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Intraspecific Variation In Two Cosmopolitan Myxomycetes, Didymium Squamulosum And Didymium Difforme (physarales: Didymiaceae)

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INTRASPECIFIC VARIATION IN TWO COSMOPOLITAN MYXOMYCETES, DIDYMIUM SQUAMULOSUM AND DIDYMIUM DIFFORME (PHYSARALES: DIDYMIACEAE)

INTRASPECIFIC VARIATION IN TWO COSMOPOLITAN MYXOMYCETES, DIDYMIUM SQUAMULOSUM AND DIDYMIUM DIFFORME (PHYSARALES: DIDYMIACEAE)

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ABSTRACT

The myxomycetes (plasmodial slime molds or myxogastrids) are one of three groups considered to be true slime molds (class Eumycetozoa *sensu* Olive 1975). Two vegetative states—amoebae and plasmodia—along with a spore-producing fruiting body characterize the life cycle of the myxomycetes. These organisms are associated with decaying plant material and are found in all terrestrial habitats worldwide. A number of species are considered cosmopolitan, being found worldwide, where they are associated with a diversity of microhabitats and substrates. A review of the literature, including molecular investigations in all three groups of slime molds, is presented, and this is followed by four original studies of the intraspecific variation that exists in two cosmopolitan species of myxomycetes. Molecular intraspecific variation in these two species, *Didymium squamulosum* (Alb. & Schwein.) Fr. and *Didymium difforme* (Pers.) S.F. Gray, was investigated using DNA sequence analysis. Initially, 14 specimens of *Didymium squamulosum* from widely distributed localities were examined, using the internal transcribed spacers (ITS) of nuclear ribosomal DNA (nrDNA). Although this genetic marker was found to be too variable for continued analysis, it did offer the first evidence that significant intraspecific variation exists within cosmopolitan species of myxomycetes. An additional genetic marker located within the mitochondrial small subunit (mtSSU) was investigated for 96 collections of *Didymium squamulosum* from worldwide localities and 56 collections of *Didymium difforme* distributed among three widely separated regions. For both species, conclusions were derived from molecular analyses using Bayesian methods and a haplotype network from TCS. It was concluded in both species that for this genetic marker no clear geographical assemblages emerged. While some sequences formed groups based on biogeography, there were a number of instances in which sequences from specimens that originated from distant geographical localities were more closely related to each other than to sequences from specimens obtained in nearby localities. In *Didymium squamulosum*, four morphological characters were observed for each collection and mapped onto the gene tree produced using Bayesian methods. While this species is known to have great diversity in morphology, no patterns emerged which would suggest that observed morphological diversity was related to molecular variation. This is the first

molecular evidence that morphological diversity in a cosmopolitan species of myxomycete is the result of phenotypic plasticity rather than genetic divergence. Further evidence for phenotypic plasticity was obtained from an effort to culture each specimen of *Didymium squamulosum* spore-to-spore on agar, which resulted in only two successful cultures. In both cases, the fruiting bodies exhibited a degree of variation in morphological diversity that was different from the original specimen that had developed under natural conditions in the field.

This dissertation is approved for
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DEDICATION

This body of work is dedicated to four different people who participated in this endeavor in very different, but significant ways. To my mother, Rebecca Winsett, the first PhD in the family, your investment in my education was more than money, and I am beyond grateful. Sophia Sweeney, my friend and colleague, you cannot know the influence our conversations and time together had on this work. How fortuitous it was we took the same class all those semesters ago. Dr. Rosalyn Davis, you encouraged my confidence and kept all things in perspective. My success is due in no small part to you. Finally, although his contribution to my education and success cannot be described in this short space, I wish to include Dr. Steve Stephenson in this dedication. It was a privilege to work with you.

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Winsett, K. E., S.L. Stephenson. 2008. Using ITS sequences to assess intraspecific genetic relationships among geographically separated collections of the myxomycete *Didymium squamulosum.* Revista Mexicana de Micología 27: 59-65 *Published*

Chapter 1

Introduction and Hypotheses

The myxomycetes (myxogastrids or plasmodial slime molds) are terrestrial protists associated with decaying plant material. Along with the dictyostelids (cellular slime molds) and the protostelids, the myxomycetes make up a group of organisms commonly referred to as slime molds or in a more technical taxonomic sense the Eumycetozoa (*sensu* Olive 1975). It is known that these organisms feed upon bacteria and other unicellular organisms and probably play a significant role in nutrient cycling in terrestrial ecosystems (Stephenson and Stempen 1994). In their life cycle, myxomycetes have two vegetative stages. These are an amoeboflagellate—a unicellular form that can be an amoeba or a biflagellated cell depending upon environmental conditions—and a plasmodium, a multinucleate cell that can, and in at least one order often does, reach macroscopic dimensions. Following the plasmodium stage, fruiting bodies containing spores are formed. In most species, these fruiting bodies are macroscopic, reaching dimensions in the range of several to tens of millimeters in total extent. The myxomycetes are dispersed by spores, with an average spore diameter across all species of about 10 µm (Schnittler and Tesmer 2008). The size of these spores, along with some experimental evidence, supports the assumption that the dispersal of spores and thus the distribution of species is largely dependant upon wind (Tesmer and Schnittler 2007, Schnittler and Tesmer 2008, Stephenson et al. 2008).

A wind-based dispersal mechanism would explain the apparent global distribution of many species of myxomycetes. These cosmopolitan species are known as such because a similar morphology is found worldwide. Underlying genetic patterns, however, are more complex than the shared morphology would suggest, but intraspecific molecular patterns were previously unknown. It has been shown that some species are in actuality species complexes made up of both sexual and asexual strains and thus are multiple biological species (El Hage et al. 2000, Clark 2000). Complexes of asexual species are independently evolving units amassing molecular and potentially morphological variation independent of other strains within the same species (Clark 2000). The purpose of this dissertation research project was to develop a molecular dataset to describe intraspecific variation in two cosmopolitan species of myxomycetes.

For both species, collections to be examined were chosen to represent the global distribution of these organisms. These data were analyzed to determine if underlying geographical patterns existed that are not apparent when looking at morphology alone.

The majority of this work described herein addresses the cosmopolitan species *Didymium squamulosum* (Alb. & Schwein.) Fr. This species clearly exhibits morphological variation, and was also the subject of study that used isozymes in an effort to uncover any patterns that correlated to mating types and morphology (Martin and Alexopoulos 1969, El Hage et al. 2000). The present project builds upon the previous research by usingDNA sequence analysis to determine species boundaries and intraspecific variation. The body of work described herein represents the first intraspecific analysis of a myxomycete using molecular techniques.

A second cosmopolitan species (*Didymium difforme* [Pers.] S.F. Gray) was included that does not show the same range of macroscopic morphological diversity that has been observed in *Didymium squamulosum*. However, *D. difforme* is also found worldwide, and the DNA sequence analysis was applied to this species to determine if patterns of diversity would occur in a data set that included multiple collections from three very different geographical regions of the world.

HYPOTHESES

This project was directed towards evaluating the hypotheses below.

(1) Both cosmopolitan species form biogeographical clades based upon the analysis of DNA sequence data.

H_0: Analysis of DNA sequence data show the clades to be organized such that they are not based upon biogeography.

(2) Multiple sequences from the same part of the world are more closely related to each other than to sequences from isolates obtained in other parts of the world.

H_0: Sequences from multiple isolates collected in the same region of the world are associated with other isolates from different locations.

(3) Variation in morphological characters coincides with the clades on the gene tree constructed for *Didymium squamulosum*.

H_0: Variation in morphological characters does not occur according to the molecular patterns observed in each gene tree.

Following the introduction, the description of this dissertation project begins with a review of the literature that relates to the biology of these organisms and outlines all of the information previously available on the molecular biology of myxomycetes.

Chapters Three through Five of this dissertation address the intraspecific variation of a single species, *Didymium squamulosum.* Analysis of this species was initially attempted by sequencing the internal transcribed spacers (ITS) of the nuclear ribosomal DNA (rDNA). These results were published in the journal *Revista Mexicana de Micología* in 2008, and included as Chapter Three, formatted appropriately as a manuscript to this journal. The amount of variation produced by this marker was too high to be useful, and it was not used in subsequent investigations. However, geographical patterns emerged through analysis of short regions of the small and large subunit of the ribosomal DNA that flank the ITS as well as the entire 5.8S ribosomal DNA that separates the first and second ITS, suggesting that further analysis using an appropriate marker would more adequately describe these patterns. Chapter Four describes the use of another marker, a hypervariable region of the mitochondrial small subunit (mtSSU) along with a description of the variation that occurs in the morphology of specimens of *D*. *squamulosum* for a larger set of collections of this species than for the set used in the examination of ITS genetic variation. This chapter also contains the largest global dataset of collections for molecular analysis of any single species of myxomycete to date. The analysis of a subset of this dataset—all of the specimens from New Zealand—is described in Chapter Five. This subset includes a series of specimens of *D. squamulosum* collected within a single area (approximately 20 by 300 meters) at the same locality. This represents the first molecular investigation of a local population of myxomycetes.

The mitochondrial marker, mtSSU, was also used to analyze the variation within another cosmopolitan species of myxomycete, *Didymium difforme*, the description of which is found in Chapter Six. Chapter Seven consists of a synthesis of the findings of this project and concluding

remarks describing how these results fit in the larger picture of myxomycete biology, the biology of cosmopolitan organisms, and the ubiquity theory.

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Chapter 2

Introduction to the Myxomycetes

A. Biology of the Organism

1. Introduction

The myxomycetes are one of three groups of organisms traditionally recognized as true slime molds (class Eumycetozoa *sensu* Olive 1975). One group, the myxomycetes, are eukaryotic amoeboid organisms with trophic stages that feed upon bacteria and other microorganisms associated with decaying plant material in all types of terrestrial habitats. There are approximately 900 species of myxomycetes known worldwide (Lado 2001). They apparently occur in any terrestrial habitat where vegetation is present, including the Antarctic peninsula as well as all types of temperate and tropical habitats. Many species have a global distribution, occurring in many varied ecosystems worldwide (Stephenson and Stempen 1994). Until their recent inclusion into the molecular phylogeny of eukaryotic organisms (Baldauf and Doolittle 1997) our knowledge of all three groups of slime molds had been accumulated within two quite different fields of science—mycology (the study of fungi) and zoology (the study of animals). As a result, throughout the history of study for these organisms, a number of terms have been used collectively to describe the group. For the sake of clarity, nomenclatural terms, specified below, will be used throughout this work when discussing the slime molds.

In some cases, the taxonomic nomenclature and the common nomenclature are similar (e.g., Dictyostelia and dictyostelids), and in the present work each group will be refered to using the most commonly used names for the group in question, both individually and as a whole. The affinity of the group to the fungi was suggested by such early taxonomic terms as Myxogastres and Myxomycetes, originally proposed to imply a relationship to the Gasteromycete (puffball) fungi (Martin 1960, Martin and Alexopoulos 1969). The terms myxomycete and myxogastria remain common labels for one group of slime molds. In this work, “myxomycetes” will be used when referring to the plasmodial slime molds. In the mid-nineteenth century De Bary introduced the term Mycetozoa for the slime molds, reflecting his suggestion that the organisms were more closely related to the animals and not the plants and fungi (Martin 1959, Martin and Alexopoulos

1969). All three of these terms were suggested for the slime molds before the discovery and recognition of the two other groups, the Dictyostelia (herein referred to as dictyostelids or cellular slime molds) and the Protostelia (herein referred to as protostelids). Olive (1975) suggested the use of the term Eumycetozoa to include all three groups of slime molds, but although this term has been used as a formal taxon name, it will be used in this work as the common form "eumycetozoans" when informally referring to all three groups of slime molds collectively.

Many species of myxomycetes do not seem to have geographical barriers with respect to their distribution. The existence of these so-called cosmopolitan species, which have broad distributions on multiple continents and in varied habitats, is theoretically possible as a result of long distance dispersal. The large numbers and small size of the spores they produce allow them to travel long distances by various mechanisms, including wind currents.

It is not yet known, however, if this high dispersal potential, which in theory would allow for movement of spores between continents, is commonly realized. In fact, it has been hypothesized that individual species of myxomycetes are not genetically ubiquitous meaning genetic grouping would have localized distribution not evident by the common morphology worldwide. For example, results obtained from two studies (El Hage et al. 2000, Clark and Stephenson 2003) on the reproductive systems of *Didymium squamulosum* indicated that both non-heterothallic (presumably apomictic or asexual) and heterothallic strains of this species occurred in nature, suggesting that the *Didymium squamulosum* morphospecies is actually a complex of sibling biological species. These data imply there is much more to be understood about a particular species than can be perceived by a consideration of morphology alone.

The approximately 900 species described for the myxomycetes (Lado 2001) are based solely on the morphological species concept rather than the biological or phylogenetic species concepts. The phylogenetic species concept, which designates species based upon shared evolutionary history, is not used in part because of the deficiency in molecular phylogenetic data for the group. These taxa are described based upon patterns and differences in the morphological variation of the fruiting body. It is more difficult to determine myxomycete taxa based upon other species concepts because of the lack of understanding of the complex life

history of these organisms. The biological species concept (Mayr 1942) poses problems for the myxomycetes because asexual (apogamic) strains and two forms of sexual strains (homothallic and heterothallic) can occur within the same morphological species. In a documented case, the reproductive strategy changed *within* a strain in laboratory conditions from heterothallic to apogamic (Collins 1961, Collins 1963, Collins 1964, Collins 1976, Therrien et al. 1977, Collins 1979, Mulleavy and Collins 1979, Clark and Landolt 2001, Clark and Stephenson 2003, Clark et al. 2004).

With the advances made in the phylogenetic study of organisms, the phylogenetic species concept wherein a species is recognized as a taxon that includes those individuals with shared evolutionary history (Taylor et al. 2000) has become increasingly important. The functionality of this concept as it relates to myxomycetes is questionable with so little phylogenetic data available at this time. However, it seems that the goal to provide a classification based on shared evolutionary history is not in opposition to the goals of the morphological species concept, which classifies taxa based upon morphological characters, especially as data are collected to confirm or contradict traditionally taxonomically useful characters as in line with the constructed molecular phylogenies.

The morphological species concept will probably continue to be appropriate for general use with the myxomycetes for efficiency of communication considering the common morphologies are found worldwide and phylogenetic or mating type information is more difficult to determine. However, it is recognized that this does not offer the most detailed information relating to the true life history and relationships within and among species, especially as it is known that, as already mentioned, multiple biological species exist within a single morphological species (Clark 2000).

Studies of myxomycete genetics have revealed the presence of both sexual and asexual lines within a morphological species (Collins 1979, Clark 2000, El Hage et al. 2000). An apomictic line is genetically isolated, so that any accumulation of mutations leading to morphological variation would drive speciation. However, it is not practical to test and describe each apomictic strain because the variation from the typical form of the species would be minor or, perhaps, not detected (Clark 2000). For all practical purposes, the morphological species

concept is the best and strongest method for describing the different species of myxomycetes. However, the knowledge of the complexity of reproductive strategies and genetic variation, coupled with morphological variation, could theoretically provide considerable insight into the population biology and mechanisms of distribution within species of myxomycetes if patterns undetected by analysis of morphology alone are better documented and understood more completely.

The morphological species concept, as it applies to myxomycetes, unites a broad range of traits, including multiple accepted states per character within individual species. It is assumed, based on such evidence, that ecological biotypes within these broadly defined species must occur (Stephenson et al. 2008). Moreover, it is known that some cosmopolitan species are actually genetically isolated, presumably apomictic, lines separated from other such clonal lines by geography (El Hage et al. 2000, Stephenson et al. 2008). A morphological species is known to exhibit variations in morphology from one region to the next. Because of this and that apomictic lines are capable of independent evolution, it stands to reason there may be a relationship between intraspecific morphological variation and genetic variation within morphospecies (Stephenson et al. 2008).

Most myxomycete species are globally distributed, but interestingly, about 50% of the described species are known from fewer than five localities worldwide. For most of these, the species in question is known from only the single type locality (Schnittler and Mitchell 2000). These authors suggested that morphological variants in existing species are being described as species new to science. In light of the known intraspecific genetic variation, however, the occurrence of these apparently new species could also suggest that independent evolution in clonal isolates or geographically isolated populations is occurring, such that morphological variations are collected over time, ultimately producing a morphological biotype—a group of individuals that exhibit the same morphological character variations (Clark 2000, El Hage et al. 2000). Although the rate at which such a process takes place in myxomycetes is not known, it is likely that these taxonomic entities are adapting to local ecological or environmental conditions.

It is not known whether these local assemblages represent the situation that exists for the majority of myxomycete populations or if a mechanism of long-distance dispersal is also an important factor in establishing and maintaining local populations of myxomycetes. Myxomycete spores are small enough be easily dispersed by wind, both locally and over considerable distances by intercontinental wind patterns (Finlay 2002, Stephenson et al. 2008). While the latter is a currently accepted method of distribution, it is not known whether it is the predominant method for dispersal, since distribution patterns have also been related to ecological characteristics, including the type of substrate, of the environment within which a species is found (Stephenson et al. 1993, Stephenson et al. 2008).

The purpose of the project described herein was to obtain baseline data, the first intraspecific molecular data for any myxomycete, on two species of myxomycetes—*Didymium squamulosum* (Alb. & Schw.) Fr. and *Didymium difforme* (Pers.) S.F. Gray—using DNA sequence analysis of genes (nuclear and mitochondrial) with perceived utility for studies of closely related myxomycetes (Martín et al. 2003; Dennis Miller, pers. comm.). Both of these species appear to occur worldwide and thus are considered cosmopolitan, and both are morphological species with a relatively broad range of acceptable character state variations within the species description (Martin and Alexopoulos 1969). Data were collected to describe the distribution of these species and then to examine the possible role of long distance dispersal as a significant factor for the distribution patterns of cosmopolitan species.

2. Life Cycle of the Myxomycetes

The generalized life cycle of a myxomycete begins with spore germination and then proceeds through two vegetative stages, the first being a uninucleate amoeboid stage and the second a multinucleate plasmodium (plural: plasmodia). This is followed by the reproductive stage, which involves production of fruiting structures (Figure 1). The life cycle can proceed though different pathways that can include a range of forms including two possible resting stages and be completed under various types of ecological conditions, most of which are not fully understood (Clark 2000).

a. Spore germination

The myxomycete spore is an important structure for the classification and identification of different taxa. Recent molecular systematics studies support the traditional view that spore morphology is a conserved character—a character that has remained stable through time—thus supporting the higher-order taxa traditionally recognized in the myxomycetes (Martin and Alexopoulos 1969, Fiore-Donno et al. 2005).

Based upon laboratory germination experiments on agar, it is known that spore germination within a particular species of myxomycete involves a complex of biotic and abiotic factors (Smart 1937, Alexopoulos 1963, Gray and Alexopoulos 1968). Laboratory germination and development through the entire life cycle has as yet not been achieved for most myxomycete taxa. Successful germination and culture on agar has been reported for only about 10% of all known species (Haskins and Wrigley de Basanta 2008). The fact that so many taxa have not yet been successfully brought into culture suggests that the optimal environmental conditions, the combination of biotic and abiotic conditions to support growth, are complex. From experimental data (Smart 1937), it is known that species are not limited to one specific set of conditions, and the variety of successful combinations of these factors even within a single species explains the exploitation of so many microhabitats in every major biome on Earth.

b. Amoeboflagellate

The spore germinates to give rise to an amoeba or swarm cell (bi-flagellate cell). These cells have very little observable variation among different taxa; however, species have been placed into artificial groups based upon the behavior of these cells, including the extent of the alternation between the flagellate and amoeboid states (Ross 1957). Observations have revealed that species show different behaviors, with some germinating as amoebae then releasing flagella for a period of time and others germinating with flagella or producing these structures very soon after emergence from the spore. Some species can, for an undefined number of times, switch between the amoeboid and the flagellate stages, whereas others show

behavior that allows production of flagella only once and reversion to an amoeba is permanent (Alexopoulos 1963).

The amoeboflagellates represent the first trophic (or feeding) stage of myxomycetes. They engulf microorganisms for food, often feeding upon bacteria or fungal spores. It is assumed that most species are generalists (Alexopoulos 1963, Gray and Alexopoulos 1968); however, agar culture experiments show that for some, as yet unknown reason, some species can display food preferences (Gray and Alexopoulos 1968, Haskins and Wrigley de Basanta 2008).

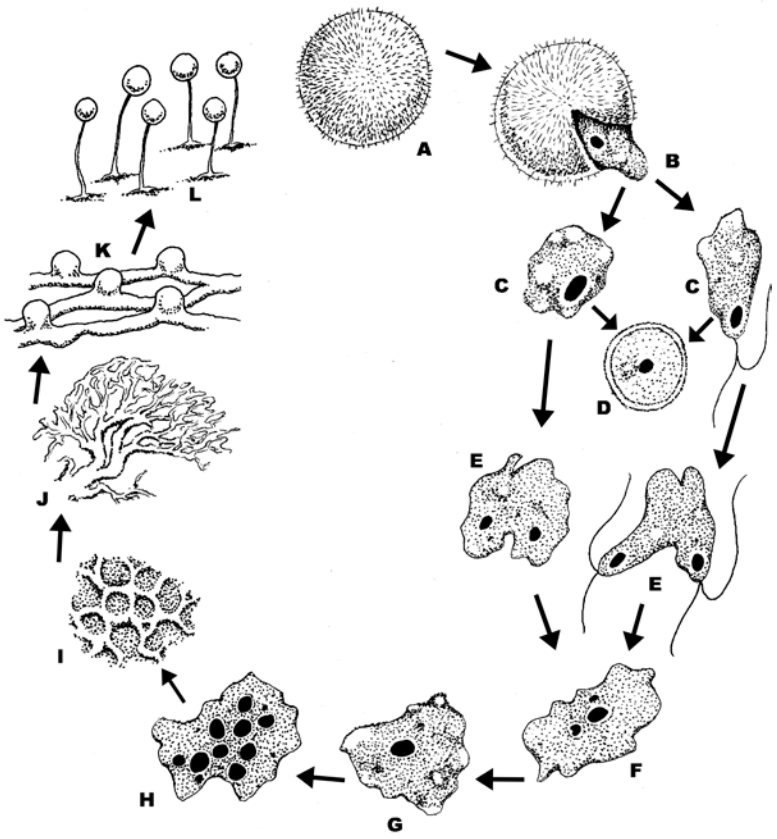

Figure 1. Generalized life cycle in the myxomycetes. A-B. A protoplast emerges from the spore. C. The protoplast can take the form of an amoeba or a flagellated cell during the first trophic stage. D. Under dry conditions or in the absence of food, amoebae form a microcyst, or resting stage. E-F. Compatible amoebae fuse to form a zygote (G). H. The nucleus of the amoeba divides by mitosis and each subsequent nucleus also divides without being followed by cytokinesis, thus producing a single large cell (J), the plasmodium, that represents the second trophic stage. Under adverse conditions, the plasmodium can form the second type of resting stage found in myxomycetes, the sclerotium (I). K-L. Fruiting bodies are formed from the plasmodium. During fruiting body formation, spores are produced. Adapted from Stephenson (2003)

Amoeboflagellates multiply by mitosis and can produce large clonal populations in laboratory culture (Collins and Ling 1964). It is hypothesized that myxomycete amoeboflagellates are found in great numbers in the soil, but very little ecological information is available for amoeboflagellate populations due to the difficulty in assessing populations and identifying the taxa present (Feest and Madelin 1985a, 1985b; Feest 1987; Madelin 1990). Available experimental data (Feest 1987) indicate that the assemblage of myxomycete amoebae makes up 15-50% of the total amoebae in the soil, suggesting that in this stage of the life cycle, these organisms have a significant ecological role. Large populations may occur, making this a singularly important life cycle stage for long-term maintenance of populations (Clark 1992).

While amoeboflagellates are indistinguishable among taxa, significant differences in nuclear content, particularly relating to haploid or diploid states, are common within species (Therrien et al. 1977, El Hage et al. 2000). In the generalized life cycle, the protoplast emerging from the spore as a haploid cell that has the potential of functioning as a gamete; as such, compatible cells can fuse to form a zygote. Within the myxomycetes, however, it has been shown that amoeboflagellates can also occur as diploid cells that are not products of nuclear fusion but rather from apogamic (asexual) strains (Therrien et al. 1977)

Heterogeneity in nuclear content—amoebae of a species can be haploid or diploid upon emergence from the spore—is particularly significant when considering species concepts for myxomycetes. Diploid protoplasts would be clones of the parent individual and thus would represent a potentially separate biological species, and any mutations would be passed along to offspring, accumulating only in this clonal line (Clark and Landolt 2001). Further complicating the genetic life cycle is evidence that a single morphological species can include both sexual and apomictic strains. As a result, populations encountered in nature would seemingly consist of complexes of sibling biological species (Therrien et al. 1977, Clark and Landolt 2001). Furthermore, in laboratory experiments, diploid, apogamic strains can originate from a cross of haploid cells from two sexual strains (Mulleavy and Collins 1979). This suggests that an understanding of the generalized life cycle is too simplistic, and it cannot be assumed that

individuals (in the case of apogamic strains) or populations of heterothallic or homothallic (sexual) strains will always adhere to the same life cycle through time.

In light of such variations, the life history of a myxomycete is still not fully understood. Much of our current understanding of the generalized life cycle and its variations has resulted from observations involving only a few species that are particularly well suited to laboratory culture, leaving out the opportunity for obtaining similar data from 90% of the species in this entire group of organisms.

c. Plasmodium Formation

The plasmodium is the second trophic stage of the myxomycete life cycle. Mitosis occurs without cytokinesis so that a multinucleate cell is produced which continues to go through the cell cycle without cytokinesis to distribute the nuclei and cytoplasm into different cells. Because of this, plasmodia can grow to be quite large, easily becoming a cell that is several centimeters to a meter or more in total extent.

In the generalized life cycle, amoeboflagellates serve as gametes, producing—upon fusion—a diploid zygote. The development of a plasmodium has been seen to occur through several methods (Ross 1957, Alexopoulos 1963). It is assumed that the most common method involves the diploid nucleus dividing by mitosis without subsequent cytokinesis, thus producing a multinucleate cell. This—along with the growth stages of the cell cycle—serves to increase the size of the cell, and continued growth is based on further mitotic divisions that occur in each nucleus thereafter over the life of the plasmodial stage (Ross 1957, Alexopoulos 1963). Other observations of plasmodium formation show that fusion of other zygotes from the same population of amoebae and coalescence with other plasmodia from that population of amoebae contributes to the growth of a plasmodium (Alexopoulos 1963).

Three types of plasmodia are found in the myxomycetes. These are the phaneroplasmodium, aphaneroplasmodium and the protoplasmodium. Each of the five taxonomic orders traditionally recognized for myxomycetes (described in more detail later in this dissertation) is characterized by a particular type of plasmodium. The most frequently encountered type of plasmodium in nature is the phaneroplasmodium of the Physarales because

of its often vivid colors and (in many instances) relatively large size. Cytoplasmic streaming is characteristic of this type, allowing the plasmodium to creep along its substrate. A fan-shaped feeding front allows the plasmodium to spread in different directions searching for food. The phaneroplasmodium gives rise to one to many fruiting bodies. In contrast, the protoplasmodium, characteristic of the Echinosteliales and Liceales, does not have cytoplasmic streaming and usually produces only one fruiting body per plasmodium. The third type is the aphaneroplasmodium of the Stemonitales and Trichiales, which has characteristics of both the phaneroplasmodium and protoplasmodium. It lacks or exhibits very little cytoplasmic streaming but produces one to many fruiting bodies per plasmodium (Gray and Alexopoulos 1968, Martin and Alexopoulos 1969, Olive 1975, Collins 1979).

The ecological importance of the plasmodium is evident in the ability of this structure to move about its microhabitat and the trophic relationships that exist within biological communities, where it feeds on bacteria and other single-celled organisms. Moreover, it is possible that the motility of the plasmodium is the mechanism by which an amoebal population moves from its feeding ground to the substrate upon which fruiting occurs, if they are not the same (Ing 1994). Although the type of plasmodium observed for different taxa is a conserved character, the diversity in morphology displayed by plasmodia is not used to differentiate among species of myxomycetes.

d. Microcysts and Sclerotia

The feeding stages of the myxomycete described above can also revert to resting stages when unsuitable environmental conditions are encountered. The circumstances that trigger the formation of these resting structures are not completely understood. However, it has been shown in culture that dry conditions, a shortage of food or variation from normal temperatures can prompt the formation of both microcysts and sclerotia (Alexopoulos 1963, Gray and Alexopoulos 1968, Olive 1975, Stephenson and Stempen 1994).

A microcyst forms from an amoeboflagellate. The cyst is a round, sturdy structure that can withstand extreme temperatures or dry conditions and germinate to an amoeboflagellate again upon reoccurrence of appropriate conditions (Gray and Alexopoulos 1968, Stephenson and

Stempen 1994). A plasmodium forms a sclerotium with the same basic purpose as a microcyst. The sclerotium (plural: sclerotia) is made up of numerous macrocysts that contain various numbers of nuclei and are surrounded by a membrane. Upon the return of favorable growing conditions, the macrocysts reunite and the structure reverts to a normal plasmodium (Alexopoulos 1963). These resting stages within the life cycle of myxomycetes almost certainly play a significant role in the distribution of myxomycetes because they are small enough to be distributed by mechanisms such as wind or movement of soil. They are also able to survive conditions that do not favor growth for extended periods of time causing the persistence of species in many different habitats. These strategies are particularly important for cosmopolitan species, given the variety of habitats in which they live (ranging from deserts to tropical rainforests and temperate forests) and thus the wide range of conditions that may not always be conducive to the growth and fruiting of myxomycetes.

e. Sporulation and Sporocarps

The sporocarp, or fruiting body, is the mature, spore-disseminating structure in the myxomycete life cycle that is derived from the plasmodium. These are the structures used to classify and describe the distribution of myxomycetes because (1) they are often the only visible forms in nature or moist chamber cultures (the standard laboratory technique by which substrate material is kept in conditions to induce growth and fruiting of myxomycetes present in the material in microscopic form) and also because (2) they have the necessary diversity of characteristics needed for classification not found in the vegetative stages. Sporulation occurs during the last stage of development, and the spores that are produced represent the only cells in a fruiting body (Alexopoulos 1963). The stipe (stalk), columella, capillitium, and peridium are sterile, non-cellular portions of the fruiting body, albeit exceptionally important for identification (Martin and Alexopoulos 1969, Olive 1975, Stephenson and Stempen 1994, Clark 2000). Various abiotic factors, such as decreasing moisture and temperature, are generally considered factors to initiate sporulation, wherein a plasmodium develops into fruiting bodies and the nuclei are encased in a spore (Alexopoulos 1963, Martin and Alexopoulos 1969, Collins 1979, Stephenson and Stempen 1994).

The fruiting bodies produced by myxomycetes contain the entire suite of characters by which species are classified. These structures may be stalked or sessile and among the different taxa are exceedingly diverse in morphology (Martin and Alexopoulos 1969). Although a mechanism for phenotypic plasticity is not known, morphological species do show a range in the character states accepted for the taxon in question, and these have not been linked definitively to microhabitat variation or substrate nor linked to genetic variation (Martin and Alexopoulos 1969, Clark 2000, El Hage et al. 2000).

3. Mechanisms of Long Distance Dispersal

There are several points in the life cycle of the myxomycetes where dispersal is possible. It is assumed that the most important means of dispersal is the movement of spores by some vector or physical factor (Alexopoulos 1963). Additionally, macrocysts and microcysts, the two other resting stages of the life cycle, are capable of withstanding a period of unfavorable environmental conditions and then return from dormancy when either the overall environment or the more limited microenvironment is returned to an appropriate state for growth, feeding and maturation. These can be transported over short distances by some vector and re-emerge for further growth in a different habitat or microhabitat. To a lesser extent, the exploitation of the habitat and associated microhabitats by amoebae and plasmodium moving through the environment represent alternate forms of dispersal, although probably not a likely mechanism for moving any individual a significant distance.

With the majority of myxomycete species considered as cosmopolitan (found worldwide), there is a general acceptance of the idea that species, most likely by movement of spores, are dispersed over long distances (Stephenson et al. 2008). The average diameter of the myxomycete spore is 10.3 µm as calculated for a data set that considered nearly all the described species. The vast majority of species have spores that fall within the range of 5 to 15 µm, with only a few examples producing spores >15 µm in diameter. With this small average size, it is hypothesized that myxomycete spores have great mobility, especially by wind (Tesmer and Schnittler 2007, Schnittler and Tesmer 2008, Stephenson et al. 2008).

The limited data available on the presence of myxomycete spores in samples from airborne material does provide evidence for wind as a known vector. Myxomycetes spores have been identified in studies of samples of air spora at concentrations as high as 520 spores per cubic meter and, at one site in the central United States, represented 0.5% of the yearly total of airborne spores collected (Surratt and Levetin 2005, Gillum and Levetin 2008). The similarities between spores of different species mean that species cannot be identified by spore morphology alone. Identification by using molecular technique was attempted, and nine taxa were found in samples of material collected from a rooftop collection site offering further evidence that myxomycetes are dispersed by air (Kamono et al. 2009). No study to examine the possibility of long distance dispersal has been done, however the evidence that spores are traveling through air as seen in the previously mentioned studies and known wind events causing intercontinental transport of dust, which includes spores and other microorganisms (Kellogg and Griffin 2006) suggests that dispersal of species over long distances is possible.

The effect of long distance dispersal would explain the abundance of cosmopolitan species within the myxomycetes since it would serve as a mechanism by which species are distributed worldwide. Many species, including those studied here (*Didymium squamulosum*. and *Didymium difforme*, are found in vegetated terrestrial habitats at high and low latitudes as well as high and low elevations and in many varied habitats (Eumycetozoan Database, University of Arkansas). A largely airborne mechanism for the dispersal of myxomycete spores would be an obvious explanation for the exploitation of habitats that are geographically dispersed worldwide. Indirect evidence for airborne dispersal is gathered frequently in studies of the biodiversity of myxomycetes when species are found in moist chamber cultures from substrate material collected above the ground. Such aerial litter is comprised of the dead, but still attached, leaves and twigs of plants. Bark samples from living trees are generally collected at breast height (approximately 1.5 m), and the results obtained suggest that species derived from moist chamber cultures prepared with this material were “caught” in these natural spore traps (Tesmer and Schnittler 2007).

The potential for dispersal in myxomycetes is significant. Estimates have put the spore numbers for most species of myxomycetes at 10^5 to 10^6 spores per fruiting body (Schnittler and Tesmer 2008). Plasmodia of myxomycetes can produce multiple fruiting bodies, thus yielding estimated spore counts in the millions from a single individual organism. Each spore has the potential to produce a plasmodium and fruiting bodies, thus the reproductive potential coupled with the dispersal potential for airborne spores suggests a clear mechanism for cosmopolitan species.

Uninhibited dispersal over long distances would result in a cosmopolitan distribution of myxomycetes. Many species are known to have wide distributions in which the same morphological species is found on multiple continents and isolated islands (Martin and Alexopoulos 1969, Stephenson et al. 2007, Stephenson et al. 2008). However, some of the distribution data revealed that a few species seem to have a tropical or a temperate distribution or appear to favor a specific substrate. These data suggest that either dispersal is limited or habitat preference occurs in these organisms. Whether this is by physical limitations, variation in ecological conditions or some combination of both is not known. Species do occur that seem to show a preference for a narrow set of microhabitat conditions such as occurrence on a specific type of substrate or within a specific microhabitat (Stephenson et al. 2008). However, these data are based on the production of fruiting bodies on a particular substrate, either in nature or in moist chamber culture, and having such fruiting bodies is the only method to date for identifying myxomycetes to species.

It is possible that distribution is more complicated than currently understood. For example, in moist chamber cultures, it is possible to observe plasmodia that never produce fruiting bodies (pers. observ.). It is assumed in these cases that the conditions present were not appropriate for the production of fruiting bodies. It is possible, then, that amoebae or plasmodium occur in a given habitat or microhabitat without sporulation ever taking place, providing an example of the opportunity for incomplete information about the biodiversity of a given habitat or the distribution of a species worldwide.

4. Morphology and Taxonomy of Myxomycetes

Myxomycetes are classified on the basis of a morphological species concept that describes and groups taxa based on a series of macroscopic and microscopic characteristics of the sporocarp, capillitium and spores.

Although the morphological diagnosis for particular species is traditionally supported, little effort has been made to map the diagnostic characters onto groups established by molecular phylogenetic analysis. So, in spite of the perceived robustness of the morphological classification scheme, there is very little information for the myxomycetes that has clearly established the true value of these characters as methods for reconstructing the relationships among taxa. In dicytostelids (cellular slime molds), recent analyses involved mapping morphological characters onto a phylogenetic tree in order to assess their value in providing evidence of shared evolutionary history among taxa (Schaap et al. 2006). Twenty characters taken from taxonomic traits, modes of behavior, and fruiting body size and shape were mapped to a molecular phylogenetic tree built using most of the described species of the Dictyostelia. Interestingly, most characters traditionally used to diagnose species did not show a pattern that agreed with the evolutionary clades determined by the molecular phylogeny constructed from the DNA sequences obtained for the small subunit rDNA and α-tubulin genes.

In myxomycetes, relatively few data have been generated to address the same question of the alignment between taxonomically valuable characters and evolutionary history. Fiore-Donno et al. (2005) suggested a phylogenetic hypothesis, a prediction of evolutionary relationships, based upon elongation factor 1-α and small subunit ribosomal RNA in which the order-level taxa (Echinosteliales, Stemonitales, Physarales, Trichiales, and Liceales) recognized in the traditional morphological classification system are resolved, and in this hypothesis, two clades are apparent on the basis of spore color (a dark-spored clade and a clear-spored clade). In the higher order taxa, color of spores seems robust as a diagnostic character, which does support the traditional use of spore characteristics in the classification of myxomycetes. It remains to be seen if other taxonomically valuable characters and further DNA and protein analysis continue to support the traditional classification system.

