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# Shifts in Myxomycete Community Structure in Selected Microhabitats Across Nutrient Treatments in a Lowland Tropical Forest of Panama

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Shifts in Myxomycete Community Structure in Selected Microhabitats Across Nutrient  
Treatments in a Lowland Tropical Forest of Panama

A dissertation submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy in Biology

by

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## **Abstract**

Myxomycetes (plasmodial slime molds) are abundant amoeboid predators of bacteria and other microorganisms. They are found worldwide, largely in association with decaying plant material in terrestrial habitats. Their consumption of bacterial prey puts microbial predators such as myxomycetes in a key position in various ecosystem processes wherein they help regulate the flow of nutrients (e.g., N and C) through the ecosystem. The importance of microbial predators in nutrient cycling and plant productivity is well established. Given the extent to which tropical ecosystems influence global nutrient fluxes, along with the ecologically significant role that myxomycetes play in these processes, there is a pressing need to learn more about this understudied community.

This dissertation comprises an important first step toward developing an understanding of the ecological role of myxomycetes, with two major contributions. Identifying species is a fundamental step toward characterizing the diversity of a community. Given the unique challenge of identifying species of myxomycetes imposed by their unique biology, this dissertation begins in chapter two with a review of the myxomycete species concept. The various species concepts used (or potentially used) to identify species of myxomycetes are discussed. In chapter three, the challenges that myxomycete identification pose are described in the context of an ecological study. Therein, the use of a long-term nutrient fertilization experiment is described within which the effects of three major macronutrients, N, P and K on the myxomycete community in a lowland tropical forest of Panama are investigated. Interestingly, very little evidence supports the presence of a nutrient limitation to the myxomycete community, despite the many limitations that have been identified for other groups in this ecosystem (e.g., plants). The unexpected results provide the opportunity to again discuss the complications of species

identification and enumeration of myxomycetes for in-depth studies. The results also highlight the unique biology of myxomycetes and provide new insights into their ecology. Finally, in chapter four, a holistic approach is employed to describe a species new to science that was discovered during the course of this dissertation work in Panama. Overall this dissertation highlights the importance of myxomycete taxonomy in an ecological framework.



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Throughout my dissertation research I have been fortunate to be part of several collaborative works and would like to thank some of those collaborators that have not yet been mentioned especially Drs. Franck Carbonero, Dmitry Leontyev, Carlos Rojas and also, Marjorie Cedeño. I also thank my many fellow graduate students that have provided me with friendship, support, nonjudgmental scientific conversation and laughter. It is thanks to my friends that my time here in Arkansas has been such an unforgettable journey.

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## **Dedication**

I dedicate this dissertation to my parents, Beverly and Anthony Walker.

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## **List of Published Papers**

Both of the published chapters cited below are included in this dissertation in the styles corresponding to each of the journals in which they are published.

### **Chapter IV - published.**

Walker LM, Leontyev D, Stephenson SL (2015) *Perichaena longipes*, a new myxomycete from the Neotropics. *Mycologia* 107:1012–1022.

### **Chapter II - published.**

Walker LM, Stephenson SL (2016). The species problem in myxomycetes revisited. *Protist* 167:319–338.



## **I. Introduction and the motivation behind this dissertation research**

Myxomycetes (also known as plasmodial slime molds or myxogastriids) are a monophyletic group of microbial eukaryotes in the supergroup Amoebozoa (Cavalier-Smith 1998; Fiore-Donno et al. 2005; Shadwick et al. 2009; Fiore-Donno et al. 2010; Adl et al. 2012). Their life cycle usually involves two trophic stages—one consisting of microscopic uninucleate amoeboid flagellates and the other of a multinucleate plasmodium—along with a reproductive stage somewhat similar to the spore-producing fruiting bodies of certain macrofungi, albeit much smaller. Due to their resemblance to fungi and because they commonly occur in similar microhabitats (e.g., decaying plant material), myxomycetes were traditionally studied by mycologists (Martin and Alexopoulos 1969). In fact, the name myxomycete was derived from the words “*myxa*” (meaning slime) and “*mycetes*” (referring to fungi) (Link 1833). Anton de Bary (1859) was the first to provide a formal description of myxomycetes and it appears that he was also the first to recognize that myxomycetes are more closely related to amoeboid protists than they are to fungi. Now, in all phylogenies, myxomycetes are well recognized as protists belonging to the supergroup Amoebozoa (Cavalier-Smith 1998).

Myxomycetes, together with the Dictyostelia (dictyostelids) and members of the genus *Ceratiomyxa*, form a larger clade referred to as the Macromycetozoa (Fiore-Donno et al. 2010). The myxomycetes are the most speciose group within the Macromycetozoa, with more than 900 described morphospecies (Lado 2005–2015, which is the source of the nomenclature used throughout this dissertation). Molecular data have confirmed the division of myxomycetes into two clearly defined groups, historically distinguished by spore color and still commonly referred to as the dark-spored and bright-spored clades (Fiore-Donno et al. 2005), or more recently recognized as the superorders Columellidia and Lucisporidia, respectively (Cavalier-Smith

2013). Within the two main groups of myxomycetes five orders are traditionally recognized. The dark-spored clade is comprised of the orders Echinosteliales, Physarales and Stemonitales, while the orders Liceales and Trichiales are located in the bright-spored clade (e.g., Martin and Alexopoulos 1969; Olive 1970). Traditional myxomycete taxonomy (including the aforementioned five orders) is entirely based upon a morphological species concept, wherein morphological features of mature fruiting bodies are used to delimit species (note that the fruiting body is the only stage of the life cycle that can be used for species level identification). Despite currently accumulating molecular data indicating that the morphological species concept does not accurately reflect the evolutionary relationships within the group at this time, it is still the most commonly used method of species identification and delimitation for practical purposes. The debate over the most appropriate species concept in the myxomycetes is quite complex and is therefore covered in considerable detail in chapter two of this dissertation.

The generalized life cycle of a typical myxomycete, as first described by de Bary (1887) and recently illustrated in a number of monographs and papers (e.g., Stephenson and Stempen 1994; Walker and Stephenson 2016), usually involves two strikingly different trophic stages. The first trophic stage is a uninucleate amoeba with or without flagella (the term “amoeboflagellate” encompasses both types) and the second is a distinctive multinucleate structure called a plasmodium. Plasmodia are mobile and may range in size from a few micrometers to more than a meter across in some species. Given appropriate stimuli, the plasmodium gives rise to one or to many spore-containing fruiting bodies (or sporophores). Fruiting bodies range in size between 0.5 and 4.0 mm in height and across the whole group display a very wide range of shapes and colors.

Amoeboflagellate cells and plasmodia of myxomycetes are typically recognized as predators of bacteria and, to a lesser extent, fungi (Stephenson 2011). However, plasmodia are also known to feed upon larger prey such as yeasts and filamentous fungi (Stephenson and Stempen 1994) as well as algae (Lazo 1961). Myxomycetes can be found worldwide, largely in association with plant material in terrestrial environments (Stephenson and Stempen 1994). Some of the most commonly recognized myxomycete substrata or microhabitats include, leaf litter on the forest floor, leaf litter that is still attached or has fallen but not yet reached the floor (aerial litter), decaying woody tissues, the bark of living trees and soil. Although most widely recognized in terrestrial environments, myxomycetes have also been reported to occur in both natural and manmade aquatic habitats (Page 1988; Walker et al. 2003; Walochnik et al. 2004; Lindley et al. 2007), as well as in some even less expected microhabitats such as in the coelomic cavity of sea urchins (Dyková et al. 2007). Because myxomycetes have been found in virtually every major terrestrial habitat examined to date, they are generally considered to be cosmopolitan organisms (Stephenson et al. 2008). Some groups of myxomycetes however, like many other protists, display a moderate level of endemism (Foissner 2006), and patterns in their biogeographic distributions are recently becoming more apparent (e.g., Estrada-Torres et al. 2012; Aguilar et al. 2013). Some major factors known to drive distribution patterns are temperature and moisture (Alexopoulos 1963) and pH of the substratum (e.g., Härkönen 1977; Stephenson 1989; de Basanta 2000). At the landscape level, too, several trends have been identified. For example, despite the general trend of many organisms to increase in diversity with decreasing latitude (Hillebrand 2004), myxomycete biodiversity appears to be higher in temperate forests as compared to tropical forests (Stephenson et al. 1993). Different ecosystems tend to support different assemblages of myxomycetes as well. For example, members of the

genera *Physarum* and *Didymium* appear to be among the most abundant myxomycetes encountered in tropical regions, while species of *Cribraria* and *Trichia* are some of the more predominant taxa in temperate regions (Stephenson et al. 1993; Stephenson et al. 2000). The melting snowbanks in alpine and subalpine zones host a distinct assemblage of myxomycetes representing approximately 100 species, largely from the dark-spored clade (Novozhilov et al. 2013). Large-scale trends such as these are likely to be attributed to niche preference at the microhabitat level and can limit myxomycete distribution. *Diachea arboricola*, for example, is considered truly corticolous (living on the bark of living trees) as it has been found only on this substrate (Keller et al. 2004). Another distinct assemblage of myxomycetes of approximately 100 species are coprophilous (occurring on dung), and as many as 16 of these species have either been reported to occur only on dung or have been rarely reported on other substrates and therefore may be truly coprophilous (Eliasson 2013). Not surprising, within substrate types an even finer scale niche preference can be observed, such as in the lignicolous myxomycetes (living in association with dead woody tissues) upon which different assemblages are consistently found in association with particular stages of woody decay (Liu et al. 2015 and references therein).

Most of our knowledge concerning myxomycetes has been obtained through either laboratory culture or field observations. Both of these methods of study, however, have their own set of difficulties that can at times, greatly alter the interpretation of results and thereby bias our understanding of myxomycetes, particularly in natural settings. Anyone who has carried out a field survey of myxomycetes knows that finding fruiting bodies is sometimes incredibly painstaking and is in no way complete for any given locality. The vast majority of myxomycete fruiting bodies are only one to two millimeters in height, and 20 to 40% are less than 0.5 mm in

height (Novozhilov et al. 2000). Given their small size and the knowledge that at least some species can be found fruiting everywhere from the underside of a leaf on the forest floor, to the top of the forest canopy, and everywhere in between, in addition to their fragile and ephemeral nature, a truly complete survey would be incredibly difficult. Many myxomycete surveys also include a laboratory culture component such as moist chamber culture (Martin and Gilbert 1933) as a supplement to field surveys, largely for this reason (Novozhilov et al. 2000). Many species, however, rarely (if ever) form fruiting bodies in natural settings, while still others may not form fruiting bodies in moist chambers for various reasons (e.g., the size limitation imposed by the size of the Petri dish). Taken together, these difficulties make in-depth quantitative studies of natural populations of myxomycetes exceedingly difficult. Consequently, an accurate and complete picture of myxomycete habitat preference, community structure and life history strategies is not yet available.

The use of molecular data in myxomycete classification and study has occurred rather late (Fiore-Donno et al. 2005) compared to its use in other groups of organisms. Yet, already several examples exist of molecular studies revealing information that was never before possible to obtain. Consider, for example, the soil microhabitat from which myxomycete isolations are particularly difficult and within which enumeration of myxomycetes was previously possible only by tedious culture methods such as the most probable number technique (MPN). Recently, however, transcriptomic approaches have identified macromycetozoans (the group comprised of myxomycetes, Dictyostelia and *Ceratiomyxa*) to be the most abundant amoeboid predators in some soils (Urich et al. 2008; Geisen et al. 2015). And with the use a targeted environmental sequencing approach, Fiore-Donno et al. (2016) confirmed the abundance of myxomycetes in particular in the soil and also revealed an unprecedented level of genetic diversity in that

microhabitat. Feest and Campbell (1986) reported numbers  $>20,000 \text{ cm}^3$  in samples of temperate soils. Other than abundance, culture based studies have also suggested that myxomycetes actually spend the majority of their lives as amoebae in soil (Feest 1987; Stephenson and Feest 2012). Because myxomycetes were previously identifiable only through morphology of the fruiting body, specimens enumerated or isolated from the soil (or any microhabitat) that could not be brought to formation of fruiting bodies in culture, could not be identified. However, now armed with molecular approaches such as high-throughput sequencing of target genes (e.g., small subunit rDNA), it is now possible to also identify species diversity at a level that was never before possible.

In addition to the ways that molecular data will confirm and enhance existing knowledge, in other cases these data will surely overturn or transform our current understanding of myxomycetes. A recent study by Clissmann et al. (2015) investigating the bright-spored myxomycetes associated with decaying beech logs, simultaneously employed molecular analyses with both moist chamber and field observation methods. From the same logs, these authors found only two species that were identified with both the moist chamber culture and targeted sequencing of the SSU rDNA gene. Upon deeper investigation of the gene sequences, the authors noted the abundance of sequences from large myxomycete species such as *Reticularia lycoperdon*, *Lycogala epidendrum* and *Fuligo septica* that presumably due to their size, very rarely occur in moist chambers. These authors also identified a number of sequences from species that fruit extremely rarely, such as *Prototrichia metallica*, *Arcyodes incarnata*, *Calomyxa* sp. and *Cornuvia* sp. In addition, these and other molecular data identify the presence of numerous sequences that do not correlate to known myxomycete sequences and may represent completely new species yet to be described (Clissmann et al. 2015; Fiore-Donno et al. 2016).

The possibilities that molecular data can provide are sure to greatly expand upon our knowledge of myxomycetes. However, it is crucial that these data can be incorporated into the already available knowledge in order to build upon that foundation. To do so requires that there is agreement between the traditional morphological identification of species (as used with moist chamber or observational studies) and newly emerging concepts that identify species using molecular data. At a time when molecular data are accumulating at ever-faster rates, and in many cases challenging the longstanding concepts of species, this is an urgent matter. This is the subject of chapter two of this dissertation entitled, *The species problem in myxomycetes revisited*.

Identification of species is an important task as species arguably represent one of the most basic units used to study ecology and particularly to measure biodiversity. Measuring components of biodiversity (e.g., species abundance and richness) within and between communities helps us understand the world around us. Given the importance of this topic, in chapter two several species concepts will be discussed, highlighting their strengths and weaknesses when applied to myxomycetes. The chapter includes a thorough background describing myxomycete life history strategies as well as the most recently available data regarding the myxomycete phylogeny. Due to the increasing use and application of molecular sequence data, chapter two represents a timely reassessment of the species concepts in myxomycetes.

Evidence documenting the importance of protozoan predators to ecosystem health, with the fact that myxomycetes are among the most abundant being well established, indicates that myxomycetes are essential components of terrestrial food webs (e.g., Ekelund and Ronn 1994; Adl and Gupta 2006). The majority of data concerning the ecological role of myxomycetes are based upon studies of temperate ecosystems. Given the extent to which tropical ecosystems

contribute to global nutrient cycling and ecosystem health, and because the tropics are likely to be more threatened by global change, the matter is arguably more urgent in tropical forests (Zhou et al. 2013). Therefore, there is a vital need to characterize and document this important community, its composition, and its response to changes in nutrient status. The overarching goal is ultimately to understand the role of myxomycetes in nutrient cycling and ecosystem function in tropical forests. Chapter three of this dissertation, entitled, *The response of myxomycete communities to 14 years of N, P and K addition in a lowland tropical rain forest* is a first step toward that goal.

Chapter three describes the use of a long-term nutrient fertilization to investigate myxomycete ecology in a lowland tropical forest of Panama. In collaboration with the Smithsonian Tropical Research Institute (STRI), a large, fully factorial NPK fertilization experiment was utilized, allowing a first glimpse into this litter-inhabiting myxomycete community while simultaneously gathering baseline data relating to possible nutrient limitations. Continuously fertilized since 1998, these plots represent an extremely valuable resource for elucidating possible nutrient limitations and foundational concepts in patterns and dynamics of myxomycete diversity in the litter microhabitat.

To characterize the effects of nutrient fertilization on the litter-inhabiting myxomycete community, samples of leaf litter and small woody debris were collected on two separate occasions one year apart. Utilizing traditional moist chamber cultures, the following hypotheses were tested: (1) myxomycete abundance and diversity will be greater on the P plots as compared to other treatment and control plots due to a previously identified limitation of this nutrient to both the above and below-ground communities at this site (Wright et al. 2011; Turner and Wright 2013), and (2) that myxomycete abundance and diversity will be significantly lower on



the N plots compared to the other plots due to a decreased soil pH and the apparent lack of N limitation to this community.

The NPK fertilization experiment used herein represents the longest running nutrient addition study in any old-growth lowland tropical forest in the world (Wright et al. 2011). This is also the first example of a K fertilization experiment in any old-growth tropical forest and is now also the very first to incorporate protozoans. The information derived from this project should spur further ecological research on myxomycetes and other protozoans, in Panama and around the world.

The final chapter of this dissertation is entitled *Perichaena longipes, a new myxomycete from the Neotropics* and it contains the description of a new species of myxomycete that was found in abundance during the course of the dissertation research being conducted in Panama. In line with the guidelines proposed in chapter two of the dissertation, the description includes a detailed morphological description and comparison with other morphologically similar species of *Perichaena*. Additionally, in order to verify the molecular distinctness of this taxon, a 5' portion of the nuclear small subunit rDNA from this and several closely related species, were sequenced. Finally, the description concludes by providing a new dichotomous key for morphological identification of all currently recognized stipitate and sub-sessile species of *Perichaena*.

The goal of the dissertation research described herein is to expand our knowledge of myxomycetes in two major respects. First, by providing an updated and timely discussion concerning myxomycete taxonomy and classification and second by providing a first time glimpse into the ecology of litter-inhabiting myxomycetes in the nutrient-poor lowland tropical forests of Panama. Taken together, this work highlights the importance of taxonomy in the

framework of ecology for myxomycetes. The dissertation closes with concluding remarks that summarize the major contributions that have been made herein and suggest future directions.

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## **II. The species problem in myxomycetes revisited**

Walker LM, Stephenson SL (2016). The species problem in myxomycetes revisited. *Protist* 167:319–338.

### **Abstract**

Species identification in the myxomycetes (plasmodial slime molds or myxogastriids) poses particular challenges to researchers as a result of their morphological plasticity and frequent alternation between sexual and asexual life strategies. Traditionally, myxomycete morphology has been used as the primary method of species delimitation. However, with the increasing availability of genetic information, traditional myxomycete taxonomy is being increasingly challenged, and new hypotheses continue to emerge. Due to conflicts that sometimes occur between traditional and more modern species concepts that are based largely on molecular data, there is a pressing need to revisit the discussion surrounding the species concept used for myxomycetes. Biological diversity is being increasingly studied with molecular methods and data accumulate at ever-faster rates, making resolution of this matter urgent. In this review, currently used and potentially useful species concepts (biological, morphological, phylogenetic and ecological) are reviewed, and an integrated approach to resolve the myxomycete species problem is discussed.

### **Introduction**

“A particular kind of biological entity” is the definition of a species as proposed by Nixon and Wheeler (1990), and this conforms to the general concept used by most modern biologists. However, a debate has been ongoing since the time of Linnaeus as to the “best” definition of what truly constitutes a species. Haldane (1956) defined a species as "a name given to a group of

organisms for convenience, and indeed of necessity." For practical reasons, the recognition of species is necessary for communication and the general exchange of knowledge and, to be most effective, a species should be defined in a way that is universally accepted yet subject to change as additional data become available (Adl et al. 2007; Cavalier-Smith 1998). Defining a species is of monumental importance because species are the fundamental units of ecology, carrying out unique roles in their particular niches, communities and ecosystems (Cohan and Perry 2007). Therefore, it is not surprising that reaching an agreement on just how to define a species has proven to be so problematic. Over the years, this debate has led to the creation of more than two dozen different species concepts (Wilkins 2006), ranging from the biological species concept initially proposed by Mayr (1942) to more recent concepts based largely or completely on molecular data (Vasilyeva and Stephenson 2010).

The definition of a species in the myxomycetes (plasmodial slime molds or myxogastriids) has presented particular challenges. With a complex life cycle, several different life strategies, varying levels of phenotypic plasticity and genetic variation, the myxomycetes challenge every major species concept that has been put forward. In this paper, the species concepts currently used for myxomycetes will be discussed. Other potential concepts that could be used, the problems associated with each, the implications of these problems on a larger scale and suggestions that might yield a possible solution also will be considered.

## **Background**

The myxomycetes form a single well-supported clade within the supergroup Amoebozoa (Adl et al. 2012; Fiore-Donno et al. 2010a; Lahr et al. 2011a). Myxomycetes, together with the Dictyostelia (dictyostelids) and members of the genus *Ceratiomyxa*, form a larger clade referred

to as the Macromycetozoa (Fiore-Donno et al. 2010a). Although the members of the Macromycetozoa are defined on the basis of their macroscopic fruiting bodies, this is not an apomorphic character, since the formation of fruiting bodies has many origins and is widespread throughout the eukaryotes (e.g., Adl et al. 2012; Brown et al. 2012; Shadwick et al. 2009). The myxomycetes, along with their sister group *Ceratiomyxa*, are generally defined-by the presence of a flagellated stage in their life cycle and an often relatively large acellular plasmodial stage (Fiore-Donno et al. 2010a).

The myxomycetes are the most speciose group within the Macromycetozoa, with more than 900 described morphospecies (Lado 2005–2015, which is the source of the nomenclature used herein). Myxomycetes are traditionally recognized as predators of bacteria and (to a lesser extent) fungi that occur in association with decaying plant material (Stephenson 2011). As a whole, myxomycetes are cosmopolitan organisms and have been found in virtually every major terrestrial habitat examined to date. However, recent work indicates that some groups of myxomycetes (Aguilar et al. 2013; Estrada-Torres et al. 2013), like many other protists (Foissner 2006), display a moderate level of endemism, and patterns in their biogeographic distributions are becoming ever more apparent. Myxomycetes commonly occur in all types of forests, where they have been isolated from all of the microhabitats found in forest ecosystems (e.g., the bark surface of living trees, coarse woody debris, ground litter and aerial litter). Myxomycetes also appear to be among the more abundant organisms in soil. In fact, recent evidence indicates that macromycetozoans are the most abundant amoeboid predators in some soils (Urich et al. 2008; Geisen et al. 2015), and their importance in that habitat is no longer questioned.

The generalized life cycle of a typical myxomycete, as first described by de Bary (1887) and recently illustrated in a number of monographs and papers (e.g., Stephenson and Stempen



1994; Everhart and Keller 2008), encompasses two strikingly different trophic stages. The first is a uninucleate amoeba with or without flagella (the term “amoebflagellate” encompasses both types) (Fig. 1, C1) and the second is a distinctive multinucleate structure, the plasmodium (Fig. 1, H1). Plasmodia are motile and range in size from a few micrometers to more than a meter across in some species. Plasmodia often contain many thousands of synchronously dividing nuclei. Given appropriate stimuli, the plasmodium gives rise to one or more (as many as several hundred or even a few thousand in some species) spore-containing fruiting bodies (also referred to as sporophores), most of which range between 0.5 and 4.0 mm in height (Fig. 1, A). Most of what is known about the myxomycete life cycle has been derived from studies of just two species (*Physarum polycephalum* and *Didymium iridis*), but current evidence suggests that most other species follow a similar general pattern (citations).

Bacteria appear to represent the main food resource for both trophic stages (amoebflagellate cells and plasmodia) in the myxomycete life cycle, but plasmodia are also known to feed upon larger prey such as algae (Lazo 1961), yeasts and the spores, hyphae and fruiting bodies of filamentous fungi (Stephenson and Stempen 1994). Interestingly, plasmodia are also capable of engulfing amoebflagellates and small plasmodia of the same or other species of myxomycetes (Clark and Haskins 2012).

Under adverse conditions such as drying of the microhabitat, a drop in temperature and/or a lack of suitable food organisms, a plasmodium may convert into a hardened, resistant structure called a sclerotium (Fig. 1, G), which can quickly revert to the active stage upon the return of favorable conditions (Stephenson and Stempen 1994). Similarly, amoebflagellate cells<sup>1</sup> can

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<sup>1</sup> The term "amoebflagellate" encompasses both amoeboid and flagellate forms. The two forms are reversible but it is unclear if the flagellated form is able to directly form a microcyst or if the flagellum must first be resorbed. However, flagellates can develop directly from microcysts.

undergo a reversible transformation to dormant structures called microcysts (Fig. 1, C1). All three of these resting stages, sclerotia, microcysts and spores, are capable of resisting relatively harsh conditions and remain viable for various periods of time. This is likely an important strategy for survival of myxomycetes in the often environmentally dynamic habitats (e.g., deserts) in which some species tend to occur.

The above represents a very generalized model of the life cycle in a “typical” myxomycete (Fig. 1), but there are a number of important variations of this basic model that complicate things. Examples of sexual and asexual, heterothallic (possessing mating types) and non-heterothallic (not possessing mating types) life cycles are found (Table 1). In some instances, a single lineage has been characterized by more than one reproductive strategy, and can even switch from one strategy to another and then back again (e.g., Clark 1995). Clark and Haskins (2010) reported that 14 of the 51 morphospecies (largely overrepresented by members of the order Physarales) of myxomycetes in which the reproductive system had at that time been subjected to detailed examination were documented as displaying both heterothallism and non-heterothallism. Twenty-nine of the same 51 morphospecies investigated were found to display only a non-heterothallic reproductive system, thus suggesting that the 'typical' myxomycete life cycle is probably over simplistic.

Heterothallic myxomycetes generally follow the basic life cycle outlined above, with a single locus multi-allelic mating system controlling the fusion of two haploid amoebflagellate cells, first to form a diploid zygote which then matures into a coenocytic plasmodium. Meiosis is assumed to take place in the maturing spores, which then germinate to produce the next generation of uninucleate (haploid) amoebflagellate cells. Heterothallic lineages can possess as many as 16 mating types in a single morphospecies, as reported for *Physarum polycephalum* by

Moriyama and Kawano (2010). Mating types may be considered to represent a situation similar to that of separate sexes, in that they block the fusion of “like” gametes (i.e. they are presumed to promote out-crossing). However, the application of the term “sexes” is best reserved for organisms with anisogamous life cycles. Therefore, many myxomycete morphospecies with a heterothallic mating system are composed of a number of biological sibling species. As a result, although they may be identical in morphology, they are unable to interbreed (due to mating type incompatibility) and are therefore on separate evolutionary trajectories (Clark 1995). Groups of such sibling species appear to be common and have been thoroughly investigated and documented in at least 10 morphospecies, including *Arcyria cinerea*, *Didymium iridis*, and *D. squamulosum* (e.g., Betterley and Collins 1983; Clark and Collins 1976; Clark et al. 2002; El Hage et al. 2000). Identifying sibling species in natural populations of myxomycetes is more difficult, but with newly available molecular methods, evidence has been obtained to support the occurrence of sibling species in natural populations of *Trichia varia* in Germany (Feng and Schnittler 2015) and some species in the Stemonitales and Physarales associated with the snowbank habitat in northwestern Russia (Novozhilov et al. 2013b).

Myxomycetes with non-heterothallic modes of reproduction, which are not known to possess mating types, are largely apogamic (non-sexual, no ploidal change) (Fig. 1, C3) rather than homothallic (sexual, ploidal cycle) (e.g., Clark and Haskins 2010). These apogamic lines are able to convert directly from amoebflagellates into plasmodia without ploidal change or the need for fusion with other amoebflagellates (Clark 1997; Collins 1980; Therrien et al. 1977), allowing formation of essentially immortal clonal lines (Clark 1992). Although observed numerous times in laboratory cultures, the first evidence to suggest asexual clonal lineages of

myxomycetes occurring in natural populations was only recently documented for several morphospecies within the genus *Lamproderma* (Fiore-Donno et al. 2011).

Further complicating things, a number of the aforementioned apogamic, diploid (non-heterothallic) isolates (all members of the Physarales or Stemonitales) have been reported to revert to heterothallism in culture (Collins and Therrien 1976; Collins 1980, Collins et al. 1983; Collins and Gong 1985; Collins and Tang 1988; Yemma et al. 1980). According to Clark and Haskins (2013) the most reasonable explanation for these revertant cultures is that they were the product of a form of heterothallic reproduction called automixis, for which incomplete meiosis resulted in the production of genetically identical diploid descendants. This would explain the common overlap of heterothallic and non-heterothallic isolates in a single population and also supports the concept that most myxomycete morphospecies are complexes composed of a sexually reproducing core of biological sibling species and a cluster of asexual, isolated clonal lines (Clark 2000). Presumably, apogamic isolates can more successfully colonize a new habitat than sexual isolates (Schnittler and Tesmer 2008). The former appear better adapted to efficiently exploit new and especially ephemeral habitats due to their smaller size and faster sporulation times (Betterley and Collins 1983; Clark and Haskins 2013). Exploiting new habitats in isolation could (and probably does) allow genetic and/or morphological divergence to take place due to niche specific adaptation (Schnittler and Mitchell 2000), but the ability to revert back to heterothallism should maintain the cohesion of the species complex and be advantageous for the survivability of the species overall. However, it seems possible that these apogamic clonal lines could also accumulate mutations, ultimately causing them to lose the ability to revert back to heterothallism (Collins et al. 1983; Clark and Haskins 2010). With an inability to revert to heterothallism, the genetic isolation would presumably be permanent and lead toward continued

divergence of the two lineages. Lending strength to this concept, morphologically distinct, reproductively isolated, non-heterothallic isolates are occasionally collected from ephemeral habitats such as aerial leaf litter and inflorescences. These collections conform to the general morphospecies description except for their miniature size, and when grown in culture, a number of these collections have been shown to retain their miniature form (e.g., *D. iridis* [Clark et al. 2001] and *P. pusillum* [Clark et al. 2004]).

Other variations on the reproductive cycle in myxomycetes include rarely seen “selfing” (Collins 1961), in which heterothallic amoebflagellates are able to produce haploid plasmodia without crossing. There also appear to be rare sterile reproductive systems that cannot form fruiting bodies at all, examples of which have been observed for some isolates of *Didymium iridis* (Collins 1961), *Physarum flavicomum* (Henney 1967), *P. rigidium* (Henney and Henney 1968) and *Stemonitis flavogenita* (Collins et al. 1983). However, just because fruiting bodies are not recorded for a particular isolate does not mean that the isolate in question cannot fruit, only that fruiting has not been observed. The lack of fruiting body formation could be the result of inappropriate growth conditions, the absence of genetically compatible amoebflagellates (in heterothallic isolates), or a genetic mutation (Collins et al. 1983). Alternatively, it is possible that these isolates have lost the ability to form fruiting bodies, suggesting that their life cycle could consist entirely of the amoebflagellate<sup>2</sup> stage (Fig. 1, C2). Consider for example the previously recognized genus *Hyperamoeba*, all members of which have been shown to belong to the dark-spored clade of myxomycetes (Fiore-Donno et al. 2010b). Isolates once assigned to this genus have never been observed to form fruiting bodies, which is one reason they were not initially

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<sup>2</sup> Division of amoebflagellates occurs only in the non-flagellated form. Because these forms are reversible, this means the flagellum must first be resorbed before the cell undergoes division.

regarded as myxomycetes. To more fully understand the lack of fruiting in some myxomycetes will require further study.

