods are used for fungi occurring on large woody debris or snags (Heilmann-Clausen, 2001), dung (Nyberg and Persson, 2002; Richardson, 2001), fruit (Rogers, 1979; Callan and Carris, 2004), animal corpses (Benjamin et al., 2004; Evans and Samson, 1982, 1984; Hywel-Jones, 1997; Sagara, 1995), and those closely associated with particular animals or plants, including commensals or mutually beneficial symbionts (Stone et al., 2004; Rand, 2004; Summerbell, 2004) and pathogens (Barron, 2004; Callan and Carris, 2004; Summerbell, 2004).

10.5.2.1   *Sporocarps on Large Woody Debris*

For fungi that fruit on large woody debris, it is often most efficient to use a log-based sampling method (Lodge et al., 2004; Huhndorf et al., 2004). The logs should be classified into diameter and decay classes, tree species (or at least conifers vs. dicotyledonous plants), and whether they are upright, suspended, or on the ground. This allows for selection of several representative logs (replicates) from each type (stratified sampling). A good example of this method was employed by Heilmann-Claussen (2001) in Denmark; in that study, the logs were classified into age classes based on historical aerial photographic records. Logs may be located and quantified per unit area using several different methods, including line transects and the point-quarter method (Lodge et al., 2004).

10.5.2.2   *Sporocarps on Leaf Litter, Twigs, and Small Branches*

For fungi fruiting on fine debris, it is generally recommended to use a plot-based or band-transect method. While relatively large plots have been used for fungi growing on fine litter (25 to 1000 m^2, 5 to 100 m on a side; e.g., Schmit et al., 1999; Straatsma et al., 2001), it is often more efficient to use smaller, 1 m^2 plots distributed along transect lines (Cantrell, 2004; Lodge and Cantrell, 1995; Lodge et al., 2004). If the fungi to be surveyed are so small or cryptic that they cannot be recognized without the aid of a microscope, such as small ascomycetes on small wood and leaves, the debris can be collected from a quarter of the area in the plot on each sample date and returned to the laboratory for closer examination (Cantrell, 2004; Huhndorf and Lodge, 1997; Huhndorf et al., 2004; Lodge et al., 2004).

10.5.2.3   *Sporocarps on Soil and Ectomycorrhizal Associates of Trees*

Saprotrophic fungi that fruit on soil and ectomycorrhizal fungal symbionts of tree roots generally require large plots or survey bands in order to detect a majority of the species that are actually present on the site (Lodge et al., 2004; O'Dell et al., 1999; Straatsma et

al., 2001). Some species in these groups fruit only rarely (Lodge, 1996; O'Dell et al., 1999; Schmit et al., 1999), and their occurrence in different plots is often patchy (Straatsma et al., 2001; Schmit et al., 1999). Because ectomycorrhizal fungi are associated with only certain types of trees and shrubs, the distribution of the plant community should also be considered when selecting plots. Forest types are often related to topography, so rectangular plots (e.g., 10×50 m) that are oriented at right angles to the slope are often better than square plots for obtaining a homogeneous sample of the same plant association.

10.5.3 Moist Chambers

Moist chambers are used to stimulate fruit body production on substrata that have been collected from the (Krug, 2004). This method is most often used for fungi growing on leaves or small woody debris, such as ascomycetes (e.g., Polishook et al., 1996; Rambelli et al., 2004) and slime molds (e.g., Snittler and Stephenson, 2000), and fungi growing on dung (Bills and Polishook, 1993; Krug et al., 2004; Richardson, 2001; Rossman et al., 1998). The substrata are usually placed on moist paper towels in an inflated plastic bag or in a container with a lid. The samples are then examined for the presence of fruit bodies periodically for 2 to 6 weeks.

10.5.4 Culturing

10.5.4.1 *Endophytes*

Endophytic fungi are those that live inside of live plant parts without causing disease, though some may in fact be latent pathogens or mutualistic symbionts (Caroll, 1988, 1995; Viret and Petrini, 1994). Symptomless but fully expanded leaves are normally collected, but petioles, twigs, branches, and roots have also been studied for endophytes (Caroll, 1988, 1995). Typically, the plant parts are surface sterilized using a 95% ethanol wash followed by immersion for 2 to 5 min in dilute (0.5%) sodium hypochlorite, and a sterile distilled water rinse. Small sections of surface-sterilized plant tissue (smaller pieces are better — Carroll, 1995; Stone et al., 2004; but 1- to 2-mm pieces or segments are often the practical, lower limit) are then placed on agar media containing growth inhibitors in Petri dishes (Lodge et al., 1996). The antibiotics and growth inhibitors prevent bacterial growth and slow the growth of fast-growing endophytes that could otherwise inhibit slower-growing fungi. Typical media include Malt Extract Agar (MEA) with 250 mg/l oxytetra-cyline (Lodge et al., 1996) and MEA with 35 μm/ml rose bengal, 50 μm/ml streptomycin, and 50 μm/ml chloramphenicol added after autoclaving (Bayman et al., 1997, 1998).