Morphological heterogeneity within a species can occur if variation in response to different environmental conditions exists, and although these variations often occur in material harvested from moist chamber cultures in which substrate material is incubated in the laboratory to simulate appropriate life cycle conditions for myxomycetes, intraspecific variation in fruiting body characteristics is known from those found in nature as well (Martin and Alexopoulos 1969).

Characteristics used for classification are derived from the fruiting bodies because of the much more extensive suite of characters available as compared to the amoeboflagellates and plasmodia. As noted, parts of the myxomycete fruiting body used for classification and identification include the spores, capillitium, stipe (or stalk) and peridium.

The spores are found in a range of sizes and colors, and they also show significant diversity in ornamentation among the taxa of myxomycetes (Martin and Alexopoulos 1969). In some groups, the spores occur within the fruiting body in association with a series of threads, collectively known as the capillitium. A true capillitium is not found in every group of myxomycetes, but when this structure is present, it offers conserved characters for classification. Among these are differences not only in size and shape, but also, in a manner similar to spores, unique states of ornamentation. The structure and characteristics of the peridium, which surrounds the spore mass, also offer a few, often macroscopic, characters that can be used for classification. The two species addressed in this dissertation project belong to the order Physarales defined largely by the presence of calcareous deposits that occur on the surface of the peridium (Martin and Alexopoulos 1969). The stipe, or stalk, is the subject of ambiguity within some species. In the myxomycetes, a stalk can be present or absent for different taxa, but it is also observed within some species, including *Didymium squamulosum*, that a stalk can be found in a range of lengths.

As described above, many species of myxomycete are considered cosmopolitan.Within the cosmopolitan morphospecies, it is not at all uncommon to observe generous variation in the states that exist for taxonomically important diagnostic characters (Martin and Alexopoulos 1969). The plastic nature of morphological characteristics in myxomycetes is not at all understood and

may be a result of environmental factors, an underlying genetic mechanism that has yet to be uncovered, or some combination of both (Clark 2000).

B. Review of Molecular Investigations in the Eumycetozoa

1. Introduction

The nature of microbial species is different than that for macroorganisms. With fewer morphological characters, there are fewer opportunities to observe morphological variation as genetic distance occurs between lineages. Most morphological microbial species are cosmopolitan meaning they seem to have no geographical barriers and are found in locations that have appropriate habitat (Finlay 2004). Molecular methods have shown that diversity in microorganisms is more complex than morphological analysis would imply (Epstein and López-García 2008). Molecular analysis of morphological species has revealed diversity within species, and analysis of environmental samples has uncovered previously unknown lineages of protists that could be novel phylum-level lineages (Epstein and López-García 2008). The concept of cryptic diversity, diversity within a morphological species that is indistinguishable by morphology alone, as it applies to protists is also a result of major molecular study of microorganisms (Slapeta et al. 2005). As a result of the addition of molecular study of microorganisms, it is clear that morphology alone cannot adequately describe microbial species. More consideration, therefore, must be put into reconciling genetic and morphological components in the study of protists including the slime molds.

Because slime molds spend the majority of their life cycle as microscopic amoebae and plasmodia that are indistinguishable at the species level, the future of the study of slime mold distribution, population structure and species concepts must be linked to meaningful molecular signals and analysis. Most of the molecular data available for any of the three groups of slime molds is related to the construction of significant hypotheses for phylogenetic relationships among and within the taxa that traditionally make up the eumycetozoa (Baldauf and Doolittle 1997, Baldauf et al. 2000, Fiore-Donno et al. 2005, Schaap et al. 2006, Fiore-Donno et al. 2008). The use of molecular biology to study the ecology of these organisms and the intraspecific relationships that exist among the various taxa is in its infancy.

2. Slime Molds and the Eukaryotic Phylogeny

The eumycetozoa were not clearly placed within the eukaryotic tree of life until well into the twentieth century. Since the first formal description of a species of myxomycete, scientists have held the position that these organisms were related to the fungi due to the presence of a fruiting body suggestive of the fruiting structures produced by some fungi (Martin and Alexopoulos 1969). The full life cycle of a myxomycete was first described in the mid-nineteenth century by de Bary to include the amoeboid and plasmodial stages, which gave evidence that the myxomycetes were more closely related to animals than to plants or fungi (Martin 1958, Martin and Alexopoulos 1969). Despite these investigations into the life history of myxomycetes, the connection to the fungi was accepted well into the twentieth century (Martin 1949, Whittaker 1959, Martin 1961, Martin and Alexopoulos 1969, Whittaker 1969). Whittaker (1969) acknowledged the differences between the plasmodial slime molds and the fungi, proposing the Myxomycota as a subkingdom within the Fungi (Whittaker 1969, Baldauf and Doolittle 1997). Because Whittaker's Kingdom Protista was proposed for the classification of what were considered to be mostly unicellular organisms, it was not an appropriate descriptor for all the life stages of myxomycetes. The multicellularity of slime molds, and, perhaps, to some extent, simply tradition (because the organisms do not fit easily into any of the kingdoms), the eumycetozoa were considered non-mycelial fungi. The myxomycetes were also included as the Mycetozoa in Copeland's (1956) Kingdom Protoctista.

At the same time, scientists who studied these organisms aligned them with either the fungi or the protozoa. Olive (1970, 1975) classified all three groups of slime molds—myxomycetes, dictyostelids and protostelids—with the protozoa and proposed a taxon (the Eumycetozoa) that included all three groups. The controversy of the classification of eumycetozoa remained unresolved until well into the twentieth century.

However, this controversy over the proper placement of eumycetozoans has been solved through the examination of molecular-based phylogenetic data. Early molecular phylogenetic efforts showed the eumycetozoa, based upon the taxa included in the particular investigation, to form a monophyletic sister group to the fungi; however, they did not fall within the groups of

protists also being included in the analysis (Baldauf and Doolittle 1997). Conclusive molecular phylogenetic data from further investigations with more taxon sampling aligned the eumycetozoa with other organisms from the protists, and thus it is now accepted that the slime molds would, in the traditional five-kingdom system, be categorized with other microscopic amoebae in Whittaker's Kingdom Protista (Baldauf et al. 2000, Baldauf 2003).

With the addition of DNA and protein analysis, the higher-level classification of eukaryotes is now much more complex than the four eukaryotic kingdoms would suggest (Baldauf 2003, Simpson and Roger 2004, Adl et al. 2005, Keeling 2005, Parfrey et al. 2006, Baldauf 2008). The most recent kingdom-level phylogeny of the eukaryotes has organisms with eukaryotic cells (containing a nucleus and organelles) divided into six supergroups (Figure 2) rather than the previously accepted "Whittaker system" of four kingdoms—Plantae, Animalia, Fungi and Protista (Whittaker 1969, Baldauf 2003, Adl et al. 2005).

The kingdom-level supergroups are based upon a composite of morphological and molecular phylogenetic data, but the division of the protists into separate super-groups is largely derived from recent molecular phylogenetic studies that have taken advantage of variation not seen in the limited number of available morphological characters of microorganisms (Adl et al. 2005, Simpson and Roger 2005, Parfrey 2008). The phylogeny of the eukaryotic domain is now divided into the Amoebozoa, Chromalveolata, Excavata, Opisthokonta, Plantae, and Rhizaria (Figure 2).

The protists are now, on the basis of molecular phylogenetic evidence, spread throughout the eukaryotic domain in at least five of the six supergroups, including the Opisthokonta, Amoebozoa, Chromalveolata, Rhizaria and Excavata. The general amoeboid morphology shared with slime molds is found in at least four supergroups—the Amoebozoa, Chromalveolata, Excavata and Rhizaria (Parfrey et al. 2008). This is a significant change from the original protist kingdom designation, but not a wholly surprising one. Organisms within this group were placed within the protists because they obviously were not animals, plants or fungi. Whittaker (1969) separated all essentially unicellular eukaryotic organisms into the Protista, which made the taxon for all practical purposes a "grab-bag" to encompass the diversity of eukaryotic microorganisms

(Simpson and Roger 2004). All true slime molds are found in the supergroup Amoebozoa (Adl et al. 2005, Shadwick et al. 2009, Fiore-Donno et al. 2010). Most members share an amoeboid life stage, but amoeboid organisms are found in more than one of the supergroups in the eukaryotic molecular phylogeny (Baldauf 2000, Adl et al. 2005, Keeling et al. 2005, Parfrey et al. 2008). It has been suggested that no clear morphological synapomorphy, or shared derived character, can be found for the Amoebozoa, leaving molecular phylogenetic data to support its delineation as a supergroup, although molecular evidence for the monophyly of the group and internal relationships among the taxa has been inconsistant (Parfrey et al. 2008, Yoon et al. 2008, Pawlowski and Burki 2009).

Until recently, it was assumed that within the Amoebozoa the eumycetozoans still formed a monophyletic taxon composed of the dictyostelids, myxomycetes and protostelids. However recent work within eumycetozoan phylogeny has suggested a less clear relationship, thus exchanging controversy over the placement of the organisms within the eukaryotic tree of life to controversy over the relationships among the groups of slime molds themselves.

3. Eumycetozoan Phylogeny

Eumycetozoa (*sensu* Olive 1975), the true slime molds, has traditionally included the Myxogastria (myxomycetes or plasmodial slime molds), Dictyostelia (dictyostelids or cellular slime molds) and Protostelia (protostelids). All three groups are composed of amoeboid organisms capable of producing fruiting bodies under favorable conditions (Olive 1975).

Until recently, all classification within the Eumycetozoa wasdetermined by morphological traits within the life cycle (Martin and Alexopolous 1969). With the recent application of molecular techniques, it has been possible to produce new evidence to address the stability of morphology-based systematics for slime molds.

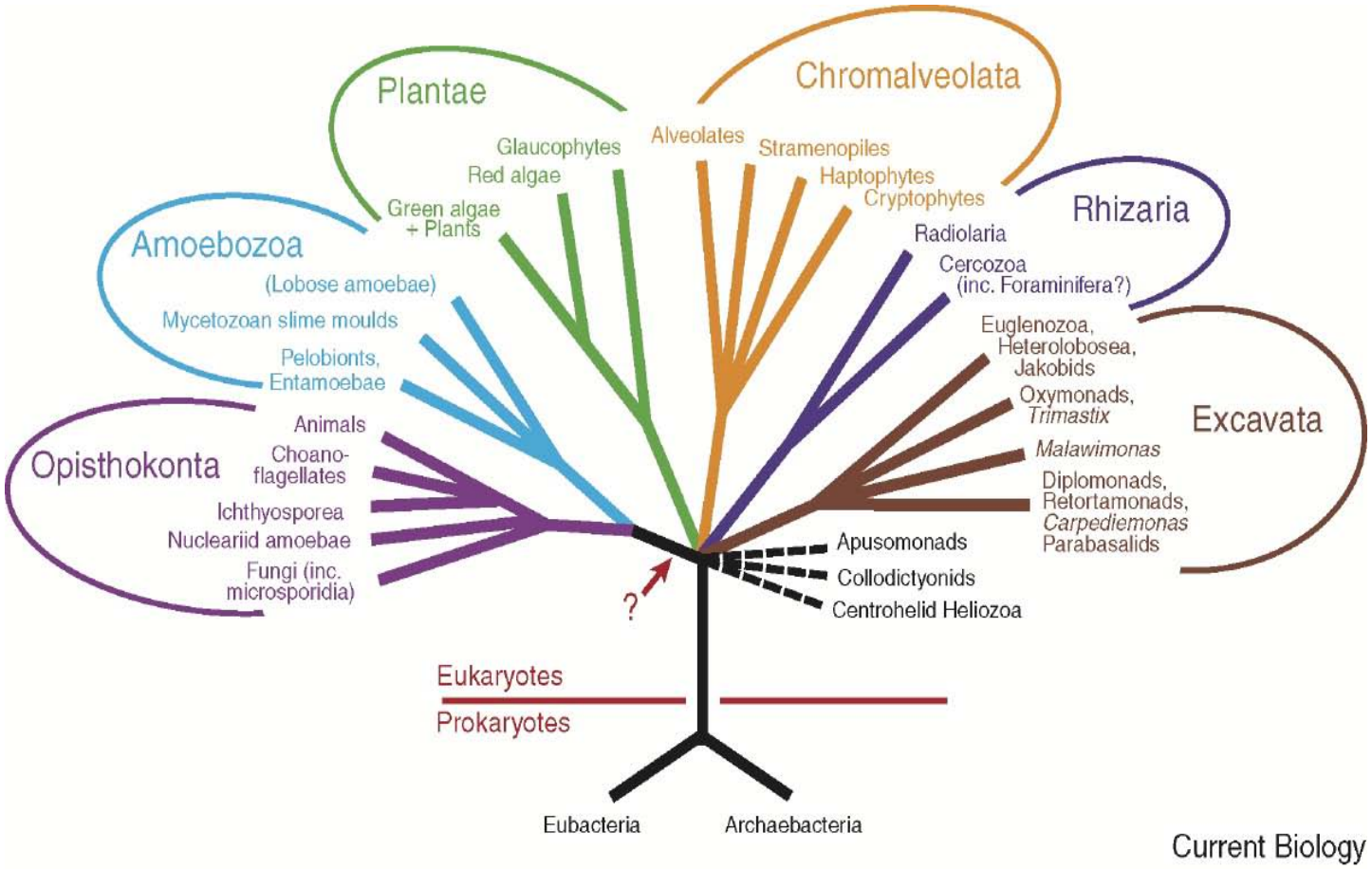

Figure 2. The eukaryotic domain as divided into kingdom-level supergroups based upon molecular phylogenetic data. The eumycetozoans are found in the Amoebozoa as a sister group to the Opisthokonta (from Simpson and Roger 2004

Early assumptions for the systematic relationships among the higher taxa of the eumycetozoa remained largely unchanged prior to the addition of DNA and protein data for the development of phylogenetic hypotheses. Olive (1975) introduced the taxon Eumycetozoa to include all three groups of slime molds and postulated a paraphyletic group, the Protostelids, basal to the monophyletic myxomycetes and dicytostelids (Spiegel et al. 1995). This prediction was particularly supported by the characteristics of the amoeboflagellate cells and the stages of the life history of the groups, because all three groups produce fruiting bodies during the reproductive stages of the life cycle (Olive 1975, Spiegel et al. 1995).

The first molecular phylogenies confirmed the taxon Eumycetozoa as monophyletic and consisting of the three traditional groups (Baldauf and Doolittle 1997, Baldauf 2003, Fiore-Donno et al. 2005). While considered an ecological guild (Schnittler et al. 2006, Schnittler and Tesmer 2008) and thus functioning in a similar manner in terrestrial ecosystems, recent molecular evidence suggests that the Eumycetozoa is, in fact, not a monophyletic taxon that excludes other taxa within the Amoebozoa. Both myxomycetes and dictyostelids are monophyletic taxa, but the taxon containing the protostelids breaks apart into at least five different clades throughout the super group Amoebozoa (Shadwick et al. 2009, Fiore-Donno et al. 2010). According to these data, there is no clade within the phylogeny of Amoebozoa that includes the traditional eumycetozoa to the exclusion of amoebae not included in the myxomycetes, dictyostelids or protostelids as described by Olive (1975). The relationships within the three groups of slime molds have also been subject to limited molecular analysis.

a. Phylogeny of the Protostelids

The protostelids were largely assumed to be the most basal group of eumycetozoans. The primitive nature of these slime molds was inferred from the simplicity of their fruiting bodies and life cycle (Spiegel 1990). This group, like the myxomycetes, has an amoeboid vegetative stage within the life cycle and fruiting bodies that hold spores. In contrast to the myxomycetes, however, the fruiting bodies produced are microscopic, generally producing only a single spore but with some species found having up to eight (Spiegel et al. 2007). Molecular investigation of protostelids originally confirmed the inclusion of protostelids within Olive's Eumycetozoa (Baldauf

and Doolittle 1997, Baldauf et al. 2000). However, the limitation of these studies is now known to be inadequate taxon sampling. The most recent phylogenies of the eumycetozoans, which include a broad sampling of protostelids, have suggested paraphyly within the group, as species are found outside the eumycetozoan clade and across the Amoebozoa phylogenetic tree (Shadwick et al. 2009, Fiore-Donno et al. 2010).

b. Phylogeny of the Dictyostelids

The significance of molecular data within the dicytostelids is not a controversy over monophyly of the taxon but rather the relationships that exist between molecular phylogenies and traditionally important morphological characters. Unlike the protostelids, the monophyly of the dicytostelids as a group is generally accepted and supported by molecular data. However, the monophyly of the genera in the traditional classification scheme based on morphology was called into question by recent molecular data. Species of cellular slime molds are assigned to three genera (*Dictyostelium, Polysphondylium* and *Acytostelium*) that are described largely upon the morphology of fruiting bodies (Raper 1984). The recent molecular phylogenetic data suggested four well-supported groups that do not correspond with any of the taxa within the dictyostelids (Schaap et al. 2006). As such, the available molecular phylogenetic data, however, call into question the monophyly of these genera. Rather, they are split into the four clades, with no clear pattern of the morphological characters traditionally used in dictyostelid classification found across the phylogenetic tree (Schaap et al. 2006).

The molecular phylogeny of the dictyostelids has been coupled with a map of traditionally important traits for classification, including the traits that separate the three genera (Schaap et al. 2006). Each clade contains a mix of character states previously used to separate genera, suggesting that rather than signifying any monophyletic relationships within taxa, fruiting body morphology is highly plastic (Schaap et al. 2006). It was further suggested by adding these data to the body of knowledge available for dictyostelids, it must be recognized that at least two of the three genera are not monophyletic (Schaap et al. 2006). The stability of this phylogeny of the dictyostelids has been recognized through its use in addition to morphological analysis in the

diagnosis of species and determination of new species (Romeralo et al. 2009, Romeralo et al., in press).

Intraspecific analysis within dictyostelid species is limited, but RFLP (restriction fragment length polymorphism) analysis of multiple isolates of *Dictyostelium discoideum* revealed variation among isolates (Francis and Eisenberg 1993). Fifty-four isolates from a single locality were digested with *Eco*RI and probed with an 800 bp fragment containing a tRNA gene. Thirty-two had unique RFLP profiles, but groups were uncovered with isolates that had the same RFLP profile. Initially, it was suggested that the multiple isolates with the same RFLP profile were evidence for gene flow within the population, which was rejected when no crosses resulted in recombinant offspring (Francis and Eisenberg 1993). The result, however, was evidence that natural populations of a slime mold have underlying genetic variation that cannot be predicted based upon morphology or variation in morphology.

c. Phylogeny of the Myxomycetes

Molecular investigations have reinforced the main thrust of the morphological classification of myxomycetes. This is the most species-rich group of eumycetozoans and they possess the most complex suite of morphological characters used to determine its classification scheme. The phylogeny of the myxomycetes (Myxogastria) has only recently been determined with a level of taxon sampling that includes all of the taxonomic orders of the myxomycetes (Fiore-Donno et al. 2005). The molecular phylogeny obtained thus far has suggested confirmation of the classification and designation of taxa at the order level originally determined by morphology of the fruiting bodies (Fiore-Donno et al. 2005).

This phylogeny by Fiore-Donno et al. (2005) (Figure 3), generated by using small subunit ribosomal DNA (SSU rDNA) and elongation factor 1-alpha (EF1-a) DNA sequence data, supported the taxonomic orders based upon morphological data (Martin and Alexopolous 1969). Four of the traditional five orders are grouped based on spore color, with a “dark-spored” clade consisting of the Stemonitales and Physarales and a “light-spored” clade including the Trichiales and Liceales. The Echinosteliales composed a separate clade, and the Ceratiomyxales, a controversial member of the myxomycetes was not considered. It has been suggested that this

order is actually more closely related to the protostelids, but the most recent investigations into the phylogeny of eumycetozoans suggested that the genus *Ceratiomyxa*, the only genus in Ceratiomyxales, is a sister group to the traditional myxomycetes that is more closely related to the myxomycetes and dicytostelids than the protostelids (Spiegel 1990, Fiore-Donno et al. 2010).

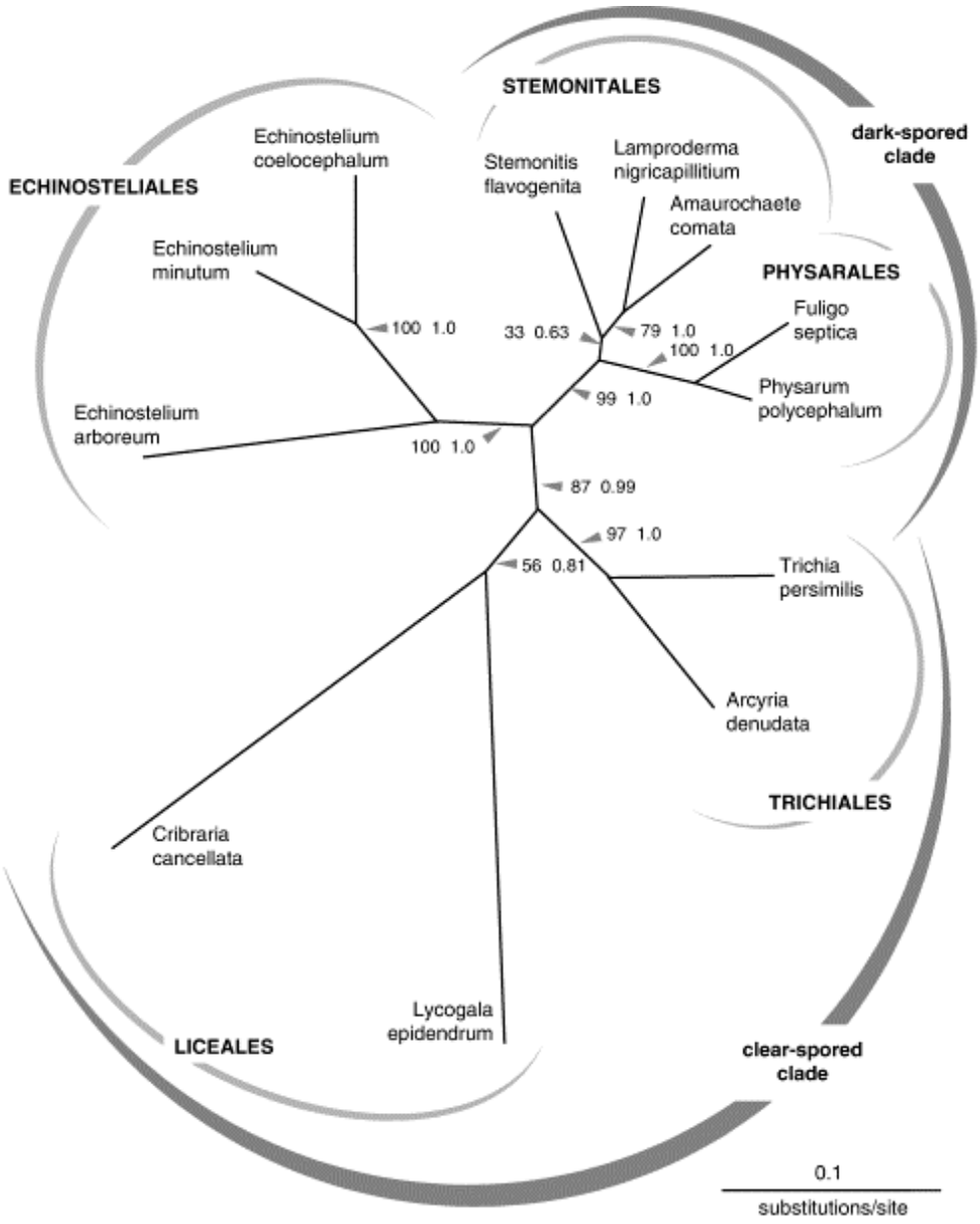

Figure 3. Phylogeny of the myxomycetes based on SSU rDNA and EF-1a data. These data support the traditional groups of slime molds at the order taxonomic level (from Fiore-Donno et al. 2005)

4. Molecular Biology and Ecology of Slime Molds

Studies of the molecular phylogeny of various taxa of eumycetozoans have provided a wealth of information about the relationships between slime molds and other eukaryotic organisms and among the slime mold taxa within each group.Using this research approach for ecological purposes has been extremely limited. The diversity and dispersal of eumycetozoans are two ecological questions that are currently only partially addressed using traditional laboratory culture techniques. Recent data obtained for myxomycetes has suggested that only a portion of the complete ecological story is known for the slime molds.

For example, molecular techniques have been used in several recent efforts to describe the eumycetozoan community in natural habitats (Kamono and Fukui 2006, Ko Ko et al. 2009). Using PCR primers designed for myxomycete SSU rDNA and DNA extracted from environmental samples, including soils, it is possible to identify species of slime molds. This suggests there is much to learn from investigating a natural habitat beyond observing the fruiting bodies that are found in nature or laboratory culture of substrate material (Kamono and Fukui 2006, Ko Ko et al. 2009). The vegetative part of the life cycle occurs as microscopic amoebae or small plasmodia. It has been thought that the soil and other decaying plant material could contain significantly more slime mold populations than are revealed by current methods for studying biodiversity of slime molds, which require the formation of fruiting bodies (Feest and Madelin 1985a, 1985b; Madelin 1990).

The study of these microorganisms in soils and other habitats using molecular markers has added a significant body of knowledge of the diversity of eukaryotic organisms in certain habitats in nature (Baldauf 2008). The molecular studies of this type that have been conducted so far are rudimentary. More examination of potential markers along with the development of a library of reference sequences are required before the utility of these molecular approaches can be maximized to understand the biodiversity of myxomycetes.

The earliest suggestion that intraspecific molecular variation might exist, but not readily observed in the morphology of the species, came from isozyme studies undertaken to examine taxonomic relationships between closely related species (Franke 1967, Franke et al. 1968,

Franke and Berry 1972). The analysis of isozymes has been used to determine taxonomic relationships among closely related taxa and to describe variation within a single species by taking advantage of variation within certain enzymes. This variation can occur as a result of multiple alleles expressed from a single locus, an enzyme produced from multiple loci or variation from post-translational processing of the polypeptide (Micales et al. 1986). The profiles from analysis of myxomycetes supported the traditional morphological classification by showing significant variation between species that were examined. However, intraspecific variation was observed for the species represented by multiple isolates in these early studies (Franke and Berry 1972, Franke 1973). It was recognized that before isozyme techniques could be used to address taxonomic questions, the range of isozyme variation within a morphological species must be understood and analysis of forty-five isolates of *Fuligo septica* produced distinctive variation within the species (Franke et al. 1968, Berry and Franke 1973). The addition of multiple isolates for a single species for isozyme analysis produced multiple profiles within the species that could neither be correlated to the geographical origin nor the ecological conditions of the isolate. Though no explanation was established for the intraspecific variation, when the profiles were compared with profiles for other species of myxomycetes, all of the isolates of *Fuligo septica* are clearly separate from other species in the Physarales (Berry and Franke 1973). Interestingly, two morphological variants, white and yellow forms, which are both recognized as *Fuligo septica*, were separated by isozyme profiles. The white forms most closely related to each other and more different from the yellow forms than any yellow isolate was to any other yellow isolate (Berry and Franke 1973). No taxonomic changes were made based upon these data and, to my knowledge, no further data were collected to confirm this pattern. However, these data represent an early suggestion that morphological variation may represent underlying divisions or groups within a species. Isozyme or allozyme analysis is limiting because the technique is less informative and less sensitive than DNA sequencing for single base variations within the gene sequence.

Morphological species of myxomycetes are known to have variable morphological characters, and it is unresolved whether or not such variations represent evidence of actual genetic distance between isolates or simply the plasticity of the character controlled by habitat

variation (Clark 2000). An attempt to study the relationship that exists between genetic variation and morphology was undertaken using isozyme analysis in *Didymium squamulosum.* Isozyme variation was found to exist among the taxa being considered, but no significant correlation between genetic variation and morphological variation was noted (El Hage et al. 2000). No other study directed towards a single species of myxomycete, and using the more advanced techniques of molecular biology including DNA sequencing, has been undertaken with the objective of investigating the question of the possible correlation between genetic variation and morphological variation. As such, the underlying mechanism for morphological variation within the same species of myxomycete is still not known, and because species can exist in nature as clonal lineages that can independently accumulate variation, it is possible the morphological variation suggests underlying genetic variation (Clark 2000).

Further isozyme analysis within a single species uncovered patterns based upon genetic cross-compatiblity wherein those isolates of *Didymium iridis* (Dit.) Fr. that are homothallic (not interbreeding with other isolates) had unique isozyme profiles dissimilar to other isolates both heterothallic and nonheterothallic, and similar isozyme profiles were found within those isolates that are heterothallic and "cross-compatible" (Betterley and Collins 1983). In this case, morphologically indistinct isolates exhibited variation in reproductive strategies (heterothallic or non-heterothallic—perhaps apomictic) and measurable variation in isozyme profiles—an indirect measure of DNA polymorphism.

Studies of genetic variation within a single species have been informative in other organisms, often generating ideas and information relating to patterns of dispersal and population-level genetic structure, thus providing the data necessary to discriminate among populations and describe possible patterns of gene flow within and between populations (Avise 2009). The results obtained from this type of study have the potential to be highly informative in myxomycetes because the microscopic stages of the life cycle in nature make it impossible to observe the movement of individuals within and between populations, to reveal the limits of populations in nature, or to assess the genetic variation that exists among individuals within populations or species. Coupled with information on the geographical locality of each isolate

being considered, it seems likely that within-species molecular investigations could enhance our understanding of the dispersal of these organisms.

Prior to this dissertation, there were no data for fine scale sequence-based studies of any species of myxomycete. Most DNA sequence-based efforts to develop any kind of understanding within the myxomycetes has been directed towards questions relating to the higher-order phylogeny. These include investigating the relationships (1) between the eumycetozoa and other eukaryotic organisms, (2) among the three groups of slime molds, and (3) within groups to understand the relationships among genera. The first study based upon DNA sequencing of multiple isolates from the same myxomycete morphospecies used nuclear ribosomal Internal Transcribed Spacer (ITS) data for *Didymium squamulosum* and is presented as one chapter of this dissertation (Winsett et al. 2008).

In myxomycetes, using molecular biology as a tool for studying the ecology of these organisms promises to add significantly to our understanding of species concepts, biodiversity both within habitats and on a global scale, and the relationships that exist among local populations and between widely separate populations over the entire range of the species.

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CHAPTER 3

USING SEQUENCES TO ASSESS INTRASPECIFIC GENETIC RELATIONSHIPS AMONG GEOGRAPHICALLY SEPARATED COLLECTIONS OF THE MYXOMCYETE *DIDYMIUM SQUAMULOSUM*

Winsett, K. E. and S. L. Stephenson. 2008. Using sequences to assess intraspecific genetic relationships among geographically separated collections of the myxomcyete *Didymium squamulosum*. Revista Mexicana de Micología 27: 59-65.

ABSTRACT

An analysis of ITS sequences of *Didymium squamulosum*, a myxomycete regarded as cosmopolitan, shows variation among geographically separated isolates. ITS 1 and 2 and 5.8S rDNA were amplified from specimens collected in widely scattered localities. The sequences were analyzed by parsimony analysis and the resulting trees show separate, moderate and well supported clades grouping some, but not all, of the geographical locations. ITS is the first molecular marker examined for intraspecific variation in myxomycetes, and although sequence analysis shows statistically significant differences among sequences from the 14 specimens considered, the heterogeneity in ITS 1 and ITS 2 appears to be too great for any more meaningful biogeographical conclusions. Variation in the 5.8S and the amount of variation found in ITS 1 and 2 may signify that the isolates examined represent a complex of sibling species, although this particular marker cannot be used to make that distinction in this myxomycete.

Keywords: *Didymium squamulosum*, ITS region, intraspecific variation

INTRODUCTION

The myxomycetes (also called plasmodial slime molds or myxogastrids) are a group of eukaryotic, phagotrophic bacteriovores usually present and often abundant in terrestrial ecosystems. The myxomycete life cycle involves two very different trophic stages, one consisting of uninucleate amoebae, with or without flagella, and the other consisting of a distinctive multinucleate structure, the plasmodium (Martin and Alexopoulos, 1969). Under favorable

conditions, the plasmodium gives rise to one or more fruiting bodies containing spores. The fruiting bodies of myxomycetes are somewhat suggestive of those produced by higher fungi, although they are considerably smaller (usually no more than 1-2 mm tall). The spores of myxomycetes are, for most species, apparently wind-dispersed and complete the life cycle by germinating to produce the uninucleate amoeboflagellate cells (Stephenson *et al*., 2008).

There are approximately 875 recognized species of myxomycetes (Lado, 2001). The majority of these are probably cosmopolitan, but a few seem to be confined to the tropics or subtropics and some others have been collected only in temperate regions of the world (Martin and Alexopoulos, 1969; Stephenson *et al.*, 2004; Stephenson *et al.,* 2008). Myxomycetes appear to be particularly abundant in temperate forests, but at least some species apparently occur in any terrestrial ecosystem with plants (and thus plant detritus) present (Stephenson *et al.*, 2008; Stephenson and Stempen, 1994).

Didymium squamulosum (Alb. & Shwein.) Fr. (Order Physarales, Family Didymiaceae) is an example of a truly cosmopolitan morphological species of myxomycete reported from numerous localities throughout the world (Figure 1) in habitats ranging from arctic tundra and subantarctic herbfields to lowland tropical rain forests and deserts (Clark and Stephenson, 2003; ElHage *et al.,* 2000; Martin and Alexopoulos, 1969; Pickering, 2006). The possible mechanism for long distance dispersal is the potential mobility of spores in this species and all known myxomycete species, which are small enough (8-11 µm in diameter) to be transported by wind currents (Martin and Alexopoulos, 1969; Stephenson *et al.,* 2008; Tesmer and Schnittler, 2007).

However, it is not yet known if this high dispersal potential, which in theory would allow for movement of spores between continents, is commonly realized. In fact, it has been hypothesized that this particular species of myxomycetes is not genetically ubiquitous. For example, results obtained from two studies (Clark and Stephenson, 2003; ElHage *et al.* 2000) of the reproductive systems of *Didymium squamulosum* indicated that both non-heterothallic (presumably apomictic) and heterothallic strains of this species occurred in nature, suggesting that the *Didymium squamulosum* morphospecies is a complex of sibling biological species.

These data suggest there is much more information to be understood within the species that cannot be perceived by a consideration of morphology alone. The purpose of the study described herein was to obtain some initial baseline data on one example of a species (*Didymium squamulosum*) regarded as cosmopolitan, using DNA sequence analysis of a gene region with perceived utility for study of closely related myxomycetes (Martín *et al.*, 2003). It was anticipated that this type of analysis in the internal transcribed spacers of the ribosomal DNA (ITS rDNA) region of *Didymium squamulosum* would provide evidence of any underlying genetic diversity and possible biogeographical patterns that may exist in cosmopolitan myxomycetes, thus representing a source of data by which to study mechanisms of dispersal, population biology and evolution within myxomycetes.

MATERIALS AND METHODS

DNA amplification and sequencing

Thirteen specimens (fruiting bodies from moist chamber culture or field collections) of *Didymium squamulosum* from widely scattered localities throughout the world (Table 1) were analyzed for biogeographically meaningful variation in the internal transcribed spacers 1 and 2 (ITS1, ITS2). For DNA extraction, 4-6 sporangia were ground in Sigma acid-washed glass beads with a sieve size of 710-1180 μm to release the spores. DNA was extracted from the spores using 150 μL of 5% Chelex suspension at 56 °C for 4 hours then 98 °C for 30 minutes in a Bio-Rad PTC-0200 DNA Engine Thermal Cycler. ITS 1 and 2, 5.8S, and flanking small and large subunit (SSU and LSU) regions of rDNA were amplified by means of the polymerase chain reaction (PCR) using amplification primers in the SSU and LSU as described by Martín *et al.* (2003) (Table 2). The DNA templates were amplified in Invitrogen Platinum Blue PCR SuperMix with the primers using a PCR protocol, which consisted of an initial activation reaction at 94 °C for 2 minutes and 35 repetitions of 94 °C for 30 sec, 52 °C for 1 min, and 72 °C for 2 min. The PCR products were transformed and cloned, using the methods of the Invitrogen TOPO TA Cloning Kit for Sequencing. Four clones were chosen for sequencing in an effort to assess any intra-clonal variation. DNA for sequencing was extracted with the Qiagen QIAprep Spin Miniprep Kit and

sequenced on a Perkin-Elmer ABI 3700 sequencer. Internal sequencing primers in the 5.8S were modified from Martín *et al.* (2003) based on *Didymium squamulosum* sequencing (Table).

Sequence alignment and analysis

The sequences were initially aligned using ClustalX (Thompson *et al*., 1997) and manually edited in SeaView (Galtier *et al*., 1996). Thirteen isolates were analyzed by Maximum Likelihood analysis of the homologous alignment of sequences in PAUP 4.0 (Swofford, 2002) using the likelihood settings from the best-fit model (TVMef+I+G) selected by Akaike Information Criterion (AIC) in ModelTest (Posada and Crandall, 1998). Branch support was determined using 500 bootstrap replicates.

