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Population Structure and Mating Dynamics in the Social Amoeba Dictyostelium discoideum

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by

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ABSTRACT OF THE DISSERTATION

Population structure and mating dynamics in the social amoeba *Dictyostelium discoideum*

by

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Professors Joan E. Strassmann and David C. Queller, Chairpersons

Successfully investigating the evolution and maintenance of sex and mating systems can often have as much to do with choosing the right study system as it has to do with asking the right questions. *Dictyostelium discoideum* has long been the focus of researchers interested in understanding a number of biological processes, such as motility, chemotaxis and development. More recently, attentions have shifted to include questions about the evolution of social and sexual interactions both within and between species. The *D. discoideum* life cycles, both asexual and sexual, are uniquely social, each requiring a costly sacrificial act. This offers an ideal system for exploring questions about kin recognition, conflict, and the evolution of multicellularity, as well as the evolution of differential sexual investment and mating types.

This dissertation focused on understanding the phylogenetic and geographical relationships between clones in *D. discoideum* and identifying the social and selective pressures that shape its mating system. I introduce this mating system in Chapter 1. In Chapter 2, I investigated genetic variation and population structure in *D. discoideum* to identify possible

factors that could affect interactions between clones. I used DNA sequence data and phylogenetic techniques to show that though *D. discoideum* clones form a monophyletic group, there is evidence of genetic differentiation among locations (F_{ST} = 0.242, P = 0.011), suggesting geographic or other barriers limit gene flow between populations. In chapter 3, I again looked for population structure, this time concentrating on gamete size and sex ratio, to understand selective pressures maintaining multiple mating types in *D. discoideum*. Evidence suggests that both balancing selection and drift are likely acting on the *D. discoideum* mating system. I found no differences in gamete size across the three mating types and also no genetic differentiation across three wild populations at the mating type locus. However, I found that mating type frequency varied across these populations, likely due to drift.

Chapter 4 focused on understanding the social dynamics of mating in *D. discoideum.* During macrocyst formation, two cells of complementary mating types fuse to form a zygote. This zygote then consumes hundreds of surrounding amoebae, likely clones of the original two cells, for use as protection and food. I varied the frequencies at which two clones of differing mating types interacted to investigate the possibility that one mating type cheats another by differentially contributing to the cannibalized cells. Contrary to previous claims that mating type I induces mating type II, coercing it to contribute disproportionately more of these cannibalized cells during macrocyst production, I found that these cells are likely contributed relative to their frequency in the population, regardless of mating type. However, I did find evidence for differential contribution to macrocyst production between some pairs of clones, suggesting that cheating can happen between partners during sex, but is rare and clone-specific.

Overall, these studies looked for evidence of underlying population structure in *D. discoideum* that could impact our understanding of social and sexual interactions in this species.

I also applied questions about the maintenance of sex usually only asked in two-sex systems to the unique social sexual interactions within *D. discoideum* in order to expand the understanding of how mating systems evolve and are maintained in nature. I developed and used new tools and techniques for observing the processes important to understanding this unique system and identified genetic and social factors that could impact how individuals interact during both the asexual and sexual life cycles.

CHAPTER 1

INTRODUCTION TO THE DISSERTATION

Background

Sexual reproduction is a term usually reserved for genetic recombination of the gametes of two parents through meiosis. Understanding the costs and benefits of sexual reproduction is one of the great questions of biology. Most eukaryotic species are facultatively sexual, a term describing the behavior of primarily asexual organisms that undergo occasional rounds of sex (Hurst & Peck 1996; Dacks & Roger 1999; Xu 2004; Lahr et al. 2011). Examples of this include the early protistan group oxymonads, the algal species *Chlamydomonas reinhardtii*, the myxogastrid plasmodial slime mold *Pyhsarum polycephalum*, and the fission yeast *Schizosaccharomyces pombe* (Collins 1975; Dacks & Roger 1999; Egel & Penny 2008; Lahr 2011). In facultatively sexual organisms, sexual reproduction is often a strategy for responding to unfavorable environmental or fitness cues (Poole et al. 2003; Hadany & Otto 2007; Horandl 2009; D'Souza & Michiels 2010). During the sexual stage, many organisms form hardy dormant structures that are more likely to survive environmental stresses like harsh winters or periods of low nutrient supply (Egel & Penny 2007; Hörandl 2009). Other species undergo sex at higher rates when they begin to starve, as seen in *Chlamydomonas reinhardtii* (Harris 1989; Hadany & Otto 2007). Grishkan et al. (2003) found that soil microfungi increased sexuality when exposed to severe drought and high salinity, suggesting that this increase in recombination promoted genotypic adaptation by increasing genetic variability. Hadany & Otto (2007) modeled an allele that induces sex in less fit individuals and found that this fitness-associated sex created an evolutionary stable system in which the fittest individuals remained asexual while the rest participated in sexual reproduction. Species demonstrating both sexual and asexual reproduction tend to gain many of the benefits from sexual reproduction, while avoiding much of the cost.

Most research on sex assumes two sexes with equal investment in them, but more sexes appear in some organisms. Fisher's principle explains that negative frequency dependent selection, in which the rare sex has higher mating potential, favors a sex ratio of 1:1 in most sexual species (Fisher 1930). However, much of what we know about sex ratios comes from analyses of two-sex systems. We have known from theory, that selection can favor the evolution of multiple sexes and that we should expect even sex ratios among them (Iwasa & Sasaki 1987). Only recently, though, have researchers really begun to explore the many natural systems that exhibit more diverse sexual strategies than the frequently observed male-female system. Mating systems can be found in many different forms, ranging from the mostly asexual bdelloid rotifer to species like the fungus *Schizophyllum commune*, which displays over 20,000 different mating types (Kothe 1996; Welch and Meselson 2000; Clark and Haskins 2010; Billiard et al. 2011, 2012). Theories predict various factors that can influence the number of sexes, or mating types. The most well-known theory for the evolution of more than two mating types comes from Iwasa and Sasaki (1987). They predicted that a new mating type that arises in the population should be favored by selection because it can mate with a larger proportion of the population. This negative frequency-dependent selection theory assumes both that there is a cost to not finding a mate and that all mating types are inter-compatible. Many other theories focus on the maintenance of two mating types, describing factors such as cytoplasmic conflict and the evolution of anisogamy as possible limitations to increases in mating type number (reviewed in Billiard et al. 2011). Although there is a clear interest in the literature as to the evolution of mating types, very few studies, if any, have addressed the question of sex ratios in natural systems displaying more than two mating types. Lacking also are studies on the evolution of

anisogamy in systems with multiple mating types. How are these mating types maintained? How, if at all, do their roles differ during reproduction?

Study System

I propose that the social amoeba *Dictyostelium discoideum* is particularly good for exploring questions about sexual reproduction and mating systems. While most studies of these single-celled eukaryotic amoebae focus on the asexual fruiting body cycle, the sexual cycle and macrocyst formation offer a unique system for studying the maintenance of a sexual stage. Dictyostelids are soil-dwelling eukaryotes that, for much of their life cycle, live as a solitary individuals feeding on bacteria and dividing mitotically until food resources are consumed and amoebae begin to starve. At this stage, amoebae can continue down one of two pathways, the asexual social cycle or the sexual cycle, both initiated by the onset of starvation. Like many microbial eukaryotes, Dictyostelids are facultatively sexual. Sex has been observed throughout the phylogeny of the social amoebas (Dictyostelia) and is considered ancestral, with any asexual species having secondarily lost the trait (Erdos et al. 1973a, b, 1975; Clark et al. 1973; Francis 1975; Chang and Raper 1981; Kawakami and Hagiwara 1999; Schaap 2006).

In this sexual cycle (illustrated in Figure 1.1), two cells of opposite mating types fuse to form a reproductive zygote, or giant cell (Saga et al. 1983). This giant cell then releases large amounts of cAMP, the same signal released in the asexual fruiting body cycle, to attract surrounding amoebae (Abe et al. 1984). In response to this signal, hundreds of amoebae aggregate and adhere to the giant cell, forming a dense clump. At the completion of aggregation, the peripheral cells produce a thick, cellulose wall called the primary wall or fibrillar sheath that surrounds the entire mass, now called a precyst (Blaskovics & Raper 1957). This wall is similar

to the slime sheath that is formed around the multicellular slug in the fruiting body life cycle. After the primary wall formation, the giant cell begins to engulf the surrounding peripheral amoebae, surrounding them with vacuoles and converting them to endocytes (Filosa & Dengler 1972). During this time, a second cellulose wall, this time produced by the giant cell, forms around the endocyte-filled giant cell (Blaskovics and Raper 1957; Filosa and Dengler 1972; Erdos et al. 1973). As the now macrocyst matures, the endocytes are completely degenerated, the giant cell shrinks and darkens, and a two-layered tertiary wall is produced (Filosa and Dengler 1972; Erdos et al. 1973). This inner membrane is similar to the walls of the spores found in the fruiting body sorus. After a period of dormancy, during which time meiosis should occur, the cyst begins to swell, splitting the tertiary wall into two separated parts (Erdos et al. 1973; Nickerson & Raper 1973). The multinucleate giant cell splits into uninucleate fragments called pro-amoebae that continue to divide to form smaller myxamoebae. After the secondary wall and outer layer of the tertiary wall break away from the inner layer, hundreds of myxamoebae break through the remaining tertiary wall, ready to begin their solitary lifestyle.

As discussed previously, reproductive zygotes, or giant cells, are produced by the fusion of two cells of opposite mating types. Though macrocyst production has been identified throughout the *Dictyostelium* phylogeny, the identification of mating types has revealed a variety of primarily single-locus systems. There are three mating types in *D. discoideum*. These three types (Type I, II, and III) are self-incompatible, but can mate with any of the other two self-incompatible types (Erdos et al. 1973; Clark et al. 1973). This type of heterothallic mating is the most common in social amoeba. Previous studies have identified similar heterothallic mating in the species *D. purpureum, D. giganteum, D. rosarium, D. monochasioides, Polysphondylium violaceum, P. pallidum* and *P. pseudo-candidum*. The number of mating types ranges from two

to four, with two being the most common (Clark et al. 1973; Francis 1975; Erdos et al. 1975; Chang & Raper 1981; Kawakami & Hagiwara 1999). Recently, the three self-incompatible *D. discoideum* mating types were sequenced, identifying three versions of a single genetic locus (Bloomfield et al. 2010). This study revealed that type I and III mating loci have completely different sequences, including a *mat-A* class gene in type I and a *mat-S* class gene in type II. The type II locus, on the other hand, is a homologous composition of the two and contains both gene classes. Successful macrocyst formation between two of these three cell types requires the presence of a *mat-A* class gene in one and a *mat-S* class gene in the other, revealing much about the mechanisms for compatibility between these three heterothallic cell types.

Though macrocyst formation has never been observed directly in nature, many factors have been observed in the laboratory that either favor or inhibit their formation. The three inhibitors most commonly discussed in macrocyst formation protocols are the presence of phosphate, light, and dry conditions (Clark et al. 1973; Erdos et al. 1973; Wallace & Raper 1979; Francis & Eisenberg 1993). The simplest and most successful protocols remove these three factors by growing clones on a specialized nutrient agar (0.1% lactose, 0.1% peptone, 1.5% agar) in an excess of a phosphate-free salt solution (0.06% NaCl, 0.075% KCl, 0.03% $CaCl_2$) and incubating in the dark (Bonner, 1947; Blaskovics & Raper 1957). There are also a few other known chemical inhibitors, as well as promoters, of macrocyst formation. Activated charcoal interferes with a variety of diffusible hormones required for macrocyst development (Weinkauff & Filosa 1965; Filosa 1979). Chloroquine and ammonia also prevent macrocyst formation, specifically preventing the production of reproductive zygotes by inhibiting cell fusion (Rivera & O'Day 1987; Fang et al. 1992). Ethylene is well-known for inducing the formation of macrocysts in conditions favoring fruiting body production (Amagai 1984; Amagai 1992), while

calcium can increase macrocyst production by enhancing giant cell formation (Chagla et al. 1980). Aside from the environmental conditions that affect macrocyst formation, the giant cell itself plays a huge part in the production of a mature macrocyst. This reproductive zygote produces an auto-inhibitor that prevents zygote giant cell formation by other cells, ensuring a remaining population of cells for use as food (O'Day et al. 1981). The zygote also produces a significant amount of cyclic AMP, a known chemoattractant, acting as a powerful aggregation center (Abe et al. 1984). Along with the factors that affect macrocyst formation, previous studies have also focused on attempts to induce germination using techniques such as temperature shock and manual breakage. Though many of the attempted techniques had little or no effect on germination, the most successful technique for increasing the rate of germination was allowing the macrocysts to age for months, suggesting that macrocyst formation truly is a dormant stage that evolved to survive through a lengthy harsh season such as winter (Nickerson & Raper 1973b).

