Brett A. Summerell

Royal Botanic Gardens – Sydney, New South Wales, Australia

Baharuddin Salleh

School of Biological Sciences, Science University of Malaysia, Penang, Malaysia

John F. Leslie

Throckmorton Plant Sciences Center, Kansas State University, Manhattan

A Utilitarian Approach to *Fusarium* Identification

Most plant pathologists, at some time in their career, must identify a culture of a *Fusarium* species. The complexity of the problem varies, depending on the host from which the culture originated and the degree of resolution required in the identification. *Fusarium* species cause a huge range of diseases on an extraordinary range of host plants. The fungus can be soil-borne, airborne, or carried in plant residue, and can be recovered from any part of a plant from the deepest root to the highest flower. In addition, *Fusarium* taxonomy has been plagued by changing species concepts, with as few as nine or well over 1,000 species being recognized by various taxonomists during the past 100 years, depending on the species concept employed. The literature stabilized significantly in the early 1980s with the publications of Gerlach and Nirenberg (12) and Nelson et al. (31), who defined morphological species concepts that were widely accepted and successfully used by numerous practitioners. These publications are best thought of as definitive signposts rather than as the end of the journey. Since that time, the application of biological (23) and phylogenetic (33) species concepts to new and existing strain collections has indicated that many of the previously described species were in need of further splitting if the species designations are to

be biologically meaningful. In many cases, formal descriptions of such species have been made (11,18,26) or old names have been resurrected and associated with groups of strains now split from previous species (43).

If these previously cryptic species were rare or of limited economic importance, then much of this activity could be viewed as a “tempest in a teapot” from a practical point of view. Unfortunately, these species can be quite important, e.g., *F. andiyazi* and *F. thapsinum* are major pathogens of sorghum that differ both from one another and from other strains in *Fusarium* section *Liseola*, with which they had previously been grouped as “*F. moniliforme*” (23,26). As these different species are recognized and strains assigned to them, often on the basis of sexual cross-fertility or similarities of DNA sequences, distinguishing morphological features often can be discerned. These morphological features serve as the basis for formal descriptions of these taxa that meet the rules of the International Code for Botanical Nomenclature, but are not necessarily easily applied in a diagnostic setting.

Our objective in this review is to outline a practical approach to identifying species of *Fusarium*, especially in relation to those that affect plants (and indirectly plant pathologists!). This approach will allow sufficient identification of most cultures for routine work in a diagnostic laboratory, and indicate when assistance from other scientists with more specialized equipment or skills should be sought. When identifying strains of *Fusarium*, spore type and morphology are commonly viewed as the most important pieces of data, but the diagnostic process can be simplified if additional information such as host plant, disease symptoms, and observations made in

the isolation and recovery process are included. As this review is targeted to plant pathologists, it focuses on problems associated with the diagnosis and identification of plant diseases caused by *Fusarium*, and the examples used relate to plant pathology. However, the principles outlined can be used when identifying *Fusarium* cultures that originate from other sources, and may find applications in studies of other fungi or plant pathogens as well.

Species Concepts in *Fusarium*

The species concept being used in the species definition essentially defines the criteria through which species can be recognized and the basis upon which they can be differentiated from one another. In *Fusarium*, there currently are three different basic species concepts being employed—morphological, biological, and phylogenetic (see Leslie et al. [24] for a more detailed discussion of these species concepts and their application to *Fusarium*). In general, morphological species concepts are based on the similarity of observable morphological characters, e.g., spore size and shape. Biological species concepts require that members of the same species are sexually cross-fertile and that the progeny of the crosses are both viable and fertile. (Horses and donkeys are in separate species because the mules that result from a cross between the two are not fertile.) Finally, DNA sequences have been used to generate characters that usually are treated cladistically to form phylogenies, and those that are part of the same monophyletic group, i.e., that have a common genetic origin, at a defined level are said to be in the same species. Although any group of sufficiently numerous characters can form the basis of a phylogenetic lineage, in practice DNA sequences of one to several

Corresponding author: Brett A. Summerell, Royal Botanic Gardens – Sydney, Mrs. Macquaries Road, Sydney, New South Wales 2000, Australia; E-mail: Brett.Summerell@rbgsyd.nsw.gov.au

Contribution no. 03-118-J from the Kansas Agricultural Experiment Station, Manhattan.

Publication no. D-2002-1206-01F

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“appropriately conserved” genes are used for this purpose. All of these species concepts are subject to errors that can result from the “Iceberg effect” (50), wherein a relatively small number of cultures are analyzed (i.e., the tips of the iceberg) and differences/similarities discernable only when large numbers of strains are analyzed are discounted or ignored. Thus, a species description based on a relatively large set of strains collected at multiple times or locations usually is more reliable than one that is based on a relatively small collection (>20 to 30) from a single field sample or from culture collections. Although sample size is not a crucial variable in making diagnostic strain identifications, the soundness of the species description can be affected by this variable.

For many species of *Fusarium*, morphological characters are the only ones that are well described and widely available. For a limited number of *Fusarium* species, we have biological species information, e.g., the mating populations in the *Gibberella*

fujikuroi and *Nectria haematococca* species complexes, and in many cases, defined, publicly available tester strains can be used to make the crosses required for identification. For still other *Fusarium* species, DNA sequence information is available to support phylogenetic species concepts, e.g., Nirenberg and O'Donnell (33). There are relatively few instances in which all three types of information exist, and in such cases these data have been used to develop a “polyphasic” species description, e.g., *Gibberella circinata* (*Fusarium circinatum*) (5,33) and *Gibberella konza* (*Fusarium konzum*) (52). In these cases, however, the species definitions are extremely robust. The most common error resulting from a morphological species description is the grouping of isolates that should be separated into a common species, or “lumping.” With biological species, the most common error is no diagnosis because the appropriate tester strains may not be available or because suitable laboratory crossing conditions

have not been identified. With phylogenetic species, the most common error is resolving isolates into more groups than are biologically meaningful, or “splitting.”