Another intriguing potential departure from the ‘typical’ myxomycete life cycle is a possible form of asexual reproduction via the formation of amoeboid cells directly from a plasmodium (Fig. 1, H2). Indira (1964 and 1969) first described this phenomenon with detailed drawings of what she observed in cultures of *Stemonitis herbatica* and *Arcyria cinerea*. Later, the phenomenon was also described and presented along with photographic evidence in a species of *Physarum* (Ross and Cummings 1967) and *S. flavogenita* (Haskins 1991). Repeatedly, under controlled conditions, tiny pseudopods were first observed to swell up and then have a constriction form behind them; the constriction continued to deepen until a uninucleate amoeboid mass was “cut off” from the larger multi-nucleate plasmodium. It is possible that these amoeboid masses are only an abnormal response to the culture environment and that this phenomenon may not be common in nature, if it occurs at all. These amoeboid masses could be plasmodial fragments formed to allow a plasmodium to increase in size to maximize the use of available resources and increase spore production (Haskins 1991) or they could be a means of releasing unwanted materials, neither of which would have genetic implications. However, a more compelling hypothesis is that these amoeboid cells are true amoeboflagellate cells capable of propagation, thus representing a novel form of asexual reproduction. In *A. cinerea*, Indira (1969) reported that the newly independent cells became flagellated within just a few minutes and swam away, although the ultimate fate of those cells was unknown. The formation of flagella on such cells has not been witnessed in any other species nor is there photographic evidence of this occurring. Although intriguing and seemingly possible, more work is needed to assess just how common this phenomenon is in myxomycetes, to verify whether or not it ever occurs in nature

and to determine what these cells are actually capable of doing (i.e. fusing with other amoeboflagellates or directly producing new plasmodia).

### **Currently Used and Potential Species Concepts**

Understanding the complexities of the myxomycete life cycle and the various modes of reproduction as described above, is crucial for understanding the problems associated with delimiting species in the myxomycetes. These life cycle complexities and variations have bearing on all of the commonly used species concepts, the four most common of which (biological, morphological, phylogenetic and ecological) are described in the sections below, followed by a discussion of their use (or potential use) in myxomycetes.

#### *The Biological Species Concept*

The biological species as proposed by Ernst Mayr (1942) defines a species as a group of “actually or potentially interbreeding natural populations which are reproductively isolated from all other such groups.” Based on the information outlined above, the biological species concept is clearly difficult to apply to myxomycetes, particularly with respect to lineages that may be exclusively asexual. This important flaw in the concept, particularly when applied to protists, was first pointed out more than a half century ago by Sonneborn (1957), a prominent protistologist working with *Paramecium* (another protist). Nonetheless, attempts to apply this species concept to protists persist.

In order to discuss the potential application of a biological species concept to myxomycetes, it is important to first define what is meant by use of the term sex. Herein we use the definition of sex put forth by Lahr et al. (2011b), which involves the presence of both meiotic

reduction of the genome *and* subsequent fusion of nuclei during karyogamy. As discussed previously, a single myxomycete morphospecies often contains multiple biological sibling species that are reproductively isolated from one another, as well as a swarm of asexual clonal lines (Clark 1995). In addition, many taxa demonstrate multiple modes of reproduction, often within the same lineage, as is the case for *Echinostelium minutum*, *Fuligo septica* and *Physarum pusillum* (Clark and Haskins 2010 and references cited therein) to name a few, and it is possible that strictly asexual lineages exist (Clark and Haskins 2011). Hence, the variation that exists within the myxomycete life cycle limits the ability to use a biological species concept outright.

Another problem with the use of the biological species concept in the myxomycetes is the need for direct evidence of sex, which is available only through isolation and laboratory culture. Currently, only about 10% of all known myxomycete morphospecies can be cultured, placing a severe constraint on taxon sampling in reproductive studies (Haskins and Wrigley de Basanta 2008). In addition, some reliance on morphology is still required initially to identify possible mating pairs to test. However, morphology is not always correlated with reproductive isolation; morphologically distinct populations sometimes can interbreed, while other morphologically similar populations cannot. If the biological species concept is to be used to test a hypothesis, then many biological species could be overlooked or over identified. To avoid these obvious constraints, most protistologists (including myxomycetologists) have historically relied on a morphological species concept (Clark 2000; Finlay 2004).

### *The Morphological Species Concept*

Traditionally, as already noted, myxomycete taxonomy has delimited species using morphological features of the fruiting body. The characters used include the general structure



and appearance of the fruiting body (Fig. 2) and its component parts (e.g., stalk, peridium, capillitium, etc.), the presence or absence of lime, and the color and ornamentation of the spores (e.g., Martin and Alexopoulos 1969). This morphological species concept assumes that a speciation event is always accompanied by a morphological change, when in fact this may not always be the case (Andersen 1998). Therefore, a single morphospecies may represent a range of distinct genotypes which may or may not be able to interbreed. This may explain why protist morphology seems to remain relatively constant for very long periods of time. Indeed, the few myxomycete fossils that have been found (e.g., species of *Arcyria* and *Stemonitis*) from Baltic amber dating from the Eocene [Dörfelt and Schmidt 2006]) show no apparent differences from modern forms (Keller 2012; Stephenson 2011). The morphological species concept also assumes that distinct morphological features reflect shared evolutionary history and isolation from other groups. However, molecular analyses have revealed that discordance between morphology and evolutionary relationships are common in microbial lineages throughout the tree of life (Lahr et al. 2014), and the myxomycetes are no exception. Numerous examples exist of convergent morphologies in the myxomycetes. Examples include the repeated loss and gain of stalk formation across the entire myxomycete clade (Fiore-Donno et al. 2012; Shadwick et al. 2009) or the repeated evolution of fruiting bodies bearing compound fruiting bodies such as in the distantly related genera of *Lycogala* (bright-spored clade) and *Brefeldia* (dark-spored clade) (Schnittler and Tesmer 2008). It should be noted that even for myxomycetes which appear to share most other characters, those species with compound fruiting bodies have almost invariably been placed in different genera from those with solitary fruiting bodies. Such a dramatic change in morphology is likely to be under the control of a very small set of genes (citations), but this has yet to be investigated to any real extent.

Another assumption of the morphological species concept is that the characters used to distinguish different taxa show only slight variation within a species (Clark 2000). However, the characters used to identify myxomycetes, like many protists, can at times be extremely variable, blurring the lines between morphospecies and even genera. There are several examples of species that consistently bridge gaps between genera, containing features characteristic of more than one genus such as *Hemitrichia leiocarpa*, which could just as easily be assigned to the genus *Arcyria*, which was the case in earlier monographic treatments of the myxomycetes (e.g., Martin and Alexopoulos 1969). Moreover, the morphological species concept assumes that the characteristics used for identification are stable. However, the conditions under which myxomycetes form fruiting bodies can greatly affect the morphology of the mature fruiting body (Schnittler and Mitchell 2000). For example, in moist chamber culture, fruiting bodies that form upside down on the lid of the Petri dish (a common occurrence) regularly have reduced or even absent stalks, which can conceal important morphological features (Novozhilov et al. 2013a). In nature too, variation among fruiting bodies is common and readily observed by collectors working across a range of habitats or even a range of microhabitats or substrata within one locality. Although such plasticity is widely acknowledged, its full extent is unknown for the vast majority of myxomycetes. In order to quantify the level of variation that normally can be expected for a particular taxon requires spore-to-spore culture under controlled laboratory conditions (Keller 1996). As one example, in their description of *Didymium annulisporum*, Keller and Schoknecht (1989) established cultures on four different substrata (sterile and unsterile dung and two types of agar) and recorded substrate specific fruiting body morphology in terms of lime content, overall size and general shape, number of fruiting bodies and variations

in the peridium. Quantification of morphological variation in this manner is a valuable supplement to any new species description.

One final problem with use of the morphological species concept in myxomycetes, is the requirement of mature, intact fruiting bodies available for study. Morphological identifications in myxomycetes cannot be made using any of the other life stages such as the amoebflagellate cells or plasmodia (Clark 2000; Lado 2005–2015; Stephenson and Stempen 1994). Therefore, when fruiting body material is limited or nonexistent, such as when spores or amoebae are isolated from environmental samples and cannot be cultured (or if fruiting body formation does not result from culture), a strictly morphological identification is not possible.

Since 1965, the number of newly described myxomycete taxa has skyrocketed, in part due to increased sampling from diverse new habitats and localities, along with an increasing trend toward splitting genera and species into smaller groups (Keller and Everhart 2008). With this increase in the number of species of myxomycetes being described, there is also an increasing number of new taxa named on the basis of limited information. Lado (2001) estimated that monotypic myxomycete genera (i.e. those containing only a single species) account for 14 of 59 (or 24%) of all genera. Meanwhile Schnittler and Mitchell (2000) compiled a database containing 1012 subgeneric taxa, of which only 866 were classified at the species level and 305 taxa were described from only a single type locality. The amount of variation that can be observed in a single, localized, clonal population clearly indicates caution should be used in defining new taxa based on just one or even a few type specimens that differ in only minor respects from already described species. Instead, multiple specimens from multiple geographic locations should be thoroughly examined before a decision is made to recognize a species as new to science.

Although the morphological species concept has been the taxonomic standard for centuries, it is now clear that this approach to myxomycete species identification is in need of revision. One particularly noteworthy example in which the morphological species concept is not reliable for species identification is in the *Didymium iridis* species complex, the first myxomycete species complex (or group of sibling species) to be investigated extensively (Betterley and Collins 1983; Clark and Stephenson 1990; Clark et al. 1991; Clark and Landolt 1993; Collins 1976). This work showed that *D. iridis* has at least three non-interbreeding sets of mating types in the heterothallic lines and also contains multiple non-heterothallic, apogamic lines. Because the three heterothallic mating types are reproductively isolated from one another, and the non-heterothallic lines are also reproductively isolated, at least nine biological sibling species comprise this one morphospecies. In addition, detailed observations have shown that there is considerable overlap in the morphology of *D. iridis* and other stalked species in the same genus. Examples include *D. bahiense*, *D. nigripes*, *D. pertusum*, *D. ovoideum*, *D. melanospermum*, *D. megalosporum* and *D. verrucosporum* (El Hage et al. 2000; Clark et al. 2001). In fact, Lado (2001) suggested that there are actually as many as 30 morphospecies contained within the *D. iridis* complex, all of which may overlap with respect to the columella and/or stalk coloration, diameter of the sporotheca, total height of the fruiting body, color of the capillitium or plasmodium, the amount of lime present, and general shape (Fig. 2). As such, the *D. iridis* complex, one of the best studied of all morphospecies of myxomycetes, is particularly in need of detailed taxonomic revision.

### *The Phylogenetic Species Concept*

Phylogeny, particularly when based upon molecular sequences, is a powerful tool that can be used to predict species boundaries as well as to test biological and morphological species concepts (Andersen 1998, Caron 2013). A phylogenetic species concept relies on the use of phylogenetic trees, where the least inclusive cluster is considered a species (or phylopecies) (Mishler and Theriot 2000). Ideally, this also corresponds to the smallest biologically important unit deemed worthy of recognition (e.g., Cracraft 1983; Mishler 2010; Nixon and Wheeler 1990).

In lieu of whole genome sequencing, which is still not feasible for most taxa, a phylogenetic species concept therefore requires the selection of an appropriate gene(s) with which to construct the phylogeny. The most widely used gene for phylogenetic reconstruction in amoebozoans in general (Nassonova et al. 2010) and for myxomycetes in particular, is the ribosomal small subunit RNA gene [SSU], and a number of new species have already been identified and renamed based in large part on SSU sequences (Fiore-Donno et al. 2010b).

Although extremely slowly evolving across many major taxa, SSU can be a highly and sporadically variable phylogenetic marker in other groups, particularly in the myxomycetes, which also contain a high load of group I introns of variable lengths (Fiore-Donno et al. 2010a and 2012; Nandipati et al. 2012; Pawlowski et al. 2012; Shadwick et al. 2009). In lieu of full-length SSU sequences, which can be a challenge to obtain due to the aforementioned introns and sequence variation, Fiore-Donno et al. (2011) identified an intron-free region of ca. 700bp in the 5' SSU that can be amplified with a single pair of PCR primers that has proven powerful in the context of both phylogenetic and ecological studies (e.g., Fiore-Donno et al. 2012; Fiore-Donno et al. 2016; Novozhilov et al. 2013b). Intraspecific SSU sequence variation can vary significantly

between clades and even between multiple isolates of a single myxomycete morphospecies from a single population. When assessing myxomycete diversity with SSU sequence data parallel to morphology, one could find identical SSU sequences for multiple collections of a single morphospecies (Fiore-Donno et al. 2011) or alternatively one may find that SSU sequence diversity is twice as great as morphological diversity (Novozhilov et al. 2013b). Therefore, it is valuable to examine multiple molecular markers when constructing phylogenetic trees, particularly in the absence of other supporting data (e.g., Parfrey et al. 2006; Taylor et al. 2000).

Elongation factor-1 $\alpha$  [EF-1 $\alpha$ ] is another commonly sequenced gene used for constructing myxomycete phylogenetic trees (e.g., Feng and Schnittler 2015; Fiore-Donno et al. 2005; Fiore-Donno et al. 2010a). Due to its role in protein synthesis EF-1 $\alpha$  is highly conserved and abundant in actively growing cells (Baldauf 1999). Furthermore, the nearly complete sequence can be amplified with a single pair of universal primers (Baldauf and Doolittle 1997). Two additional markers that have been used to construct myxomycete phylogenetic trees are the more rapidly evolving ribosomal internal transcribed spacer [ITS] and mitochondrial cytochrome c oxidase [COI] genes, although with varying levels of success. Unlike the successful use of ITS sequencing in fungi (White et al. 1990; Schoch et al. 2012) and some other groups, the power of ITS for phylogenetic reconstruction in the myxomycetes is rather limited. Myxomycete ITS sequences are extremely variable and in many cases cannot even be aligned among closely related taxa (Fiore-Donno et al. 2011; Martin et al. 2003). The high level of sequence variation limits its ability to resolve evolutionary relationships in the myxomycetes although when used as a supplementary marker it can be valuable for differentiating between some species (Baba et al. 2015; Winsett and Stephenson 2008). Similarly, when COI is used as a supplemental tool it has proven successful for some amoebae (Nassonova et al. 2010), ciliates (Barth et al. 2006;

Chantangsi et al. 2007), diatoms (Evans et al. 2007) and numerous other non-protistan groups (e.g., Hebert et al. 2004; Pawlowski et al. 2012). In myxomycetes however, success with use of the COI gene sequence has been limited due to the high levels of sporadic variation among lineages. Even with primers specifically designed to amplify the first half of the myxomycete COI, sequences can be difficult to obtain and provide varying results (e.g., Feng and Schnittler 2015; Walker et al. 2011). Therefore, both ITS and COI can be valuable as supplementary markers to distinguish between closely related species but are not reliable markers to use for reconstructing deeper evolutionary relationships among myxomycetes.

#### Integrating molecules with morphology

Although molecular sequences from a few laboratory-cultured species have been used to confidently place myxomycetes as a whole in the larger eukaryotic tree (e.g., Cavalier-Smith et al. 1996; Baldauf 1999), DNA had not been successfully recovered from field-collected material until 2003 (Martin et al. 2003). The ability to use field-collected myxomycetes as a source of DNA greatly increased opportunities in molecular phylogeny, due to the great abundance of available herbarium specimens and the limited number of species that can be cultured for DNA isolation (Clark 2000). This increased availability of molecular data has led to an increased awareness of the ecological roles of myxomycetes and an increasingly sophisticated understanding of morphological evolution in myxomycetes.

Fiore-Donno and colleagues have resolved a number of important questions relating to myxomycete phylogeny, primarily by building trees from concatenated SSU and EF-1 $\alpha$  sequence data (e.g., Fiore-Donno et al. 2005). Unlike the situation with the dictyostelids (Schaap et al. 2006), these and other molecular data have revealed some level of congruence between

molecules and morphology in myxomycetes (Myxogastria). Molecular data have confirmed the monophyly of the myxomycetes and its division into two clearly defined groups commonly referred to as the dark-spored and bright-spored clades, more recently recognized as the superorders Columellidia and Lucisporidia, respectively (Cavalier-Smith 2013). As their names suggest, the dark-spored and bright-spored clades are historically distinguished on the basis of spore color, and the taxonomic distribution of this character is largely consistent with molecular phylogeny (Fiore-Donno et al. 2005). Meanwhile, within these two clades the traditionally recognized five orders—Echinosteliales, Physarales and Stemonitales in the dark-spored clade and Liceales and Trichiales in the bright-spored clade (e.g., Martin and Alexopoulos 1969, Olive 1970) do not seem to hold together. In fact, molecular phylogenetic data indicate that the current taxonomy (based on morphology) does not accurately reflect the phylogenetic relationships that exist within both the dark-spored clade (Fiore-Donno et al. 2008; Fiore-Donno et al. 2012) and the bright-spored clades (Fiore-Donno et al. 2013), and many of these relationships are currently being revised. Figure 3 reflects this newly emerging myxomycete phylogenetic tree, based upon currently available data.

Current molecular data indicate that within the dark-spored clade (Columellidia), the monophyletic order Echinosteliales is the earliest diverging clade and branches as a sister group to a taxonomic assemblage consisting of the orders Stemonitales and Physarales (Kretzschmar et al. 2015). The genus *Barbeyella*, with its capillitium adhering to peridial plates, may represent the transitional form between Echinosteliales and the rest of the dark-spored clade. Members of the Stemonitales and Physarales do not sort into two clearly distinct groups as current traditionally used taxonomic concepts have suggested. Instead, there are three main branches including (1) the "*Meriderma* group" (from the Stemonitales), (2) the traditional Physarales



together with the "*Lamproderma* group" (also from the Stemonitales) and (3) the rest of the Stemonitales (except for the *Meriderma* and *Lamproderma* groups) (Fiore-Donno et al. 2010a; Fiore-Donno et al. 2012). Within the bright-spored clade (Lucisporidia), there are two main branches, the first consisting of a monophyletic Cribrariaceae (from the Liceales) and the second composed of the Reticulariaceae (also from the Liceales) and three other clades that consist of various members of the orders Trichiales and Liceales (Clissmann et al. 2015; Fiore-Donno et al. 2013). The recently re-erected genus *Alwisia* (Liceales, Reticulariaceae) may represent the form, closest to the last common ancestor of the second clade referred to above (Leontyev et al. 2014b; Leontyev et al. 2014c). It is readily apparent that the traditionally used morphological species concept is no longer appropriate for many of the taxa that have been considered to make up the Trichiales and Liceales (Fiore-Donno et al. 2013; Leontyev et al. 2014a; Walker et al. 2015). As such, it would seem that there exist examples of incongruence between morphology and molecular data throughout myxomycete phylogeny, particularly at the ranks of genus and species.

At the genus level, let us first consider the genus *Lamproderma* in the dark-spored clade. Traditionally regarded as morphologically distinct, the monophyly of *Lamproderma* is rejected by molecular phylogenetic analyses, which indicate instead that "Lamproderma" appears to consist of several clades intermingled with species from three other genera (*Diacheopsis*, *Colloderma* and *Elaeomyxa*) (Fiore-Donno et al. 2012). Consequently, the stalk, columella, spotted (or not) peridium and the presence of "white splinters" in the peridium, which are characters previously used to identify members of genus, are shown by SSU phylogeny and further comparative morphology to be labile at the species level. Instead, a more reliable character for delimiting species of *Lamproderma* appears to be the presence of a shining

peridium. The example mentioned above and numerous others exemplify how molecular data can guide morphological studies and lead to an increasingly sophisticated understanding of the mode and tempo of morphological evolution, which in turn will lead to a better understanding of the significance of various morphological characters used in myxomycete taxonomy. For example, the combination of molecular and morphological data indicate that the stalk of the myxomycete fruiting body is far less reliable as a taxonomic marker than has long been thought to be the case. A stalked fruiting body appears to be the ancestral state for myxomycetes because the earliest diverging groups in both the dark-spored (Echinosteliales) and bright-spored (Cribrariaceae) clades are almost always stalked. However, the stalked condition of fruiting bodies also appears to be readily lost and can encompass considerable variability in length, even within a single population (Martin and Alexopoulos 1969), which makes the stalk unreliable at many taxonomic levels (e.g., Shadwick et al. 2009). Two other characters traditionally considered important for species delimitation in various myxomycete genera—spore color and ornamentation (Kalyanasundaram 2004)—have been revealed by molecular data to be unreliable in at least some cases, such as for *Lamproderma puncticulatum* and *L. columbinum*, which show a great deal of variation in spore morphology among isolates (Schnittler et al. 2010; Fiore-Donno et al. 2011).

Understanding the surprising evolutionary instability of such morphological characters may explain why the placement of a number of species currently do not seem to agree with traditional taxonomy. For example, the lack of a capillitium is the major character used to distinguish between the traditional orders Liceales and Trichiales in the bright-spored clade. However, the phylogenetic significance of this character has been under scrutiny for quite some time (Alexopoulos 1976) because the lack of a capillitium (or any other single character) is not

universal among or uniquely restricted to the Liceales. Not only are there species within the Liceales that undeniably have a true capillitium, such as *Listerella paradoxa* (Eliasson 1977) or *Alwisia bombardata* (Leontyev et al. 2014a), but some species of *Perichaena* (Ramírez-Ortega et al. 2009 and references cited therein) in the Trichiales and even some members of the dark-spored clade of myxomycetes also lack a capillitium. An example of the latter is *Didymium eremophilum* (Blackwell and Gilbertson 1980). Although the dark-spored clade has been studied to a much greater depth and seems to be more clearly delimited, there are also morphological characters used in classical identifications within this clade that are in need of molecular analyses to verify their significance. For example, the color of lime found in fruiting bodies has been used to distinguish between several species pairs in the genus *Physarum* (e.g., *P. globuliferum* and *P. bilgramii*) (Aldrich 1982; Stephenson 2003). Another character that should be investigated in the dark-spored clade is the form of the capillitium. Although the presence or absence of capillitial lime being used to separate the Didymeaceae from Physaraceae is supported by molecular data (Fiore-Donno et al. 2009; Nandipati et al. 2012), the use of capillitial structure (calcareous tubules versus nodules) being used as a character to separate the genera *Badhamia* and *Physarum* has not been supported by molecular phylogenetic data. However, the form of the capillitium or the overlap of other characters used to distinguish between these two genera may not be surprising, given those same molecular data indicate that the genus *Physarum* is polyphyletic, containing at least three clades interspersed with members of the genus *Badhamia* (Nandipati et al. 2012).

The first use of SSU phylogeny to reclassify a "species" of myxomycete was in 2010, when the genus *Hyperamoeba* (mentioned earlier) was shown to be invalid and the species formerly assigned to the genus were transferred to various genera in the dark-spored clade of

myxomycetes (Fiore-Donno et al. 2010b). Species of *Hyperamoeba* traditionally had not been recognized as belonging to the Macromycetozoa because they never formed fruiting bodies and occupied habitats extraordinarily different from those of most Macromycetozoa. For example, "hyperamoebae" have been found in human feces (Zaman et al. 1999), the coelomic cavity of sea urchins as endosymbionts (Dykova et al. 2007), freshwater sediments (Walker et al. 2003), physiotherapy water baths and water treatment plants (Walochnik et al. 2004). However, the "hyperamoeba" SSU sequences clearly cluster within the dark-spored clade of myxomycetes (e.g., Fiore-Donno et al. 2010b; Walochnik et al. 2004). Subsequently, a more rigorous morphological examination uncovered the overlooked second flagellum, a character that strongly places these taxa among the myxomycetes (Dykova et al. 2007; Fiore-Donno et al. 2010b; Walochnik et al. 2004).

Yet another example of the power of an integrative approach to taxonomy to modify the placement of particular taxa is the myxomycete *Kelleromyxa fimicola*. This taxon was described initially in 1929 as *Licea fimicola* due to its apparent resemblance to members of the genus *Licea* in the bright-spored clade (Dearness and Bisby 1929). However, upon re-evaluation of the type specimen and other collections consisting of numerous fully matured fruiting bodies, Eliasson et al. (1991) concluded that its assignment to the genus *Licea* could not be supported and that, in fact, this taxon shares more characters (e.g., dark spores, phaneroplasmodium, and the presence of a capillitium) in common with members of the Physarales in the dark-spored clade. Noting its similarities to members of the order Physarales, a new genus was erected within the order for its temporary placement, and the taxon was renamed *Kelleromyxa fimicola* (Eliasson et al. 1991). This was finally confirmed by Erastova et al. (2013) using an SSU phylogeny that clearly placed

*Kelleromyxa fimicola* within the dark-spored myxomycete clade, confirming the significance of the Physarales-like morphological characteristics noted by Eliasson et al. (1991).

Morphology is not always able to distinguish molecular differences however. Therefore, in situations where a single gene molecular phylogeny delimits two groups but there is no corroborating evidence (e.g., additional gene markers, ecology or morphology) to support the existence of two groups, the researcher must consider whether or not there is an urgency to separate them. Over-naming taxa is a longstanding problem that can lead to undue confusion and misinformation, with the potential to slow the scientific process (Dayrat 2005). To avoid this problem, until more data are available to support further delimitation, the two isolates potentially could be accepted as two haplotypes that represent variation within the morphospecies.

Numerous examples exist in the literature of cases in which molecular data from single gene analyses suggests delimitation but the authors cannot justify the split without further investigation. For example, during their revision of the *Tubifera ferruginosa* complex Leontyev et al. (2015) identified four out of their 10 18S sequences of *Lycogala epidendrum* that appeared to represent four new species. Inspection of the fruiting bodies, however, showed no morphological variation, so rather than describe these specimens as new taxa based on partial SSU sequences, the authors instead identified the need to investigate more material and in greater detail. Similarly, Aguilar et al. (2013), who studied 125 specimens of *Badhamia melanospora* from 91 localities, identified two groups strongly distinguished by SSU sequence divergence, morphology and biogeography. However, none of the characters (morphology or geography) could a priori distinguish between the two groups, so the authors instead concluded at that time that they were likely to be cryptic species.

### *The Ecological Species Concept*

The ecological species concept is grounded in Gause's theory of competitive exclusion (1934), in which organisms must differentiate in order to avoid having exactly the same role in the environment and thus occupy the same ecological niche. This differentiation of groups to exploit different resources requires each group to locally adapt and eventually leads to an irreversible separation into ecologically distinct groups or 'ecotypes'. Ecotypes are thus considered to equate to species in the traditional sense and are defined as populations of closely related organisms which share adaptations to the local niche and whose genetic divergence is capped by one or more mechanisms (e.g., reproductive isolation) (Cohan 2002).

Due to the nature of how they are formed, ecotypes should directly represent biologically significant taxa because each one is carrying out a unique ecological role, thereby making the ecological species concept an intuitive and natural way of delimiting taxa (Ward 1998). The ecological species concept assumes that the characters which allow the occupation of a particular niche will be reflected in physiology, morphology or sequence divergence (Fenchel 2005; Kopac et al. 2014; Palys et al. 1997). However, just as with the concepts previously discussed, the thresholds used to delimit clades in this way (e.g., physiology, morphology or sequence divergence) do not always reflect distinct ecotypes (Finlay 2004; Finlay et al. 2006; Cavalier-Smith 2010; Knowles and Carstens 2007).

For the myxomycetes and many other groups of protists, an ecological species concept is not yet feasible, simply because their ecology remains poorly understood and cannot be linked reliably to physiology, morphology or genetics. Until we know more, ecological information is valuable only as a complement to other forms of data. A few hypotheses have been proposed which would link myxomycete ecology to some aspects of their morphology although not at the

species level. For example, aphanoplasmodia, which are unique to the Stemonitales, lack a protective slime sheath such as that found in phaneroplasmodia (the plasmodial type typical of many other myxomycetes). This has led to the suggestion that members of the Stemonitales or any myxomycetes with aphanoplasmodia are unlikely to be found in particularly dry environments. In fact, most taxa with aphanoplasmodia are thought to spend the majority of their plasmodial existence within substrates such as woody tissues, where they are protected from desiccation (Clissmann et al. 2015; Stephenson and Stempen 1994). In line with this hypothesis, members of the Stemonitales are almost entirely lignicolous (associated with wood). However, there are some notable exceptions such as *Lamproderma scintillans* and *Stemonitis herbatica* which are almost invariably associated with non-woody substrates (Martin and Alexopoulos 1969). Another hypothesis that could relate ecology to morphology has been proposed for some coprophilous (inhabiting dung) myxomycetes. Although the occurrence of myxomycetes on dung is not uncommon (more than 100 species have been reported to occur on dung), there are only 16 species that have either been reported to occur only on dung or that have been rarely reported on other substrates and therefore may be truly coprophilous (Eliasson 2013). Three of these possibly obligate coprophilous species (*Licea alexopouli* [Blackwell 1974], *Kelleromyxa fimicola* [Eliasson et al. 1991] and *Trichia brunnea* [Eliasson and Keller 1999]) display unique thick-walled spores that could represent an adaptation to passing through the digestive tract of herbivores. However, once again more research is needed to test this hypothesis.

Given the difficulties involved in working with protists such as myxomycetes, obtaining a more accurate picture of habitat preference or niche specialization will require the enhancement of traditional culture methods and the increased utilization of rapidly developing molecular tools such as metagenetics, metagenomics and single-cell genome sequencing. These

and other emerging molecular methods should potentially allow greater depth and breadth in detection of myxomycete diversity in a given sample, thereby avoiding, at least in part, the inherent biases associated with finding, identifying and culturing myxomycetes. The use of these techniques in conjunction with detailed environmental studies and information on temporal, geographic and chemical gradients will allow a far greater understanding of the ecological roles of myxomycetes (and other protists) than previously possible, and this is likely to affect our understanding of their taxonomy.

### **Moving Towards a Solution**

The problems associated with species delimitation are, of course, not limited to the protists or the myxomycetes but are widespread across the tree of life. The complications of rampant lateral gene transfer obscuring phylogenetic signals appear to be restricted largely to prokaryotes (Keeling and Palmer 2008) and thus, are not considered in the present discussion. However, it will be exceedingly useful to integrate an understanding of other groups of eukaryotes in which similar challenges exist, such as guidelines proposed by protistologists and those studying some other amoebozoans (e.g., Adl et al. 2007; Finlay 2004; Smirnov et al. 2011).

To improve our understanding of the evolution and biology of the myxomycetes and other eukaryotic microbes, as well as identify a suitable way to delineate taxa, it is important that molecular data be considered in conjunction with traditional taxonomic methods especially since the latter represents the foundation for the classification used in all monographs of the group published to date. The combined use of multiple types of data (e.g., morphological, molecular, and ecological) provides a natural approach to species delimitation which has been referred to as an integrative taxonomic approach (Dayrat 2005; Will et al. 2005). Based on the limited number



of studies in which an effort has been made to apply an integrative approach to myxomycete taxonomy (e.g., Feng and Schnittler 2015), developing an understanding of the entire subject of myxomycete taxonomy at the level of species and below (e.g., cryptic species and haplotypes) will require an enormous amount of research on the part of the individuals who work with these organisms.

Due to their morphological plasticity, genetic variability and the presence of several alternative reproductive strategies (sometimes within a single lineage), all of which complicate traditional species concepts, an integrated approach to myxomycete taxonomy should be particularly beneficial. This has led to the recommendation (e.g., Keller and Everhart 2008; Schnittler and Mitchell 2000) that any description of a myxomycete species should, when feasible, include (1) investigation of multiple specimens from multiple localities and associated microhabitats, (2) detailed description of the collection locality, microhabitat or substrate, and associated abiotic conditions at the time of the collection, (3) detailed information on the comparative morphology of fruiting bodies, including microscopic images, (4) spore-to-spore culture to verify the consistency of morphological and physiological traits, and (5) molecular phylogenetic trees based on multiple independent markers. A few recent and noteworthy examples of an integrative approach to myxomycete species delimitation include the description of *Physarum pseudonotabile* by Novozhilov et al. (2013a) and the revision of the *Tubifera ferruginosa* complex by Leontyev et al. (2015). Of course, some of the recommended components of this “ideal” species description are not possible in every instance. For example, the revision of the *Tubifera ferruginosa* complex by Leontyev et al. (2015) did not involve spore-to-spore culture since *T. ferruginosa* has not yet been successfully cultured. Detailed descriptions of the collection locality, microhabitat and associated abiotic conditions at the time of collection

may not always be available for every specimen. In other cases, fruiting body material may be limited, forcing the researcher to choose between the destruction of fruiting body material for DNA extraction or its preservation for microscopic and culture methods. In still other cases, obtaining high quality sequence data from specimens may not be possible, regardless of the amount of time and effort involved.