10.5.4.2 *Leaf Washes*

Leaf washes have been used to study the composition of spores on leaf surfaces. Phylloplane fungi are important in natural biocontrol of pathogens (Bélanger and Avis, 2002; Lindow et al., 2002). Epiphytic fungi, including lichens, can comprise a significant amount of biomass in some ecosystems, and they can play critical roles in food webs and nutrient cycles (Lindow et al., 2002; Lodge, 1996; Stone et al., 1996). Most studies of endophytic and decomposer fungi using the particle filtration method also obtain cultures of phylloplane organisms from surface wash water.

10.5.4.3 *Particle Filtration*

The particle filtration technique was designed by Bills and Polishook (1994, 1996) and Polishook et al. (1996) to eliminate or reduce the number of isolates derived from dormant

spores in cultures taken from decomposing plant debris. Thus, cultures derived using this method are primarily of vegetatively active mycelia. The disadvantage of any culture method is that only fungi that are capable of growing in pure culture are detected. According to the method (modified by Bills, 2000), leaf litter is air dried for 3 h before microfungal species are isolated using the particle filtration method, though leaves may be dried for a few weeks with little loss of diversity, if necessary (Paulus et al., 2003). Pretreatment of the leaf surfaces for 2 min in 0.5% NaOCl, followed by washing with sterile distilled water, was found to be effective in killing surface contaminants without reducing the abundance or diversity of fungi cultured from particles (Paulus et al., 2003).

Air-dried, decomposed leaves are pulverized at high speed, and then washed with a stream of sterile distilled water to remove spores. The particles trapped on the 105-μm mesh filter are washed several more times and then plated at several dilutions onto agar media in 90-mm Petri dishes using a flamed, bent glass rod (10 plates each of Malt–Cyclosporin Agar and Bandoni's Medium). This procedure should be carried out in a sterile hood.

At least two types of culture media should be used for the initial dilution plates, Malt-Cycloporin Agar (Malt Yeast Agar (MYA), with 10 mg of Cyclosporin A added when the medium is cool; Polishook et al., 1996) and Bandoni's Medium (4 g of L-sorbose, 0.5 g of yeast extract, and 20 g of agar per liter of distilled water). Fifty milligrams per liter of chlortetracycline and streptomycin sulfate are also added to the Malt-Cyclosporin Agar and Bandoni's Medium when the agar is cool to prevent bacterial growth. All fungi growing from particles should be isolated as they emerge to prevent them from inhibiting the growth of other fungi, and to prevent bias. The fungi can then be sorted into morphologically similar species (morphospecies) after 1 month of growth. Fungi growing from the particles can be transferred to Petri dishes or slants with Malt Yeast Agar (10 g of malt, 2 g of yeast extract, and 20 g of agar per liter of distilled water). Placing subcultures on additional media, such as catmeal (OMA), cornmeal (CMA), malt (MA), Potato Dextrose Agar (PDA) (see Rossman et al., 1998), or Potato Carrot Agar (PCA, see Paulus et al., 2003) is useful for separation and identification of strains, especially those that fail to sporulate. Common culture media can be found in Bills and Foster (2004). Autoclaved banana leaves, wheat leaves, or autoclaved leaves of the species from which the fungi were originally isolated can be added to PDA, PCA, or MEA media to promote sporulation.

10.5.5   Area-Based Plots

Area-based plots are one of the most frequently used methods in ecology to quantify the number of species per unit area, and to provide a basis for comparing areas using statistical methods. Detailed recommendations for the design of plot-based studies of macrofungi are available elsewhere (Lodge et al., 2004; Mueller et al., 2004; Molina et al., 2001; O'Dell and Lodge, 2004). Comparisons of fungal communities using plot-based methods have been made between areas receiving different treatments (Barr and Kuyper, 1993; Shaw et al., 2003), between areas under consideration for conservation or for inventorying for prioritizing conservation of rare species (Molina et al., 2001), between different plant communities or plant associations (Straatsma et al., 2001), and to study the effects of air pollutants on fungi (e.g., Fellner, 1993). Area-based plots must be used to construct species-area curves (see below).