Dissertation Overview

My dissertation research has focused primarily on more thoroughly characterizing the sexual cycle of *D. discoideum*, as well as identifying any underlying population structure that could affect its social behavior, both in the asexual and sexual cycles. In Chapter 1, I address the possibility that hidden genetic differentiation or even cryptic species may confound our understanding of social and sexual interactions in *D. discoideum*. I analyze ribosomal nuclear and mitochondrial DNA sequence data using molecular ecology techniques to address the following questions: Is *D. discoideum* a monophyletic group? Is there evidence of genetic subpopulations or even species? Does this genetic differentiation indicate geographic isolation

or some other type of barrier to gene flow? In Chapter 2, I identify the selective pressures maintaining a low number of mating types in *D. discoideum*. As discussed earlier, *D. discoideum* has three self-incompatible mating types. This is more than two, as are commonly seen in many taxa, but far fewer than the hundreds or thousands that are predicted in early theory on mating type numbers (Iwasa & Sasaki, 1987; Hurst, 1996). In order to further understand what forces drive these low numbers, I address the following questions: Do *D. discoideum* gametes of each mating type differ in size? What are the relative roles of balancing selection and drift on maintaining mating type frequencies in natural populations?

In Chapter 3, I will focus on a historical claim that macrocyst production can be induced in one mating type by another. Sex is unique in Dictyostelids in that it has both the sexual dynamic involving the fusion of two cells to form a diploid zygote that can develop and hatch, releasing hundreds of potentially recombinant offspring and the social dynamic (a possible form of parental investment) involving the cannibalization of hundreds of potentially related individuals for use as both nutrients and protection. Contributing disproportionately fewer of these sacrificed cells would correspond with a reproductive advantage for the uncooperative genotype contributing to the zygote but not to the surrounding body. This is likely the underlying mechanism behind previous reports that claimed that a Type I *D. discoideum* clone induced macrocyst production in a Type II clone (O'Day and Lewis, 1975; MacHac and Bonner, 1975). Since these experiments were performed on a single pair of clones, it is unknown whether this pattern is representative of interactions between all Type I and Type II *D. discoideum* clones or simply an isolated association. It is also unknown whether Type III interacts with either Type I or Type II clones in a similar manner. By varying the frequency of each mating type in pairwise mating experiments, I address the following questions: Is there

unfair investment in macrocyst production? If so, is this unfairness dictated by a mating hierarchy such that contribution to reproduction differs depending on which mating type is dominant in a pairing?

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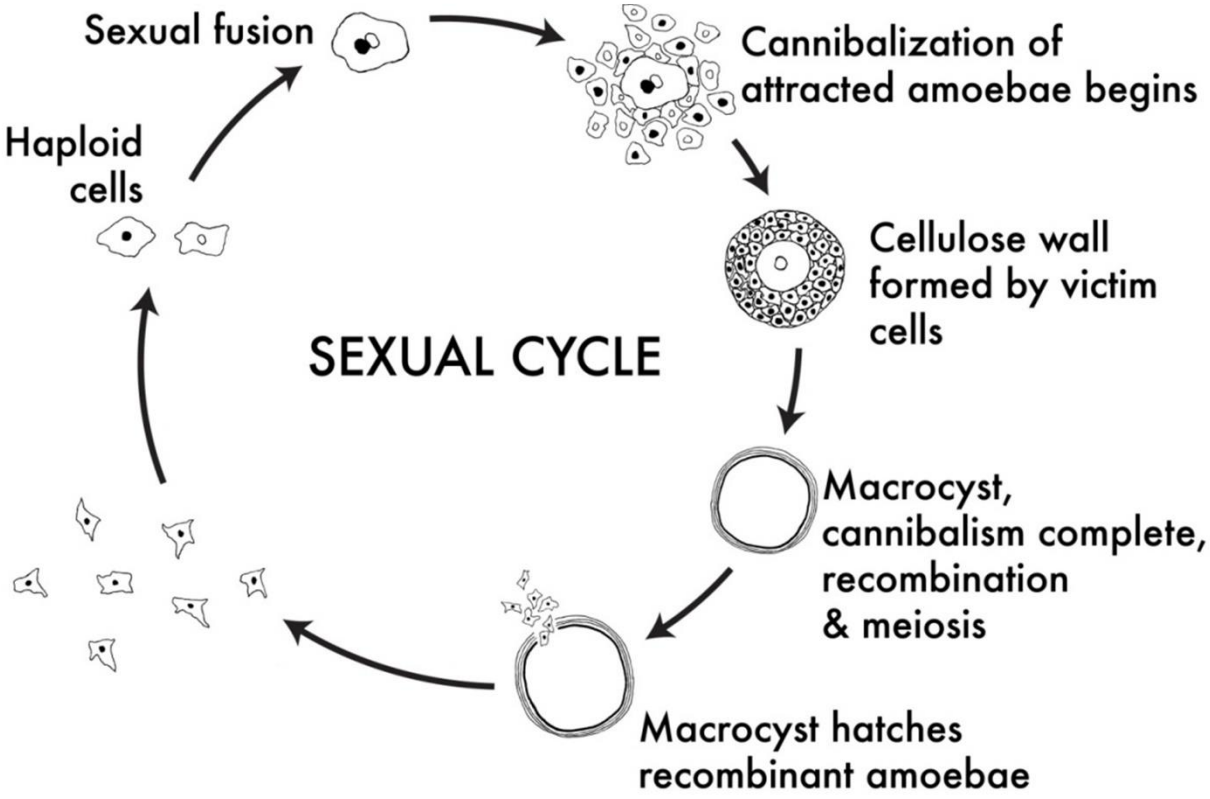
Figure 1.1. *Dictyostelium discoideum* sexual life cycle. Adapted from illustration created by David Brown & Joan E. Strassmann, CC Creative Commons Attribution - Share Alike 3.0.



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CHAPTER 2

Genetic diversity in the social amoeba *Dictyostelium discoideum*: population differentiation and cryptic species

by

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Abstract

The social amoeba *Dictyostelium discoideum* is a commonly used model organism for the study of social evolution, multicellularity, and cell biology. But the boundaries and structure of the species have not been explored. The lack of morphological traits to distinguish *D. discoideum* makes even knowing whether a given clone is *D. discoideum* a challenge. We address this with a phylogeny of a widespread collection of clones from a range of locations and including clones identified previously as potential cryptic species. We sequenced portions of nuclear ribosomal DNA and mitochondrial DNA, analyzing approximately 5500 and 2500 base pairs from the two regions respectively. We compared these sequences to known reference sequences for both *D. discoideum* and other closely related *Dictyostelium* species to create Bayesian and neighbor-joining phylogenetic trees representing the evolutionary relationships among the clones. We identified 51 unique *D. discoideum* concatenated sequences based on the combined mitochondrial and ribosomal sequence data. We also identified four unique *D. citrinum* concatenated sequences, three of which were previously classified as *D. discoideum* clones. Our analysis of the data revealed that all *D. discoideum* clones form a monophyletic group, but there are several well-supported subclades and pronounced genetic differentiation among locations (F_{ST} = 0.242, P = 0.011), suggesting the presence of geographic or other barriers between populations. Our results reveal the need for further investigation into potential tropical cryptic species.

Introduction

Microbes have become increasingly useful study systems for sociality, and have been found to exhibit surprisingly complex social behaviors (Crespi 2001; Strassmann et al. 2000; West et al. 2006, Strassmann & Queller 2011). As research on sociality in microorganisms flourishes, however, there is still a limited understanding of population structure in social microorganisms. The debate continues as to whether free-living microbes are limited by dispersal or if they are everywhere, having no population structure (Finlay 2002; Whitaker et al. 2003; Whitfield 2005; Winsett and Stephenson 2008). Extensive and accurate understanding of the evolutionary relationships among interacting individuals of the same species is necessary for forming conclusions about their social interactions. High relatedness is a key element in promoting kin selection, maintaining cooperation, and controlling cheating (Fletcher & Michener 1987; Gilbert et al. 2007; Hamilton 1964). A thorough understanding of genetic variation and population structure in social microorganisms is still lacking for most species.

Dictyostelium discoideum is the most commonly studied species of social amoeba, a eukaryotic member of the Amoebozoa with both a single-cell stage and a multicellular stage (Raper 1984; Kessin 2001; Strassmann and Queller *in press*). It has long been used as a model system for studies of cell-cell signaling, chemotaxis, cytokinesis, motility, phagocytosis, and development (Ashworth and Dee 1975; Bonner 1967, 2008; Chisholm et al. 2006; Eichinger et al. 1999; Kessin 2001; Loomis 1986). *Dictyostelium discoideum* clones were originally identified based on morphological features that differed from other known dictyostelids (Raper 1935). The presence of a cellular basal disk, stalkless migration, and other morphological and developmental traits continue to be used as identifying features for *D. discoideum* clones (Raper 1984).

The multicellular life stage of *D. discoideum* offers a unique opportunity to study the dynamics of social evolution, such as the evolution of cheating and cheating prevention (Buss 1982; Buttery et al. 2009; Ennis et al. 2000; Hudson et al. 2002; Strassmann et al. 2000; Travisano and Velicer 2004; Strassmann and Queller 2011). Since the multicellular body forms from aggregation, genetically different clones could exploit others and gain a reproductive advantage by preferentially contributing to spore production, and little is known of how these interactions relate to the evolutionary relationships within the species (Pál et al. 2000; Strassmann et al. 2000). A recent study focusing solely on within-species *D. discoideum* interactions revealed a link between genetic distance and kin recognition (Ostrowski et al. 2008). Within the species, clones were more likely to co-aggregate with genetically similar clones (based on 12 polymorphic microsatellite loci) than with more dissimilar clones. A recent study reported the role of gene pair *lagC1* and *lagB1* (also referred to as *tgrC1* and *tgrB1* respectively) in *Dictyostelium* kin recognition (Benabentos et al. 2009). These studies emphasize the importance of molecular mechanisms in sociality. Understanding the genetic structure of the organism and possible factors that could affect its behavior is a key factor in performing successful studies in this important model organism. These studies provide further evidence for the need to clarify the species status of *D. discoideum* clones. Since individual clones can be frozen and used repeatedly in multiple studies, it is important to be able to separate within- and between-species interactions.

In experiments using only *D. discoideum* as the model, cheating could be attributed to the presence of two distinct species in a mix if it turns out that one clone is actually a different, closely related species. In *D. discoideum,* wild clones readily mix to form chimeras in which some clones form a disproportionate number of spores when mixed with others (Buttery et al.

2009; Foster et al. 2002; Strassmann et al. 2000). Evidence from recent work also indicates that, at least in a laboratory setting, these chimeras are also readily formed between different species of social amoeba in which one party overwhelmingly dominates (Jack et al. 2008). These chimeras indicate that *Dictyostelium* cells potentially recognize and discriminate against other species, while still interacting with them. The recent dictyostelid phylogeny (Schaap et al. 2006) revealed the misidentification of a putative *Dictyostelium discoideum* clone (V34) showing it to be a close relative *D. citrinum*. Previous studies also suggest the existence of several genetically distinct biological species within *D. discoideum* (Briscoe et al. 1987; Evans et al. 1988). Four clones (AC4, WS526, WS584 and ZA3A) were mentioned in both studies as being different from others. Briscoe et al. (1987) based their findings on mating-type classification (i.e. asexual or homothallic as opposed to sexual), as well as on allozyme electrophoresis and a monoclonal antibody. Evans et al. (1988) also noted unusual mating characteristics in the four clones but based their findings mainly on the hybridization (or lack thereof) of DNA probes specific for DNA sequences cloned from a known *D. discoideum* type strain.

In this project, we included these asexual and homothallic clones of uncertain species identification, as well as known sexual clones from a third study, focusing on the sexual cycle of two *D. discoideum* clones (A_2Cyc^r (IR1) and WS205) (Francis 1998). We also included several *D. citrinum* clones as the sister species to *D. discoideum*, and used *D. purpureum* as the outgroup. We used the sequenced *D. discoideum* genome (Ogawa et al. 2000; Eichinger et al. 2005) as a start for sequencing informative regions of 93 *Dictyostelium* clones to address the following questions: Do clones identified as *D. discoideum* represent a single, monophyletic group? Is the species composed of multiple, genetically distinct subpopulations or even species? Is there evidence of population genetic structure by location? We use methods similar to other

studies that have revealed probable cryptic species in *D. purpureum* (Mehdiabadi et al. 2009), and revealed the absence of cryptic species in a collection of *D. giganteum* clones (Mehdiabadi et al. 2010).

Materials and Methods

Clones

We analyzed 92 clones, collected by us and others from 11 North American states, and Mexico, Costa Rica, Guatemala, and Japan (Figure 2.1, Table A1.1). These clones consisted of 89 *D. discoideum* clones, two *D. citrinum* clones representing the sister group and, as the outgroup, one *D. purpureum* clone. Included in the 92 clones were four clones (AC4, ZA3A, WS526, WS584) whose identity as *D. discoideum* was considered to be questionable (Briscoe et al. 1987; Evans et al. 1988). We acquired data from GenBank for the *D. discoideum* sequence, one *D. citrinum* sequence and the *D. purpureum* sequence. We grew the 89 other clones from pure frozen stocks (i.e. fruiting bodies developed from a single spore prior to freezing) on SM/5 agar plates (Sussman 1987, p. 26) in association with *Klebsiella aerogenes* bacteria.