RESULTS

The ITS region from 13 isolates of *Didymium squamulosum* was amplified using known primers from Martín *et al*. (2003), which are in flanking regions of the ITS 1 and 2 in the small and large subunits, thus adding approximately 132 characters to the sequence. Internal primers in the 5.8S were developed for sequencing purposes using known sequences of *Didymium squamulosum*. The sequenced region for every isolate includes all of ITS1, 5.8S and ITS2 rDNA, and length variation of the sequences among the specimens was evident in both ITS1 and ITS2, with the total amplified region ranging from approximately 1090 to 1416 base pairs. In each sequence, approximately 302 base pairs come from the rDNA genes (26S, 5.8S and 19S), thus leaving a sequence length of 788-1114 base pairs as the total length of ITS 1 and 2.

The sequence alignment offered 397 unambiguously aligned sites (22.8% of the total length), and of these, only 95 sites (5.45% of the total length) are within either ITS1 or ITS2. A graphical representation of the sequence alignment using Fingerprint (Lou and Golding, 2007) illustrates the heterogeneity of the full sequences amplified for this species (Figure 2).

The maximum likelihood phylogram displays the relationships between isolates based upon analysis using the best-fit model selected by AIC in ModelTest. Bootstrap values greater than 60 are displayed (Figure 3). Well-supported groups of isolates are found in the data analysis. Two of the well-supported groups are made up of isolates found at the same collecting sites: (a) New Zealand 26852, 26833 and (b) Alaska 7112, 7139, 7141. They are supported with

bootstrap values of 92 and 94, respectively. However, a fourth isolate from Alaska and a third isolate from New Zealand do not group with the other isolates from the geographical region.

DISCUSSION

Didymium squamulosum as a study organism

Didymium squamulosum is known to be a widely distributed species with variable morphological features (Clark and Stephenson, 2003, ElHage *et al*., 2000). In this species, as with many species of myxomycetes, it is not known if morphological variation is an expression of phenotypic plasticity or phenotypic evidence of genetic variation or divergence (Clark, 2000). A study of sporangium characteristics for isolates of *Didymium squamulosum* did not coincide with genetic variation measured by isozyme variation and the results of plasmodium fusion tests (Clark and Stephenson, 2003; ElHage *et al*., 2000). These previous studies suggest a complex of biological species within the recognized morphological species of *Didymium squamulosum*. Thus, the genetic variation within *Didymium squamulosum* is potentially a very valuable tool to understand the intraspecific diversity and the potential cryptic species-level diversity within the morphological species.

ITS as a genetic marker for geographic patterns

Internal transcribed spacers (ITS) are intervening sequences between the small and large subunits of the ribosomal DNA (rDNA). In *Physarum polycephalum* (Schwein.), rDNA occurs on a single, linear, extrachromosomal DNA molecule in the nucleolus of the cell. The large and small subunit exons are separated by two spacers and the 5.8S exon. There are two copies of each region arranged as a palindrome around a large central spacer (Campbell *et al.*, 1979, Seebeck *et al.*, 1979). These two spacers, ITS 1 and ITS 2 were successfully amplified for an intrageneric study of *Lamproderma* (Martín *et al*., 2003). Interestingly, it was reported that the alignment for this region was unambiguous for the 3 species investigated (Martín *et al*., 2003). This sequence, though, was problematic in *Didymium squamulosum* due to the extensive variation in both nucleotide and length diversity within the species limiting the usefulness of the marker for genetic study within populations. In this baseline data set, less than 6% of the entire ITS sequence was found to be homologous.

The analysis of the ITS1-5.8S-ITS2 regions carried out in the present study divided the collections of *Didymium squamulosum* into clades with some evidence of a geographical pattern. Collections from the same region (e.g., two specimens from New Zealand and three from Alaska) share a clade in the topology of the tree. However, the ITS complex is too variable to give a reliable description of the relationships among geographically disjunct populations. The majority of unambiguously aligned sites for analysis were chosen not from ITS1 or ITS2, but from the flanking large and small subunit rDNA and the 5.8S segments. The ITS regions themselves are too variable in nucleotide content and length to construct an unambiguous alignment.

Biogeographical inference

The topology of the tree from these analyses does separate the samples into well-supported clades. However, the placement of isolates in the tree does not, in general, follow a discernable biogeographical pattern (see for example, Alaska 6407 and Costa Rica 14106) .

The data obtained thus far indicate that additional study of the relationships among specimens of *Didymium squamulosum* and other myxomycetes is warranted. The variation uncovered in the ITS sequences of *Didymium squamulosum* suggest that a more complete genetic profile within the species will offer data to explore local and global dispersal patterns beyond the variation observed in the morphological characteristics of the species. However, ITS seems to have limited usefulness for investigations within a species due to extensive heterogeneity of sequences between isolates (Figure 2). It will be important to isolate a gene or gene region that will offer enough variation within a species without presenting too much and unsuitable variation for intraspecific analysis.

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Catalog Number	Specimen Origin
6407	Alaska, USA
7112	Alaska, USA
7139	Alaska, USA
7141	Alaska, USA
17019	Australia
14106	Costa Rica
24032	Hawaii, USA
29653	Malawi, Africa
18798	Malawi, Africa
26833	New Zealand
26852	New Zealand
OTA 58428	New Zealand
L029	Oklahoma, USA

Table 1. Specimens of *Didymium squamulosum* used in the study with accession numbers for the database at http://www.slimemold.uark.edu where more information for each specimen may be obtained.

Amplification primers:	
SSU 3'For (PHYS-5)	5'-GGA AGC AGA AGT CGT AAC AAG G –3'
LSU 5'Rev (PHYS-4)	5'-TTC CTC CGC TGA CTA ATA TGC –3'
Internal sequencing primers:	
Didymium 5.8S For (mod. from 5.8S-48)	5'-GCT TCG ACG AAG AGC GCA G –3'
Didymium 5.8S Rev (mod. from 5.8S-30)	5'-CTG CGC TCT TCG TCG AAG C -3'

Table 2. Amplification primers used in this study are from Martín *et al.* (2003). Internal sequencing primers in the 5.8S gene were modified from the original and designed from sequences of *Didymium squamulosum.*

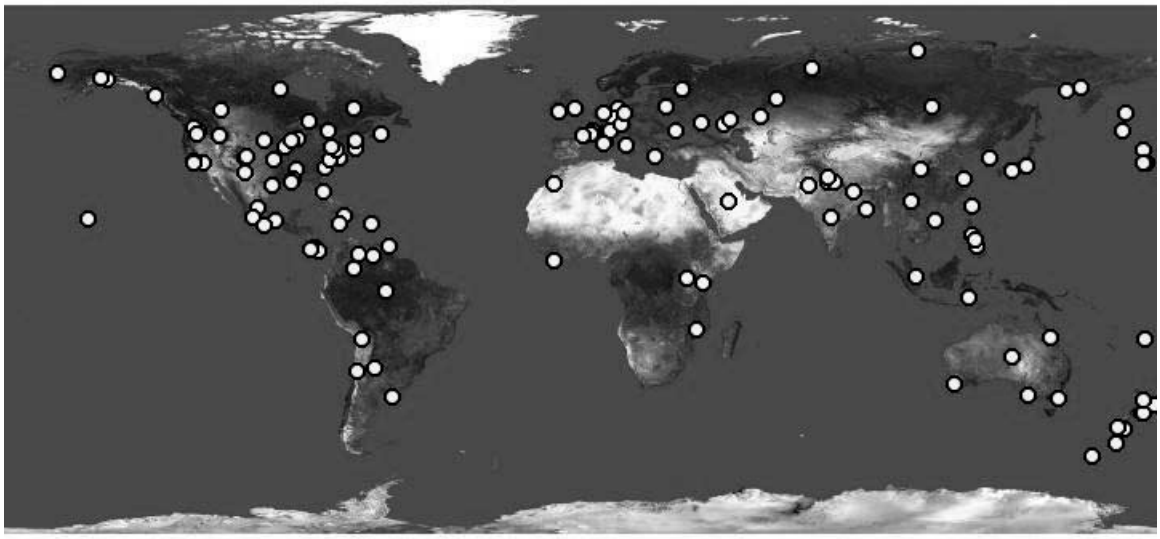

Figure 1. Recorded collection localities for *Didymium squamulosum* based upon data available at http://www.discoverlife.org (Pickering, 2006).

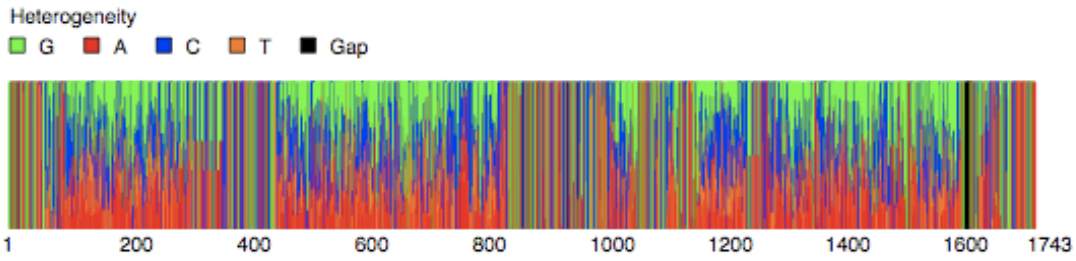

Figure 2. Fingerprint (Lou and Golding, 2007) of the entire ITS multiple sequence alignment for 13 isolates of *Didymium squamulosum*. Conserved regions are visible as straight lines; heterogeneous regions are represented by the jagged sections.

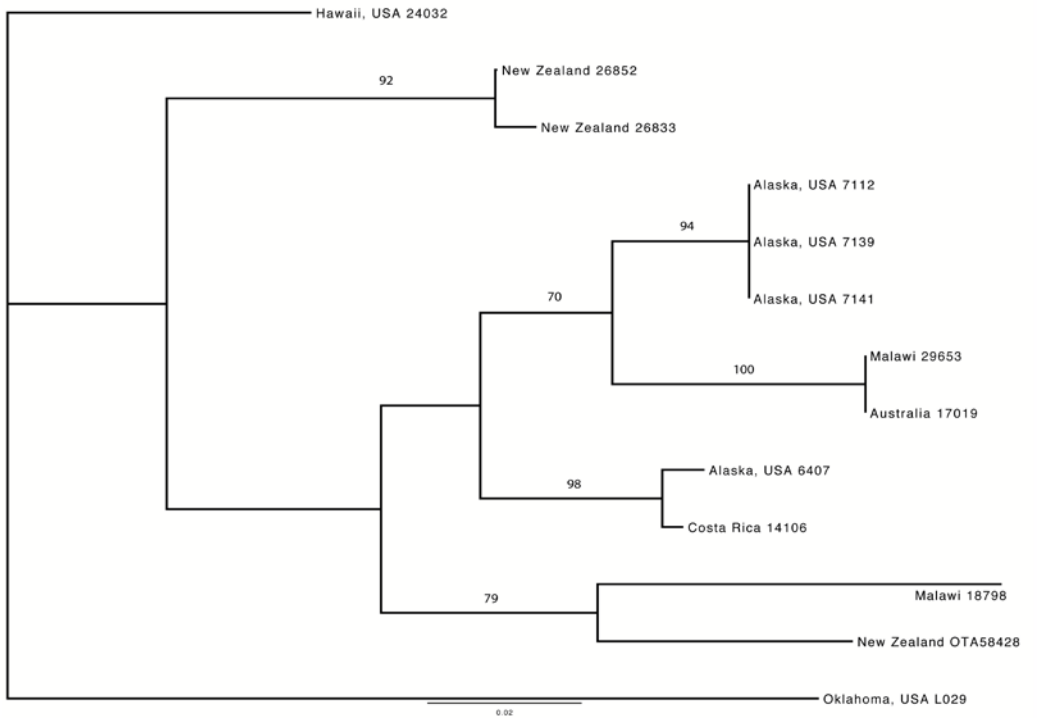

Figure 3. Maximum Likelihood phylogram of thirteen isolates; assumed model (TVMef+I+G) selected by AIC. Significant bootstrap values (greater than 60) are displayed.

Chapter 4

Global distribution, morphology, ecology and molecular diversity of *Didymium squamulosum* (Alb. & Schwein.) Fr.

A. Introduction to *Didymium squamulosum*

Didymium squamulosum (Alb. & Schwein.) Fr. (Order Physarales, Family Didymiaceae) is "remarkable for the variations which it presents in the fruiting phase" (MacBride 1922). The typical isolate of *D. squamulosum* is characterized by the presence of a light-colored fluted stalk, a dark spore mass typical of the Physarales, and a peridium covered by a white, crystalline lime crust (Martin and Alexopoulos 1969). This species is commonly found on diverse types of substrate material in a number of different ecological settings and is known to vary from the normal morphological form over a range of accepted states for taxonomically important morphological characters (Martin and Alexopoulos 1969, El Hage 2000). The underlying mechanism controlling the heterogeneity in morphology is not clear. It is thought this may be contingent upon the ecological setting in which the specimen is found or possibly related to as yet uncovered molecular variation or genetic isolation (Clark 2000, El Hage et al. 2000).

The standard description for *D. squamulosum*, as given in the most recent comprehensive monograph on the myxomycetes, is given below.

> *Didymium squamulosum* (Alb. & Schw.) Fr.
> Usually sporangiate and stalked, but varying to sessile and plasmodiocarpous, often in the same fruiting, white, gray or rarely pinkish when fresh, soon fading; sporangia appearing globose or depressed, in reality usually discoid and deeply umbilicate below, 0.3-1 mm in diameter, up to 1.5 mm in total height; peridium membranous, transparent, somewhat iridescent, usually covered with a thick, white crust of stellate lime crystals which often form a reticulate surface, the lime sometimes scanty; stalk stout, calcareous, usually more or less fluted but sometimes nearly smooth, white or ochraceous to orange or pinkish, arising from a discoid, concolorous hypothallus, often attaining two-thirds of total height, but sometimes very short and buried in the umbilicus, or lacking; columella white or pale, discoid or hemispheric, consisting of the thickened, umbilicate sporangial base, with an expanded, subglobose or flattened tip; capillitium variable, the threads slender or course, nearly simple or branching profusely, colorless or pallid, less commonly dark, often bearing conspicuous thickenings; spores black in mass, dark violaceous brown by transmitted light, minutely warted or spinulose, the warts sometimes clustered, 8-11 μm in diameter. Plasmodium colorless, white or yellow. (Martin and Alexopoulos 1969).

A molecular correlation would be suggestive of a mechanism for intraspecific variation possibly related to speciation. Because few molecular investigations have been done within any single species within the myxomycetes, there is no prior knowledge of population structure or patterns of intraspecific variation upon which any morphological variation can be mapped or any predictions made based upon morphological variation within any data set of a single morphological species.

Sporulation in myxomycetes has been linked to changes in the surrounding environment. Specifically, the transition from plasmodium to fruiting body has been linked to the drying out of the substrate, changes in the populations of food organisms, and changes in other chemical or abiotic factors (Stephenson and Stempen 1994). Changes in the environment do not have to be regular events, thus the mechanism of sporulation must be able to proceed in a variety of different conditions. The irregularity and diversity in environmental triggers could serve as a cause for heterogeneity in fruiting body morphology. At this point, any such hypothesis is merely speculation. As already noted, few studies have attempted to link morphological variation to geographic or genetic patterns, and no pattern has yet been found (Clark and Mires 1999, El Hage et al. 2000).

There are three recognized reproductive strategies in the myxomycetes (Collins 1979). A heterothallic strain requires the fusion of complementary gametes; a homothallic strain is also sexual in that the amoeboflagellate gametes are haploid but there are no complementary gametes, rather fusion takes place between any two haploid cells. The third possibility is an apogamic strain, which is diploid throughout the life cycle and thus never requires the fusion of nuclei to form a plasmodium. When asexual strains are present, these isolates are genetically distinct from any other strain. Evolutionarily speaking, such strains will accumulate mutations and variations independently of other strains within the morphological species. Any morphological variation would be passed down within the strain from parent to offspring, at which point it would be shared with other individuals of the species. In theory, morphologically distinct individuals with the morphological variation derived in this manner would be limited to the single strain and not likely to be found in other, genetically different strains. However, with the limited number of

available characters for study in the myxomycetes, the possibility of conversion on the same variation in morphology is plausible among distinct strains. Morphological variation linked to an asexual strain could also be found on a worldwide scale if a mechanism of long-distance dispersal is at work.

Didymium squamulosum is known to be a complex of biological species in which both sexual and asexual strains have been found in nature, and it has been suggested that this species exists in nature as many asexual strains with a core of sexual strains representing the different reproductive strategies known in myxomycetes (El Hage et al. 2000).

B. Global distribution

Didymium squamulosum is an example of a truly cosmopolitan morphological species of myxomycete, since it has been reported from numerous localities throughout the world and in habitats ranging from arctic tundra and subantarctic herbfields to lowland tropical rain forests and deserts (Clark and Stephenson 2003, El Hage et al. 2000, Martin and Alexopoulos 1969). The world distribution map provided herein (Figure 1) was developed from data available in the Global Biodiversity Information Facility (GBIF: http://www.data.gbif.org). The possible mechanism for long distance dispersal is the potential mobility of spores in this species and all other known species of myxomycetes species. The spores are small enough (8-11 µm in diameter) to be transported by wind currents, and the traditionally accepted mechanism for long distance dispersal in the myxomycetes relies upon the wind acting as a vector for moving spores (Martin and Alexopoulos 1969, Tesmer and Schnittler 2007, Stephenson et al. 2008).

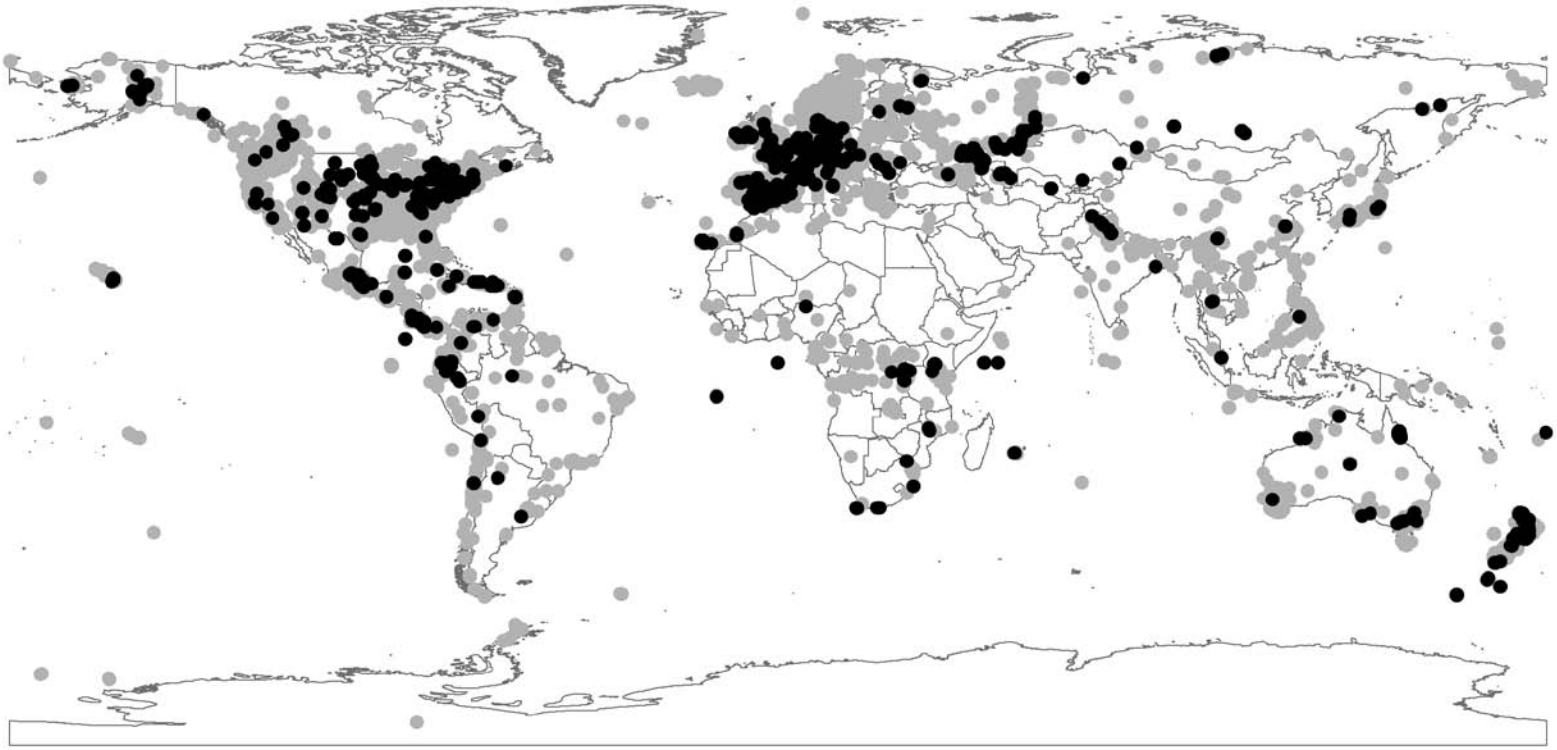

Figure 1. Worldwide distribution of *Didymium squamulosum*. Black points represent localities from which *D. squamulosum* has been collected, based upon data retrieved from the Global Biodiversity Information Facility (GBIF) (date retrieved: April 2010). These are only those collections for which information on latitude and longitude is available (approximately 64% of all collections in GBIF). Gray points represent locality data for all myxomycete collections in GBIF for which GPS georeferencing points are available (approximately 55% of all collections in GBIF).

C. Ecology

Our understanding of the ecology of myxomycetes is based upon (1) the examination of substrate material directly in the field for the presence of fruiting bodies that developed under natural conditions and (2) isolating these organisms in the laboratory using the moist chamber culture technique, which involves placing substrate material in a Petri dish with water to create a microcosm of the appropriate conditions for growth and development of myxomycetes. Because there is, as yet, no method for studying the biodiversity of myxomycetes in any stage of the life cycle beyond the fruiting bodies, ecological research is dependent upon examining a habitat at the appropriate time to find fruiting bodies and to sample, using the moist chamber culture technique, substrate material in an effort to induce fruiting of any of the microscopic life stages that may be present in the sample.

The drawback of both of these approaches for studying myxomycete ecology relates to the fact that assumptions must be made regarding the thoroughness of the sampling and the level of function that a species found actually has in the given environment. The actual Petri dish that is the basis for the moist chamber culture is an imitation of the environmental conditions that promote growth and development of the organism being studied. Any biodiversity information that is taken from the development of fruiting bodies in these moist chamber cultures only establishes the existence of the species in the habitat from which the samples were obtained. Because it cannot be established if the species was present as a spore, amoeboflagellate, microscopic plasmodium or one of the two resting stages in the life cycle of a myxomycete, it cannot be determined if the species was actually functioning in that habitat. Theoretically, myxomycete spores are capable of long distance dispersal, but it has also been observed that some species apparently have disjunct populations (Tesmer and Schnittler 2007, Stephenson et al. 2008). This begs the question of whether the actual distribution of myxomycete spores or resting stages is more widespread than myxomycetes actively functioning in a given habitat. At this time, there is no method for answering this question. The development of molecular probes for more rigorously examining a given locality, including the soil microhabitat, promises to allow

further study and to expand our ecological knowledge of these organisms (Kamono and Fukui 2006, Ko Ko et al. 2009).

The current methods available for ecological study of myxomycetes have, despite the inherent challenges involved in studying these organisms, uncovered important information about the distribution of these organisms and provide a basis for the development of more rigorous inquiries into the relationship between these organisms and their environment. In most studies of myxomycete biodiversity and ecology, substrate collections are classified into four main categories. These are aerial litter (dead plant material still attached to the plant, thus off the ground), ground litter (dead plant material on the ground), bark (outer bark from living trees, collected at breast height) and, when present, dung of herbivorous animals. It is possible to investigate even more specific substrates, depending upon on the habitat or ecosystem of interest, and such studies have included lianas (Wrigley de Basanta et al. 2008), cacti (Lado et al. 2007), and the inflorescences of tropical plants (Schnittler and Stephenson 2002).

The worldwide database of mycetozoans at the University of Arkansas (hereafter referred to as UARKM) holds the largest and most complete dataset for myxomycetes. The database, unlike GBIF, also includes important ecological information about the substrate on which a particular specimen occurred and the habitat in which the specimen was collected. This database has 888 records of *D. squamulosum,* and 883 of these have substrate and habitat information. Of these records, 488 specimens were obtained with the use of moist chamber cultures. For these records, pH of the substrate material is available in most instances. This measurement is usually taken twenty-four hours after the substrate material is wetted by placing a pH probe in the standing water remaining in the dish. For the records collected in the field, the data collected represent the snapshot in time in which it is known that the environmental conditions favored the growth and development of myxomycetes. Collections from material that developed in moist chamber may not represent the expected seasonality of the species because the species may be found as a spore, amoeboflagellate, microcyst, macrocyst and/or microscopic plasmodium, which (as noted earlier) in the case of the spore, microcyst, or macrocyst, the

individual organism is not functioning in that environment at that time. The moist chamber culture would stimulate development because more or less ideal conditions are created.

Of the collections in the UARKM database, 395 collections (44%) were collected in the field and 488 (55%) were obtained from moist chamber cultures. The collections from aerial and ground litter were more evenly distributed (44% aerial and 51% ground) in the set of moist chamber collections, whereas the field collections were less similar (21% aerial and 73% ground). However, these values are not necessarily meaningful because aerial litter is not usually productive in the field because the substrate is often too dry for fruiting body production and the fruitings tend to be generally smaller and more easily overlooked (Stephenson, pers. comm.). Therefore, *D. squamulosum* in any form on aerial substrates (microcyst, macrocyst, or spore) would produce fruiting bodies when more or less ideal environmental conditions are simulated in the moist chamber. Overall, of the collections in UARKM, 585 collections (66%) are from ground litter and 298 (34%) from aerial litter.

D. Morphological and Molecular Diversity

1. Sporocarp collections

All collections of *Didymium squamulosum* were obtained from recent ecological and biodiversity studies that yielded specimens that fruited in the field and also utilized the moist chamber culture technique to cultivate myxomycetes from substrate material collected in different geographic localities around the world (Figure 2, Table 1). As described in more detail later in this chapter, morphological diversity was documented by examining each collection through a Zeiss (Carl Zeiss, Inc., Germany) dissecting microscope. From the descriptions compiled for each collection by this examination, character states were established for four different major morphological characters that are traditionally taxonomically important—relative stalk length, lime texture on the peridium, hypothallus presence and size, and stalk color.

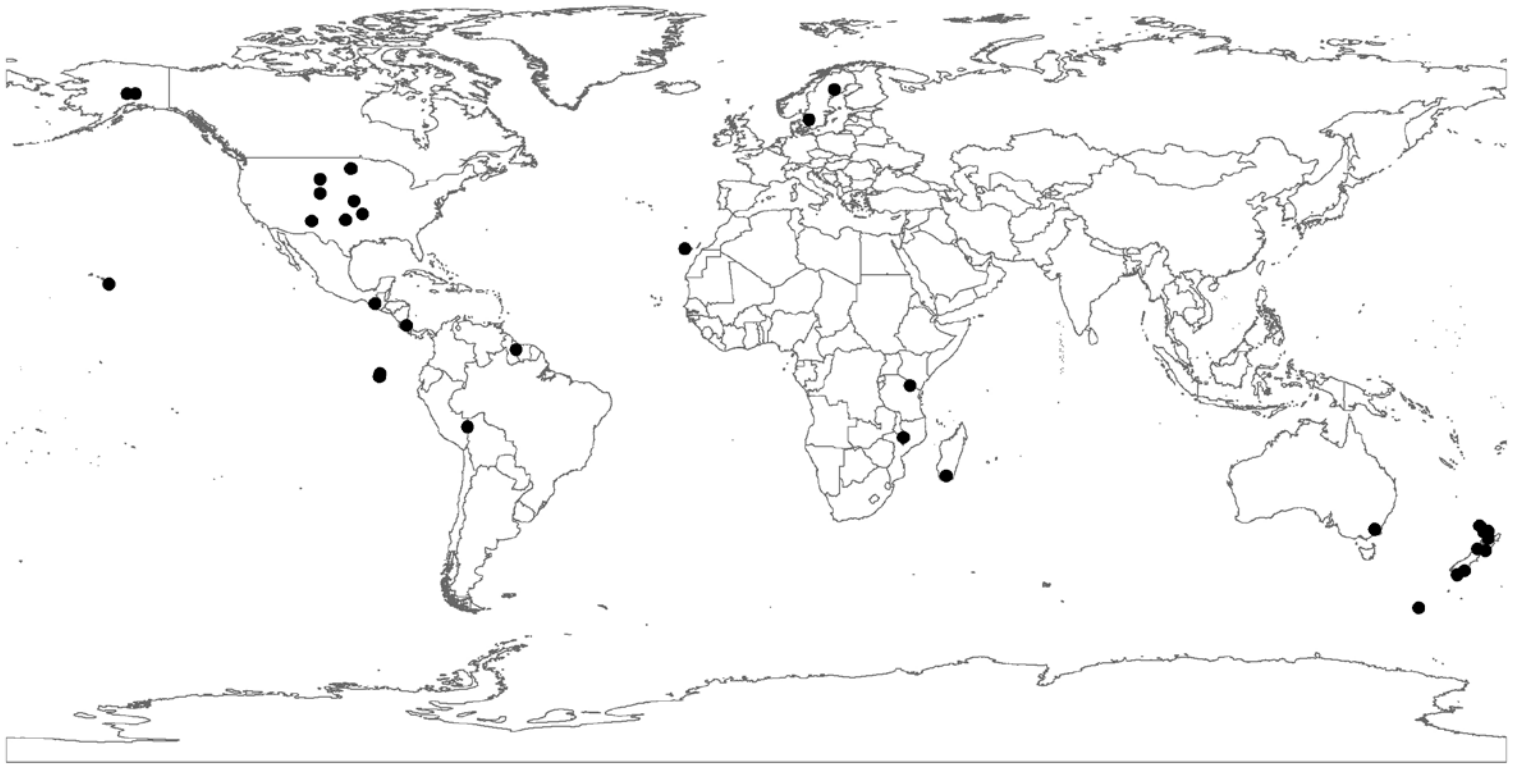

Figure 2. Map of localities from which collections used in the present study originated.

Table 1. Specimen data for collections used in the analysis of *D. squamulosum.* Collections with alpha-numeric accession numbers include the herbarium code abbreviation for the herbarium from which the collection was borrowed.

DNA Number	Collector Number	Accession Number	Genbank Number	Locality	Latitude/Longitude
5	SLS19510	19510	HQ450466	Australia	-36.43 / 148.33
6	SLS18764	24034	HQ450456	Hawaii, USA	19.89 / -155.34
8	CS149	44972	HQ129885	New Zealand	-36.942432 / 174.593573
9	SLS6393	6393	HQ450473	Alaska, USA	63.73 / -150.97
11	SLS7112	7112	HQ450500	Alaska, USA	63.73 / -148.96
12	SLS7139	7139	HQ450501	Alaska, USA	63.73 / -148.96
13	SLS7141	7141	HQ450502	Alaska, USA	63.73 / -148.96
14	L026	22208	HQ45045	Arkansas, USA	36.02168 / -94.5038
15	SLS18799	44983	HQ450491	Malawi	-15.2833 / 35.2833
18	SLS18798	18798	HQ450477	Malawi	-15.2833 / 35.2833
22	MS14106	44981	HQ450490	Costa Rica	10.42 / -84.01
24	SLS6952	6952	HQ450471	Macquarie Island	-54.50 / 158.89
25	MM23646	44978	HQ450492	Peru	-12.836447 / -69.295333
47	UE3304	GB-0039251	HQ450453	Sweden	57.7949 / 12.6337
48	UE6328	GB-0039249	HQ450489	Sweden	64.605 / 18.669
49	UE3424	GB-0039252	HQ450480	Galapagos Islands	-0.659 / -90.368
50	UE2346	GB-0039236	HQ450472	Galapagos Islands	-1.277 / -90.486
51	CS157	44973	HQ129905	New Zealand	-36.942432 / 174.593573
53	CS158	44674	HQ129886	New Zealand	-36.942432 / 174.593573
56	SLS9376	9376	HQ450493	Guyana	4.944180 / -57.635996
62	EB10390	TFC-10.390	HQ450481	Canary Islands	28.126 / -17.237
65	MH3637	H7003654	HQ450454	Tanzania	-3.29 / 36.83
66	MH3442	H7003655	HQ450479	Tanzania	-3.29/ 36.83
79	AWR492	26039	HQ450458	North Dakota, USA	46.44134 / -97.38476
80	AWR754	26301	HQ450474	Oklahoma, USA	39.08238/ -96.56455
82	AWR706	26253	HQ450457	North Dakota, USA	39.10185 / -96.56494
83	AWR716	26263	HQ450459	North Dakota, USA	34.7424 / -98.60523
84	AWR677	26224	HQ450482	Kansas, USA	44.07315 / -104.63812
85	AWR735	26282	HQ450467	North Dakota, USA	46.44134 / -97.38476
86	AWR746	26293	HQ450460	Kansas, USA	39.08238 / -96.56455
87	AWR841	26388	HQ450461	North Dakota, USA	40.81222 / -104.72448

88	AWR736	26283	HQ450476	Colorado, USA	40.812370 / -104.73484
89	AWR1059	26592	HQ450483	Colorado, USA	34.38369 / -106.67885
90	AWR1075	26607	HQ450462	North Dakota, USA	40.81222 / -104.72448
92	AWR996	26537	HQ450475	North Dakota, USA	46.44109 / -97.25319
93	AWR1040	26573	HQ450463	North Dakota, USA	46.441340 / -97.384760
94	AWR1023	26558	HQ450499	New Mexico, USA	46.44134 / -97.38476
95	AWR719	26266	HQ450484	Wyoming, USA	44.07 / -104.64
96	AWR713	26260	HQ450485	Oklahoma, USA	46.44134 / -97.38476
97	AWR738	26285	HQ450465	Kansas, USA	46.44134 / -97.38476
98	AWR762	26309	HQ450464	Kansas, USA	34.7424 / -98.60523
99	AWR1029	26564	HQ450486	Colorado, USA	40.81 / -104.73
100	AWR491	26038	HQ450468	North Dakota, USA	46.44 / -97.38
101	AWR1112	26644	HQ450488	Kansas, USA	46.44134 / -97.38476
102	AWR1160	26689	HQ450470	Kansas, USA	39.10185 / -96.56494
103	AWR533	26080	HQ450487	Wyoming, USA	46.44134 / -97.38476
104	CS23880	44690	HQ129878	New Zealand	-36.942432 / 174.593573
105	CS185	44974	HQ129880	New Zealand	-36.948750 / 174.925579
106	CS187	44975	HQ129870	New Zealand	-37.00 / 174.549074
107	CS188	44976	HQ129871	New Zealand	-36.942432 / 174.593573
108	SLS9449	28100	HQ129908	New Zealand	-36.83 / 175.58
109	SLS9394	28046	HQ129910	New Zealand	-37.00 / 174.57
110	SLS23723	44984	HQ129906	New Zealand	-38.47 / 175.57
112	SLS9423	28074	HQ129868	New Zealand	-36.83 / 175.58
113	SLS9411	28062	HQ129872	New Zealand	-36.83 / 175.58
114	SLS9591	28239	HQ129911	New Zealand	-41.35 / 174.93
116	SLS9428	28079	HQ129879	New Zealand	-36.83 / 175.58
117	SLS9503	28151	HQ129877	New Zealand	-36.97 / 174.50
120	SLS9478	28126	HQ129907	New Zealand	-46.90 / 168.13
122	SLS23890	44980	HQ129869	New Zealand	-35.653 / 173.549
123	SLS9454	28105	HQ129881	New Zealand	-36.83 / 175.58
124	SLS9378	28032	HQ129876	New Zealand	-40.90 / 173.01
125	OTA58428	OTA58428	HQ129909	New Zealand	-45.907 / 169.982
126	SLS9381	28035	HQ129873	New Zealand	-40.90 / 173.01
127	CS050	44971	HQ129875	New Zealand	-36.851553 / 174.535796
128	CS082	44647	HQ129874	New Zealand	-36.939 / 174.642

132	CR1349	31858	HQ450494	Guatemala	15.50 / -91.50
133	CR1355	31864	HQ450503	Guatemala	15.50 / -91.50
135	CR1376	31885	HQ450504	Guatemala	15.50 / -91.50
137	CR1382	31891	HQ450495	Guatemala	15.46 / -91.54
138	CR1386	31894	HQ450496	Guatemala	15.50 / -91.50
139	CR1390	31898	HQ450469	Guatemala	15.50 / -91.50
141	CR1402	31910	HQ450497	Guatemala	15.50 / -91.50
143	MS14530	44982	HQ450498	Costa Rica	10.42 / -84.01
152	SLS23253	43669	HQ450478	Madagascar	-24.107 / 45.609
200	SLS23302	43763	HQ129887	New Zealand	-36.86 / 174.52
201	SLS23307	43767	HQ129888	New Zealand	-36.86 / 174.52
202	SLS23322	43782	HQ129889	New Zealand	-36.86 / 174.52
203	SLS23330	43790	HQ129890	New Zealand	-36.86 / 174.52
204	SLS23301	43762	HQ129891	New Zealand	-36.86 / 174.52
205	SLS23299	43760	HQ129892	New Zealand	-36.86 / 174.52
206	SLS23309	43769	HQ129893	New Zealand	-36.86 / 174.52
207	SLS23312	43772	HQ129894	New Zealand	-36.86 / 174.52
208	SLS23291	43752	HQ129895	New Zealand	-36.86 / 174.52
210	SLS23298	43759	HQ129896	New Zealand	-36.86 / 174.52
211	SLS23319	43779	HQ129897	New Zealand	-36.86 / 174.52
212	SLS23287	43748	HQ129898	New Zealand	-36.86 / 174.52
213	SLS23281	43742	HQ129899	New Zealand	-36.86 / 174.52
214	SLS23295	43756	HQ129900	New Zealand	-36.86 / 174.52
215	SLS23300	43761	HQ129901	New Zealand	-36.86 / 174.52
217	SLS23288	43749	HQ129902	New Zealand	-36.86 / 174.52
220	SLS23296	43757	HQ129903	New Zealand	-36.86 / 174.52
225	SLS23316	43776	HQ129904	New Zealand	-36.86 / 174.52
226	CS130	26848	HQ129882	New Zealand	-36.86 / 174.52
229	CS218	44977	HQ129883	New Zealand	-36.86 / 174.52
230	CS23889	44979	HQ129884	New Zealand	-36.86 / 174.52

2. Molecular Methods

DNA was extracted from fruiting bodies using the Chelex method as described previously (Winsett and Stephenson 2008). Briefly, spores from four to five fruiting bodies and 150 µL of a 5% Chelex suspension were placed in a thermocycler held at 56 C for four hours and then 98 C for 30 minutes to release DNA from the other cellular material that is part of the spore. Extracted DNA was frozen in microcentrifuge tubes at -20 C until use.