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10.5.6   Transect-Based Methods

Transect-based methods are useful for studying how populations vary along environmental gradients (Van Maanen and Goubiere, 2000). Data in which a single gradient axis is of interest can be analyzed statistically using logistic regression (Van Maanen and Gouriere,

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2000) or correlation analyses. It does not matter if the gradient axis represents a single environmental gradient (e.g., a moisture gradient) or a complex environmental gradient (e.g., an elevational gradient in which temperature, moisture, and other factors vary in concert). Multivariate analyses, such as correspondence analysis, are often used to elucidate patterns in transect-based studies (see below). Transects have been used to demonstrate the association of ectomycorrhizal fungi with a particular ectomycorrhizal host that had a clumped distribution rather than an environmental gradient (Henkel et al., 2002), and to look at patterns of host specialization in relation to host diversity and dispersion patterns (Gilbert and Sousa, 2002).

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10.6 STATISTICAL ANALYSES FOR DIVERSITY STUDIES

10.6.1 Species-Accumulation Curves

One of the oldest and most common analyses of diversity data is to construct a species-accumulation curve, such as the familiar species-area curve (Rosenzweig, 1995). A species-accumulation curve is constructed by plotting the cumulative number of species found against some relevant measure of the effort used in finding them. As the cumulative number of species rises, more effort is required to find undiscovered species, which will be reflected by a leveling off in a species-accumulation graph (Figure 10.1). Species-accumulation curves are often used as an aid to determine if sampling effort has been sufficient to discover most of the species present in an area or on a substrate, or to make comparisons between sites.

When constructing a species-accumulation curve, careful consideration must be given to the variable used to measure effort. For a species-accumulation curve to be meaningful, the measurement that is used to quantify effort should be as accurate as the
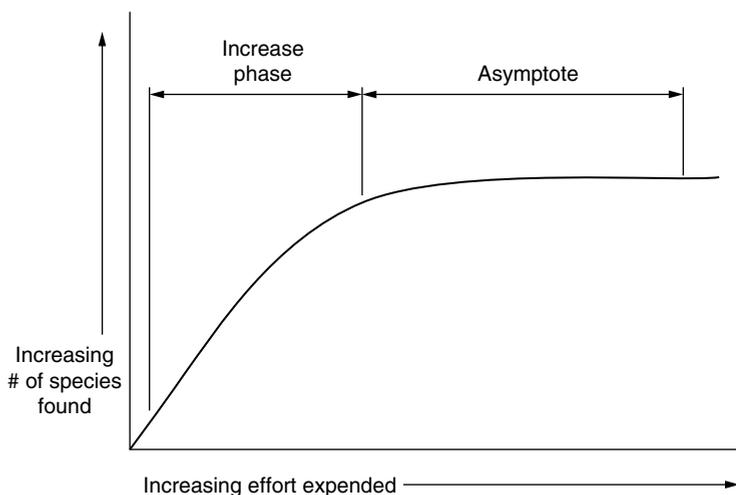

Figure 10.1 An idealized species-accumulation curve. During the increase phase, when little effort has been expended on a survey, increasing effort leads to a substantial increase in the number of species found. Eventually, most of the species are found and the species-accumulation curve reaches an asymptote where more effort results in finding few new species. In practice, few fungal surveys are extensive enough to reach the asymptote.

measurement of species richness. Different methods of collecting will give data that are more or less suited to different species-accumulation curves, as we discuss below.

10.6.1.1   Species-Area Curves

Species-area curves are likely the most widely used accumulation curve in diversity studies, particularly in studies that focus on plants. These curves are constructed based on data indicating how many species are found in areas of different sizes. They are often used as a guide to determine if sufficient area has been sampled in a biodiversity study, or to determine the size of sample plots that are needed.

Mycologists should be very cautious when using these curves, particularly if they are used to assess sampling effort or predict the total species richness of a given area. When species-area curves are used to determine the species richness of an area, the underlying assumption is that all, or nearly all, of the species have been discovered in the sampled areas used as data to construct the curve. Regardless of the sampling method used, the high species richness of fungi, their cryptic nature, and their seasonality make it likely that many species in a given area will be missed. Unfortunately, when analyzing species-area curves, it is not possible to distinguish between finding few species due to sampling a small area vs. finding few species because the area studied was not sampled well enough. Therefore, it most instances, using species-area curves will lead to an underestimate of fungal species richness, as many species will be missed in the sampled areas. The underestimation of fungal richness could potentially lead to an overestimate of the adequacy of sampling.

Despite these difficulties, there are some situations where the use of a species-area curve is useful, even if not all species have been sampled. For example, researchers may want to compare the species richness of several different areas, but only have diversity data that was collected from plots or transects of different sizes. The species richness of each site can be regressed against the area surveyed (with the variables log transformed, if necessary). Sites can then be compared on whether they are more or less diverse than would be expected given the area sampled.