DNA Sequencing

We extracted DNA from spores using a Chelex/Proteinase K extraction protocol. We amplified a non-coding region of the mitochondrial genome (mtDNA) and regions of the nuclear ribosomal DNA (rDNA) by polymerase chain reaction (PCR) using the following protocol (step 1: 2 minutes at 94ºC; step 2: 30 seconds at 94ºC; step 3: 30 seconds starting at 65ºC and decreasing 1º per cycle; step 4: 1 minute at 72ºC; step 5: 15 cycles to step 2; step 6: 30 seconds at 94ºC; step 7: 30 seconds at 50ºC; step 8: 1 minute at 72ºC; step 9: 25 times to step 6; step 10: 6

minutes at 72ºC) with the following primers (see Table A1.2). We cleaned the PCR product with USB (Cleveland, OH) ExoSAP-IT and then sequenced using PE Applied Biosystems (Foster City, CA) Big Dye 3.1 chemistry and a 3100 genetic analyzer. We analyzed approximately 5500 base pairs of the nucleotide sequence of nuclear 17S, 5.8S, 26S and 5S rDNA regions and approximately 2500 base pairs of the nucleotide sequence of mtDNA (ATPase, LSU intron, and cytochrome oxidase genes). We aligned the sequences using the programs Lasergene SeqMan v. 7.0.0 and BioEdit Sequence Alignment Editor v. 7.0.5.2. Sequences have been deposited in GenBank [Accession numbers JF930786-JF931129].

Data Analysis

We used comparative DNA sequence data to estimate gene trees/phylogenies and to measure genetic differentiation among populations. We used both Bayesian and distance (neighbor joining) methods for phylogenetic reconstruction. Our phylogenetic analyses focused on three different datasets: 1) all unique haplotypes obtained from the concatenated mtDNA sequences, 2) all unique rDNA haplotypes, and 3) all unique combined sequences obtained from concatenating mtDNA and rDNA sequences from each clone. For the Bayesian analyses, we used MrBayes v. 3.1 (Huelsenbeck & Ronquist 2001) to estimate a phylogeny for each dataset based on the GTR+Γ model of molecular evolution. This model was selected separately for the mitochondrial and ribosomal datasets using jModelTest version 0.1.1 (Posada 2008). In addition, six high-frequency, polymorphic indels in the mtDNA data were scored as standard presence/absence characters and were included in the analysis with weighting equal to the nucleotide polymorphisms. For each analysis, four Metropolis-coupled Markov chains were run for 250,000 burn-in generations followed by 1.75×10^6 generations of data collection.

Neighbor-joining phylogenies were estimated for each dataset using the proportion (p) distance model in MEGA4 (Tamura et al. 2007) with gaps and missing data excluded in pairwise comparisons. Bootstrap values were based on 1000 replicates.

To test for population genetic differentiation, we calculated F_{ST} values separately for the mtDNA and the rDNA data using the analysis of molecular variance approach (Excoffier et al. 1992) implemented in Arlequin 2.0 (Schneider et al. 2000). We also calculated pairwise F_{ST} values for populations with sample sizes greater than 7. For the pairwise comparisons, we combined the Mexican and Costa Rican populations. We included these in only the ribosomal pairwise F_{ST} calculation because the total sample size was still less than 7 for the mitochondrial data. These analyses were based on all sites with less than 5% missing/gap data. Seven clones (concatenated sequence numbers 4, 5, 7, 29, 30, 38 and 41) were excluded from the mitochondrial F_{ST} calculation due to missing data. ZA3A (concatenated sequence number 6) was excluded from both F_{ST} analyses because we did not know where it came from. We estimated P-values empirically by permuting sequences among populations 1000 times. We also calculated the pairwise distances between the phylogenetic groups in this study and compared these values to the pairwise distances between known species of Group 4 Dictyostelids, which includes all three species used in this study, using published sequence from Schaap et al. (2006). We compared only sequence data from the 17S region due to the availability of published data. We used the software program MEGA4 (Tamura et al. 2007) to estimate pairwise genetic distances between clones using the p-distance algorithm. Gaps and missing data were eliminated in pairwise sequence comparisons.

To test for the presence of potential cryptic species, we used the generalized mixed Yule-coalescent model (GMYC) developed by Pons et al. (2006). This model uses likelihood based

methods to identify shifts from between-species to within-species branching events. To implement this model, we estimated an ultrametric tree for each dataset using Beast v.1.5.4 (Drummond & Rambaut 2007) based on the GTR+Γ model of molecular evolution. All parameters were set to default values. The maximum clade credibility (MCC) tree was calculated for each tree using TreeAnnotator v.1.5.4. We used code provided by T.G. Barraclough in conjunction with R v.2.11.1 and functions from the APE library to run the model. Results were then compared to the results from both the Bayesian and Neighbor-joining phylogenetic analyses.

Results

Estimated Gene Trees/Phylogenies

Mitochondrial DNA Data. We identified 40 unique *D. discoideum* haplotypes from the mitochondrial sequence data (Table A1.1). We produced a phylogenetic tree from Bayesian analysis of the 40 haplotypes that was generally not well resolved (Figure 2.2a). When compared with neighbor-joining analysis of the haplotypes (Figure 2.2a), the Bayesian and neighbor-joining trees were similarly unresolved. The results of the GMYC analysis were not supported by the results of either phylogenetic analysis. GMYC analyses indicated that the mtDNA haplotypes disc 4, disc 7, and disc 18 were genetically distinct entities and the rest of the *D. discoideum* haplotypes were included in the same genetic cluster. Neither phylogenetic analyses differentiated *D. discoideum* from *D. citrinum*. In the Bayesian tree, the three *D. citrinum* clones group together, though not significantly, with the mtDNA haplotype disc 5 falling outside of them. In the neighbor-joining tree, two of the *D. citrinum* accessions group together with disc 4, again not significantly, with the third *D. citrinum* clone falling outside of

them. We were unable to obtain adequate mitochondrial sequence data using our sequencing primers from the *D. citrinum* clone (V34), so this haplotype was not included in the mitochondrial analysis. Three *D. discoideum* (CF3B, QS94, and QS108; Table A1.1) were not included due to a lack of adequate sequence data.

Ribosomal DNA Data. We identified 28 unique *D. discoideum* haplotypes from the ribosomal sequence data (Table A1.1). Ten of the 28 *D. discoideum* haplotypes were observed multiple times, often from multiple locations. We produced a phylogenetic tree through Bayesian analysis of the unique haplotypes (Figure 2.2b). The *D. discoideum* haplotypes formed a monophyletic group with a posterior probability of 1.0. The four *D. citrinum* clones were grouped with a posterior probability of 0.99 with *D. purpureum* as the outgroup. The results from the neighbor-joining analysis were similar to those from the Bayesian analysis. The phylogenetic trees from both analyses had similar topologies and nodal support. One noticeable difference was the placement of rDNA haplotypes disc 1, 2, and 3. Both analyses grouped the three haplotypes with strong support, however the neighbor-joining phylogenetic tree placed the group more basally than did the Bayesian analysis, making it basal to the large grouping of mainly US clones like most of the rest of the Mexican and Costa Rican clones. The results from the GMYC analyses were supported by both phylogenetic analyses. GMYC analyses showed seven genetically distinct clusters and/or entities. The rDNA haplotypes disc 1, 2, and 3 were grouped as a single cluster, disc 4, 5, 6, 7, and 8 were each genetically distinct entities and the rest of the *D. discoideum* haplotypes were grouped together as a single cluster.

Combined Mitochondrial and Ribosomal DNA Data. Overall, we identified 50 unique *D. discoideum* concatenated sequences based on the combined sequence data (Table A1.1). We also identified 4 *D. citrinum* concatenated sequences, including two that had previously been classified as *D. discoideum* (WS584 and WS526, concatenated sequences citr 1 and citr 2 respectively). We collected more than one clone of 13 of the 51 *D. discoideum* concatenated sequences, sometimes from a range of different geographic locations. We produced a Bayesian phylogenetic tree from the *D. discoideum* and *D. citrinum* concatenated sequences using the data from the *D. purpureum* as the outgroup (Figure 2.3). The four *D. citrinum* concatenated sequences formed a monophyletic group with a posterior probability of 1.0. The remaining *D. discoideum* concatenated sequences also formed a monophyletic group. They were divided into several well-supported basal clusters and a mostly unresolved larger grouping. The large grouping contained all of the U.S. clones plus one from Mexico and the one from Japan. Though it was mostly unresolved, there was evidence of some well-supported subgroupings. The Bayesian phylogeny shared a similar topology and nodal support to the tree produced from neighbor-joining analysis of the 50 unique concatenated sequences (Figure 2.3). As in the ribosomal tree, the placement of the grouping of concatenated sequence numbers 1-3 varied between trees. In the combined analysis, the group was placed more basally by the Bayesian analysis than in the neighbor-joining tree. The neighbor-joining analysis also indicated strong support for a grouping of concatenated sequences 10-12, 18 and 37 as well as for 6, 7 and 46. The neighbor-joining analysis showed less support for the grouping of concatenated sequences 4, 5 and 8, although both trees placed these three clones as the most basal of the *D. discoideum* clones. The results of the GMYC analyses were supported by both phylogenetic analyses and

suggested the presence of 10 unique clusters and/or entities within *D. discoideum* (Figure 2.1, Figure 2.3). There were two genetically distinct clusters within the larger, mostly U.S. grouping and each of the more basal Mexican and Costa Rican clones represented a genetically distinct entity.

Genetic Differentiation Among Populations

The mitochondrial data indicated no significant pattern of differentiation among populations (F_{ST} = 0.099, P = 0.111). The pairwise F_{ST} comparisons for the mitochondrial data also did not indicate strong population structure by location. However, the pairwise F_{ST} comparisons involving the North Carolina population were all significant (Table 2.1). For the ribosomal data, there was significant genetic differentiation among populations (F_{ST} = 0.263, P = 0.003). The pairwise F_{ST} comparisons for the ribosomal data, which included the added combined Mexico/Costa Rica population, were also all significant, indicating restricted movement of haplotypes between populations on a continental scale (Table 2.1).

We calculated the pairwise distances for the 17S ribosomal sequences between nearest-neighbor Group 4 Dictyostelid species and found values ranging from 0.001 to 0.037 (data not shown). We then looked at the distances between phylogenetic groups in our study. Distances between *D. discoideum* clones and *D. citrinum* clones (0.008 - 0.011) fell into the middle of the range above. Within *D. discoideum*, pairwise distances between the large mainly U.S. group and the basal entities from Mexico and Central America averaged 0.002. Distances among those basal entities averaged 0.004. These are within the range of some recognized Group-4 Dictyostelid species pairs (*D. brefeldianum* G121 – *D. mucoroides* S28B 0.001; *D. capitatum*

91HO50 - *D. pseudobrefeldianum* 91HO8 0.003; *D. sphaerocephalum* GR11- *D. mucoroides* G81 0.002; *D. brunneum* WS700 – *D. giganteum* WS589 0.004).

Discussion

In this study, we found evidence of differentiation of populations based on location and well-supported subgroups within the named species *D. discoideum*, indicating support for previous suggestions of cryptic species. We also show that some care needs to be exercised with morphological assessments of species identity, although identification by this means is usually accurate. Based on the approximately 2500 base pairs of mitochondrial DNA and 5500 base pairs of ribosomal DNA, as well as previous work, three isolates previously described as *D. discoideum* were reclassified as *D. citrinum*. After this change, data from this study show that isolates currently described as *D. discoideum* are genetically distinct from the closely related species *D. citrinum*, forming a monophyletic group.

We found significant genetic differentiation among populations based on the ribosomal DNA data. This suggests that *D. discoideum* clones are more highly related to their neighbors than to clones from more distantly located populations, in this case from other U.S. states or countries. This may be a result of geographic barriers and other limitations on interactions between populations. The popular view on microbial biogeography is that free-living microbial eukaryotes are not limited by dispersal due to their extremely large populations and small size (Fenchel and Finlay 2004; Finlay 2002). Some species of microorganisms, however, have been found to exhibit patterns of restricted geographic distributions similar to those observed in larger organisms, indicating a need for further investigation into the population structure and geographic distribution of known microbial morphospecies (Foissner 2006, 2008; Smith and

Wilkinson 2007). Recent studies have suggested that this isolation by distance can cause microbial genetic differentiation (Kim et al. 2004; Vos and Velicer 2008; Whitaker et al. 2003). The observed population structure in this study is consistent with some isolation by distance. The ribosomal DNA F_{ST} value reveals that 26% of genetic differentiation can be attributed to between-population differences, indicating what Wright would call "very great genetic differentiation" (Wright 1978).