## **Conclusions**

The myxomycetes challenge many of the currently recognized taxonomic systems, largely due to their morphological plasticity, genetic variability and the presence of several alternative reproductive strategies (sometimes within a single lineage). At present, not everyone who works with this group shares the same concepts for species recognition, even for some of the more common morphospecies that have long been recognized. Furthermore, myxomycetes face a problem that is common in protistology, that of an old and extensive set of literature (more than 3000 papers before 2001) focused solely on reporting species inventories from particular localities (Schnittler and Mitchell 2000). With such a wealth of available data, it is crucial that the research community reach some kind of agreement on an approach to species delimitation and taxonomy that can be used to simultaneously exploit the information contained in those data as well as build upon them without great complication.

Undoubtedly, the concept of a species for the myxomycetes as well as other groups of protists will remain the subject of debate well into the future. The review presented herein is certainly not intended to be the final word on the subject, and more detailed discussions concerning the species debate at broad are readily available in the published literature (e.g., Mishler 2010; Pigliucci 2003; Podani 2010). Instead, our objective was to examine the biological

side of the debate as it relates to the myxomycetes as there are clearly different issues with respect to other groups of organisms.

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## Glossary

**Amoeboflagellate** - A uninucleate cell that represents one of the two trophic stages in the life cycle of a myxomycete. The term is used to describe both amoeboid and flagellate forms; in myxomycetes, the two forms are reversible, although division occurs only in the amoeboid form, meaning the flagellum must first be resorbed before undergoing division.

**Aphanoplasmodium** - A type of plasmodium that is flat, transparent, lacks a protective slime sheath and is difficult to observe in nature; characteristic of the Stemonitales.

**Apogamic** - A form of non-heterothallic reproduction that does not involve the fusion of amoebflagellates (gametes) and lacks a haploid-diploid cycle.

**Automictic** - A form of heterothallic reproduction in which the fusion of haploid amoebflagellates (gametes) results in the production of a diploid plasmodium.

**Capillitium** - A system of sterile elements (takes numerous forms) found within the spore mass of many myxomycetes (Fig. 2D).

**Columella** - A structure that extends into the spore mass from below; in stalked fruiting bodies the columella appears to represent an extension of the stalk (Fig. 2E).

**Fruiting body** - A general term for the spore-producing structure or reproductive stage in the myxomycete life cycle; the structure within which (or in one case, on the surface of which) the spores are produced; also referred to as a fructification or sporocarp.

**Heterothallic** - Possessing mating types and having a haploid-diploid sexual reproductive cycle.

**Homothallic** - A form of non-heterothallic reproduction in which the fusion of haploid amoebflagellates (gametes) results in the production of a diploid plasmodium.

**Hypothallus** - Thin layer deposited by the plasmodium onto the substrate at the time of fruiting. The hypothallus when present has many forms and may connect multiple fruiting bodies. (Fig. 2A)

**Lignicolous** - Living on or within wood.

**Macromycetozoa** - The monophyletic group composed of the Dictyostelia, Myxogastria and *Ceratiomyxa*

**Microcyst** - A dormant, resistant structure formed by an amoebflagellate cell under adverse conditions.

**Myxogastria** - The plasmodial slime molds which produce macroscopic fruiting bodies (although excluding the other slime molds, Dictyostelia, protostelids and *Ceratiomyxa*).

**Non-heterothallic** - Not possessing mating types; non-heterothallic myxomycetes may be either homothallic (sexual, ploidal cycle) or apogamic (non-sexual, no ploidal cycle).

**Peridium** - The covering over the spore mass of a fruiting body (Fig. 2C).

**Plasmodium** - An acellular, multinucleate mass of protoplasm that represents one of the two trophic stages in the life cycle of a myxomycete.

**Phaneroplasmodium** - A type of plasmodium that may be relatively large and conspicuous enough to be observed in nature. Typically composed of a network of vein-like strands containing the streaming protoplasm within, protected by a slime sheath.

**Sclerotium** - A dormant, resistant structure formed by a plasmodium under adverse conditions.



**Stipe (or stalk)** - A structure supporting the spore mass; often elevated some distance above the substrate (Fig. 2F).

## Tables and Figures

**Table 1.** Mating systems in myxomycetes. The table does not include the complications of polyploidy or mutations.

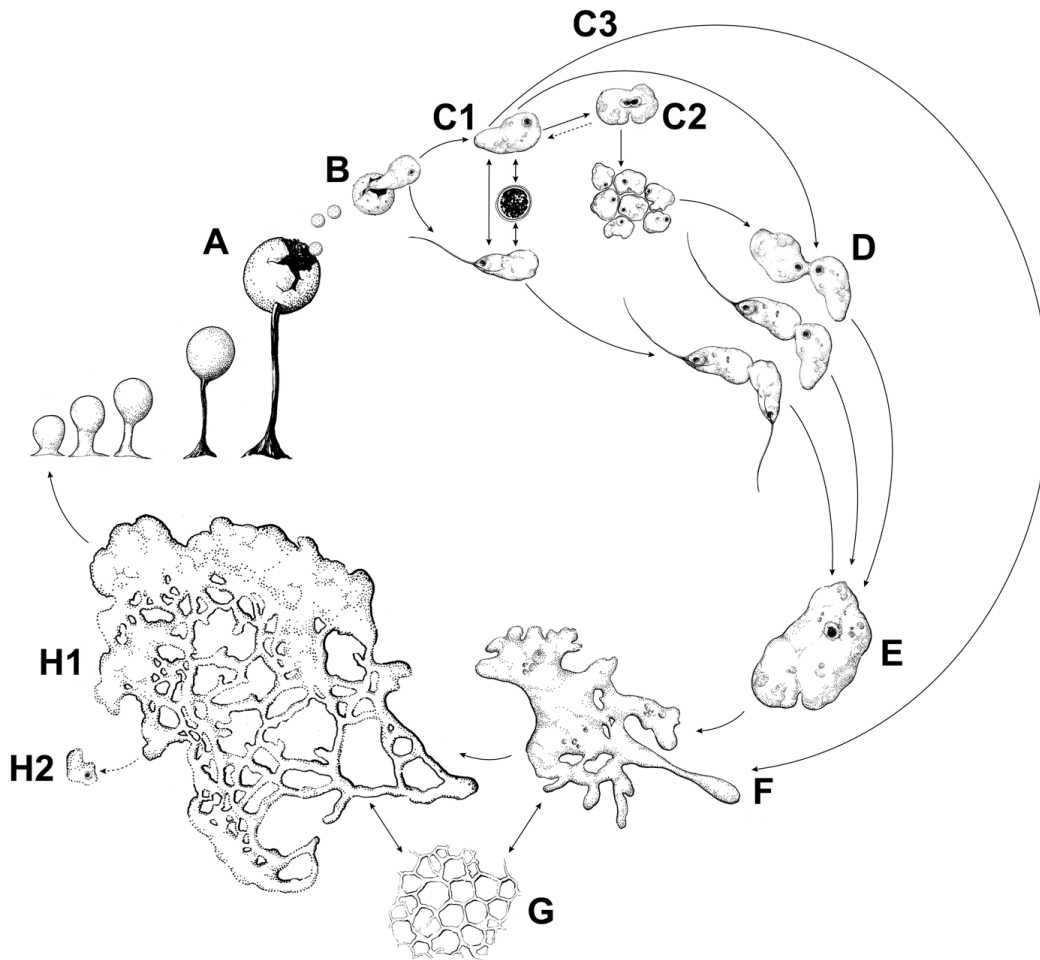
	Heterothallic			Non-heterothallic	
<b>Mating System</b>	<b>Sexual</b>	<b>Selfing<sup>c</sup></b>	<b>Automictic</b>	<b>Apogamic<sup>a</sup></b>	<b>Homothallic<sup>b</sup></b>
<b>Mating types</b>	Yes	Yes	Yes	No	No
<b>Syngamy required to produce plasmodia</b>	Yes	No	Yes	No	Yes
<b>Meiosis</b>	Yes	No	Incomplete	No	Yes
<b>Ploidy:</b>					
Spores and amoebflagellates	1n	1n	1n	1n or 2n	1n
Zygote	2n	-	2n	-	2n
Plasmodia	2n	1n	2n	1n or 2n	2n
Resultant spores and amoebflagellates	1n	1n	2n	1n or 2n	1n
<b>Mating system of resultant amoebflagellates</b>	<b>Sexual</b>	<b>Sexual, facultatively apogamic<sup>d</sup></b>	<b>Apogamic until reversion to sexual</b>	<b>Apogamic</b>	<b>Homothallic</b>

<sup>a</sup> Apogamic clonal lines can be produced by several different mechanisms, from heterothallic and non-heterothallic lines

<sup>b</sup> True homothallism has not been confirmed in myxomycetes

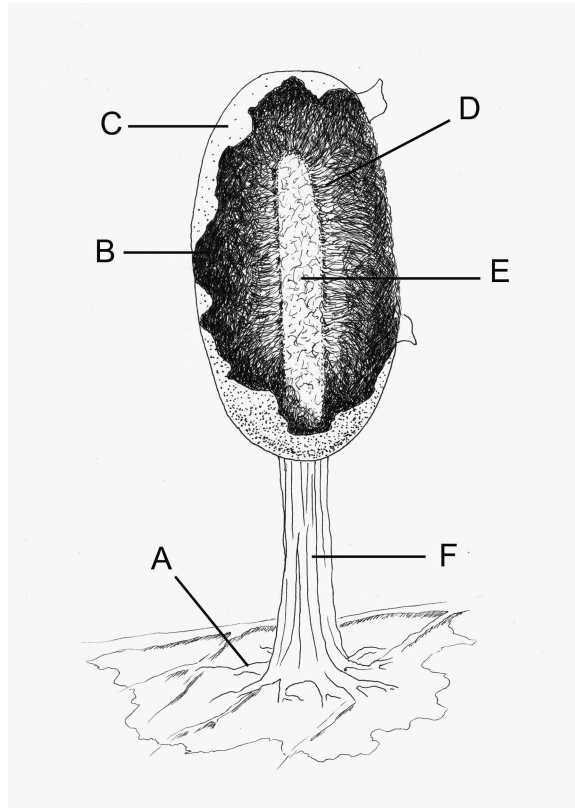
<sup>c</sup> Selfing occurs secondary to a normal sexual cycle; sexuality is not lost in the isolate

<sup>d</sup> Amoebflagellates retain their sexuality but lack mating types; they can produce plasmodia with or without crossing

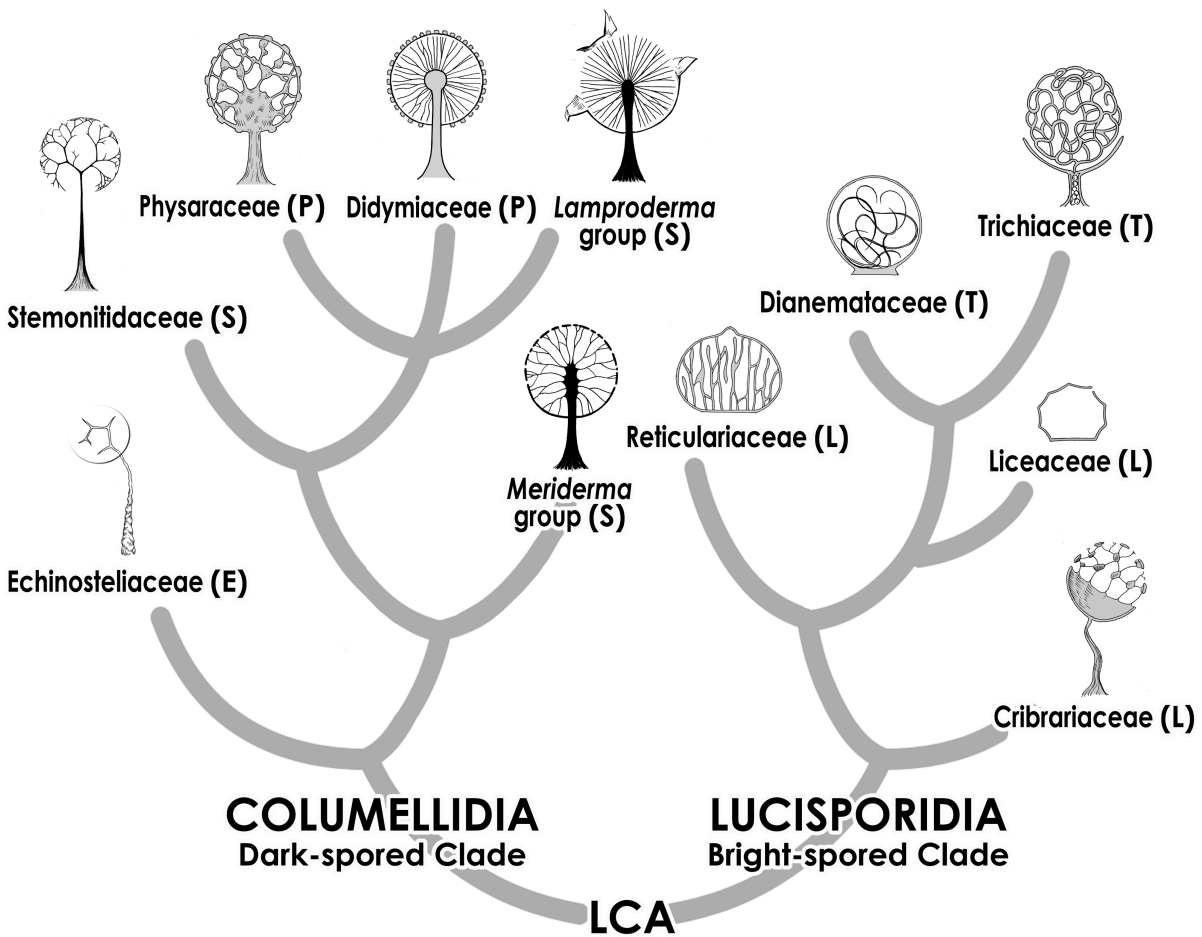


**Figure 1.** Life cycle (not drawn to scale) of a "typical and simplified" heterothallic myxomycete, with a few possible alternate cycles also indicated. Ploidy level is indicated only for the "typical" heterothallic life cycle and is placed in parentheses throughout this figure caption (for a list of alternative mating cycles and associated ploidy levels see Table 1). (A) Spores dispersing from a mature fruiting body. (B) Uninucleate amoebflagellate (haploid) emerging from a spore. (C1) Depending upon the environmental conditions, amoebflagellates may or may not have flagella; under adverse conditions, amoebflagellates may convert into a resistant structure called a microcyst. (C2) The non-flagellated form is able to undergo cellular division. The dotted line from the dividing cell (C2) back to the general amoebflagellate stage (C1) represents a hypothetical alternative life cycle in which a lineage no longer forms plasmodia or fruiting bodies. (C3) Representation of an apogamic life cycle in which the amoebflagellate is able to directly form a plasmodium without the need for fusion with other amoebflagellates. (D) Fusion of two compatible amoebflagellates (haploid) to form an amoeboid zygote (diploid). (E) The zygote (diploid) will feed and grow by undergoing multiple rounds of mitosis without

**Figure 1. (Cont.)** cytokinesis, first to create a single large multinucleate cell (F) and then a plasmodium (diploid). (H1) Plasmodium displaying a vein-like network in which the cellular contents are shuttled throughout. Given appropriate stimuli, the plasmodium gives rise to mature fruiting bodies (A). During the formation of fruiting bodies, meiosis occurs to produce four spores (haploid). (H2) Another hypothetical alternate life cycle in which amoebflagellates are produced by the plasmodium and could potentially act as gametes. (G) Under adverse conditions, a plasmodium may convert into a hardened, resistant structure called a sclerotium. (Drawing courtesy of Angela Mele.)



**Figure 2.** Structural components of a typical fruiting body. (A) Hypothallus (B) Spore mass and capillitium (C) Peridium (D) Capillitium (E) Columella (F) Stalk (or stipe) (Drawing adapted from Stephenson [2003]).



**Figure 3.** Illustrated representation of myxomycete SSU phylogeny. Tree topology was derived from SSU phylogenies in the literature (e.g., Cavalier-Smith et al. 2013; Clissmann et al. 2015; Fiore-Donno et al. 2010a; Fiore-Donno et al. 2012; Fiore-Donno et al. 2013; Kretzschmar et al. 2015; Leontyev et al. 2014a; Leontyev et al. 2015; Walker et al. 2015). The tree is rooted by the last common ancestor (LCA). Because the SSU phylogeny does not completely correspond to traditionally recognized taxonomy for the group based largely on morphological data, each taxon named on the tree is followed by a letter in parentheses that corresponds to the order from which the taxon is currently ascribed [(E) Echinosteliales, (S) Stemonitales, (P) Physarales, (L) Liceales, (T) Trichiales]. (Drawing courtesy of Dmitry Leontyev.)

### **III. The response of myxomycete communities to 14 years of N, P and K addition in a lowland tropical rain forest**

#### **Abstract**

Myxomycetes (plasmodial slime molds) are abundant protozoan predators that feed on bacteria and other microorganisms. In doing so, these organisms help mediate the flow of nutrients, playing a critically important role in the functioning of global ecosystems. Nutrient availability is a primary constraint on the productivity and distribution of organisms in tropical forests. As global temperatures rise and atmospheric CO<sub>2</sub> increases, nutrient availability will become increasingly important in lowland tropical forests. The extent to which nutrient limitation affects the myxomycete community is unknown. To increase our understanding of myxomycete ecology and possible nutrient limitations, this project took advantage of a long-term nutrient fertilization experiment in the lowland forests of Panama in order to investigate the impacts of increased levels of nitrogen (N), phosphorus (P) and potassium (K) on the litter-inhabiting myxomycete community. Two hypotheses were tested herein. The first hypothesis, that myxomycete abundance and diversity would increase with an increase in available P and NPK, was only marginally supported. The second hypothesis that myxomycete abundance and diversity would decrease in response to N fertilization was not supported at all. A large number of previous studies have identified multiple nutrient limitations to various members of this forest community; therefore, the lack of response for the myxomycete community was unexpected. Further work is needed to resolve whether the myxomycete community is indeed unaffected by nutrient fertilization at this site or if the experimental methods employed herein were unable to strongly and consistently support the hypotheses due to the unique biology and ecology of myxomycetes.

## **Introduction**

Tropical forests are major contributors to the global cycling of nutrients, accounting for approximately one-third of global terrestrial net primary production (NPP), and they contain up to 55% of global carbon stocks (Cleveland et al. 2011; Pan et al. 2011). The cycling and storage of nutrients in tropical forests is largely dependent upon the decomposition of plant materials, and the layer of litter on the forest floor represents what is presumably the largest and most labile source of nutrients. Hence, the microbial community that is responsible for decomposition of the plant litter on the forest floor plays a significant role in global nutrient cycling. Recent evidence suggests that changes in the microbial decomposer community can have significant effects on the rate of decomposition, altering available nutrient ratios and thereby immediately impacting carbon cycling (Cornelissen et al. 2007). Given the importance of tropical forests and nutrient cycling, it is urgent that we gain an understanding of the factors underlying rates of primary production and decomposition in these ecosystems. Our understanding of the role microbial communities in these processes is particularly limited. Given that the microbial community is largely responsible for regulating the flow of nutrients through forest ecosystems via decomposition of organic matter, this is an especially urgent priority.

The macronutrients nitrogen (N), phosphorus (P) and potassium (K), are a necessity for growth and metabolism; therefore, all forest ecosystems are largely dependent upon their availability (Sterner and Elser 2002; Hartman and Richardson 2013). The availability of these and other nutrients in soil is largely a product of their modes of formation. P and K are primarily derived from underlying bedrock over the course of soil development and are lost as soils age, largely due to erosion and leaching (Walker and Syers 1976). In contrast, N is largely absent from bedrock and is instead generated via biological fixation and is accumulated from the



atmosphere over time (Vitousek et al. 1986; Hedin et al. 2009). Therefore, the old and highly weathered soils in tropical forests are generally considered to be rich in N but poor in P and other rock-derived nutrients such as calcium (Ca) and K (Vitousek 1984; Vitousek and Sanford 1986; Townsend et al. 2007; Vitousek et al. 2010). However, the soil nutrient pool is not always a direct reflection of nutrient availability to plants, as evidenced by studies indicating that despite large pools of soil N, tropical forest productivity can still be limited by N (LeBauer and Treseder 2008). Therefore, it is possible that N rich but P and K poor soils in tropical forests are actually limited by N in addition to P and K. Nutrient limitation can occur independently, although multiple nutrient limitation is probably most often the case (Vitousek et al. 2010 and references therein). Not surprisingly, trees have species-specific nutrient requirements and respond differently to nutrient limitation (Mayor et al. 2014). Moreover, the alleviation of one nutrient limitation is likely to lead quickly to another (Davidson and Howarth 2007; Vitousek et al. 2010).

Scientists have long pondered how tropical ecosystems are able to maintain such high rates of NPP when growing upon such nutrient limited soils. Furthermore, in light of currently increasing levels of atmospheric CO<sub>2</sub>, rising temperatures and nutrient deposition, primary production in lowland tropical forests will be increasingly limited by the availability of nutrients (Vitousek 1984, Cleveland et al. 2011; Wright 2013). And at a time when much research effort is being invested into predicting the effects of anthropogenic change to the global ecosystem, it is crucial that we fill in the gaps in our knowledge regarding nutrient cycling in tropical forests.

Attempts at understanding nutrient cycling have led to numerous nutrient fertilization experiments which generally identify process-limiting nutrients as those that increase the rate of some biological process such as growth or decomposition after fertilization (Chapin et al. 1986;

Vitousek et al. 2010). From meta-analyses of these nutrient fertilization studies, some generalities concerning nutrient limitation have emerged, including (1) the widespread limitation of K to tree growth (Tripler et al. 2006), (2) that regardless of latitude, N limits NPP aboveground (LeBauer and Treseder 2008) and, (3) a co-limitation of N and P often limits plant biomass or productivity (Elser et al. 2007). Unfortunately, however, of the many nutrient fertilization studies, very few of these studies have focused on lowland tropical forests (Cleveland et al. 2011; Wright et al. 2011).

To help fill this void, in 1998 S. Joseph Wright and colleagues, in collaboration with the Smithsonian Tropical Research Institute (STRI), established a large-scale NPK factorial fertilization experiment in an old-growth, lowland tropical forest in Panama. Since its establishment the experiment has been continually fertilized and monitored and was still underway when this dissertation was being prepared. Established primarily to investigate how plants cope with nutrient limitation in nutrient-poor tropical soils, these plots represent an extremely valuable opportunity to evaluate numerous other components of a tropical forest ecosystem as well, such as, the litter-inhabiting microbial community. Indeed, various nutrient limitations and co-limitations have been demonstrated to the plant and, to a lesser extent, the microbial communities at this study site (e.g., Wright et al. 2011; Sayer et al. 2012; Turner and Wright 2013).

The bulk of research that has focused on the microbial decomposer community has largely focused on the fungal communities as well as bacteria. While fungi do represent the largest pool of decomposer microbes, there is often a tendency of the research community to consider all 'microbes' as a single unit, disregarding the importance of other members of this trophic system with which they are less familiar. Protozoan predators such as myxomycetes are

one such group that is often overlooked in the context of the decomposer food web and the "microbial loop" (Clarholm 1985).

The importance of diversity in the microbial community as a whole, however, is becoming increasingly apparent. For instance, research has shown that closely related strains of some protist predators have significantly different effects on bacterial community structure (e.g., Ronn et al. 2002; Glücksman et al. 2010). It has also been recognized that increased diversity in the soil microbial community increases rates of decomposition and other essential processes (e.g., Stout 1980; Gessner et al. 2010; Wilkinson et al. 2012). Given the importance of amoeboid grazers of bacteria such as myxomycetes, to plant performance (Bjørnlund et al. 2012) and organic matter decomposition (Wang et al. 2009) in the nutrient-limited tropics, there is a critical need to develop an understanding of the myxomycete community and its possible nutrient limitations.

The myxomycetes (plasmodial slime molds) form a monophyletic group within the amoebozoan supergroup (Adl et al. 2012). Together with the Dictyostelia (dictyostelids) and members of the genus *Ceratiomyxa*, they form a larger clade called Macromycetozoa (Fiore-Donno et al. 2010). Macromycetozoans (the most speciose of which are the myxomycetes) are often the dominant soil protozoans (Adl and Gupta 2006; Urich et al. 2008), with myxomycetes alone exceeding 20,000 cells per cm<sup>3</sup> in some temperate soils (Feest and Campbell 1986; Feest 1987). In addition to their abundance in the soil, they are also abundant and more commonly studied in the overlying litter layer, decaying wood, bark of living trees and numerous other microhabitats associated with plant materials (Stephenson 2011). As a whole, myxomycetes are cosmopolitan organisms and have been found in virtually every major terrestrial habitat

examined to date. However, finer-scale understanding of myxomycete community structure and ecology is greatly needed.

Through the consumption of bacteria, fungi and other microbes, myxomycetes and other protozoan and nematode predators (Rønn et al. 2012), are responsible for mediating the flow of nutrients from decomposers to the soil to plants and higher trophic levels. Nutrients immobilized in microbial biomass are released through predation and made available for plant and higher trophic level uptake (Bonkowski and Clarholm 2012). The positive effects of protozoan grazers are well established and include increases in plant growth, organic matter decomposition and soil fertility (Ekelund and Rønn 1994; Adl and Gupta 2006; Anderson 2012). Protozoans such as myxomycetes are therefore a critical component of terrestrial ecosystems (Adl and Gupta 2006) and, as such, they deserve greater attention.

### **Research Objectives and Hypotheses**

To understand the role of myxomycetes (or any group of organisms) in any ecosystem process, it is fundamental first to identify the players involved and then to characterize their diversity (Anderson 2012; Crotty et al. 2012). This was the main goal of the study described herein, to generate a body of data on the myxomycete community associated with forest litter in a lowland tropical rain forest in Panama and to investigate how it is affected by changes in nutrient status. Utilizing a long-term nutrient fertilization study allowed the investigation of possible limitation and co-limitation of three major macronutrients N, P and K and how these nutrients affect the litter-inhabiting myxomycete community.

Two main hypotheses were investigated. The first hypothesis was that myxomycete abundance and diversity will be greater on the P plots as compared to other treatment and control

plots. Tropical soils are commonly low in available P (Vitousek 1984) and indeed a limited supply of available P ( $<1 \text{ mg P kg}^{-1}$ ) has been shown to exist for control plots at this site (Turner et al. 2012). Furthermore, fast growing microbes are commonly considered to be limited by the supply of P (Elser et al. 1996). In line with this hypothesis, Turner and Wright (2013) reported a significant increase in total microbial carbon (13%), N (21%) and P (49%) and a concurrent decrease in phosphatase activity (by 65%) with the addition of P but not N or K, dramatically demonstrating P as a limiting factor to the soil microbial community at this site. Additionally, aboveground, P and NPK fertilization increased decomposition of organic matter (Kaspari et al. 2008; Sayer et al. 2012). Where decomposition is increased, myxomycete abundance and diversity is also expected to increase due to the presumed increase in the number and diversity of microbial decomposer prey (Feest and Madelin 1988; Stephenson and Landolt 1996). In fact, Kaspari et al. (2008) suggested that the increased decomposition identified on these plots is directly enhanced by the fertilization of the microbial community, and these authors have reported that bacterial community composition does shift in response to the various treatments (Kaspari et al. 2010). Also, in temperate forests, which are not typically limited by available P, soil myxomycete abundance consistently and positively correlates with increased concentrations of soil P (Feest and Campbell 1986; Feest 1987; Feest and Madelin 1988).

The second hypothesis was that myxomycete abundance and diversity would be significantly lower on the N plots as compared to the other plots. Substrate pH is one of the main factors determining myxomycete abundance and distribution patterns in nature (Stephenson 1989), with most species demonstrating a relatively strict pH tolerance range between 5 and 8 (Martin and Alexopoulos 1969). The soil pH on the N plots is on average 0.5 units lower than on other plots (Sayer et al. 2012). This lower pH often falls well below the lower tolerance limit of

pH 5, thereby presumably limiting myxomycete occurrence. Also, the tropics are generally thought to be rich in N and no limitation to the aboveground plant community (Wright et al. 2011) or the soil microbial community (Turner and Wright 2013) has been identified at this site.

## **Materials and Methods**

### *Study Site*

Experimental plots are located on the Gigante Peninsula of Lake Gatun, which forms the Panama Canal Waterway within the Barro Colorado Nature Monument in the Republic of Panama. Situated approximately 1 km inland from Lake Gatun (9°6'30.7" N, 79°50'36.9" W), the elevation at the study site grades from 25 m in the southwest to 61 m in the northeast corner (Yavitt et al. 2009). The annual rainfall is approximately 2.5 m/yr, 90% of which falls during the rainy season from May through mid-December (Windsor 1990). Mean monthly temperature is 81°F in April and 79°F in the other 11 months of the year. Relative humidity is >75%. The plant species composition and structure (canopy heights > 40 m) are typical of seasonally evergreen old-growth (>200 years) lowland tropical forests. The dominant tree species are members of the Leguminosae and Bombacaceae; overall, approximately 18% of the tree species are deciduous (Condit et al. 1996).

### *Experimental Design*

The factorial NPK nutrient fertilization experiment includes eight treatments (2 x 2 x 2) replicated four times. Due to a gradient in soil properties and plant community composition (S. J. Wright, *unpublished data*) the four replicates were placed perpendicular to the afore-mentioned 36 m elevation gradient with which they are associated (Yavitt et al. 2009). The eight treatments

were blocked within each replicate to contrast the N, P, K and NPK against the NP, NK, PK and control treatments to create a balanced incomplete-block design in order to decrease the error associated with the spatial variation (Winer et al. 1971).

Experimental plots are situated on a north to south rectangular quadrat of approximately 38.4 ha, with each plot measuring 40 x 40 m with a distance between each plot of 30 to 40 m (Yavitt et al. 2011). Fertilization of the experimental plots has been conducted here since 1998 under the direction of S. Joseph Wright at STRI. Nutrients (dry, granular fertilizers) were added by hand four times per year, with N as coated urea ( $(\text{NH}_2)_2\text{CO}$ ), P as triple superphosphate ( $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ ), and K as potassium chloride (KCl), in annual concentrations of 125 kg N.ha<sup>-1</sup>.yr<sup>-1</sup>, 50 kg P. ha<sup>-1</sup>.yr<sup>-1</sup>, and 50 kg K. ha<sup>-1</sup>.yr<sup>-1</sup>, respectively (Wright et al. 2011). The N addition is equal to 69% of annual input from fine litter-fall at a nearby site and 470% of the P (Yavitt et al. 2004). Although soil nutrient content was not measured in the research described herein, other sources detail the significant increases in extractable soil nitrate and phosphate as a result of N and P additions and indicate that the nutrient treatments are indeed increasing the availability of the respective nutrients (e.g., Yavitt et al. 2009; Turner et al. 2013). The experiment also includes a micronutrient treatment that will not be addressed herein. Each of the four yearly nutrient additions (in equal amounts) were added in the wet season, with six to eight weeks between each application. The nutrients were applied by casting handfuls of the dry fertilizer while systematically walking through each plot. To ensure the most homogeneous application, a different systematic pattern was followed at each of the four applications for each plot in a given year. Most of the above detail concerning the experimental design is drawn from Yavitt et al. (2009, 2011).