A number of species-area curves for fungi have been published. Interestingly, species-area curves constructed from data collected during a single sampling occasion are more likely to level off than those constructed from long-term sampling. Guevara and Dirzo (1998) studied macrofungi in an evergreen cloud forest in Mexico. They did two samplings, one in May and one in September, along two transects. Looking at each transect in each month, they found that once approximately 100 m^2 was sampled; the species-area curve leveled off, but they did not make a species-area curve with the combined data from both months. Brunner et al. (1992) studied macrofungi living in two *Alnus* forests in Alaska by collecting from two 1000 m^2 plots in each forest. After sampling nine times during a single growing season, they determined that surveying 2000 m^2 was not sufficient to fully sample these communities, but felt that 3000 m^2 would be sufficient. Bills et al. (1986) inventoried ectomycorrhizal–basidiomycete communities on twelve 256 m^2 plots split between red spruce and hardwood forests in West Virginia. After collecting on approximately 27 occasions over 3 years, they found no leveling off in the species-area curve.

Lodge and Cantrell (1995), working in a tropical forest in Ecuador, surveyed twenty-four 1 m^2 plots divided between two transects for litter-decomposing agarics. Each plot was only sampled once. They found that the species-area curve for these plots leveled off at about 20 m^2. They then looked at the overlap between the two transects. The overlap was approximately 50%, which indicates a good sampling of the community as a whole

(Coddington et al., 1991). Cantrell (2004) found similar results in a survey of tropical discomycetes in Puerto Rico and the Dominican Republic.

10.6.1.2   *Species-Substrate Unit Curves*

Species-substrate unit curves are accumulation curves that use the number of substrate units sampled as a measure of effort. In practice, a wide variety of studies have made use of this type of curve. This can include large substrate units sampled in nature, such as macrofungi on logs (Lindblad, 2001), or the number of substrate units incubated in a laboratory setting (Yanna et al., 2001). Care must be taken to ensure that the unit used to measure effort is appropriate — are the units that are sampled actually equivalent, or do they differ greatly in size and quality? For example, fungi were found to fruit differentially among substratum types and diameter classes of woody substrates in a subtropical wet forest in Puerto Rico (Lodge, 1996; Huhndorf and Lodge, 1997). Even if substrate units are physically similar, they could differ if sampled at different times of the year or in different habitats.

10.6.1.3   *Species-Collection Curves*

Species-collection curves plot the number of species found against the number of collections made in order to find them. Collections can be defined as any sample of fungus that can be identified — a fruiting body, an isolate, etc. One of the biggest advantages of constructing a collection curve is that the number of collections processed can be used to determine other useful information. For instance, the monetary cost of processing a single collection could be calculated, and the species-collection curve could be used to determine how much of a monetary investment is required to find a given number of species. Similarly, the amount of time needed to process a collection, or the number of collections that can be found in a single collection trip, can be used to cast the species-accumulation curve in terms of the amount of time that needs to be invested to find a given number of species (Longino and Colwell, 1997).

   The biggest drawback to species-collection curves is the need to collect and identify every specimen encountered. To make an accurate curve, every occurrence of every species must be recorded. This can place an extra burden on inventories, as otherwise researchers may not bother to record common species once they have already been found.

10.6.2   Analysis of Species-Accumulation Curves

One of the most common ways of analyzing species-accumulation curves is to inspect the curve to determine if it levels off as effort increases. For example, Lodge and Cantrell (1995) examined a species-area curve to determine that 24 plots of 1 m^2 are sufficient to sample the diversity of agarics living in tropical forest litter. Tofts and Orton (1998) used species-effort curves to determine that 21 visits to a Caledonian pine forest were not sufficient to find all agarics, but they did show a leveling off in the curve when only species that are restricted to Caledonian pinewoods are considered.

   Some ecologists recommend using statistical techniques to determine the asymptote of a given curve. Unfortunately, there is no consensus on how this should be done. Several statistical and ecological issues complicate extrapolation. Statistically, estimating diversity from a species-effort curve requires an extrapolation beyond the data (He and Legendre, 1996). To extrapolate beyond the data, it is necessary to choose a particular equation for the species-effort curve. Numerous equations have been proposed (see review in Colwell and Coddington, 1994; Christen and Nakamura, 2000), and different methods may be

appropriate for different community structures (Keating et al., 1998). Currently, there is no generally accepted method to determine which species-effort model is appropriate for any given data set.

Extrapolating a species-effort curve has also been questioned on ecological grounds. Extrapolating assumes that given a large enough effort, an asymptote in the species-effort curve would actually be reached (He and Legendre, 1996). However, as sampling effort increases, it becomes more likely that the sampling expands beyond a relatively homogeneous community. This is particularly true for species-area curves, and it has recently been suggested that, in general, species-area curves do not reach an asymptote at scales above 1 ha (Williamson et al., 2001).