A 400 base pair hypervariable region of the mitochondrial small subunit (mtSSU) was amplified using the PCR primers mtCore1 (5′–TAG TGT TAT TCG TGA TGA CT–3′) and mtCore2 (5′–CTC GAA TTA AAC CAC AT–3′). The DNA templates were amplified using a 25 µL reaction (12.5 µL Promega GoTaq® Green Master Mix, 8.5 µL dH_2O, 1 µL each primer and 2 µL template DNA) using a PCR protocol, which consisted of an initial activation step at 94 C for two minutes (per instructions for Master Mix) and 35 repetitions of 94 C for 30 sec, 40 C for one min, and 72 C for one minute. The PCR amplicon was extracted from the PCR product by a modified gel electrophoresis and gel extraction method (modified from Dentinger et al. [2009]). A 20 µL portion of each PCR product was run in a 1% agarose gel prepared with 1X TA buffer (20X: 193.6 g Trizma base, 45.7 mL glacial acetic acid, dH_2O up to 2L, dilute to 1X) with ethidium bromide included at 80 V for approximately 30 minutes. The resulting band was cut from the gel and placed in a filtered micropipette tip cut to fit into a 1.5 mL microcentrifuge tube. The tube with the piece of gel sitting on the filter was frozen to break up the agarose matrix then spun in a centrifuge at 13 000 rpm for 10 minutes. The filtered pipette tip was removed from the tube, leaving the extracted DNA within the flow-through.

DNA samples were sequenced on a Perkin-Elmer ABI 3700 sequencer (Perkin Elmer Applied Biosystems Division, Waltham, Massachusetts) using amplification primers. Resulting chromatograms were checked and edited using Sequencher v4.3 (Gene Codes Corporation). All sequences were submitted to GenBank (Accession numbers HQ129868-HQ129911 and HQ450453 - 450504). Sequence alignments were performed using Clustal X and then manually edited in Seaview (Larkin et al. 2007, Gouy et al. 2010). Summary statistics for the sequences including the number of haplotypes, haplotype diversity, nucleotide diversity, and number of

pairwise differences were calculated using DnaSP v. 5 (Librado and Rozas 2009). This software was also used to test for neutrality of mutation through Tajima's (1989) D statistic and the D* and F* statistics of Fu and Li (1993). A gene tree was produced using Bayesian Markov Chain Monte Carlo methods using the software package BEAST v.1.5.4 (Drummond and Rambaut 2007). The alignment in nexus format was imported into BEAUTi, to format the appropriate XML file to be used by BEAST. The alignment was analyzed using estimated base frequencies and a chain length of 1 000 000. Performance suggestions for operators that were included in the output from BEAST were used to make operator modifications in BEAUTi to optimize the data set. The updated XML file produced by BEAUTi was imported into BEAST and reanalyzed. This step was repeated until the operator values were optimized according to the performance suggestions in the BEAST output file. The resulting ".trees" output was formatted using TreeAnnotator v.1.5.4 and saved as a ".tre" file that was viewed and edited in Fig Tree v. 1.3.1. A gene geneology (haplotype) network was constructed using TCS (Clement et al. 2000).

3. Agar Culture

An effort was made to germinate all isolates in agar culture using known methods (Haskins and Wrigley de Basanta 2008). Spores were placed directly onto the surface of Half Strength Bacto Corn Meal Agar (CM/2) (8.5g Bacto corn meal extract agar and 12.5 g plain Bacto agar in 1 L of distilled water [Haskins and Wrigley de Basanta 2008]). Following germination, and plasmodium formation, cultures were fed *E. coli* and sterilized crushed rolled oats to generate substantial plasmodia. Plasmodia were allowed to fruit and fruiting bodies along with substrate agar were cut out and glued onto herbarium quality paper trays fit into pill boxes (e.g., Fisher Scientific 03-505A) to create a voucher for each culture

4. Results and Discussion

a. Molecular Data

A 398 base pair region of the mitochondrial small subunit (mtSSU) was amplified and sequenced for 96 collections of *Didymium squamulosum* from worldwide localities (Table 1). All sequences were analyzed using BEAST, DnaSP and TCS softwares to describe intraspecific variation.

In DnaSP (Librado and Rozas 2009), all 96 sequences were analyzed with alignment gaps excluded, leaving a total of 375 sites, 297 of which were invariable and 78 polymorphic. Of the polymorphic sites, 41 were parsimony informative. Summary statistics are found in Table 2. The nucleotide diversity (π) was 0.02917 with an average number of nucleotide differences (*k*) of 10.939. Thirty haplotypes were calculated with a haplotype diversity of 0.841. Sixteen haplotypes were singletons, eleven represented only two sequences. The most common haplotype—haplotype 13—occurred 35 times. Haplotypes are displayed by collection in Table 3 and by locality in Table 4. Three tests of neutrality in which the null hypothesis is that mutations are neutral were calculated: Tajima's D (1989) and Fu and Li's D* and F* (1993). When the entire data set was analyzed, Tajima's D was not significant, while Fu and Li's D* and F* were significant. Therefore, according to one statistic, the null hypothesis cannot be rejected, but according to the others, it can be rejected.

Table 2. Summary statistics for mtDNA polymoprhisms in sequences of *Didymium squamulosum* in New Zealand. n_s is the number of sequences; h_n is number of haplotypes; H_d ($\pm$SD) is haplotype diversity $\pm$ standard deviation; π (k) is nucleotide diversity and mean number of nucleotide differences.

n_s	**h_n**	**H_d ($\pm$SD)**	**π (k)**	**Fu and Li's D*(P)**	**Fu and Li's F*(P)**	**Tajima's D (P)**
96	30	0.8406 (0.032)	0.02917 (10.93860)	-3.39704 (P<0.02)	-2.90835 (P<0.05)	-1.05993 (ns)

Table 3. Collections of *Didymium squamulosum* used in this study and the haplotype group to which each belongs as calculated by DnaSP (Librado and Rozas 2009).

DNA Number	Collector Number	Accession Number	Country	Haplotype Group
5	SLS19510	19510	Australia	5
6	SLS18764	24034	Hawaii, USA	4
8	CS149	44972	New Zealand	12
9	SLS6393	6393	Alaska, USA	8
11	SLS7112	7112	Alaska, USA	29
12	SLS7139	7139	Alaska, USA	30
13	SLS7141	7141	Alaska, USA	30
14	L026	22208	Arkansas, USA	3
15	SLS18799	44983	Malawi	23
18	SLS18798	18798	Malawi	11
22	MS14106	44981	Costa Rica	20
24	SLS6952	6952	Macquarie Island	7
25	MM23646	44978	Peru	23
47	UE3304	GB-0039251	Sweden	1
48	UE6328	GB-0039249	Sweden	19
49	UE3424	GB-0039252	Galapagos Islands	16
50	UE2346	GB-0039236	Galapagos Islands	7
51	CS157	44973	New Zealand	19
53	CS158	44674	New Zealand	12
56	SLS9376	9376	Guyana	24
62	EB10390	TFC-10.390	Canary Islands	12
65	MH3637	H7003654	Tanzania	2
66	MH3442	H7003655	Tanzania	12
79	AWR492	26039	North Dakota, USA	5
80	AWR754	26301	Oklahoma, USA	9
82	AWR706	26253	North Dakota, USA	5
83	AWR716	26263	North Dakota, USA	5
84	AWR677	26224	Kansas, USA	17
85	AWR735	26282	North Dakota, USA	12
86	AWR746	26293	Kansas, USA	5
87	AWR841	26388	North Dakota, USA	5
88	AWR736	26283	Colorado, USA	10
89	AWR1059	26592	Colorado, USA	17
90	AWR1075	26607	North Dakota, USA	5
92	AWR996	26537	North Dakota, USA	9
93	AWR1040	26573	North Dakota, USA	5
94	AWR1023	26558	New Mexico, USA	17
95	AWR719	26266	Wyoming, USA	17
96	AWR713	26260	Oklahoma, USA	17
97	AWR738	26285	Kansas, USA	5
98	AWR762	26309	Kansas, USA	5
99	AWR1029	26564	Colorado, USA	17
100	AWR491	26038	North Dakota, USA	12
101	AWR1112	26644	Kansas, USA	18
102	AWR1160	26689	Kansas, USA	5
103	AWR533	26080	Wyoming, USA	17
104	CS23880	44690	New Zealand	12
105	CS185	44974	New Zealand	12

106	CS187	44975	New Zealand	12
107	CS188	44976	New Zealand	12
108	SLS9449	28100	New Zealand	22
109	SLS9394	28046	New Zealand	22
110	SLS23723	44984	New Zealand	20
112	SLS9423	28074	New Zealand	1
113	SLS9411	28062	New Zealand	12
114	SLS9591	28239	New Zealand	17
116	SLS9428	28079	New Zealand	14
117	SLS9503	28151	New Zealand	12
120	SLS9478	28126	New Zealand	21
122	SLS23890	44980	New Zealand	4
123	SLS9454	28105	New Zealand	12
124	SLS9378	28032	New Zealand	12
125	OTA58428	OTA58428	New Zealand	13
126	SLS9381	28035	New Zealand	13
127	CS050	44971	New Zealand	12
128	CS082	44647	New Zealand	12
132	CR1349	31858	Guatemala	25
133	CR1355	31864	Guatemala	17
135	CR1376	31885	Guatemala	17
137	CR1382	31891	Guatemala	26
138	CR1386	31894	Guatemala	17
139	CR1390	31898	Guatemala	5
141	CR1402	31910	Guatemala	27
143	MS14530	44982	Costa Rica	28
152	SLS23253	43669	Madagascar	15
200	SLS23302	43763	New Zealand	12
201	SLS23307	43767	New Zealand	12
202	SLS23322	43782	New Zealand	12
203	SLS23330	43790	New Zealand	12
204	SLS23301	43762	New Zealand	12
205	SLS23299	43760	New Zealand	12
206	SLS23309	43769	New Zealand	12
207	SLS23312	43772	New Zealand	12
208	SLS23291	43752	New Zealand	12
210	SLS23298	43759	New Zealand	12
211	SLS23319	43779	New Zealand	12
212	SLS23287	43748	New Zealand	12
213	SLS23281	43742	New Zealand	12
214	SLS23295	43756	New Zealand	12
215	SLS23300	43761	New Zealand	12
217	SLS23288	43749	New Zealand	12
220	SLS23296	43757	New Zealand	12
225	SLS23316	43776	New Zealand	12
226	CS130	26848	New Zealand	12
229	CS218	44977	New Zealand	12
230	CS23889	44979	New Zealand	12

Table 4. Haplotype designations by locality with the frequency of each haplotype noted in parentheses.

Locality	**Haplotype (frequency)**
Alaska	8(1), 29(1), 30(2)
Arkansas	3(1)
Australia	5(1)
Canary Islands	12(1)
Colorado	10(1), 17(2)
Costa Rica	20(1), 28(1)
Galapagos Islands	7(1), 16(1)
Guatemala	5(1), 1(3), 25(1), 26(1), 27(1)
Guyana	24(1)
Hawaii	4(1)
Kansas	5(4), 17(1), 18(1)
Macquarie Island	7(1)
Madagascar	15(1)
Malawi	11(1), 23(1)
New Mexico	17(1)
New Zealand	1(1), 4(1), 12(33), 13(2), 14(1), 17(1), 19(1), 20(1), 21(1), 22(2)
North Dakota	5(6), 6(2), 9(1)
Oklahoma	9(1), 17(1)
Peru	23(1)
Sweden	1(1), 19(1)
Tanzania	2(1), 12(1)
Wyoming	17(2)

In the gene tree produced by Bayesian analysis (Figure 3), three (I, II and III) significant clades (posterior probability greater than or equal to 0.75 or 75%) are resolved that include 11, 12, and 37 collections, respectively. None of the three groups is made up of collections from a single locality or region. Rather, they include a mix of sequences from geographically disparate localities. Group I includes seven sequences that originated from collections representing the grasslands of the central United States, but along with these are sequences representing one collection from New Zealand and three from Guatemala. Group II is also mostly made up of sequences representing collections from the grasslands (10 sequences), but includes two sequences, one each from Australian and Guatemalan collections.

Group III is made up of sequences that mostly represent New Zealand (34 of 37). Included in this group, however, are three sequences from quite disparate localities, including the Galapagos Islands, Canary Islands and Tanzania. The large number of sequences from New Zealand may be misleading because it includes all of the isolates collected at the same time in a single locality (NZ200-208, 210-215, 217, 220, 225-226), and all of these may in fact represent a single individual. The collecting locality was approximately 20 by 300 m. An apparently single individual of a myxomycete (likely a species of *Didymium*) has been reported to occur over an area at least 1.3 km^2 as determined by plasmodial fusion test. In the latter, an individual is determined by whether or not plasmodia arising from different fruiting bodies fuse to form a single plasmodium (Stephenson et al. 2004). *Didymium squamulosum* does—possibly frequently—occur as an asexual strain in nature (El Hage et al. 2000). If this is the case at this site, then all of the above-mentioned sequences are multiple sequences from the same individual. However, without plasmodial fusion tests, there is currently no way to determine if all of these collections represent one individual. As described later in this chapter, spores from only five collections from this locality germinated in laboratory culture and none of these formed plasmodia, making it impossible to carry out the plasmodia fusion tests.

Within the other tip clades, there are multiple examples of sequences from disparate geographical localities grouped together, including groups represented by both hemispheres. For example, group IV is made up of a sequence from a Costa Rican collection and two from New

Zealand collections. Group V is made up of three sequences, one each from Kansas, Sweden and New Zealand. Group VI included two sequences representing collections from the Galapagos Islands off the coast of Ecuador in South America and Macquarie Island in the Subantarctic.

Three general regions of the world were represented by the most collections sequenced for this data set. Forty-four collections were from New Zealand, 23 from the central United States grasslands, and nine from Central America. None of these groups of collections were resolved into unique clades. For example, New Zealand collections were found in eight different tip clades, Central American collections were found in seven places on the tree, and the grasslands collections are found in five different places on the gene tree.

All of the haplotypes determined by DnaSP (Librado and Rozas 2009) correspond to clades on the tree. No single haplotype with multiple sequences is found in two or more separate significant clades on the tree.

A haplotype network (Figure 4) constructed by TCS (Clement et al. 2000) using all of the sequences also corresponds to the haplotypes calculated by DnaSP. The haplotype network resolves three major groups separated by multiple steps. Groups one and two are separated by 10 steps, and 17 steps separate groups two and three. In the network, haplotype numbers are the same as those found in the DnaSP output (Table 3) because the sequences grouped together are the same. Comparing haplotype numbers to locality, it was determined that group one consists of nine different localities, group 2 has 16 different localities, and group three has seven different localities. No single group contains all of the collections from and of the three regions (Central America, the central United States grasslands, and New Zealand). Sequences from Central American collections are found in all three groups, sequences from the grasslands collections are found in groups one and two, and sequences from New Zealand collections are found in all three groups.

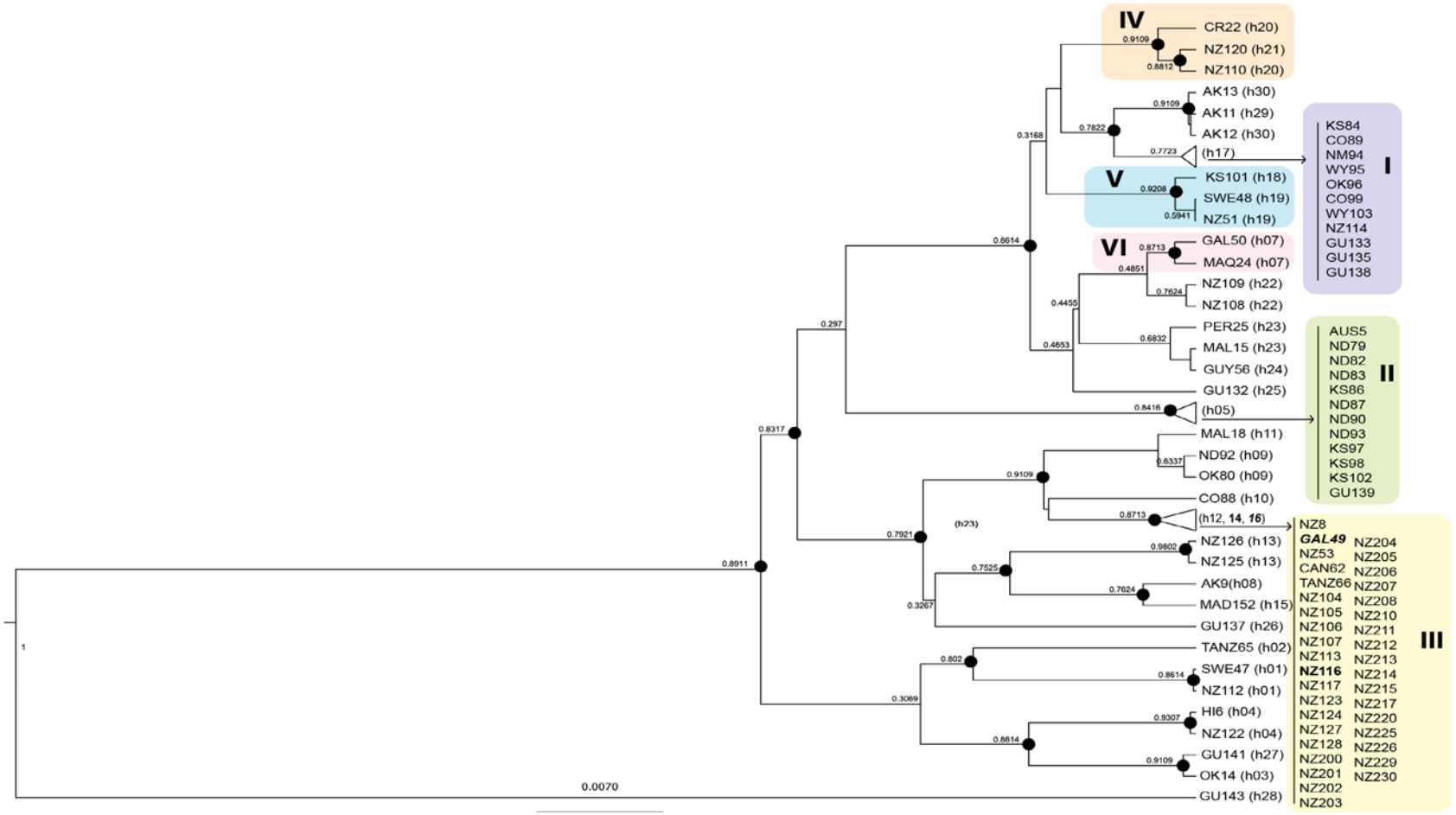

Figure 3. Unrooted gene tree of the ~400 bp section of mitochondrial small subunit for 96 collections of *Didymium squamulosum* collected worldwide. Node labels are posterior probability values to 4 significant digits. Significance of posterior probabilities increases as number approaches 1 or 100%. Nodes with black dot are significant (≥75%) Collapsed clades had no internal nodes with PP values gre ater than 20%. Other nodes with PP values less than 20% are not marked. Haplotype designations for each sequence are indicated in parentheses.

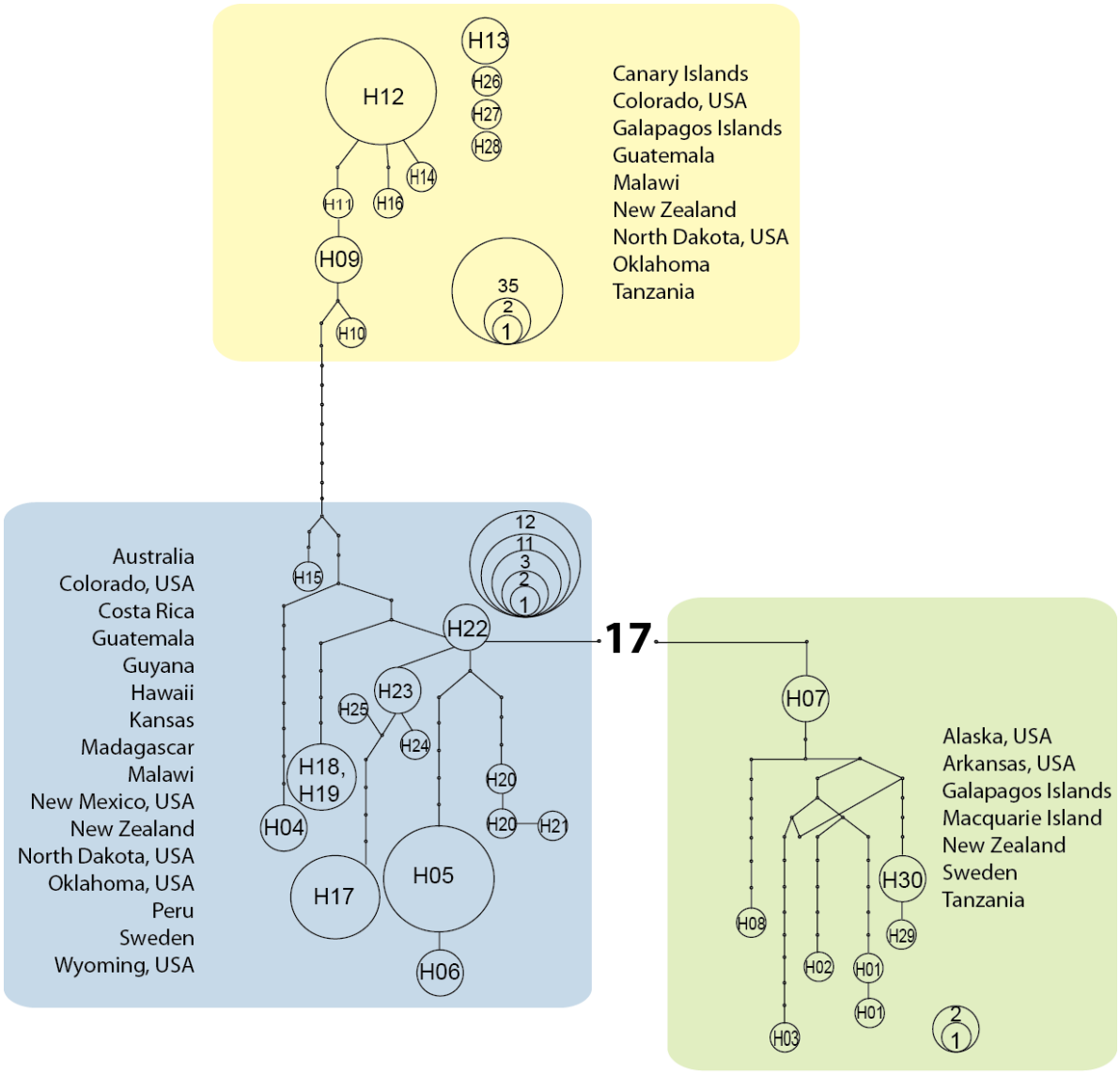

Figure 4. Gene geneology for the mitochondrial small subunit locus sequences estimated by TCS (Clement et al. 2000) with maximum connection steps set at 20 steps. A small circle (unit branch) represents one mutation with the site in the alignment where the mutation occurred noted on the branch. Square and oval sizes are proportional to the sequences represented by the haplotype. Haplotype numbers corresponds to those given in Table 3.

b. Morphology

The mechanism determining the variable morphology within cosmopolitan species of myxomycetes is, as yet, unexplained. It is possible that these variations correlate to genetic distance where, in a species such as *Didymium squamulosum* in which multiple asexual lines have been determined, genetic variation has accumulated in separate lines over time so that it can be seen in phenotypic variation (Clark 2000). Because this species is found in a variety of habitats and on a variety of substrates, it is also possible that variation could be explained as phenotypic plasticity, where the variation in the environment causes variation in phenotype.

It is evident from the species description from Martin and Alexopoulos (1969) as well as the observed characters in the series of collections used for this molecular study, that *Didymium squamulosum* shows a range of morphological variation with respect to morphological characters, particularly those characters used to typify the species (e.g., stalk shape, peridium lime and color). Four characters—each found with multiple states as summarized in Table 5—that are used for diagnosing a fruiting body as this species were described for the series of collections included in this study (Table 6).

Table 5. Character states established for four different major morphological characters that are traditionally taxonomically important and known to vary within *D. squamulosum.*

Relative stalk length	Lime texture	Hypothallus	Stalk Color
Long	Flaky	Evident and large	White
Average	Grainy	Evident and small	Brown
Short	Smooth	Not Evident	Two-toned
			Orange

Table 6. Observations of the morphology in major characters of the fruiting body for the collections used in molecular analysis (n=96).

DNA #	Collector #	Accession #	Country	Substrate	Relative stalk length	Peridium lime	Stalk color	Hypothallus
6	SLS18764	24034	Hawaii, USA	Aerial litter	Average	Smooth	Orange	Not evident
8	CS149	44972	New Zealand	Palm petiole	Average	Grainy	White	Evident
9	SLS6393	6393	Alaska, USA	Ground litter	Short	Grainy	White	Small
11	SLS7112	7112	Alaska, USA	Ground litter	Short	Grainy	White	Small
18	SLS18798	18798	Malawi	Aerial litter	Long	Smooth	White	Not evident
22	MS14106	44981	Costa Rica	Ground litter	Long	Flaky	White	Not evident
24	SLS6952	6952	Macquarie Island	Living petioles	Short	Grainy	White	Evident
25	MM23646	44978	Peru	Litter	Long	Flaky	Brown	Not evident
48	UE6328	GB-0039249	Sweden	Ground litter	Short	Grainy	White	Evident
50	UE2346	GB-0039236	Galapagos Islands	Ground litter	Short	Flaky	White	Small
51	CS157	44973	New Zealand	Palm petiole	Long	Smooth	White	Evident
53	CS158	44674	New Zealand	Palm petiole	Average	Grainy	White	Small
79	AWR492	26039	North Dakota, USA	Aerial broadleaf	Average	Smooth	White	Evident
80	AWR754	26301	Oklahoma, USA	Aerial broadleaf	Average	Smooth	White	Evident
82	AWR706	26253	North Dakota, USA	Aerial grass	Average	Smooth	White	Not evident
83	AWR716	26263	North Dakota, USA	Aerial broadleaf	Average	Smooth	White	Evident
84	AWR677	26224	Kansas, USA	Aerial broadleaf	Average	Flaky	Orange	Small
85	AWR735	26282	North Dakota, USA	Aerial grass	Long	Smooth	White	Evident
86	AWR746	26293	Kansas, USA	Aerial broadleaf	Average	Smooth	White	Evident
87	AWR841	26388	North Dakota, USA	Aerial broadleaf	Average	Smooth	White	Not evident
88	AWR736	26283	Colorado, USA	Aerial broadleaf	Long	Smooth	Two-toned	Evident

89	AWR1059	26592	Colorado, USA	Aerial grass	Average	Flaky	Two-toned	Evident
90	AWR1075	26607	North Dakota, USA	Aerial broadleaf	Short	Smooth	White	Evident
92	AWR996	26537	North Dakota, USA	Aerial broadleaf	Long	Smooth	Two-toned	Evident
93	AWR1040	26573	North Dakota, USA	Aerial grass	Short	Smooth	Orange	Evident
94	AWR1023	26558	New Mexico, USA	Aerial broadleaf	Short	Flaky	Two-toned	Evident
95	AWR719	26266	Wyoming, USA	Aerial grass	Short	Flaky	Brown	Small
96	AWR713	26260	Oklahoma, USA	Aerial grass	Short	Flaky	White	Not evident
97	AWR738	26285	Kansas, USA	Ground broadleaf	Average	Smooth	White	Evident
98	AWR762	26309	Kansas, USA	Aerial broadleaf	Short	Smooth	White	Evident
100	AWR491	26038	North Dakota, USA	Aerial grass	Average	Smooth	White	Evident
101	AWR1112	26644	Kansas, USA	Aerial broadleaf	Short	Smooth	White	Evident
102	AWR1160	26689	Kansas, USA	Aerial broadleaf	Average	Smooth	White	Evident
103	AWR533	26080	Wyoming, USA	Aerial grass	Average	Flaky	Two-toned	Not evident
104	CS23880	44690	New Zealand	Palm petiole	Average	Smooth	White	Not evident
105	CS185	44974	New Zealand	Palm petiole	Average	Smooth	White	Small
106	CS187	44975	New Zealand	Palm petiole	Average	Smooth	White	Evident
107	CS188	44976	New Zealand	Palm petiole	Average	Smooth	Brown	Not evident
108	SLS9449	28100	New Zealand	Palm frond	Short	Grainy	White	Evident
109	SLS9394	28046	New Zealand	Palm frond	Average	Flaky	White	Small
110	SLS23723	44984	New Zealand	Aerial litter	Long	Flaky	Brown	Not evident
112	SLS9423	28074	New Zealand	Ground litter	Average	Smooth	White	Small
113	SLS9411	28062	New Zealand	Palm frond	Average	Smooth	White	Evident
114	SLS9591	28239	New Zealand	Palm frond	Average	Smooth	Two-toned	Not evident
116	SLS9428	28079	New Zealand	Palm frond	Long	Flaky	White	Evident

117	SLS9503	28151	New Zealand	Palm frond	Long	Smooth	White	Evident
120	SLS9478	28126	New Zealand	Aerial litter	Short	Smooth	Brown	Evident
122	SLS23890	44980	New Zealand	Palm frond	Long	Grainy	Brown	Evident
123	SLS9454	28105	New Zealand	Palm frond	Average	Smooth	White	Evident
124	SLS9378	28032	New Zealand	Palm frond	Average	Smooth	Brown	Evident
125	OTA58428	OTA58428	New Zealand	Ground litter	Short	Smooth	White	Not evident
126	SLS9381	28035	New Zealand	Palm frond	Average	Smooth	White	Small
127	CS050	44971	New Zealand	Palm frond	Average	Grainy	Brown	Evident
128	CS082	44647	New Zealand	Ground litter	Long	Grainy	White	Evident
132	CR1349	31858	Guatemala	Ground litter	Short	Flaky	Two-toned	Evident
133	CR1355	31864	Guatemala	Aerial litter	Short	Flaky	Two-toned	Not evident
135	CR1376	31885	Guatemala	Aerial litter	Short	Flaky	Two-toned	Not evident
138	CR1386	31894	Guatemala	Aerial litter	Short	Flaky	Brown	Not evident
139	CR1390	31898	Guatemala	Ground litter	Average	Smooth	White	Evident
141	CR1402	31910	Guatemala	Aerial litter	Short	Smooth	Two-toned	Evident
143	MS14530	44982	Costa Rica	Ground litter	Average	Grainy	White	Evident
200	SLS23302	43763	New Zealand	Palm frond	Average	Grainy	Brown	Evident
201	SLS23307	43767	New Zealand	Palm frond	Average	Grainy	White	Evident
202	SLS23322	43782	New Zealand	Palm frond	Average	Smooth	White	Small
203	SLS23330	43790	New Zealand	Palm frond	Average	Grainy	Orange	Small
204	SLS23301	43762	New Zealand	Palm frond	Long	Smooth	White	Small
205	SLS23299	43760	New Zealand	Palm frond	Long	Smooth	White	Small
206	SLS23309	43769	New Zealand	Palm frond	Long	Smooth	Brown	Small
207	SLS23312	43772	New Zealand	Palm frond	Long	Smooth	White	Small
208	SLS23291	43752	New Zealand	Palm frond	Average	Smooth	Brown	Evident
210	SLS23298	43759	New Zealand	Palm frond	Average	Grainy	White	Evident
211	SLS23319	43779	New Zealand	Palm frond	Average	Grainy	White	Evident
212	SLS23287	43748	New Zealand	Palm frond	Average	Grainy	Brown	Evident
213	SLS23281	43742	New Zealand	Palm frond	Long	Smooth	White	Evident
214	SLS23295	43756	New Zealand	Palm frond	Average	Smooth	White	Evident
215	SLS23300	43761	New Zealand	Palm frond	Average	Grainy	Two-toned	Not evident

217	SLS23288	43749	New Zealand	Palm frond	Average	Grainy	Two-toned	Not evident
220	SLS23296	43757	New Zealand	Palm frond	Average	Grainy	White	Evident
225	SLS23316	43776	New Zealand	Palm frond	Average	Smooth	White	Small
229	CS218	44977	New Zealand	Palm frond	Average	Grainy	White	Small
230	CS23889	44979	New Zealand	Palm frond	Average	Smooth	White	Evident

These characters also represent the most obvious (i.e., macroscopic) expressions of morphological variety within the morphospecies. The variation in stalk length was categorized using obvious demarcations in relative size when compared to the other collections. The three sizes as illustrated in Figure 5 were long (which were also characterized by a thin stalk), average (which were more stout than the long stalks), and short (which included the collections that were nearly sessile as both forms were found in most collections characterized by short stalks).

The peridium that covers the spore mass has an outer layer of calcium carbonate (generally called “lime”) that can be seen to display different textures (Table 5, Figure 6). These textures tended to remain constant within a collection, but varied between collections.

The hypothallus is a thin layer of acellular material deposited on the substrate underneath a fruiting. Three general types of hypothalli—evident, evident and small, and not evident—were apparent in these collections (Table 5, Figure 7). An evident hypothallus was much wider than the base of the stalk and appeared white due to lime deposits. A small hypothallus was barely wider than the base of the stalk, but also white or mostly white with lime deposits. When a hypothallus was not evident, there were no lime deposits beneath the stalk. The hypothallus is most likely present but transparent, so that only the color of the substrate is seen beneath the stalk.

The stalk of *Didymium squamulosum* is generally white because of lime deposits, but four different colors were observed in these collections (Table 5, Figure 8). The orange, brown and two-toned stalks were without readily apparent lime. However, in all cases, lime was deposited on the peridium, so the abiotic components from which the lime is derived were not absent from the environment.

The spores of *Didymium squamulosum* are dark brown by transmitted light. The description from Martin and Alexopoulos (1969) states that the ornamentation is minute, either shaped as warts or small spines with an average diameter of 8-11 μm. Micrographs of spores (Figure 9) from a selection of collections that are variable in other characters show little demonstrable variation in spore morphology, and fit within the expected average diameter. All spores have small warts and a common color. Variation on the edges of each spore such as the

perceived ridges on B and the difficulty seeing the warts on B, F or G are artifacts from creating the micrograph. Spore H appears to have a thick outside coat. This spore is from one of the oldest specimens, so it may be that the spore is partially dehydrated with the protoplast pulling away from the edge of the spore.

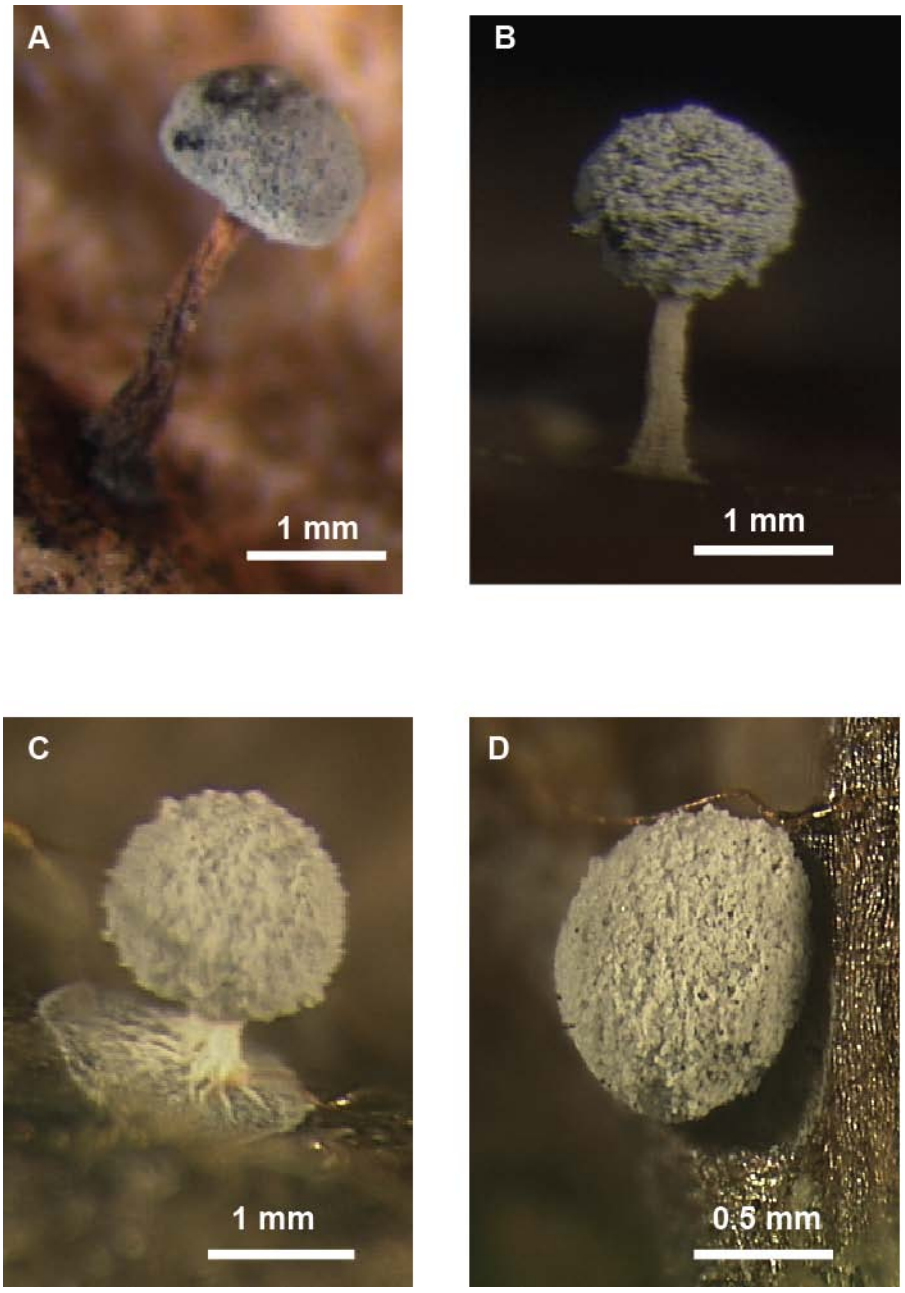

Figure 5. Illustration of the variation that exists in relative stalk length within collections of *Didymium squamulosum*. A. long; B. average; C. short; and D. nearly sessile stalks.

