Zygolabs: Zygomycete Fungi In Teaching And Research

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Preface

ZyGoLife is an interdisciplinary research consortium focused on advancing research and education of zygomycete fungi. ZyGoLife is funded by the National Science Foundation as part of the Genealogy of Life Program (DEB-1441604, DEB-1441715, DEB-1441677, DEB-1441728). It is based in numerous laboratories and institutions with research expertise in systematics, ecology, cell biology, genomics, and evolutionary biology.

Zygomycetes are an important group of fungi with respect to evolutionary origins of terrestrial fungi, ecological processes in nature, and industrial uses by humans. They are, however, one of the more understudied groups of fungi. To advance the study of zygomycetes, ZyGoLife is producing *ZyGoLabs: Zygomycete Fungi in Teaching and Research*. It is our hope that this effort will advance teaching and research of zygomycetes, and that it will entice more teachers to incorporate them in the classroom and more mycologists – professors and students alike – to study them. The title of the book is an homage to *Zoosporic Fungi in Teaching and Research*, which is how many of us first learned the mycology of flagellated fungi.

This version of *ZyGoLabs: Zygomycete Fungi in Teaching and Research* is a prelease draft and served as the basis for the ZyGoLife Workshop held at the annual Mycological Society of America meeting on July 15, 2017 at the University of Georgia, Athens GA. It will be further developed over the near future and released as a formal publication.

ZyGoLabs: Zygomycete Fungi in Teaching and Research is dedicated to Gerald L. Benny, Kerry L. O'Donnell and Robert W. Lichtwardt. They carried the torch in zygomycete fungi research over the past 40 years, providing the foundation for today's researchers in zygomycete biology. This publication and indeed the ZyGoLife research consortium would not be possible without them.

Chapter 1

Overview of zygomycete fungi

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1.1 Overview of Kingdom Fungi

Fungi are frequently described as four groups – chytridiomycetes, zygomycetes, ascomycetes and basidiomycetes – that are defined by morphologies associated with reproduction. The chytridiomycetes are recognized based on their production of zoospores, characterized by a single posterior, whiplash flagellum. The zygomycetes are characterized by gametangial conjugation and the production of zygospores (Fig. **??**a), aseptate (coenocytic) hyphae, and asexual reproduction typically by sporangia (Fig. **??**b). The ascomycetes and basidiomycetes are diagnosed by the production of asci and basidia, respectively, possession of regularly septate hyphae, and a dikaryotic nuclear phase in their life cycle. The classification of Kingdom Fungi used here recognizes eight phyla (Fig. **??**, Table **??**) with the chytrids comprising three paraphyletic lineages including Cryptomycota/Microsporidia, Chytridiomycota and Blastocladiomycota. The zygomycetes are also paraphyletic and are classified in two phyla, Zoopagomycota and Mucoromycota. The monophyly of ascomycetes and basidiomycetes has been confirmed and they are classified as phyla Ascomycota and Basidiomycota, respectively, of the subkingdom Dikarya.

1.2 Zygomycete fungi

Genome-scale phylogenies do not support the monophyly of zygomycetes and reject the zygospore as a synapomorphy for them (**?**). Rather the zygospore, as it is currently defined, arose in the MRCA of Zoopagomycota, Mucoromycota, Ascomycota and Basidiomycota, and lost in the MRCA of Dikarya (Ascomycota+Basidiomycota). Most zygomycetes are characterized by coenocytic hyphae and sporangial asexual reproduction, but lineages exist that are characterized by septate or compartmentalized hyphae (Fig. **??**c) and/or asexual reproduction by formation of conidia (Fig. **??**d). Importantly, it is with the emergence of the zygomycete fungi that we observe a loss of the fungal flagellum and the rise of the terrestrial, filamentous fungi. It is assumed that this loss of the flagellum in Kingdom Fungi corresponds to the transition to terrestrial environment and the emergence of terrestrial ecosystems.

1.3 Zoopagomycota

Zoopagomycota is sister to Mucoromycota+Dikarya. It comprises three subphyla, Zoopagomycotina, Kickxellomycotina and Entomophthoromycotina. The primary ecologies of the phylum include pathogens and commensals of animals, parasites of other fungi and amoebae, and rarely as plant associates. The placement of Zoopagomycota as sister to the remainder of nonflagellated fungi suggests that diversification with animals and nonplant hosts occurred at least as early as diversification with terrestrial plants. Also, the loss of the flagellum in fungi corresponds to other modifications including the loss of the centriole. Most nonflagellated fungi of Mucoromycota, Basidiomycota and Ascomycota possess an organelle unique to fungi, the spindle pole body (SPB), which serves as the microtubule organizing center necessary for chromosome segregation during nuclear division. In contrast, Zoopagomycota lineages retain a functional centrosome that possesses a degenerate 9+2 microtubular system (**?**). There is some evidence that *Olpidium*, a genus of zoosporic fungus that retains its flagellum and infects nematodes and plant roots of Brassicaceae, may be closely related to Zoopagomycota (**?**).

Zoopagomycotina contains a single order Zoopagales (Ch2, Ch3). Species of the order include predators of nematodes (e.g., *Stylopage*) and nematode eggs (e.g., *Rhopalomyces*), predators of amoebae (e.g., *Stylopage*, *Zoopage*), and mycoparasites of mucoralean fungi (e.g., *Syncephalis*). Hyphae are small in diameter, coenocytic, and they form haustoria on or within their hosts. Asexual reproduction is by conidia or sporangia according to species, and where known sexual reproduction is by production of zygospores. Many of these fungi are obligate symbionts and thus difficult to obtain in axenic culture, and for this reason there exists a paucity of molecular and genomic data.