### *Sampling*

Samples of both leaf litter (LL) and small pieces of woody debris (SWD) were collected in triplicate and from three sites within each of the 32 experimental plots. The three sites were the center of the plot, and two sites approximately 10 m to the northeast and southwest from the center point. At each of these sites, samples of leaves and small woody debris were haphazardly collected by hand and placed into separate paper bags where they were allowed to air-dry before being shipped to the University of Arkansas for use in preparing moist chamber culture.

Sampling was performed twice, in late summer and during the rainy season of both 2012 and 2013. The sampling procedure was identical between years, although not all of the treatments were sampled in both years. In 2012, due to the preliminary nature of the project, only five of the eight treatments were sampled (N, P, K, NPK, and an untreated control), whereas in 2013, all eight factorial NPK treatments (above treatments plus NK, NP and PK) were included. The M treatments were also sampled in 2013, but they will not be discussed herein.

### *Moist Chamber Culture*

From each of three sampling sites per plot, three moist chamber cultures were prepared for each type of substrate (LL or SWD), so that each plot was represented by 18 moist chamber cultures (9 per substrate) for a total of 360 in 2012 and 576 in 2013. Moist chamber cultures are a simple, reliable and cost effective method for isolating myxomycetes from leaf litter and similar substrates (Stephenson and Stempen 1994) and represent the standard protocol for ecological studies of litter-inhabiting myxomycetes (e.g., Stephenson 1989). In brief, each bag of dried substrate was emptied onto a clean piece of paper and placed by hand into a Petri dish previously fitted with a filter paper lining (to absorb and retain moisture). Each Petri dish was filled with



approximately equal amounts of tissues (judged visually). Although in the field an effort was made to haphazardly sample from the forest floor without inserting personal bias, at this step in the protocol, bias was intentionally applied in order to ensure thorough representation of the sample between the three replicate plates. To do so, while the entire sample was laid out upon the clean paper, the contents were visualized and an attempt was made to equally represent the different types of tissue and different leaves of substrate in each sample (no attempt was made to identify the plant materials beyond such things as the color, shape or size of the dry tissues). In the majority of cases, leaves and twigs or bark were broken apart by hand as required to get them to fit into the Petri dish. After filling all of the Petri dishes with dry substrate, deionized water was liberally applied and allowed to incubate at room temperature overnight. On day two, the pH of the water was recorded with a pH meter, and a surface electrode before draining all of the excess water. Moisture within the moist chambers was then maintained relatively constant via the filter paper; more deionized water was added throughout the incubation period as needed. Moist chambers were incubated under ambient light at room temperature (ca. 70 - 72 F) and monitored using a stereomicroscope at least once per week for the following three to six months.

#### *Identification of Myxomycetes*

Fruiting bodies collected from moist chamber were identified on the basis of morphology (e.g., Martin and Alexopoulos 1969); nomenclature follows Lado (2005 - 2015). These records were then used to calculate myxomycete abundance and diversity. All fruiting bodies collected were deposited in the herbarium (UARK) of the University of Arkansas (Fayetteville, AR 72701).

### *Data Collection*

For the first three months of moist chamber incubation, during weekly microscopic inspection, all fruiting bodies were collected and prepared as herbarium specimens, and the presence of plasmodia was noted (through visualization of a plasmodium, plasmodial tracks or an encysted form of plasmodium called a sclerotium). The plates were then monitored bi-weekly until fruiting body formation occurred. In plates in which fruiting bodies were never observed but plasmodia were present, the plate was recorded to be positive for myxomycetes but with an abundance of only one, and the taxonomy was recorded as unknown (plasmodia can not be used to identify myxomycete species and therefore cannot be identified nor quantified).

After the first three months in which all fruiting bodies were collected and retained for the herbarium, if the plates were still producing either fruiting bodies or if plasmodia were still present, the plates were monitored for up to another 3 months. During this extended period of monitoring, however, fruiting bodies were recorded and collected from a plate only if they represented a species not previously recorded for that plate.

Due to the complex nature of the myxomycete life cycle, if any particular species was found more than one time in one plate, it could not be ruled out that (i) the two separate fruitings were not initially derived from the same plasmodium that had fragmented during moist chamber incubation, thereby representing a single collection or individual (Eliasson 1981; Stephenson 1988), or (ii) that they were clones of apogamic lineages (which is common in myxomycetes) and therefore not representative of true abundance in the sample at the time of collection. In this situation, apogamic spores that were shed within the plate from a single fruiting body would be able to form new plasmodia and fruiting bodies without sexual fusion.

Due to the complications that myxomycete life history strategies impose on cultural studies, two separate data files were generated (for each year and each substrate). The first data set (DS1) contains all recorded fruitings or the presence of plasmodia if fruiting bodies were not formed. Therefore, DS1 includes the total number of fruitings observed during the entire period of incubation. This file contains all records and the total number of fruitings that occurred in a plate for each species (ignoring the potential bias of clonality and plasmodial separation). Data set two (DS2) was prepared for analyses in accordance with traditional methods; collections that were not assigned a positive morphological identification were removed unless they represented the only record of any myxomycete presence on the plate (allowing their inclusion to count toward abundance but not to alter the measurement of diversity), and all duplicate records for any one species per plate were also removed (to account for the biases of clonality and plasmodial separation). Unless noted otherwise, all analyses and results presented herein were generated using DS2.

### *Data Analysis*

Litter and small woody debris represent remarkably different microhabitats, and commonly support distinct microbial communities (Novozhilov et al. 2000; Liu et al. 2015). To confirm this pattern at the study site and to determine whether the two substrates should be analyzed together or separately in order to test our hypotheses, coefficient of community (CC) and percent similarity (PS) indices were calculated (Ellenberg and Mueller-Dombois 1974; Gauch 1982). The CC considers the presence or absence of each species calculated with Equation 1, where  $a$  and  $b$  are the number of species total between the two communities and  $c$  is the number of species in common between the two communities.

$$(1) CC = \frac{2c}{(a + b)}$$

The CC index ranges from 0 (no species are shared) to 1.0 (all species are shared). The PS considers the relative abundance of each species in addition to its presence or absence and is calculated with Equation 2, where *min* is the lesser of the two relative abundances of each species (*a*, *b*, . . . *x*) in the two communities.

$$(2) PS = \Sigma \min(a, b, \dots x)$$

The PS index ranges between 0 (no species shared) to 1.0 (all species shared and in equal relative abundances). EstimateS Software (Colwell 2006) was used to evaluate completeness of the survey by assessing the relationship between the numbers of species identified and sampling effort for each substrate in each year. The same software was also used to estimate total richness with the non-parametric Chao 2 estimator (Chao 1987) for each treatment. Species richness was used as a proxy for species diversity to analyze the effect of fertilization treatments. Three-way analysis of variance (ANOVA) was performed for each response variable and each substrate in the 2013 data set to evaluate the effect of fertilization to the myxomycete community (one-way ANOVA was used for 2012 data). A critical P value of 0.05 was employed for all statistical tests in this study. The blocks were included as a factor for the 2013 data but not for the 2012 data, due to the incomplete sampling in that year. Significant ANOVAs ( $p < 0.05$ ) were followed by multiple pairwise comparisons of means with a Tukey's test to control for Type I error otherwise associated with the use of multiple tests (Quinn and Keough 2002). Repeated-measures (RM) ANOVA were performed for each response variable to evaluate temporal variation between the two years (only the five treatments that were included in both years of sampling, were included in this analysis). The three-way ANOVAs for treatment effects were carried out with R Studio v.

0.99.447 (2015); all remaining analyses were carried out in JMP<sup>®</sup> Pro 12.1.0 (SAS Institute Inc, Cary, NC).

Nonmetric multidimensional scaling (NMDS) was used to visualize the Bray-Curtis dissimilarity matrix of community compositions among the fertilization plots and the significance of treatment effect on the ordination was tested with a PERMANOVA. Both the NMDS and PERMANOVA were conducted using the Vegan package (Dixon 2003; Oksanen et al. 2007) in R Studio v. 0.99.447 (2015).

## **Results**

### *Moist Chamber Cultures*

Over 90% of the 936 moist chamber cultures that were prepared over the two-year sampling period were positive for myxomycetes (the presence of plasmodia or fruiting bodies were recorded). Species accumulation curves (Fig. 2) indicate that sampling was nearly complete in both years for the small woody debris (SWD) but considerably less so for the leaf litter (LL) moist chamber cultures. A total of 3,432 records (occurrences) were made in DS1, but after removing all duplicates, DS2 contains just 1,939 records (Table 1); over 2,000 collections of fruiting bodies were deposited into the herbarium of the University of Arkansas.

A total of 91 morphospecies were identified throughout the study (Table 3), comprising a wide diversity of the entire group, including 29 genera, 11 families and all 5 orders traditionally recognized by Olive (1970). Species rank abundance curves (Fig. 2) show a trend that is characteristic of microbial communities (Caron and Countway 2009), wherein the large majority of records are rare or singletons. Thirty-four of the species recorded had not previously been reported to occur in Panama, bringing the list of species records for that country to 151

(according to lists published by Piepenbring [2006] and Myxotropic [www.myxotropic.org, accessed on 12-1-2015]) (Table 4). At least one of these species, *Perichaena longipes*, was completely new to science and has been formally described (Walker et al. 2015).

### *Substrate Comparison*

To determine whether or not the two substrates (SWD and LL) should be analyzed together or separately, the coefficient of community (CC) and percent similarity (PS) values of the two myxomycete communities were calculated using only the larger 2013 data set. The CC calculated herein was 0.642 on an index that ranges from 0 (no species are shared) to 1.0 (all species are shared). The PS, which also takes the relative abundance of each species into account, was 0.548 on an index that ranges from 0 (no species shared) to 1.0 (all species shared and in equal relative abundances). The cumulative richness was 112, but only 53 of those were shared in common between LL and SWD substrates; the other 59 species were unique to one substrate or the other.

Furthermore, the community compositions were quite different between the two substrates. Considering only the 10 most abundant species found on each substrate in 2013 (Table 2), which represent 63% and 53% of all records found on LL and SWD substrates, respectively, only five of these species were shared (*Arcyria cinerea*, *Diderma effusum*, *Lamproderma scintillans*, *Perichaena chrysosperma* and *Cribraria microcarpa*). When this subset of data, which accounts for the majority of all records but includes only the 10 most abundant species for each substrate, was used to calculate the PS, the PS value drops to just 0.446. Narrowing in on only the most abundant species in this manner highlighted some well-known patterns in niche specificity. For example, *Lamproderma scintillans*, which is commonly

associated with the LL substrate, was identified 71 times on LL samples herein, accounting for 16% of all records for that substrate. In the SWD samples, however, *L. scintillans* is not ranked among the 10 most abundant, as it was only recorded 10 times (from a total of 766 records) on that substrate during moist chamber culture, accounting for just 1.0% of all records for SWD samples. Similarly, the SWD moist chamber cultures were dominated by species commonly associated with woody tissues, such as *Stemonitis fusca*.

In 2012, the LL and SWD substrate pH differed significantly (albeit, barely) ( $p = 0.0447$ ), with a higher average pH of LL of 6.4 compared to an average of 6.2 for SWD. Because the sampling in 2013 included eight treatments versus only five in 2012, the difference in pH between substrates was tested for the full dataset with all eight treatments and also on a dataset that included only the five treatments (as in the 2012 dataset). In both cases, however, the substrate pH in 2013 did not significantly differ between LL and SWD.

### *Treatment Effects*

Surprisingly, the only significant effect of nutrient fertilization to the myxomycete community was identified in the SWD samples, and only during the preliminary study in 2012, wherein, myxomycete abundance (Fig. 4A) and richness (Fig. 4B) were significantly different among treatments in the SWD community ( $p = 0.0308$  and  $p = 0.0372$ , respectively). The ad hoc Tukey's test identified a marginally significant difference in myxomycete abundance, where the average increased from 17.5 occurrences on the N plots to 32.25 on the P plots ( $p = 0.0466$ ). Species richness was significantly different between the N and NPK plots ( $p = 0.0299$ ), where average richness was 8.25 species on the N plots and 17.25 on the NPK plots. No treatment

effect to myxomycete abundance or richness was identified in 2012 LL (Fig. 4) or in either substrate in 2013 (Fig. 5).

There was no statistically significant difference in substrate pH among the fertilization treatments (Fig. 6) for either year of this study, nor was there any significant correlation between substrate pH and species abundance or richness. The pH for both LL and SWD substrate samples did not differ by replicate in 2012 (Fig. 7A), however, it did differ significantly among the four replicates in 2013 ( $p < 0.001$ ) (Fig. 7B).

The NMDS plots (Fig. 8) of 2013 myxomycete community compositions did not indicate divergence in response to fertilization, and this was supported by a lack of significance of the PERMANOVA. To investigate the treatment effect at the species level, the ten most abundant species for each substrate were compiled to generate Figure 9. One observation that stands out in Figure 9B is the complete lack of both *Stemonitis fusca* and *Comatricha tenerrima* on the N plots, despite the fact that these two species represent the second and fifth most abundant species occurring on the SWD substrate at this site. Chi-square tests were then used to test for significantly different distributions of each of the top 10 species across all 8 treatments for each substrate. In the LL, *Perichaena longipes* (the species newly described during the course of this dissertation project) is the only species among the top 10 that is distributed significantly different ( $p = 0.02118$ ) among the treatment plots. In the SWD, only *Arcyria denudata* had a significantly different ( $p = 0.0039$ ) distribution among the fertilization treatments. Despite the absence of *S. fusca* and *C. tenerrima* on the N plots, they did not significantly differ among all eight treatments in SWD.



### *Temporal Effects*

Myxomycete species abundance (Fig. 10) and richness (Fig. 11) were compared for the five nutrient treatments (N, P, K, NPK and untreated control) that were sampled in both 2012 and 2013 with a RM ANOVA. The only significant temporal effect of nutrient fertilization was identified for myxomycete species richness in the LL microhabitat ( $p = 0.0288$ ). There was no temporal effect of fertilization to myxomycete abundance in the LL nor was a temporal effect identified for species richness or abundance in the SWD.

A marginally significant temporal effect of nutrient fertilization to substrate pH (Fig. 12) was identified in the SWD samples ( $p = 0.0434$ ), for which the overall average pH rose from 6.2 in 2012 to 6.6 in 2013. There was no temporal effect of nutrient fertilization to substrate pH in the LL samples, where the pH also rose, although to a lesser degree, from an average of 6.4 in 2012 to 6.6 in 2013.

### **Discussion**

Being directly responsible for the decomposition of the vast majority of the earth's organic matter, microbes are recognized as drivers of numerous terrestrial ecosystem processes (e.g., global C cycle and N mineralization) with the potential to moderate and/or accentuate global change (Treseder et al. 2011; Anderson 2012; Crotty et al. 2012). Protist grazers such as myxomycetes play a pivotal role in these processes by regulating the flow of nutrients to higher trophic levels (Bonkowski 2004; Adl and Gupta 2006). Quantifying the abundance and species composition of this community is the first step towards integrating the role of these organisms into our conceptual understanding of ecosystem processes (Crotty et al. 2012; Anderson 2012). Tropical forests contribute significantly to the global cycling of nutrients (Vitousek and Sanford

1986; Cleveland et al. 2011), making them especially important in this regard. Given this fact and in view of current climatic scenarios, understanding the role of protozoans in global cycling of nutrients is of utmost importance and necessary in order to accurately incorporate them into predictive models of global biogeochemical cycles (Caron et al. 2009; Treseder et al. 2012; Yang et al. 2013).

The goal of this project was to identify correlations between the myxomycete community and nutrient status, in order to allow the formulation of directly testable hypotheses and the predictability of relationships that exist between myxomycetes and nutrient status (Cleveland et al. 2011; Fierer et al. 2011). The effects of trophic cascades on resource consumption can be strong, and the species diversity within and among different trophic levels can be a powerful influence on both top-down and bottom-up effects (Srivastava et al. 2009). Furthermore, the interconnectedness of organisms and processes within and among each thread of the food web increases the difficulty of elucidating a specific role for any one group and is beyond the scope of this research. Instead, the data generated herein were intended to serve as a first step towards developing a more complete understanding of myxomycete ecology in the tropics, particularly in terms of their response to nutrient availability, one of the key constraints on the productivity and distribution of above and below ground organisms in tropical forests (Cleveland et al. 2011).

The nutrient fertilization experiment utilized in this study has been ongoing since 1998, and over the years has been the focus of a large number of research studies, most of which were focused on the above-ground plant community. From these studies, a great deal of information regarding this forest ecosystem and its response to nutrient addition has been obtained and it is with these data that the hypotheses relating to myxomycetes were formed. The plant communities at this site are limited by multiple nutrients. The P fertilization plots saw a

substantial increase in leaf litter fall (Wright et al. 2011; Turner et al. 2015) and an increase in decomposition with P alone, or P with N and K (NPK) was noted after ten years of nutrient addition (Kaspari et al. 2008; Sayer et al. 2012). Also, the addition of K alone or in combination with N (NK) significantly increased seedling growth rates (Wright et al. 2011). The addition of N alone, however, had no significant effects on the aboveground plant community, although it did significantly decrease the average soil pH (Sayer et al. 2012). Also belowground, the addition of K alone or with N (NK) reduced fine root biomass after ten years of fertilization (Wright et al. 2011).

Less work at this site has focused on the microbial community although according to Turner and Wright (2013), the soil microbial community is also strongly limited by P. After ten years of fertilization those authors identified significant increases in total microbial carbon (13%), N (21%) and P (49%), and a concurrent decrease in phosphatase activity (by 65%) with the addition of P but not N or K. Phosphatase increases the available pools of P when that nutrient is limiting (Marklein and Houlton 2012), therefore the strong decrease in phosphatase activity with P fertilization indicates an alleviation from P limitation. Together those results dramatically demonstrated P as a limiting factor to the soil microbial community at this site. This may not be surprising since the control soils at this site are reportedly low in available P (Turner et al. 2012).

Given all of the effects of nutrient addition that have been identified at this site to above- and belowground communities, the data generated herein indicating that myxomycetes are not greatly affected by nutrient fertilization was quite surprising. The hypotheses relating to myxomycetes were grounded upon the previously demonstrated nutrient limitations identified at this site as described above. For instance, the first hypothesis, that myxomycetes would increase

in abundance and diversity with P addition, is based on the assumption that the increased plant growth on these plots will have cascading effects throughout the ecosystem, such as through an increase in available C and N in the soil due to increased exudate production and higher tissue nutrient contents. Similarly, where decomposition is increased on the P and NPK plots, the hypothesis that myxomycete abundance and diversity will also increase is largely based upon the assumption that the increased decomposition means an increase the availability of microbial prey. This first hypothesis, however, was only marginally supported, and only in the 2012 SWD dataset.

The second hypothesis, that myxomycete abundance and diversity would decrease on the N plots due to a significantly lower soil pH on the N plots, that often falls below the myxomycete tolerance range of between 5 and 8 (Martin and Alexopoulos 1969), was not at all supported. Substrate pH is well recognized as a major factor that determines the distribution of myxomycetes (Stephenson 1989). Furthermore, N limitation has not been identified at this site for other plant (Wright et al. 2011) or microbial communities (Turner and Wright 2013). This hypothesis, however, could only be based upon the incorrect assumption that the decreased soil pH on the N plots would be mirrored in the overlying litter layer (i.e., the microhabitats being studied herein). The litter substrate pH recorded in the present project does not have a significantly different pH than the other treatments however. It does seem likely, however, that the soil and litter myxomycete communities are highly connected. If true, it would be expected that the low pH of the soil on N plots might in fact decrease the myxomycete abundance and diversity in the litter but that the actual treatment effect is occurring in the soil. Although there are no data to indicate that this is the case, and it could not be directly tested herein, the following anecdotal observations have lead to the author's assumptions. In tropical forests

frequent rains (especially in the wet tropics) continually return spores, microcysts or other above ground sources of myxomycetes, back into the soil. Also, work from temperate soils indicates that myxomycetes spend the majority of their lives in the soil as amoebae (Feest 1987; Stephenson and Feest 2012). Soil pore space does not allow enough room for fruiting body formation; therefore, myxomycetes in the soil would need to emerge to or above the soil surface in order to form fruiting bodies and complete their life cycle (for sexual strains). The litter microhabitat just above the soil surface would provide such a location, substrate and the space necessary to produce spore-bearing fruiting bodies. Although the connectedness of the soil and litter microhabitats has yet to be explicitly demonstrated, it is worth noting that despite their presence in soil as identified by amoebae or plasmodia on agar or in traditional moist chamber cultures (wherein soil is the substrate), it is notoriously difficult to obtain fruiting bodies. However, Rollins and Stephenson (2013) described a modified moist chamber method, in which sterile straw was placed over the soil to serve as a substrate (and perhaps also serving as a moisture gradient) on which to fruit. These authors were able to obtain fruiting bodies from 56% the modified soil moist chambers, a rate much higher than expected for traditional agar-based culture methods, illustrating the ability of myxomycetes to exit the soil and form fruiting bodies. The direct testing of this hypothesis would be a very valuable contribution to the current understanding of myxomycete ecology.

### *Species Composition*

The hypotheses mentioned above were tested by measuring total myxomycete community abundance and species richness. However, due to the distribution pattern of the myxomycete data, which is composed of just a few abundant species and many rare or single species, it is

possible that treatment effects may have been present but that they were being masked due to the distribution patterns of the species present. Several additional analyses using the larger and experimentally complete dataset from 2013 were performed in order to rule out the possibility of a treatment effect being concealed by the distribution pattern. First, NMDS (Fig. 9) and PERMANOVA were performed to investigate overall shifts in the total community composition in response to nutrient fertilization. However, these analyses did not identify any community composition divergence in response to fertilization. Next, to investigate treatment effects at the individual species level, Figure 10 was generated to visually inspect the distribution of only the ten most abundant species for each substrate. One interesting pattern stood out, the complete lack of *Stemonitis fusca* and *Comatricha tenerrima* on the N plots from SWD, despite their large abundance on all other treatments. Finally, to ensure that the high abundance of *Arcyria cinerea*, which accounts for 18% and 17% of all records for 2012 and 2013, respectively, was not masking treatment effects, all analyses were repeated using a dataset in which all of the *A. cinerea* occurrences were removed. As one of the most abundant and cosmopolitan species of myxomycetes worldwide, *Arcyria cinerea* likely has a wide niche tolerance and is therefore less sensitive to shifts in various environmental parameters or resources. The removal of *A. cinerea*, however, did not statistically alter the results of any analyses, indicating that the high abundance of *A. cinerea* is not concealing a treatment effect.

### *Temporal Effects*

Very little is known regarding temporal variation in myxomycete community composition. The data reported herein were collected from in-depth sampling conducted over two years using the same procedures, and thus provided a novel opportunity to investigate inter-annual variation in

the litter-inhabiting myxomycete community. Analysis of inter-annual variation between two years is something that has not been done in the tropics and only rarely in other ecosystems (Stephenson and Stempen 1994).

Using a RM ANOVA for the five nutrient treatments (N, P, K, NPK and untreated control) the effect of nutrient treatment to myxomycete abundance and richness between the two years of sampling was tested. The effect of nutrient treatment significantly differed only between the two years for myxomycete richness in the LL ( $p = 0.0288$ ). There was no temporal effect of fertilization to myxomycete abundance in the LL nor was a temporal effect identified for species richness or abundance in the SWD.

#### *Possible Effect Detection Error*

Experimental design.—The balanced incomplete-block design utilized herein attains greatest statistical power when sampled in full (i.e., to include all eight treatments). Due to the experimental design, only when sampled in full, can the model account for the spatial variation associated with the 36 m elevation gradient, thus reducing the statistical error and thereby increase the power. So it is surprising that the only treatment effect identified in this study, was in the 2012 dataset, and no treatment effect was identified in the 2013 dataset with which the full experimental power of the design is harnessed. The error associated with the elevation gradient is due to a shift in plant community composition and varying soil chemical properties, especially pH, across the gradient (Yavitt et al. 2009; Wright et al. 2011). Those authors noted a pH gradient from the Southwest to Northeast corners, with the average soil pH shifting from 4.5 to 7.5, respectively. Although the blocking factor cannot be applied for the incomplete sampling in 2012, when applied to the statistical model in 2013, the power of the ANOVAs did indeed

increase as evidenced by significant P values for the blocking factor in each test and also by the decrease in the residual error when the blocks were added into the models.

Because of the dense lowland tropical forest present at the experimental site, wind is not likely to be a predominant mechanism of spore dispersal for myxomycetes. Therefore, the possibility that easily wind-borne spores have been exchanged throughout the experimental area too readily to detect any treatment effect is probably not a factor. Furthermore, the distance between each treatment plot is no less than 20 m at any point. Within each plot, samples were collected from a central location and 10 m in two directions; therefore, the closest any two collection sites could have been is approximately 40 m. Although wind is not likely to be dispersing spores, there is of course a great deal of movement of ground water, which may disperse spores. In addition to other natural mechanisms such as animals serving as vectors, there is also a great deal of human traffic at this study site that could potentially be moving spores between all of the plots as well.

Separate analysis of substrates.—The two substrates, LL and SWD host distinct myxomycete communities as indicated by the low PS and CC values (0.548 and 0.642, respectively); and therefore, the data generated from each substrate were analyzed independently. The two communities together, however, do represent the forest floor litter habitat as a whole. Therefore, all analyses of treatment effects were also performed on datasets in which the two substrates were combined. The outcome of the analyses containing both substrates, however, always identified the same pattern as was observed in the SWD dataset when analyzed by itself, and in no instances affected the statistical outcome of the test, perhaps due to the comparatively large



number of records for the SWD compared to the LL samples. Therefore, analysis of the dataset that contained the two substrates together (LL and SWD) will not be discussed further.

Experimental limitations.—The traditional methods of moist chamber culture and morphological identification of species, together represent the standard protocol for ecological studies of myxomycetes (Stephenson and Stempen 1994). Moist chamber culture is a simple, and cost effective method for isolating and identifying myxomycetes from forest floor litter and many other substrates (e.g., Stephenson 1989). However, each of these methods also comprise their own portion of difficulty and bias that could have potentially limited the ability to detect a treatment effect herein. Some of the biases associated with the use of moist chamber cultures for ecological study are inherent and unavoidable, including the unnatural setting of a Petri dish in a laboratory, and the associated alteration of the original ecological niche, which likely favors some species while simultaneously discouraging others. For example, some large species commonly observed in the field such as *Lycogala epidendrum* rarely, if ever, form fruiting bodies in moist chambers due to the size restriction imposed by the chamber (Novozhilov et al. 2000). Another important complication in the ecological study of myxomycetes is their complex life cycle, which is further compounded by the use of moist chamber cultures. For any particular species that is found more than one time in the same plate, it can not be ruled out (i) whether the two fruitings were initially derived from the same plasmodia that has previously fragmented during moist chamber incubation or (ii) that they were clones from apogamic lineages. Being unable to distinguish whether or not two or more occurrences of the same species represent an abundance of one or of more remains problematic for ecological studies that rely on the counts of abundance. Due to this complication in moist chamber analyses, in the present study two data

sets were generated. In the initial dataset (DS1) containing 3,432 records, every occurrence of every species was recorded, regardless of any previous occurrence of the same species (ignoring the potential bias of clonality and plasmodial separation). The second dataset (DS2) was then generated by removing any duplicate occurrences for each particular species in each plate, thereby limiting the abundance of each species on any one plate, to one, despite the number of times it was observed in that plate decreasing the number of records to 1,939. Because DS2 represents the traditional handling of myxomycete data, all analyses described herein were performed with DS2. However, after the surprising results were obtained, that of no treatment effect, and to ensure that the traditional data handling method was not obscuring a treatment effect, all analyses were repeated using DS1 but otherwise as described in the Materials and Methods section of this chapter. The use of this larger dataset, however, did not alter any of the statistical outcomes of analyses. Again, only marginally significant treatment effects were identified, and only in 2012 SWD. Given the depth of this research project, which possibly comprises the largest dataset of myxomycetes from any one locality, this could be considered an encouraging result as it supports the use of traditional methodologies in data collection because the inclusion of multiple occurrences of a particular species from one moist chamber culture did not significantly alter the results.

Despite the controversy surrounding the use of a morphological species concept to identify species of myxomycetes (e.g., Walker and Stephenson 2016), it was determined to be the most effective approach for this study for several reasons. At the time of this study, the traditional morphological species concept was still the most commonly employed method of myxomycete identification. In light of this and given that the myxomycete taxonomy at that time was still based almost entirely upon a morphological concept, incorporating data obtained herein

with the very large set of data in the publicly available literature would be the most straightforward and meaningful. A molecular phylogenetic approach using environmental sequencing is the only other method that could have potentially been considered for use in this project. Environmental sequencing shows immense potential for use in the study of myxomycete ecology; however, the amount of sequencing required for this large-scale, in-depth ecological study made this option cost prohibitive. In addition, because such an approach in the myxomycetes was still in its infancy, the resources available (e.g., reference sequence databases) were still limited, which would have limited the possible resolution of taxa and weakened interpretation of results. Because there was no evidence available at that time to indicate that a sequencing approach (or any other approach for that matter) would be a more accurate or thorough assessment of diversity, no approach other than that used herein could be justified.

It is unclear, however, whether or not the moist chamber culture method and morphological identification of species were able to fully capture a myxomycete response to fertilization (if present). The addition of a field observation survey would have been a valuable addition to this work as it would have captured more of the diversity at this site by avoiding culture bias. However, given the large size of this experiment, the addition of such observational work would have been cost and time prohibitive. It is very possible that moist chamber culture was unable to provide an accurate enough view of the true myxomycete abundance and diversity to be used for quantitative analyses; however, it is unlikely that the morphological identification of species has affected the outcome of the study to any large extent. Furthermore, all specimens were independently identified by two people. These were the author of this dissertation, and the author's advisor, Dr. Steven L. Stephenson (in situations where the lead author was unable to complete the identification, then the specimens were identified only by her advisor). As a leading

expert in the field of myxomycete taxonomy, Dr. Stephenson confirmed the identification of every specimen before it was placed into the herbarium at the University of Arkansas. Although some errors have certainly been made, given the expertise of those making the identifications and the large number of records, it is unlikely that any errors made in morphological identification contributed to the overall outcome of analyses. The possible implications of morphology concealing cryptic diversity is not addressed herein.

According to the literature, the level of sampling conducted herein should be adequate for evaluating the myxomycete community. Novozhilov et al. (2000) suggested that for an area of 0.1 ha, the species accumulation curve typically flattens out around 30 samples subjected to moist chamber cultures, and therefore this should be sufficient to capture the more common species. Each of the treatment plots in this experiment are slightly smaller at just 0.08 ha and from each plot, three samples of each substrate (LL and SWD) were collected and each sample was used to establish three moist chamber cultures for a total of 18 moist chambers per plot (nine of each substrate). Although slightly less than the recommendation, each plot herein was replicated four times; therefore, the total number of moist chambers for each treatment was 72. When all nine treatments were sampled in 2013 this meant a total of 648 moist chambers were established. This is a large number of moist chambers for one to manage and was only possible by partitioning the samples into two groups to incubate separately. Indeed, this level of sampling seems to be appropriate for the SWD samples, which according to the Chao2 richness estimator were sampled to 76% of completion in 2012 and 85% in 2013. The LL samples on the other hand do not appear to have captured the true richness. The Chao2 richness estimator hypothesizes that in 2012 the LL samples were only sampled to 52% of completion and 44% in 2013.