Dictyostelium discoideum is found in regions separated by large geographic barriers, including distance, rivers, and mountain ranges. However, isolates from the United States, one isolate from Mexico and the only isolate from Japan, as observed in this study, were found to be genetically similar, indicating that the geographic barriers isolating populations are not absolute. Multiple vectors for dispersal may contribute to the widely observed genotypes in addition to explaining the population structure indicated by the significant F_{ST} value. *Dictyostelium discoideum* is well-adapted for dispersal through the production of spores. Short-range dispersal is most commonly achieved when spores are carried by water or consumed or carried by soil invertebrates (Huss 1989; O'Dell 1979). The formation of the slug is also beneficial for local dispersal of cells (Kuzdzal-Fick et al. 2007). These short-range methods of dispersal are limited, however, and can therefore explain the observed population structure by location. Many microbes achieve wider dispersal by getting picked up and carried by air. *Dictyostelium* sori are usually too heavy to be carried by air. Aerial dispersal is therefore an improbable means of dispersal for *D. discoideum* (Cavender 1973), and this may largely explain the observed population structure. More distant travel can be facilitated by larger organisms that carry *D. discoideum* spores externally on wet fur or feathers or internally after ingestion of a substrate on which fruiting bodies are attached (Suthers 1985).

Based only on the prediction that *D. discoideum* individuals are limited by dispersal, the isolate from Japan should have been the most diverged from the rest of the species. However, this was not the case. Though more Japanese isolates would be needed to get a clearer understanding of the evolutionary relationships of isolates from that population and populations from other locations, from the data in this study, differentiation of populations within North America were more supported by the Bayesian tree. Of the eight sequenced clones from Mexico and Costa Rica, only one was observed, along with all of the U.S. clones, within the large undifferentiated clade of *D. discoideum*. The rest were all found in well-supported basal groups that included only one other clone collected from an unknown location. One hypothesis for this is that the clones analyzed from Mexico and Costa Rica that fell into the well-supported basal groups were actually one or more cryptic species genetically distinct from both *D. discoideum* and the closely related *D. citrinum*.

Observations of the raw sequence data revealed relatively large variation within and between the Mexican and Costa Rican populations in DNA segments that were observed to be highly conserved in the other *D. discoideum* concatenated sequences (data not shown). Comparisons of the percentage of divergence between the Mexican and Costa Rican clones and the rest of the *D. discoideum* clones also revealed more variation within and between the Mexican and Costa Rican populations, as evidenced by the longer branch lengths between these clones (Figure 2.3). This further supports the theory that cryptic species may be present. It also emphasizes the need to further sample these regions of Mexico and Costa Rica, as the addition of more individuals might better explain the evolutionary relationships in these regions, either by filling in the gaps or further defining cryptic species.

Sexual incompatibility between groups, if present, could also provide further evidence for cryptic species, however using current methods for testing mating compatibility may prove unreliable. Though most research is concentrated solely on the asexual stage that all *Dictyostelium* clones share, the less commonly observed sexual stage contains both sexual self-incompatible and homothallic, or self-compatible types (Erdos et al. 1973). The sexual stage of *D. discoideum* is less common in nature than the asexual stage, and is often difficult to recreate in a laboratory setting, however genetic exchange does occur (Francis 1998; Wallace and Raper 1979). Preliminary data suggest that macrocysts form both within and between the identified clusters, however viability has yet to be tested, and therefore these results neither confirm nor disprove the existence of cryptic species (unpublished data).

The presence of homothallic clones also complicates these results. Two of the Mexican and Costa Rican clones (AC4 and ZA3A, concatenated sequences disc 4 and disc 6 respectively) were previously labeled as possible unknown species in previous studies (Briscoe et al. 1987; Evans et al. 1988, Table A1.1). Both Briscoe and Evans noted that clones AC4 and ZA3A expressed the self-compatible mating type. Based on preliminary data, the homothallic mating type was unique to AC4 and ZA3A and, as of yet, was not found in any of the other clones in this study (unpublished data). Though these results may not reflect what occurs in nature, the data imply that homothallism may be a trait unique to the Mexican and Costa Rican clones that was lost in the larger *D. discoideum* clade. This trait also makes identifying cryptic species based on mating incompatibility difficult, as current methods do not differentiate between selfing and interactions with other clones.

We know from previous work that in *D. discoideum*, genetically similar clones are more likely to group together in an aggregate than more genetically dissimilar clones (Ostrowski et al.

2008). We also observed, in this study, phylogenetic clustering of genetically similar *D. discoideum* clones by location and uncovered well-supported phylogenetic subgroups within the species. Interestingly, patterns similar to these have been found in another dictyostelid species. Mehdiabadi et al. (2009) found three distinct phylogenetic groups within the species *D. purpureum* and that interactions, both in the sexual stage and in the aggregation stage, were more likely to occur between clones from within each group than between. These findings suggest that morphological species identification is not enough to answer questions about kin selection, altruism, and other questions about social evolution. Although *D. discoideum* is an important and useful tool for studying biological phenomena, researchers should be wary of clones identified as *D. discoideum* based wholly on phenotype due to the possible existence of cryptic species. Further investigation into the Mexican and Costa Rican clones is needed to support our hypothesis that some or all of these clones should be renamed as a species separate from *D. discoideum.* It is plausible, however, that the named species *D. discoideum* consists of one or more cryptic species.

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Table 2.1. Pairwise Fst values between populations of *Dictyostelium discoideum* with sample sizes greater than 7 individuals. Asterisks (*) indicate significant pairwise comparisons with single asterisks indicating a significant value of $p<0.05$ and double asterisks indicating $p<0.001$.

	MA	NC	TX	VA	MX/CR
Ribosomal DNA					
Massachusetts (MA)	-				
North Carolina (NC)	0.319**	-			
Texas (TX)	0.500**	0.211*	-		
Virginia (VA)	0.315**	0.131*	0.116*	-	
Mexico/Costa Rica (MX/CR)	0.243**	0.285**	0.256**	0.433**	-
Mitochondrial DNA					
Massachusetts (MA)	-				
North Carolina (NC)	0.327**	-			
Texas (TX)	0.005	0.112*	-		
Virginia (VA)	-0.011	0.197*	-0.031	-	

Figure 2.1. Geographic locations of *Dictyostelium discoideum* clones used in this study. Reference strains are not included. Also not pictured are one *D. discoideum* clone from Japan and one from an unknown location. Locations of possible taxonomic groups are identified by colored circles. In locations where more than one possible taxonomic group was present, proportions of each are represented.

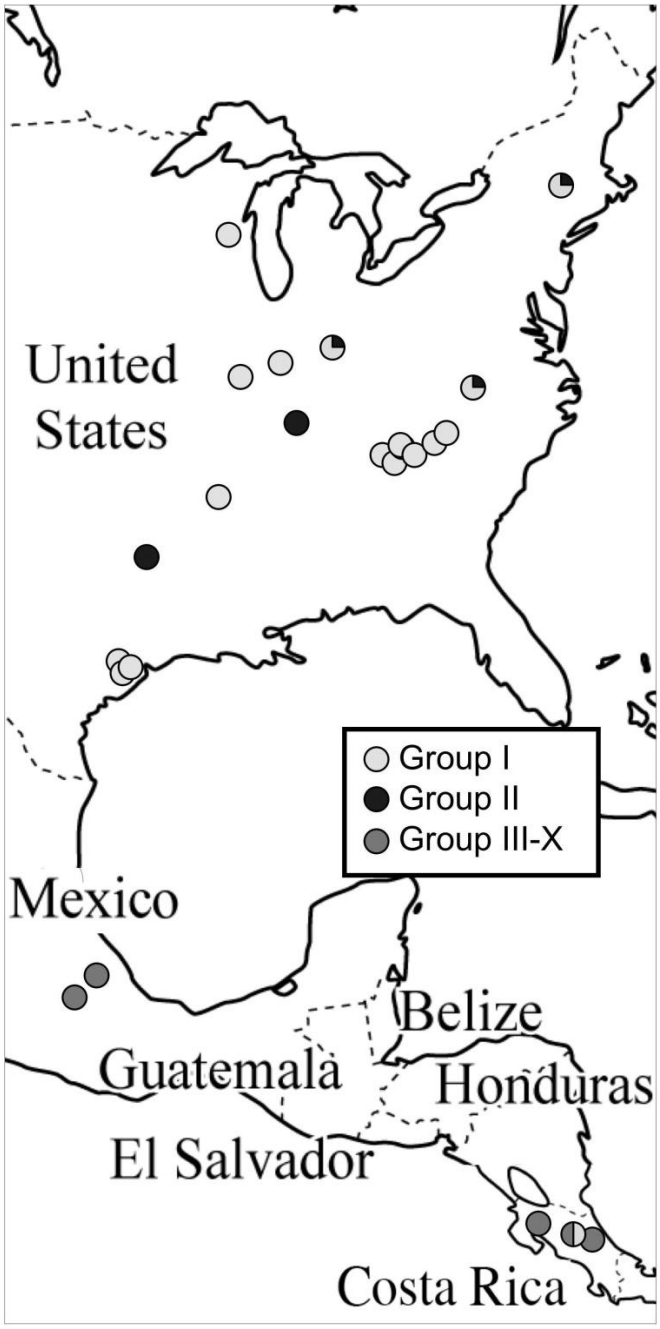


Figure 2.2. Bayesian phylogenetic trees created from (a) mitochondrial and (b) ribosomal DNA sequence data, with Bayesian posterior probablilities and cooresponding neighbor-joining bootstrap values. Each terminal branch represents a haplotype. Taxon names correspond to combined mitochondrial and ribosomal DNA concatenated sequences. Branch lengths are not drawn to scale. Symbols represent locations of origin for each clone represented by the concatenated sequence.