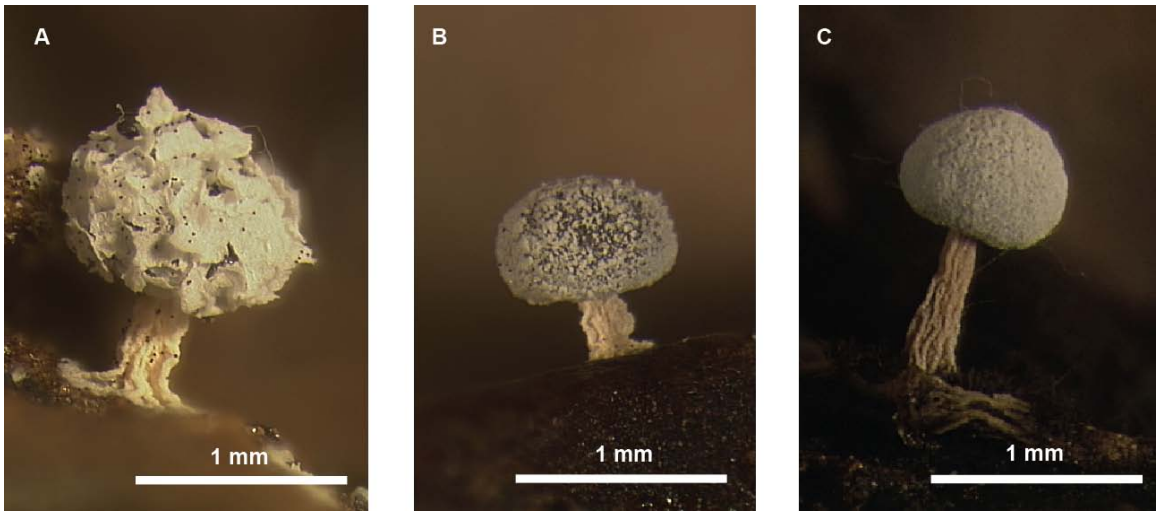

Figure 6. An illustration of the variation in peridium texture found in *Didymium squamulosum*: A. flaky, B. grainy, and C. smooth.

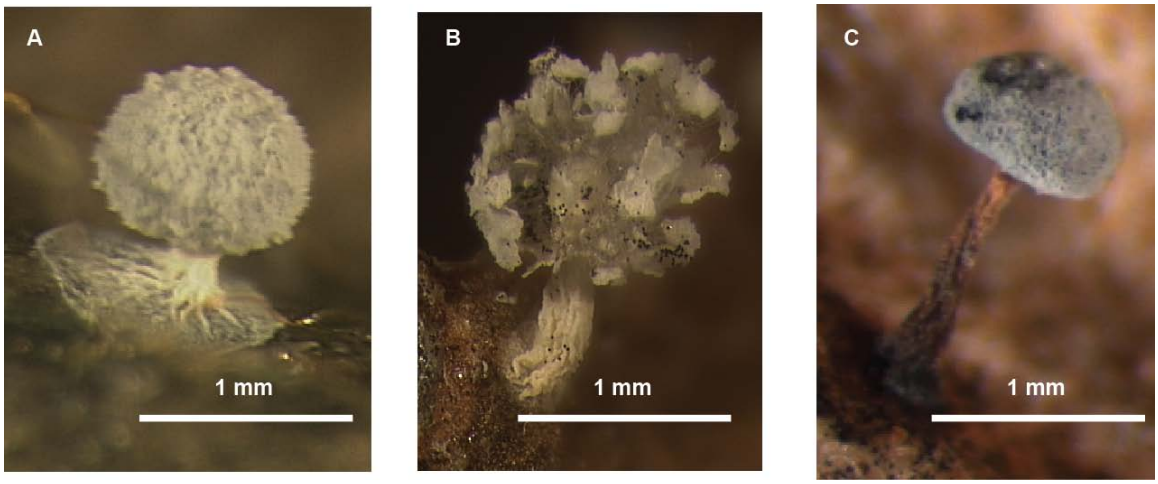

Figure 7. An illustration of the variation that occurs for the hypothallus found in collections of *Didymium squamulosum*: A. evident, B. evident but small, and C. not evident.

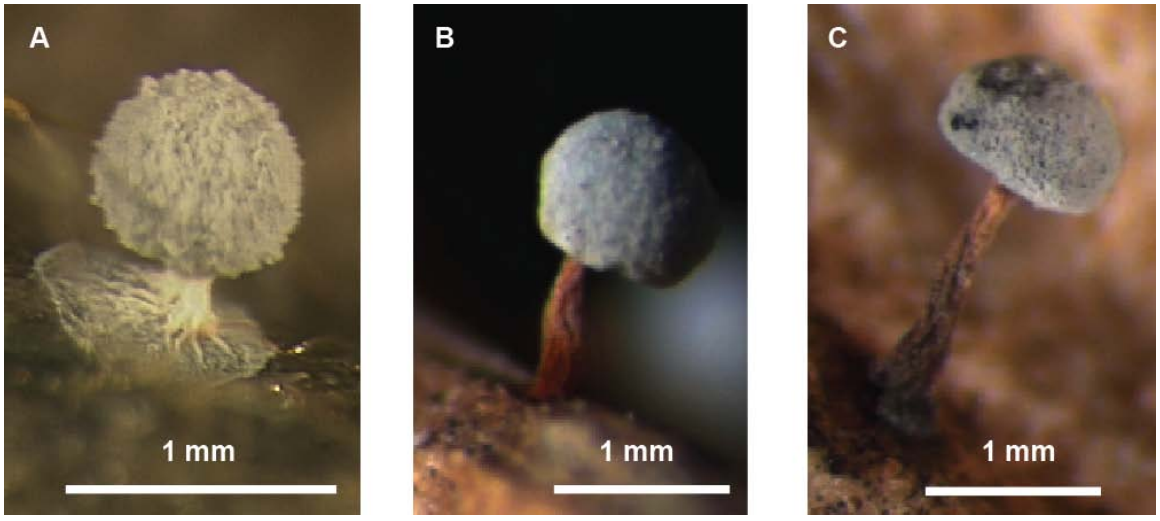

Figure 8. An illustration of the variation that exists in stalk color: A. white, B. orange and C. two-toned.

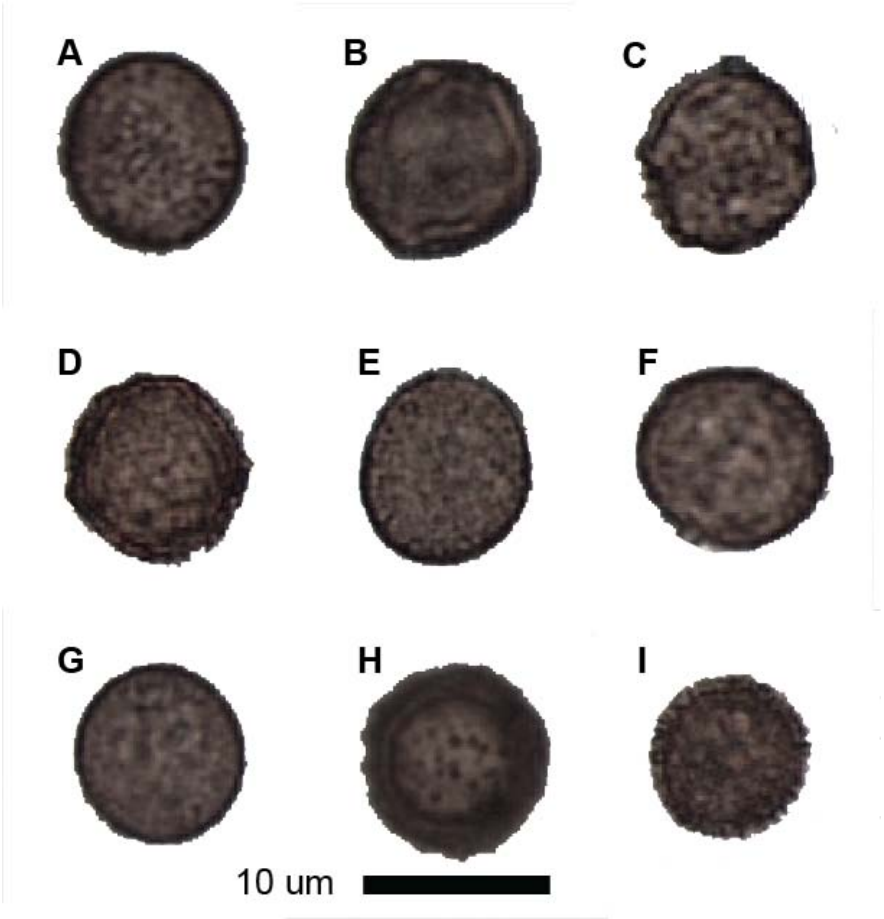

Figure 9. DIC 63x oil micrographs of spores from herbarium specimens of *Didymium squamulosum.* A. 14106, B. 14530, C. 23723 D. 26260, E. 26282, F. 26573 G. 31891, H. CS158, I. CS182b.

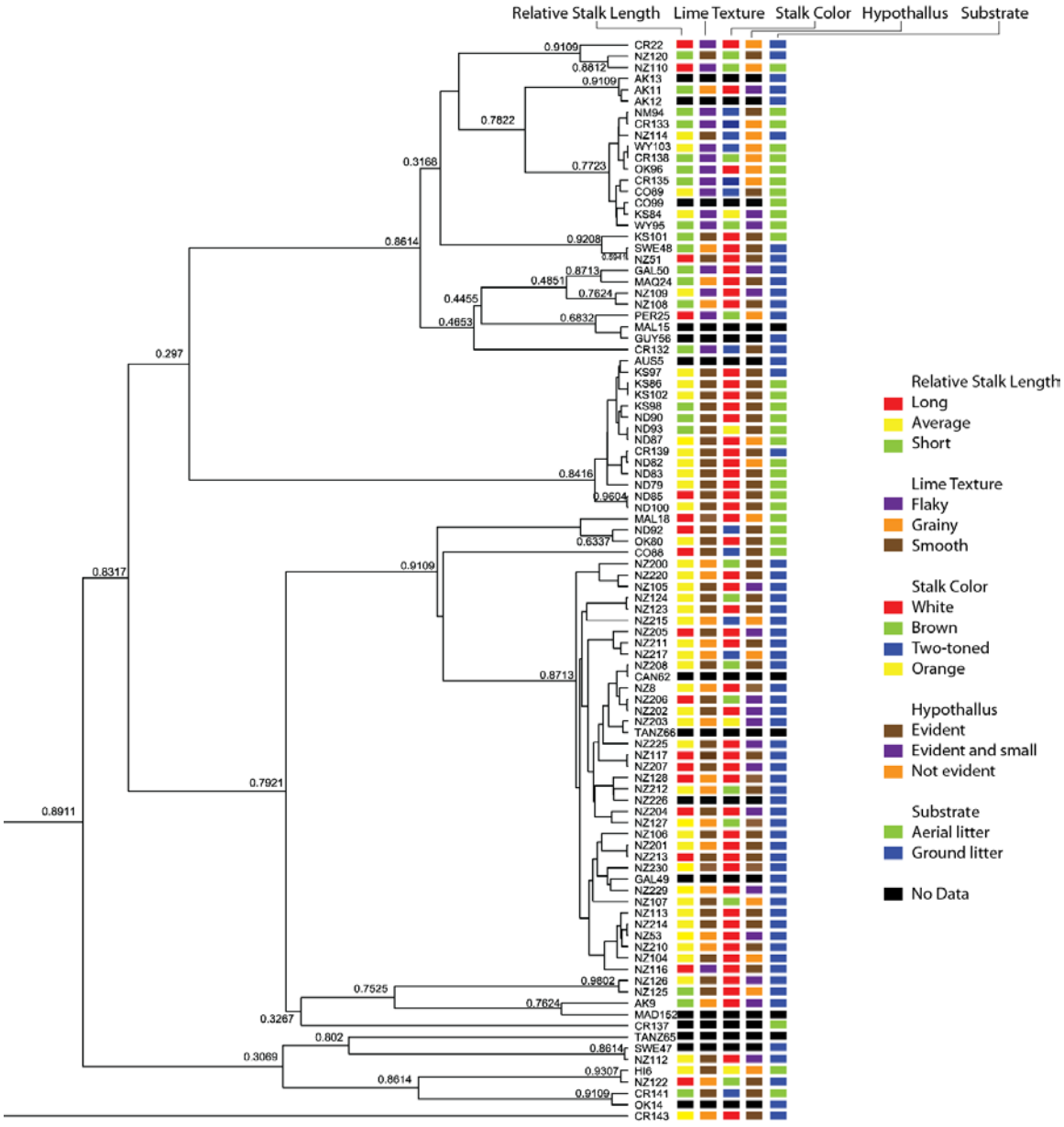

Figure 10. Morphological character states mapped onto the unrooted tree from Bayesian analysis of DNA sequence data for 96 collections of *Didymium squamulosum.*

There were no patterns among the morphological data such that all collections exhibiting a certain character state also were characterized by some other character state. Moreover, no character state appeared to be associated with particular substrate types. The character states did not correspond to any of the significant clades that resulted from the Bayesian analysis of the DNA sequence data from 96 collections of the species (Figure 10). All of the specimens from the local population in New Zealand (NZ200-225), which were collected at the same time and have the same DNA sequence for this marker, showed variation in all traits. Relative stalk length was found as both average and long, the lime on the peridium found as both smooth and grainy, stalk color included all four states, and the hypothallus varied in size and was absent in three of the eighteen collections.

The variation found within collections further suggests that morphological variation is not associated with genetic variation. The characters described for each collection were not always stable within the collection. For example, stalk length varied within collections (Figure 11). In this case, these fruiting bodies are the same individual and the same age, but the length of the stalks is different between the two fruiting bodies.

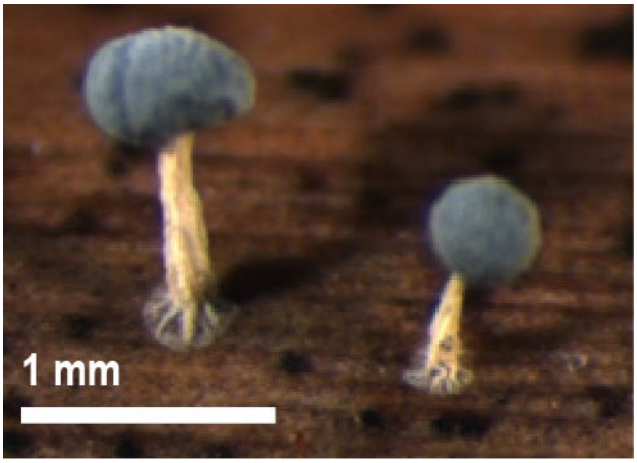

Figure 11. The extent of the variation in stalk length found within collections.

As mentioned earlier, an effort was made to germinate all collections in culture to compare the original morphology of the fruiting bodies formed in nature or that appeared in moist

chamber cultures to fruiting bodies grown under standard culture conditions. This was done to attempt to control the environment in which each isolate forms fruiting bodies. The collections used in this study came from a variety of habitat types and fruited on a variety of different substrates. If environment is playing a role in the phenotypic variation, controlled culture conditions should eliminate the different conditions that would affect the phenotype causing observable variation. Spore germination occurred for 15 collections (Table 7). Only two of these produced fruiting bodies. When these two collections of fruiting bodies were compared to the original morphology, variation in the four characters examined was observed. Collection 26573 produced a clearly white stalk—caused by calcium carbonate deposits on the surface—in culture, thus differing from the specimen that fruited on a natural substrate, which appeared orange—with little or no calcium carbonate on the surface (Figure 12).

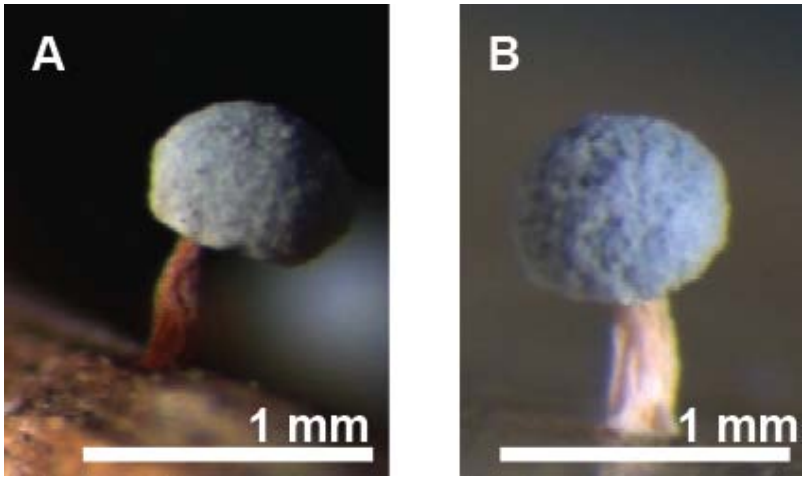

Figure 12. Fruiting bodies from the same isolate. The fruiting body on the left developed on a natural substrate. The fruiting body on the right developed in agar culture using standard culturing conditions.

Collection 26283 also varied in stalk color between agar culture and the fruiting that occurred on a natural substrate. However, the stalk did not appear white in either case. Instead, in culture the stalk was one color rather than the two-toned situation characteristic of fruiting bodies from the natural substrate. In both cases, the hypothallus was evident on the natural substrate but not in agar culture. However, this could be due to the structure being difficult to see

on agar. Although these are only two of the 96 collections cultured from spore to spore on agar, they do provide evidence suggesting that phenotypic variation is a result of the plastic nature of the characters in different environments rather than stable variation due to genetic distance.

In light of the possibility that morphological variation is determined by environmental conditions, this aspect of the biology of *Didymium squamulosum* could be further studied by spore-to-spore culture in a variety of laboratory culture conditions. For example, a natural substrate-based agar such as a leaf litter infusion or an addition of a sterilized piece of an appropriate substrate such as a leaf petiole to the laboratory culture could be used. Any evidence of morphological variation in this additional culture would be further evidence that the character states observed are affected by some combination of environmental factors.

Table 7. Collections that were cultured from spore to spore. Abbreviations in column of culturing information are: G—spores germinated in agar culture; P—plasmodium formed in agar culture; FB—fruiting bodies formed in agar culture. Character descriptions are listed as herbarium specimen/specimen obtained fromagar culture.

DNA #	Accession #	Culture	Country	Substrate	Relative stalk length	Peridium lime	Stalk color	Hypothallus
80	26301	G	Oklahoma, USA	Aerial broadleaf				
82	26253	G, P	North Dakota, USA	Aerial grass				
85	26282	G, P	North Dakota, USA	Aerial grass				
86	26293	G	Kansas, USA	Aerial broadleaf				
88	26283	G, P, FB	Colorado, USA	Aerial broadleaf	Long/ long	Smooth/ smooth	Two-toned/ orange	Evident/ not evident
92	26537	G	North Dakota, USA	Aerial broadleaf				
93	26573	G, P, FB	North Dakota, USA	Aerial grass	Short/ average	Smooth/ smooth	Orange/ white	Evident/ not evident
132	31858	G	Guatemala	Ground litter				
135	31885	G	Guatemala	Aerial litter				
141	31910	G	Guatemala	Aerial litter				
200	43763	G	New Zealand	Palm frond				
203	43790	G	New Zealand	Palm frond				
207	43772	G	New Zealand	Palm frond				
208	43752	G	New Zealand	Palm frond				
211	43779	G	New Zealand	Palm frond				

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Biodiversity occurrence data provided by: BCCM/MUCL - (Agro)Industrial Fungi and Yeasts Collection Banc de dades de biodiversitat de Catalunya-FungaCat, Banc de dades de biodiversitat de Catalunya-LiqueCat, Database of Fungal Specimens Maintained at the Selected Japanese Herbaria, Myxomycetes Collection of National Museum of Nature and Science, Danish Mycological Society fungal records database, UCD BOTANICAL CONSERVATORY, Herbarium of Oskarshamn (OHN), Fungi (S), Botany (UPS), ATCC Fungi Collection Catalog, DSMZ Fungi Mycology herbarium Oslo (O), CBS Filamentous Fungi Database, Pollichia Pilzherbar, Biologiezentrum Linz, Nationaal Herbarium Nederland - Leiden Branch, Harvard University Herbaria, The Myxomycetes Collections at the Botanische Staatssammlung Munchen - Collection of Hermann Neubert, The Myxomycetes Collections at the Botanische Staatssammlung Munchen - Collection of Martin Schnittler, The Fungal Collection at the Staatliches Museum fur Naturkunde Gerlitz, The Myxomycetes Collections at the Botanische Staatssammlung Munchen - Main Collection, Herbarium GJO, Water Colours of Fungi by Fritz Wohlfarth at the Botanische Staatssammlung Munchen, Herbarium WU Bishop Museum Natural History Specimen Data, CBS fungi strains, BCCM/IHEM - Biomedical Fungi and Yeasts Collection Australian National Herbarium (CANB), CABI Bioscience Genetic Resource Collection, Flora Mycologica Iberica Real Jardin Botanico Madrid: MA-Funhist, IFO Filamentous Fungi, The Myxomycetes Collection at the V. L. Komarov Botanical Institute St. Petersburg, Gothenburg Herbarium - General (GBIF: IH: GB: Herbarium), Real Jardin Botanico Madrid: MA-Fungi Planetary Biodiversity Inventory Eumycetozoan Databank, International Collection of Microorganisms from Plants - New Zealand National Microbial Collection, NZFUNGI - Collections of the New Zealand National Fungal Herbarium (PDD), National Botanic Garden Belgium – Myxomycetes, Phanerogamie USU-UTC Specimen Database SysTax, CABI Bioscience Fungus Collection, Botanical Museum Copenhagen Mycology Herbarium, Herbarium Slovenian Fungal Database - Boletus informaticus, Japan Collection of Microorganisms(Fungi), Departamento de Investigacion y Experiencias Forestales de Valonsadero de la Junta de Castilla y Leon: JCYL-Fungi, Genebank National Institute of Agrobiological Sciences Biological Resource Center, Department of Biotechnology National Institute of Technology and Evaluation RBGE Herbarium (E), Fungi Collection in BCRC

Herbarium of The New York Botanical Garden Zoological specimens, RBGE Living Collections, Herbario micologico de La Universidad de La Laguna Tenerife: TFC_Mic Registros biologicos en areas protegidas obtenidos de documentos impresos, ANTARCTIC PLANT DATABASE New Zealand Biodiversity, Recording Network Plants of Papua New Guinea, Herbier de la Guyane, CONN GBIF data Paleobiology Database Ibaraki Nature Museum Fungi collection (1) Generalitat Valenciana. Banco de Datos de la Biodiversidad de la Comunitat Valenciana, BDBCV - II Semana de la Biodiversidad (Castellon Spain) 2007 BDBCV - II Semana de la Biodiversidad (Castellon Spain) 2007, EDIT - ATBI in Mercantour/Alpi Marittime (France/Italy), Inventaire national du Patrimoine naturel (INPN) Gurgltal (Tarrenz), Tag der Artenvielfalt im Taubental, The Collection of Lichenicolous Fungi at the Botanische Staatssammlung Munchen, Biological and palaeontological collection and observation data MNHNL Schwanheimer Wald Heinersdorfer Sumpfwiese, Irish Records from the Fungal Records Database of Britain and Ireland, GEO-Hauptveranstaltung (NLP Harz / Hochharz), Water Colours of Fungi by Konrad Schieferdecker at the Botanische Staatssammlung Munchen Naturschutzgebiet Heiliger Hain (Wahrenholz), Fungi Collection Dalbekschlucht Staatsforst Rehna - Woitendorfer Wald im Biospherenreservat Schaalsee GEO-Hauptveranstaltung (Insel Vilm), East Ayrshire Countryside Ranger Service - East Ayrshire Species Database Fungal Specimens collected by HabitatVision (Jacob Heilmann-Clausen) Fungi (GBIF-SE:Artdatabanken), Take a Pride in Fife Environmental Information Centre - Records for Fife from TAPIF EIC Fungi BioFokus Fungi field notes Oslo (O), Notes from the Mycology Herbarium Oslo (O), Mycology herbarium Bergen (BG) Mycology Herbarium Trondheim (TRH), Fungus collection of the Jyvaskyla University Museum, Herbarium (AMNH). (Accessed through GBIF Data Portal, data.gbif.org, 2010-04).

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Chapter 5

Intraspecific genetic variation among isolates of *Didymium squamulosum* from a single geographic locality

A. Introduction

Myxomycetes (myxogastrids or plasmodial slime molds) are terrestrial protists with micro- and macroscopic life stages that include an amoeboflagellate, a plasmodium and an aerial, spore-bearing structure usually referred to as a fruiting body. The vegetative stages—amoeboflagellate and plasmodium—are both motile and occur within the substrate, where they feed on bacteria. Under conditions that are still not fully understood, fruiting bodies are produced by plasmodia and spore formation occurs within the former (Stephenson and Stempen 1994). It is not possible to identify species from features of their amoeboflagellates or plasmodia because these stages are identical across species, so our understanding of the distribution of myxomycetes in nature relies upon fruiting body formation on the substrates within which these organisms occur.

The biology of myxomycetes is such that the study of relationships within a particular species is not straightforward. The life cycle has four distinct stages. The first of these is the spore, which germinates to give rise to an amoeboflagellate (Alexopoulos 1963, Martin and Alexopoulos 1969). In sexual strains, two compatible amoeboflagellates function as gametes to produce a diploid zygote, which undergoes multiple mitotic divisions to develop into the plasmodium, which is essentially a single large multinucleate cell with streaming plasmodium. Finally, fruiting bodies are derived from the plasmodium, with spores formed from the nuclei and cellular material within each developing fruiting body. The dispersal potential of this group of organisms is greatly enhanced by various aspects of this life cycle, since there are several possible points of dispersal—the spore, the amoeboflagellate and the plasmodium. A single fruiting body (also sometimes called a sporocarp) can produce on order of magnitude 10^5–10^6 spores (Schnittler and Tesmer 2008). With the usual production of more than one sporocarp per fruiting, the total spore potential exceeds several millions for a typical fruiting.

The protoplast of any particular spore emerges as an amoeboflagellate, which then divides by mitosis and subsequent cytokinesis in the substrate (soil or decaying plant material), thus producing a clonal population of amoebae. It is unknown how long the amoeboflagellate stage persists in nature. With the presence of sufficient food organisms, amoeboflagellate populations may grow very large. In culture, the number of amoeboflagellates can be on order of magnitude 10^3-10^4 per Petri dish. In an asexual strain, as is common in *Didymium squamulosum* (El Hage et al. 2000), each amoeboflagellate has the potential to produce a plasmodium. In asexual strains, the amoeboflagellate emerges from the spore as a diploid cell and thus is able to begin mitosis without fusion with a compatible cell to form a zygote. Again, one can only speculate—based upon behavior in culture—as to how long an isolate remains in the plasmodium stage and how large a single plasmodium can become. It has been observed that a plasmodium can remain without fruiting in culture for weeks or months if provided with adequate growing conditions. It also has been observed in culture that the larger a plasmodium grows, the more fruiting bodies are generally produced. As such, the number of plasmodia and the size of the plasmodia found in nature would affect the reproductive potential of an isolate.

Because there is no way of easily determining whether or not fruiting bodies found in close proximity on the same substrate are the same individual, derived from a clone, or are actually separate individuals in a population, the relationships within species of myxomycetes are poorly understood. It is known that heterothallic isolates (sexual isolates with more than one mating type required for zygote formation) and homothallic strains (sexual isolates with one mating type that may actually be apomictic or asexual) occur within the same morphospecies. However, the distribution of any of these genetic types in nature has been subjected to very little study because the study of populations in myxomycetes is not straightforward. This is due to the genetic complexity within a species makes a population difficult to define.

When collecting myxomycetes in the field, it is not uncommon to find multiple fruitings of the same species in the same locality. Rather than defining this as a population, there is some evidence to suggest that each fruiting is part of the same individual instead of a group of related individuals (Stephenson et al. 2004). The presence of homothallic or apomictic isolates within a

morphospecies and the high reproductive potential through more than one stage of the life cycle means it is possible for multiple isolates to be genetically identical, thus further complicating the definition of a population. Unless otherwise noted, the term “population” will be used herein to denote a group of amoeboflagellates or fruiting bodies, whether or not they actually represent a collection of different individuals in the same locality. The definition of an individual in myxomycetes is also a poorly understood concept. It is known that a single plasmodium can produce multiple fruiting bodies that in theory are parts of the same individual. This is easily recognized in culture where the entire plasmodium is visible and contained within a defined space. In nature it is not uncommon for plasmodia to be cryptic, occurring within or underneath a substrate rather than at the surface.

In a case where fruiting bodies of the same species are found at different locations on, for example, the same log on the forest floor, it is assumed they result from the same isolate. However, this is might not be the case. There are several possible explanations for this result based upon what is known about the life cycle of myxomycetes. First, a single plasmodium could have expanded to include the entire substrate and then fruited, so that the multiple fruiting bodies occur at different points along the substrate. Second, spores from a fruiting body on the substrate could have been dispersed, with a number of the spores from that fruiting body landing on or near the substrate in question. If multiple spores germinated, this would populate the substrate with the same strain of the species. Third, amoeboflagellates divide by mitosis and cytokinesis and travel along the substrate in search of food organisms. The amoeboflagellates could have formed plasmodia in different parts of the substrate, resulting in multiple fruitings of the same genetic strain. Fourth, a plasmodium could have been somehow divided or cut apart. Each of the resulting portions continues to move about in search of food organisms, ultimately producing fruiting bodies in different locations along the substrate. Fifth, the fruitings at different locations on the log actually could have developed from spores derived from completely different isolates of the same morphospecies.

Evidence for the relationship that exists among separated groups or clusters of fruiting bodies is limited to assumptions based upon observations of myxomycetes in laboratory culture.

No molecular (DNA-based) evidence is available to offer insights as to the relationship among fruiting bodies of a single species in a given locality or a particular substrate. Previous examinations of isolates from a given locality have used the presence or absence of fusion between plasmodia as the test for whether or not they represented the same individual. For example, in one such study it was found that a single genetic strain of an unknown species (but possibly *Didymium nigripes* [Link] Fr.) occurred over an area at least 1.3 km^2 (Stephenson et al. 2004). No fruiting bodies were obtained for any the strains tested, making it impossible to verify the species involved. However, it can be inferred from these data that clonal reproduction of a single individual occurred, either derived from an apomictic individual or cell division by amoebae, resulting in a large group of amoeboflagellates from the same spore found over a large area. Knowing that cosmopolitan species can exist as both homothallic and heterothallic sexual strains and asexual strains (El Hage et al. 2000), it is impossible to know just which genetic mechanism was involved from observation of morphology alone.

As noted above, the most widely used non-molecular method for determining what constitutes an individual in species of myxomycete is based on whether or not plasmodia from different isolates will fuse to form a single plasmodium. Fusion of plasmodia is controlled by a polygenic system involving approximately 20 loci that must have the same phenotype for fusion to occur (Ling and Clark 1981). Therefore, the assumption is made that fusion only occurs between identical isolates (Ling and Clark 1981, El Hage et al. 2000, Stephenson et al. 2004). This method is not appropriate for all myxomycetes because it has been possible to achieve growth of only about 10% of all species in laboratory culture (Haskins and Wrigley de Basanta 2008). Also, plasmodial fusion determines only if two isolates are the same individual and does not provide any information as to the degree of relationship between individuals if it is determined that the isolates are not the same. It has been possible in other organisms, such as fungi, to use DNA sequence analysis not only to derive information about local populations but also the degree to which isolates are related to each other and biogeographical significance of the genetic variation (see Vilgalys and Sun 1994, Wu et al. 2000, Zervakis et al. 2004), making this type of analysis more robust in determining any population structure that may exist within a set of isolates.

The study reported herein represents an effort to examine localized geographical patterns in myxomycetes using DNA sequence data, taking advantage of a series of collections of *Didymium squamulosum* (Alb. and Schwein.) Fr. (Physarales: Didymiaceae) that fruited in nature and were collected on the same day from the same locality. *Didymium squamulosum* is a common an apparently cosmopolitan morphospecies. However, it is unusual to find >15 fruitings at the same time and place, with the fruiting presumably representing a local population, as was the case in this instance.

In this study, twenty-one collections (seventeen collected on the same day) of *Didymium squamulosum* from a single locality in New Zealand were examined using molecular sequence analysis to determine the relationship that existed among discrete isolates of a single morphospecies These were compared with twenty-five other isolates collected at other localities throughout New Zealand.

B. Methods

1. Site Description

Forty-four collections of Didymium squamulosum from New Zealand were used for this study (Table 1). Sixteen collections representing specimens that fruited under natural conditions in the field were obtained from a single locality in the Waitoro Reserve of the Waitakere Ranges Regional Park (-36.861783, 174.517283) near Auckland, New Zealand on 31 October 2009. All of these collections were found within an area of approximately 20 by 300 m but physically separated from each other as a result of occurring on different substrates (i.e., these are not multiple collections derived from a fruiting that obviously arose from the same plasmodium). Three other collections (CS130, CS218 and CSAK181) were obtained in the same locality as the series of specimens collected on 31 October 2009 but on other dates in previous years. The remaining twenty-three collections were from other localities throughout New Zealand (Table 1 and Figure 1). Vouchers for all of these collections are deposited in the myxomycete collection at the University of Arkansas. All collection data can be accessed through the worldwide slime mold database at http://slimemold.uark.edu.

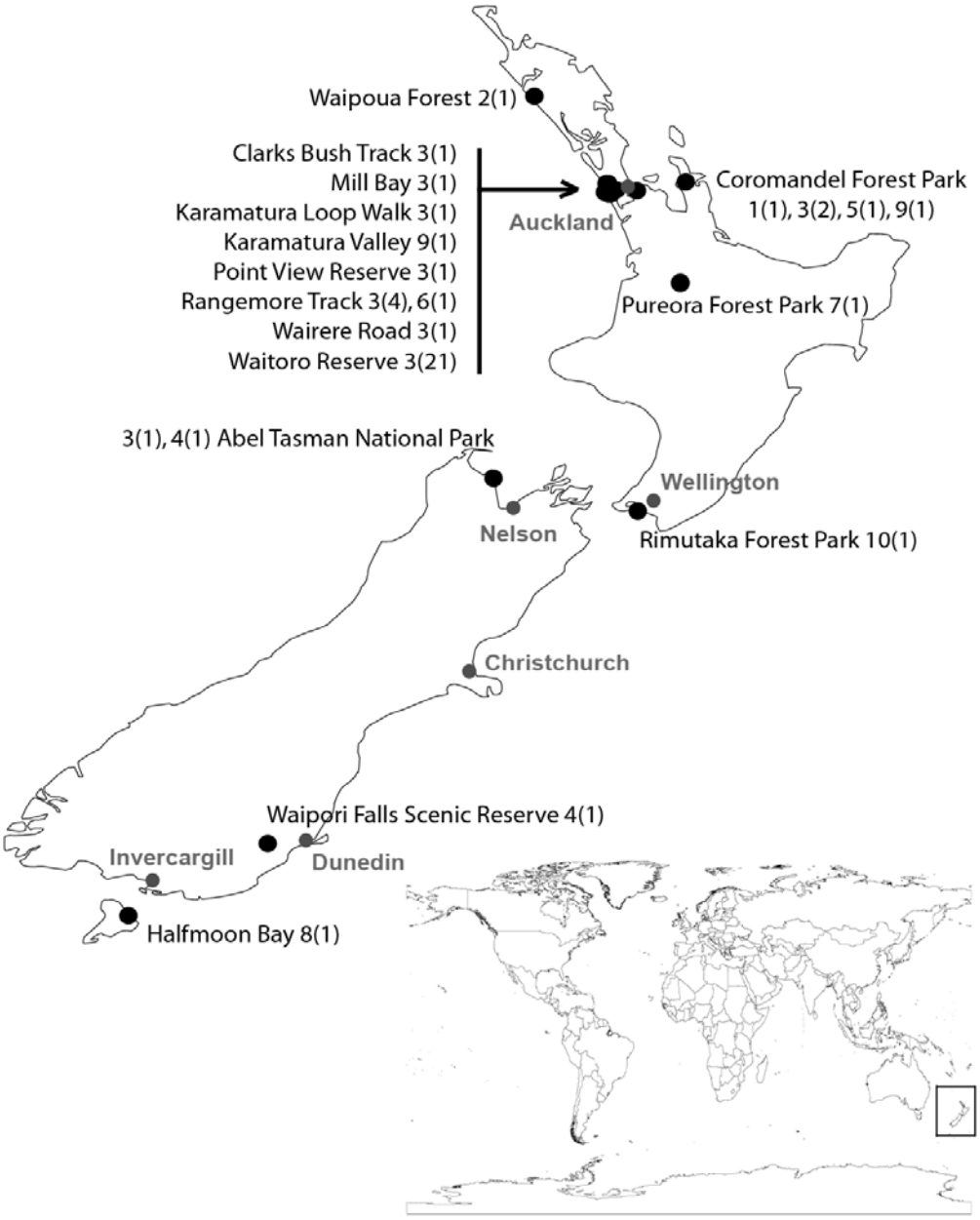

Figure 1. Collection localities for all isolates used in this study. Haplotype number followed by the frequency of that haplotype at that locality in parentheses is presented with the name of the locality.