Many plant pathologists think that such concepts and approaches are somewhat pedantic and best left to mycologists to sort out. Yet, for a number of taxa within *Fusarium*, the approach taken can result in different outcomes with important practical consequences. The best example of this dilemma is the widely known and reported maize pathogen, *F. moniliforme*. For decades, this fungus was associated with cob and stalk rot in maize and stalk rot and grain mold in sorghum, as well as with the production of mycotoxins such as fumonisins and moniliformin, compounds whose common names are derived from the species name. Depending upon the species concept employed, different numbers of species of *Fusarium* are associated with these problems. If a morphological species concept is used, then a single morphological entity, *F. moniliforme* sensu lato, is

Fig. 1. Typical disease symptoms caused by various species of *Fusarium*. A, Fusarium wilt of banana caused by *F. oxysporum* f. sp. *cubense*. B, Fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici*. C, Stem rot of vanilla caused by *F. oxysporum*. D, Mango malformation caused by *F. manginifera*. E, Fusarium wilt of Canary Island date palm caused by *F. oxysporum* f. sp. *canariensis*. F, Bakanae disease of rice caused by *Fusarium fujikuroi*. G, Stalk rot of sorghum caused by *F. thapsinum*. H, Root rot of *Aglaonema commutatum* caused by *Fusarium solani*. I, Cob rot of maize caused by *F. verticillioides*. All photos by authors except D, by Randy Ploetz, E, by Suzanne Bullock, and G, by Larry Claflin.

associated with all of these problems. If the biological species concept is used, then two species, *F. verticillioides* and *F. thapsinum* (17), along with a significant number of sterile strains that belong to neither species can be identified. The recent acceptance of the change of name *F. moniliforme* to *F. verticillioides* to conform to nomenclatural rules allows these two entities to be clearly distinguished. Recently, molecular analysis, which follows a phylogenetic species concept, has allowed the recognition of an additional species, *F. andiyazi* (26), from the sterile strains identified in the biological species approach, but the sexual stage associated with *F. andiyazi* has not yet been formed under laboratory conditions. Cursory microscopic examination of cultures of any of these three species would be insufficient to differentiate them even though there are important biological differences. *F. verticillioides* causes cob and stalk rots on maize and produces large amounts of fumonisin, while *F. thapsinum* is associated with sorghum stalk rot diseases and produces very little fumonisin but high levels of moniliformin, and *F. andiyazi* causes significant disease in sorghum seedlings but produces little if any fumonisin or moniliformin and is not acutely toxic to ducklings (J. F. Leslie, K. A. Zeller, S. C. Lamprecht, J. P. Rheeder, and W. F. O. Marasas, unpublished).

Overall Identification Strategy

The critical question for all who identify *Fusarium* cultures is, "How much information is needed?" Part of the problem in identifying *Fusarium* is that the answer to this question changes depending on the species being identified and the use to which the answer will be put. Often the most difficult part of the diagnostic identification of a *Fusarium* culture is to clearly define the question being asked, to determine the amount of information needed to answer that question, and to know if the information obtained suffices to answer the question.

The first step in the identification process is to clearly describe the plant disease and the symptoms observed on the diseased plant, and to note the weather conditions under which the disease occurred. Next, isolation and recovery protocols are evaluated to determine the spectrum of species likely to be recovered. Finally, the strains of interest are purified and then evaluated by using morphological, molecular, and cross-fertility criteria. These steps are evaluated in more detail in the text that follows.

The Diseased Plant, Disease Symptoms, and Distribution

Strains of *Fusarium* species can cause an extraordinarily broad range of plant diseases (Fig. 1). The most important are the crown and root rots, stalk rots, head and grain blights, and vascular wilt dis-

eases that are well known to most pathologists (30,48), but lesser known diseases such as malformation disease in mango (38) and bakane disease in rice can have important local economic impact. The nature of the disease provides important clues as to the species that will be recovered, and often limits the range of species that need to be distinguished (Table 1). Thus, the possible outcomes for most routine diagnoses can be identified quickly, as the number of species associated with particular hosts or disease symptoms often is relatively limited. However, if symptoms are novel or unusual for the diseased host, the information provided by this approach may not be particularly valuable.

Fusarium species recovered from both natural and agricultural ecosystems have distinct climatic preferences (1,2,6). The climate, and even local variations in weather, can limit the range of species observed, even if several are present, and influence their relative frequency. In broad terms, there are species that prefer tropical climates, hot arid climates, or temperate climates, and a fourth group that has a cosmopolitan range. A concern with many of the species in the fourth group is that they may be sets of sibling species, which are morphologically indistinguishable but genetically distinct, that have yet to be adequately resolved. For example, maize stalk rot from plants in relatively warm, dry areas such as Kansas is most likely caused by *F. verticillioides*, while the same disease in cooler moister areas such as Minnesota or the highlands of Mexico is more likely to be caused by *F. subglutinans*. In wheat, *F. culmorum* can cause crown disease in cooler temperate areas, while *F. pseudograminearum* is more common in warmer subtropical and arid regions (1).

Isolation Procedures

Isolation protocols have a significant impact on the recovery of *Fusarium* species from diseased plants. It is common to recover more than one species of *Fusarium* from individual pieces of diseased plant material. The isolation technique selected should maximize recovery of the "true" pathogen while restricting the recovery of "weed" fungi, especially secondary pathogens and saprophytes such as those found in intensively cultivated soil (25). As a number of *Fusarium* species are commonly recovered as weeds (Table 2), from both plant parts and soil, the identification of the causal agent always requires care. Where possible, Koch's postulates should be repeated with such questionable strains before they are reported as pathogens. Such tests, however, are usually far beyond the scope of routine, utilitarian laboratory diagnoses.