## Conclusions

The work presented herein does not strongly support the hypothesis of a nutrient treatment effect on the litter-inhabiting myxomycete community at this site. The only detected effect of nutrient limitation to the myxomycete community was detected in the 2012 data and only in the SWD microhabitat. However, when repeated exactly in 2013, no significant effect was identified which leads to the question of whether or not the effect in 2012 was real? Furthermore, the RM ANOVA testing for a temporal effect did not detect a significant difference between the two years, for either the SWD or the LL. The experimental design and level of sampling conducted appear to be within an appropriate range to test the hypotheses, but it is possible that the moist chamber culture method may not be a suitable method for use in such quantitative studies, especially on the small experimental area considered in the present study (36 ha). Despite the treatment effects identified in numerous other studies for other groups of organisms (as cited throughout this chapter), the myxomycetes do not seem to follow the same pattern of treatment effects, or at least not to the same intensity.

Microbial populations have been shown to increase in abundance and level of activity as well as shifting in community composition in response to fertilization at this site (Kaspari et al. 2008; Kaspari et al. 2010; Turner and Wright 2013). The lack of a strong response in the myxomycete community, which is intimately linked with the total microbial community, would suggest that the myxomycetes have some mechanism of survival making them less prone to nutrient limitation. Myxomycetes are generally considered to feed on a wide variety of bacteria, mycelial fungi, detritus, and yeast (Martin and Alexopoulos 1969; Stephenson and Stempen 1994). Perhaps their diet is general enough that despite changes in the available microbial prey (due to the afore mentioned shifts in the microbial community in response to treatments),

myxomycetes remain unaffected as long as prey or other resources are available. Selective predation has been identified in many protist predators, including some amoebae (e.g., Rønn et al. 2002; Rosenberg et al. 2009; Bell et al. 2010). However, similar data for individual myxomycete amoebae (the stage which myxomycetes appear to spend the majority of their lives) were not found in the literature review herein. A great deal of research is available, however, highlighting the ability of myxomycete plasmodia (the multinucleate, trophic stage) to alter their foraging strategy in order to maintain a preferred nutrient balanced diet (e.g., Dussutour et al. 2010). Furthermore, of the three species of myxomycete whose plasmodia have been investigated thus far (*Didymium iridis*, *Didymium bahiense* and *Physarum polycephalum*), each species appears to use a different foraging strategy which the authors suggest is an adaptive response to different preferred food sources (Yip et al. 2014; Latty and Beekman 2015). Whether a similar situation exists for the uninucleate, amoeboid stage is not clear. Moreover, because in moist chamber culture it is largely unclear whether the species present were initially in an amoeboid or plasmodial form at the time of collection, it is unclear how this information can be applied to the data generated in the present study. There are currently approximately 900 described myxomycete morphospecies (Lado 2005–2015), if indeed a wide diversity of food preference and foraging strategies are present, that could also help explain the maintenance of such a high diversity for the group as well as the common coexistence of numerous species in the same microhabitat.

The data reported herein for myxomycete ecology are rather inconclusive at this stage, yet the observations made are very intriguing and should certainly warrant further investigation.

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## Tables and Figures

**Table 1.** Species richness and abundance (number of occurrences) by substrate (LL = leaf litter, SWD = small woody debris) for each year of the study. Data set 1 is the original data recorded before removing duplicate species occurrences by plate to generate Data Set 2.

	<b>Data set 2 (DS2)</b>		<b>Data set 1 (DS1)</b>
	<b>Richness</b>	<b>Abundance</b>	<b>Abundance</b>
<b>2012</b>			
LL (n = 20)	43	195	444
SWD (n = 20)	69	509	961
<b>2013</b>			
LL (n = 32)	75	469	845
SWD (n = 32)	86	697	1182

**Table 2.** The ten most abundant species for each substrate in 2013 (LL = leaf litter, SWD = small woody debris).

<b>Species</b>	<b>LL</b>	<b>SWD</b>
<i>Arcyria cinerea</i>	76	123
<i>Diderma effusum</i>	52	29
<i>Lamproderma scintillans</i>	71	0
<i>Perichaena chrysosperma</i>	14	35
<i>Perichaena depressa</i>	10	35
<i>Cribraria microcarpa</i>	11	27
<i>Stemonitis fusca</i> var. <i>nigrescens</i>	0	37
<i>Comatricha tenerrima</i>	0	33
<i>Collaria arcyrionema</i>	0	30
<i>Arcyria denudata</i>	0	29
<i>Arcyria leiocarpa</i>	0	26
<i>Didymium nigripes</i>	22	0
<i>Perichaena longipes</i>	15	0
<i>Comatricha pulchella</i>	13	0
<i>Diderma hemisphaericum</i>	13	0
Total number of records	297	404

**Table 3.** List of all species recorded in the present study, including both years of data

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<i>Arcyria afroalpina</i>	<i>Didymium effusum</i>	<i>Physarum aeneum</i>
<i>Arcyria cinerea</i>	<i>Didymium iridis</i>	<i>Physarum album</i>
<i>Arcyria denudata</i>	<i>Didymium nigripes</i>	<i>Physarum bivalve</i>
<i>Arcyria insignis</i>	<i>Didymium ochroideum</i>	<i>Physarum cinereum</i>
<i>Arcyria leiocarpa</i>	<i>Didymium squamulosum</i>	<i>Physarum compressum</i>
<i>Arcyria marginoundulata</i>	<i>Echinostelium minutum</i>	<i>Physarum crateriforme</i>
<i>Arcyria pomiformis</i>	<i>Enerthenema papillatum</i>	<i>Physarum decipiens</i>
<i>Badhamia affinis</i>	<i>Hemitrichia calyculata</i>	<i>Physarum galbeum</i>
<i>Calomyxa metalica</i>	<i>Hemitrichia leiocarpa</i>	<i>Physarum globuliferum</i>
<i>Ceratiomyxa fruticulosa</i>	<i>Hemitrichia pardina</i>	<i>Physarum lakhanpalii</i>
<i>Clastoderma debaryanum</i>	<i>Hemitrichia serpula</i>	<i>Physarum lateritium</i>
<i>Collaria arcyrionema</i>	<i>Lamproderma arcyrionema</i>	<i>Physarum melleum</i>
<i>Collaria</i> sp. A	<i>Lamproderma scintillans</i>	<i>Physarum nigripes</i>
<i>Comatricha ellae</i>	<i>Licea belmontiana</i>	<i>Physarum oblatum</i>
<i>Comatricha laxa</i>	<i>Licea biforis</i>	<i>Physarum pusillum</i>
<i>Comatricha lurida</i>	<i>Licea eleanorae</i>	<i>Physarum roseum</i>
<i>Comatricha nigra</i>	<i>Licea kleistobolus</i>	<i>Physarum superbum</i>
<i>Comatricha pulchella</i>	<i>Licea operculata</i>	<i>Physarum tenerum</i>
<i>Comatricha</i> sp. A	<i>Licea rufocuprea</i>	<i>Physarum virescens</i>
<i>Comatricha tenerrima</i>	<i>Lycogala conicum</i>	<i>Physarum viride</i>
<i>Comatrichia pulchella</i>	<i>Macbrideola decapillata</i>	<i>Physarum superbum</i>
<i>Comatrichia tenerrima</i>	<i>Macbrideola ovoidea</i>	<i>Stemonitis fusca</i> var. <i>nigrescens</i>
<i>Craterium aereum</i>	<i>Macbrideola</i> sp. A	<i>Stemonitis herbatica</i>
<i>Cribraria microcarpa</i>	<i>Macbrideola</i> sp. C	<i>Stemonitopsis typhina</i>
<i>Cribraria tenerrima</i>	<i>Macbrideola synsporus</i>	<i>Trichia favoginea</i>
<i>Cribraria violacea</i>	<i>Metatrichia vesparia</i>	
<i>Diachea bulbillosa</i>	<i>Paradiacheopsis fimbriata</i>	
<i>Diachea leucopodia</i>	<i>Perichaena chrysosperma</i>	
<i>Diderma effusum</i>	<i>Perichaena depressa</i>	
<i>Diderma hemisphaericum</i>	<i>Perichaena longipes</i>	
<i>Didymium anellus</i>	<i>Perichaena pedata</i>	
<i>Didymium difforme</i>	<i>Perichaena vermicularis</i>	

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**Table 4.** An updated species list for The Republic of Panama. Species identified herein are in column A, column B contains the records compiled by Piepenbring et al. (2006) and column C represents the species records on Myxotropic [www.myxotropic.org, accessed on 12-1-2015]

<b>Species</b>	<b>A</b>	<b>B</b>	<b>C</b>
<i>Arcyria afroalpina</i>	1		
<i>Arcyria cinerea</i>	1	1	1
<i>Arcyria denudata</i>	1	1	1
<i>Arcyria insignis</i>	1	1	
<i>Arcyria leiocarpa</i>	1	1	
<i>Arcyria marginoundulata</i>	1		
<i>Arcyria pomiformis</i>	1	1	
<i>Badhamia affinis</i>	1	1	
<i>Calomyxa metalica</i>	1		
<i>Ceratiomyxa fruticulosa</i>	1		1
<i>Clastoderma debaryanum</i>	1		1
<i>Collaria arcyrionema</i>	1		1
<i>Collaria sp. A</i>	1		
<i>Comatricha ellae</i>	1		
<i>Comatricha laxa</i>	1	1	
<i>Comatricha lurida</i>	1		
<i>Comatricha nigra</i>	1	1	
<i>Comatricha pulchella</i>	1	1	
<i>Comatricha sp. A</i>	1		
<i>Comatricha tenerrima</i>	1		
<i>Comatrichia pulchella</i>	1		
<i>Comatrichia tenerrima</i>	1		
<i>Craterium aereum</i>	1		
<i>Cribraria microcarpa</i>	1	1	1
<i>Cribraria tenerrima</i>	1		
<i>Cribraria violacea</i>	1	1	1
<i>Diachea bulbillosa</i>	1	1	
<i>Diachea leucopodia</i>	1		
<i>Diderma effusum</i>	1	1	1
<i>Diderma hemisphaericum</i>	1		
<i>Didymium anellus</i>	1		
<i>Didymium difforme</i>	1		
<i>Didymium effusum</i>	1		
<i>Didymium iridis</i>	1	1	
<i>Didymium nigripes</i>	1	1	
<i>Didymium ochroideum</i>	1		
<i>Didymium squamulosum</i>	1	1	
<i>Echinostelium minutum</i>	1		

**Table 4. (Cont.)**

<b>Species</b>	<b>A</b>	<b>B</b>	<b>C</b>
<i>Enerthenema papillatum</i>	1		
<i>Hemitrichia calyculata</i>	1	1	1
<i>Hemitrichia leiocarpa</i>	1		
<i>Hemitrichia pardina</i>	1		
<i>Hemitrichia serpula</i>	1	1	1
<i>Lamproderma arcyryonema</i>	1		
<i>Lamproderma scintillans</i>	1	1	
<i>Licea belmontiana</i>	1		
<i>Licea biforis</i>	1		
<i>Licea eleanorae</i>	1		
<i>Licea kleistobolus</i>	1		
<i>Licea operculata</i>	1	1	
<i>Licea rufocuprea</i>	1		
<i>Lycogala conicum</i>	1	1	
<i>Macbrideola decapillata</i>	1		
<i>Macbrideola ovoidea</i>	1		
<i>Macbrideola sp. A</i>	1		
<i>Macbrideola sp. C</i>	1		
<i>Macbrideola synsporus</i>	1		
<i>Metatrichia vesparia</i>	1		
<i>Paradiacheopsis fimbriata</i>	1		
<i>Perichaena chrysosperma</i>	1	1	
<i>Perichaena depressa</i>	1	1	1
<i>Perichaena longipes</i>	1		
<i>Perichaena pedata</i>	1		
<i>Perichaena vermicularis</i>	1	1	
<i>Physarum aeneum</i>	1		
<i>Physarum album</i>	1		1
<i>Physarum bivalve</i>	1	1	
<i>Physarum cinereum</i>	1	1	
<i>Physarum compressum</i>	1	1	
<i>Physarum crateriforme</i>	1		
<i>Physarum decipiens</i>	1		
<i>Physarum galbeum</i>	1		
<i>Physarum globuliferum</i>	1	1	1
<i>Physarum lakhanpalii</i>	1		
<i>Physarum lateritium</i>	1		
<i>Physarum melleum</i>	1	1	
<i>Physarum nigripes</i>	1		
<i>Physarum oblatum</i>	1	1	1

**Table 4. (Cont.)**

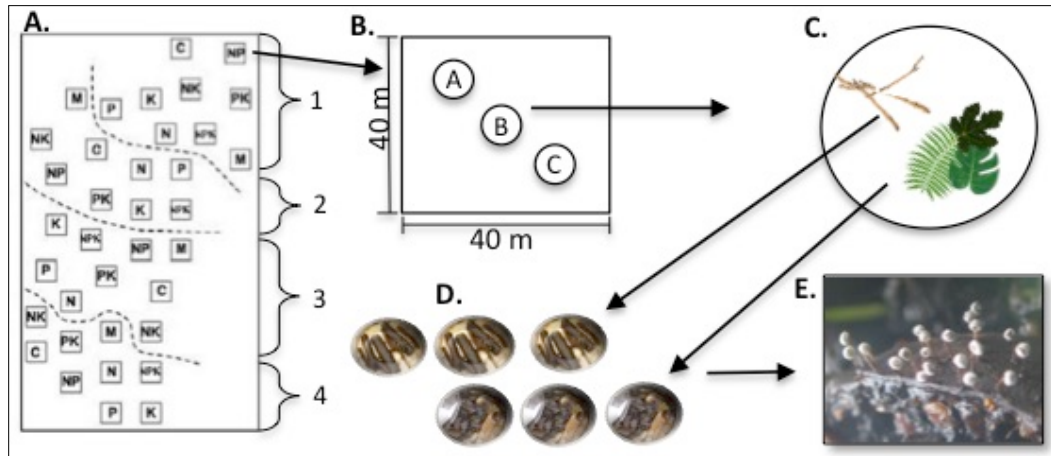
<b>Species</b>	<b>A</b>	<b>B</b>	<b>C</b>
<i>Physarum pusillum</i>	1	1	1
<i>Physarum roseum</i>	1		
<i>Physarum superbum</i>	1		
<i>Physarum tenerum</i>	1	1	1
<i>Physarum virescens</i>	1		
<i>Physarum viride</i>	1	1	1
<i>Pysarum superbum</i>	1		
<i>Stemonitis fusca var. nigrescens</i>	1		
<i>Stemonitis herbatica</i>	1		
<i>Stemonitis nigrescens</i>	1	1	
<i>Stemonitopsis typhina</i>	1	1	1
<i>Trichia favoginea</i>	1	1	1
<i>Willkommangea reticulata</i>	1	1	
<i>Arcyria magna</i>		1	
<i>Arcyria minuta</i>		1	1
<i>Arcyria nutans</i>		1	
<i>Arcyria oerstedii</i>		1	1
<i>Badhamia gracillis</i>		1	
<i>Brefeldia maxima</i>		1	
<i>Ceratiomyxa fruiticulosa</i>		1	
<i>Ceratiomyxa morchella</i>		1	
<i>Ceratiomyxa sphaerosperma</i>		1	1
<i>Clastoderma debaryanum</i>		1	
<i>Collaria acrcyonema</i>		1	
<i>Comatricha afroalpina</i>		1	1
<i>Cornuvia serpula</i>			1
<i>Craterium leucocephalum</i>		1	
<i>Craterium paraguariense</i>		1	
<i>Cribraria argillacea</i>		1	
<i>Cribraria aurantiaca</i>		1	1
<i>Cribraria cancellata</i>		1	
<i>Cribraria intricata</i>		1	1
<i>Cribraria languescens</i>		1	1
<i>Cribraria pachydietyon</i>		1	
<i>Cribraria piriformis</i>		1	
<i>Cribraria tenella</i>		1	1
<i>Diachea leucopoda</i>		1	
<i>Diachea radiata</i>		1	
<i>Dictydiaethalium plubeum</i>		1	
<i>Diderma hemispaericum</i>		1	
<i>Diderma rugosum</i>		1	1

**Table 4. (Cont.)**

<b>Species</b>	<b>A</b>	<b>B</b>	<b>C</b>
<i>Diderma spumarioides</i>		1	
<i>Didymium clavus</i>		1	1
<i>Didymium intermedium</i>		1	
<i>Didymium verrucosporum</i>		1	
<i>Echinostelium minutum</i>		1	
<i>Enteridium lycoperdon</i>		1	
<i>Enteridium splendens</i>		1	
<i>Fuligo septica</i>		1	
<i>Hemitrichia clavata</i>		1	1
<i>Hemitrichia minor</i>		1	
<i>Licea minima</i>		1	
<i>Licea pedicellata</i>		1	1
<i>Licea pusilla</i>		1	
<i>Licea tuberculata</i>		1	
<i>Lycogala epidendrum</i>		1	
<i>Lycogala exiguum</i>		1	
<i>Lycogala flavofuscum</i>		1	
<i>Metatrichia vesparium</i>		1	
<i>Perichaena corticalis</i>		1	
<i>Physarella oblonga</i>		1	1
<i>Physarum auriscalpium</i>		1	
<i>Physarum bogoriense</i>		1	
<i>Physarum didermoides</i>		1	1
<i>Physarum echinosporum</i>		1	
<i>Physarum fulgens</i>		1	
<i>Physarum leucopus</i>		1	
<i>Physarum nutans</i>		1	
<i>Physarum penetrale</i>		1	1
<i>Physarum psittacinum</i>		1	1
<i>Physarum pulcherripes</i>		1	
<i>Physarum serpula</i>		1	
<i>Physarum stellatum</i>		1	1
<i>Stemonaria longa</i>			1
<i>Stemonitis axifera</i>		1	1
<i>Stemonitis flavogentia</i>		1	
<i>Stemonitis fusca</i>		1	1
<i>Stemonitis pallida</i>		1	1
<i>Stemonitis splendens</i>		1	
<i>Stemonitis virginensis</i>		1	1
<i>Stemonitopsis aequalis</i>		1	
<i>Stemonitopsis hyperopta</i>		1	1

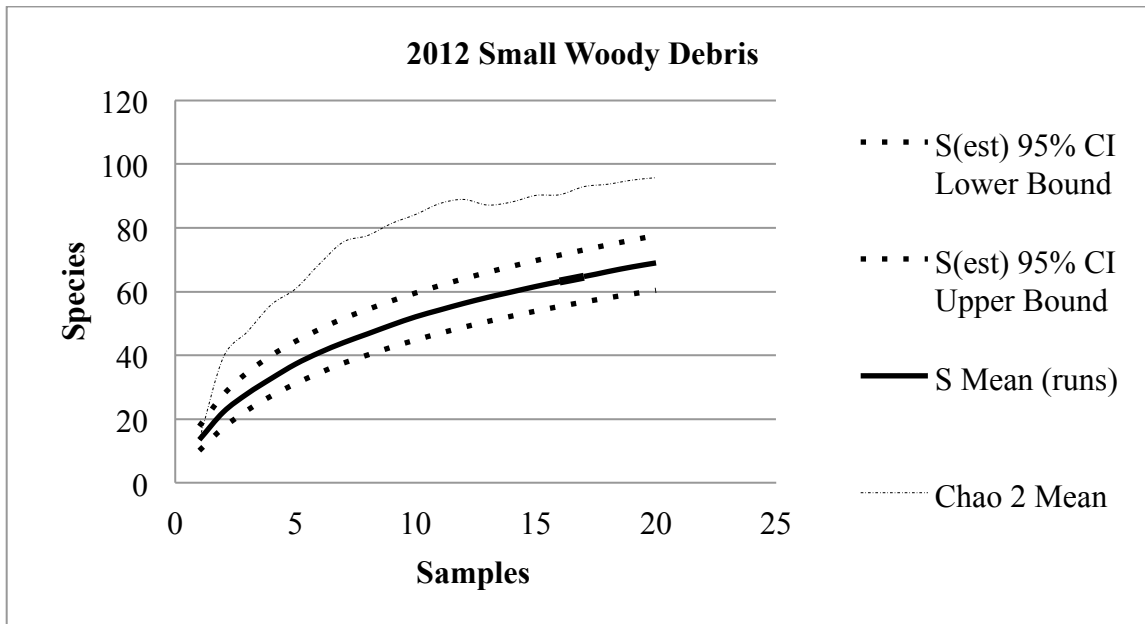
**Table 4. (Cont.)**

<b>Species</b>	<b>A</b>	<b>B</b>	<b>C</b>
<i>Symphytocarpus longus</i>		1	
<i>Tubifera ferruginosa</i>		1	
<i>Tubifera microsperma</i>		1	

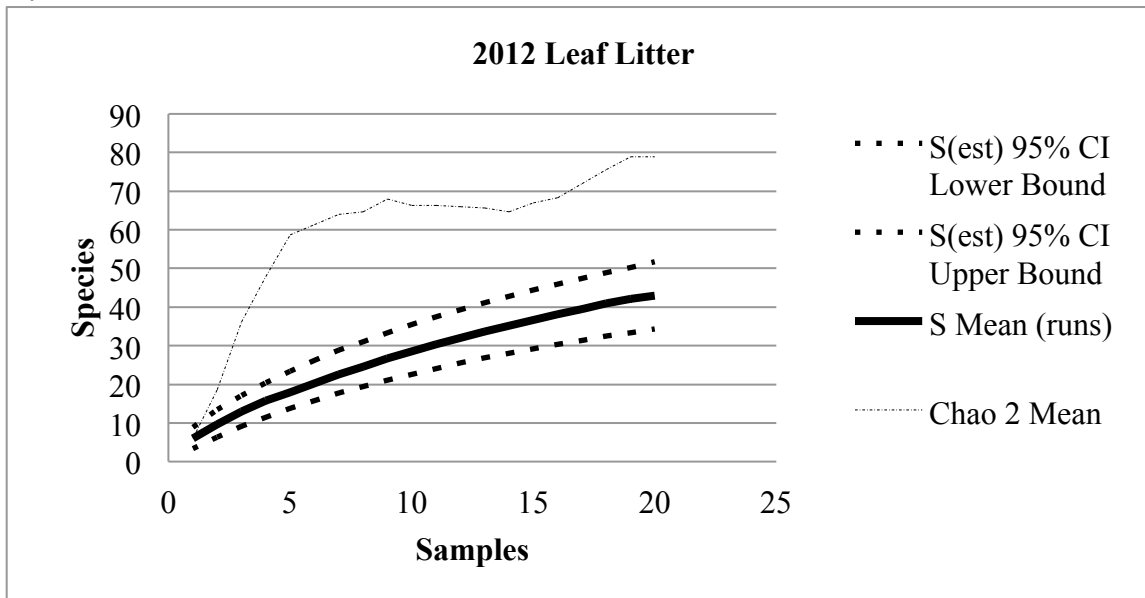


**Figure 1.** A. Layout of the nutrient fertilization plots, total area 38.4 ha. Each plot is labeled according to the nutrient treatment(s) (N = Nitrogen, P = Phosphorus, K = Potassium, M = Micronutrient, C = Control). The four replicates are identified along the right side of this diagram. B. Each plot, 40 x 40 m, sampled at three sites, 10 m apart C. Leaf litter and small woody debris sampled at each site D. Litter samples are used to establish three moist chamber cultures for each substrate E. Myxomycete identification and community analysis using the morphological species concept of recovered fruiting bodies.

A.

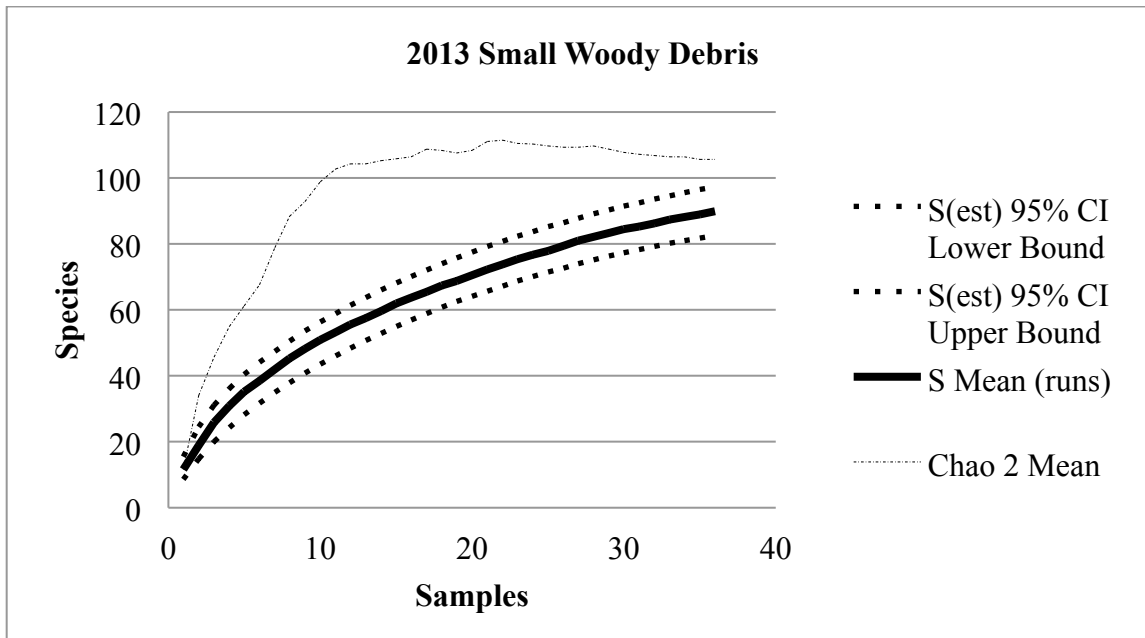


B.

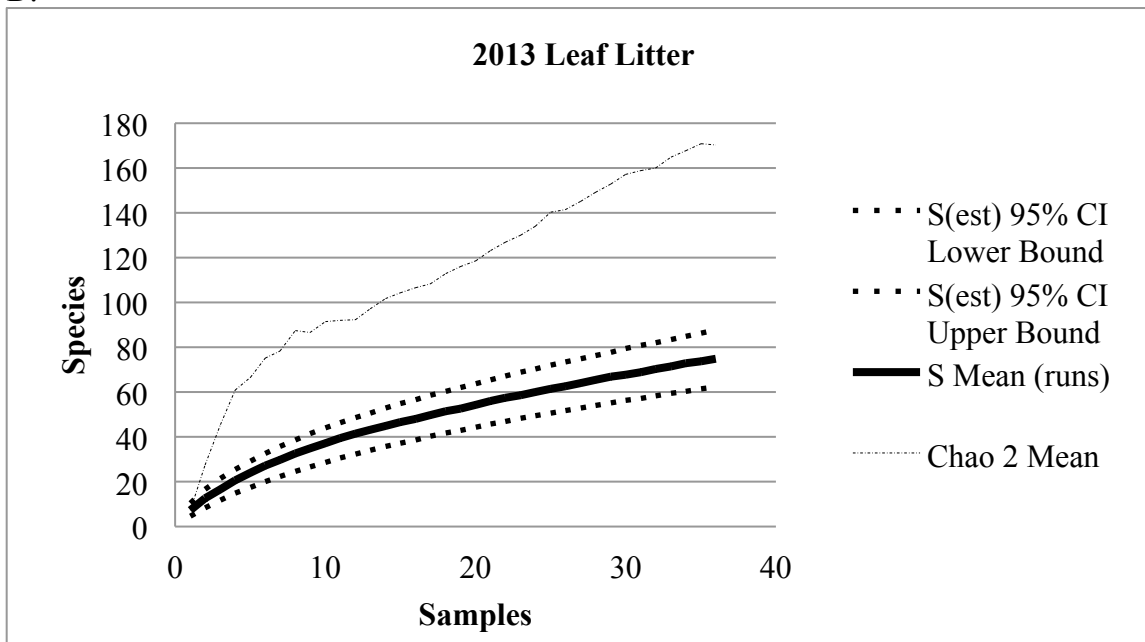


**Figure 2.** Species accumulation curves for A. 2012 small woody debris, B. 2012 leaf litter, C. 2013 small woody debris and D. 2013 leaf litter

C.



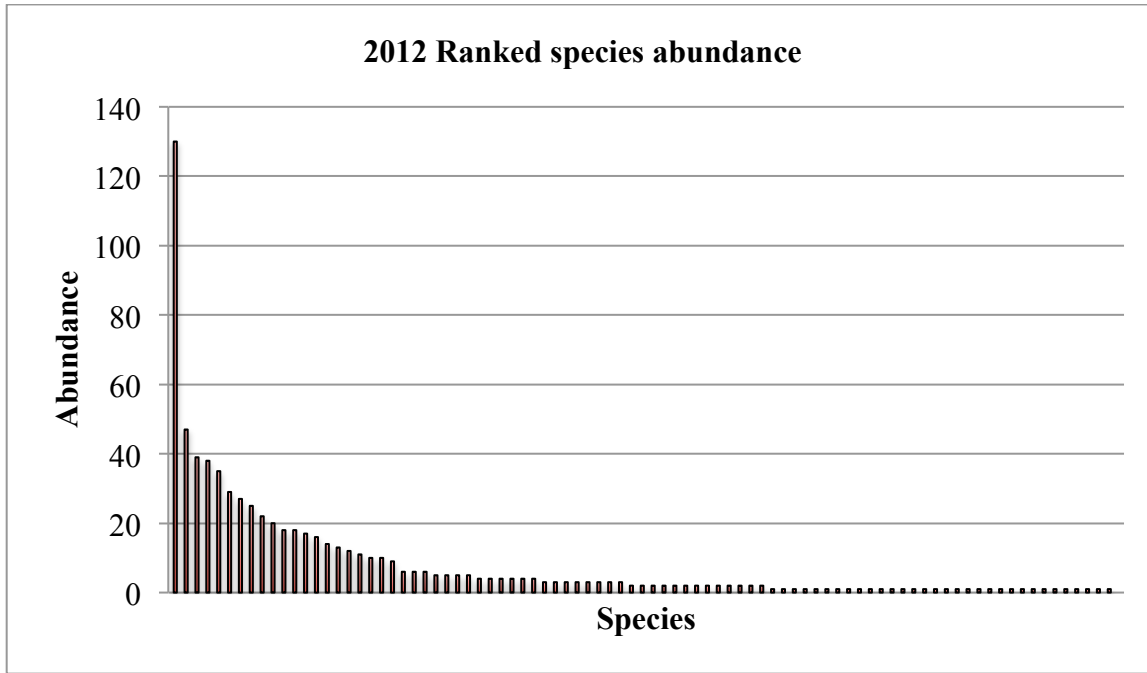
D.