Table 1. Identification and locality data for 44 isolates of *Didymium squamulosum* from New Zealand used in this study.

DNA Number	Collector number/ Accession number (UARK)	GenBank Accession number	Locality	Lat/Long	Haplotype designation
8	CS149/ 44972	HQ129885	Rangemore Track, Scenic Drive, Waitakere Ranges	-36.942432/ 174.593573	3
51	CS157/ 44973	HQ129905	Rangemore Track, Scenic Drive, Waitakere Ranges	-36.942432/ 174.593573	6
53	CS158/ 44674	HQ129886	Rangemore Track, Scenic Drive, Waitakere Ranges	-36.942432/ 174.593573	3
104	CS23880/ 44690	HQ129878	Rangemore Track, Scenic Drive, Waitakere Ranges	-36.942432/ 174.593573	3
105	CS185/ 44974	HQ129880	Point View Reserve, Manukau, Auckland	-36.948750/ 174.925579	3
106	CS187/ 44975	HQ129870	Karamatura Loop Walk, Huia, Waitakere Ranges	-37.00/ 174.549074	3
107	CS188/ 44976	HQ129871	Rangemore Track, Scenic Drive, Waitakere Ranges	-36.942432/ 174.593573	3
108	SLS9449/ 28100	HQ129908	Coromandel Forest Park on the Coromandel Peninsula	-36.83/ 175.58	9
109	SLS9394/ 28046	HQ129910	Karamatura Valley, Waitakere Ranges	-37.00/ 174.57	9
110	SLS23723/ 44984	HQ129906	Pureora Forest Park	-38.47/ 175.57	7
112	SLS9423/ 28074	HQ129868	Coromandel Forest Park on the Coromandel Peninsula	-36.83/ 175.58	1
113	SLS9411/ 28062	HQ129872	Coromandel Forest Park on the Coromandel Peninsula	-36.83/ 175.58	3
114	SLS9591/ 28239	HQ129911	Rimutaka Forest Park near Wellington	-41.35/ 174.93	10
116	SLS9428/ 28079	HQ129879	Coromandel Forest Park on the Coromandel Peninsula	-36.83/ 175.58	5
117	SLS9503/ 28151	HQ129877	Mill Bay, Waitakere Ranges	-36.97/ 174.50	3

120	SLS9478/ 28126	HQ129907	Roadside near Halfmoon Bay, Stewart Island	-46.90/ 168.13	8
122	SLS23890/ 44980	HQ129869	Waipoua Forest, Northland	-35.653/ 173.549	2
123	SLS9454/ 28105	HQ129881	Coromandel Forest Park on the Coromandel Peninsula	-36.83/ 175.58	3
124	SLS9378/ 28032	HQ129876	Abel Tasman National Park	-40.90/ 173.01	3
125	OTA58428	HQ129909	Waipori Falls Scenic Reserve, Otago	-45.907/ 169.982	4
126	SLS9381/ 28035	HQ129873	Abel Tasman National Park	-40.90/ 173.01	4
127	CS050/ 44971	HQ129875	Wairere Road, Waitakere Ranges	-36.851553/ 174.535796	3
128	CS082/ 44647	HQ129874	Clarks Bush Track, Titirangi, near Auckland	-36.939/ 174.642	3
200	SLS23302/ 43763	HQ129887	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
201	SLS23307/ 43767	HQ129888	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
202	SLS23322/ 43782	HQ129889	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
203	SLS23330/ 43790	HQ129890	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
204	SLS23301/ 43762	HQ129891	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
205	SLS23299/ 43760	HQ129892	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
206	SLS23309/ 43769	HQ129893	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
207	SLS23312/ 43772	HQ129894	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
208	SLS23291/ 43752	HQ129895	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
210	SLS23298/ 43759	HQ129896	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3

211	SLS23319/ 43779	HQ129897	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
212	SLS23287/ 43748	HQ129898	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
213	SLS23281/ 43742	HQ129899	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
214	SLS23295/ 43756	HQ129900	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
215	SLS23300/ 43761	HQ129901	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
217	SLS23288/ 43749	HQ129902	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
220	SLS23296/ 43757	HQ129903	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
225	SLS23316/ 43776	HQ129904	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
226	CS130/ 26848	HQ129882	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
229	CS218/ 44977	HQ129883	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3
230	CSAK181/ 44979	HQ129884	Waitoro Reserve, Waitakere Ranges	-36.86/ 174.52	3

2. Molecular Methods

DNA was extracted from sporocarps using the Chelex method as described previously (Winsett and Stephenson 2008). Briefly, spores from four to five sporocarps and 150 µL of a 5% Chelex suspension were placed in a thermocycler held at 56 C for four hours and then 98 C for thirty minutes to release DNA from the other cellular material that is part of the spore. Extracted DNA was frozen in microcentrifuge tubes at -20 C until use.

A 400 base pair hypervariable region of the mitochondrial small subunit (mtSSU) was amplified using the PCR primers mtCore1 (5′–TAG TGT TAT TCG TGA TGA CT–3′) and mtCore2 (5′–CTC GAA TTA AAC CAC AT–3′). The DNA templates were amplified using a 25 µL reaction (12.5 µL Promega GoTaq® Green Master Mix, 8.5 µL dH_2O, 1 µL each primer and 2 µL template DNA) using a PCR protocol, which consisted of an initial activation step at 94 C for two minutes (per instructions for Master Mix) and 35 repetitions of 94 C for 30 sec, 40 C for one min, and 72 C for one minute. The PCR amplicon was extracted from the PCR product by a modified gel electrophoresis and gel extraction method (modified from Dentinger et al. [2009]). A 20 µL portion of each PCR product was run in a 1% agarose gel prepared with 1X TA buffer (20X: 193.6 g Trizma base, 45.7 mL glacial acetic acid, dH_2O up to 2L, dilute to 1X) with ethidium bromide included at 80 V for approximately 30 minutes. The resulting band was cut from the gel and placed in a filtered micropipette tip cut to fit into a 1.5 mL microcentrifuge tube. The tube with the piece of gel sitting on the filter was frozen to break up the agarose matrix then spun in a centrifuge at 13 000 rpm for 10 minutes. The filtered pipette tip was removed from the tube, leaving the extracted DNA within the flow-through.

DNA samples were sequenced on a Perkin-Elmer ABI 3700 sequencer (Perkin Elmer Applied Biosystems Division) using amplification primers. Resulting chromatograms were checked and edited using Sequencher v4.3 (Gene Codes Corporation). All sequences were submitted to GenBank (Accession numbers HQ129868-HQ129911). Sequence alignments were performed using Clustal X and then manually edited in Seaview (Larkin et al. 2007, Gouy et al. 2010). Summary statistics for the sequences including the number of haplotypes, haplotype diversity, nucleotide diversity, and number of pairwise differences were calculated using DnaSP

v. 5 (Librado and Rozas 2009). This software was also used to test for neutrality of mutation through Tajima's (1989) D statistic and the D* and F* statistics of Fu and Li (1993). A gene tree was produced using Bayesian Markov Chain Monte Carlo methods using the software package BEAST v.1.5.4 (Drummond and Rambaut 2007). The alignment in nexus format was imported into BEAUTi, to format the appropriate XML file to be used by BEAST. The alignment was analyzed using estimated base frequencies and a chain length of 1 000 000. Performance suggestions for operators that were included in the output from BEAST were used to make operator modifications in BEAUTi to optimize the data set. The updated XML file produced by BEAUTi was imported into BEAST and reanalyzed. This step was repeated until the operator values were optimized according to the performance suggestions in the BEAST output file. The resulting ".trees" output was formatted using TreeAnnotator v.1.5.4 and saved as a ".tre" file that was viewed and edited in Fig Tree v. 1.3.1. A gene geneology (haplotype) network was constructed using TCS (Clement et al. 2000).

In addition, we attempted to sequence the nuclear small subunit of the ribosomal DNA (SSU rDNA) for each of the isolates to provide additional information for the patterns found in this analysis. However, only a few successful amplifications occurred and no sequences were obtained for this locus from any PCR product.

3. Agar Culture

An effort was made to germinate spores from all isolates in agar culture using standard methods (e.g. Haskins and Wrigley de Basanta 2008). Spores were placed directly onto the surface of two types of agar—half-strength cornmeal agar (CM/2) and 0.75% water agar. Spores from only two collections germinated. However, neither plasmodium nor fruiting bodies were observed. It was, therefore, impossible to carry out any genetic experiments to determine if any of the isolates represented the same individual on the basis of information obtained using the plasmodium fusion test or examining single spore isolations to determine if the isolates were not heterothallic for this dataset.

C. Results and Discussion

A 385 base pair region of the mitochondrial small subunit (mtSSU) was amplified and sequenced for forty-four isolates of *Didymium squamulosum* from New Zealand. There were a total of 40 variable sites, 12 of which were singleton polymorphisms, and 28 parsimony-informative sites. Summary statistics are presented in Table 2.

Table 2. Summary statistics for mtDNA polymoprhisms in sequences of *Didymium squamulosum* obtained from specimens collected in New Zealand. Note that n_s is the number of sequences; h_n is number of haplotypes; H_d ($\pm$SD) is haplotype diversity $\pm$ standard deviation; π (k) is nucleotide diversity and mean number of nucleotide differences.

n_s	h_n	H_d ($\pm$SD)	π (k)	Fu and Li's D*(P)	Fu and Li's F*(P)	Tajima's D (P)
44	10	0.440 (0.094)	0.01889 (7.23467)	-0.65838 (ns)	-0.84524 (ns)	-0.80687 (ns)

A BLASTn (Zhang et al. 2000) search of these sequences with the nucleotide collection in GenBank resulted in the greatest similarity with sequences available for *Physarum polycephalum* (Schwein.) mitochondrial genome and the mitochondrial small subunit gene for *P. polycephalum.*

Seven collecting localities representing 29 of the 44 isolates were located close together in the Waitakere Ranges near Auckland, New Zealand (Figure 3). Sixteen of these were collections obtained from the same locality on the same date (the Waitoro Reserve on 31 October 2009), with an additional three that were obtained at the same locality but on different dates. Of the other localities, three were located on the North Island, two on the South Island and one on Stewart Island (Figure 1).

These forty-four sequences represented ten haplotypes, seven of which were singletons. Haplotype designations for each sequence are included in Table 1, and haplotype frequencies by locality are found in Table 3. Haplotype designations are an arbitrary number between one and ten that correspond to the output from DnaSP—but have no specific meaning except to separate the sequences into the ten categories. The most frequent haplotype, haplotype three, occurred 33 times and was found at nine localities. Except for one sequence (NZ116) that was a singleton haplotype, haplotype groups correspond to the tip clades of the gene tree produced using BEAST (Figure 2).

Table 3. Frequency of each haplotype by collection locality.

Locality	Haplotype number (frequency)
Abel Tasman National Park	3(1), 4(1)
Clarks Bush Track	3(1)
Coromandel Forest Park	1(1), 3(2), 5(1), 9(1)
Halfmoon Bay	8(1)
Karamatura Loop Walk	3(1)
Karamatura Valley	9(1)
Mill Bay	3(1)
Point View Reserve	3(1)
Pureora Forest Park	7(1)
Rangemore Track	3(4), 6(1)
Rimutaka Forest Park	10(1)
Waipori Falls Scenic Reserve	4(1)
Waipoua Forest	2(1)
Wairere Road	3(1)
Waitoro Reserve	3 (21)

Nucleotide diversity (π) was 0.01889, and the average number for nucleotide difference (*k*) was 7.23467. All three test statistics for neutral mutations—Tajima (1989) D and Fu and Li (1993) D* and F*—were not significant; thus, the null hypothesis that mutations are neutral (are not under a selective pressure) cannot be rejected.

The haplotype network constructed using TCS shows the distance between haplotypes, and, except for one (haplotype nine), confirms the haplotype designations from DnaSP. In the TCS output (Figure 4), haplotype nine is divided such that each sequence is a separate haplotype separated by two mutations in the DNA sequence.

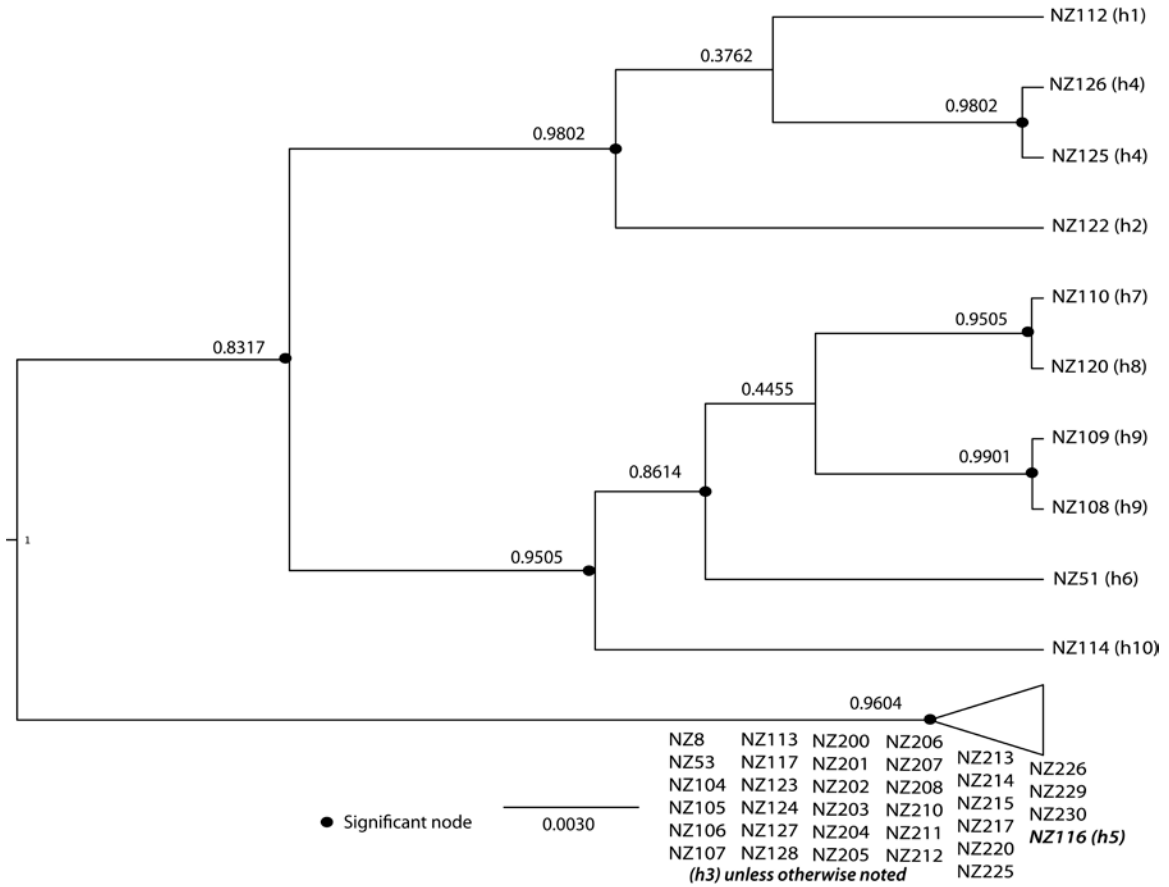

Figure 2. Unrooted gene tree of a 385 bp section of the mitochondrial small subunit for 44 isolates of *Didymium squamulosum* collected in New Zealand. Node labels are posterior probability values to 4 significant digits. Significance of posterior probabilities increases as number approaches 1 or 100%. Collapsed clade of 34 sequences (listed) had no internal nodes with PP values greater than 15%. Haplotype designations for each sequence are indicated in parentheses.

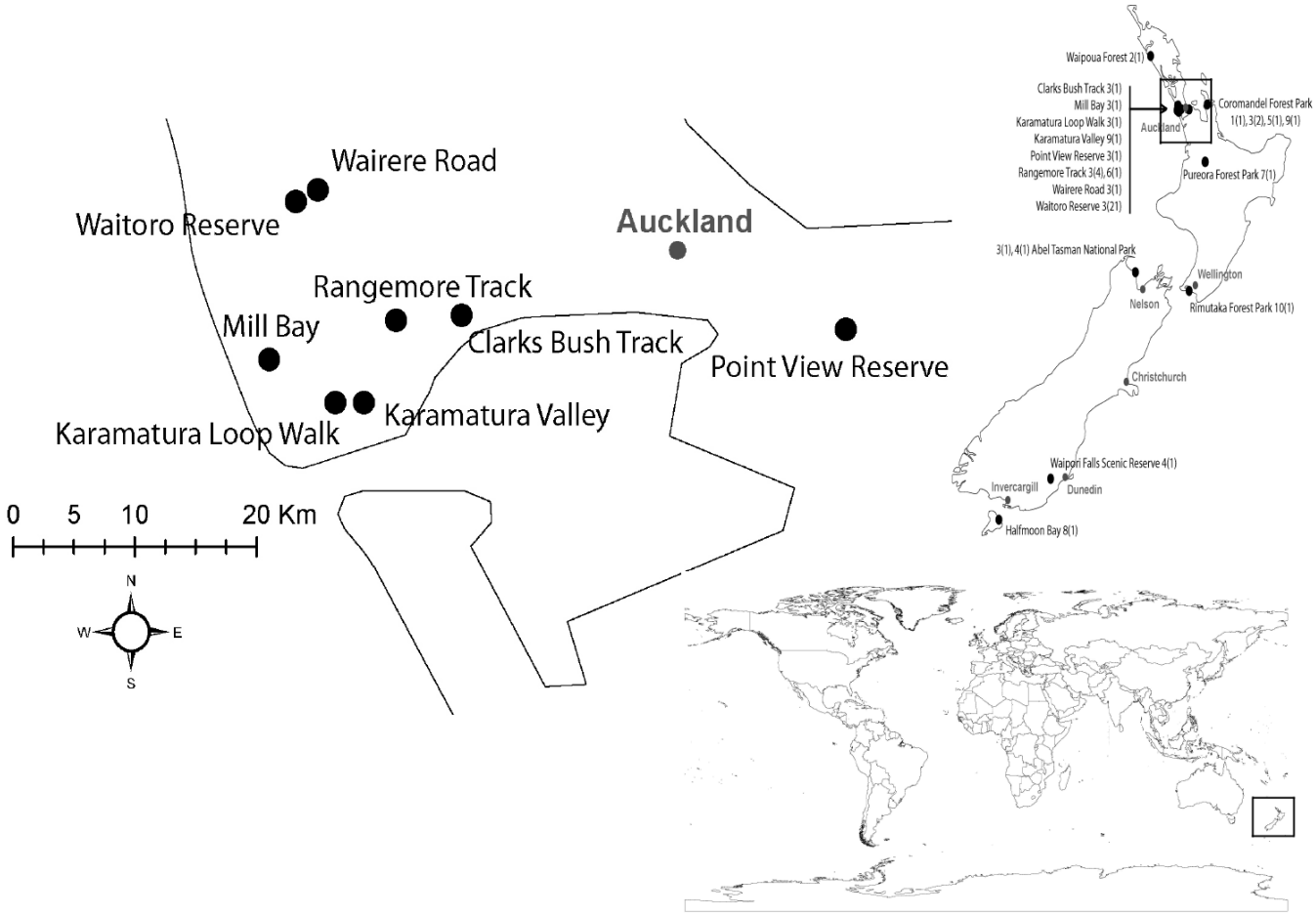

Figure 3. Map of the collecting localities near Auckland. All localities except for the Point View Reserve are in the Waitakere Ranges Regional Park.

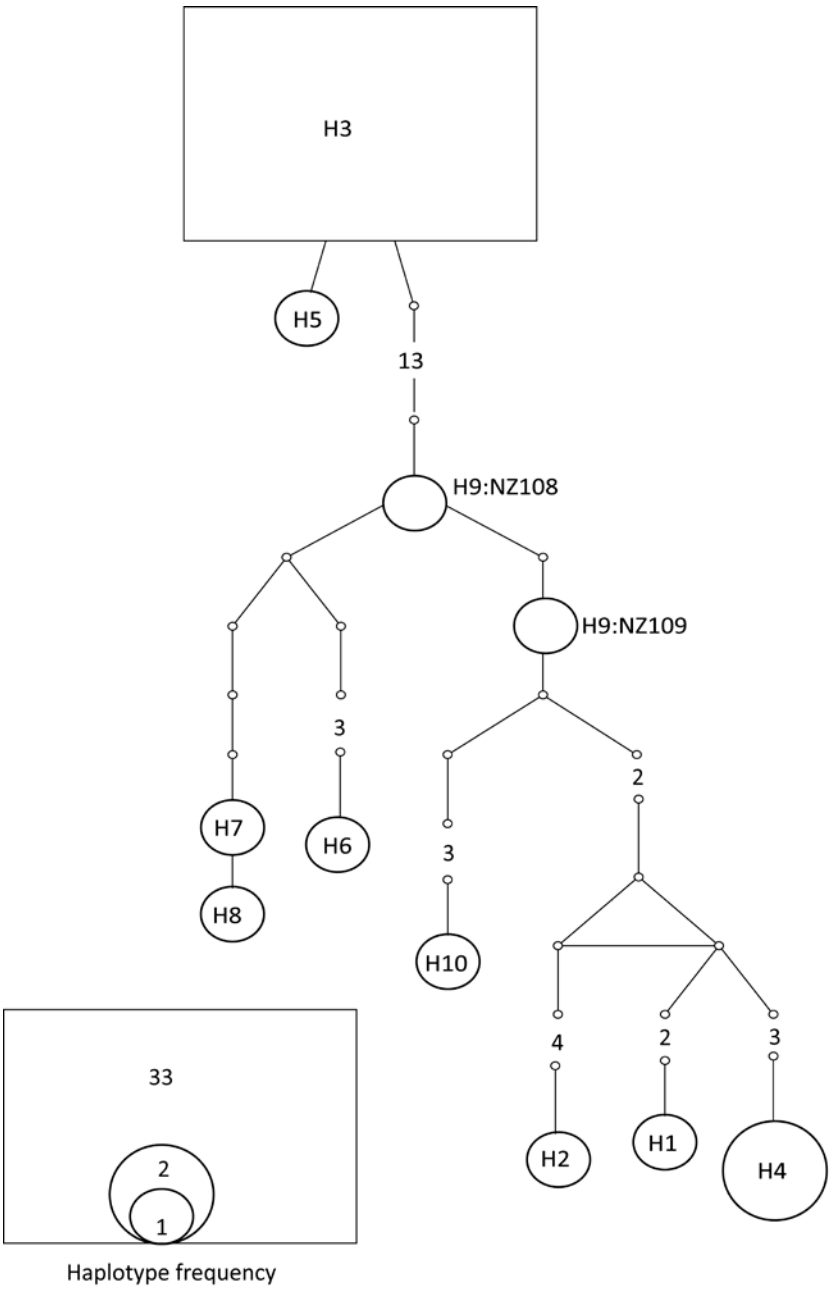

Figure 4. Gene geneology for the mitochondrial small subunit locus sequences estimated by TCS (Clement et al. 2000) with maximum connection steps set at 16. A line segment represents one mutation, and rectangle and oval sizes are proportional to the sequences represented by the haplotype. Haplotype number corresponds to numbers in Table 1.

The largest clade (seen as the collapsed clade in Figure 2) in this analysis contained 32 sequences including all of the isolates from the Waitoro Reserve. However, this clade is limited neither to the isolates from Waitoro nor to isolates from nearby localities in the Waitakere Ranges

Regional Park. Five of the 32 sequences (NZ104, NZ113, NZ123, NZ124, NZ116) are from localities outside of the Waitakere Ranges including one sequence from a collection taken from Abel Tasman National Park, which is located on the South Island (Figure 1). The sequences in the major clade did not represent all of the collections from Waitakeres Ranges Regional Park. Two sequences (NZ51 and NZ109) from collections taken inside the Waitakeres Ranges Regional Park were divergent from the rest. One site, the Karamatura Valley, was represented by only one collection, and the sequence from this collection (NZ109) was not included with the majority of sequences from this area but with an isolate collected from the Coromandel Forest Park (NZ108), some 100 km away by a direct path.

For those localities represented by multiple sequences, only sequences representing the Waitoro Reserve in the Waitakeres Ranges were grouped together in this analysis. The other localities represented by multiple sequences were found in different clades on the gene tree. Four collections from Coromandel Forest Park were divided such that two sequences (NZ113, NZ116) were included with the large clade and the remaining two (NZ108, NZ112) were resolved into two different clades on the gene tree and were also different haplotypes. Only four of the five sequences (NZ8, NZ53, NZ104 and NZ107) from isolates collected along the Rangemore Track within the Waitakeres Ranges Regional Park were found in the large clade. The fifth (NZ51) was more closely related to isolates from as far away as the Rimutaka Forest Park (NZ114) on the southern end of the North Island (Figure 1).

Clades were not grouped by island, either. Neither the collections from the North and South Islands nor the sequence from Stewart Island formed groups separated from the sequences from collections taken from the other islands. For example, the sequence from the collection from Pureora Forest Park (NZ110) is most closely related to the sequence representing the collection from Stewart Island. In the large clade of 32 sequences was a single sequence from an isolate collected in Abel Tasman National Park, which is on the northern end of the South Island and approximately 500 km away by a direct path. The other sequence from an isolate collected in Abel Tasman National park is more closely related based on this DNA sequence

analysis to an isolate collected in the Waipori Scenic Reserve on the southern end of the South Island, more than 500 km away by a direct path.

Many species of myxomycetes, including *Didymium squamulosum,* are widely distributed, being found worldwide and in many different types of habitats. It is recognized that cosmopolitan morphospecies such as *Didymium squamulosum* exhibit morphological variation in taxonomically valuable characters in the fruiting body stage of the life cycle. While these facts are recognized, there are very little data, morphological or genetic, that describe intraspecific variation within a species. It is known that *Didymium squamulosum* is a species complex made up of different biological species due to the existence of asexual lines within the morphological species (El Hage et al. 2000).

Molecular (DNA-based) data measuring variation within *Didymium squamulosum* is limited to a single study using DNA sequences of the nuclear ribosomal internal transcribed spacers (ITS) for a series of collections of *Didymium squamulosum* as reported by Winsett and Stephenson (2008). The data obtained from this previous study was limited by the marker chosen for analysis, as it was shown that the ITS region in *Didymium squamulosum* is too variable for meaningful analysis because unambiguous alignment of both spacers was impossible. However, analysis of data from the 5.8S and short regions of the small and large subunits that flank the ITS region revealed the existence of a few groups of sequences, which suggested the occurrence of geographical patterns within the species. In some cases, isolates of *Didymium squamulosum* collected in the same general locality were more similar to each other than they were to isolates from more distant locality. Whether the divergence of sequences from collections representing nearby localities was due to the hypervariability of the marker or the distribution of different genetic types within the species complex was not determined.

In the present study, we applied another molecular marker to a series of collections of *Didymium squamulosum* to determine if biogeographical patterns could be detected and also to assess the extent of the variation within a series of collections taken from the same locality on the same date. *Didymium squamulosum* is known to occur in both sexual and asexual strains, and without a mating type study and single spore isolations, it is impossible to know whether fruiting

bodies of the same morphospecies in the same locality represent a population or a clonal isolate. This series of collections, however, exhibited identical sequences for the hypervariable region of the mtSSU used in this study, suggesting they are very closely related if not part of a clonal lineage. These results seem to suggest that the *Didymium squamulosum* collections obtained at this locality represent either a population that is one large clone or a population that is the result of local spore dispersal resulting in such a high degree of relatedness at this locus. However, when sequences from other isolates of the same morphospecies collected throughout New Zealand were included in the analysis, the isolates from this single locality, the local population, did not group together to the exclusion of other isolates from very different localities (FIG 2).

It is known that myxomycete spores are dispersed by several different methods, including wind. The latter is assumed to be a significant factor in the dispersal of spores, accounting for wide distributions of species of myxomycetes (Stephenson et al. 2008). Myxomycete spores have been found in airborne sampling of spores, so it is known that spores do travel by air currents at least at heights of fifteen meters above the ground (Gillum and Levetin 2008, Kamono et al. 2009). However, it is not known to what extent long distance dispersal accounts for the dispersal of myxomycete spores or if localized dispersal of spores and movement of amoeboflagellates and plasmodia is a more common occurrence. The observed genetic relationship among the isolates from the Waitoro Reserve suggests local dispersal is responsible for the distribution of *Didymium squamulosum* at that locality.

The large clade made up of gene sequences from 32 collections does not, as it first appears, contain data only from isolates collected in a localized area making them more closely related to each other than to isolates collected from a greater distance. However, the majority of sequences in this clade are from either the same locality, as is the case for the series of collections from Waitoro Reserve, or nearby localities in the Waitakere Ranges near Auckland, suggesting that local dispersal is quite common. While theoretically spores this size (myxomycete spores are on average between 10-15 μm) can travel long distances on air currents, local dispersal by interruption of travel through the air, short distances traveled by

spores due to small disturbances in the microhabitat, or local movement of amoeboflagellates or plasmodium may be very common modes of dispersal.

To expand this research, the development of data sets that include multiple specimens from a single locality like the number of specimens obtained at Waitoro would allow for a more rigorous comparison of geographically isolated groups of specimens. At this point in our understanding of myxomycete biology, there is no way to determine the total biodiversity present at any locality or the specific time at which any individual species will produce fruiting bodies. Because of this, it is impossible to plan to collect a specific number of species at any locality. As such, we must take advantage of a series of collections as they are found, similar to the circumstances involving the collections found at Waitoro Reserve. With the recent global inventory of myxomycetes, there are many more specimens of common species to use for analysis. The influx of newer or fresher material is significant for the development of data sets through molecular biology because of the higher probability of positive results from DNA extraction and PCR. Ongoing biodiversity research in the national parks in the United States and natural areas around the world also provide a source for fresh material of cosmopolitan myxomycetes, and the development of molecular probes that could be used to identify species from environmental samples of substrate material (see Kamono and Fukui 2006, Ko Ko et al. 2009) will significantly advance our ability to study the diversity within populations and species.

The data considered herein are from only one of the many cosmopolitan species that exist in the myxomycetes. In order to develop a better model for the population biology and amount of intraspecific variation within cosmopolitan myxomycetes, it will be important to address the questions of long distance dispersal and intraspecific polymorphism in other species that are known to have a global distribution.

It is also important to note that these data represent only one gene region in the genome of the organism. Replicating the analysis using another gene would enhance the strength of these data. It has already been shown that another common locus used for evaluation of intraspecific variation, the internal transcribed spacer (ITS) region in the nuclear ribosomal DNA, is not appropriate for intraspecific investigation in myxomycetes (Winsett and Stephenson 2008).

At this point, in the biology of these organisms, few other gene options have been investigated for their value in this type of study as most molecular biology in myxomycetes to date has been in the higher order phylogenetic relationships (Fiore-Donno et al. 2005, 2010). To more thoroughly investigate intraspecific or population relationships, it is important to identify and verify the value of other markers for this type of study.

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Chapter 6

Global distribution, morphology, ecology and molecular diversity of *Didymium difforme* (Pers.) S.F. Gray

A. Introduction to *Didymium difforme*

Like *Didymium squamulosum,* the description of *Didymium difforme* (Pers.) S.F. Gray clearly suggests that it displays variation in morphology that could be indicative of genetic diversity.

> *Didymium difforme* (Pers.) S.F. Gray
> Fruiting body a sporangium, sessile, gregarious, flat-pulvinate, 0.3-1 mm broad, varying to short, netted or effused plasmodiocarps up to 25 mm in length, smooth, white; peridium double, the outer wall crustose, *Diderma*-like, composed of densely aggregated lime crystals, sometimes lacking, the inner wall delicate, purplish or colorless, iridescent; capillitium usually scanty, sometimes profuse, of brown or nearly colorless, dichotomously branching threads, often rather coarse below, slender above; columella lacking or represented by the purplish, thickened calcareous base; spores black in mass, dark purple-brown or purplish gray by transmitted light, minutely warted or smooth, 11-14 µm in diameter; sometimes with a thicker portion forming a cap at one side. Plasmodium colorless or yellow (From Martin and Alexopoulos [1969]).

Variation occurs in the structure of the fruiting body (sporocarp), which can be found as single sporangia to plasmodiocarps, with the published descriptions indicating a size range that can reach 25 mm. While known to be more commonly found as plasmodiocarps, the three records of *D. difforme* reported in one study (Schnittler 2001) occurred only in the form of small sporocarps. The major taxonomically important characters of the fruiting structure—including such characters as the capillitium, peridium and spore ornamentation and size—can exhibit a range of variation, as noted in the most recent comprehensive monograph on the myxomycetes.

The morphological species concept in myxomycetes lends itself to the false description of variants from the norm as new species (Clark 2000, Clark 2004). Without knowing the mechanism by which the morphological characters are affected by environmental conditions or having some experimental data on the extent of differences between morphological variants and “normal” isolates, assumptions cannot be made as to the classification of these collections. It has been recognized that variation under natural conditions may play a part in the development of typical or atypical fruiting bodies of particular species of myxomycetes. As early as 1901, culturing

experiments determined the stability of morphological variation in collections of *D. difforme* (Lister 1901). Variations in culture conditions, including abiotic factors such as moisture and biotic factors such as the assemblages of bacteria and fungi present in the same culture, have been shown to produce morphological variation in *D. difforme* (Cayley 1929). These factors represent only a very small part of the natural environment in which myxomycetes are found, which suggests the possibility that many different conditions and combinations of environmental characteristics could be important in the normal or abnormal development and growth of a particular species.

It cannot be ruled out that genetic variation plays a part in the morphological variation observed within *D. difforme* or any other species of myxomycete, because as yet no data exist to describe the intraspecific molecular variation within *D. difforme*. Early isozyme studies as described previously (see Chapter 2) were able to provide general conclusions that intraspecific variation exists within other species but did not reveal any correlation between morphological variation and enzyme or protein variation (Franke et al. 1968, Franke and Berry 1972, Franke 1973, Berry and Franke 1973, Betterley and Collins 1983).

There are not enough data presently available to determine if morphological variation can be attributed to an accumulation of genetic variation, and thus, stable characteristics that would be observed in further fructifications of the same isolate or if the isolate shows variation in different microhabitats or microclimates due to plasticity of the phenotype (Clark 2000, Clark 2004).

In other species of this genus (e.g., *Didymium iridis* [Ditmar] Fr., *Didymium megalosporum* Berk. & M.A. Curtis, *Didymium ovoideum* Nann.-Bremek., and *Didymium squamulosum* [Alb. & Schwein.] Fr.) for which more isolates have been examined, both heterothallic and non-heterothallic lines were shown to exist, thus indicating that myxomycete morphospecies may be complexes of heterothallic (sexual) lines associated with a number of non-heterothallic (presumed apomictic) clonal lines (El Hage et al. 2000, Clark 2004). Clonal lines would evolve independent of other lines, meaning they can independently accrue variation, which may ultimately affect morphology. The isolates for which the reproductive system has

been studied were found to be non-heterothallic—either homothallic or apomictic—wherein cultures started from single spores went through the entire life cycle spore to spore (Cayley 1929, Clark 2004). For these isolates, it is possible that any morphological variation could be the result of independent evolution within the strain; however, no experimental evidence exists to understand the cause or stability of any morphological variation found within the morphospecies.

B. Global distribution of *Didymium difforme*

Didymium difforme is a cosmopolitan species found worldwide (Figure 1). The global distribution of the species appears to be centered in temperate regions of the northern hemisphere. However, where significant studies of myxomycete biodiversity have been carried out, the species usually has been recorded.

C. Ecology of *Didymium difforme*

The habit of *D. difforme* is not limited to any particular type of substrate, and the species has been found on bark and dung as well as aerial litter and ground litter. The Global Biodiversity Information Facility (GBIF) is an organization that makes biodiversity data freely available on the Internet. This database includes nearly 200 000 records of myxomycetes and 1 600 records of *D. difforme* with georeferencing and collector data. The limitations of these data, however, include the absence of ecological information about the referenced specimens. The largest available database of myxomycetes, which is also one of the largest in the world, was developed at the University of Arkansas. This database—included in GBIF—does include ecological data and contains more than 840 records for *D. difforme*. The database at the University of Arkansas (hereafter referred to as UARKM) is the largest collection of myxomycete records with associated ecological data in the world. As such, it represents a previously unavailable dataset for making broad conclusions about the ecology of particular species of myxomycetes. Ecological conclusions are extrapolated from the worldwide UARKM database and the historical literature.