Working from fresh and freshly diseased plant material usually reduces the number of secondary pathogens and saprophytes present in a sample. The longer the plant has been diseased, and the longer the sample has been awaiting analysis, the more likely it is that fungal species other than the pathogen, including other *Fusarium* species, will have invaded the diseased area. This problem is particularly acute for soilborne diseases, such as wilt diseases and root rots, and these diseased samples should be expected to yield a range of fungi other than the original causal organism. Among *Fusarium* species, *F. equiseti* and *F. semitectum* are noted saprophytic colonizers that can be regularly recovered as secondary invaders of diseased tissue. In many cases, these fungi are reported as pathogens or causal agents of diseases based on the recovery of these species from diseased material, but Koch's postu-

Table 1. *Fusarium* species commonly recovered as causes of diseases from economically important host plants

Plant species	<i>Fusarium</i> species
Banana	<i>F. oxysporum</i> f. sp. <i>cubense</i>
Cotton	<i>F. oxysporum</i> f. sp. <i>vasinfectum</i>
Legumes	<i>F. avenaceum</i> and formae speciales of <i>F. oxysporum</i> and <i>F. solani</i>
Maize	<i>F. graminearum</i> , <i>F. proliferatum</i> , <i>F. subglutinans</i> , <i>F. verticillioides</i>
Rice	<i>F. fujikuroi</i>
Sorghum	<i>F. andiyazi</i> , <i>F. proliferatum</i> , and <i>F. thapsinum</i>
Vegetables	Formae speciales of <i>F. oxysporum</i> and <i>F. solani</i>
Wheat/barley/oats	<i>F. culmorum</i> , <i>F. graminearum</i> , and <i>F. pseudograminearum</i>

Table 2. Species of *Fusarium* regularly recovered from various parts of diseased plants as saprophytes

Plant part	<i>Fusarium</i> species
Roots and stem bases	<i>F. acuminatum</i> , <i>F. avenaceum</i> , <i>F. compactum</i> , <i>F. equiseti</i> , <i>F. proliferatum</i> , <i>F. oxysporum</i> , <i>F. solani</i>
Leaves and aerial parts	<i>F. proliferatum</i> , <i>F. semitectum</i>
Flowers	<i>F. semitectum</i>
Seed and grain	<i>F. chlamydosporum</i> , <i>F. equiseti</i> , <i>F. poae</i> , <i>F. semitectum</i>

lates either cannot be or were not completed with the recovered strains. The frequent reporting of species such as *F. equiseti* and *F. semitectum* as pathogens of a broad range of plant species supports this conclusion, and also means that reports of the pathogenicity of these species should be viewed with caution unless supported with data that go beyond the recovery of these species from diseased plant material.

The type of disease often dictates the isolation procedure used. The most obvious part of the plant from which to recover the fungus is the part being affected by the disease. However, this task must be approached with an open mind, as some symptoms can be a response to infection at another point. As a general rule, if there is a lesion or disease focus, attempt to isolate the fungus from the leading edge of this region, as this is the point at which the fungus is most likely to be actively growing and easily recovered. Also, in general and to the extent feasible, more samples are better than fewer, since if the "real" pathogen is lost in a larger group of secondary invaders, the more samples taken, the more likely the correct causal agent is to be identified from the crowd. Careful examination of the diseased plant also can provide hints for the best isolation procedure to use. We usually examine plant material both before and after washing it, as a thorough wash can dislodge diagnostic characters, e.g., the presence of sporodochia or

perithecia, on the outside of the stem. We also often cut stems and examine cross sections or cuts through the lumen of the stem to determine if vascular browning has occurred or if hyphae are present in the lumen. Such symptoms can be diagnostic for vascular wilts of a range of species caused by *F. oxysporum*, or for crown and stem rots of wheat and barley caused by *F. pseudograminearum* or *F. culmorum*.

In some situations, the pathogen is well defined, and the question being asked is one of quantity, i.e., how frequently is the pathogen recovered, rather than quality, i.e., to which species do the pathogenic isolates belong. In such cases, there may be isolation techniques that can be used to recover a pathogen from numerous samples while still providing enough information to the identity of the pathogen to allow the collection of meaningful data. For example, with the wheat crown rot pathogen, *F. pseudograminearum*, there is a selective medium (7) on which many fungi and bacteria cannot grow, but the pathogen can be tentatively identified by colony morphology. These identification schemes always must be validated through a more detailed examination of a randomly selected subset of isolates, and usually are developed in response to a need to be able to quickly identify and quantify the amount of a particular species or set of species. However, it is only through such identification processes that the relatively large-scale experiments

needed for some bioclimatic modeling studies, for example, can be undertaken (1).

Recovering a *Fusarium* Species

The successful culturing of a *Fusarium* species leads to further decisions that can significantly influence the final result. The first decision is whether the *Fusarium* isolate cultured is of importance and its analysis worthy of additional time and material resources, e.g., is the strain a pathogen? The quality and quantity of the plant samples available usually guide this decision. If the diseased material is of a high quality, i.e., it is representative and has not degraded significantly, and if there are sufficient samples to ensure consistency in the recovery of the pathogen, then this decision should be easy. Unfortunately not all samples are of this type, and it is not uncommon for a client to expect the plant diagnostician to perform a miracle by recovering only the causal agent and producing an unquestionable diagnosis on the basis of a limited sample of very suspect material. In these instances, experience plays an important role in the diagnostic decision, and the formal tasks of exhaustive isolation, strain recovery, and subculturing often are replaced by a more pragmatic decision-making process grounded in the familiarity of the diagnostician with hosts, diseases, and the frequency with which proven pathogenic organisms have been recovered from similar samples.

Fig. 2. Colony morphology of *Fusarium* species on potato dextrose agar. The top plate in each pair is the upper surface and the lower plate is the under surface. A, *F. poae*. B, *F. oxysporum*. C, *F. acuminatum*. D, *F. nelsonii*. E, *F. subglutinans*. F, *F. nygamai*. G, *F. pseudonygamai*. H, *F. lateritium*. I, *F. thapsinum*. J, *F. decemcellulare*. K, *F. verticillioides*. L, *F. culmorum*.

Fig. 3. Flow chart of identification protocol used for identifying *Fusarium* species.

The combination of host plant, the part of the plant from which the strain was recovered, climatic region, local weather data, and isolation protocol often greatly reduce the number of *Fusarium* species expected to be recovered. In at least some cases, a tentative diagnosis can be made on this information alone, with examination of cultural morphology serving to confirm the inferred result, rather than being the primary means of identification.