Kickxellomycotina comprises four orders, Asellariales, Dimargaritales, Harpellales and Kickxellales (Ch2, Ch4). Species of Kickxellomycotina possess hyphae that are regularly compartmentalized by bifurcate septa that are occluded by a lenticular plug (Fig **??**c). Asellariales and Harpellales (Ch4) are associated with digestive tracts of aquatic stages of arthropods and comprise two of the four orders that have been treated previously as Trichomycetes (Lichtwardt 1986); the other two orders, Amoebidiales and Eccrinales, are members of Mesomycetozoea, not Kingdom Fungi (**?**, **?**). Asellariales has filamentous, branched thalli and reproduce asexually by disarticulation of the thalli into arthrospores (Ch 4). They occur in the digestive tracts of marine, aquatic and terrestrial species of isopods and Collembola where they are thought to function as commensals. Harpellales has branched or unbranched filamentous thalli and reproduce by trichospores, asexual spores with hair-like appendages (Ch. 4). They attach to the hindgut of aquatic stages of arthropods via a holdfast and are generally considered to be in a commensal relationship with their host. Dimargaritales species are haustorial parasites of other fungi with the best-known species occurring on mucoralean hosts (**?**), and Kickxellales (Ch 2) includes mycoparasites and saprobes isolated from soil. Both Dimargaritales and Kickxellales produce unique sporangia called merosporangia (Fig. **??**e). These are cylindrical sporangia that arise from a bulbous structure, and one or more sporangiospores may occur in chains within the sporangium.

Entomophthoromycotina (Ch. 5, Ch. 6) contains three classes each with a single order: Basidiobolomycetes and Basidiobolales, Entomophthoromycetes and Entomophthorales, and Neozygitomycetes and Neozygitales (**?**, **?**, **?**). These fungi are associated with animals as either commensals isolated from animal dung or as pathogens and parasites of insects. Many species are commonly isolated from soil and maintained in pure culture, which is consistent with a saprobic life cycle phase. Basidiobolales is typically isolated from amphibian dung although species are known to occur on the dung of other vertebrates. They produce conidiophores that forcibly eject a primary conidium, which if it lands on an appropriate substrate will germinate to form a mycelium, or if not, undergo repetitive germination, producing a second conidium (Ch 6). Under some conditions nonforcibly discharged capilliconidia are produced from forcibly discharged primary conidia. Capilliconidia adhere to the outer surface of insects. Dispersal is then achieved when spore-carrying insects are ingested by insectivorous animals, and after surviving gut passage, the fungus is subsequently excreted with the feces. The phylogenetic placement of Basidiobolales with molecular and genome scale data is problematic. In all current datasets, it is characterized by long and unstable branches and its relationship to other Entomophthoromycotina is unambiguous at this time (**?**). Entomophthorales, literally, insect destroyers, comprises pathogens of insects. Like Basidibolales, they also produce forcibly discharged conidia (Ch 5). They infect their hosts via spores and multiply within the host as one to two-celled hyphal

bodies, which also can function as gametangia. Upon the host's death, the fungus ruptures through the cuticle segments producing forcibly discharged primary conidia. Frequently, infected hosts alight in perched or elevated positions, a phenomenon known as summit disease, which is thought to be an induced behavior or adaptation for spore dispersal of the pathogen (**?**). Neozygitales are pathogens of insects and mites. They were classified as a family within Entomophthorales, but were distinguished from Entomophthorales based on shape and size of chromosomes (**?**), although inadequate molecular data currently exist to test this hypothesis. *Neozygites* produces adhesive capilliconidia similar to that of *Basidiobolus.*

1.4 Mucoromycota

Mucoromycota consists of three subphyla including Glomeromycotina, Mortierellomycotina and Mucoromycotina. Unlike Zoopagomycota, Mucoromycota is characterized by plant associations and plant based ecologies (e.g., mycorrhizae, root endophytes, decomposers, etc.). Some do exist as parasites of animals and other fungi, but these all represent opportunistic infections of hosts with compromised immune systems or relatively recent derivations from saprobic ecologies (**?**). Mucoromycota is the sister group to Dikarya, which is also characterized by dominant plant associated life styles, suggesting that the MRCA of Mucoromycota and Dikarya corresponds to the origin of modern fungal-plant associations, or at least the evolutionary potential for such relationships.

Glomeromycotina (Ch. 7) consists of the arbuscular mycorrhizae and *Geosiphon*, a symbiont of cyanobacteria (**?**). Arbuscular mycorrhizae are the most common form of mycorrhizae on the planet, and arbuscule fossils are present among the first land plant fossils (**?**), confirming an ancient symbiosis. As such they are a central taxon in the development of hypotheses concerning the evolution of early land plants and terrestrial ecosystems. Despite this importance, they have been an enigma with respect to phylogenetics of Kingdom Fungi. Morphologically, they resemble zygomycetes in the production of coenocytic hyphae and terminal or subterminal spores that resemble azygospores, asexually formed zygospore-like structures produced terminally on a single hypha or suspensor cell. Sexual reproduction has never been observed for the group, preventing analysis of morphological characters traditionally used in classifications. Early molecular phylogenies based on the small subunit ribosomal DNA (SSU DNA) resolved the arbuscular mycorrhizae – with varying statistical support depending on the analysis – as separate from the zygomycetes and sister to Dikarya (**?**). However, genome-scale phylogenies and genome content analyses strongly support the arbuscular mycorrhizae as a member of Mucoromycota (**?**). Currently there are four orders of Glomeromycotina, Archaeosporales, Diversisporales, Glomerales and Paraglomerales with *Geosiphon* being classified in Archaeosporales (**?**).