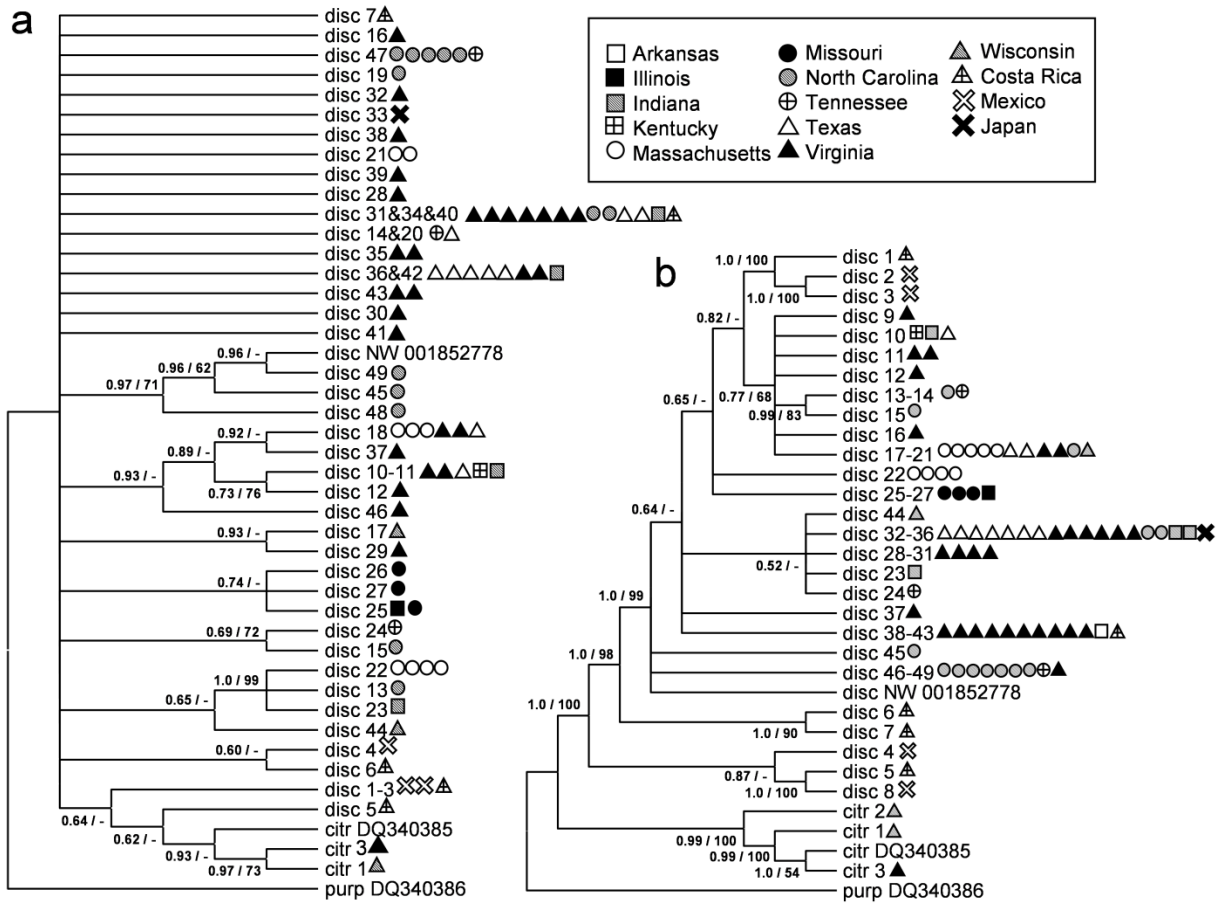
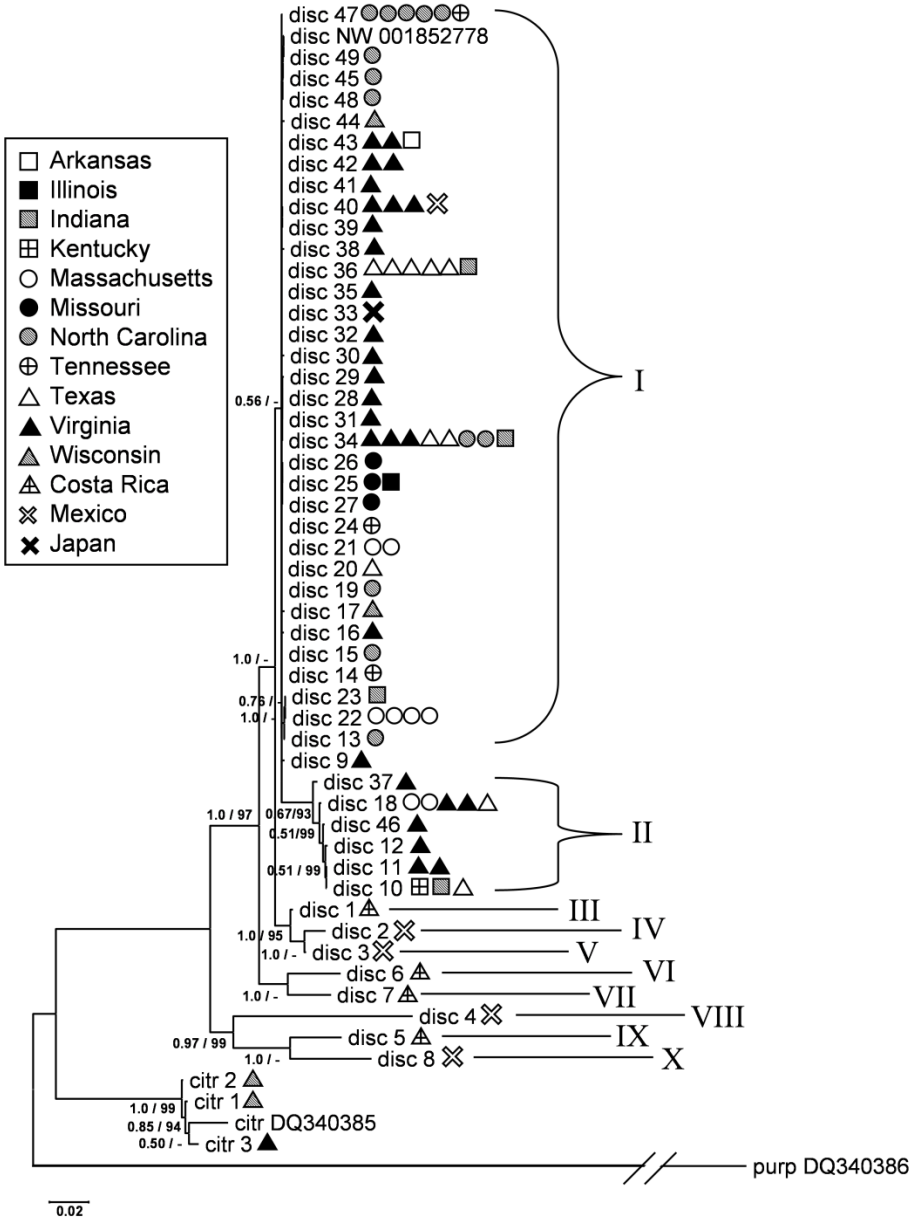


Figure 2.3. Bayesian phylogenetic tree created from combined mitochondrial and ribosomal DNA sequence data, with Bayesian posterior probabilities and cooresponding neighbor-joining bootstrap values. Taxa represent concatenated sequences. Bar indicates distance in terms of substitutions per site. Symbols represent locations of origin for each clone represented by the concatenated sequence. Numbered brackets and lines coorespond with genetic clusters and entities determined by GMYC analyses.



CHAPTER 3

Sex ratio and gamete size across eastern North America in *Dictyostelium discoideum*, a social amoeba with three sexes

by

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Abstract

Theory indicates that numbers of mating types should tend towards infinity or remain at two. The social amoeba, *Dictyostelium discoideum*, however, has three mating types. It is therefore a mystery how this species has broken the threshold of two mating types, but has not increased towards a much higher number. Frequency dependent selection on rare types in combination with isogamy, a form of reproduction involving gametes similar in size, could explain the evolution of multiple mating types in this system. Other factors, such as drift, may be preventing the evolution of more than three. We first looked for evidence of isogamy by measuring gamete size associated with each type. We found no evidence of size dissimilarities between gametes. We then looked for evidence of balancing selection, by examining mating type distributions in natural populations and comparing genetic differentiation at the mating type locus to that at more neutral loci. We found that mating type frequency varied among the three populations we examined, with only one of the three showing an even sex ratio, which does not support balancing selection. However, we found more population structure at neutral loci than the mating type locus, suggesting that the three mating types are indeed maintained at intermediate frequencies by balancing selection. Overall, the data are consistent with balancing selection acting on *D. discoideum* mating types, but with a sufficiently weak rare sex advantage to allow for drift, a potential explanation for why these amoebae have only three mating types.

Introduction

Research on the evolution and maintenance of sex and sex ratios in eukaryotes has historically focused heavily on those systems exhibiting two distinct mating types, one male and one female. But more than two mating types occur in some species. Recently, researchers have begun to explore the many natural systems that exhibit more diverse sexual strategies. In nature, the observed numbers of mating types in systems that have evolved past two can range from low numbers like those seen in many ciliates (3-15 mating types; Collins & Gorovsky, 2005; Phadke & Zufall, 2009), and the acellular slime mold *Physarum polycephalum* (≥13 mating types; Collins & Tang, 1977) to hundreds or even thousands of mating types like those seen in many fungal species (Kothe, 1996; Billiard et al., 2011; 2012). The fungus *Schizophyllum commune* is the most commonly recognized example of a high number of mating types due to its tetrapolar mating type system, with over 20,000 allele combinations currently estimated (Raper, 1966; Kothe, 1996). Variation in mating systems is also common in plants, where self-incompatibility alleles can range from fewer than 10 to an estimated 200 (Lawrence, 2000; Castric & Vekemans, 2004; Busch et al., 2014).

With all this diversity, it is important to understand how differing numbers of mating types can evolve and be maintained in natural systems. Theory predicts that the number of mating types should tend towards infinity or remain at two (Iwasa & Sasaki, 1987). In their model suggesting large numbers of mating types, Iwasa and Sasaki propose that a new mating type that arises in the population should be favored by selection because it can mate with a larger proportion of the population. This negative frequency-dependent selection theory assumes both that there is a cost to not finding a mate and that all mating types are inter-compatible. Plant theory for numbers of self-incompatibility alleles also centers on negative frequency-dependent

selection for explaining how new alleles arise in populations and why we see so many (Wright, 1939). Iwasa and Sasaki (1987) also constructed a model for why only two mating types might remain. In this model, individuals or gametes can wait, without cost, for a suitable mate, and populations tend to lose all but two mating types most likely due to drift. More recent theory focuses on explaining more actively why we often only see two mating types (reviewed in Billiard et al., 2011). The evolution of anisogamy, cytoplasmic conflict leading to uniparental organellar inheritance, and high selfing rates that reduce the cost of finding a mate are just a few of the hypothesized constraints on the evolution of more than two mating types.

Dictyostelium discoideum shows evidence of intermediate numbers of mating types. These social amoebae and other members of the Dictyosteliidae produce a sexual structure called a macrocyst, the diploid fusion product of two haploid cells of different mating types (Blaskovics & Raper, 1957; Filosa & Dengler, 1972; Erdos et al., 1973a,b; O'Day, 1979; O'Day & Durston, 1979; Saga & Yanagisawa, 1983; O'Day & Keszei, 2012; Bloomfield, 2013). Dictyostelia exhibit a variety of mating strategies with evidence of homothallic, or self-compatible species, as well as systems of 2, 3 and 4 mating types (Erdos et al., 1973a, 1975; Clark et al., 1973; Francis, 1975; Cavender et al., 1981, 2005; Chang & Raper, 1981; Kawakami & Hagiwara, 1999). The most commonly studied of these, *D. discoideum*, has three self-incompatible mating types determined by a single locus with three alleles, which cannot mate with themselves but can mate with either of the other two types (Erdos et al., 1973a; Clark et al., 1973; Bloomfield et al., 2010). We know that sex is common in nature from evidence of rapid decay in linkage disequilibrium with distance along the chromosome and recombinant genotypes in wild populations (Flowers et al., 2010). However, direct evidence from hatching macrocysts in the lab has been challenging to obtain. Though much of the process has been documented, many

aspects of the *D. discoideum* mating system are still yet to be understood. One such missing element is a clearer understanding of how the number and distribution of its mating types fit in with the theory that explains mating type evolution in the rest of the eukaryotes. What keeps *D. discoideum* at three?

The possible selective pressures maintaining low numbers of mating types in microbial eukaryotes are likely to vary across lineages, as indicated in ciliates (Phadke & Zufall, 2009). However, even in ciliates, the forces driving patterns of mating type numbers and their distributions remain unclear. Overall, this field is vastly understudied across microbial eukaryotes. Since this question has never been addressed in *Dictyostelium*, we investigated how three mating types are maintained in *D. discoideum*, considering two common characteristics of mating systems, anisogamy and negative frequency-dependent selection at the mating type locus. First, physical differences between gametes, most notably size differences, have been associated with the evolution and maintenance of two-sex systems (Randerson & Hurst, 2001; Bulmer & Parker, 2002). This type of reproduction, labeled anisogamy, can result from disruptive selection favoring increases in both the size and number of gametes. Once this happens, it removes the frequency-dependent advantages of a rare sex, as gametes are no longer universally compatible. Small gametes only mate with large gametes and vice versa. While anisogamy is common in multicellular organisms, the opposite, isogamy, is more often found in unicellular organisms where vegetative structures are less complex and increased gamete size yields less of a reproductive fitness gain (Parker et al., 1972; Knowlton, 1974; Bell, 1978). Size differences between *D. discoideum* gametes could suggest differentiation and/or specialization of mating types that would make intermediate mating types unfavorable and limit the evolution of more mating types.

Second, we focused on two manifestations of negative frequency-dependent selection at the mating type locus. First, mate availability is extremely important for reproduction and can be a limiting factor. Similar to the theory predicting the evolution of an infinite number of mating types (Iwasa & Sasaki, 1987), equal sex ratios are predicted to be caused and maintained by a frequency-dependent selection favoring the rarer sex (Fisher, 1930; Wright, 1939). Deviations, though rare, can be caused by a variety of factors such as local mate competition, mate attractiveness, maternal condition and environmental dynamics (Hamilton, 1967; Charnov, 1982; West, 2009). Evenness is expected to persist even in systems with multiple mating types (Orias & Rohlf, 1964; Iwasa & Sasaki, 1987). It is not known if all three of the *D. discoideum* mating types persist in all natural populations or if they do, at what frequencies. Skewed mating type distributions could indicate differential pressures on sex allocation suggesting that larger numbers of some mating types may result from other sources of selection or drift.

Second, unlike neutral alleles, genes responsible for sex determination or mating compatibility are generally under balancing selection. Evidence for this is fairly ubiquitous in sexual species, most notably in self-incompatibility alleles in plants (Vekemans & Slatkin, 1994) and mating compatibility genes in fungi (May et al., 1999). Balancing selection contributes to both allelic diversification and the maintenance of ancient alleles. Allelic diversification, as proposed by models for the evolution of high numbers of sex determination alleles in which rare types are favored in the population, has been discussed previously (Wright, 1939; Iwasa & Sasaki, 1987). But, balancing selection also tends to maintain alleles for mating compatibility in a population over long periods of evolutionary time (reviewed in Delph & Kelly, 2014). In *D. discoideum*, we know from the very divergent sequences of the alleles at the mating type locus, that the mating types have been diverging in the species for a very long time (Bloomfield et al.

2010). This suggests that balancing selection is acting on the mating types. It is unknown if the distributions of mating type alleles found in each population also show evidence of balancing selection.

Here, we investigated two questions: Do *D. discoideum* gametes of each mating type differ in size? What are the relative roles of balancing selection and drift on maintaining mating type frequencies in natural populations? To answer these questions, we identified the mating types of 170 individual clones from three well-sampled natural populations and measured the gamete sizes from a representative subset of two of these populations. We show evidence of isogamy, not anisogamy, and evidence that while balancing selection appears to be maintaining the frequencies of the three mating types when compared to more neutral markers, sex allocation varies across populations.