Subculturing, Purification, and Growing Conditions

If a culture of *Fusarium* has been found that warrants further work, then it must be subcultured and purified before the identification process proceeds further. These steps may appear both mundane and self-evident, but many diagnosticians make critical mistakes because they have not been taken. A common mistake is to try to identify the culture directly from the isolation medium. The most common medium for recovering *Fusarium* species is PPA, a peptone-PCNB medium (29) that is semi-selective for *Fusarium*. Colonies growing on this medium for more than 2 to 3 weeks usually die due to the accumulation of toxic metabolites that are byproducts of the fungal growth on this medium. Similarly, cultures on many rich media, e.g., potato dextrose agar (PDA), do not produce the uniform macroconidia that are necessary for an accurate identification. In such cases, spores characteristic of many different species can appear to be recovered from a single culture. Finally, cultures that have not been purified often are mixed, and the resulting irregular mixture of morphological characters leads, at best, to inaccurate diagnoses and, at worst, to both nonsense and frustration.

In some cases, conidia from colonies on PPA can be used for starting single-spore cultures from which identifications can be made. However, some species yield only abnormal cultures if started from single spores from PPA-grown colonies. Unless there is previous reason to expect colony stability, colonies should be transferred from the isolation medium to a nutritionally weak medium, e.g., water agar with a bit of sterilized plant tissue, and spores from the resulting colonies used for the single-spore subculturing process. Macroconidia, the large banana-shaped spores most researchers associate with *Fusarium*, microconidia, and ascospores all can be used to initiate these subcultures. Microconidia are the most common spore type produced by some species, but macroconidia usually are preferred because their larger size makes them easier to handle. Single spores are separated either by dilution plating or by micromanipulation on water agar and incubated overnight. Germ-lings are subcultured from the water agar to a medium for growth, identification, and/or preservation. In some instances, it may be necessary to purify cultures by isolating a hyphal tip instead of a single spore, as some species, e.g., *F. graminearum*, occasionally produce sparse macroconidia and others, e.g., *F. longipes*, mutate readily when subcultured as single spores. We cannot overemphasize the importance of attempting to identify only purified cultures; we have both witnessed and received cultures from researchers who were having great difficulty identifying a *Fusarium* culture because they were working with a mixed culture(s).

Accurate identification of a culture requires growing it on at least two media—carnation leaf-piece agar (CLA) and PDA.

CLA is a natural substrate medium made by floating γ -irradiated sterilized carnation leaf-pieces on a water agar base (10). Many species of *Fusarium* readily form sporodochia of robust, uniform macroconidia on the carnation leaf-pieces that are particularly useful for identification purposes (or for use in making single-spore subcultures). PDA cultures are used primarily to assess pigmentation and gross colony morphology (Fig. 2). Cultures growing on PDA should generally be regarded as “dead ends” and should not be subcultured further. If further subcultures are necessary, then single spores or mass transfers from CLA plates should be used instead. We usually use cultures growing in 6-cm-diameter plastic petri dishes on CLA and PDA to make initial evaluations, and then make decisions as to whether the fungus should be grown on Spezieller Nährstoffarmer Agar (SNA) (32) or a defined liquid medium. Cultures grown on SNA are usually evaluated for microconidia, which may be more abundant and diverse on this medium, and for chlamydospores, which often are more common and are produced more rapidly on this medium. Cultures grown in liquid medium are usually used as a source of DNA for DNA sequencing or for studies of molecular genetic diversity.

Incubation conditions for *Fusarium* vary depending on the laboratory and the facilities available. Critical parameters are temperatures of 20 to 25°C and the presence of light, particularly some exposure to UV (black) light. A diurnal (day/night) cycle often is used and probably is beneficial, although some species, e.g., some members of the *Gibberella fujikuroi* clade (33), are reported to form critical diagnostic characters only in total darkness or under

Fig. 4. Formation and types of microconidia produced by *Fusarium* species. A, Microconidia produced in short chains (*F. brevicatenulatum*). B, Microconidia produced in long chains (*F. decemcellulare*). C, Microconidia produced in false heads (*F. circinatum*). D, Napiform microconidia in false heads (*F. konzum*). E, Oval microconidia (*F. babinda*). F, Pyriform microconidia (*F. anthophilum*). G, Clavate microconidia (*F. anthophilum*). H, Fusiform microconidia (*F. semitectum*). I, Napiform microconidia (*F. poae*). J, Globose microconidia (*F. anthophilum*).

continuous UV or white light. For PDA cultures, light usually is not essential but does increase the total amount of pigment and the speed with which the pigments are produced. Light is more important for CLA cultures, as it increases sporodochial production.

The Essence of Morphological Identifications

After a purified colony is subcultured to CLA and PDA, it usually is incubated for 7 to 10 days, depending on its rate of growth, before any attempts are made to identify it. Macroconidia, microconidia, and chlamydospores can all be of importance in species identification, and a pictorial guide of the different spore types (7,12,31) should be procured before attempting an extensive or significant set of identifications. We provide the identification protocol that we follow (Fig. 3), but other protocols can be used as well. Consistency and completeness, i.e., being sure to collect all of the data needed, are the critical characteristics of this process.

1. Record gross colony morphology features.

- What is the color of the PDA culture?
- What pigments are produced in the agar?
- Are sporodochia obvious in the center of the PDA plate or on the carnation leaf-pieces? If so, what color are they?
- How thick and high is the hyphal growth?

2. Examine the CLA plate in situ under the 10× objective (×100 total magnification) of a compound microscope. If such examinations are made on a routine basis, we recommend purchasing a metallurgical objective (20× objective; ×200 total magnification), which has a longer working distance and a greater depth of field than does a comparable lens that is commonly used for making biological observations.

- Place the plate containing the CLA culture on the stage of the microscope and examine the features of the fungus directly. Increasing magnification from ×100 to ×200 (by switching from the 10× to the 20× objective) may be necessary in some cases for a detailed examination. Determine if microconidia are present, and if so note their shape, size, and the manner in which they are formed (Fig. 4; This figure is not exhaustive and others types of microconidia also may be observed). The terminology used to describe these microconidia can be confusing, and reference to a good mycological dictionary, e.g., Kirk et al. (15), may be necessary to reduce confusion and to ensure precision in these descriptions.