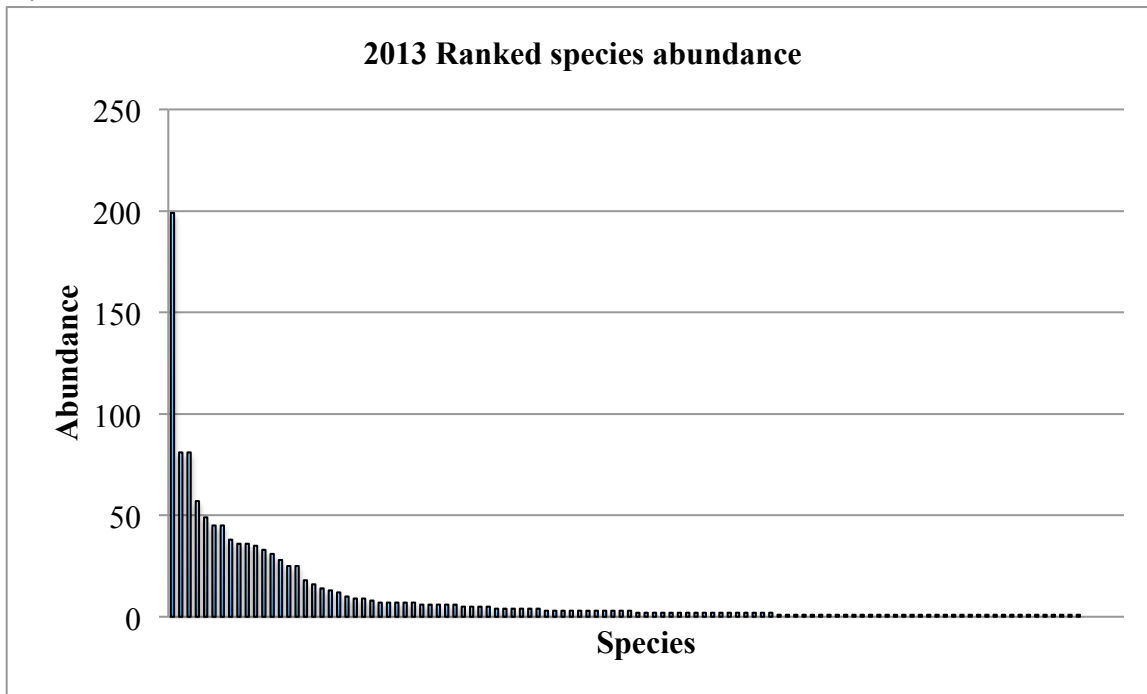
**Figure 2 (Cont.).** Species accumulation curves for A. 2012 small woody debris, B. 2012 leaf litter, C. 2013 small woody debris and D. 2013 leaf litter



A.

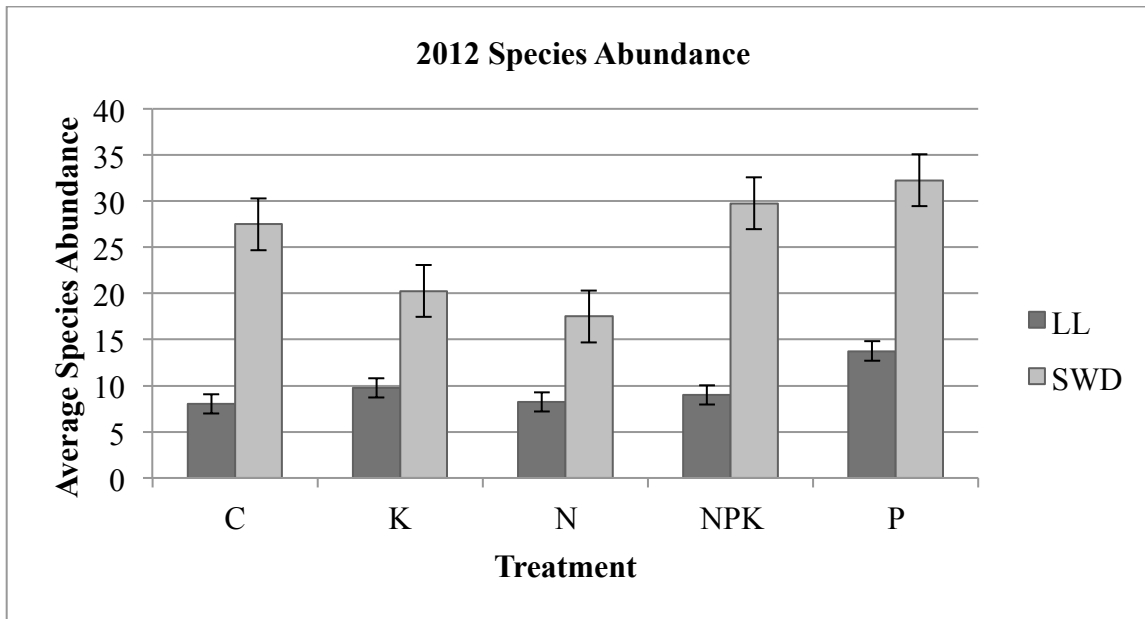


B.

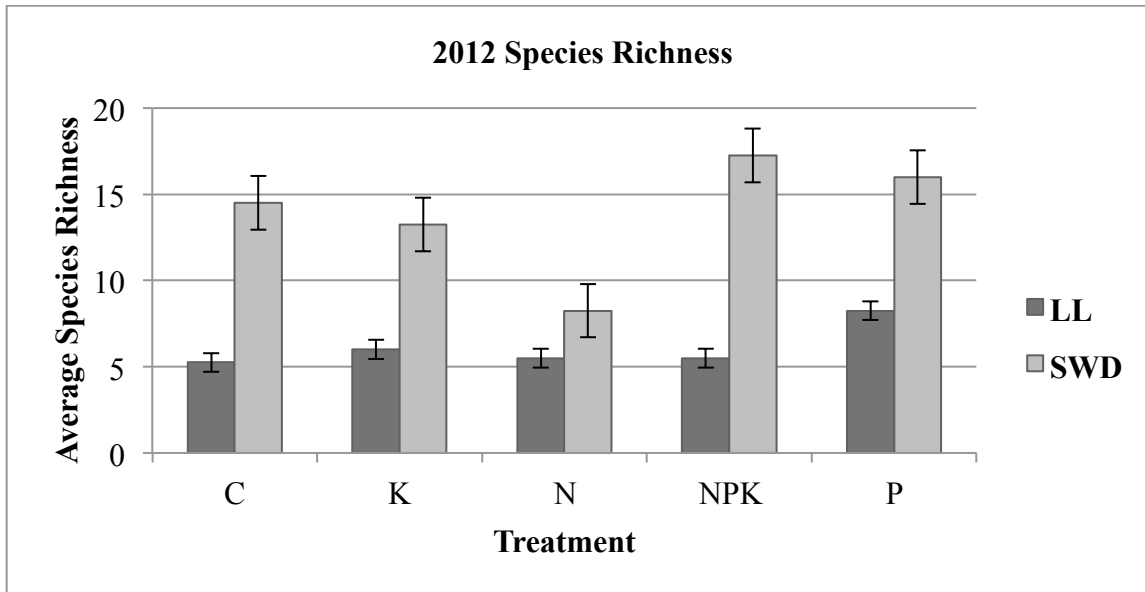


**Figure 3.** Total number of records of each species (leaf litter and small woody debris substrates are combined) for A. 2012 and B. 2013

A.

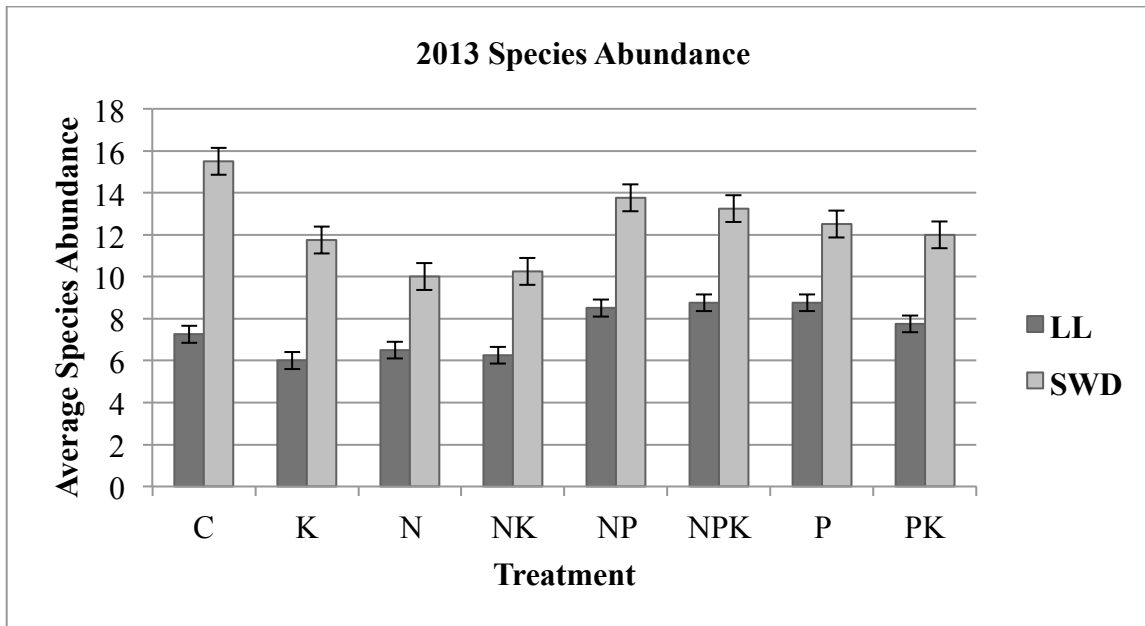


B.

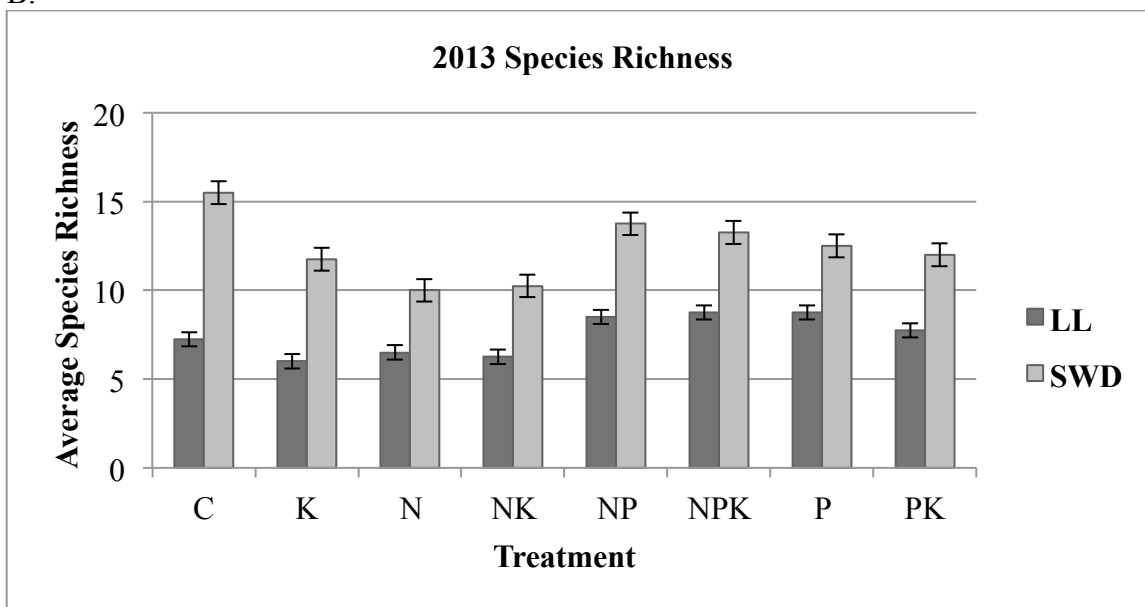


**Figure 4.** Distribution of average myxomycete (A) abundance and (B) richness by substrate and among treatments in 2012. Bars represent standard error of the average for each treatment (LL = leaf litter, SWD = small woody debris).

A.

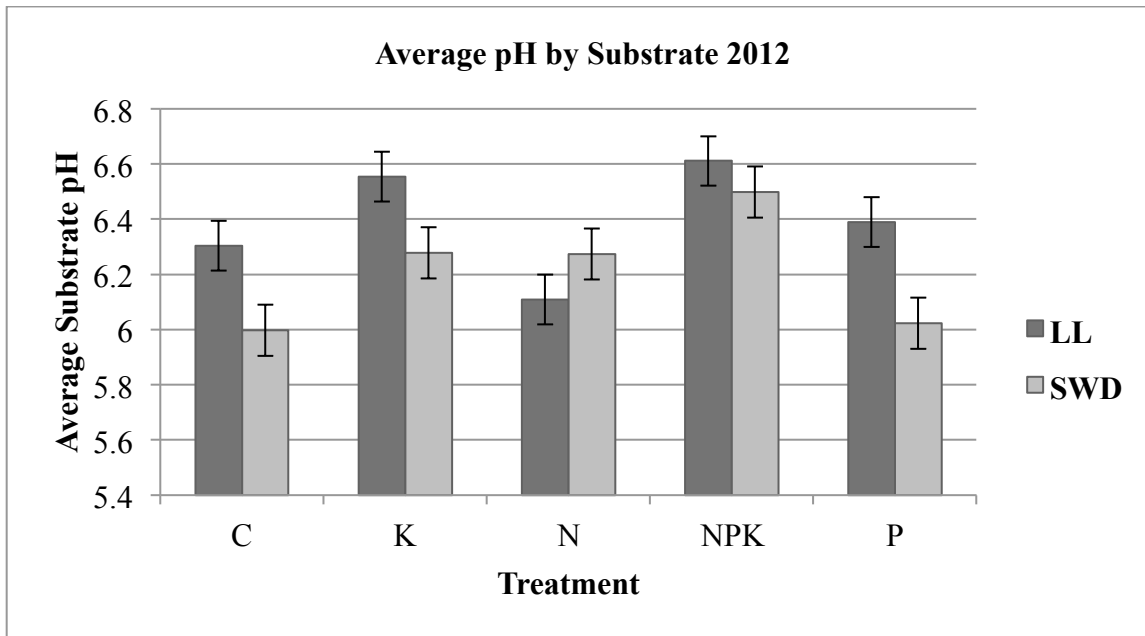


B.

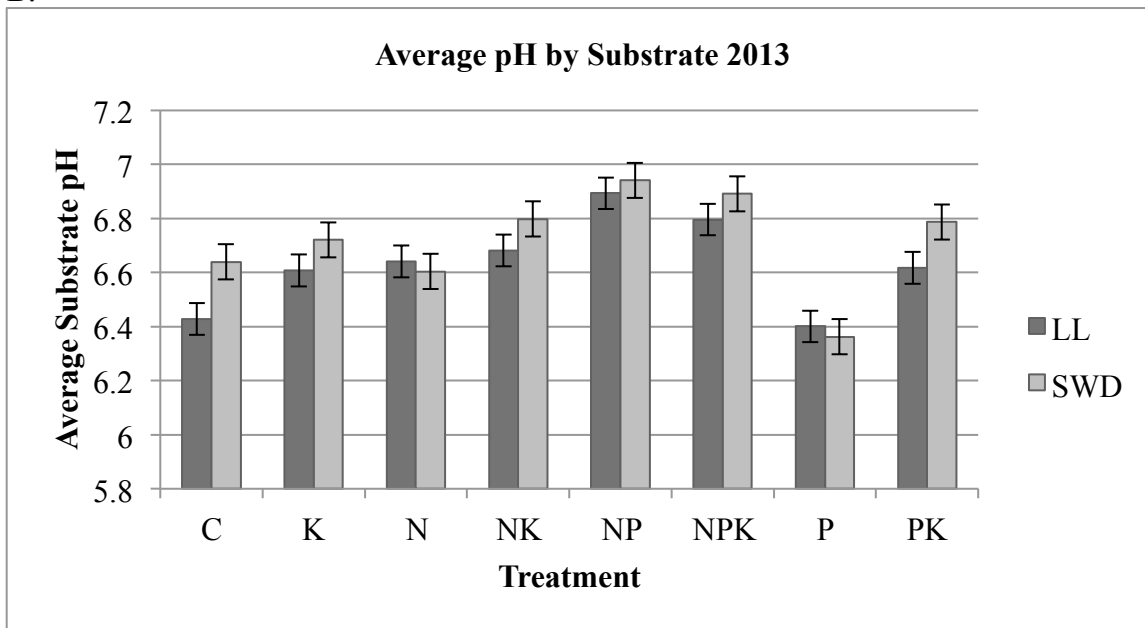


**Figure 5.** Distribution of average myxomycete (A) abundance and (B) richness by substrate and among treatments in 2013. Bars represent standard error of the average for each treatment (LL = leaf litter, SWD = small woody debris).

A.

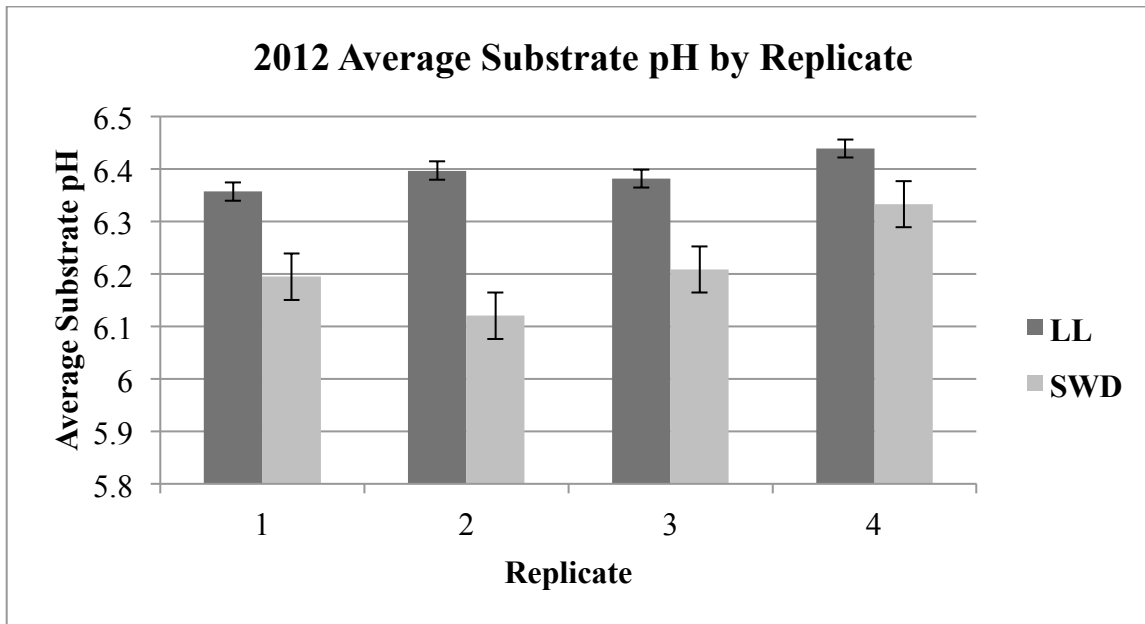


B.

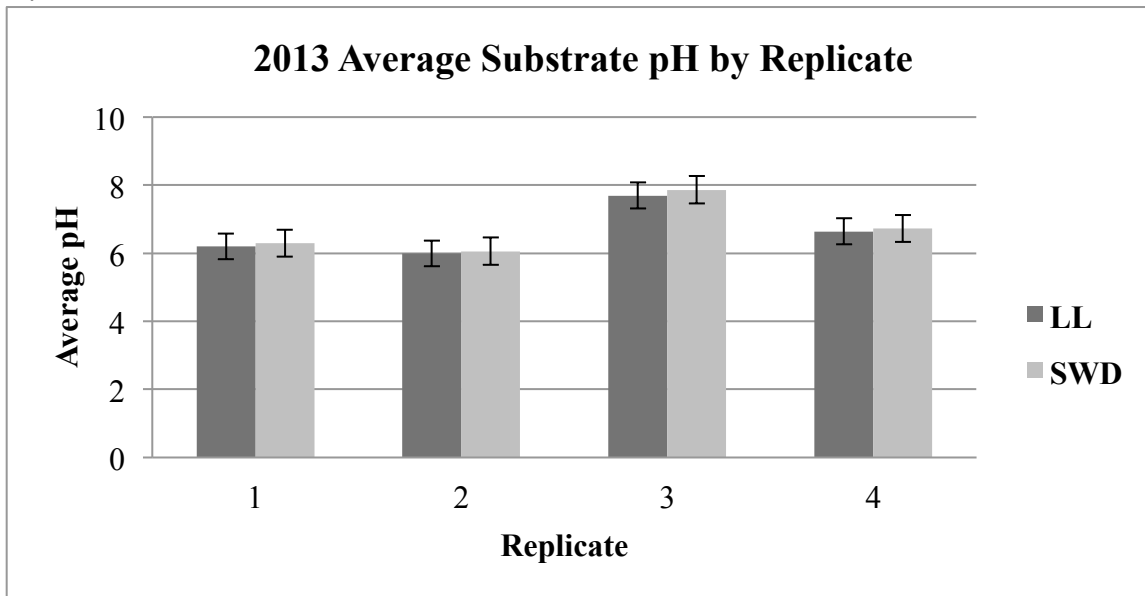


**Figure 6.** Distribution of average substrate pH in (A) 2012 and (B) 2013 by substrate and among treatments. Bars represent standard error of the average for each treatment (LL = leaf litter, SWD = small woody debris).

A.

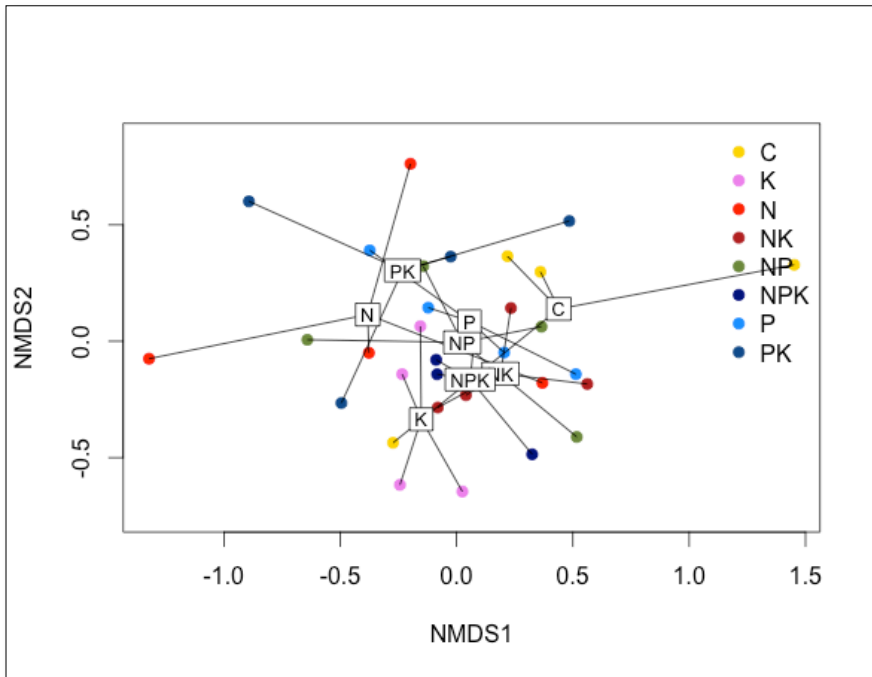


B.

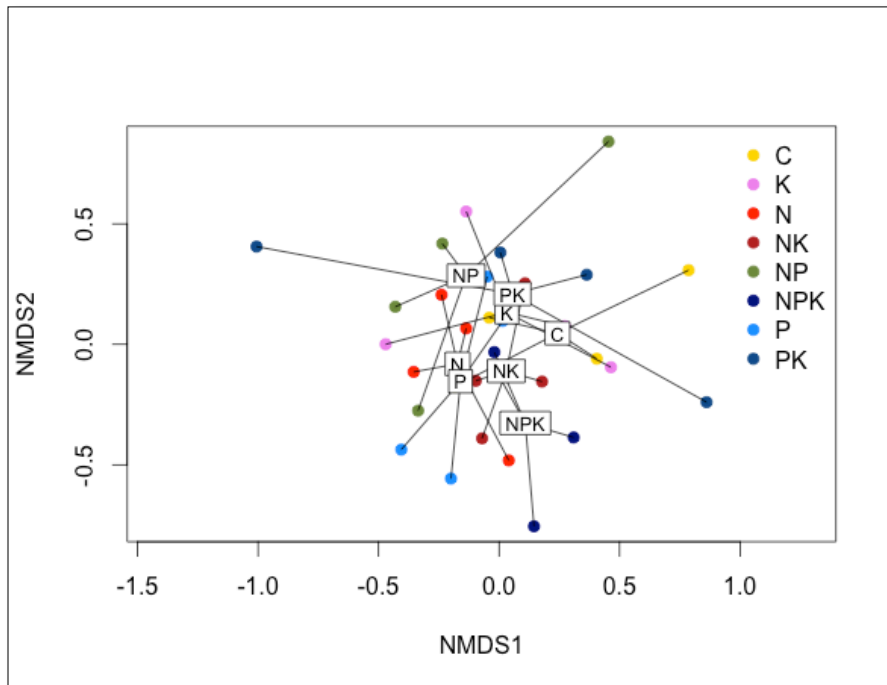


**Figure 7.** Distribution of average substrate pH in (A) 2012 and (B) 2013 for each replicate, by treatment. Bars represent standard error of the average for each replicate (LL = leaf litter, SWD = small woody debris).

A.



B.

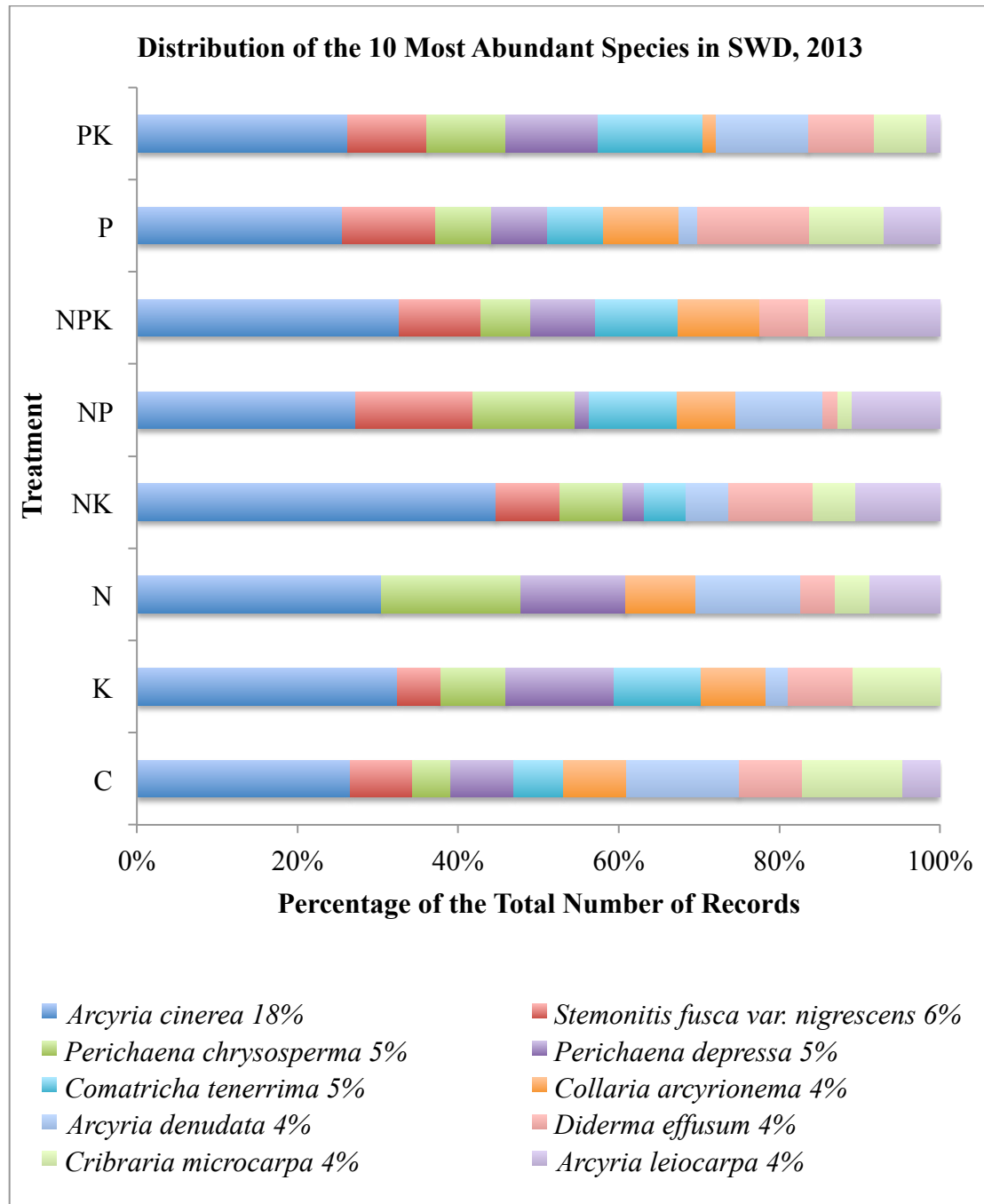


**Figure 8.** NMDS plot illustrating the divergence in community composition of myxomycetes between in (A) leaf litter and (B) small woody debris in nutrient treatments and the control for the 2013 dataset. The plot was produced using Bray-Curtis measure of similarity. Replicate plots for each nutrient treatment are connected by bars and each treatment is labeled at its centroid.

A.



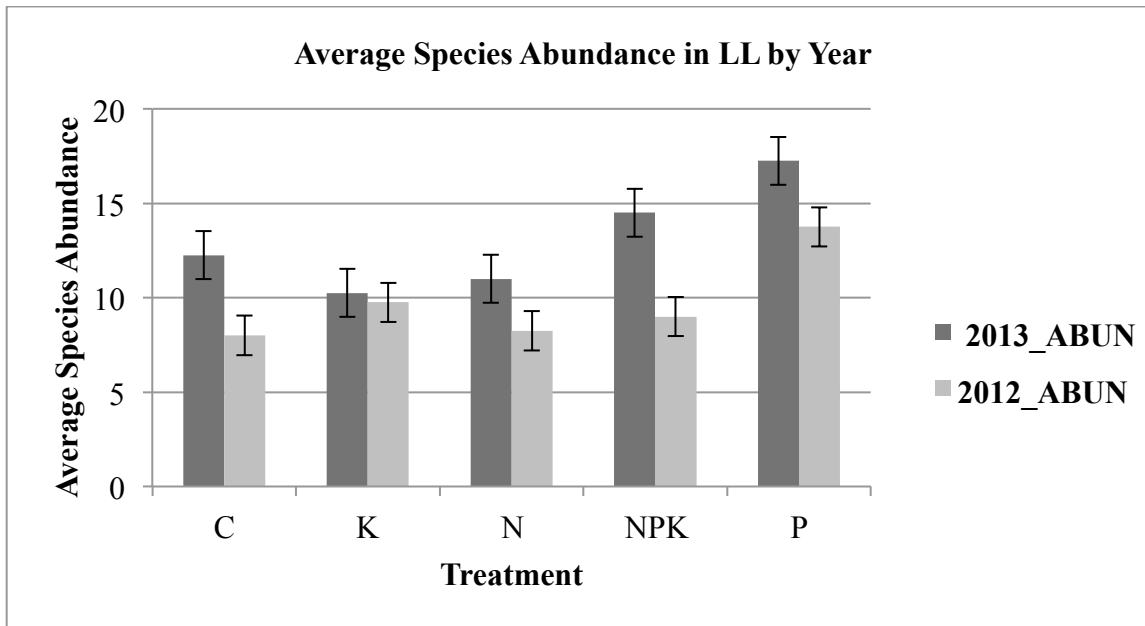
B.