Materials and Methods

Study Populations

To look at mating type distributions, we identified the mating types of *Dictyostelium discoideum* clones from frozen stocks originally isolated from soil samples. We analyzed 170 clones, collected from four geographic locations: 87 near the Mountain Lake Biological Station in Virginia (Fortunato et al., 2003), 47 from the Houston Arboretum in Houston, Texas and 36 from two locations in North Carolina (Table A2.1). We analyzed a subset of the 170 clones, focusing only on the Virginia and Texas populations, to measure gamete size. Before all analyses, we grew the clones from clonal frozen stocks on nutrient agar plates with the bacterial food source *Klebsiella pneumoniae*.

In choosing our clones, we accounted for the possibility of oversampling issues affecting our results. Many more isolates were collected from the populations we focused on here than were used in this study. We used information on soil sample, mating type and microsatellite allele markers to make sure our list of clones was comprised of independent samples. Isolates from different soil samples were assumed to be independent samples but duplicate isolates from a single soil sample were excluded whenever they showed the same mating type and the same genotypes at five microsatellite loci.

Gamete Size Measurement

To measure gamete size, we sampled multiple clones from each of the three self-incompatible mating types from two populations. Because two haploid cells fuse to form the reproductive zygote during the sexual cycle of *D. discoideum*, we measured the size of cells prepared in the absence of a compatible mating partner but in conditions conducive for sexual fusion, to get at their size right before fusion. These fusion-competent cells are considered at this point to be gametes (Saga et al., 1983; O'Day et al., 1987; Urushihara & Muramoto, 2006). Specifically, we plated $2x10^5$ spores on LP agar plates (0.1% lactose, 0.1% peptone, 1.5% agar) in an excess of Bonner's salt solution (SS: 0.06% NaCl, 0.03% $CaCl_2$, 0.075% KCl) with *K. pneumoniae* and incubated the plates in the dark for 3 days at 22° C. We then collected the resulting dark-grown cells and measured the cell diameters using a Nexcelom Cellometer Auto 1000 (Lawrence, MA). We used the default settings with the exception of a cell size minimum set to 5 um and a maximum set to 15 um. In each population, we measured 160 cell diameters from each of four to six clones per mating type.

For comparison, we also measured the size of cells grown in conditions conducive for fruiting body conditions in order to get at vegetative cell sizes when clones are not preparing for

sexual fusion. We plated $2x10^5$ spores on SM/5 agar plates with *K. pneumoniae* and allowed the plates to grow on a bench for ~36 hours. We collected pre-aggregate vegetative cells in buffer and used the same methods as previous for measuring cell diameters.

Mating Type Identification and Microsatellite Analysis

We developed mating type specific primers (see Table A2.2) based on the published mating type gene sequences identified by Bloomfield et al. (2010). Each mating type expresses a unique set of genes (Type I: matA; Type II: matC, matB, matD; Type III: matS, matT), allowing for the development of a gene presence/absence assay for mating type identification. We repeated techniques described in Douglas et al. (2011) for DNA extraction, amplification and sequencing. We extracted DNA from spores using a Chelex/Proteinase K protocol and amplified, by polymerase chain reaction (PCR), regions of the mating type genes using the primers we developed. We ran the PCR product on a 1% agarose gel to identify presence/absence of bands as an indication of mating type. To verify the use of this method to identify mating types, we also checked the accuracy of approximately 15% of our results using either Sanger sequencing and/or mating compatibility tests. We used methods similar to those available on dictyBase for the compatibility tests (http://dictybase.org/techniques/media/mating_types.html, Basu et al., 2013). We plated spores from two *D. discoideum* clones together in an excess of SS buffer on LP agar plates with *K. pneumoniae* and incubated the plates in the dark for at least one week. Presence of macrocysts at this point indicated mating compatibility. Based on these assessments, we found our methods to be an excellent technique for identifying the presence of mating type genes.

To look for balancing selection on the three mating types, we compared F_{ST} at the mating type locus to that at more neutral microsatellite loci. Lower F_{ST} at the mating type locus would

mean that its alleles were maintained at more even frequencies across populations than the neutral loci, and thus represent evidence for balancing selection on that locus. We acquired data for microsatellite allele sizes at 5 select loci for 168 *D. discoideum* clones from populations in Virginia (104 clones), Texas (40 clones) and North Carolina (24 clones) from Smith (2004; Table A2.1). Of those 168 clones, 139 overlapped with the clones we looked at in this study.

Statistical Analyses

Gamete Size: Unless otherwise indicated, all statistical analyses were performed using R software (version 3.2.3.) (R Core Team, 2015). We implemented a Welch's two sample t-test to compare the diameters of gametes to vegetative cells. To analyze the relationships between cell diameter measurements and both geographic origin and mating type, we fitted separate linear mixed-effects models to the gametic and vegetative datasets using the "lme" function from the R package "nlme" (Pinheiro et al., 2014). We treated geographic origin and mating type as fixed effects and clone identity as the random effect. Based on AIC and BIC scores, this model fit the data better than a model including the interaction effects of geographic origin and mating type. We used Type III tests to estimate the significance of the fixed effects. Though our data appeared to have a normal distribution based on the kurtosis and skewness, they failed the Shapiro-Wilk test of normality. Because of this, and because our errors were also not normally distributed, we implemented techniques based on Anderson & ter Braak (2003) where we applied permutation tests to the residuals under a reduced model. We used R code written for Noh & Henry (2015) that permuted residuals from fitting a model of only the effect not being tested. For example, the permutation test for mating type resampled residuals of a model that included only population origin as the fixed effect. The permuted p-values we report reflect the

proportion of times the F-value of the resampled data were larger or equal to the F-value of the real data.

Mating Type Frequency: To analyze the evenness of the frequencies of mating types within populations, we performed chi-squared goodness-of-fit tests using R software. We corrected for multiple comparisons by implementing the Benjamini-Hochberg procedure for controlling false discovery rates (Benjamini & Hochberg, 1995). The reported significant results remained significant after this correction. We examined the standardized residuals from statistically significant tests to identify the mating types that were more or less prevalent than expected.

Population Differentiation: We compared the differences between populations both in mating type frequencies and microsatellite allele frequencies by calculating estimates of F_{ST} using FSTAT version 2.9.3 (Goudet, 2001) and Hedrick's G'_{ST} (Hedrick, 2005) using the R package "diveRsity" (Keenan, et al., 2013). The latter is a standardized measure of genetic differentiation that can account for the high mutation rates and diversity of microsatellites, addressing the underestimation of genetic structure observed using only F_{ST} (Meirmans & Hedrick, 2011). Estimates of F_{ST} range from 0.0 to 1.0, but when there are large numbers of alleles at a locus, a value of 1.0 can never be reached even with complete differentiation. This is due to within-population diversity. Hedrick's G'_{ST} corrects for this by dividing the differentiation estimate by the maximum value it could take given the numbers of populations and alleles.

Results

Gamete sizes do not differ by mating type, but Texas gametes are smaller

We measured a total of 4640 gamete cells, representing 14 clones from Virginia (5 Type I, 4 Type II, 5 Type III) and 15 clones from Texas (6 Type I, 4 Type II, 5 Type III). We also measured 4800 vegetative cells, representing 15 clones from Virginia (5 Type I, 5 Type II, 5 Type III) and 15 clones from Texas (6 Type I, 4 Type II, 5 Type III). We did not detect evidence of cell size differences between mating types in either cell type (gamete: $F_{2,25} = 0.38$, $P_{perm} = 0.68$; vegetative: $F_{2,26} = 0.43$, $P_{perm} = 0.64$; Fig. 3.1A-3.1B). Overall, we found that gametes were significantly larger than vegetative cells (mean 9.99 and 9.32 microns, respectively; $t_{45} = 5.33$, $p < 0.0001$; Fig. 3.1C). Gametes from Virginia, averaged 10.23 microns and were significantly larger than gametes from Texas at an average of 9.77 microns ($F_{1,25} = 4.78$, $P_{perm} = 0.01$; Fig. 3.1D). We did not see this geographic difference between vegetative cells (Virginia = mean 9.37 microns, Texas = mean 9.24 microns; $F_{1,26} = 0.43$, $P_{perm} = 0.64$).

Frequencies of mating types are unequal and vary between locations

We identified the mating types of individual clones collected at well-sampled populations from four distinct geographic regions. In total, we identified 77 Type I, 39 Type II and 55 Type III individuals (Fig. 3.2, Table A2.1). Overall, the distribution of mating types differed from the balancing selection expectation of equal frequencies ($\chi^2 = 12.8$, df = 2, p = 0.01). Examining the standardized residuals from the chi-square test revealed that this departure is due to the identification of significantly more than expected Type I individuals and significantly fewer than expected Type II individuals (Table A2.3). Within individual populations, we found a range of distributions. In the population near Mountain Lake Biological Station, Virginia, we found an even distribution of mating types (34 Type I, 25 Type II, 28 Type III; $\chi^2 = 1.45$, df = 2, p = 0.48). The population in Houston, Texas significantly differed from an even distribution, with significantly fewer observed Type II individuals (22 Type I, 8 Type II, 18 Type III; $\chi^2 = 6.5$, df =

2, p = 0.04). Due to low sample numbers, we combined two populations in North Carolina. We identified 10 Type I, 3 Type II, and 2 Type III individuals in Linville Falls, NC and 11 Type I, 3 Type II, and 7 Type III individuals in Little Butts Gap, NC. Overall, we again found an uneven distribution of mating types when we combined these two populations, with significantly more than expected Type I individuals but significantly fewer than expected Type II individuals (χ^2 = 10.5, df = 2, p = 0.005).

Balancing selection maintains mating type distributions across populations

When we compared the three geographic populations to each other, we found no significant genetic differentiation in mating type frequency by geographic location (F_{ST} = 0.01, G'_{ST} =0.05; Table 3.1). We found substantially higher levels of genetic differentiation at the microsatellite loci (Mean: F_{ST} = 0.10, G'_{ST} = 0.55, Range: F_{ST} = 0.10-0.13, G'_{ST} = 0.32-0.77). Both the F_{ST} and G'_{ST} estimates for the mating type locus fell well below all the respective 95% confidence intervals for the microsatellite loci, suggesting strong evidence for balancing selection.

Discussion

Here we give the first empirical evidence for isogamy in *D. discoideum*. Individuals of each of the three mating types expressed in *D. discoideum* produce gametes that are indistinguishable in size. Because *D. discoideum* has evolved multiple mating types and lives primarily in a unicellular form, we were not surprised to find a lack of evidence for mating type-specific gamete size differences. Unicellular species are commonly isogamous, with gametes that are usually undifferentiated in form and sex-determination mechanisms that are regulated only at the molecular level by a mating type locus (Billiard et al., 2011; Bachtrog et al., 2014).

This observation may be due to the relatively short incubation time in unicellular organisms between fertilization and maturation of a zygote compared to the ultimately much larger multicellular organisms, such that there is less of a fitness advantage for increased zygote size and therefore no disruptive selection on gamete size (Knowlton, 1974). In anisogamous organisms, where there is a pull between increasing the number of gametes and increasing the size of the gametes in order to produce more and larger zygotes, two mating types result, one small but abundant, one large but limited. In this case, any intermediate type is likely to be disfavored. Since gametes in *D. discoideum* are identical in size, there would be no intermediate type and new types could have the selective advantage described by Iwasa and Sasaki (1987). This is consistent with the fact that we see more than two mating types in *D. discoideum.*

We also found evidence for balancing selection acting on the frequencies of the mating types when we compared population genetic differentiation at the mating type locus to that at presumably neutral microsatellite loci. Mating types and other self-incompatibility or self-recognition genes tend to evolve under balancing selection (reviewed in Fijarczyk & Babik, 2015). In *D. discoideum*, we observed no evidence of population structure at the mating type locus (F_{ST} = 0.01) but evidence of moderate genetic differentiation at the neutral microsatellite loci (F_{ST} = 0.10), with the estimate at the mating type locus falling well below the 95% confidence interval for the microsatellite loci. Though this in itself is strong evidence for balancing selection at the mating type locus, we expected the F_{ST} values for the microsatellite loci could be underestimated due to the tendency of microsatellites to have high mutation rates and diversity (Balloux et al., 2000). Because of this, we used an alternative method to further estimate genetic differentiation at these markers that addresses this problem. We calculated estimates for Hedrick's G'_{ST}, a measure specifically designed to correct the underestimation of

microsatellite data, for both the microsatellite loci and the mating type locus. The new estimate still showed about a ten-fold increase in population differentiation at the microsatellite loci compared to the mating type locus (Microsatellite: $G'_{ST} = 0.55$; Mating: $G'_{ST} = 0.05$), further strong evidence that mating types are maintained by balancing selection.