The only example of extrapolating of species-area curve for fungi that we are aware of is that of Guevara and Dirzo (1998). They used two equations, the logarithmic and the Clench, to determine if they had sampled sufficient area for macrofungi on two sampling occasions. Both equations gave similar results, indicating that they had collected over 90% of the species. Unfortunately, they did not continue collecting to determine if the predictions of the equations were accurate.

A more recent use of species-effort curves is to extrapolate the amount of effort needed to find a given number of additional species (Keating et al., 1998). This is similar to predicting the asymptote of a species-effort curve, but rather than the curve being extrapolated to the point where it levels off, it is extrapolated to the point where an arbitrary number of new species have been found. This technique raises the same statistical concerns as determining the asymptote of a species-effort curve. However, if the curve is not being extrapolated very far in comparison to the data collected, the statistical problems are less troubling, as different curve-fitting techniques will probably give similar results.

10.6.3   Nonparametric Species Richness Estimators

In recent years, biostatisticians have realized the limits of species-effort curves to estimate species richness and have worked to develop simpler, more accurate estimators. Numerous nonparametric methods have been designed to do this. These methods are nonparametric in that they do not assume any particular distribution of common and rare species in a community. In general, these methods make use of data on the abundance of each species that has been detected. Depending on the collecting methods used, abundance of species can be measured by number of collections of a species, number of substrate units a species is found on, number of subplots a species is found on, number of cultures made of a species, etc. Colwell and Coddington (1994) provide a useful review of many of these estimators. Additional estimators have been derived by Solow and Polasky (1999) and Shen et al. (2003).

Several nonparametric estimators use the total number of species found, the number of species found once, and the number of species found twice to determine the number of species that have yet to be discovered in the area or on the substrate being studied. For example, one of the simpler estimators is $S = S_{obs} + a^2 / 2b$, where S is the estimated number of species, S_{obs} is the number of species observed, a is the number of species found only once, and b is the number of species found exactly two times (Chao, 1984). As the example makes clear, these estimators are attractive to ecologists because they are easy to calculate and rely on information that is easily gleaned from a biodiversity inventory.

Given the large number of available estimators, several researchers have attempted to determine which is the most appropriate for real data sets. Chiarucci et al. (2003) used an extensive data set measuring plant species richness and location from dunes in southwestern Australia. They tested four most commonly used estimators and concluded that none of them perform well: "the estimates obtained can hardly be expected to be accurate

and are not likely to be easy to interpret." To provide an accurate estimate, the estimators needed more data than will likely be available and were consistently biased. Chiarucci et al. (2003) also provide a comprehensive review of the literature, testing the performance of nonparametric estimators, which supports their conclusions that the performance of the estimators is disappointing.

The only study that has evaluated these estimators for fungal data sets is that of Schmit et al. (1999). They surveyed macrofungi for 3 years from plots in an oak forest and compared the predicted species richness based on data from the first year to the actual species richness found in the study. Seven species richness estimators were tested, and all of them gave predictions that were consistently too low and that increased as the amount of data increased.

Based on the limited results available, there seems to be two problems with the application of these estimators to fungi or any other hyperdiverse taxonomic group. The first is that more data are required to provide a reliable estimate of species richness than is generally available in a mycological study (Chiarucci et al., 2003 and references therein). Second, nonparametric estimators assume that the detectability of a species does not change during the time that the study is being conducted. Numerous mycological studies have demonstrated that the abundance of fungal species varies from year to year (e.g., Murakami, 1989; Perini et al., 1989; Schmit et al., 1999), which violates this key assumption. Despite these disappointing results, new methods of estimating species richness are being developed, and new techniques are being used to evaluate them. Undoubtedly, the use of nonparametric estimators will continue to be an active research area that has the potential to provide considerable insight into patterns of species richness of fungi.

10.6.4   Multivariate Methods

In order to understand fungal diversity, it is important to do more than just measure the species richness of fungi at various locations. In recent years, many investigators have worked to identify environmental and biological factors that influence fungal community structure. In practice, the community structure that is examined is either the patterns of presence and absence of species in various sites (= communities) or the patterns in the abundance of species in various sites.

The most important analytical tools for analyzing community structure are multivariate statistics such as cluster analysis and ordination techniques. In general, multivariate methods allow the investigator to group or order a number of sites based on their similarity, which is determined by analyzing a large number of variables. In the context of diversity studies, multivariate analysis is generally used in situations where investigators have information from several sites on the presence or abundance of fungal species, and it sometimes incorporates data on other variables (rainfall, soil chemistry, plant community structure, etc.) as well. In general, these techniques work best when a consistent sampling methodology has been used so that the data from each site are truly comparable. However, multivariate methods can also be used as part of a meta-analysis synthesizing data from several studies, provided due thought is given as to which technique is most appropriate.