The relationship of Glomeromycotina to the other subphyla of Mucoromycota is unresolved, with some analyses resolving it as sister to Mortierellomycotina+Mucoromycotina, while others resolve it as sister group to Mortierellomycotina (**?**). The taxon sampling for both Glomeromycotina and Mortierellomycotina is sparse and expanded taxon sampling is needed to fully test these rival hypotheses. Mortierellomycotina, and its sole order Mortierellales, are commonly isolated soil fungi (Ch 8). They produce zygospores and sporangia similar to some species of Mucorales, the order in which they were previously classified, but molecular phylogenetics (**?**) and genome-scale (**?**) phylogenies both strongly support the taxon as representing a distinct subphylum. These fungi have been demonstrated as root endophytes of plants, but their effect on the host fitness remains unknown. Mortierellales are also prolific producers of fatty acids, in particular arachidonic acid. Both Glomeromycotina and Mortierellomycotina possess intimate relationships with bacteria, and while facultative, show high levels of specificity and cospeciation (**?**), the fungus tends to grow better when cleared of the bacterium (**?**).

Mucoromycotina (Ch 9, Ch 10) contains the remainder of known zygomycete species and is classified in three orders: Mucorales, Umbelopsidales and Endogonales (**?**). Mucorales is one of the more commonly isolated groups of fungi, as many are fast growing, early colonizers of carbon rich substrates. Because many species culture relatively easily, Mucorales are well represented in culture collections and their zygospores and sporangia are well documented. They include taxa that cause economically significant pre- and postharvest diseases of fruits (e.g., *Gilbertella, Mucor, Rhizopus*). They also significantly impact humans both benefi-

cially through their use in industrial production of food (e.g., tempeh, *Rhizopus*) and compounds used as food supplements (e.g., beta-carotene, *Blakeslea*), and antagonistically as rare but increasingly diagnosed human mycoses (e.g., *Mucor, Apophysomyces*). It is among Mucorales that sexual reproduction in fungi was first demonstrated and numerous species of Mucorales exhibit phototropic responses to light (Ch. 9, Ch. 10), making them important eukaryotic model organisms (e.g., *Mucor mucedo, Phycomyces blakesleeanus*). Umbelopsidales was recently described for *Umbelopsis* (**?**), a genus of soil-inhabiting fungi that also occurs as root endophytes. Endogonales are saprobic or ectomycorrhizal depending on the species (81). Saprobic species occur in heavily decayed woody substrates while mycorrhizal species associate with both early diverging land plants and vascular plants (Fig. **??**f, Bidartondo et al. 2011). They have been argued as important organisms in the colonization of land by green plants (Field et al. 2014) and represent an independent origin of mycorrhizae relative to both Glomeromycotina and Dikarya.

Table 1.1: Classification of Kingdom Fungi.

Phylum	Subphylum	Class
Cryptomycota M.D.M. Jones & T.A. Richards 2011 (=Rozellomycota Doweld (2011))		
Microsporidia		
Blastocladiomycota T.Y. James (2007)		Blastocladiomycetes Doweld (2001)
Chytridiomycota Hibbett et al. (2007)		Chytridiomycetes Caval.-Sm. (1998) Monoblepharidomycetes J.H Schaffner (1909) Neocallimastigomycetes M.J. Powell (2007)
Zoopagomycota Gryganski et al. (2016)	Zoopagomycotina Benny (2007) Kickxellomycotina Benny (2007)	
	Entomophthoromycotina Humber (2007)	Basidiobolomycetes Doweld (2001) Neozygitomycetes Humber (2012) Entomophthoromycetes Humber (2012)
Mucoromycota Doweld (2001)	Glomeromycotina Spatafora & Stajich (2016) Mortierellomycotina Hoffm., K. Voigt & P.M. Kirk (2011) Mucoromycotina Benny (2007)	Glomeromycetes Caval.-Sm. (1998) Moretierellomycetes Caval.-Sm. (1998)
Ascomycota (Berk.) Caval.-Sm. (1998)	Pezizomycotina O.E. Erikss. & Winka (1997)	Arthoniomycetes O.E. Erikss. & Winka (1997) Coniocybomycetes M. Prieto & Wedin (2013) Dothideomycetes O.E. Erikss. & Winka (1997) Eurotiomycetes O.E. Erikss. & Winka (1997) Geoglossomycetes Zheng Wang, C.L.Schoch & Spatafora (2009)

Phylum	Subphylum	Class
		Laboulbeniomycetes Engler (1898)
		Lecanoromycetes O.E. Erikss. & Winka (1997)
		Leotiomycetes O.E. Erikss. & Winka (1997)
		Lichinomycetes Reeb, Lutzoni & Cl. Roux (2004)
		Orbiliomycetes O.E. Erikss. & Baral (2003)
		Pezizomycetes O.E. Erikss. & Winka (1997)
		Sordariomycetes O.E. Erikss. & Winka (1997)
		Xylonomycetes Gazis & P. Chaverri (2012)
	Saccharomycotina O.E. Erikss. & Winka (1997)	Saccharomycetes G. Winter (1880)
	Taphrinomycotina O.E. Erikss. & Winka (1997)	Archaeorhizomycetes Rosling & T.Y. James (2011)
	Neolectomycetes O.E. Erikss. & Winka (1997)	Pneumocystidomycetes O.E. Erikss. & Winka (1997)
		Schizosaccharomycetes O.E. Erikss. & Winka (1997)
		Taphrinomycetes O.E. Erikss. & Winka (1997)
Basidiomycota R.T. Moore (1980)	Agaricomycotina Doweld (2001)	Agaricomycetes Doweld (2001)
		Dacrymycetes Doweld (2001)
		Tremellomycetes Doweld (2001)
		Wallemiomycetes Zalar, de Hoog & Schroers (2005)
	Pucciniomycotina R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)	Agaricostilbomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
		Atractiellomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
		Classiculomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
		Cryptomycocolacomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
		Cystobasidiomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
		Microbotryomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)