But, according to theory, isogamy and balancing selection allow for the evolution of an infinite number of mating types, not just for the transition from 2 to 3 that we see in *D. discoideum*. Though balancing selection may maintain the overall diversity of mating types across populations, we also see evidence of drift acting on individual populations, suggesting that the advantage of rare mating types may be weak. Microbial eukaryotes with multiple mating types are expected to reach a stable equilibrium where all mating types are equal in a population. The few known examples come from ciliates, where equal frequencies of multiple mating types have been observed empirically and predicted theoretically (Orias & Rolf, 1964; Doerder et al., 1995). These equal frequencies are also common for self-incompatibility alleles in plants (reviewed in Castric & Vekemans, 2004). However, in *D. discoideum*, the overall frequencies of the three mating types were not equal, with fewer observed Type II individuals. Between locations, the frequencies of the three mating types also differed, with only one of the three populations, Virginia, showing equal frequencies of the three sexes. Differences in mating type frequencies between populations most likely reflect drift in the face of weak selection. Though less common, this pattern of drift is not unusual to mating type systems, having also been observed at self-incompatibility loci in plants (Campbell & Lawrence, 1981; Kato & Mukai, 2004). Thus the data are consistent with balancing selection but with a common sex disadvantage that is so weak that it is unable to maintain allele frequencies that are even or

uniform across populations. Such a weak rare sex advantage might also explain why the number of sexes has remained low.

Conclusions and Implications for Future Research

Since relatively little is known about macrocysts in *D. discoideum* compared to the more commonly studied fruiting body, the intent of this study was to further characterize aspects of the sexual cycle that could shed light on how low numbers of mating types are maintained. In doing so, we found evidence of isogamy and balancing selection, both conducive for the evolution of multiple mating types. However, we also found evidence for drift acting on the mating types that could explain why we only see three mating types. Returning to the original models proposed by Iwasa and Sasaki (1987), in which a common sex disadvantage promotes the evolution of many mating types but drift can reduce that number to just two, we suspect that the missing piece to this puzzle may be a more thorough understanding of the cost of mating (or not) in *D. discoideum.* These models predict a very large number of mating types to evolve if common mating types suffer a fitness cost for not having as many potential mating partners, but only two if they do not. We know that mating in *D. discoideum* is a potentially costly event in itself. Though not addressed here, macrocyst formation is a uniquely social process that differs from the sexual cycles in other organisms. Upon formation, hundreds of amoebae are attracted to and then cannibalized by the diploid zygote, a potentially altruistic act. Understanding the social contract involved in sex and macrocyst formation in *D. discoideum* and the costs of not participating could further our understanding of how the mating system is maintained.

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Table 3.1. F_{ST} and G'_{ST} values show differentiation in mating type frequencies and microsatellite allele frequencies between populations of *Dictyostelium discoideum*. We included the 95% confidence intervals for each of the overall microsatellite loci differentiation estimates.

Locus	**F_{ST}**	**G'_{ST}**	**# of alleles**
Microsatellite Loci			
Dict5	0.097	0.592	15
Dict13	0.128	0.770	17
Dict19	0.104	0.315	7
Dict23	0.086	0.672	22
Dict25	0.097	0.668	21
Average	**0.103**	**0.548**	**16.4**
95% CI	0.091-0.116	0.475-0.609	
Mating Type Locus			
Mat	**0.009**	**0.051**	**3**

Figure 3.1. Gametes are larger in Virginia, but are the same across mating types. Plots show cell diameter for A) gametes of each mating type, B) vegetative cells of each mating type, C) vegetative cells compared to gametes, and D) game cells divided by geographic population. Asterisk represents statistical significance. N represents number of clones from which 160 cell diameters were measured.

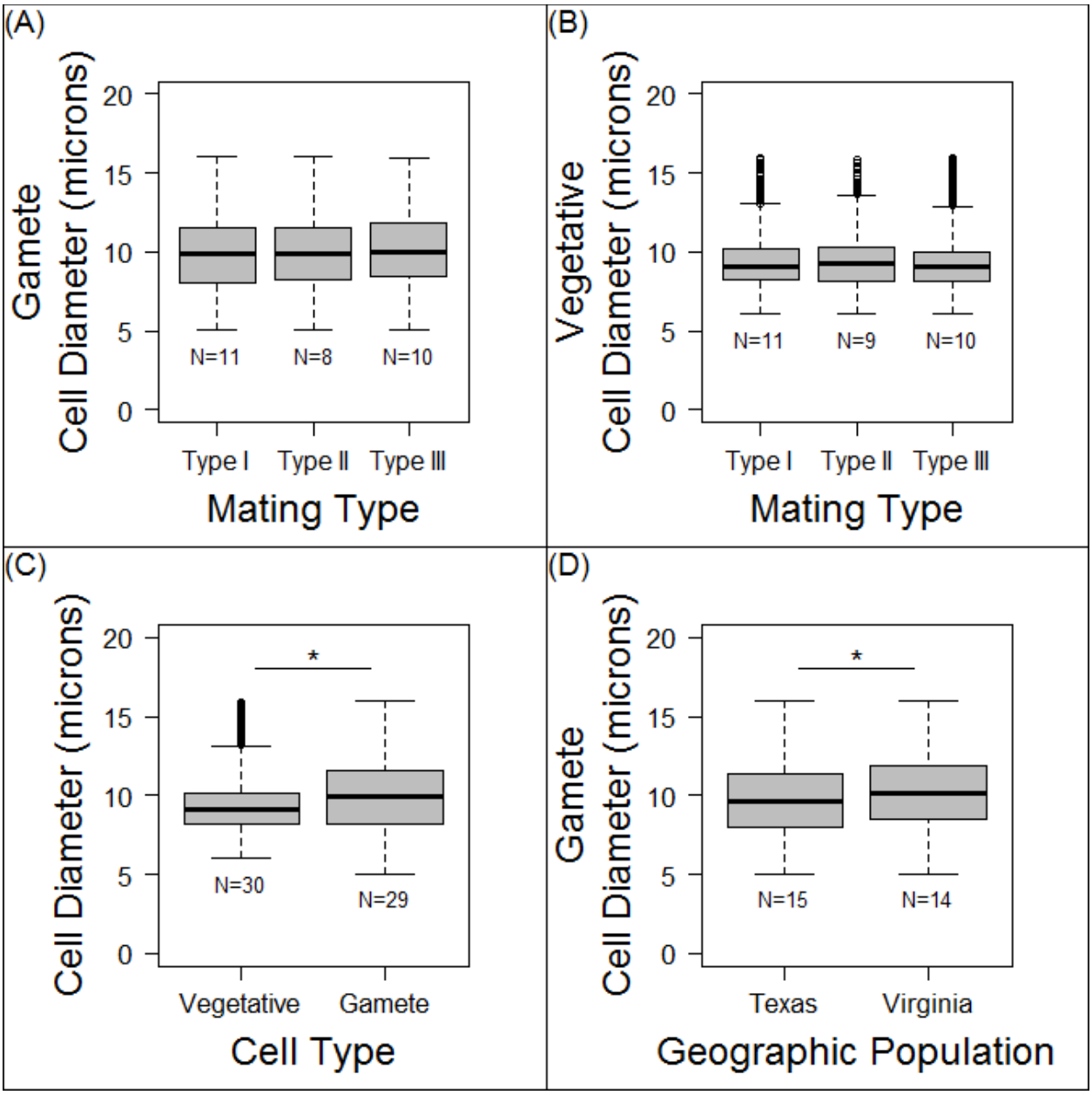
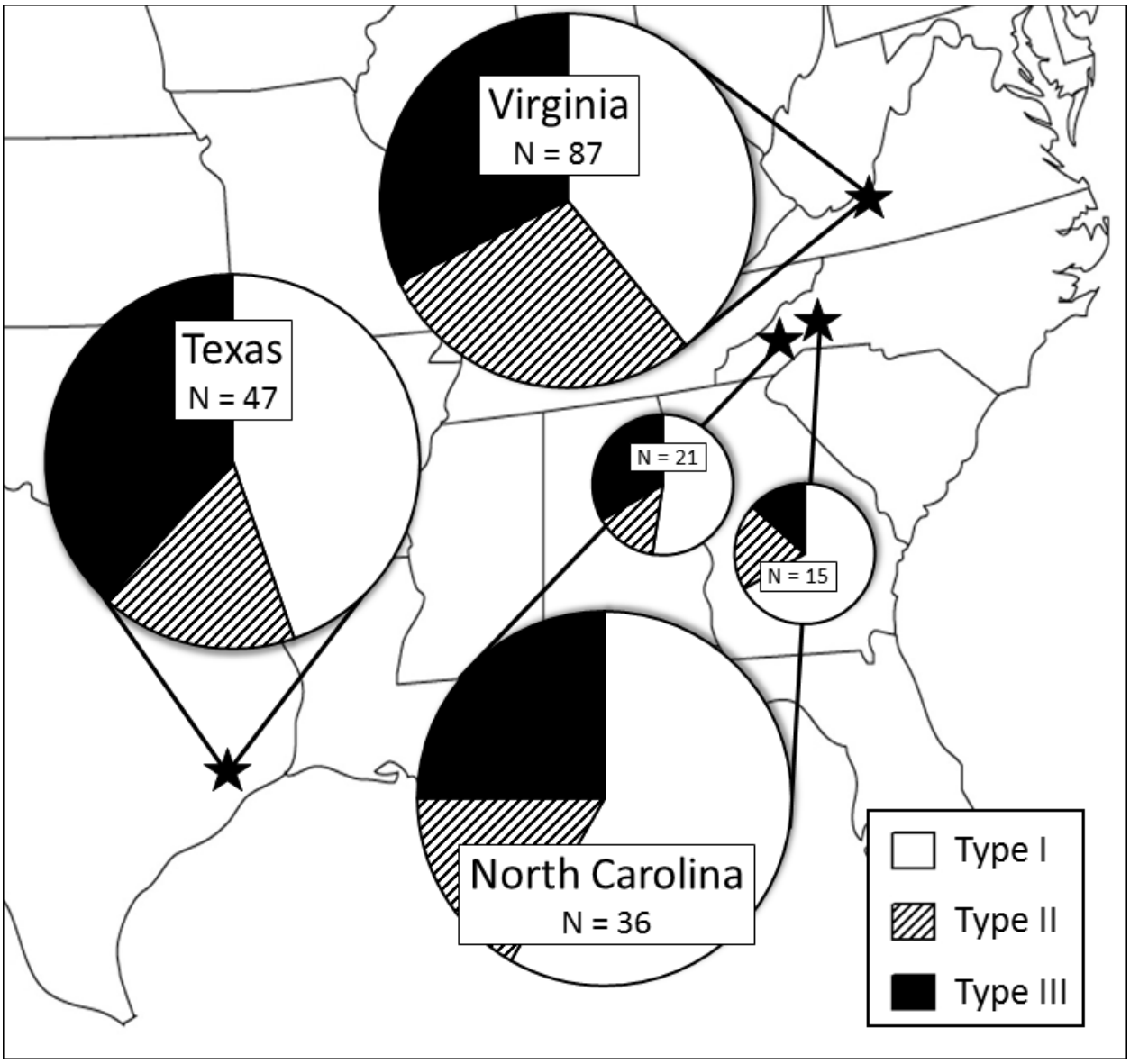


Figure 3.2. Mating type proportions vary by population. The pie charts show the distributions of mating types within each of the four geographic populations, with the large pie for North Carolina representing the combined totals from the two populations represented individually by the smaller pies. Stars indicate approximate locations of sampling sites.



CHAPTER 4

Social amoebae mating types do not invest unequally in sexual offspring

by

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Abstract

Unequal investment by different sexes in their progeny is common, including differential investment in the zygote or differential care of the young. The social amoeba *Dictyostelium discoideum* has a sexual stage in which isogamous cells of two of the three mating types fuse to form a zygote which then attracts hundreds of other cells to the macrocyst. The latter cells are cannibalized and so make no genetic contribution to reproduction. Previous literature suggests that this sacrifice may be induced in cells of one mating type by cells of another, resulting in a higher than expected production of macrocysts when the inducing type is rare and a reproductive advantage for this social cheat. We tested this hypothesis in 8 field-collected trios of clones of each of the three *D. discoideum* mating types by measuring macrocyst production at different pairwise frequencies. We found evidence that supported differential contribution in only two clone pairs, so this pattern is rare and clone-specific. In general, we found that each of the mating types contributes cells relative to their proportion in the population. We also found a significant quadratic relationship between partner frequency and macrocyst production, suggesting that when one clone is rare, macrocyst production is limited by partner availability. We were also unable to replicate previous findings that macrocyst production could be induced in the absence of a compatible mating partner. Overall, mating type-specific differential investment during sex is unlikely in microbial eukaryotes like *D. discoideum*.

Introduction

Understanding differences in investment during reproduction has been crucial to understanding the evolution of sex and sexual roles in eukaryotes (Trivers 1972). At the gametic level, the most commonly recognized example of dramatic differences in investment is the sperm and the egg, evolved primarily due to tradeoffs between gamete number and gamete size (Parker et al. 1972; Birkhead et al. 2008; Claw and Swanson 2012). Nutrient provisioning to the zygote also commonly differs between parents. In many species, nutrients are provided to the embryo maternally, either directly, for example through a placenta, or indirectly through the production of a nutrient-rich yolk (Callard and Ho 1987; Guraya 1989; Valle 1993). Differences in post-zygotic investment, or parental care, have also evolved in a variety of ways in eukaryotes due to disruptive selection on the sexes (Clutton-Brock 1991).