A confusing array of multivariate techniques have been developed that can be used to study fungal diversity, and careful thought must be given to choosing the correct one. Luckily, ordination techniques are widely used in the biological sciences, and there is a large statistical literature analyzing the properties of the various techniques. The choice of analysis technique is driven by the data at hand and the goal of the particular study.
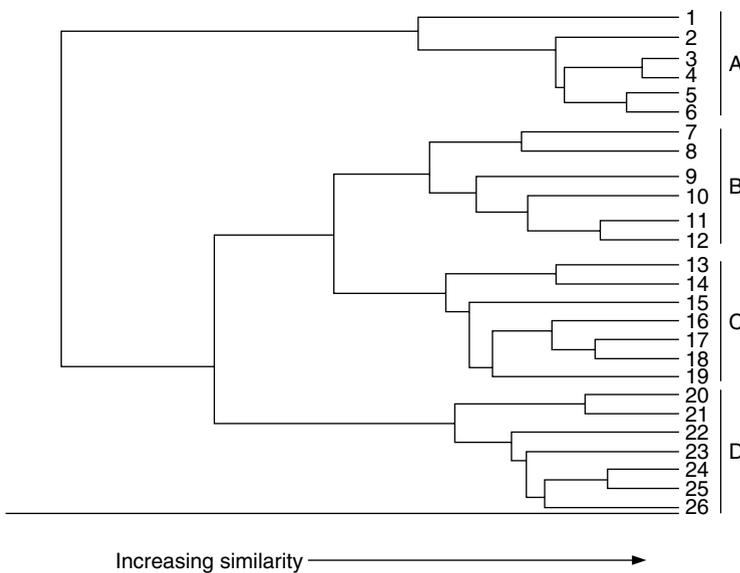
Figure 10.2   An idealized cluster analysis. In this diagram, 26 sites have been clustered based on the similarity of their fungal communities. The closer to the right-hand side of the graph that two communities are linked, the more similar they are. The 26 sites cluster into four major groups (A to D). Group B sites are most similar to group C sites, and group A sites are the least similar to those in the other three groups.

10.6.5   Exploratory Analysis

Oftentimes, investigators will wish to use data to group sites, hosts, etc., based on similarities in fungal communities. In some instances, the investigators will have an *a priori* hypothesis about fungal communities they wish to test; methods for doing so are described in Section 10.6.6. This section deals with analytical tools that are useful when investigators are performing an exploratory study and do not have a specific hypothesis to test.

10.6.5.1   *Cluster Analysis*

One of the most common multivariate techniques is cluster analysis. In cluster analysis, a number of cases, such as sites or hosts, are grouped based on variables such as the presence or absence or abundance of species. Cases that are closely connected on the cluster diagram are the most similar to one another (Figure 10.2). Cluster analysis provides a grouping of cases that is hierarchical, and the analysis shows the places of each case in a series of clusters and subclusters, each more inclusive, but with less overall similarity than the ones below it.

For example, Laganá et al. (1999) used cluster analysis to show the relationship between the macrofungal communities found in Italian forests. They combined data from 30 survey plots in five different forest types and demonstrated that fungal communities sampled from different locations within a single forest type (e.g., evergreen oak wood or chestnut woods) cluster closely together. At a higher level in the hierarchy, the fungal communities from low-elevation deciduous forests clustered together to the exclusion of high-altitude and coniferous forests. Press Perini et al. (1995) used cluster analysis to compare the plant and macrofungal communities in a number of Italian fir forests. They

demonstrated that forests that had similar plant communities also had similar macrofungal communities.

10.6.5.2 Principal Components Analysis

Principal components analysis (PCA) is one of several methods that are used to reduce variation on many axes into a more manageable, smaller set of axes that allows the researcher to more easily visualize the data. Each of the new axes is a linear combination of the original axes. The Euclidean distances among cases and among variables along the new axes are the same as those in the original data set. The scores for the first axis explain the maximum amount of the variability in the data set that can be elucidated by a single such variable. The scores for the second axis explain the maximum amount of the variation that was not elucidated by the first axis; the scores for the third explain the maximum variation that was not elucidated by the first two; and so on for additional axes. Each axis explains less of the overall variation in the original data set than those that preceded it.

In fungal ecology, one use of this technique is to analyze what is known as a site x species matrix. In this type of matrix, the columns are locations where fungi have been found, such as plots that have been surveyed, host tree species, or experimental treatments. Rows are the species that have been found in at least one site, host, or treatment. Each cell in the matrix is either the abundance of the species or, in the case of a presence–absence matrix, 1 if the species is present and 0 if it is not. PCA is used in cases in which abundance data are available for each species. The axis scores of the sites are then plotted and sites are grouped by visual inspection.