Phylum	Subphylum	Class
		Mixiomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
		Pucciniomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
		Tritirachiomycetes Aime & Schell (2011)
	Ustilaginomycotina Doweld (2001)	Exobasidiomycetes Begerow, M. Stoll & R. Bauer 2007
		Malasseziomycetes Denchev & T. Denchev 2014
		Moniliellomycetes Q.M. Wang, F.Y. Bai & Boekhout (2014)
		Ustilaginomycetes E. Warming (1884)

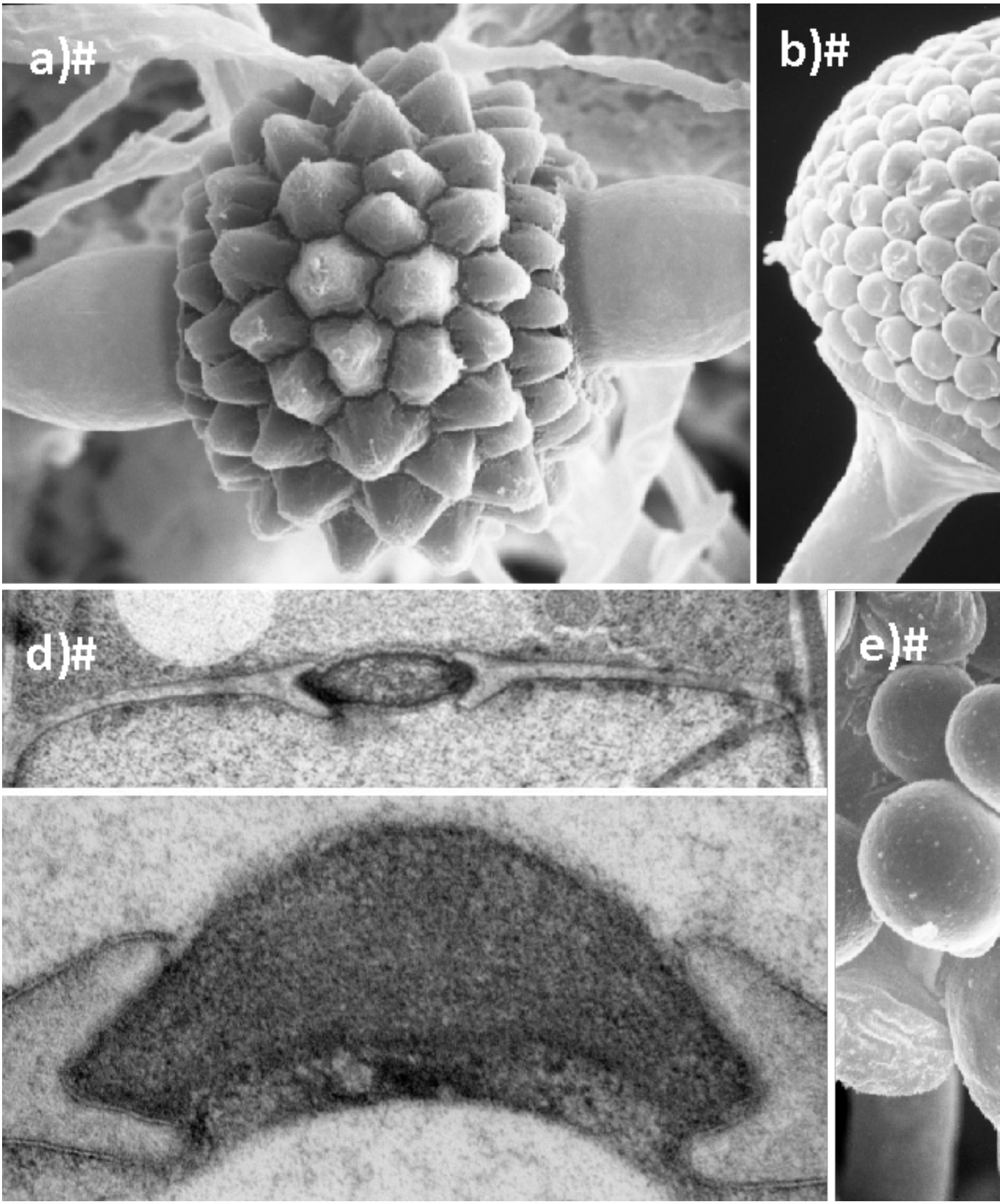

Figure 1.1: Zygomycete morphologies. a) Zygosporangium of *Cunninghamella homothallica*. b) Sporangium of *Rhizopus stolonifer*. c) Merosporangium of *Kickxella alabastrina* d) Bifurcate septum with lenticular plug, *Coemansia*. e) Primary conidium of *Conidiobolus coronatus* with secondary microconidia. (Photos by K. O'Donnell, Zygomycetes in Culture.) f) Endogone flammicorona sporocarp, zygospores (inset)