The UARKM database contains 844 records of *Didymium difforme* with data on the ecological conditions under which this species was collected. Of these records, 623

specimens were obtained with the use of moist chamber cultures, a laboratory technique by which substrate material is placed in a Petri dish with filter paper and kept continually moist creating what is effectively a microcosm of the ideal microclimate for the growth and development of myxomycetes. For these records, pH of the substrate materials is available in most instances. This measurement is usually taken twenty-four hours after the substrate material is wetted by placing a pH probe in the standing water in the dish. Three collections are from moist chamber cultures for which no pH data were recorded, and the remaining 218 records are assumed to be specimens that fruited in the field under natural conditions, since the standard procedure used by all of the collectors listed is to determine the pH for all moist chamber culture dishes.

For the records obtained in the field, the data collected represent the snapshot in time during which it is assumed that the environmental conditions favored growth. It is well known that myxomycetes show a seasonal preference for fruiting, although beyond recognizing that the particular season presents ideal conditions for fruiting, there is no known understanding of the specific factors, abiotic and biotic, that account for the seasonality. Collections from material that developed in moist chamber cultures may not represent the expected seasonality of the species because the species may be found as a spore, amoeboflagellate, microcyst, macrocyst and/or microscopic plasmodium, which in the case of the spore, microcyst, or macrocyst, the individual organism is not functioning in that environment at that time. The moist chamber culture would stimulate development because favorable conditions are created.

The literature describes the usual habitat of *D. difforme* as herbaceous litter, including dead leaves and “decaying herbaceous stalks” and dung from herbivorous animals (Lister 1925, Martin and Alexopoulos 1969). Although not specifically stated, I interpret the second substrate listed above as being what is regularly termed aerial litter—dead plant material still attached to the plant above the ground. For 842 records in the worldwide database, substrate material is listed. Aerial litter is listed for 551 records, ground litter for 269 records, dung for 17 records, and bark for 6 records. While no assumptions can be made for the sampling scheme by which all of

the substrate materials were sampled, a conclusion can be drawn that *D. difforme* shows a marked preference for decaying herbaceous litter, with over 97% of all collections in this database having been collected from herbaceous substrates. For the collections on herbaceous litter (820), 67% were found on aerial litter and the remaining (33%) on ground litter. Again, without the benefit of a statistically appropriate understanding of the sampling scheme, it is not possible to analyze these data in a statistical fashion. However, the number of collections found on aerial substrates is just over twice the number from ground litter, suggesting a tendency for this species to fruit on aerial substrates. The percentages are similar whether the specimen was collected in the field or harvested from moist chamber cultures (Table 1). This preference for aerial litter is noted in the literature, although for much smaller datasets in specific geographical areas (e.g., Schnittler and Stephenson 2002). As previously stated, specimens from moist chamber cultures develop from microscopic stages of the life cycle that may or may not be actually functioning on the substrate when it is collected but rather exist as one of the three resting stages—spore, microcyst or macrocyst. It is not possible to determine how this species occurred on the substrate prior to fructification in the moist chamber culture. However, finding the species fruiting in approximately the same proportions of aerial and ground litter substrates suggests some growth preference for the complex of environmental characteristics represented by the aerial litter microhabitat, although by no means to the exclusion of other substrates

Table 1. Collections of *Didymium difforme* from UARKM, with percentage distribution per substrate type calculated from the subset of collections with information on substrate indicated as part of the record.

All collections	**Substrate**	**Number**	**Percent**
	Aerial litter	551	65.4
	Bark	6	0.71
	Dung	17	2.0
	Ground litter	269	31.9
Total collections		843	
Field collections			
	Aerial litter	140	63.3
	Dung	2	0.9
	Ground litter	79	35.7
Total collections		221	
Moist chamber			
	Aerial litter	411	66.1
	Bark	6	0.96
	Dung	15	2.4
	Ground litter	190	30.5
Total collections		622	

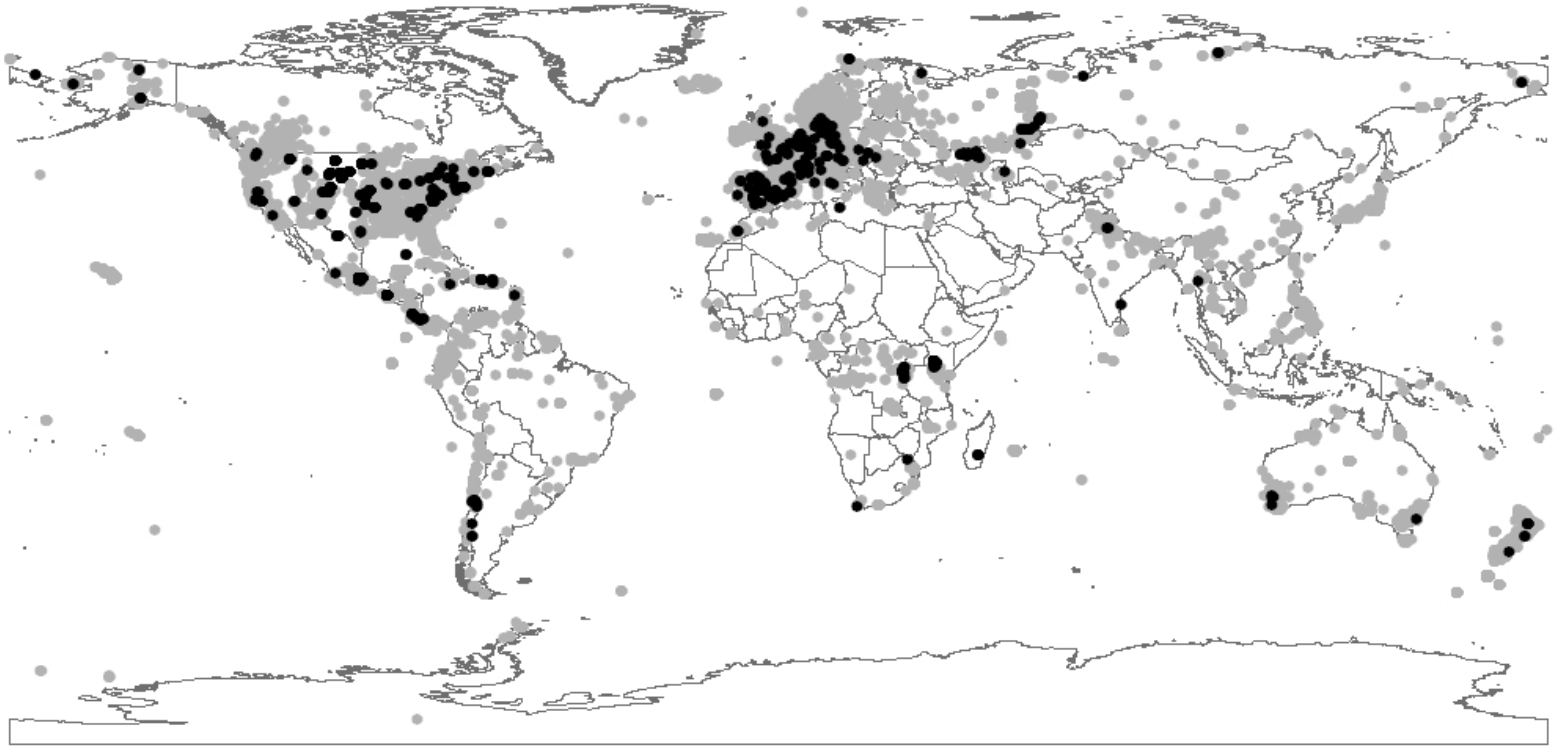

Figure 1. The global distribution of *Didymium difforme.* Black points represent collection locations from which *D. difforme* has been recorded, based upon data retrieved from the Global Biodiversity Information Facility (GBIF) (date retrieved: April 2010). Only those collections for which latitude and longitude are available (approximately 64% of all collections in GBIF) are indicated. Gray points represent locality data for all myxomycete collections in GBIF for which georeferencing points are available (approximately 55% of all collections in GBIF).

D. Morphological and Molecular Diversity

1. Sporocarp Collections

All collections of *Didymium difforme* used in the present study were from recent ecological and biodiversity surveys that utilized the moist chamber culture technique to cultivate myxomycetes from substrate material collected in different geographical locations around the world. Fifty-four collections were sequenced for a DNA marker in the mitochondrial small subunit. Twenty-four of these collections are from Kenya, four collections from Costa Rica, sixteen from Mexico, seven from North Dakota in the United States, and one each from Wyoming, Kansas and Oklahoma in the United States (Figure 2, Table 2).

Sporocarps of seven collections (Figure 3 A-G: 35095, 36292, 27224, 36250, 36259, 36297, 36204) used in this study display the range in variation of color and shape variation found in the collections of *Didymium difforme.* The blue tint of E and F is a photographic artifact and not the actual color of the sporocarps. Note instead, the variations in brown tint found on the sporocarps, which is assumed to be a result of aging. Images are to the scale of Image C unless a separate scale bar is included on the image. It is known that sporocarps of *Didymium difforme* can occur as sporangia (single sessile sporocarps) or plasmodiocarps (sessile branched or netted fruiting body) (Martin and Alexooulos 1969, Stephenson and Stempen 1994). Though this is an example of phenotypic variation, examination of the sporocarps used in this study showed that sporangia and plasmodiocarps were often found in single collections (fruitings) (e.g., Figure 3: E). Rather than this type of variation possibly indicating genetic distance, in these collections, it was a plastic character in which phenotypic variation was most likely determined by some ecological factor related to the substrate or microenvironment at the time of fruiting.

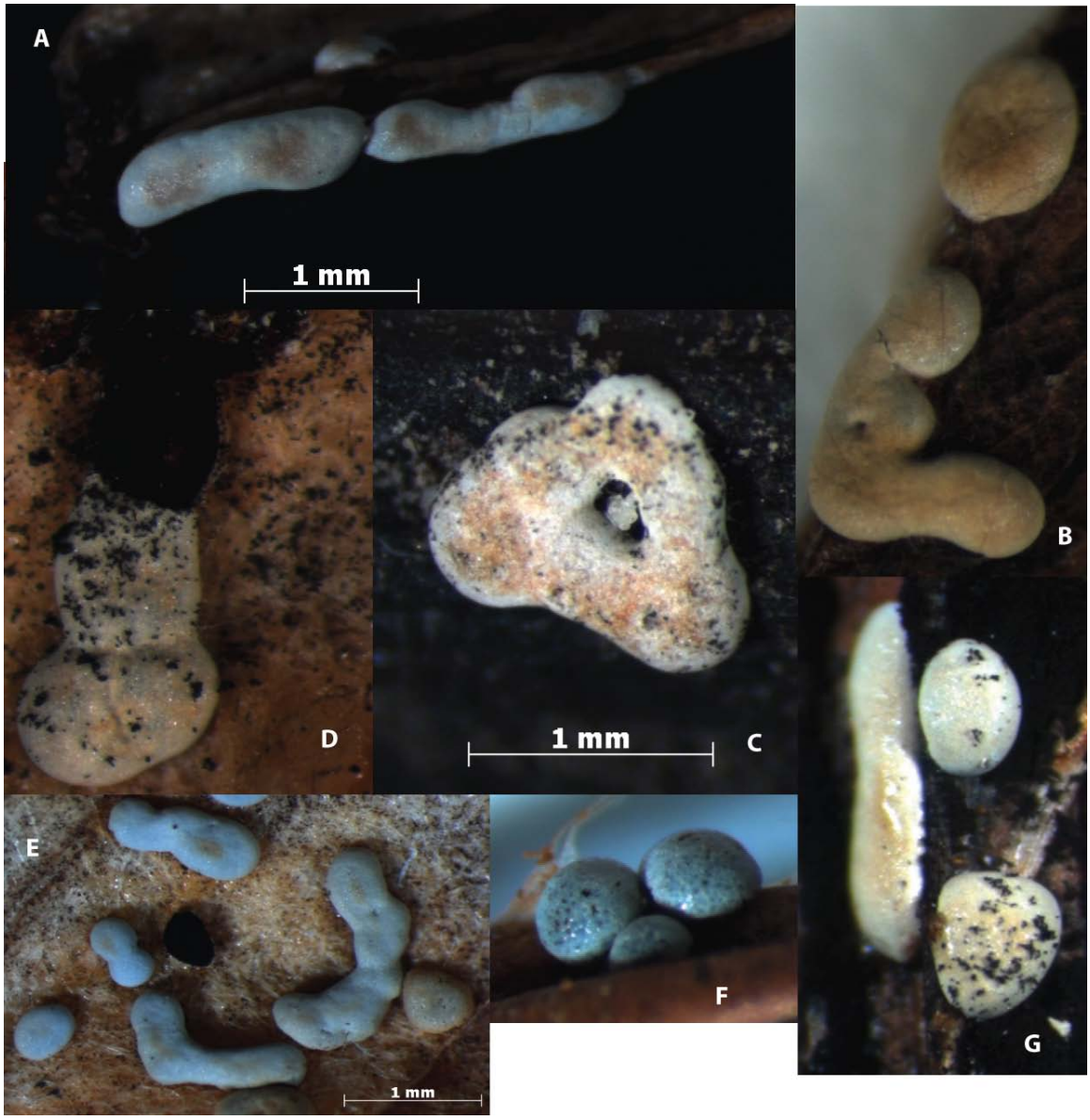

Figure 3 A-G. Sporocarps of seven collections (A-G: 35095, 36292, 27224, 36250, 36259, 36297, 36204) used in this study display the range in variation of color and fruiting body shape found in the collections of *Didymium difforme.* The blue tine of E and F is a result of the lighting and camera and not the actual color of the sporocarps. Note instead, the variations in brown tint found on the sporocarps, which is assumed to be a result of aging. Images are to the scale of Image C unless a separate scale bar is included on the image.

Table 2. List of specimens of *Didymium difforme* used in this study. All vouchers are herbarium specimens deposited in the University of Arkansas (UARK) myxomycete collection with data available online at http://slimemold.uark.edu.

DNA Number	Country	Collector Number	Accession Number	GenBank Number	Haplotype
1	Kenya	GN116	22223	HQ450426	8
2	Kenya	GN117	22224	HQ450438	6
3	Kenya	GN118	22225	HQ450418	6
4	Kenya	GN122	22228	HQ450416	6
5	Kenya	GN157	23705	HQ450430	6
9	Kenya	GN313	27316	HQ450450	13
10	Kenya	GN401	27404	HQ450397	1
12	Kenya	GN484	27487	HQ450404	4
15	Kenya	GN690	27693	HQ450452	13
16	Kenya	GN1694	36451	HQ450401	3
19	Kenya	GN1986	36743	HQ450433	11
24	Kenya	GN2630	38970	HQ450402	3
25	Kenya	GN2695	39035	HQ450417	6
26	Kenya	GN2722	39062	HQ450429	10
28	Kenya	GN2849	39216	HQ450419	6
31	Kenya	GN2922	39289	HQ450422	6
34	Kenya	GN3066	39433	HQ450420	6
35	Kenya	GN3097	39464	HQ450449	3
37	Kenya	GN3135	39502	HQ450428	9
41	Kenya	GN3337	39704	HQ450399	3
42	Kenya	GN3369	39742	HQ450448	3
47	Kenya	GN3496	39869	HQ450405	4
49	Kenya	GN3534	39907	HQ450403	4
50	Kenya	GN3568	39941	HQ450442	6
51	Kenya	GN3598	39971	HQ450400	3
53	Costa Rica	CR1767	34694	HQ450423	6
54	Costa Rica	CR1791	34718	HQ450431	6
55	Costa Rica	CR1800	34727	HQ450406	5
57	Costa Rica	CR1943	35146	HQ450421	6
58	Mexico	CR2047	36259	HQ450409	7
59	Mexico	CR2062	36274	HQ450446	6
60	Mexico	CR1889	35098	HQ450447	12
61	Mexico	CR2085	36297	HQ450427	8
62	Mexico	CR2080	36292	HQ450443	6
63	Mexico	CR2038	36250	HQ450414	6
64	Mexico	CR1992	36204	HQ450424	6
65	Mexico	CR2066	36278	HQ450439	6
67	Mexico	CR2055	36267	HQ450444	6
69	Mexico	CR1886	35095	HQ450435	5
70	Mexico	CR1882	35091	HQ450432	6
71	Mexico	CR1881	35090	HQ450407	5
72	Mexico	CR1877	35086	HQ450440	6
74	Mexico	CR1895	35084	HQ450441	6
75	Mexico	CR1861	35070	HQ450445	6
76	Mexico	CR1221	30625	HQ450425	6
80	Mexico	CR1126	30569	HQ450398	2
82	North Dakota	AWR1191	26718	HQ450410	6

84	North Dakota	AWR535	26082	HQ450451	13
87	North Dakota	AWR995	26536	HQ450413	6
88	North Dakota	AWR1088	26620	HQ450437	6
91	North Dakota	AWR690	26237	HQ450415	6
93	North Dakota	AWR1199	26724	HQ450436	6
94	North Dakota	AWR1187	26715	HQ450408	6
96	Wyoming	AWR1271	27224	HQ450412	6
98	Kansas	AWR1063	26596	HQ450411	6
100	Oklahoma	AWR1151	26681	HQ450434	6

Figure 2. The isolates of *Didymium difforme* sequenced in this study were collected from Kenya, Central America and several localities in the grasslands of the central United States.

2. Molecular Methods

DNA was extracted from sporocarps using the Chelex method as described previously (Winsett and Stephenson 2008). Briefly, spores from four to five sporocarps and 150 µL of a 5% Chelex suspension were placed in a thermocycler held at 56 C for four hours and then 98 C for thirty minutes to release DNA from the other cellular material that is part of the spore. Extracted DNA was frozen in microcentrifuge tubes at -20 C until use.

A 400 base pair hypervariable region of the mitochondrial small subunit (mtSSU) was amplified using the primers mtCore1 (5′–TAG TGT TAT TCG TGA TGA CT–3′) and mtCore2 (5′–CTC GAA TTA AAC CAC AT–3′). The DNA templates were amplified using a 25 µL reaction (12.5 µL Promega GoTaq® Green Master Mix, 8.5 µL dH_2O, 1 µL each primer and 2 µL template DNA) using a PCR protocol, which consisted of an initial activation step at 94 C for two minutes (per instructions for Master Mix) and 35 repetitions of 94 C for 30 sec, 40 C for one min, and 72 C for one minute. The PCR amplicon was extracted from the PCR product by a modified gel electrophoresis and gel extraction method (modified from Dentinger et al. [2009]). A 20 µL portion of each PCR product was run in a 1% agarose gel prepared with 1X TA buffer (20X: 193.6 g Trizma base, 45.7 mL glacial acetic acid, dH_2O up to 2L, dilute to 1X) with ethidium bromide included at 80 V for approximately 30 minutes. The resulting band was cut from the gel and placed in a filtered micropipette tip cut to fit into a 1.5 mL microcentrifuge tube. The tube with the piece of gel sitting on the filter was frozen to break up the agarose matrix then spun in a centrifuge at 13 000 rpm for 10 minutes. The filtered pipette tip was removed from the tube, leaving the extracted DNA within the flow-through.

DNA samples were sequenced on a Perkin-Elmer ABI 3700 sequencer (Perkin Elmer Applied Biosystems Division) using amplification primers. Resulting chromatograms were checked and edited using Sequencher v4.3 (Gene Codes Corporation). All sequences were submitted to GenBank (Accession numbers HQ129868-HQ129911). Sequence alignments were performed using Clustal X and then manually edited in Seaview (Larkin et al. 2007, Gouy et al. 2010). Summary statistics for the sequences including the number of haplotypes, haplotype diversity, nucleotide diversity, and number of pairwise differences were calculated using

DnaSP v. 5 (Librado and Rozas 2009). This software was also used to test for neutrality of mutation through Tajima's (1989) D statistic and the D* and F* statistics of Fu and Li (1993). A gene tree was produced using Bayesian Markov Chain Monte Carlo methods using the software package BEAST v.1.5.4 (Drummond and Rambaut 2007). The alignment in nexus format was imported into BEAUTi, to format the appropriate XML file to be used by BEAST. The alignment was analyzed using estimated base frequencies and a chain length of 1 000 000. Performance suggestions for operators that were included in the output from BEAST were used to make operator modifications in BEAUTi to optimize the data set. The updated XML file produced by BEAUTi was imported into BEAST and reanalyzed. This step was repeated until the operator values were optimized according to the performance suggestions in the BEAST output file. The resulting ".trees" output was formatted using TreeAnnotator v.1.5.4 and saved as a ".tre" file that was viewed and edited in Fig Tree v. 1.3.1. A gene geneology (haplotype) network was constructed using TCS (Clement et al. 2000).

In addition, we attempted to sequence the nuclear small subunit of the ribosomal DNA (SSU rDNA) for each of the isolates to provide additional information for the patterns found in this analysis. However, only a few successful amplifications occurred and no sequences were obtained for this locus from any PCR product.

3. Agar Culture Methods

An effort was made to germinate all isolates in agar culture using standard methods in order to compare the morphology of all specimens in a common environment so as to determine if any variation observed in herbarium specimens could be due to the environment in which the specimens developed (Haskins and Wrigley de Basanta 2008). Spores were placed directly onto the surface of Half Strength Bacto Corn Meal Agar (CM/2) (8.5 g Bacto corn meal extract agar and 12.5 g plain Bacto agar in 1 L of distilled water). Following germination and plasmodium formation, cultures were fed *E. coli* and sterilized crushed rolled oats to generate substantial plasmodia. Plasmodia were allowed to fruit and fruiting bodies along with substrate agar were cut out and glued onto herbarium quality paper trays fit into pill boxes (e.g., Fisher Scientific 03-505A) to establish vouchers for each culture.

4. Results and Discussion

a. Molecular data

Fifty-six sequences were obtained from isolates of *D. difforme* from different geographical locations. The amplified region was a nearly 400 base pair (bp) sequence in the mitochondrial small subunit that was known to be variable within a species (Dennis Miller [University of Texas at Dallas], pers. comm.). A BLAST search in GenBank (Zhang et al. 2000) revealed only two similar sequences, the complete mitochondrial genome (AB027295.1) and the small subunit gene (X75591.1). The region of the small subunit amplified in this study is located within the small subunit gene 740 bases from the beginning of the available sequence, which is 1814 bp in length. The sequence length from the amplified region ranged from 370 bp to 483 bp. However, much of the variation in length was attributed to incomplete sequencing of the PCR product as the missing sections resulting in length variation were at the ends of the sequences so these sections were removed.

A total of 368 sites were analyzed for 56 sequences of the hypervariable region of the mtSSU for isolates of *Didymium difforme* from three different regions of the world—Central America (Costa Rica and Mexico), the central United States grasslands and Kenya. Of the 368 sites, 73 were polymorphic with 66 sites parsimony informative. Summary statistics describing the set of sequences are found in Table 4. Based on these sequences, 13 haplotypes emerged, seven of which included only one sequence (singleton haplotypes). Haplotype designations for each sequence are found in Table 2. Haplotype designations are an arbitrary number between one and thirteen that correspond to the output from DnaSP, but have no specific meaning except to separate the sequences into the 13 categories. The largest haplotype included 32 sequences of isolates found in all three regions. Nucleotide diversity (π) was 0.03065, and the average nucleotide difference (*k*) among the sequences was 8.79545. Two of the three test statistics for neutral mutations Fu and Li's (1993) D* and F* were not significant suggesting that all mutations are neutral. Tajima's (1989) D was significant with a P value less than 0.05. It has been suggested that Tajima's test is more powerful than Fu and Li's statistics in cases of population growth and genetic hitchhiking (Fu 1997), thus these results may indicate an alternative

hypothesis that the population is increasing or the locus sequenced is linked to another that is the object of selection as the mechanism responsible for these polymorphisms.

Summary statistics and test statistics for neutrality were also calculated for each of the three regions (Central America, central United States grasslands, and Kenya) independently (Table 4). The sequences from isolates collected in Kenya represented the highest number of haplotypes (9). Central American isolates included six haplotypes, and the grasslands represented two haplotypes. The test statistics for neutrality were not significant for Kenya, but in the sequences from both Central America and the grasslands, these statistics were significant, thus suggesting that the mutations are not neutral. However, the sample sizes for the subsets are 25 individuals or less, which is considered too low for appropriate statistical power, thus rejecting the null hypothesis that the polymorphisms are neutral may not be the appropriate course of action (Simonsen et al. 1995).

The results of the molecular analysis of the mtDNA marker indicated that several groups of isolates were apparent (Figure 4). Interestingly, the groups were not all composed of isolates from a single geographical region. In *D. difforme* three major, apparently significant groups (clades) were resolved. Two of the clades (I and III) are made up of two and three isolates, respectively. The remaining clade (II) is made up of the majority of the specimens included in the analysis and is further resolved in Figure 5. Major clades were numbered with Roman numerals for ease of communication. These numbers do not reflect any level of classification.

Clade I was composed of two isolates, one from Kenya and the other from Mexico, with a significant posterior probability (PP) of 99%. Clade III groups two Mexican isolates with one from North Dakota in the United States, with a significant PP score of 97%. The largest significant clade of *D. difforme* sequences contained 51 sequences that were significantly grouped with a 97% PP. Figure 5 is a subset of the full tree showing Clade II.

Table 4. Summary statistics for mtDNA polymoprhisms in sequences of *Didymium difforme*. n_s is the number of sequences; h_n is number of haplotypes; H_d (±SD) is haplotype diversity ± standard deviation; π (k) is nucleotide diversity and mean number of nucleotide differences.

Location	n_s	h_n	H_d (±SD)	π (k)	Fu and Li's D*(P)	Fu and Li's F*(P)	Tajima's D (P)
Kenya	25	9	0.817 (0.055)	0.04122 (11.83000)	-0.79084 (ns)	-1.29175 (ns)	-1.75712 (ns)
Central America	21	6	0.552 (0.122)	0.01691 (4.85238)	-3.92687 (P<0.02)	-4.06715 (P<0.02)	-2.46275 (P<0.001)
North American grasslands	10	2	0.200 (0.154)	0.03275 (9.40000)	-2.51341 (P<0.02)	-2.72698 (P<0.02)	-2.11847 (P<0.001)
Total	56	13	0.662 (0.069)	0.03065 (8.79545)	0.90327 (ns)	-0.16997 (ns)	-1.84919 (P<0.05)

Clade II is further divided into three significant (PP>70%) groups (IV, V and VI). Clade VI, with a PP of 94%, was the only significant group having sequences from a single geographical region, with nine sequences from specimens collected in Kenya. Clades IV and V made up one large group that was separated from the Kenyan group (VI). Clade IV included four sequences, all from Central America, with three from Mexico and one from Costa Rica. The three Mexican sequences, however, did not group together. Two Mexican sequences are grouped with the Costa Rican sequence, with a PP of 89%. Clade V included 38 sequences. The group of 38 sequences was supported as a single group with a significant PP of 92%. Only three of the interior nodes, however, had significant level (greater than 70%) of PP. Two Kenyan isolates were grouped with a Costa Rican isolate with 84% PP, a single Mexican and one Kenyan isolate were grouped with a PP of 94%, and two Kenyan isolates were grouped with 99% PP. The remaining sequences were resolved into clades within the larger group V. However, the PP values were not significant, averaging only 18%. Group V was composed of sequences from a wide geographical range that encompassed Kenya, Central America (Costa Rica and Mexico), and the central United States (Kansas, North Dakota, Wyoming and Oklahoma).

Haplotype designations were congruent to the gene tree constructed for these sequences. The significant (>75% PP) tip clades on the gene tree (Figures 3 and 4) were composed of a single haplotype (e.g., III, IV, and VI) or included all the sequences for a certain haplotype along with singleton haplotypes (e.g., Ken 26-Ken19-Ken05-CR54 and clade V).

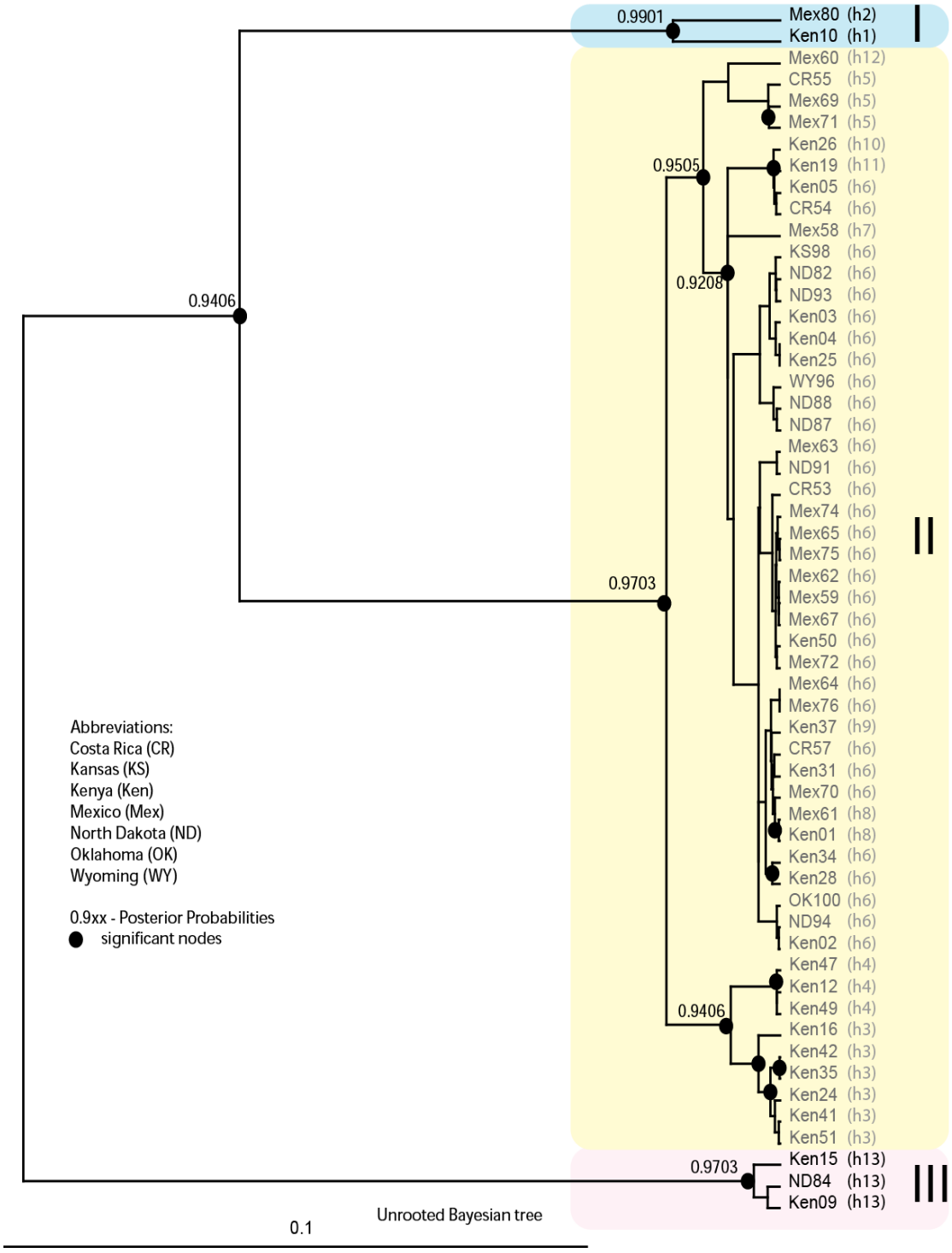

Figure 4. An unrooted Bayesian tree showing relationships among the 56 isolates used in this study. Node labels are posterior probabilities. As the number approaches one, the significance of the node increases.

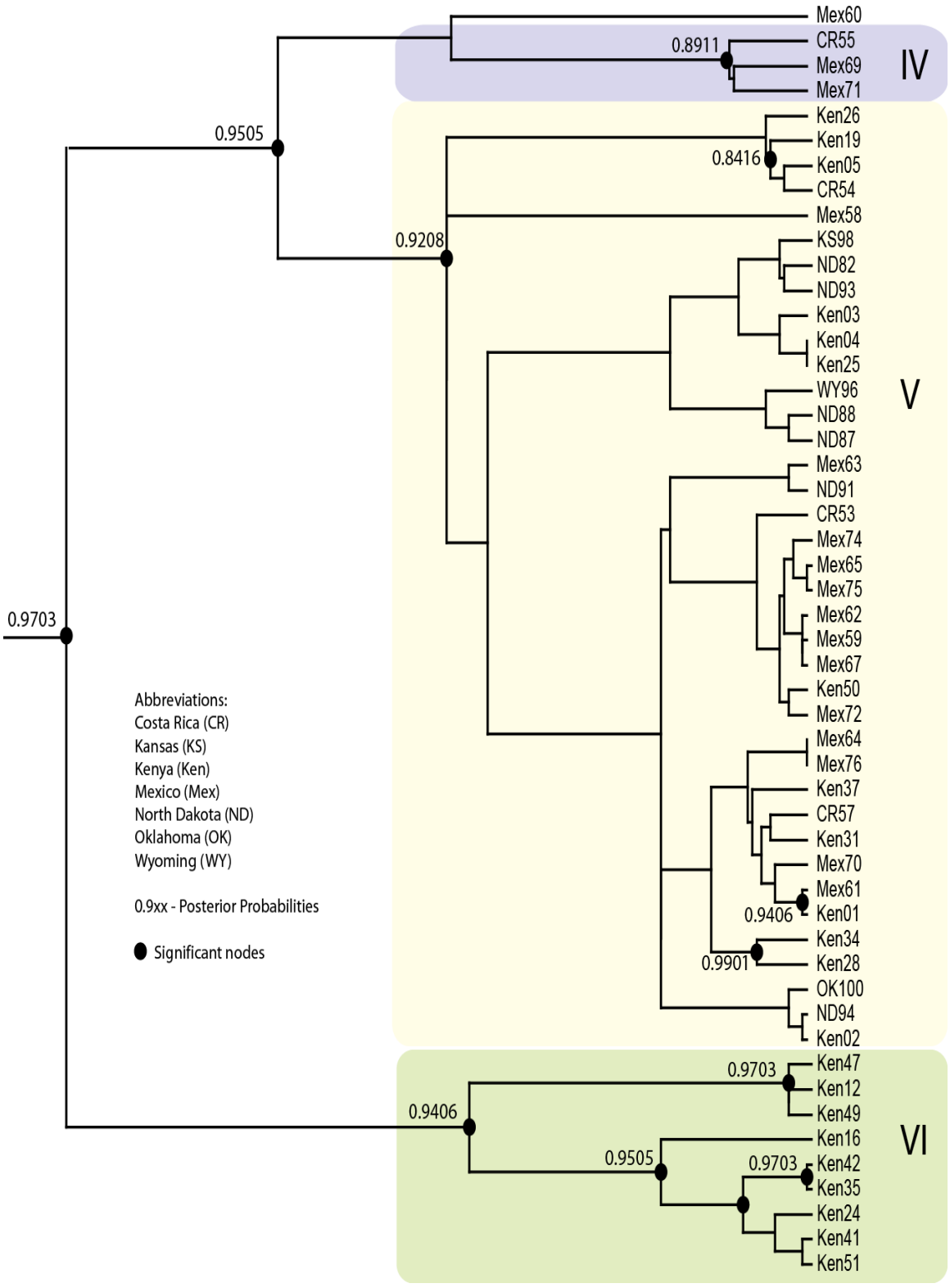

Figure 5. A subset (Clade II) of the tree in shown in Figure 4.