For example, Newton and Haigh (1998) studied the relationship between ectomycorrhizae and their host in Great Britain. They constructed a matrix with tree genera as the columns and ectomycorrhizal species that associate with the trees for the rows. PCA demonstrated that there were strong similarities in ectomycorrhizal species found on genera in the Fagaceae (*Quercus*, *Fagus*, and *Castanea*), between coniferous genera (*Pinus*, *Picea*) and *Alnus*, *Populus*, and *Salix*.

A second use for PCA is to aid in analyses that include measurements of numerous environmental factors. Oftentimes, when an investigator takes several soil chemistry-, climate-, or vegetation-related measurements at numerous sites, it can be difficult to determine which of those factors influence fungal communities. By using a principal components analysis, the investigator can reduce many environmental variables to a more manageable number of axes (Figure 10.3). A PCA also provides loadings, which tell the investigator the contribution of the variables in the original data set to the axis scores created by the analysis. This can allow an investigator to determine which species or environmental factors in the original data set are most important in differentiating the sites. For example, Hansen (1988) used PCA to study the relationship between soil chemistry and the macrofungal community found on plots in a Swedish beech forest. The study combined a previously published macrofungal survey with measurements of 31 environmental variables in the survey plots. PCA was used to reduce the environmental variables to three principal coordinates that were related to fungal community structure. The analysis showed that saturation of the soil with bases, percent clay in the soil, and percent organic matter in the soil had the biggest impact on the occurrence of macrofungal species.

10.6.5.3 Principal Coordinates Analysis

In usage, principal coordinates analysis (PCO), also known as metric multidimensional scaling, is similar to PCA. Like a PCA, PCO reduces the number of variables in a
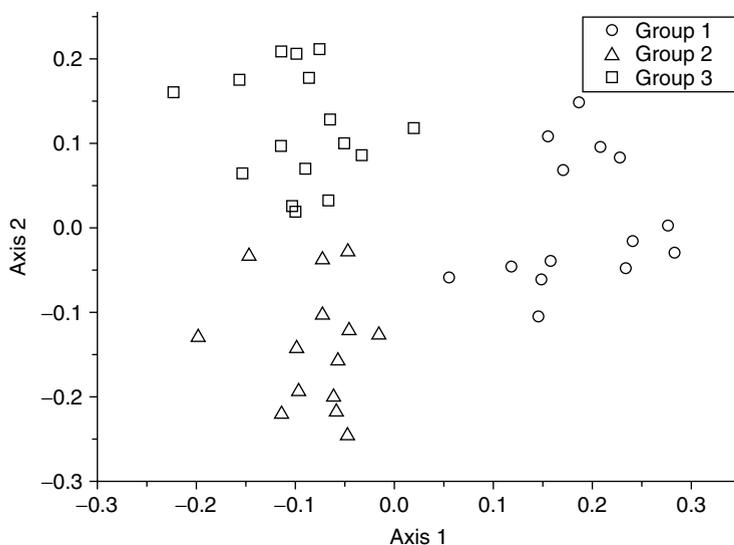
Figure 10.3   An ordination graph from a PCA. In this graph, sites from three different groups (e.g., vegetation types or experimental treatments) are plotted. The axis scores are calculated based on the abundance of each fungal species at each site. The graph shows that the sites from each of the groups cluster together on the graph. Axis 1 separates the sites in group 1 from those of the other two groups, while axis 2 separates the sites in group 2 from those in group 3.

multivariate analysis so that the similarities and the differences between the cases can be more easily visualized. There are several differences between the two analyses, however. One of the most important practical differences is that PCO can use abundance data or presence–absence data. PCO is better than PCA for presence–absence data, as a variety of distances measure can be used, including those designed specifically for presence–absence data. One drawback of PCO is that it does not supply axis loadings, which makes it more difficult to determine which variables have the greatest influence on the results. Peter et al. (2001) used PCO to demonstrate dramatic shifts in macrofungal fruiting in Norway spruce forest on plots that had been fertilized with nitrogen. Packham et al. (2002) used PCO to demonstrate that the same environmental factors that influence distribution of vascular plants in Tasmanian eucalypt forests also influence the distribution of macrofungal species.