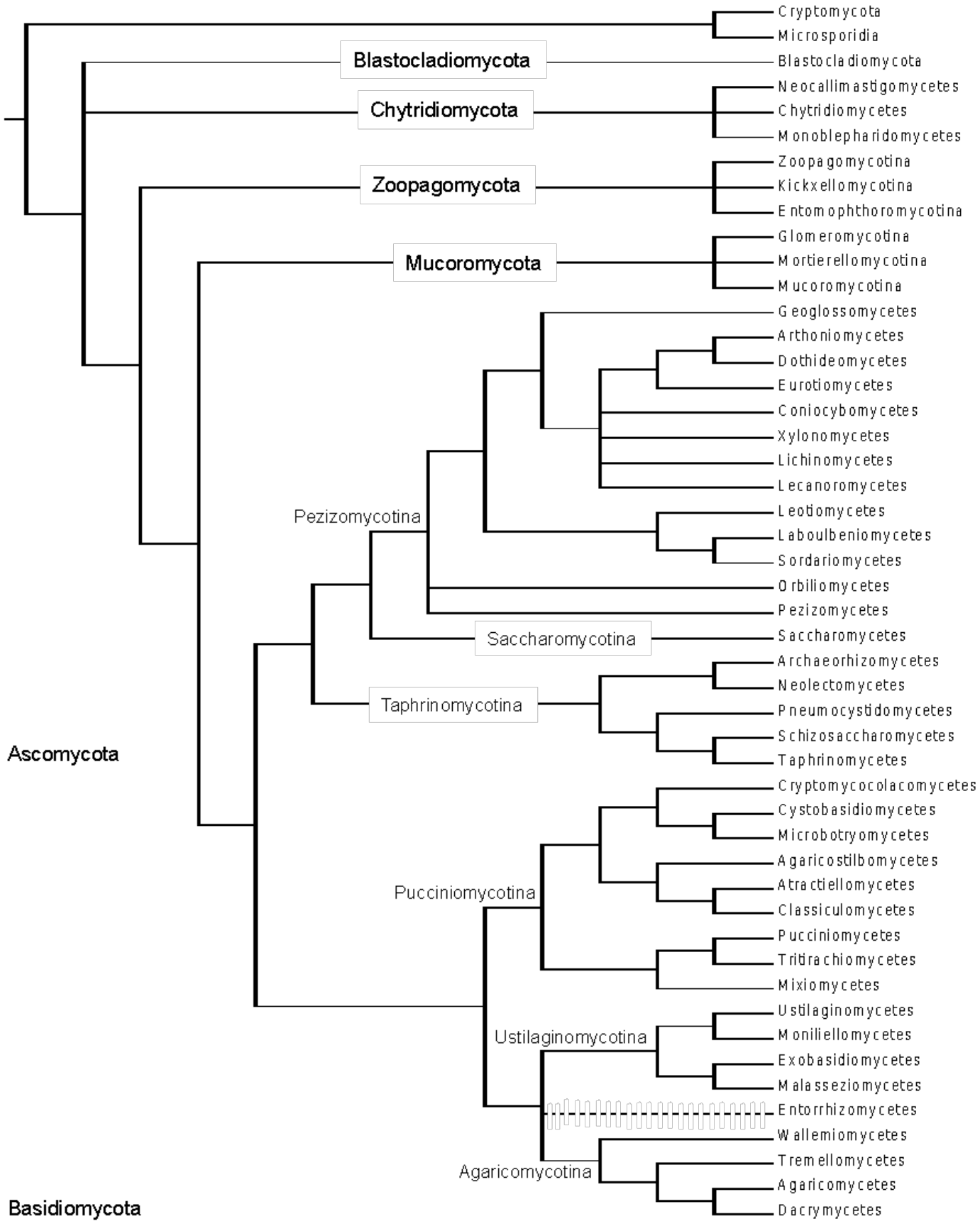

Figure 1.2: Fungal Tree of Life

Chapter 2

A laboratory guide for the observation, isolation, and culturing of zygomycetes with an emphasis on selected taxa of Zoopagomycotina

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2.1 Introduction

Zoopagomycotina (phylum Zoopagomycota (**?**)) comprises endo- or ectoparasitic fungi that attack other fungi (mycoparasites) or small animals such as nematodes, rotifers, and amoebae (**?**). Most species can be obtained from soil or leaf litter, but some are found in herbivore dung. Ectoparasitic and predaceous species penetrate the host via a haustorium whereas endoparasitic species produce thalli directly inside the host. Predaceous taxa in the genera *Acaulopage* and *Zoophagus* utilize short lateral hyphae coated with a sticky adhesive to trap prey (**?**; **?**). Others such as species of *Amoebophilus* and *Cochlonema* produce gluey spores that adhere to the host and later germinate and penetrate the host cuticle. Sexual reproduction is unknown for most species. Homothallic and heterothallic forms have been inferred based on observations of zygospore formation, but the mating system remains essentially unknown for this subphylum (**?**). Indeed, many aspects of the basic biology of these fungi such as dispersal mechanisms, host/parasite interactions, geographic distribution, and life cycle remain unclear (**?**).

The subphylum Zoopagomycotina is among the least studied groups of fungi due in large part to the obligate nature of their parasitic associations. These fungi cannot be found unless the appropriate host organism is also present in the sample. Even if the host is present, it may take up to several months of incubation for some of these parasites to appear on culture plates (**?**; **?**). Once obtained, maintenance of these co-cultures over time is labor intensive and often unsustainable due to the unknown nutritional or habitat requirements of the host organisms and/or the parasites. Furthermore, the majority of species have not been obtained in axenic culture which means that molecular phylogenetic studies are challenging for many taxa. As a result, although five families are named, the evolutionary relationships between taxa have not been tested. However, mycoparasitic members of the Piptocephalidaceae are some of the easiest to collect from soil or dung and also to grow in culture due to their association with common host fungi (**?**). The Piptocephalidaceae contains three genera (*Kuzhuaea*, *Piptocephalis*, and *Syncephalis*) that are all

haustorial mycoparasites of fungi (primarily members of the Mortierellomycotina and Mucoromycotina) (**?**; **?**). *Piptocephalis* species can be recognized by the typically tall, aerial, dichotomously branched hyphae and sporophores. In contrast, *Syncephalis* species usually form networks of thin, cobweb-like aerial hyphae that produce short, unbranched sporophores. *Syncephalis* sporophores are generally more difficult to observe under the dissecting microscope relative to *Piptocephalis* species, but the tufts of cottony hyphae are an easily recognized feature of *Syncephalis*.