- If microconidia are present, determine the nature of the conidiogenous cells, i.e., the cells in the hyphae on which the microconidia are borne. There are two

common types—monophialides and polyphialides (Fig. 5). Monophialides have a single opening in the conidiogenous cell, while polyphialides have two or more. It is often difficult to make this determination at this stage, and this feature may need to be re-evaluated following observations of a microscope slide preparation.

- Chlamydospores usually can be observed in the hyphae, on the surface of the agar, or embedded in the agar. They are more common in older cultures than in younger ones, and often occur more abundantly on media such as mud agar (19) or SNA than on CLA. Chlamydospores normally form in older cultures and may be in hyphae or conidiospores, and it may be necessary to re-examine cultures for up to a month to determine definitively if chlamydospores are present. Cultures retained for such lengthy periods of time are susceptible to contamination with culture mites and should be incubated in an area separate from that used for the initial recovery of strains from infected plant material (often the source of the mites) and from where fresh cultures for identification, which also can be easily contaminated, are kept.

- True chlamydospores, pseudochlamydospores, and swollen cells appear similar and can be easily confused. True chlamydospores have a thick wall, a warty appearance (verruucose), and light coloration, usually yellow-brown. True chlamydospores are an important survival structure for many species, especially those that are soilborne. Chlamydospores can be formed in chains, clumps, or singly and can be found in the hyphae above, on, or below the agar

surface or within the carnation leaf-pieces. Pseudochlamydospores have been described, so far, for only a single species, *F. andiyazi* (26), and their value as a diagnostic character has not yet been widely evaluated. Pseudochlamydospores are thin and smooth walled and are found either singly or as short chains in the hyphae. Swollen cells are found in a number of species, especially in section *Liseola*. By definition, they are found in the hyphae and usually are less round in shape than are either pseudochlamydospores or chlamydospores.

3. Make microscope slide preparations for detailed examinations of macroconidia, microconidia (if present), and the conidiogenous cells producing the microconidia (if present). Mounts should be made of scrapes of sporodochial macroconidia, and also of scrapes of the agar surface away from the sporodochia (usually for observation of chlamydospores, microconidia, and microconidiophores). In some cases, a mount from aerial mycelia also is needed. We normally use plain water mounts, but some researchers use stain to highlight the features. If water mount preparations are used, then the microscope must be adjusted so that all of the features of the hyaline structures can be clearly observed. The use of differential interference microscopy (Nomarski) can be useful in discerning the hyaline features of these structures. Similarly, photographs obtained with a digital camera often are easier to compare than are comparative slides from the same or different cultures.

- **Macroconidia.** These should be the first structure examined. Preferably, they should be from sporodochia on the CLA plate, as these spores will be the most

Fig. 5. Conidiogenous cells of *Fusarium* species. A, Microconidia borne on monophialides (*F. oxysporum*). B, Microconidia borne on polyphialides (*F. chlamydosporum*). C, Monophialide of *F. solani*. D, Polyphialide of *F. denticulatum*.

consistent in terms of shape and size. Key macroconidial characters are the shape of the spore, its size, the number of septa, and the shape of the apical and basal cells. Take away an overall impression and do not focus on rare, unusual shapes or sizes or small nuances in size or shape. First impressions are very important, often are correct, and generally increase in importance with experience. The shape of the macroconidia ranges from short and squat to highly curved and elongate with needle-like spores (Fig. 6). Size differences are important, but only in a gross sense, i.e., not differences of a few microns. The apical cell of the macroconidium may be rounded, needle-like, or whip-like. The basal cell, or foot cell, can be barely notched or resemble an upside-down foot. The combined characters of the macroconidia often are sufficient to identify the species to which the isolate belongs.

- **Microconidia.** Variations in microconidial spore morphology often are easier to see in a microscope water mount (Fig. 4) than in the in situ observations of the producing culture. Results from the in situ observations should be confirmed at this time.

- **Conidiogenous cells.** As with the microconidia, the difference between monophialides and polyphialides often is clearer in a microscope water mount (Fig. 5) than in the in situ observation of the producing culture. Again, results from in situ observations should be confirmed at this time.

4. **Secondary characters.** The most commonly used secondary characters are odor and growth rate. Usually these are used to confirm a diagnosis based on other characters rather than to make an initial identification. Growth rates may be based on linear growth in a race tube (41) or on radial growth in a petri dish (7), usually at 25 or 30°C. The two rates are not directly comparable, and if growth rate is used as a character, then a comparison with the correct reference

data set is essential. Other useful secondary characters can be physiological data on toxins and other metabolites produced (49), but such data are not commonly available for routine diagnoses.

5. **Organizing the data.** The data collected on an isolate at this stage often are qualitatively diverse and quantitatively large. We usually use data recording sheets, such as those found in Burgess et al. (7), which have separate fields for each of the important characters, to organize results in a consistent manner and to provide a place for making systematic notes of both routine and unusual properties of a culture.

6. **Making an identification.** At this stage, there is enough information to identify some species accurately but not others. The three most commonly used manuals, Gerlach and Nirenberg (12), Nelson et al. (31), and Burgess et al. (7), are all based solely on morphology, are somewhat dated in their content, and lack information on many of the more recently described species. All of these manuals outline a process for making an identification that is based on a series of keys or tabular groupings. Traditionally, the genus *Fusarium* has been separated into sections, with the species within a section sharing important morphological characters. Although the sections are not monophyletic on the basis of DNA sequence characters, they are a useful means for sorting cultures from morphological identification characters, e.g., presence/absence of chlamydospores, microconidia, or various types of conidiogenous cells. A further guide to the morphological identification of cultures is beyond the scope of this presentation, and the previously identified manuals should be relied upon for guidance until an updated manual becomes available. With the data collected thus far, at least 21 species can be identified (Table 3, column 1) regularly and reliably, but differentiating many of the remaining species requires additional information or

experience. The species that can be identified at this stage are a mixture of well-defined species with strong morphological characters that we think will withstand further scrutiny, e.g., *F. poae* and *F. chlamydosporum*, as well as species that are relatively poorly defined and likely to be polyphyletic, e.g., *F. compactum* and *F. lateritium*, but have not yet warranted intensive research. Studies of species in the latter group that are based on molecular data could alter the placement of such species on this list.