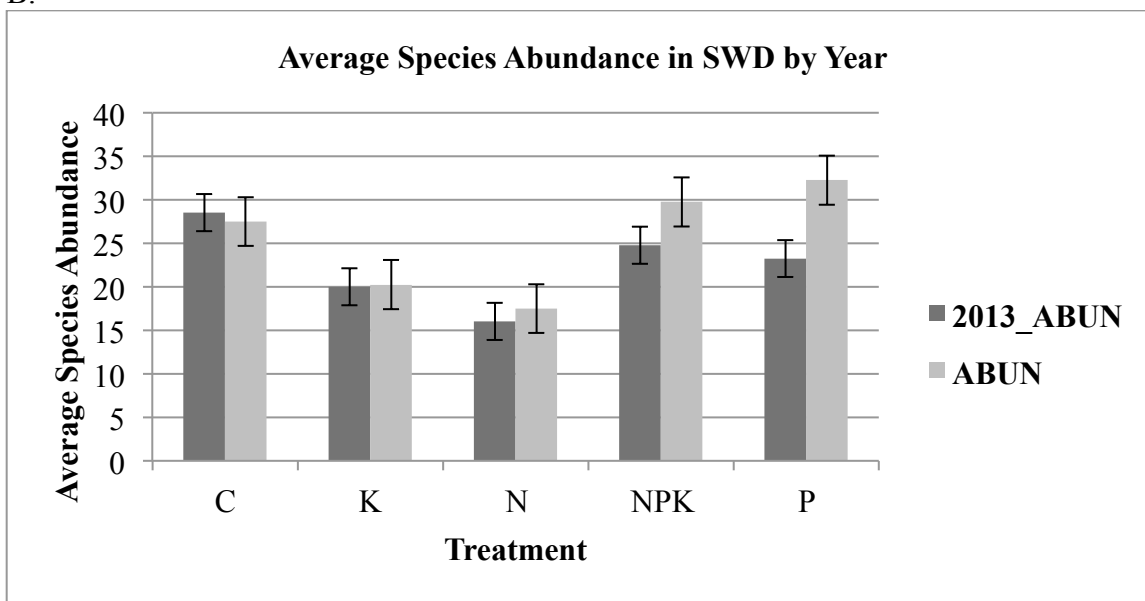
**Figure 9.** The distribution of the ten most abundant species in 2013 for (A) leaf litter and (B) small woody debris.



A.

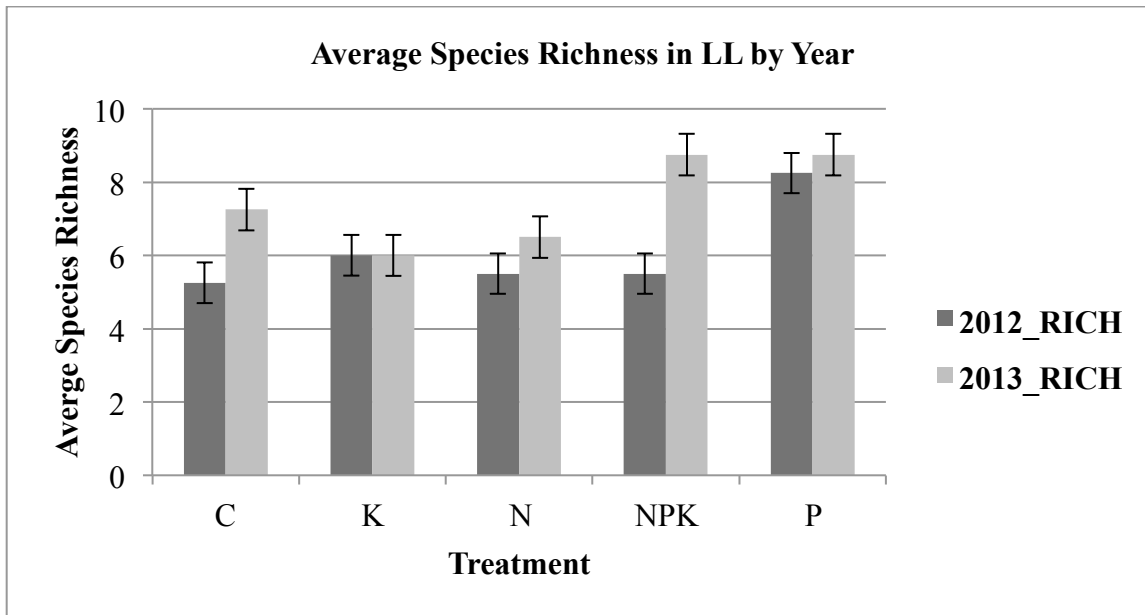


B.

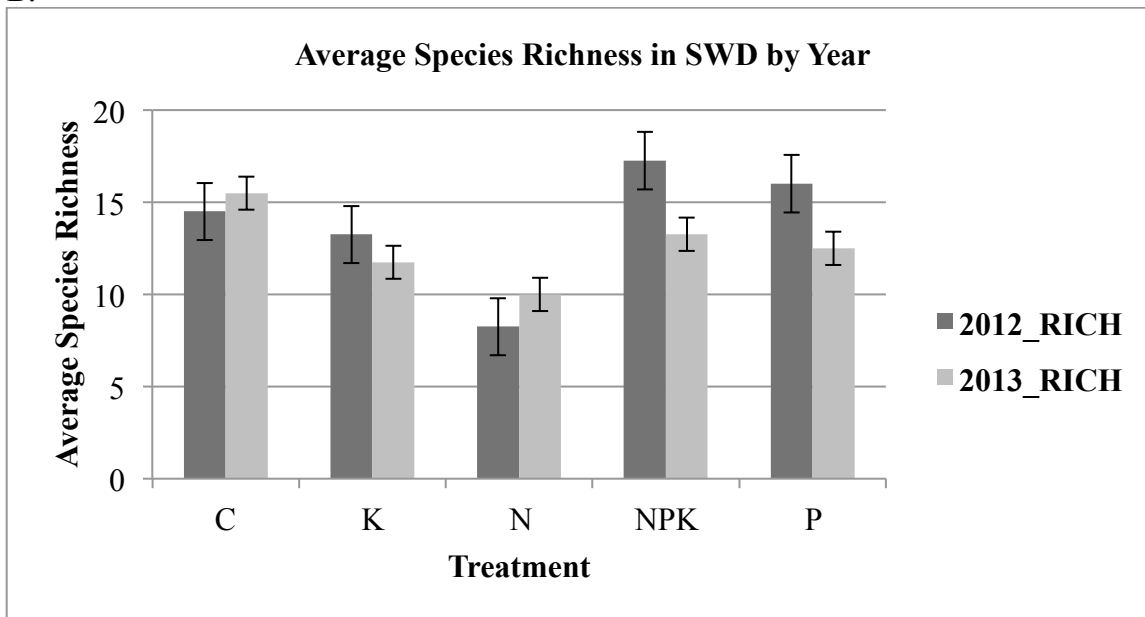


**Figure 10.** Distribution of average species abundance in (A) LL and (B) SWD by year and among treatments. Bars represent standard error of the average for each treatment. (LL = leaf litter, SWD = small woody debris).

A.

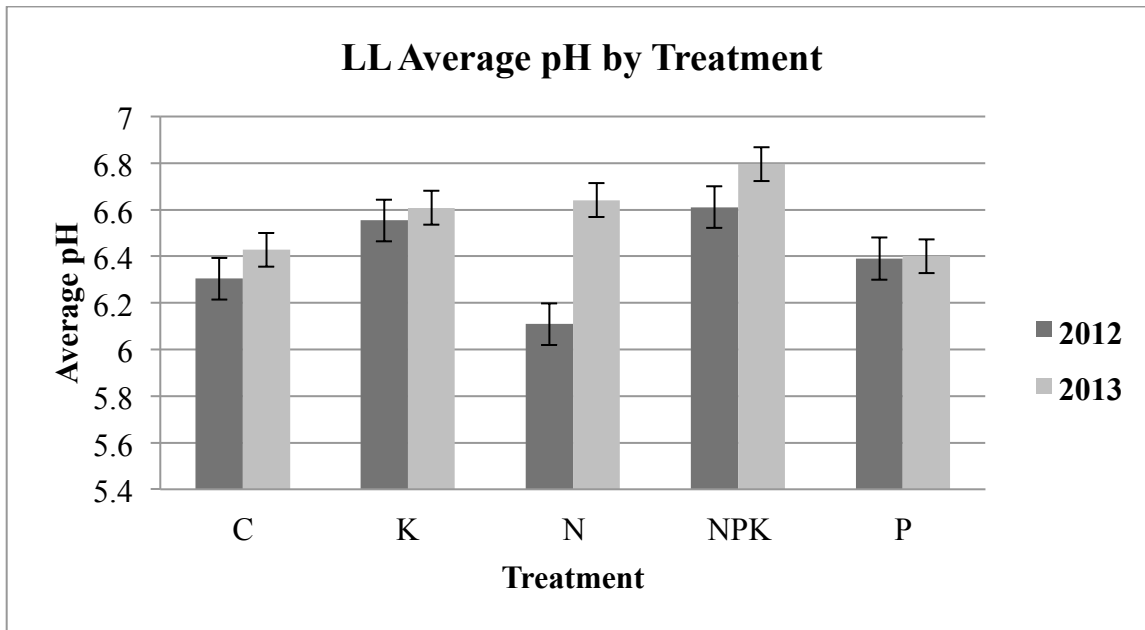


B.

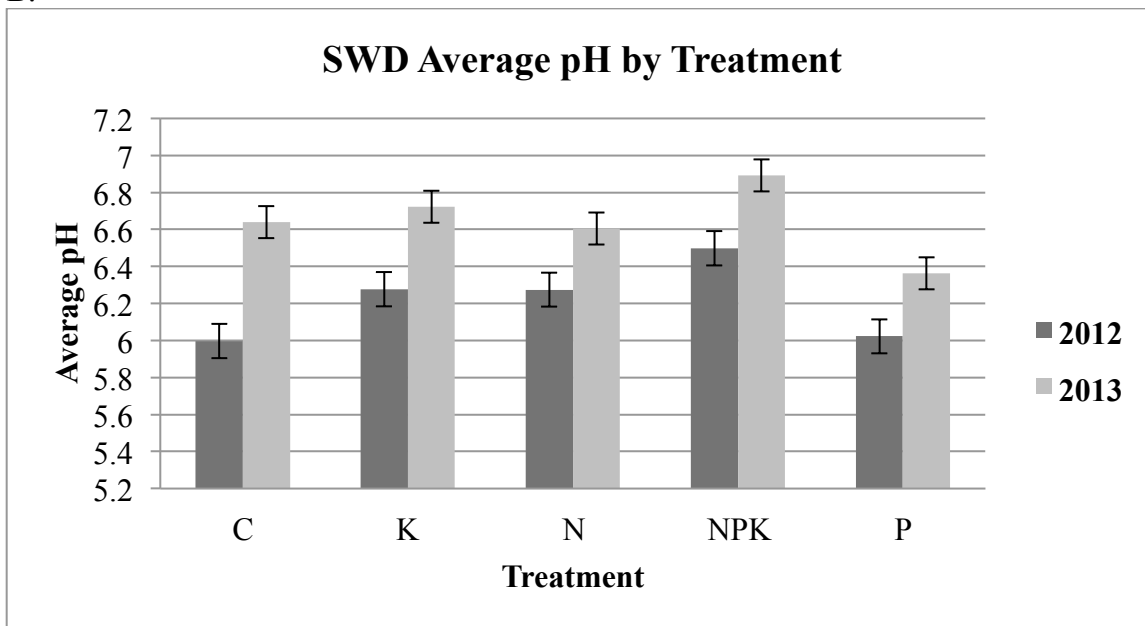


**Figure 11.** Distribution of average species richness in (A) LL and (B) SWD by year and among treatments. Bars represent standard error of the average for each treatment. (LL = leaf litter, SWD = small woody debris).

A.



B.



**Figure 12.** Distribution of average substrate pH in (A) LL and (B) SWD by year and among treatments. Bars represent standard error of the average substrate pH for each treatment. (LL = leaf litter, SWD = small woody debris).

#### **IV. *Perichaena longipes*, a new myxomycete from the Neotropics**

Walker LM, Leontyev D, Stephenson SL (2015) *Perichaena longipes*, a new myxomycete from the Neotropics. *Mycologia* 107:1012–1022.

##### **Abstract**

A new species of myxomycete, *Perichaena longipes*, is described from 56 sporocarp specimens that appeared in moist chamber cultures prepared with samples of decaying plant materials collected in Panama, Costa Rica and Brazil. This new species is distinguished from the morphologically similar species *P. pedata* on the basis of the much longer stipe, lighter peridium and the unique ornamentation of the capillitium. The nuc 18S ribosomal DNA sequences obtained from four specimens of *P. longipes* support the distinction of this new taxon and its separation from *P. pedata*. Furthermore, maximum likelihood phylogeny supports earlier evidence that species currently within the genus *Perichaena* do not form a monophyletic clade. Instead they appear to form three separate branches within the bright-spored clade. The first clade includes *P. longipes* together with several species of *Trichia* and *Metatrachia*, the second includes *P. pedata* and *P. chryosperma*, and the third clade is composed of *P. corticalis*, *P. depressa* and *P. luteola*.

##### **Introduction**

The genus *Perichaena* (order Trichiales, Myxomycetes) was erected by E.M. Fries in 1817 and currently encompasses 32 species (Lado 2005–2014). The genus is represented by both sporocarpic and plasmodiocarpic forms generally characterized by a thick, persistent peridium and spores that are yellow to red-brown in mass. Other than a few exceptions, members of the genus have a well-developed, typically branched capillitium, which is roughened, warted or

spiny to minutely annulate and lacks spiral bands (Poulain et al. 2011). The capillitial elements are normally irregular and when viewed by scanning electron microscopy (SEM) are sometimes covered by pits (Lado et al. 2009). Some species in the genus appear to have either a very wide or a very restricted distribution, whereas still others are known from only a single type locality. Examples of the latter, described solely on the basis of morphology, are *Perichaena frustrifilaris* Q. Wang, Y. Li and J.K. Bai (Wang et al. 2000), *P. grisea* Q. Wang, Y. Li and J.K. Bai (Wang et al. 2000) and *P. membranacea* Y. Li, Q. Wang and H.Z. Li (Li et al. 1990).

The genus *Perichaena* is currently assigned to the order Trichiales and is usually placed within the family Arcyriaceae, members of which are characterized by a tubular capillitium with no spiral bands (Neubert et al. 1993, Poulain et al. 2011). However, some authors place all forms with a tubular capillitium, including *Perichaena*, within the Trichiaceae, irrespective of capillitial ornamentation (Martin and Alexopoulos 1969, Nannenga-Bremekamp 1991).

A recent phylogeny of the bright-spored myxomycetes, based on a study of the full length nuc 18S ribosomal DNA (18S) and elongation factor (EF1 a) genes, indicated that three species of *Perichaena* (*P. corticalis* [Batsch] Rostaf., *P. depressa* Lib. and *P. luteola* [Kowalski] Gilert) fall into the same cluster as *Trichia* Haller, *Metatrachia* Ing and *Oligonema* Rostaf., whereas species of *Arcyria* F.H. Wigg. represent another phylogenetic branch (Fiore-Donno et al. 2013). Therefore the placement of the genus *Perichaena* in the family Arcyriaceae definitely was not supported by molecular data. However, the validity of the genus *Perichaena* itself did seem to hold because the three species included in that study did form a monophyletic clade (Fiore-Donno et al. 2013, Clark and Haskins 2014). All three species included are characterized by sessile, spherical sporocarps, minutely warted capillitium and similarly ornamented spores. Other species of *Perichaena* with stipitate or plasmodiocarpic sporocarps or other types of capillitium

and spore ornamentation hitherto had not been included in molecular phylogenetic analyses.

During a larger project by the first author within the Barro Colorado Nature Monument in Panama, a large series of stipitate specimens of *Perichaena* were collected, initially considered to be unusually long-stiped representatives of *Perichaena pedata* (Lister and G. Lister) G. Lister ex E. Jahn (Jahn 1919) but differing from the latter by the long stipe and distinctive ornamentation of the capillitium and spores. Later additional specimens of the same morphotype were identified from material collected in Costa Rica and Brazil. The unique set of morphological features and their stability between substrates and geographical regions suggested that the specimens probably represented a new species of *Perichaena*. However, it is commonly recognized that some morphological characters of myxomycetes, such as the length of the stipe, may be only the result of phenotypic plasticity (Nannenga-Bremekamp 1991). Therefore we wished to substantiate these taxonomic combinations with a comparison of the genetic variation within the group before erecting a new taxon.

The main purpose of this study was to confirm the distinctness of the proposed new species and also to understand its approximate phylogenetic position within the bright-spored clade of myxomycetes. To this end we sequenced a 59 region of the 18S gene, recently demonstrated as a useful barcode marker for myxomycetes (Fiore-Donno et al. 2012, 2013). This same locus also was sequenced in two other morphologically distinct species, *Perichaena pedata* (a stipitate form) and *P. chrysosperma* (Curr.) Lister (a plasmodiocarpic form). In addition, we generated sequences from four specimens of *Arcyria cinerea* (Bull.) Pers. and one from *A. leiocarpa* (Cooke) Masee to be included in the phylogeny. The publicly available sequences of three other species of *Perichaena* and another 14 species of Trichiales were included in the alignment to generate a more representative phylogeny.

## **Materials and Methods**

### *Field Sampling*

The initial (and largest) series of specimens were obtained from moist chamber cultures prepared with samples of dead plant material collected in the Barro Colorado Nature Monument (BCNM) in the Republic of Panama (9°06'31"N, 79°50'37"W). The site is a typical old-growth (>200 y), lowland (25–61 m) moist tropical forest (Wright et al. 2011) with a 4 mo dry season, an average annual rainfall of approximately 2600 mm and a mean monthly temperature of 26 C (Yavitt et al. 2011). Soils at this locality are Endogleyic Cambisols, which are highly weathered, moderately acidic and have a high clay content (Koehler et al. 2012).

Later several collections of the new morphotype from nearby Costa Rica were obtained from the UARK herbarium. These specimens from also were recovered from moist chamber cultures of samples of dead plant material 2 y prior, also by the first author. The collection site is in the Sarapiquí region, at the La Selva Biological Research Station (10°25'52"N, 85°59'47"W). Forests at this site are primarily old-growth, lowland, tropical wet forests with an average annual rainfall of approximately 4000 mm and temperatures of 19–31 C.

Finally a single specimen of the new morphotype was obtained from a third locality in Brazil (01°45'N, 61°08'W). This specimen appeared in a moist chamber culture of aerial leaf litter collected by I.L. Coehlo in a tropical wet forest in Caracaraí, Roraima, Brazil, as part of a separate survey underway in the laboratory of the third author.

### *Moist Chamber Cultures*

Culture methods are described here in detail only for those samples collected in Panama; equivalent methods were used in the two other surveys that also yielded specimens of this

putative new species. The sample materials (forest floor leaf litter and pieces of small woody debris) used to prepare the moist chamber cultures were collected in Jun 2012 and Aug 2013 by the first author. All samples were placed in small paper bags in the field, returned to the laboratory and air-dried. Afterward they were shipped to the University of Arkansas at Fayetteville for processing with the use of the traditional moist chamber culture technique (Stephenson and Stempen 1994). Over 2 y a total of 1008 moist chamber cultures were established and monitored 3–6 mo each. The total number of fructifications was more than 3500, 46 of which represent the putative new species. From Costa Rica we obtained an additional nine herbarium specimens, along with the one from Brazil. Therefore the putative new species is represented by a total of 56 specimens.

### *Microscopy*

Air-dried specimens were studied with a Zeiss Axioskop 2 Plus dissecting stereomicroscope. Temporary water slides and permanent slides prepared with polyvinyl lactophenol were studied with a Leica MSV226 light microscope (LM) equipped with differential interference contrast. The freeware program CombineZP (Hadley 2010) was used to create a composite digital image from several stacked images. Microscopic measurements were made with the program Axio Vision 4.8.0.0 (Carl Zeiss Imaging Solutions GmbH). Scanning electron microscopy (SEM) was carried out with an FEI Nova Nanolab 200 FIB/SEM microscope. Air-dried sporocarps were sputter-coated with gold-palladium to form a 5 nm cover and studied at 5–15 kV. All microscopy was carried out at the University of Arkansas.

Twenty-five spores and capillitial threads from five sporocarps were measured to estimate the range of variation. Size of the sporotheca, stipe and hypothallus were measured for



15 sporocarps. The range of variation for these main parameters is given as (minimum–) mean minus standard deviation–mean plus standard deviation (–maximum). Colors are according to the Munsell scale (Munsell 1905).

### *DNA Sequencing*

DNA was extracted from 5–6 sporocarps with the Invitex Spin Food Kit II (Stratec Molecular GmbH, Germany). Sporocarps were frozen at -80 C in 1.5 mL centrifuge tubes containing acid-washed glass beads 0.7–1.1 mm diam (Sigma Chemicals, USA). Frozen samples were vortexed 1 min at 30 Hz with a Wig-L-Bug grinding mill (Reflex, USA). We followed the protocol recommended by the manufacturer except for the final step, where DNA was eluted in 50 mL buffer (instead of 200 mL).

Partial sequences of the 18S (ca. 550 bp intron-free segment of the 5' end) were amplified with various primer combinations as proposed by Fiore-Donno et al. (2013). The primary primers used were S1F: AACCTGGTTGATCCTGCC (forward) and SU19R: GACTTGTCCTCTAATTGTTACTCG (reverse) although in some cases, such as if the initial primer pair was not working favorably, other primers and primer combinations were used. All primers and the combinations used for obtaining each sequence are provided (Supplementary material I).

The PCR reaction was carried out in 40 cycles (95 C, 2.5 min; 52 C, 30 s; 72 C, 1 min), regardless of primers. Results of the PCR were verified by electrophoresis in an agarose gel in TA buffer stained with GelRed<sup>TM</sup> Nucleic Acid Gel Stain (Biotium, Hayward, California). The amplicons were purified with MSB Spin PCRapace (Stratec Molecular GmbH, Germany) following the manufacturer's protocols except for eluting in 20 mL elution buffer (instead of 10

mL) in the final step. The amplification of the product for sequencing was carried out in 40 cycles (96 C, 70 s, 53 C, 5 s, 60 C, 4 min) with the same primers used for the initial PCR reaction of each specimen. Sequencing was performed on an Applied Biosystems 3130xl Genetic Analyzer at the University of Arkansas DNA Resource Center. Sequences were generated bi-directionally, assembled with the automatic function in Sequencher<sup>®</sup> 5.2 (Gene Codes Corp., Ann Arbor, Michigan) and manually inspected before alignment.

Four partial 18S sequences were obtained from the alleged new species—two from Panamanian specimens (LMW 2574 [UARK 54115]; LMW 2869 [UARK 54447]) and two from Costa Rican specimens (LMW 26151 [UARK 47993]; LMW 26264 [UARK 48715]). To compare the sequences of the new taxon with those of closely related taxa, we also sequenced the partial 18S of four specimens of *Arcyria cinerea*, one specimen of *A. leiocarpa*, one specimen of *P. chrysosperma* and three specimens of *P. pedata* (Supplementary material I). Although a total of 13 sequences were generated, only nine appeared to be unique. The other four sequences were identical to a sequence obtained from the same species, thus representing the same 18S genotype (see below) and were eliminated from analyses. All of the new sequences were deposited in GenBank under accession numbers (GBa) KP241117– KP241129.

### *Sequence Alignment*

The nine newly obtained sequences were aligned with 18 sequences of other members of the Trichiales studied by Fiore-Donno et al. (2013). These sequences represented the following taxa: *A. cinerea*, *A. denudata* (L.) Wettst., *A. globosa* Schwein., *A. incarnata* (Pers. ex J.F. Gmel.) Pers., *A. marginoundulata* Nann.-Bremek. and Y. Yamam., *A. stipata* (Schwein.) Lister, *Hemitrichia calyculata* (Speg.) M.L. Farr, *Metatrichia floriformis* (Schwein.) Nann.-Bremek., *M.*

*vesparia* (Batsch) Nann.-Bremek. ex G.W. Martin and Alexop., *P. corticalis*, *P. depressa*, *P. luteola*, *Trichia alpina* (R.E. Fr.) Meyl., *T. decipiens* (Pers.) T. Macbr. (two different genotypes), *T. sordida* Johannesen, *T. persimilis* P. Karst. and *T. varia* (Pers. ex J.F. Gmel.) Pers. Two representatives of the family Reticulariaceae (*Tubifera applanata* Leontyev and Fefelov and *T. ferruginosa* [Batsch] J.F. Gmel.) by Leontyev et al. (2014) were used as outgroup. An alignment of the 29 sequences was generated in MEGA 5.1 (Hall 2011, Tamura et al. 2011) with the automatic procedure implemented in multiple sequence comparison by log-expectation (MUSCLE). Minor changes in the automatic alignment were made by hand in BioEdit (Hall 1999). The alignment is available on TreeBASE, submission 17633 (treebase.org).

### *Phylogenetic Analyses*

We used 434 positions (out of a total of 791) that were aligned unambiguously; most portions of the variable helices did not align. Analyses of the aligned sections were carried out with maximum likelihood (ML) algorithm using MEGA 5.1 (Hall 2011, Tamura et al. 2011). The evolutionary model was chosen in MEGA 5.1 option find best dna/protein model as GTR with gamma-distributed rate variation across sites with a proportion of invariable sites. Branch support was estimated with 1000 bootstrap replicates.

### **Results**

Twenty-seven 18S sequences from members of the Trichiales, including the nine obtained in the present study, were used to construct the ML phylogeny. The topology appeared stable and was not dependent on (i) the inclusion or exclusion of sequences (ii) the usage of different alignment algorithms (Clustal W, MUSCLE or MAFFT) or (iii) the use of whole length sequences (when

available) or only the unambiguously aligned positions. The main purpose of the study was to confirm the genetic distinctiveness of the putative new species and its approximate position within the bright-spored clade. All other conclusions relating to the topology of the tree should be considered as preliminary.

Our phylogeny revealed that all studied representatives of the Trichiaceae form a monophyletic clade with full bootstrap support. This clade is subdivided on three branches. The first branch represents the genera *Trichia* (except for *T. decipiens*), *Metatrichia* and *Perichaena*, which at this time cannot be clearly held together by any single morphological character(s). The second branch corresponds to the genus *Arcyria*, which can be defined by mostly stalked sporocarps with “cellulate” stalks (stalks filled with spore-like cells), and a net-forming capillitium without spiral bands. The third branch consists of *T. decipiens* and *H. calyculata*, both of which are defined by stalked cellulate sporocarps and a capillitium with spiral bands. This branching pattern within the Trichiaceae corresponds fully to the phylogeny based on full-length sequences of both 18S and EFI a in the bright-spored myxomycetes (Fiore-Donno et al. 2013). This branching pattern indicates that among the main genera of the Trichiaceae only *Arcyria* seems to be monophyletic, with all other genera distributed among different branches and thus presumably characterized as para- or polyphyletic.

The five sequences of *Arcyria cinerea* included in this study (four generated in the lab of the third author and one derived from Fiore-Donno et al. [2013]) did not form a single cluster. Instead three of them clustered with *A. denudata*, *A. leiocarpa* and *A. marginoundulata*; another clustered with *A. stipata* and *A. globosa*; and the last one formed its own subbasal branch.

The putative new species appeared to be represented by two similar yet unique partial 18S genotypes (p distance 0.08, the calculation is explained below), each of which was

represented by two specimens from the same locality (Panama or Costa Rica). Together these two genotype sequences form a monophyletic group within the *Trichia-Metatruchia-Perichaena* clade. However, the putative new species did not cluster with the other species of *Perichaena*, including the morphologically similar *P. pedata*. Instead the six species of *Perichaena* included in this study formed three independent branches, one consisting of the sessile sporocarpic species (*P. corticalis*, *P. depressa*, *P. luteola*), another by the stipitate *P. pedata* and the (usually) plasmodiocarpic *P. chryosperma* and the third by the proposed new stipitate species.

### **Taxonomy**

***Perichaena longipes*** L.M. Walker, Leontyev and S.L. Stephenson, sp. nov. FIG.1 MycoBank MB810916

*Typification:* PANAMA. PANAMA: Barro Colorado Nature Monument, Gigante Peninsula, (9°06'31"N, 79°50'37"W), 50 m. Old-growth tropical moist forest, on forest floor leaf litter in moist chamber culture (pH 6.7), 10 Aug 2013, *L.M. Walker LMW 2574* (HOLOTYPE. UARK 54007; GBa KP241126).

*Etymology:* The name *longipes* (from the Latin *longus* – long, *pes* – leg) refers to the stipe, the most conspicuous feature of the new species.

*Diagnosis:* Sporocarps stipitate, 0.5–0.8 mm tall, solitary or sometimes scattered in small loose groups (Fig. 1a–e). Stipe long, straight or slightly inclined, plicate, dark brown (2.5R1/2) to black, ocher-yellow to yellow-brown (2.5Y6/8) in transmitted light (Fig.1f), 0.3–0.7mm long, 25–80 mm diam. Hypothallus discoid, concolorous with the stipe, 0.1–0.4mm diam (Fig. 1c).

Sporotheca globose, 0.15–0.25 mm diam, light yellow to tan (7.5–10Y7/4–6), darker at the base, smooth, sometimes with a weak iridescent shimmer (Fig. 1a, b). Columella absent. Peridium single, tough and persistent, warted on the inner surface (Fig. 1g, h). Capillitium bright yellow in transmitted light (7.5Y8/8–10), tubular, (2.6–)3.2–5.8(–6.2) mm diam, branched and anastomosed (Fig. 1i, j), densely covered with papillate, branched, coral-like projections (Fig. 1k–n), free ends scanty, short, obtuse, sometimes with a short acuminate tip on a swollen base (Fig. 1j), the small pits (ca. 0.5 mm) sometimes are present between papillae as observed under SEM (Fig. 1l). Spores free, (7.5–)7.8–9.1(–10.7) mm, light yellow to tan in mass (7.5–10Y8/6), almost hyaline in transmitted light (Fig. 1g, i, j), as observed under LM spores appear smooth, whereas under SEM they are verrucose, with a flat cap on the tip of each wart, these caps are star-like as observed from above (Fig. 1p, q).

*Habitat and distribution:* Sporocarps of *Perichaena longipes* appeared primarily on forest floor leaf litter (47) but also occurred on small pieces of coarse woody debris (7) or on aerial leaf litter (1) in moist chamber cultures. Considering that it was recorded from localities extending from Costa Rica to Brazil, the species seems to occur in similar microhabitats throughout the Neotropics. Sporocarps of *P. longipes* always appeared in moist chamber cultures relatively late, usually after at least 4 wk of continuous culture, and sporocarps were either solitary or scattered (but never gregarious). The average pH of the moist chamber cultures in which *P. longipes* appeared was 6.5(5.0–8.1). These values are fairly typical for the substrates upon which most myxomycetes occur in nature (Stephenson and Stempen 1994).

*Other specimens examined:* PANAMA: same location, substrate and date as HOLOTYPE, *L.M. Walker LMW 268* (UARK 53971), *LMW 1850* (UARK 53985), *LMW 2007* (UARK 51762), *LMW 2754* (UARK 54115), *LMW 2777* (UARK 54129). COSTA RICA: Sarapiquí, La Selva Biological Research Station, (10°25'52"N, 85°59'47"W), 100 m. Primary tropical wet forest, on leaf litter and pieces of small woody debris in moist chamber culture (pH 6.2), 21 Jan 2012, *L.M. Walker LMW 26151* (UARK 47993; GBa KP241120); same location, substrate and date as previous, *L.M. Walker LMW 26264* (UARK 48715; GBa KP241121). BRAZIL: Caracaraí, Roraima, (01°45'N, 61°08'W), 233 m. Primary Amazon forest, on aerial litter in moist chamber culture (pH 5.3), 1 Feb 2014, *I.L. Coelho ILC 30961* (UARK 54507). All of the 47 remaining specimens were also deposited at UARK, although they have not necessarily been examined with the same degree of detail as those listed above.