In this exercise we provide information and guidance for obtaining fresh materials of Zoopagomycotina in the laboratory. Our methods emphasize common mycoparasitic taxa in the genera _Piptocephalis _and *Syncephalis* because these are ubiquitous and may therefore be the easiest taxa in Zoopagomycotina to observe in a classroom laboratory setting. These fungi can also be co-cultured on their hosts and kept for a longer term than the predaceous species. However, some of the techniques outlined here may also be used to obtain predaceous Zoopagomycotina fungi that attack small animals such as nematodes, rotifers, and amoebae. Several other sources are helpful for methods and tips for growing zygomycete fungi in the lab and may be also be helpful for this laboratory. In particular, Benny (**?**) and Benny et al. (**?**) contain figures and additional information on zygomycete culturing techniques as well as recipes for culture media and additional historical reference lists. For more complete information on nematode-trapping fungi and the zygomycete species that attack other small animals see Barron (**?**), Drechsler (**?**) and Duddington (**?**).

Below we go over the process of collecting Zoopagomycotina fungi in four different sections:

1. Soil and Dung Collection
2. Incubation of Soil and Dung
3. Viewing and Identification of Mycoparasitic Fungi
4. Isolation of Fungi from Samples

Two appendices are included to provide commonly used media recipes and methods for preserving dual cultures of mycoparasites for longer term storage. Preserved cultures are useful for creating culture collections that can be grown out and used as demonstration cultures for students to view as examples during laboratory activities.

2.2 Supplies

Although the list of supplies may vary slightly depending on the species of Zoopagomycotina that you hope to see, below is the generic list of all supplies used for obtaining Zoopagomycotina from soil and dung:

- Deep plastic or glass petri dishes with lids (e.g. 100 x 80 mm Pyrex glass crystallizing dishes). If lids are not available, then clear plastic or glass plates or Parafilm may be used as covers
- Standard size plastic and/or glass petri dishes
- Sterile filter paper discs
- Distilled water
- Low nutrient agar media (see appendix)
- Trowel, spoon, or similar device for scooping soil
- Clean plastic and paper bags
- Lab gloves
- Antibacterial antibiotics and benomyl (see appendix)
- Dissecting microscope
- Light microscope
- Bunsen burner or alcohol lamp
- Sharpie or other permanent markers
- Parafilm
- Microscope slides and coverslips
- Tissue stain for slide preparations, such as lactophenol cotton blue
- 70% or 95% ethanol for bench and tool sterilization

- Some or all of these flame-resistant isolation tools: fine-tipped forceps, tungsten wire loop, insect minuten pin with handle, nichrome wire with handle, small metal spatula, scalpel (Fig. **??**)

2.3 Soil and Dung Collection

Different taxa are commonly associated with different substrates (e.g. soil vs. litter vs. dung) so multiple substrates can be used in order to maximize Zoopagomycotina diversity. For example, *Syncephalis* species are more common from soil samples (**?**), whereas predaceous taxa like *Zoopage* species may be more common in decaying leaf litter (**?**).

2.3.1 Soil Collection

1. Find a location to sample. Nutrient rich and moist locations such as a gardens, compost heaps, beneath trees or shrubs, etc. may yield better results. Mesic forest habitats often yield many species of Zoopagomycotina.
2. Use a clean spoon or trowel to scoop a small amount (up to 250 mL/1 cup) of topsoil and place in a small, clean plastic bag. If desired, also collect decaying leaf litter laying on the soil.
3. Keep soil and leaf litter refrigerated until ready to plate on media. (Although few studies have empirically tested the effects of refrigeration, anecdotal evidence suggests that fresher soil yields the highest diversity).

2.3.2 Dung Collection

Rodent and rabbit dung are rich sources of *Piptocephalis* and *Syncephalis* species but isolates have also been obtained from the dung of horses, cows, raccoons, squirrels, goats, and bats. The most important aspect of dung collection is that it must be relatively fresh. The dung should still have moisture and have a shiny, brown appearance. Old dung is often drier, is white or green, and has a "crusty" appearance.

1. The type of dung collected will depend on the surrounding habitat. Wooded areas are typically good for collecting dung from a variety of small mammals such as squirrel, deer, rabbit, raccoon, and mouse. Even arthropod dung (e.g. cockroach or earwig) has produced some interesting Zoopagomycotina taxa and could be tested. Local farms with herbivores may be a valuable source as well. For identification of scats see Halfpenny (**?**) or similar source.
2. Place the dung in an appropriate container and refrigerate until ready to place in a moist chamber. Firm dung (e.g. mouse or rabbit pellets) should be kept in paper bags rather than in plastic bags.

   Note: Field collected dung could potentially contain nematodes or other disease agents so it should be handled with laboratory gloves. In some areas of the USA (e.g. the Southwest) rodents may act vectors for Hanta virus so it may not be appropriate to use rodent dung from all locations.

2.4 Incubation of Soil and Dung

Two main approaches have been used to view and isolate Zoopagomycotina species from soil and dung, incubation and plating. These approaches are outlined briefly here.