The utility of species identifications based solely on morphological criteria will depend on the question the identification results are needed to help answer. Returning to the earlier example with *F. verticillioides*, *F. thapsinum*, and *F. andiyazi*, based on morphological criteria alone, most diagnosticians would find it difficult to distinguish these strains, and the best name to be applied would be *Fusarium moniliforme* sensu lato, i.e., *F. moniliforme* in the broad sense. For a basic diagnosis, this result might suffice and would be more meaningful and useful than "*Fusarium* sp.," which is the most likely alternative, but in other cases, e.g., analysis of mycotoxin production, quarantine, or many journal publications, such a limited diagnosis would be inadequate, if not confusing. Separating species clusters such as these requires either sexual cross-fertility, which could be used to positively identify *F. verticillioides* or *F. thapsinum*, or analysis of molecular markers, which could be used to identify *F. andiyazi* (Table 3). Until enough cultures of these species have been examined for easily recognized morphological characters to be discerned, data other than traditional morphology will be needed to make some species-specific diagnoses both accurately and with confidence.

Beyond Morphology

Although morphological observations may not suffice for a complete identification, a great deal of information usually has been obtained on a culture by this stage

Fig. 6. Macroconidia of *Fusarium* species. A to D, Variation in macroconidial shape and length. A, *F. decemcellulare*. B, *F. longipes*. C, *F. culmorum*. D, *F. chlamydosporum*. E to H, Variation in basal cells of macroconidia. E, *F. culmorum*. F, *F. crookwellense*. G, *F. avenaceum*. H, *F. longipes*. I to L, Variation in apical cells of macroconidia. I, *F. culmorum*. J, *F. decemcellulare*. K, *F. verticillioides*. L, *F. longipes*.

of the process, and the possible species identities usually are relatively few. For species that cannot be reliably identified solely on the basis of morphology, the relative cost of the additional work must be weighed against the need for the additional information. In some laboratories, the additional work is not possible, and these researchers, who often are from less-developed countries, must find appropriate colleagues with whom they can collaborate to answer their questions.

There are two general techniques that can be used to extend the range of questions being asked. One is studies of sexual cross-fertility, and the second is the analysis of DNA sequences. Cross-fertility studies are relatively inexpensive in terms of reagents and technical sophistication, but relatively expensive in terms of the waiting time required for an answer and the need for adequate incubator space. DNA studies are more demanding of equipment, reagents, and technical expertise, but answers can be obtained more rapidly and for more species than can be obtained with

studies of sexual cross-fertility.

Sexual cross-fertility. The ability to cross and to produce the teleomorph (sexual or perfect state, e.g., Fig. 7) with standard testers of defined species groups is the ultimate assurance of a correct species identification. Most analyses of cross-fertility for species identification presently are done with strains that are members of the *Gibberella fujikuroi* species complex (21,22) and are made by crossing an unknown strain as the male parent with a standard tester strain of a known species and mating type, which serves as the female parent. Tester strains for the known mating populations in the *G. fujikuroi* species complex are available from the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City).

Crosses usually follow the general protocol described by Klittich and Leslie (16), in which the female strain is inoculated on carrot agar and allowed to colonize the plate for a week before being spermatized with a spore suspension of the male parent. The spermatization occurs by working the

spore suspension of the male parent into the mycelium of the female parent with a glass rod. Plates are incubated for 4 to 6 weeks after fertilization in a lighted (at least some UV light required) incubator. During the incubation, it is important to keep temperatures below 25°C and to prevent the sealing of the plates, either deliberately (using plastic film) or accidentally (with condensation and water films). In some cases, such as *G. circinata*, temperature control must be even tighter, and temperatures outside of a rather narrow range may not be conducive to sexual reproduction. The number of crosses to be made with each unknown culture can be reduced by half if existing molecular protocols (14,47) are used to determine whether the strain in question carries the *MAT-1* or the *MAT-2* allele at the mating type locus. Positive crosses are definitive, but negative crosses (which must have proper controls to be reliably interpreted!), while indicating that a strain is not a particular species, are not necessarily informative beyond this result.

Bands, bands, and more bands. Many techniques ranging from restriction enzyme digests of genomic DNA to the sequencing of appropriate genes can be used to identify and distinguish cultures that are morphologically inseparable. The best choice usually is based on the equipment available and whether there is a database to which the information obtained can be compared.

For many researchers, direct sequencing of one or more genes can be used to make species assignments. In this process, one or more genes is amplified and sequenced. Some of the more commonly sequenced genes for *Fusarium* include β -tubulin, histone, and elongation factor-1 α (34–37,46). The resulting sequence is then compared with similar sequences from strains in related species. The limitations to

Table 3. *Fusarium* species^z that can be identified based on different types of data collected as part of the evaluation process outlined in Figure 3

Morphology alone (species list A)	+ Sexual cross-fertility (species list B)	+ DNA-based data (species list C)
<i>F. acuminatum</i> , <i>F. avenaceum</i> , <i>F. chlamydosporum</i> , <i>F. compactum</i> , <i>F. crookwellense</i> , <i>F. culmorum</i> , <i>F. decemcellulare</i> , <i>F. dimerum</i> , <i>F. equiseti</i> , <i>F. graminearum</i> , <i>F. longipes</i> , <i>F. merismoides</i> , <i>F. oxysporum</i> , <i>F. poae</i> , <i>F. pseudograminearum</i> , <i>F. scirpi</i> , <i>F. semitectum</i> , <i>F. solani</i> , <i>F. sporotrichioides</i> , <i>F. torulosum</i> , <i>F. tricinctum</i>	<i>F. circinatum</i> , <i>F. fujikuroi</i> , <i>F. proliferatum</i> , <i>F. sacchari</i> , <i>F. sambucinum</i> , <i>F. subglutinans</i> , <i>F. thapsinum</i> , <i>F. verticillioides</i>	Remainder of <i>Gibberella fujikuroi</i> species complex Many formae speciales of <i>F. oxysporum</i> and <i>F. solani</i>

^z Only commonly recovered species are listed, for brevity.