10.6.5.4   Correspondence Analysis

Correspondence analysis (CA) is very similar to PCA and PCO. Unlike PCA, though, correspondence analysis can make use of any type of data, and unlike PCO, the chi-squared distances among the cases and variables from the original data set are preserved in the new axes. A frequently used variation on correspondence analysis is detrended correspondence analysis (DCA). Many ordination techniques, including PCA and PCO, are subject to what is referred to as either the arch or horseshoe effect. This is seen when the scores from the first two axes are plotted and the cases show an arch-shaped pattern, which indicates that the two axes are not independent. This is most likely to occur in studies where sampling has been done along an important ecological gradient and there is considerable turnover of species along that gradient. Detrended correspondence analysis corrects this problem and has become one of the most commonly used ordination tech-

niques in ecology, including fungal ecology (e.g., O'Dell et al., 1999; Ferrer and Gilbert, 2003; Heilmann-Clausen, 2001; Høiland and Bendiksen, 1996; Lindblad, 1997; Termorshuizen, 1991).

Correspondence analysis has shown that different microfungal communities are found on different parts of decaying palm fronds (Yanna et al., 2001). Salerni et al. (2001) used CA to demonstrate that the distribution of macrofungal species in Italian oak forests is influenced by soil moisture and pH.

DCA has been used to examine the factors that influence fungal community structure. Three studies have demonstrated that as logs undergo decay, there is a change in the fungi and myxomycetes that fruit on them (Høiland and Bendiksen, 1996; Lindblad, 1997; Heilmann-Clausen, 2001). It has been shown that there are differences in the communities that decay wood from different host trees (Ferrer and Gilbert, 2003). DCA has also been used to demonstrate that land use (Termorshuizen, 1991) and precipitation and vegetation (O'Dell et al., 1999) influence ectomycorrhizal community structure.

10.6.6   Hypothesis Testing

Ecologists are often faced with a situation in which they have a site by species matrix and data on an environmental factor that they believe will explain the differences in species presence or abundance between sites. Several ordination methods have been developed that allow researchers to test hypotheses given this type of data. Two of the most commonly used methods are canonical correspondence analysis (CCA) and discriminant function analysis (DFA). Unlike the ordination methods listed above, these two methods are designed to highlight differences in the data among groups or along gradients. CCA is used to determine the relationship between an environmental gradient and community structure. In CCA, the axes are drawn to maximize their correlation with a linear combination of the predictor variables and species data. The output from a CCA can be plotted like that of other ordination techniques, except that in addition to plotting the various sites or species, the environmental variables can also be plotted in the form of arrows that emanate from the origin of the graph. The direction of the arrow indicates a line on the graph along which the variable has its greatest change, and the size of the arrow indicates the magnitude of that change. Kernaghan and Harper (2001) used CCA to demonstrate that elevation has a large impact on the distribution of ectomycorrhizal macrofungi in the Rocky Mountains.

DFA, on the other hand, is used when the sites are believed to fall into distinct categories. The site by species matrix is used in the analysis as well as the *a priori* group designations. The analysis then compares the grouping derived from the data with the *a priori* grouping and determines which sites are classified in the same group by both methods.

10.6.7   Methods to Examine Fungal Distribution

10.6.7.1   *Mantel's Test*

Oftentimes investigators are interested in determining if differences in community structure between several sites are mirrored by differences in other environmental factors. One way to determine this is to use the Mantel's test. The Mantel's test compares distances between sites measured using one data set to the distances measured using a second data set. The distances used could be a community distance measure, such as the Jaccard Index, physical distance, a distance measure based on climatic data (e.g., difference in rainfall), distances based on an ordination analysis, etc. The analysis compares the two sets of distances and determines if sites that are distant based on one set of measurements are significantly more distant based on the second set of measurements than would be expected by chance alone.

Schmit et al. (1999) used the Mantel's test to provide evidence that at the scale of tens of meters, the closer that two plots are to one another, the greater overlap they have in their macrofungal communities.

10.7 CONCLUSIONS

Classical methods can be used with a wide variety of analytical techniques to characterize communities, test hypotheses, and study the ecology of individual fungal species. Due to the low cost, ease of identifying species, and ability to sample wide areas or many pieces of substrata, classical techniques will remain an important part of fungal ecology. Species lists and environmental information from several classical studies can be combined to analyze fungal distributions at wider spatial scales. However, species that fail to produce reproductive structures are often missed when sampling fungi with classical techniques — a problem not found with molecular techniques.

Combining classical and molecular techniques may allow mycologists to gain a much greater understanding of fungal distribution. In particular, by using these two techniques, it will be possible to compare the species that are merely present in a site or on a substrata with those that are able to reproduce on that site or substrata. Similarly, it will be possible to compare the geographic or host range over which a fungal species occurs to that over which it reproduces. Comparisons such as these will make it possible to determine the relative importance of different substrates or habitats for the maintenance of fungal populations. It will also be possible to compare the ecological roles played by species that differ in their frequency of reproduction. Studies such as these will provide fascinating new insights into the ecology and population dynamics of fungal species.

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