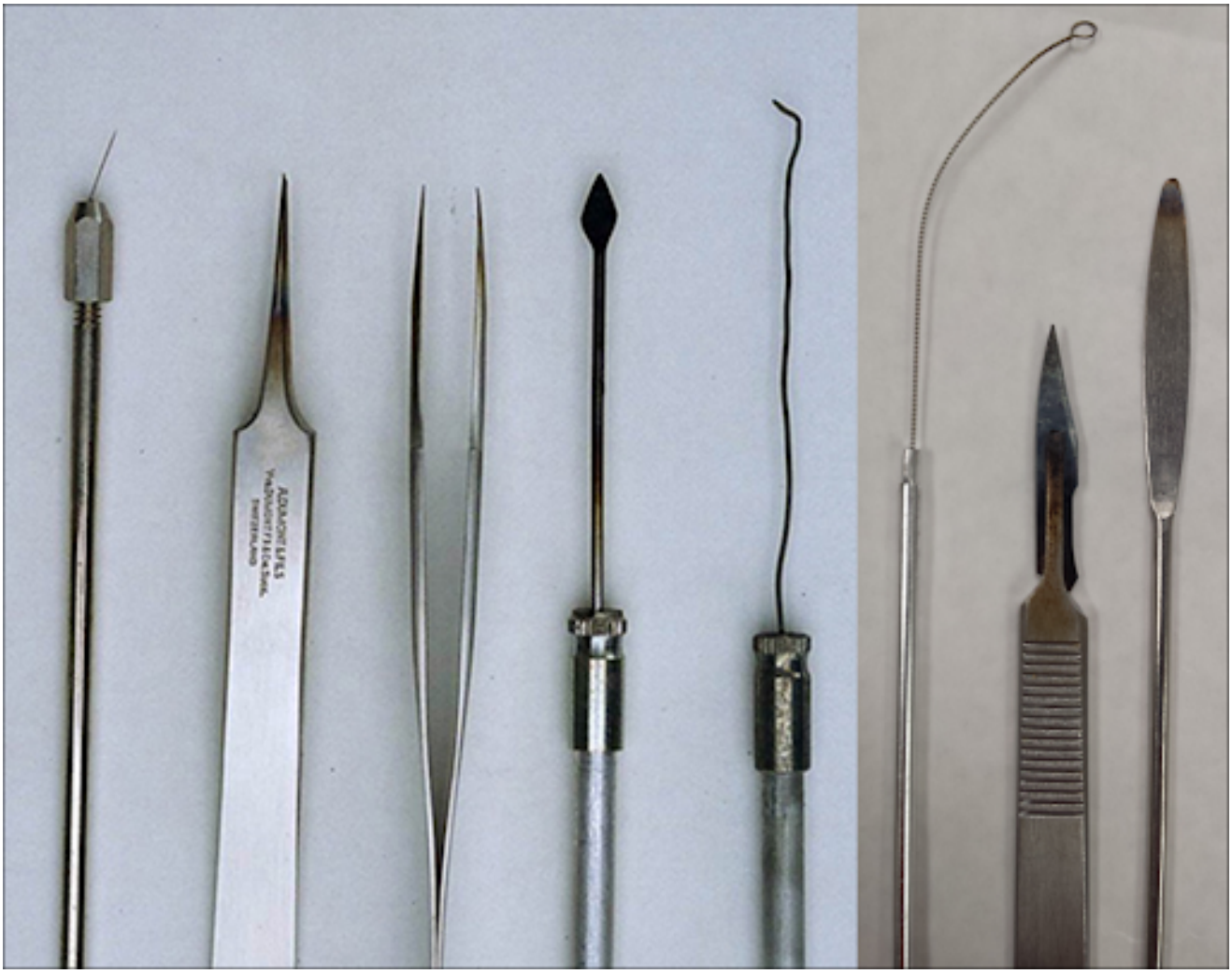

Figure 2.1: Tools used for isolating, transferring, and culturing microfungi. From left to right: insect minuten pin with pin vice handle, two examples of fine-tipped forceps, a harpoon-like mini spatula with vice handle, nichrome wire with a vice handle, tungsten wire loop, metal-handled scalpel, and small spatula. Fine-tipped forceps are useful for collecting segments of hyphae (e.g. for slide making or isolation in culture) whereas the insect minuten pin, nichrome wire, and loop may be used for transferring spores. The scalpel or spatulas are needed for cutting agar plates.

2.4.1 Soil Plating

1. Choose a low-nutrient media plate to use for isolation. The choice of plate will depend on the desired fungal groups. For mycoparasitic taxa it is typical to use plates that include antibacterial antibiotics and the anti-fungal compound benomyl to reduce growth by 'molds' (e.g. *Aspergillus* and *Penicillium* spp.). For predaceous fungi we recommend water agar plates without antibiotics or benomyl and with plenty of moisture. Host animals such as nematodes or amoeba are often more prolific if extra water is added to the plate (see Appendix below for agar and antibiotic recipes).
2. Use a spoon or small scoop to gather 1-2 grams of soil or litter and gently sprinkle evenly across one side of the plate (Fig. **??**). One side of the plate is left clear of soil to enable easier viewing of the fungi.
3. Label the plate with the collection code, date, media type. Store in a dark cabinet or container for several days. Do not seal the plate with Parafilm.
4. The plates should be observed under a dissecting microscope to look for fungi.

2.4.2 Dung Incubation in Moist Chambers

1. Plastic or glass petri dishes lined with filter paper are used for dung incubation. Standard size (i.e. 100 x 15 mm) petri dishes are too shallow for most types of dung, but may be appropriate for mouse or rat dung. Deeper dishes are better for allowing the growth of tall, aerial fungi and are best for larger dung like cow or horse (Fig. **??**).
2. Divide the dung into smaller portions that will fit in the center of the dishes. For very small dung (e.g. mouse), several pellets may be evenly spaced across the dish. The idea is to leave enough space around the dung for fungi of interest to grow out.
3. Wet the filter paper with enough distilled water to saturate the paper (but avoid having standing water in the bottom of the dish because this will promote bacterial growth). Do not parafilm the dishes.
4. Moist chambers can be incubated for several days before fungi appear, but they should be checked regularly to ensure the filter paper stays moist and to watch for overgrowth by other organisms (e.g. bacteria, *Aspergillus* and *Penicillium* spp).