Fig. 7. Perithecia and ascospores of teleomorphs of *Fusarium*. A, Perithecia of *Gibberella zeae*. B, Perithecia of *Gibberella moniliformis*. C, Perithecia of *Haemanectria haematococca*. D, Ascospores of *Gibberella zeae*. E, Ascospores of *Gibberella moniliformis*. F, Ascospores of *Haemanectria haematococca*.

this analysis have been cost, which is decreasing rapidly, and the availability of sequence data for the necessary comparisons in an accessible data bank (and the number of sequences available is increasing relatively rapidly). In most cases, DNA for such analyses is amplified from a genomic DNA mini-preparation, which can be recovered by using a commercially available kit. The PCR-amplified DNA is then submitted to a sequencing lab, and the sequence returned as a computer file that can be manipulated and compared with other sequences from standard databases such as GenBank. The diagnostic value of a single sequence often is inadequate, but once several genes have been sequenced, their diagnostic value can be very high. Direct sequencing also is useful for determining the genetic and/or evolutionary distances and relationships between a set of species. In many cases, only one, or at most a handful, of strains has been sequenced for any single species, so the possibility of variation within a species usually has not been addressed. Similarly, the number of differences required for two strains to be considered different species requires calibration and validation with data independent from the sequences themselves to have biological meaning. For example, the phylogenetic lineages in *G. zeae* described by O'Donnell et al. (36) could be considered separate species, based on DNA sequence data, if they had not previously been shown to be cross-fertile by Bowden and Leslie (4). Thus, exact matches in sequence analysis, especially if several genes are used, generally are definitive for species identification, but if there are mismatches, then the significance of the mismatches must be determined before a diagnosis is made.

We also have used amplified fragment length polymorphisms (AFLPs) as the first step in this second sorting process. AFLPs sample the genome broadly, in terms of sequences analyzed, usually resulting in a large number of polymorphic markers that can be analyzed. AFLPs can be used to group like strains together as well as to group strains with reference strains of individual species. Based on our experience, strains that share >70% of the bands in an AFLP profile are in the same species, those that share <40% of the bands are in different species, and those that share between 40 and 70% of the bands in the profile often represent taxa in which speciation is in progress. Thus, by running an unknown isolate alongside a set of reference strains, it often is relatively easy to identify a strain to species with a high degree of certainty. AFLPs have the advantage that access to a DNA sequencing facility is not required, and also that they simultaneously provide data on variation within a population that is usually difficult, if not impossible, to obtain from direct sequencing. However, AFLPs generally are not useful for answer-

ing questions related to the genetic and/or evolutionary distance between different species, as the amount of intraspecific variation usually buries the interspecific signals. AFLPs and direct sequencing are by no means the only molecular techniques that can be used for these purposes, however, and other techniques can be (and have been) used by other researchers to make such identifications.

For diagnostic purposes, specific PCR-based amplifications will probably be the wave of the future. Such simplified PCR reactions when combined with a very simple DNA extraction process, such as that of DuTeau and Leslie (8), are likely to become as common for identifying fungal pathogens as ELISAs have become for identifying viruses. Unfortunately, such diagnostics are not yet well developed for most *Fusarium* species, and those that have been described, e.g., Möller et al. (27), usually have not been broadly validated on a large number of isolates of the target species or tested against representative strains of a large number of species other than the target. Further research in this area should lead to the development and deployment of molecular diagnostics for at least some *Fusarium* species in the not too distant future.

The Special Case of *F. oxysporum*

F. oxysporum is responsible for an enormous range of plant diseases, usually involving a vascular wilt syndrome. The majority of the isolates causing vascular wilts are specific strains that infect only a small number of host plants and have been differentiated from each other using the subspecific term *formae speciales* (f. sp.). For example, the strains commonly attacking banana are *F. oxysporum* f. sp. *cubense*; cotton, *F. oxysporum* f. sp. *vasinfectum*; and tomato, *F. oxysporum* f. sp. *lycopersici*. Morphologically these strains are identical, and they also cannot be differentiated from nonpathogenic or saprophytic strains, of which there is a huge diversity, especially in soil. From a diagnostic point of view, the separation of this species into *formae speciales* has important diagnostic and quarantine implications. Identification of these strains has traditionally involved pathogenicity testing with sets of host differentials appropriate for the *formae speciales* in question. However, these tests are time-consuming to set up and can require long periods of time, e.g., 4 to 6 months for *F. oxysporum* f. sp. *canariensis* (39), before they can be scored definitively.

The vegetative compatibility group (VCG) technique (20,40) works well for some *formae speciales*, such as *F. oxysporum* f. sp. *cubense* (28), but not for others, such as *F. oxysporum* f. sp. *asparagi* (9). When the number of VCGs in the *formae special* of interest is known to be relatively small, the VCGs can be an effective

diagnostic technique. In such cases, the tester strains needed are usually easy to obtain. However, if there are a large number of VCGs, or if the form species of interest has not already been evaluated for the VCG diversity that it contains, then VCG analyses are more suitable as a research tool than as a diagnostic protocol.

Species- and form species-specific PCR-primer pairs for making identifications of strains of *F. oxysporum* are of commercial and regulatory importance. Again, research into the identification of such primers is well underway, but the reliability and validation of these PCR primers in large-scale tests remains unproven and a subject for future research. We expect significant developments in this field during the next few years.

Differences in Identification of Temperate and Tropical Species

The tropics are those areas situated between the Tropic of Cancer and the Tropic of Capricorn and generally are associated with higher precipitation and temperature (hence higher humidity), and 12-h day/night. The economies of countries in these regions often are heavily dependent on natural resources and agriculture.

As with their counterparts in temperate regions, *Fusarium* species in the tropics are quite diverse in terms of numbers of species, distribution, host range, and virulence (13). However, there are some notable distinctions between species found in tropical and temperate regions. Plant diseases caused by *Fusarium* spp. in the tropics are becoming more significant with the introduction of intensive and high-yielding production systems and genetically uniform cultivars (42,51). *F. oxysporum* and *F. solani* are the most common plant pathogens in this region. They also are widely dispersed soil inhabitants throughout the tropics, and the vascular wilts they cause are the most economically important diseases caused by *Fusarium* in the region. Members of the *G. fujikuroi* clade are frequently associated with agricultural produce or plants, particularly those in the family Poaceae and with newly introduced crops from outside the tropical region, such as asparagus.