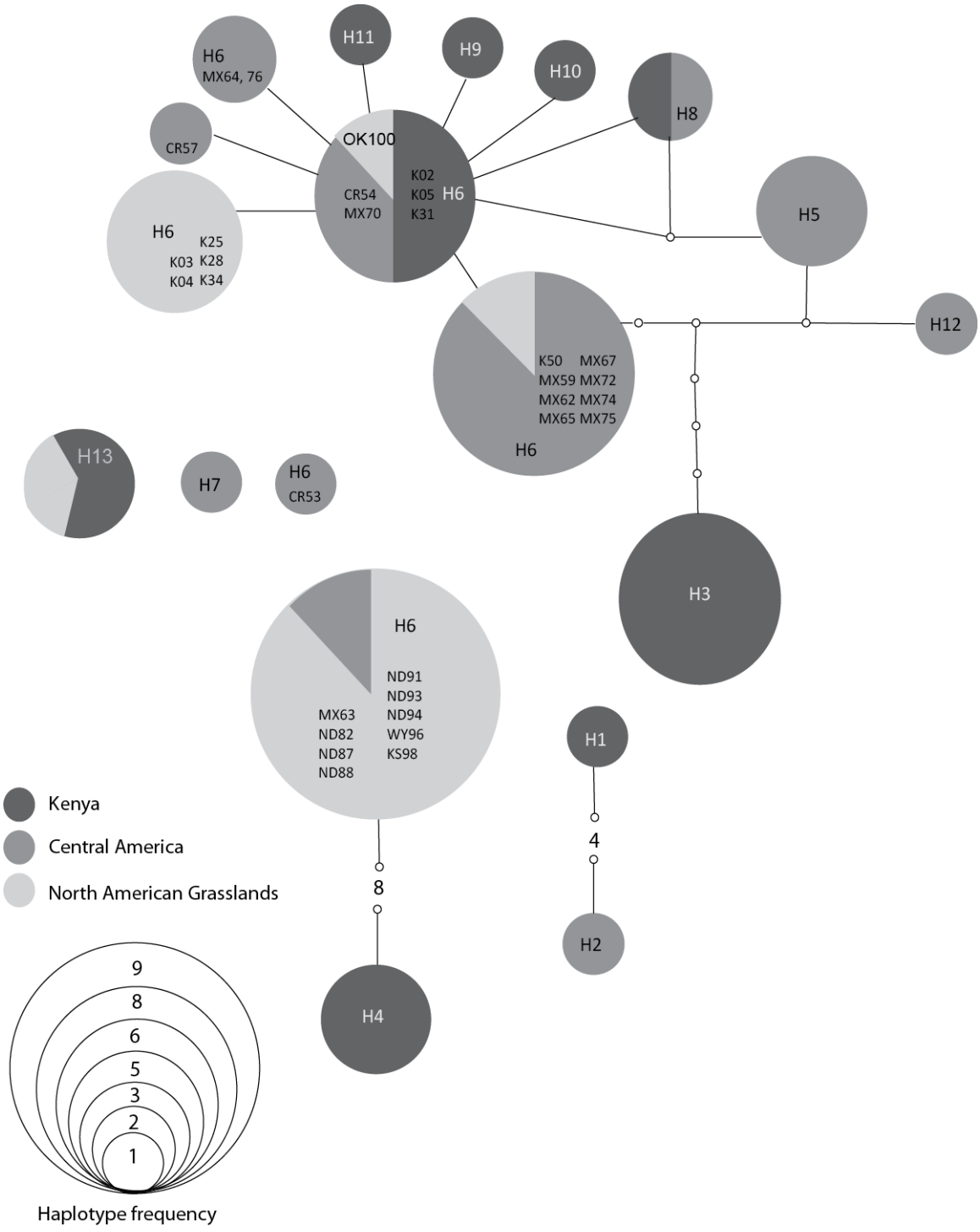

Figure 6. Gene geneology for the mitochondrial small subunit locus sequences estimated by TCS (Clement et al. 2000). Line segments indicate one mutation. Square and oval sizes are proportional to the sequences represented by the haplotype. Haplotype number corresponds to those given in Table 2.

The overall observation from this analysis of sequence data for isolates of *D. difforme* from different geographical locations is that no true geographical pattern emerges, although there are several small groups that contain isolates from the same location. One example is clade VI, which is composed only of Kenyan isolates. However, other isolates from Kenya are significantly grouped with isolates from distant geographical locations such as Mexico, Costa Rica and North Dakota. As such, it can be assumed that these sequences are more similar to those sequences from Central American isolates than those originating from material collected at a much closer geographical location. All three localities included multiple haplotypes (Table 4). The sequences representing collections from Central America had six haplotypes, the grasslands had two and Kenyan collections had nine haplotypes. No locality was made up of a single haplotype and multiple haplotypes were found in more than one locality, further supporting the assertion that no geographical affinity separated sequences from location from sequences representing the other locations.

The TCS (Clement et al. 2000) constructed for these sequences (Figure 6) separates the sequences into six different networks. Three groups are not connected any other haplotype network, which means the steps necessary to connect them (which correspond to mutations) are greater than the limit of steps used in the analysis (in this case 10). Like the gene tree constructed using BEAST, the haplotypes and haplotype groups illustrated in the network analysis do not correspond to a geographical pattern with isolates separated by great distance occurring in the same haplotype group.

Table 4. Haplotype frequency by locality.

Locality	**Haplotype (frequency)**
Central America	2(1), 5(3), 6(14), 7(1), 8(1), 12(1)
Central United States grasslands	9(6), 13(1)
Kenya	1(1), 3(6), 4(3), 6(9), 8(1), 9(1), 10(1), 11(1), 13(2)

Didymium difforme has a global distribution, and as an apparently cosmopolitan species, it is assumed that this can be achieved through mechanisms related to long-distance dispersal by wind (Stephenson et al. 2008). Because of the size of myxomycete spores—approximately 10 µm on average—it is theoretically possible for them to travel indefinitely in this way (Finlay 2002,

Schnittler and Tesmer 2008). If this method of dispersal is indeed common, then cosmopolitan morphospecies are expected and the possibility of ubiquity—cosmopolitan genetic identity—emerges as the most likely situation rather than the more restricted biogeographical patterns found in higher animals and plants (Finlay 2002). The data presented above are, with a few exceptions, geographically incongruous with isolates of a cosmopolitan morphospecies from very distant locations with the same or similar sequence identity at this locus of the mitochondrial genome. A first observation would be that these data support the idea of long-distance dispersal wherein spores traveled long distances—between continents—and were deposited in another geographical location, giving rise to a new population of the species in question.

Theoretically, this long distance dispersal is possible because of the combination of small spore size and an immense reproductive potential resulting from the huge numbers of spores produced by each fruiting body. The average myxomycete fruiting body produces on order of magnitude 10^5 to 10^6 spores (Schnittler and Tesmer 2008). From each plasmodium more than a single fruiting body—and often as many as ten or more fruiting bodies—will form in *D. difforme*, thus increasing the likelihood that random events, such as a passing wind current or water flow, will pick up spores and deposit them elsewhere.

With the similarities in sequences among isolates from very distant collecting locations, these data would support the long-distance dispersal concept. There are, however, some suggestions of geographical patterns. The group of Kenyan isolates collected together with a very high posterior probability to the exclusion of other isolates introduces the idea of local genetic patterns or populations. A population in myxomycetes is a difficult concept to circumscribe because of the variety of reproductive strategies—both sexual and asexual—possible within an individual morphospecies and the large numbers of amoebae that can result from mitosis, beginning with the protoplast that emerges from a single spore. In asexual isolates, each of these amoebae is capable of producing a plasmodium and, by extension, the millions of spores found in the fruiting bodies that are derived from one plasmodium. The amoebae and plasmodium are also both mobile over short (albeit still unknown) distances, so all of the fruiting bodies in a single area are likely to be from the same individual, rather than a collection of

individuals. In this way, it would be expected that a pattern of diversity would emerge such that those isolates collected in a single area would be more closely related, as shown by DNA sequence analysis of an appropriate molecule.

The Kenyan clade would suggest the occurrence of a population of fruiting bodies from the same individual within the same area. However, these specimens were not collected in the same locality, but in localities as far as 40 km apart. Other specimens of *D. difforme* were collected in the same locality as those in group VI but are found in different parts of the tree. In this way, the groups of sequences are illogical. Those from the same collecting localities do not group together with similar sequences at the mitochondrial locus. The other groups in this analysis tell the same story. Sequences from isolates collected in the same location do not necessarily group together, but instead group with sequences from collections from distant localities, further suggesting the occurrence of long distance dispersal of spores by a mechanism, presumed most likely to be wind.

b. Agar Culture

An effort was made to culture, using standard methods, all collections to compare resulting fruiting bodies for variation, However, it was not successful because only 16 collections germinated on agar and no fruiting bodies were observed (Table 5). Seven germination plates resulted in plasmodium formation. It is known that the color of the plasmodium in *Didymium difforme* can be colorless or yellow. All of the plasmodia observed in standard culture were yellow. With no fruiting bodies occurring in agar culture, it was not possible to make a comparison between the herbarium specimen and fruiting bodies that developed from spores of the herbarium specimen on agar.

Table 5. Data from agar culture of specimens of *D. difforme* for the collections that germinated in agar culture.

DNA Number	Locality	Collector number	Accession number	Color of the plasmodium
59	Mexico	CR2062	36274	Plasmodium not observed
62	Mexico	CR2080	36292	Plasmodium not observed
67	Mexico	CR2055	36267	Plasmodium not observed
70	Mexico	CR1882	35091	Plasmodium not observed
71	Mexico	CR1881	35090	Plasmodium not observed
72	Mexico	CR1877	35086	Yellow
75	Mexico	CR1861	35070	Yellow
76	Mexico	CR1221	30625	Plasmodium not observed
80	Mexico	CR1126	30569	Plasmodium not observed
82	North Dakota	AWR1191	26718	Plasmodium not observed
84	North Dakota	AWR535	26082	Plasmodium not observed
88	North Dakota	AWR1088	26620	Yellow
91	North Dakota	AWR690	26237	Yellow
96	Wyoming	AWR1271	27224	Yellow
98	Kansas	AWR1063	26596	Yellow
100	Oklahoma	AWR1151	26681	Yellow

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Biodiversity occurrence data provided by: BCCM/MUCL - (Agro)Industrial Fungi and Yeasts Collection Banc de dades de biodiversitat de Catalunya-FungaCat, Banc de dades de biodiversitat de Catalunya-LiqueCat, Database of Fungal Specimens Maintained at the Selected Japanese Herbaria, Myxomycetes Collection of National Museum of Nature and Science, Danish Mycological Society fungal records database, UCD BOTANICAL CONSERVATORY, Herbarium of Oskarshamn (OHN), Fungi (S), Botany (UPS), ATCC Fungi Collection Catalog, DSMZ Fungi Mycology herbarium Oslo (O), CBS Filamentous Fungi Database, Pollichia Pilzherbar, Biologiezentrum Linz, Nationaal Herbarium Nederland - Leiden Branch, Harvard University Herbaria, The Myxomycetes Collections at the Botanische Staatssammlung Munchen - Collection of Hermann Neubert, The Myxomycetes Collections at the Botanische Staatssammlung Munchen - Collection of Martin Schnittler, The Fungal Collection at the Staatliches Museum fur Naturkunde Gerlitz, The Myxomycetes Collections at the Botanische Staatssammlung Munchen - Main Collection, Herbarium GJO, Water Colours of Fungi by Fritz Wohlfarth at the Botanische Staatssammlung Munchen, Herbarium WU Bishop Museum Natural History Specimen Data, CBS fungi strains, BCCM/IHEM - Biomedical Fungi and Yeasts Collection Australian National Herbarium (CANB), CABI Bioscience Genetic Resource Collection, Flora Mycologica Iberica Real Jardin Botanico Madrid: MA-Funhist, IFO Filamentous Fungi, The Myxomycetes Collection at the V. L. Komarov Botanical Institute St. Petersburg, Gothenburg Herbarium - General (GBIF: IH: GB: Herbarium), Real Jardin Botanico Madrid: MA-Fungi Planetary Biodiversity Inventory Eumycetozoan Databank, International Collection of Microorganisms from Plants - New Zealand National Microbial Collection, NZFUNGI - Collections of the New Zealand National Fungal Herbarium (PDD), National Botanic Garden Belgium – Myxomycetes, Phanerogamie USU-UTC Specimen Database SysTax, CABI Bioscience Fungus Collection, Botanical Museum Copenhagen Mycology Herbarium, Herbarium Slovenian Fungal Database - Boletus informaticus, Japan Collection of Microorganisms(Fungi), Departamento de Investigacion y Experiencias Forestales de Valonsadero de la Junta de Castilla y Leon: JCYL-Fungi, Genebank National Institute of Agrobiological Sciences Biological Resource Center, Department of Biotechnology National Institute of Technology and Evaluation RBGE Herbarium (E), Fungi Collection in BCRC Herbarium of The New York Botanical Garden Zoological specimens, RBGE Living Collections, Herbario micologico de La Universidad de La Laguna Tenerife: TFC_Mic Registros biologicos en areas protegidas obtenidos de documentos impresos, ANTARCTIC PLANT DATABASE New Zealand Biodiversity, Recording Network Plants of Papua New Guinea, Herbier de la Guyane, CONN GBIF data Paleobiology Database Ibaraki Nature Museum Fungi collection (1) Generalitat Valenciana. Banco de Datos de la Biodiversidad de la Comunitat Valenciana, BDBCV - II Semana de la Biodiversidad (Castellon Spain) 2007 BDBCV - II Semana de la Biodiversidad (Castellon Spain) 2007, EDIT - ATBI in Mercantour/Alpi Marittime (France/Italy), Inventaire national du Patrimoine naturel (INPN) Gurgltal (Tarrenz), Tag der Artenvielfalt im Taubental, The Collection of Lichenicolous Fungi at the Botanische Staatssammlung Munchen, Biological and palaeontological collection and observation data MNHNL Schwanheimer Wald Heinersdorfer Sumpfwiese, Irish Records from the Fungal Records Database of Britain and Ireland, GEO-Hauptveranstaltung (NLP Harz / Hochharz), Water Colours of Fungi by Konrad Schieferdecker at the Botanische Staatssammlung Munchen Naturschutzgebiet Heiliger Hain (Wahrenholz), Fungi Collection Dalbekschlucht Staatsforst Rehna - Woitendorfer Wald im Biospherenreservat Schaalsee GEO-Hauptveranstaltung (Insel Vilm), East Ayrshire Countryside Ranger Service - East Ayrshire Species Database Fungal Specimens collected by HabitatVision (Jacob Heilmann-Clausen) Fungi (GBIF-SE:Artdatabanken), Take a Pride in Fife Environmental Information Centre - Records for Fife from TAPIF EIC Fungi BioFokus Fungi field notes Oslo (O), Notes from the

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Chapter 7

Conclusions and Future Directions

There is no full account of the genome of any myxomycete, although the examination of the nuclear genome and annotation of the transcriptome of *Physarum polycephalum* is in progress (http://genome.wustl.edu/). The advancement of this knowledge will greatly affect the capability and promise of molecular analysis within the myxomycetes. However, even nuclear content in the mxyomycetes is more complicated than a genome from a single species would suggest. Different species have different numbers of chromosomes, and studies of chromosome detection have suggested that chromosome numbers are another source of intraspecifc variation (Collins 1979). It is almost certain that this variation is due to differences in ploidy, but major differences exist among species, with chromosome counts ranging from eight in *Didymium squamulosum* to 124 in a diploid cell of *Echinostelium minutum* (Collings 1979). Within a species, variation in ploidy may indicate variation in reproductive strategy, denoting the presence of both sexual (with haploid gametes) and apomictic (agametic with diploid amoebae emerging from spores). If a method for uncovering these differences can be developed, even further information about patterns of intraspecific variation can be collected and directed towards ecological and evolutionary studies. Previous methods relied upon stained chromosome counts in culture, which are unreliable, considering the minuteness of each chromosome (Collins 1979). However, what can be concluded from existing data is that it will be meaningful to study fine-scale variation at the intraspecific and intrageneric levels, not only at the sequence level as described in the studies of this dissertation, but also at the whole genome level. There is a considerable amount of information relating to the evolution and function of these organisms left to understand.

Understanding intraspecific diversity within species of myxomycetes will allow us to address a number of biological questions about these organisms that, as yet, remain unanswered. So much of our current understanding of myxomycetes is based upon morphology, especially physical characteristics of the fruiting bodies. However, it is recognized that there is much more to be understood about myxomycete biology beyond what can be observed from the morphology of these organisms (Clark 2000).

This dissertation project was the first effort to characterize the genetic variation in a myxomycete using molecular techniques. The challenge in this group of organisms was to determine the appropriate molecule for assessing intraspecific variation and possible geographical patterns within a particular species. All previous molecular studies of myxomycetes addressed higher-order phylogeny, thus there was no information available about molecular markers that would be useful for showing that intraspecific variation existed. Previous attempts to study intraspecific variation used isozyme patterns to determine variation within geographically separated isolates of a single species, as described in Chapter Two (Franke 1967, Franke et al. 1968, Franke and Berry 1972, Berry and Franke 1973, El Hage et al. 2000). No geographical or ecological assemblages emerged when multiple isolates for a single species were examined. One morphological pattern emerged when two color variants of *Fuligo septica* were separated by isozyme profiles into separate groups within the species (Berry and Franke 1973). This provided the first genetic evidence that morphological variants within a single species could be due to genetic distance rather than ecological variation. Chapter Four details that the genetic marker used in this dissertation did not point to the variation at any morphological character as being a signal to a genetic variant or biotype.

There is no standard gene or DNA sequence that can be used in all groups of organisms for the purpose of answering population or evolutionary questions. Across the genomes of eukaryotic organisms, there are variations in rates of evolution, which would directly affect the utility of a particular marker (Zhang and Hewitt 2003). One example is the internal transcribed spacer (ITS) regions within ribosomal DNA. This marker is widely used in fungi (e.g., Lickey et al. 2002, Zervakis et al. 2004) with great success, but in a previous study (Chapter Three) was found to be too hypervariable for use in the myxomycete, *Didymium squamulosum*. This serves as an example of the situation in which we lack a common marker for studies addressing similar questions, and a certain amount of trial and error may accompany the exploration of markers within those groups that do not have any precedence set for intraspecific study using molecular (DNA) markers.\

Prior to the studies described herein, multiple sequences of any marker from the same species of myxomycete were quite limited, with no published records, thus making it difficult to decide on an appropriate marker for intraspecific analysis. Multiple sequences in GenBank were limited to markers used for higher-order phylogeny (e.g., elongation factor 1-alpha, nuclear small subunit ribosomal DNA and beta tubulin) that would in all likelihood not be appropriate for an intraspecific study.

As noted above, the first markers examined for the purpose of understanding intraspecific diversity in myxomycetes were the ITS sequences, which are fully described in this dissertation. However, the utility of ITS in the species *Didymium squamulosum* was discovered to be low. Conclusions from these sequences were mostly drawn from the small and large subunits of the ribosomal DNA that flank the spacers and the 5.8S ribosomal DNA that occurs between the spacers rather than the ITS regions themselves. The initial study using ITS examined thirteen sequences representing collections of *Didymium squamulosum* collected from a series of localities worldwide. Despite the hypervariability among the sequences, the analyses of the flanking and intervening regions of ribosomal DNA suggested, however, that geographical patterns may exist within the species. Two significant groups of sequences were revealed that grouped sequences from the same location together. The first grouped two sequences representing collections from New Zealand, and the second grouped three sequences representing collections from Alaska. In both cases, these sequences were more closely related to each other than to other sequences from different localities. However, the pattern was complicated because other sequences from both localities included in the dataset were found to be more significantly related to sequences from distant localities rather than the other sequences from the same locality. For example, the third New Zealand sequence was more closely related to one from Malawi, and the fourth Alaskan sequence was more closely related to one from Costa Rica. A larger dataset using this marker was not developed because of its limited utility. Instead, an attempt to discover a different, more useful marker was directed towards the mitochondrial DNA.

The second target gene region was within the mitochondrial small subunit gene (mtSSU). An approximately 400 base pair region was amplified from within the gene because of its known hypervariability within a species (Dennis Miller, University of Texas at Dallas, pers. comm.). Based on a sequence of *Physarum polycephalum* available in GenBank (Accession number X75591.1), the beginning of the target region is at position 689 (Mahendran et al. 1994). This marker was applied to a global dataset of collections of *Didymium squamulosum* and a dataset of multiple collections from three worldwide localities of *Didymium difforme*.

As presented in Chapters Four, Five and Six, the sequences for this marker for both species offered a similar pattern to that uncovered by the ITS sequences. In both species, there were groups of sequences revealed that grouped sequences from the same geographical localities as well as sequences representing collections from very different geographical localities. There were even some instances in which collections from localities in different hemispheres were grouped together.

The dataset for *Didymium squamulosum* includes sequences from collections representing twenty-two different localities. Some localities, for example, Arkansas, were represented by only one sequence but were included in order to develop a global perspective for the intraspecific variation within the species. As was the case for the pattern revealed using ITS, sequences from the same locality grouped together, but on the whole, the geographical pattern was marked by sequences from collections representing disparate geographical localities grouping together in the analysis. A subset of this dataset for *Didymium squamulosum* was also analyzed separately. This consisted of all of the sequences representing collections from New Zealand. Within this localized geographical region, the same pattern again emerged. Some sequences from the same localities grouped together, and a number of instances occurred in which sequences from the same locality were more closely related to sequences representing collections from distant localities.

All of the collections for *Didymium difforme* were selected from three specific geographical regions. These were Central America (Costa Rica and Mexico), the central United States grasslands (Kansas, Oklahoma, North Dakota and Wyoming) and the region within and

near Aberdare National Park in Kenya. The three regions, while large in area, are separated from each other by great geographical distance. The purpose of the analysis carried out on this dataset was specifically to examine multiple collections from single regions to see if localized patterns emerged wherein the isolates from each region were more closely related to each other than to those derived from collections representing the other regions. In effect, this was an effort to assess the evidence for the existence of some barrier to long-distance dispersal, which would help drive such a pattern. Regional groups were not uncovered. Rather, like the patterns for *Didymium squamulosum* using both ITS and mtSSU, some geographical groups emerged, but the pattern was broken up by statistically significant groups that included sequences from collections representing the other regions.

Another study, which was not anticipated at the onset of this dissertation work but forms the basis for Chapter 5, was the investigation of multiple sequences representing collections taken from a single local population. A series of collections of *Didymium squamulosum* was obtained on the same day from a relatively small (ca 20 by 300 m) area in a forest on the North Island of New Zealand. It was possible to sequence eighteen of these collections for the mtSSU marker. In addition, three collections taken from the same location, but on a different date, were also sequenced and included in the analysis. All of the sequences from these collections were identical, suggesting that localized populations exist, and, in addition, some mechanism of local dispersal is at work. The biology of myxomycetes, specifically *Didymium squamulosum*, complicates the overall conclusions from these data. This species occurs in nature in both sexual and asexual lineages. It could not be determined which of these was the case, but it is possible that the collections taken from this locality (the Waitoro Reserve) represent an asexual lineage that has grown in size and number at this locality. That all of these isolates fruited at the same time in the field further suggests a high degree of relationship, even to the point that they represent the fruiting bodies of the same genetic entity. Within these isolates, morphological variation was observed. Four characters for which morphological variation is known to occur were examined for the fruiting bodies from the herbarium collections used for sequence analysis (see Chapter 4). Variation in each character occurred, and no pattern was evident to connect the

morphological to the genetic variation suggesting that microenvironment more than genetic distance plays a part in morphological variation, which may be evidence for some mechanism of phenotypic plasticity. Within the isolates from the Waitoro Reserve that were identical at the sequence level, variation in all four characters occurred further suggesting phenotypic plasticity. Only five of these collections germinated in culture, and none of them produced fruiting bodies so an attempt at a common garden experiment to look for variation in standard culture conditions was unsuccessful.

The overarching pattern that emerges from a consideration of these data is the occurrence of random, inexplicable assemblages of sequences. In all four datasets, there is no clear geographical association among the specimens. In the one dataset, Chapter Four, for which some morphological characters were compared to the groups of sequences, no clear association between morphological variation and groups formed by relationships among DNA sequences was evident. On close examination of the data in all datasets, however, a few groups marked by evident geographical similarities did emerge. For example, in the dataset for *Didymium difforme* found in Chapter Six, clade VI was made up of all sequences from Kenya. No sequences representing collections from any other region were included. However, this clear geographical assemblage was accompanied by the presence of sixteen other sequences from the same region of Kenya that fell in other parts of the gene tree suggests a complex situation.

When taken in the context of myxomycete biology, an elegant explanation emerges. Myxomycetes produce spores for dispersal that across the group, average about 10 μm in diameter. Organisms with dispersal structures of this size are theoretically unlimited in the distance that the spores could travel on wind currents, especially in light of the large-scale wind events that occur to transport dust and soil long distances (Finlay 2002, Fenchel and Finlay 2003, Kellogg and Griffin 2006, Tesmer and Schnittler 2007). What has not been found is the actual evidence for occurrence of long distance dispersal. The data presented in this dissertation suggest that the mechanism for the dispersal of species of myxomycetes may be a combination of both local and long-distance movement of individuals. In fungi, which also are characterized by the dispersal of small, robust spores, geographical assemblages and biogeographical patterns

are known to occur (Halling et al. 2008, Lumbsch et al. 2008). The most informative marker for intraspecific and intrageneric studies of fungi and fungal biogeography has been established to be ITS. Because fungi have a similar possible long-distance dispersal mechanism—namely spores—it could be logically assumed that similar patterns of molecular diversity would occur in myxomycetes. Since such patterns did not emerge from these data, the possibility exists that the mtSSU marker used in the present research is not ideal for studying intraspecific diversity. However, there are no other comparable sets of data to refute or confirm this fact.

If the validity of this marker is accepted, however, when put into the terms of the life cycle, these data suggest that an individual myxomycete dispersed by spores can achieve long-distance dispersal on wind currents in addition to a local dispersal though movement of spores by direct or indirect transport from such vectors as water or insects. Fruiting bodies of myxomycetes, including the species considered in the studies described herein, can produce thousands or millions of spores during each fruiting event. It is possible, then, for any fruiting event to have spores distributed by both mechanisms. Other opportunities for local dispersal exist through the vegetative stages of the life cycle. The amoebae and plasmodium are both mobile vegetative stages that have the capability of moving through the habitat or microhabitat in search of food. Local dispersal, then, can be achieved through these life stages as it is not necessary for all amoebae resulting from a germination event or all of the plasmodia from any germination event be present in order for fruiting to occur. In other words, the multiple amoebae that form through mitosis and the multiple plasmodia that can form from the fusion of any two of these amoebae can each be the source of an individual fruiting event. In this way, a species can move through a particular microhabitat over relatively short distances. Over time, the distances between occurrences of the same species at a given locality can increase.

These facts relating to the myxomycete life cycle also have the ability to confound an elegant explanation for the patterns encountered in this study. As described in Chapter Two of this dissertation, the general life cycle is complicated by the existence of several different reproductive strategies. It is known that sexual and apogamic forms exist in both of the species *(Didymium squamulosum* and *Didymium difforme*) considered in these studies. What this means

is that within these species, reproduction can occur sexually, reported as heterothallism, in which compatible mating types in haploid nuclei of two cells fuse to form a zygote that continues through the life cycle. Apogamy, which is thought to be common in *Didymium squamulosum*, is characterized by amoebae that can form plasmodia without nuclear fusion. It is thought that these are asexual strains in which amoebae emerge from the spore with diploid nuclei. In the limited experimental data, it has also been shown that some species have mixed systems with allopatric and sympatric distributions (Clark 2000).

Because of the co-occurrence of both asexual and sexual lineages within a particular species, there is the possibility that any collection examined using DNA sequencing is a lineage undergoing independent evolution. It is also, therefore, possible that multiple collections represented on any gene tree would be evolutionarily isolated. At present, the only method for determining the nuclear condition that exists for a nucleus is through the use of single spore isolations on agar. Such single spore isolations on agar germinate. If the isolate goes through the entire life cycle from spore to spore without requiring amoebae from another spore, heterothallism can be ruled out. All amoebae originating from the mitosis of one original amoeba are genetic clones.

It is not possible to make such observations in all isolates even in a species such as *Didymium squamulosum* that is known to grow easily in culture. As indicated in the data presented in Chapter Four, it is not possible to germinate spores from every isolate of this species. Only 10% of all known species of myxomycetes have been grown in laboratory culture (Haskins and Wrigley de Basanta 2008). Others have been attempted but either never successfully germinated or did not go through the entire life cycle in culture.

The reproductive strategy has a considerable bearing on the biology of a particular population in species of myxomycetes. An apomictic lineage, even if represented by multiple collections from the same area, would not be a population in the strict sense. Rather it would be what can be regarded as essentially a single individual. It would not be possible to determine the age of the lineage (i.e., if it developed from a spore of another individual of the same asexual lineage) nor its relationship to other collections in the general area. In addition to the passive

movement of spores and resting stages, myxomycetes have two active mechanisms of movement and growth. Amoebae multiply by mitosis until an unknown trigger causes plasmodia formation. In culture, tens of thousands of amoebae can develop before plasmodial formation. In an asexual species, any—or, theoretically, all—of these amoebae can form a plasmodium. The movement of the amoebae through a substrate coupled with the movement of the plasmodium across the substrate in question can translate into the development of multiple clutches of fruiting bodies in a single area. These are not different individuals, but separate instances of the same individual. A single plasmodium can also be split into two or more portions by some event, with each part of the plasmodium continuing to grow until fruiting occurs. It is not known why or the purpose behind the persistence of diversity in reproductive strategies or how selection acts upon it.

The local population specifically described and analyzed in Chapter Five is an example of these complex circumstances. These collections were far enough apart that they were obviously not derived from a single, undisturbed plasmodium (S. L. Stephenson, pers. comm.), but that does not mean some, or perhaps all, represent the same individual due to circumstances as described above. That all of these collections fruited at approximately the same time could be an indication that they are very closely related if not members of an asexual lineage or the same individual. As mentioned, these collections could be the fruiting bodies of an asexual lineage at this locality. In this case, some of the fruiting bodies may have originated from different sporulation events. They would, however, still be genetically identical. The proliferation of an asexual lineage is likely considering the other collections from the same locality included in the analysis. All three collections offered the same identical sequences as the collections from 31 October 2009 though they were collected years earlier.

The hypotheses advanced to guide this research are listed below.

(1) Both cosmopolitan species form biogeographical clades based upon the analysis of DNA sequence data.

H_0: Analysis of DNA sequence data show the clades to be organized such that they are not based upon biogeography.

(2) Multiple sequences from the same part of the world are more closely related to each other than to sequences from isolates obtained in other parts of the world.

H_0: Sequences from multiple isolates collected in the same region of the world are associated with other isolates from different locations

(3) Variation in morphological characters coincide with the clades on the gene tree constructed for *Didymium squamulosum*.

H_0: Variation in morphological characters do not occur according to the molecular patterns seen in each gene tree.

At the conclusion of this dissertation project, it is not possible to reject any of the null hypotheses. While some geographical clades emerged in the analyses of these sequence data, there was no regularly occurring or consistent pattern to geographical assemblages, thus it is not possible to assert that geograpahical proximity is an indication of relationship.

Related to this conclusion, it is also not possible to reject the null hypothesis for the second of these hypotheses. In both *Didymium squamulosum* and *Didymium difforme*, where multiple sequences were available for a single region, no groups emerged that included all of the collections from that region. In the analysis of *Didymium difforme*, collected from three distinct regions, no region segregated out into its own group. For example, the collections from Kenya, which are physically separated from the collections of the other two regions by the western part of the African continent as well as the Atlantic Ocean, were not genetically separated at this locus. Collections from Kenya occurred across the tree in groups that also included collections originating from Central America and the central United States.

The third hypothesis is most fully addressed with the investigation of *Didymium squamulosum*. In this case, four morphological features that are recognized to be variable within the species were observed for each collection, and the character states mapped onto the gene tree produced by analysis of sequence data. As evident in the data presented in Chapter Four, none of the character states was limited to or characteristic of any of the clades revealed through molecular analysis. Because of this, it is not possible to reject the null hypothesis.

This dissertation describes the first study to address intraspecific variation of species of

myxomycetes using a molecular approach. Ultimately, having data on the intraspecific variation that exists for these organisms will shed light upon a number of questions relating to myxomycete biology, including geographical distributions and long distance dispersal. Furthermore, there are a number of yet unresolved questions regarding morphological and reproductive diversity within a particular species. One of the species (*Didymium squamulosum*) considered in this dissertation is known to have significant morphological diversity that previously has been the basis for description of taxonomic varieties within the morphospecies (Martin and Alexopoulos 1969). It has been proposed that many, if not most, of the described species known only from the type locality or a few localities may be no more than examples of the morphological variation that emerges in certain ecological situations (Schnittler and Mitchell 2000). There is no resolution for the question of whether morphological variation occurs as a result of genetic variation or phenotypic plasticity.

It has been suggested that phenotype of myxomycetes can vary based upon microenvironmental conditions. Observations on specimens in tropical forests suggest, for example, that individuals growing in these habitats tend to have a longer stalk, presumably to raise the spore mass above the film of moisture that usually exists on tropical substrates (Stephenson et al. 2004). Some experimental data is described in this dissertation that suggests that variation in morphology is most likely linked to the microenvironment in which sporulation and fruiting body formation occurs. Further evidence for this was found when other collections of *Didymium squamulosum* that were not included in the DNA sequencing effort were also grown from spore to spore in agar culture. In more than one example from agar culture of *Didymium squamulosum*, the fruiting bodies formed in the laboratory culture varied in at least one of the characters examined, which is described in Chapter 4. In one case, an isolate that had an orange stalk when it fruited in moist chamber culture, produced a white stalk characteristic of the species when grown in agar culture. In another example, an isolate in which the lime covering the peridium was smooth, had a wavy or flaky texture when grown in agar culture, also a state more characteristic for *Didymium squamulosum.* While few, these data suggest that microenvironmental conditions play a role in the development of morphological characters for

Didymium squamulosum.

It is difficult to use the agar culture technique to further examine this because of the difficulty in getting isolates to grow in culture. When the spores of 100 isolates of *Didymium squamulosum* were sowed in agar, the fraction that germinated represented only 35% of the original isolates (Winsett, unpublished data). Of these, only nine produced fruiting bodies in culture. As stated previously, only about 10% of all known myxomycete species have been successfully grown in culture. Taking together the variation in germination rate within species and the small fraction of species grown in culture, it may not be possible yet to fully investigate phenotypic plasticity in myxomycetes.

Many ecological questions including further analysis of intraspecific variation will require the addition of molecular techniques for greater resolution. Compared to other groups of organisms, the molecular study of myxomycetes especially for ecological purposes and population biology is in very early stages. There are only approximately 800 sequences for myxomycetes (excluding those from this dissertation) in GenBank (Benson et al. 2005). This number also includes the sequences for two complete genome projects for the mitochondria genome of *Physarum polycephalum* and a partial nuclear genome for the same species. The different markers analyzed for this group are few and are nearly all markers used for molecular phylogeny incuding the most common marker, the nuclear ribosomal small subunit. Before this dissertation, no species was represented by more than two or three collections for any marker. In this dissertation, two genes were advanced as possible markers for studying intraspecific diversity.

The first, ITS, is too variable for meaningful analysis at the population level. The second, a mitochondrial marker, was shown to be variable within a species but not variable to the point of absolute ambiguity of homologous alignment like ITS. The mitochondrial marker, however, is only about 400 base pairs long, which may be too short to be used alone for addressing ecological questions including biogeographical patterns, long distance dispersal and population structure. However, the only way to determine if a potential marker is an appropriate one is to use it to analyze another set of collections and compare the resulting conclusions. In other

groups, mitochondrial markers have been shown to provide significant information (Avise 2009). In addition to ITS, the only other markers that have been studied in myxomycetes were nuclear small and large subunit and elongation factor 1 alpha, but as already mentioned, they were used for phylogenetic analysis and do not appear to be appropriate for a fine scale study. Choosing another marker would represent another "shot in the dark" as characterization of other genes is quite limited.

Further directions for study at the species and intraspecific levels will require analysis of molecular markers such as DNA sequence analysis can address biodiversity and the functional role of myxomycetes in their local environment. Because of the microscopic phases of the myxomycete life cycle, it is possible that we have a limited understanding of the biodiversity and distribution of species based upon the methods now inuse that depend upon the formation of fruiting bodies. DNA sequence analysis from aerial spore traps (Kamono et al. 2009) and environmental samples (Kamono and Fukui 2006, Ko Ko et al. 2009) suggest that a molecular analysis of the habitats in which myxomycetes occur, a more detailed understanding can emerge about the species existing in an ecosystem even when fruiting bodies are not visible. It has been suggested recently using molecular analysis of soil that the mycetozoa may be one of the most numerous groups of soil-inhabiting organisms in the ecosystem further corroborating the results from culture data (Feest and Madelin 1985a, b; Feest 1987; Urich et al. 2008). As previously stated, culturing techniques are unreliable so a molecular approach to ecosystem diversity and functional understanding in future studies will be informative. At this point, no marker is considered to have the ability to delimit myxomycete groups at the species level. Informative markers, including microsatellites, have yet to be determined for the myxomycetes. Fine-scale studies of closely related taxa are represented only by the studies described for this project. Continuing molecular study at the species level should uncover a marker that will be meaningful at the level of species enhancing the ability to study soil and microhabitat diversity.

There are several reasons why we need to develop a more complete understanding of myxomycete biology, and some of these are connected to one another. One such reason is to understand the breadth of a species. At the present time, the only viable method for expanding

our understanding of true myxomycete biodiversity is to understand the diversity of informative loci within and between species. Using this knowledge, it would be possible to probe appropriate microhabitats for the vegetative structures—amoebae and plasmodium—for species identification from soil or substrate material.

A second, and equally important, reason to understand the biology of myxomycetes is to be able to determine the role these organisms play in the ecosystem. This type of understanding will require answers to questions relating to such things as “how much?” or “how many?” for particular whole ecosystems or parts of ecosystems. For example, how many myxomycete amoebae are in the soil? Related to this is the importance of biodiversity of myxomycetes. As myxomycete biology stands now, there is no way to identify a myxomycete amoeba to species. Does the lack of any real morphological variability in this stage of the myxomycete life cycle affect the ecological role or significance of these organisms in the ecosystem? For example, it is possible that due to unknown variation available only through molecular analysis, each species has a nutritional niche, focusing on specific types of food rather than exhibiting generalism, feeding on any prokaryote available.

These overarching questions require focus on different points of study within the biology of these organisms. It may not be necessary to understand the diversity or local assemblages of mating types or reproductive strategies when focusing on questions of trophic requirements. However, to understand the biodiversity of myxomycetes locally or worldwide is to understand the scope of the genetic variation of any or all species. To that end, to understand the true ecological possibilities or extent requires the understanding of the true biodiversity of myxomycetes.

This dissertation represents a first step towards understanding the extent of the genetic variation that exists for a single myxomycete, using data obtained using molecular techniques. For these organisms, each gene tested was an attempt to probe the genome for an appropriate locus to understand the relationships between species. It remains to be seen how the future of intraspecific and intrageneric molecular studies of diversity will advance, but I consider these chapters to represent a significant attempt to further this area of biology, and to establish a baseline of data from which further analysis can be launched.

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