2.5 Viewing and Identification of Mycoparasitic Fungi

2.5.1 Slide Preparation to Examine Fungi

Slides should be made of fungi of interest for identification purposes. Fungal hyphae can be collected with a sterilized fine instrument (e.g. loop) and placed in a drop of distilled water or ethanol on a microscope slide. If using ethanol, a drop of 2% KOH should be added to rehydrate the hyphae. A drop of stain such as lactophenol cotton blue may be placed next to the cover slip and allowed to diffuse across the sample. If slides are to be kept, excess liquid should be removed from the slide by placing a Kimwipe or paper towel beside the coverslip and allowing it to absorb the excess. Clear fingernail polish can be applied around the edges of the coverslip to create a seal. The area around the coverslip should be as clean and dry as possible to ensure the polish adheres.

2.5.2 Morphological Features of Mycoparasitic Genera

Piptocephalis: Common hosts: *Cokeromyces, Umbelopsis, Mucor*, and *Mortierella* (Fig. **??**)

Morphology (Fig. **??**): ***Syncephalis***: Common hosts: *Mucor, Mortierella, Zygorhynchus*, and *Rhizopus* (Fig. **??**)

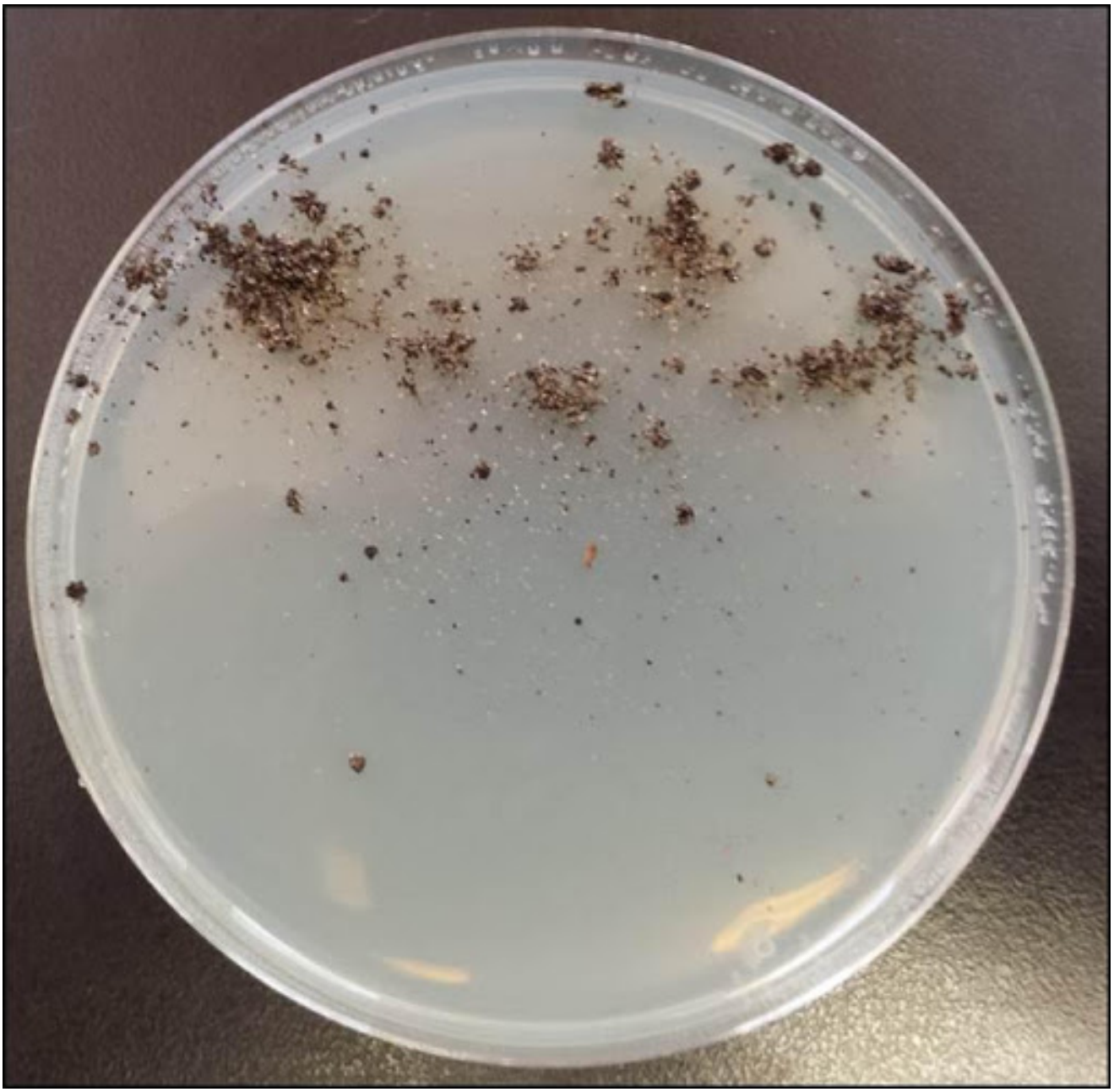

Figure 2.2: Example of a soil sample sprinkled on an agar plate. A small amount of soil spread on half of the plate helps to minimize overgrowth of fungi as well as enable easier visualization of fungi that grow out over the agar. This example has a minimal amount of soil, but more could be used depending on the size and consistency of the soil particles.