## **Discussion**

### *Limitations*

This study may be considered limited by the use of partial gene sequences of only one gene and by the limited number of sequences used in building the phylogeny. However, the topology of the tree presented is stable (see above) and fully corresponds to the topology obtained by Fiore-Donno et al. (2013), who used full-length sequences of two genes (18S, EF1a). This provides additional evidence of the validity of the 59 domain of 18S rDNA as a molecular barcode for species delimitation in myxomycetes. However, we still consider the phylogeny presented here as preliminary.

### *Validity of the Genus*

Our 18S phylogeny clearly supports the separation of *Perichaena longipes* from all other species of myxomycetes. However, *P. longipes* appeared to be much closer to *Trichia alpina*, *T. varia* and *Metatrachia floriformis* than to other species of *Perichaena*, and this relationship is supported by a high bootstrap value (0.95). This fact calls into question the appropriateness of classifying *P. longipes* as a member of the genus *Perichaena*. However, the new species cannot be assigned to *Trichia* or *Metatrachia* because both genera are characterized by having mostly unbranched capillitial threads that are ornamented with spirals (Martin and Alexopoulos 1969). Our species has a branched and anastomosed capillitium ornamented with coral-like papillae and small pits; a type of ornamentation considered to be typical for the genus *Perichaena* (see above). Therefore we observe here the evident contradiction between morphology and phylogeny. The situation becomes even more complicated when we acknowledge that all studied genera of the Trichiaceae, except *Arcyria*, do not appear to be monophyletic in this phylogeny. Instead *Perichaena* appears to be paraphyletic, represented by three different clades. Members of the genus *Trichia* are found in two of the three main branches of the Trichiales and therefore appear to be polyphyletic, as was indicated by Fiore-Donno et al. (2013). Finally, two members of the genus *Metatrachia* reveal varying results, either appearing to be sister or not sister to members of *Trichia* (results of Fiore-Donno et al. [2013] and the phylogeny generated herein, respectively). Therefore, based on the phylogenetic data, it appears that numerous divisions within the Trichiaceae should be re-evaluated, although not in the context of this study due to the small sampling and the inclusion of only a partial gene sequence from a single gene. Such a revision should only be carried out with a multiple gene phylogeny that includes a significant number of specimens for each species. Instead, with the information available herein, we can



only follow the current morphological concept for genera within the Trichiaceae, which assigns our new species to the genus *Perichaena*.

### *One or Two Species?*

Sequences of the 18S were generated from four different specimens of *P. longipes*. The two specimens collected in Panama appear to have an identical 18S genotype, as do the two specimens collected in Costa Rica; however, there is an 8% sequence divergence between the two genotypes. This brings us to question whether these two 18S genotypes represent one or two different species. To answer similar questions in another group of Lucosporideans, Leontyev et al. (2015) proposed an approach for calculating the p distances between all 18S genotypes. This distance index is calculated as the proportion (p) of nucleotide sites at which two sequences are different and varies from 0 (sequences are identical) to 1 (sequences share no common nucleotides) (Hall 2011, Tamura et al. 2011). It was shown that there exists a natural gap between p values when comparing specimens of the same species (low p values) to those of different species (high p values) (Leontyev et al. 2015). We could not carry out these calculations in the present study because most of the species are represented only by one specimen. We, however, can compare our data with the natural threshold values proposed by Leontyev et al. (2015) to distinguish among species within the bright-spored myxomycetes. The value proposed for distinguishing species within the bright-spored myxomycetes is  $P = 0.11-0.15$ , indicating that if the difference between two partial 18S genotypes is lower than 0.11 then they both belong to the same species whereas if the calculated p value is higher than 0.15 they belong to different species (Leontyev et al. 2015). Therefore the value  $P = 0.08$  obtained by comparing sequences of *Perichaena longipes* from Panama and Costa Rica corresponds to only

intraspecies genetic diversity and does not suggest the separation into separate species. This conclusion is supported by analyses reported herein that showed no morphological differences between specimens from Panama and Costa Rica.

When the same analysis is applied to the morpho-species *A. cinerea* the results are different. Herein, *A. cinerea* is represented by five specimens, each of which corresponds to its own 18S genotype (see FIG. 2). All of them have p distances much higher than mentioned species threshold (p 0.37–0.62). This in addition to the morphological diversity often seen in this morphospecies (not examined in great detail here) is further evidence to a proposed idea that *A. cinerea* not a single species but instead may be a species complex (Clark et al. 2002).

### *Morphological Analysis*

The distinctive set of morphological characters together with the unique 18S sequences indicate that *P. longipes* is a species of myxomycete new to science. It is assigned to the genus *Perichaena* on the basis of the thick and persistent peridium along with the presence of a well-developed, irregular, branching and ornamented capillitium lacking spiral bands, the surface of which is covered by pits when viewed by SEM (Novozhilov et al. 2008, Lado et al. 2009, Poulain et al. 2011). The bright yellow spores also validate the placement of *P. longipes* into the higher-level, bright-spored clade of the myxomycetes (Fiore-Donno et al. 2013).

As noted in the taxonomic diagnosis, the single most notable feature of *P. longipes* is the long stipe of the sporocarp. Although a stipitate sporocarp is not the most common expression for species of *Perichaena*, it is by no means unusual. At least six other species of *Perichaena* are characterized by a well-developed stipe. These are *P. heterospinospora* Novozhilov, Zemlyanskaya, Schnittler and S.L. Stephenson (Novozhilov et al. 2008), *P. papulosa* C.H. Liu

and J.H. Chang (Liu et al. 2007), *P. pedata*, *P. polygonospora* Novozhilov, Zemlyanskaya, Schnittler and S.L. Stephenson (Novozhilov et al. 2008), *P. pulcherrima* Petch (Petch 1909) and *P. reticulospora* H.W. Keller and D.R. Reynolds (Keller and Reynolds 1971). Four other species—*P. areolata* Rammeloo (Rammeloo 1984), *P. calongei* Lado, D. Wrigley and Estrada (Lado et al. 2009), *P. chrysosperma*, and *P. stipitata* Lado, Estrada and D. Wrigley (Estrada-Torres et al. 2009)—have reduced or short stipes. However, it should be noted that the latter are not always stipitate but instead may display a variety of sporocarp types, even within a single fruiting. In contrast, *P. longipes* appears to form only erect, stipitate sporocarps, because there was never any evidence of sessile or plasmodiocarpic forms in our collections. Therefore in many instances *P. longipes* may be distinguished from other species of *Perichaena* simply on the basis of the much greater length of the stipe. When comparing stipe lengths to the two most morphologically similar species (*P. pedata*, *P. stipitata*), the stipe in *P. longipes* is commonly more than twice the length found in either of these other species. Moreover, the stipe of *P. stipitata* usually has a frosting of lime and thus appears white (Estrada-Torres et al. 2009). It is noteworthy that in the wet tropics, a number of species of myxomycetes have been observed to possess longer stipes when compared to the same species found in temperate regions of the world, presumably because the greater height above the substrate may aid in more effective drying of spores (Schnittler and Stephenson 2000). This is another reason that multiple characters should be evaluated to support the status of a separate species for *P. longipes*. Because of the general morphological similarities between *P. longipes*, *P. pedata* and *P. stipitata* although to a lesser extent in the second instance. A quick reference (Table I) is included herein for morphological comparison between the three species.

The sporotheca of *P. longipes* is similar to those of many other stipitate species of

*Perichaena*, excluding the sporocarp of *P. polygonospora*, which is much smaller (0.05–0.1 mm), lacks a capillitium and has a unique polygonal spore shape (Novozhilov et al. 2008). The sporocarp of *P. longipes* is light yellow to tan, darker at the base, and the peridium is smooth, sometimes with a weak iridescence (Fig. 1a, b). Most other stipitate species of *Perichaena* are darker and/or do not have a smooth peridia surface. For instance, the sporotheca of *P. papulosa* is brownish orange and has an apical wart (Liu et al. 2007), *P. polygonospora* is buff or buff-yellow and covered with orange-brown protuberances (Novozhilov et al. 2008) and *P. calongei* is yellow to dark brown, with dark lines marking the edges of the peridia plates of dehiscence (Lado et al. 2009). Another stipitate species, *P. areolata*, although more similar in color (light brown to yellow) to *P. longipes* differs in that the peridium has a mottled appearance and consists of two layers versus the single layer in *P. longipes* (Lado et al. 2009).

A peridium with a single layer also occurs in *P. pedata*, *P. stipitata*, *P. polygonospora*, *P. heterosporospora* and *P. papulosa*. However, in *P. longipes* the peridium is relatively tough, persistent and the inner surface is densely and irregularly verrucose. None of the other stipitate species of *Perichaena* have a similar peridium. *Perichaena pedata* and *P. stipitata* do have a peridium in which the inner surface is ornamented, but the ornamentation is different in all three instances (Table I). The inner peridium of *P. pedata* consists of a few low, rounded ridges, whereas in *P. stipitata* the ornamentation is composed of large ocellate elements (Estrada-Torres et al. 2009). *Perichaena areolata*, *P. chrysosperma* and *P. polygonospora* also have a verrucose inner surface of the peridium, but they are distinctly different in overall morphology. The published descriptions of *P. papulosa*, *P. reticulospora*, *P. heterosporospora* and *P. pulcherrima* do not include any information on the structure of the inner peridial surface.

The capillitium of *P. longipes*, av. 3.2–5.8 mm diam, is among the largest found in any of the stipitate species of *Perichaena*. The relatively large size is explained in part by the well-developed ornamentation, which cannot be confidently excluded from consideration when the diameter is measured. In addition to the large size of the capillitium, the ornamentation is unlike that of any other described species in the Trichiaceae. The surface of the capillitium in *P. pedata* is irregularly and densely ornamented with papillate, branched, coral-like projections that are separated by pits (ca. 0.5 mm diam) visible only by SEM (Fig. 1k–n). In both of the species (*P. pedata*, *P. stipitata*) that are the most morphologically similar to *P. longipes* (Table I), the ornamentation is very different. In *P. pedata* the capillitium is ornamented with regularly and sparsely distributed spines and there are no pits between them (Estrada-Torres et al. 2009). *Perichaena stipitata* has a capillitium characterized by ornamentation consisting of large craters (3.8–6.8 mm) when viewed by SEM (Estrada-Torres et al. 2009) and there are no spines or other projections.

The difference between *P. longipes* and the other stipitate species with respect to spore size and ornamentation is not strong. However, for each pair of species being compared it is easy to find at least one distinguishing character. The spores of *P. pedata* are only sparsely warted, whereas those of *P. stipitata* and *P. longipes* are densely warted. This causes the spores of *P. stipitata* to be similar to those of *P. longipes*; however, they are considerably larger, 12.0–15.0 mm vs. 7.8–9.1 mm in *P. longipes* (Poulain et al. 2011).

The morphology of the warts covering the spores of the new species is a noticeable feature because these structures have flattened, star-shaped tips when viewed by SEM (Fig. 1p, q). Flat tips like this are unknown in other stipitate species except for *P. papulosa*, *P. chryso sperma* and *P. calongei*. However, they are common in the genus *Trichia* (*T. contorta*, *T.*

*munda* [Lister] Meyl., *T. sordida*, *T. varia*), a taxon to which our new species seems to be closely related.

The stipitate species of *Perichaena* discussed in this paper are included in the key.

### Key to the Stipitate and Subsessile Species of *Perichaena*

- 1 Spores polygonal ..... *P. polygonospora*
- 1' Spores globose or subglobose to ovate ..... 2
- 2 Spores banded-reticulate ..... *P. reticulospora*
- 2' Spores not banded-reticulate but with ornamentation ..... 3
- 3 Spores ornamented with scattered tall pyramidal spines ..... *P. heterospinospora*
- 3' Spores densely ornamented with warts or spines, but less than 0.5  $\mu\text{m}$  high ..... 4
- 4 Spore diam 12–18  $\mu\text{m}$  ..... 5
- 4' Spore diam 7–12  $\mu\text{m}$  ..... 6
- 5 Stipe calcareous, grayish or brown to black, spore diam 12–15  $\mu\text{m}$  ..... *P. stipitata*
- 5' Stipe not calcareous, brown or red brown, spore diam 14.5–18  $\mu\text{m}$  ..... *P. pulcherrima*
- 6 Peridium single ..... 7
- 6' Peridium double ..... 9
- 7 Sporocarp with apical protuberance, dehiscence leaving a cup-like base ..... *P. papulosa*
- 7' Sporocarp without an apical protuberance..... 8
- 8 Peridium with irregular dehiscence, capillitial tubules less than 3.5  $\mu\text{m}$  diam with scattered small spines ..... *P. pedata*
- 8' Peridium persistent, capillitial tubules between 3.2 and 5.8  $\mu\text{m}$  and densely ornamented with papillate, branched, coral-like projections ..... *P. longipes*

- 9 Sporocarp subsessile to sessile or plasmodiocarpic, capillitial tubules with long spines,  
2.9–5.5  $\mu\text{m}$  long ..... *P. chrysosperma*
- 9' Sporocarp subsessile to sessile but not plasmodiocarpic, capillitial tubules with spines  
less than 3  $\mu\text{m}$  long ..... 10
- 10 Peridium marked with dark lines along edges of the plates of dehiscence, capillitial  
tubules with spines, granules, or pits ..... *P. calongei*
- 10' Peridium not marked with dark lines, dehiscence not along plates, capillitial tubules with  
regularly distributed spines ..... *P. areolata*

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## Tables and Figures

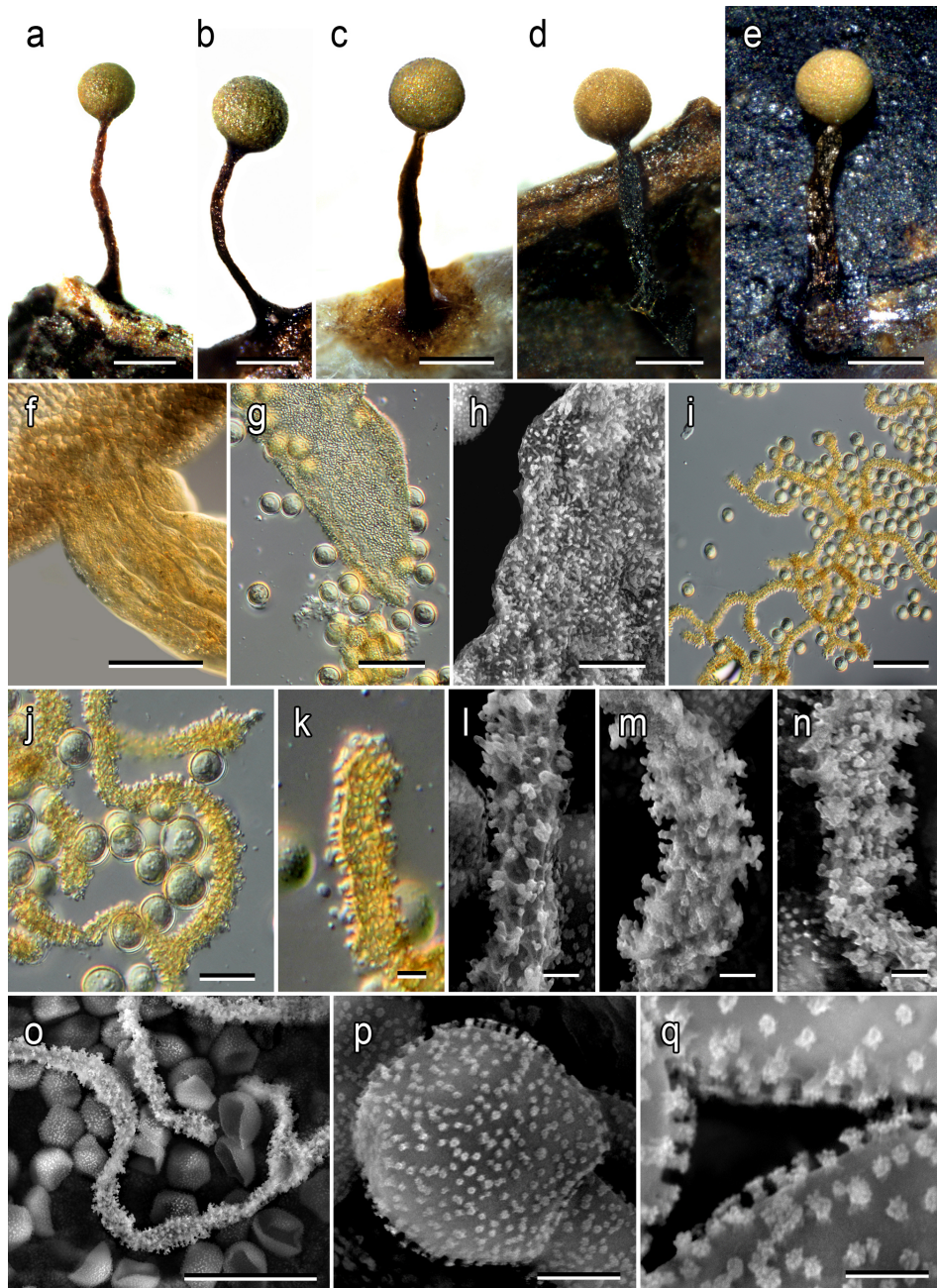
**Table 1.** Morphological characters of three stipitate species of *Perichaena*<sup>a3</sup>

	<i>Perichaena longipes</i>	<i>Perichaena pedata</i>	<i>Perichaena stipitata</i>
Total height (mm)	0.5–0.8	0.2–0.8	0.08–0.38
Diam of sporocarp (mm)	0.15–0.25	0.2–0.5	0.1–0.5
Color of sporotheca	Bright yellow to ochraceous	Ochraceous or fawn	Orange yellow to dark brown
Shape of sporotheca	Globose to subglobose	Subglobose	Subglobose to subhemispheric
Color of stipe	Dark brown to blackish	Dark brown to blackish	Calcareous and white, or brown to black without calcium
Length of stipe (mm)	0.3–0.7	0.45–0.60	0.1–0.5
Diam of stipe (mm)	0.25–0.80	1/2 to twice the diam of the sporophore	0.05–0.38
Structure of stipe	Plicate	Stout and roughened	Filled with crystalline deposits and refuse matter, sometimes striated
General structure of capillitium	Branching, tubular, free ends are scanty and obtuse	Profuse and branching	Scanty, branching, tubular, few free ends
Color of capillitium	Yellow	Yellow	Yellow
Ornamentation of capillitium	Densely ornamented with irregular spines and warts	Small, regular, scattered spines	Irregular with large holes (3.8–6.8 $\mu\text{m}$ )
Ornamentation of capillitium by SEM ( $\mu\text{m}$ )	Pits ( $\sim 0.5 \mu\text{m}$ )	Not reticulate or pitted	Holes (3.8–6.8)
Diam of capillitium ( $\mu\text{m}$ )	3.2–5.8	1.5–3.5	1.4–3.6
Spore size ( $\mu\text{m}$ )	7.8–9.1	9.0–11.0	12.0–15.0

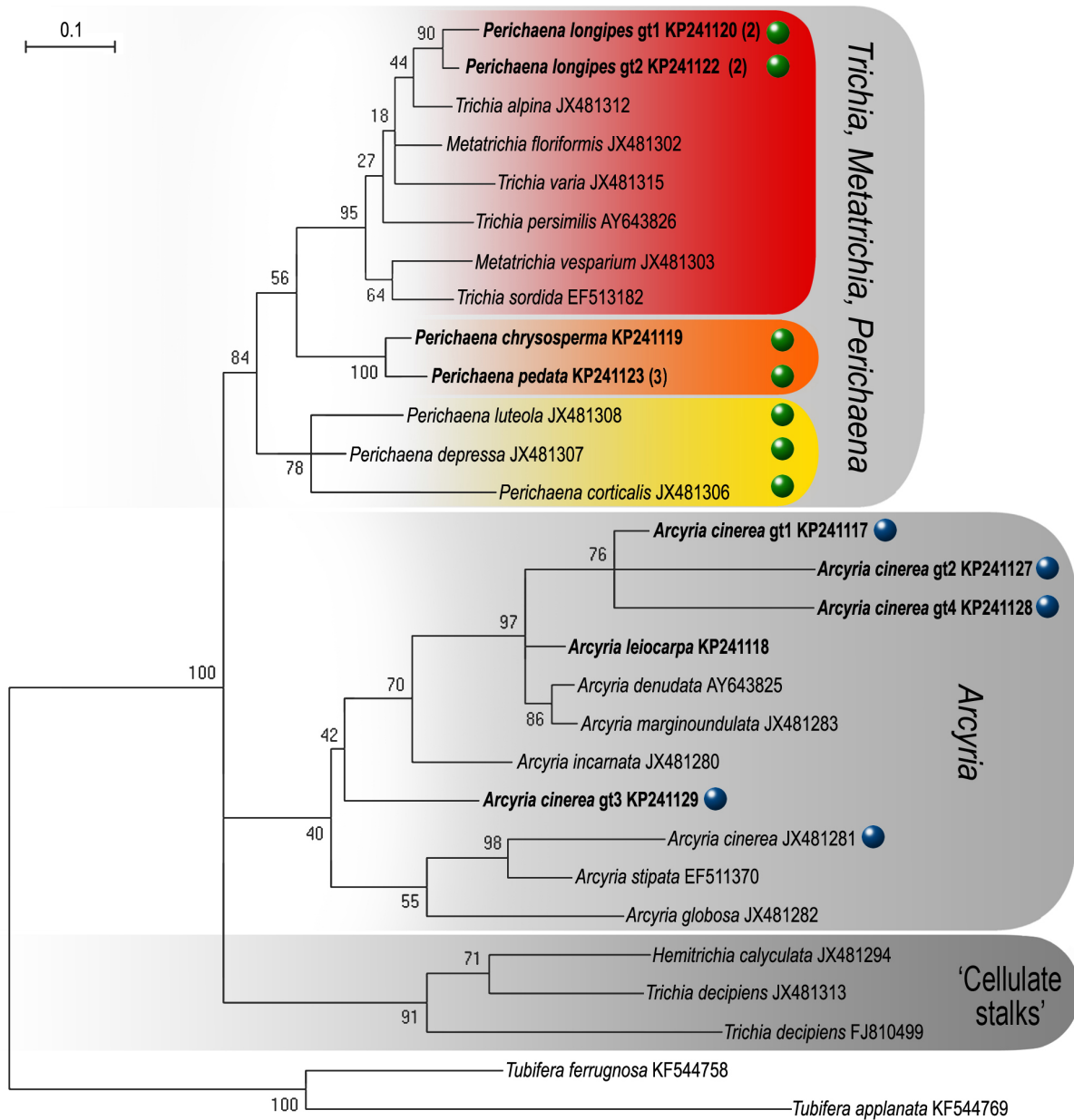
<sup>4a</sup> Characters of *P. longipes* are given according to available herbarium material, whereas characters of *P. pedata* and *P. stipitata* are given according to Estrada-Torres et al. (2009) in conjunction with Poulain et al. (2011).

**Table 1. (Cont.).**

	<i>Perichaena longipes</i>	<i>Perichaena pedata</i>	<i>Perichaena stipitata</i>
Color of spore mass	Bright yellow	Bright yellow	Orange yellow
Spore ornamentation	Prominent and abundant warts, flattened at apex and resembling a star shape	Minutely warted	Very flattened warts
Peridium	Single, thick, persistent	Single, thick, persistent	Single, membranous,
Inner peridial ornamentation	Densely verrucate	Short, rounded ridges and various sparse verrucate elements	Ocellate and weakly wrinkled



**Figure 1.** *Perichaena longipes*. a–e. General view of sporocarps. f. Stipe and the base of sporotheca as viewed in transmitted light. g. Peridium and spores in transmitted light. h. Inner surface of the peridium. i–j. Capillitium and spores. k. Optical section of a capillitial thread. l–n. Details of the ornamentation of capillitial threads. o. Spores and capillitium as viewed under SEM. p. Spore. q. Detail of spore ornamentation; bar. Specimens: a, b, h, j–q. *LMW 2574* (UARK 54007); c–g, i. *LMW 1850* (UARK 53985). Bars: a–e = 200  $\mu\text{m}$ ; f = 500  $\mu\text{m}$ ; g = 30  $\mu\text{m}$ ; h = 5  $\mu\text{m}$ ; i = 50  $\mu\text{m}$ ; j = 10  $\mu\text{m}$ ; k = 2  $\mu\text{m}$ ; l–n = 2  $\mu\text{m}$ ; o = 20  $\mu\text{m}$ ; p = 2  $\mu\text{m}$ ; q = 0.5  $\mu\text{m}$ .



**Figure 2.** Phylogenetic position of *Perichaena longipes* within the Reticulariaceae. The tree is based on partial 18S sequences (791 bp, 434 aligned positions retained) constructed with MEGA5.1 and rooted with the genus *Tubifera*. ML bootstrap replicates are shown for each branch. Each species name is accompanied by the GBA number of the 18S rDNA gene sequence used in the phylogeny (one representative for each genotype if more than one). Green (or dark) circles mark representatives of the genus *Perichaena* and the blue (or light) ones, the morphospecies *Arcyria cinerea*. Sequences obtained in this study are shown in bold. The number of identical 18S genotypes (obtained in this study) are given in parentheses (if present).

**Supplemental Material 1.** Identifications, 18S genotypes, GenBank accession numbers and localities of the specimens examined in the present study.

## V. Concluding Remarks

The research described herein has greatly expanded our knowledge of myxomycetes in two major respects, first by providing an updated and timely discussion concerning myxomycete taxonomy and classification including a much needed outline to present the various myxomycete life history strategies and their potential impacts on the biology of and research relating to myxomycetes and second, allowing, for the first time, a glimpse into the ecology of litter-inhabiting myxomycetes in the nutrient-poor lowland tropical forests of Panama and their response to nutrient addition. This research together highlights the importance of taxonomy in the framework of ecology. Within each of the three chapters in this dissertation, specific contributions to the field of myxomycete research were made.

Chapter two of this defense titled *The species problem in myxomycetes revisited* (Walker and Stephenson 2016) is a review of species concepts that may be applied to the myxomycetes. Because of the numerous alternative life history strategies and morphological plasticity commonly observed in the group, the myxomycetes challenge every proposed species concept to date. It has been several years since the last thorough discussions of this topic were written (e.g., Clark 2000; Keller and Everhart 2008) but more important than the amount of time that has passed is the technological advances that have taken place since that time. Traditional taxonomists are dwindling in numbers while molecular microbiologists are taking their place across the research landscape. There is now one fully sequenced myxomycete genome, which is that of the model organism *Physarum polycephalum* (Minx et al. 2015) and it is now possible to directly target myxomycetes from environmental samples to sequence in a high-throughput manner (Clissmann et al. 2015; Fiore-Donno et al. 2016). As our reliance on these technologies increase it is important that the discussion concerning the concept of a species continues and



ensure that the definition of a species remains compatible with new sources of data, at least to the extent that the great deal of historical data (based upon morphological information) remains relevant and can be built upon.

Furthermore, chapter two provides several specific, physical contributions to the literature. One is a newly generated figure of the myxomycete life cycle (courtesy of Angela Mele, Ch. 2, Figure 1) which not only describes a 'typical' myxomycete life cycle, but it also includes several alternative strategies as well as a hypothetical life cycle that to my knowledge has not before been included in any life cycle figure. This hypothetical cycle first described by (Indira 1964, 1969) is the generation of amoeboid cells directly from a plasmodium which could potentially act as gametes. Additionally, a table was generated (Ch 2, Table 1) to summarize the many years of tedious culture and microscopic research (cited throughout the text), which has led to a better understanding of myxomycete life history strategies. This table details numerous potential life history strategies (but without the potential complications of polyploidy or mutation), which will be a valuable resource for researchers in numerous areas of myxomycete research. Finally, chapter two ends by supplying an updated list of ideal components to include for new species descriptions going forward. Although not entirely different from previous recommendations (e.g., Schnittler and Mitchell 2000; Keller and Everhart 2008), the discussion surrounding molecular phylogenetics and molecular markers were enhanced herein based upon technologies that had emerged since the publication of those earlier works).

Chapter three of this dissertation titled *The response of myxomycete communities to 14 years of N, P and K addition in a lowland tropical rain forest*, described the first long-term nutrient fertilization experiment in the tropics to include protists. The data collected during that study which included over 3,500 records and over 2,000 fruiting body collections, comprises

what may be the largest data set of its kind for any one site. In collaboration with the Smithsonian Tropical Research Institute in Panama, a large, fully factorial NPK fertilization experiment was utilized allowing for the investigation of the litter-inhabiting myxomycete community at this site, and also the possible limitation of three major macronutrients (N, P and K) to that community. That nutrient fertilization experiment is a significant contribution to the broader research community as it represents the longest running nutrient addition study in any old-growth lowland tropical forest in the world (Wright et al. 2011). This was also the first example of a K fertilization treatment experiment in any old-growth tropical forest and is the very first to incorporate protozoans. Experiments such as these derive their ultimate value from the accumulation of large amounts of data.

Indeed, a large amount of information is available for various communities (e.g., trees, arthropods, fungi) that have been studied at this site. Despite the abundance of evidence that numerous other communities at the experimental site are limited by the supply of various nutrients (e.g., Wright et al. 2011; Sayer et al. 2012; Turner et al. 2013) no consistently significant effect of nutrient addition to the litter-inhabiting myxomycete community was identified. Only a marginal treatment effect was identified and only in one of two datasets generated in the context of that study. In 2012 myxomycete abundance and richness in small woody debris increased in response to P addition alone and P in combination with N (NP) as well as K (PK) although to a slightly lesser degree. The addition of N or K alone, however, did not appear to increase abundance or richness in the myxomycete community. Although both of those findings were in line with the hypotheses, when repeated in 2013, no treatment effects were identified in the myxomycete litter-inhabiting community. Furthermore, the nutrient fertilizations did not appear to effect the myxomycete species composition in the litter. The

surprising and unexpected results obtained in that study raised numerous valuable questions concerning myxomycete ecology and biology as well as more fundamental questions surrounding the methodologies employed for myxomycete studies. Furthermore, the data generated therein holds many possibilities for further study. The large amount of publicly available data for other communities and systems at the experimental site could be analyzed in combination with data obtained herein to test new hypotheses (e.g., myxomycete response to various soil characteristics, plant communities, etc.). Additionally, due to the very large number of fruiting bodies collected at the field site (> 2,000), which are securely stored in the herbarium at the University of Arkansas, many opportunities await further morphological and genetic studies. In fact, I have already utilized these collections to generate a small subunit rDNA sequence database. This database was generated for use in my ongoing high-throughput, environmental targeted sequencing research approach to investigate the soil-inhabiting myxomycete community at this site.

The final chapter of this dissertation is titled *Perichaena longipes, a new myxomycete from the Neotropics* and describes a new species found during the dissertation research in Panama (Walker et al. 2015). This species was abundant in samples from Panama and was also identified in Costa Rica as well as Brazil, indicating that it has a relatively wide distribution throughout the Neotropics further supporting the significance of this contribution to the literature. Additional contributions from this chapter include a new dichotomous key for morphological identification of the stipitate and sub-sessile species of *Perichaena*. Nine small subunit rDNA sequences were also generated therein and made publicly available. And the phylogeny produced within that study combined with the unique morphology of *P. longipes*, provided insight into the evolution and taxonomy of myxomycetes and more specifically

provided support for the non-monophyly of *Perichaena* within the Trichiales as first indicated by (Fiore-Donno et al. 2013).

All together my dissertation provides interesting new information about tropical litter-inhabiting myxomycetes. This work proposes intriguing new questions regarding myxomycete biology and evolution and also highlights other long-standing and fundamental questions. In-depth ecological study of myxomycetes is only recently becoming a possibility as new technologies continually emerge. Therefore, throughout this dissertation, discussions concerning the species concept in myxomycetes as well as those challenging the traditional methods of study are quite timely and should aid in future research of myxomycetes.

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