Due to deficiencies in resources, plant pathologists in the tropics have encountered problems in identifying morphologically similar *Fusarium* species. In the past, there was a tendency to name pathogenic *Fusarium* species on the basis of their host (e.g., *F. batatas*, *F. coffeanum*, *F. cubense*, *F. javanicum*, and *F. sacchari*), which has added significantly to the problems associated with identification (42). The name, per Snyder and Hansen, *F. roseum* was widely used to identify many tropical *Fusarium* isolates that produced bright red pigments in the culture media (3,44,45). Consequently, much of the recorded information on plant diseases caused by *Fusa-*

rium in the tropics lacks modern specificity, and many records and disease lists should be viewed with skepticism. In addition, there are many instances in which a tropical strain is identified to a temperate species for which it is the “best fit” or “most like,” but which on further study turns out to represent a new species. This problem points to the need to retain cultures, or to deposit them in an appropriate collection, so that such unusual cultures can be re-examined with more sophisticated techniques or in the light of refined species concepts. Certainly a great deal of additional research on tropical *Fusarium* diseases, and the identity of the species that cause them, is still needed.

There also are difficulties associated with recovering *Fusarium* species in the tropics. Usually these relate to the ability to recover clean isolates from diseased material. Temperature and humidity conditions in the tropics often lead to more rapid decay of diseased plants, resulting in samples that are more heavily colonized by secondary fungal and bacterial invaders. This secondary contamination probably is one reason for the numerous records of *F. oxysporum* and *F. solani* as plant pathogens of diverse crops in many tropical countries. Thus, samples should be taken at the earliest stages of colonization or when disease symptoms first appear, and stringent pathogenicity tests should be performed with recovered isolates that do not conform to previously described and tested disease profiles.

Conclusions

Our primary objective in this review was to illustrate the rationale and complexity that underlie the accurate identification of *Fusarium* species and the necessity to follow an identification process logically and systematically through all of the steps involved. The history of *Fusarium* biology is littered with publications and reports that are now difficult to interpret because such logic was not applied, and as a consequence the species report is either ambiguous or completely wrong. Unfortunately, such publications still occur today. The identification process is simplified if this logic is applied, the steps outlined are carefully followed, and the limitations on the information that can be acquired are clearly understood and identified. The key is to collect enough of the information necessary to make an acceptable identification. Unfortunately, the amount of information needed for such a goal varies by species and the use to which the diagnosis will be put. There is not yet a substitute for the judgment of a skilled diagnostician in determining the amount of information necessary to make an acceptable diagnosis, because it is clear that “one size” in terms of *Fusarium* diagnostics clearly does not fit all. We encourage you to seek assistance wherever possible, to attend training work-

shops or research meetings that focus on *Fusarium* diseases, and to deposit cultures in recognized culture collections and disease herbaria. By so doing, you will help increase the collective knowledge of this complex fungal genus, which will help achieve control of the many plant diseases caused by the fungus.

Acknowledgments

We thank Suzanne Bullock (Royal Botanic Gardens-Sydney) for microscopy and the preparation of the plates for this manuscript, Matt Whittington (Royal Botanic Gardens-Sydney) for the preparation of the flow chart in Figure 3, and Larry Clafin (Kansas State University), Randy Ploetz (University of Florida), and Suzanne Bullock for the use of photographs in Figure 1. Work in JFL's laboratory was supported in part by the Kansas Agricultural Experiment Station and by the Sorghum and Millet Collaborative Research Support Program (INTSORMIL) AID/DAN-1254-G-00-0021-00 from the U.S. Agency for International Development. JFL thanks the Australian-American Fulbright Commission and The Royal Botanic Gardens-Sydney for the award of fellowships to support his sabbatical leave in Australia during which time this manuscript was prepared.

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Brett A. Summerell

Dr. Summerell is a senior research scientist at the Royal Botanic Gardens, Sydney, Australia. He received his B.Sc. Agr. (1985) and Ph.D. (1988) degrees from the University of Sydney. He was appointed to the Royal Botanic Gardens, Sydney, in 1989 and currently has responsibility for managing their conservation and horticultural research programs. His main research interests are focused on the biology, ecology, and taxonomy of *Fusarium*, and on studies of fungi that cause diseases of Australian plants. He also is involved in capacity-building projects in plant pathology in Southeast Asia. He currently serves as a vice-president of the International Mycological Association, and he served as chair of the International Society of Plant Pathology Committee on *Fusarium* from 1993 to 1998.

Baharuddin Salleh

Dr. Salleh is a professor of plant pathology at the Science University of Malaysia in Penang. He received B.Sc. and M.Sc. degrees in plant pathology from Gadjah Mada University in Indonesia, and a Ph.D. in plant pathology from Reading University in the United Kingdom in 1981. His academic career has been split among research, teaching, and university administration. He has been the head of the plant pathology department, and deputy dean and dean of the School of Biological Sciences. In 1997, he was the founding director of the National Research Institute for Higher Education. He recently finished a 3-year term as the deputy vice chancellor (academics) of his university. His research on various aspects of plant pathology, particularly those related to taxonomy, pathogenicity, and toxigenicity of *Fusarium* species in Southeast Asia, has continued throughout his career. Currently, he maintains the largest collection of live *Fusarium* strains collected from diseased plants in Southeast Asia.

John F. Leslie

Dr. Leslie is a professor in the plant pathology department at Kansas State University. He received a B.A. in biology from the University of Dallas in 1971 and a Ph.D. in genetics from the University of Wisconsin-Madison in 1979. He spent 2 years as a postdoctoral researcher working with *Neurospora* at Stanford University, and was first introduced to *Fusarium* in his subsequent position as a research scientist with the International Minerals and Chemicals Corporation in Terre Haute, IN, from 1981 to 1984. In 1984, he moved to Kansas State University, where his research focused on the genetics and population biology of *Fusarium*. Dr. Leslie also has been involved in extensive international agricultural research on sorghum through the INTSORMIL Collaborative Research Support Program. He recently completed a sabbatical in Australia as a Senior Fulbright Scholar with joint tenure at the Royal Botanic Gardens in Sydney and at the Faculty of Agriculture of the University of Sydney.