However, in microbial eukaryotes, differences in parental investment are likely to be rare. Sexual roles in microbes tend to show no signs of disruptive selection. Species frequently express more than two mating types and gametes are generally identical in form and mass (Parker et al. 1972). Still, evidence for dissimilarities between microbial mating types suggests that investment can vary even in these species. Differential investment, in the form of differential inheritance, is found in the plasmodial slime mold *Physarum polycephalum*. This species exhibits hierarchical mitochondrial inheritance based on the mating type alleles of the parents (Moriyama and Kawano 2003). In the Volvocines, increased gamete differentiation evolved with increasing vegetative complexity (Knowlton 1974; Bell 1978). Unicellular genera like *Chlamydomonas* are isogamous, reproducing through the fusion of gametes identical in size. Alternatively, colony-forming genera like *Volvox* produce two types of sexual gametes that differ in size and structure.

The cellular slime mold *Dictyostelium discoideum* offers an exciting system for investigating the potential for differential contribution during reproduction in a microbial system. This unicellular eukaryote shares many of the traits of species that show no evidence for disruptive selection. In *D. discoideum*, there are three self-incompatible mating types that are identical in size and distinguishable only by a unique set of genes at a single genetic locus (Bloomfield et al. 2010; Douglas et al. 2016). However, the product of a single mating, termed a macrocyst, is formed through a uniquely social process in which the nutrients required for the reproductive success of the zygote come from cannibalized cells that could be contributed by either parent. Initially, two cells of differing mating types fuse to form a diploid zygote, called a giant cell (Saga et al. 1983). This giant cell attracts surrounding amoebae by secreting large quantities of the chemoattractant, cyclic adenosine monophosphate (cAMP) (O'Day 1979; Abe et al. 1984). As many of these attracted peripheral cells begin to get consumed by the giant cell through phagocytosis, the rest seal their fate by producing a cellulose wall that permanently joins them with the giant cell in a structure called a precyst (Blaskovics and Raper 1957; Filosa and Dengler 1972; Erdos et al. 1973a). As two more cellulose walls get formed around what will become a mature macrocyst, the rest of the peripheral cells are also cannibalized through phagocytosis by the giant cell.

Since, under conditions conducive for sex, hundreds of *D. discoideum* amoebae get phagocytized for each new zygote, we can ask questions about conflict between partners at this stage. Analogous to yolk production, the peripheral cells contribute materially, but not genetically, to the success of haploid sexual offspring that hatch out from the macrocyst (Okada et al. 1986; Filosa and Dengler 1972; Nickerson and Raper 1973). However, unique to *D. discoideum* and other dictyostelids, this contribution is a form of cellular sacrifice or altruism,

which is familiar in another context in *Dictyostelium*. For decades, *D. discoideum* has been a model organism for social evolution because, in the asexual social cycle, starved amoebae aggregate, attracted again to cAMP, to form a fruiting body that is composed of a spherical ball of spore cells held up by a stalk of dead cells (Kessin 2001; Strassmann and Queller 2011). Because there is such a large cost to participating in both macrocyst and fruiting body formation, clones can be exploited, or cheated, by contributing disproportionately to the respective sacrificed cells in either process.

While a number of examples of cheating to fruiting body formation have been observed in *D. discoideum* (described in Strassmann and Queller 2011), differential contribution to macrocyst production has been observed between only one pair of clones, NC4 and V12 (O'Day and Lewis 1975; MacHac and Bonner 1975; Lewis and O'Day 1977; Bozzone and Bonner 1982). In these studies, V12, a Type II clone, invested disproportionately more to macrocyst formation by contributing most or all of the phagocytized peripheral cells. This behavior was thought to be induced in V12 by a diffusible pheromone that was produced by cells of the Type I clone NC4 and could affect V12 even in the absence of NC4 cells. This phenomenon was not limited to *D. discoideum*, with other species also showing signs of inducible macrocyst production (Lewis and O'Day 1976; Lewis and O'Day 1979). However, subsequent studies have called into question the claim by these early studies that the diffusible pheromone could induce macrocyst formation in the physical absence of a sexually compatible mate as they were unable to replicate the original findings (Erdos et al. 1973b; Wallace 1977; Bozzone and Bonner 1982). These original studies were also limited to single representatives of mating types, so the generality of their findings to other *D. discoideum* clones is unknown. There could be dominance effects between clones that average out between mating types as a whole. Regardless

of the potential flaws of the early studies, the suggestion that mating types play separate roles in macrocyst production still remains a part of the current understanding of how *D. discoideum* and other *Dictyostelium* cells of different mating types interact (reviewed in O'Day and Keszei 2012 and Bloomfield 2013).

This study investigates this potential for unequal investment in macrocyst production by each of the three mating types in *D. discoideum*. We also test whether induction of one mating type by another might be an underlying mechanism. We propose that the process most likely to be influenced or cheated during macrocyst production is how many phagocytized peripheral cells a given clone contributes. Since it is difficult to measure who contributes because the cells get cannibalized, we will instead compare macrocyst production at varying partner frequencies. We tested for expected consequences in terms of macrocyst numbers based on three hypotheses for how peripheral cells are contributed (illustrated in Fig. 4.1): (1) that peripheral cells are contributed in proportion to the frequency of each partner, (2) that they are contributed equally and (3) that one partner potentially cheats another by contributing disproportionately fewer than its fair share. Also, because *D. discoideum* has more than two mating types and no Type III clones have ever been evaluated for levels of investment during macrocyst production, we assessed whether a mating hierarchy exists such that contribution to reproduction differs depending on which mating type is dominant in a pairing.

Materials and Methods

Clones

We tested pairwise macrocyst production among trios of *D. discoideum* clones each from the same population. We tested 24 clones in total (8 clones each of the three mating types), from

three geographic populations: 3 trios from Houston, TX (29° 46’ N, 95° 27’ W), 3 trios from Little Butts Gap trail in North Carolina (35°46’ N, 82°20’ W), and 2 trios from near Mt. Lake Biological Station, VA (37°21’ N, 80° 31’ W) (Table A3.1). The mating types of each of the clones used in this study were either previously identified or identified using the techniques from Douglas et al. (2016). We only selected clones that were compatible (i.e. produced macrocysts) with each of the other two clones in a given trio. During the selection process, we encountered pairs of clones that together produced no macrocysts even though they exhibited different mating types at the mating type locus (Table A3.2-A3.4).

We also tested our ability to measure differential macrocyst production by comparing macrocyst production between clones NC4 and V12, the focal pair in the literature on macrocyst induction in *D. discoideum* (O’Day and Lewis 1975; MacHac and Bonner 1975; Keith E. Lewis and O’Day 1977; Bozzone and Bonner 1982). We obtained these clones from the Dicty Stock Center (http://dictybase.org/StockCenter/StockCenter.html; Fey et al. 2013). Because a number of strains labeled as either NC4 or V12 have been deposited over the years, we selected five unique pairs to test for differential macrocyst production after initially checking for compatibility (Table A3.1, A3.5). We also chose to test our methods on *D. discoideum* clones WS205 and IR1 because we previously observed macrocyst production when WS205 was rare and IR1 was common, but not the reverse, suggesting WS205 may induce macrocyst production in IR1 (unpublished data). WS205 is a Type I wild clone and IR1 is a Type II axenic mutant (free of bacteria) of parental strain WS582 that still contains all Type II mating type genes. These clones were also obtained from the Dicty Stock Center. Clones were grown from frozen stock on nutrient agar plates using *Klebsiella pneumoniae*, also from the stock center, as the bacterial food source.

Assay to measure differential macrocyst production

The relative contributions of two mating types to the macrocyst are difficult to assess directly. However, measuring macrocyst production at varying partner frequencies has been shown to be an excellent indicator of differential contribution (Bozzone and Bonner 1982). To test that our methods could identify differential macrocyst production, an indication of differential contribution to peripheral cells similar to the type described in previous literature, we compared macrocyst production between *D. discoideum* clones NC4 & V12 and also between WS205 & IR1, at seven starting population frequencies (100:0, 99:1, 90:10, 50:50, 10:90 and 1:99, 0:100). We performed two replicates. To investigate differential macrocyst production in wild *D. discoideum* clones, we compared pairwise macrocyst production among eight trios of *D. discoideum* clones, each containing one representative of each mating type. The same seven starting population frequencies were tested as in the paired experiment, but each clone was tested separately against the two other clones in the trio. We performed one replicate for each trio of clones.

We performed all of our experiments in 24-well plates with 1 mL of equal parts Lactose-Peptone agar (LP: 0.1% lactose, 0.1% peptone, 1.5% agar) and Bonner's salt solution (SS: 0.06% NaCl, 0.03% $CaCl_2$, 0.075% KCl). To each well, we added a total of 5 x 10^3 *D. discoideum* with 10 µL of OD 2.0 A_{600} *K. pneumoniae* as food. We sealed each plate with black electrical tape to maintain humidity inside and then stored them in a dark incubator at 22°C for one week to ensure the completion of all macrocyst production. We then counted the number of macrocysts in each well using an inverted microscope.

Diffusion chambers

To test for induced macrocyst production without physical contact between the cells or the ensuing sexual reproduction, we set up diffusion chambers modeled after the experiment described by Lewis and O'Day (1977). The purpose of these chambers is to grow clones separately, but still allow for the exchange of volatile compounds (illustrated in Fig. 4.2). We conducted these experiments on the pairs of clones used to test our methods for identifying differential macrocyst production (NC4 & V12 and WS205 & IR1). We also tested one trio from the larger experiment (V315B1, V331B1 and V341C2). We placed three small 30 x 10 mm Petri plates in one 100 x 15 mm Petri plate. We filled the small plates with 6 mL of equal parts LP agar and SS buffer and added 2.5 x 10^4 *Dictyostelium* spores with *K. pneumoniae* as food. For each pair of clones tested, A and B, we added spores to the three small plates in the following five combinations: (1) two clone A and one B, (2) two clone B and one A, (3) three clone A, (4) three clone B, and (5) one clone A, one clone B and one with both clones to verify that macrocysts can be made in our conditions. We sealed the lid of the large plate with black electrical tape and stored them in a dark incubator at 22°C for at least one week. We then checked for the presence of macrocysts using an inverted microscope.

Statistical analyses

Statistical analyses were performed using R software (version 3.2.2.) (R Core Team, 2015). We applied separate linear mixed-effects models to the data from crosses between the five strains each of NC4 and V12 and between WS205 and IR1 using R package "nlme" (Pinheiro, et al. 2016). We looked at how the initial percent of the predicted inducer affected macrocyst production. We treated percent inducer as the fixed effect and strain pair (for NC4 x

V12) or block (for WS205 x IR1) as the random effect. We compared models that included only the linear term for percent inducer to models that also included the quadratic term and chose the former based on AIC and BIC scores. We used Type III tests to estimate the significance of the fixed effect. Due to non-normality, we square root transformed the data, which then passed the Shapiro-Wilk test of normality.

We applied similar methods to analyze macrocyst production between the trios of wild clones. We again applied linear mixed-effects modeling to analyze how macrocyst production is affected by the frequency of a given partner (Type I in Type I x Type II, Type I in Type I x Type III, and Type II in Type II x Type III). We treated frequency as a fixed effect and the identity of the clones in a given pairing as a random effect. We also included the quadratic term for frequency. Based on AIC and BIC scores, this model fit the data better than a model that also included geographic population or the model that only assessed a linear effect of frequency. We cube root transformed the data to normalize it. Linear or quadratic best-fit regression curves were calculated in R based on the model that best fit the data. Linear and quadratic functions for macrocyst production between individual clone pairs were compared using an analysis of variance test. Bonferroni correction was used to adjust for multiple comparisons. We report the corrected p-values.

Results

Measuring macrocyst production at differing ratios of mating types can be used as an indirect way to identify contributions to the cannibalized peripheral cells. In these experiments, we varied pairwise population composition to examine predictions of different hypotheses on numbers of macrocysts produced. Fig. 4.3 shows how we would expect macrocyst production to

vary by population composition based on three hypotheses for how each mating type contributes to the cannibalized peripheral cells and will be used for comparison with the actual results. In Fig. 4.3A we show the prediction for proportional fairness, in which each mating type contributes a number of cells to be consumed by the zygote that is directly proportional to the number of cells of that mating type in the population. In this scenario, our null hypothesis, there is potentially no limitation on macrocysts since cells are sacrificed at rates relative to their own frequency and thus, maximum macrocyst production is possible across all ratios. In Fig. 4.3B, we show the prediction for absolute fairness, in which each mating type contributes an equal number of peripheral cells. Since the rarer mating type will be depleted first, in this first alternative hypothesis, macrocyst production is then proportional to the number of cells of the rarer type, with very few macrocysts being produced when one type is rare (10%) and even fewer when one type is very rare (1%). Unfairness, or cheating, our second alternative hypothesis, is shown in Fig. 4.3C, in which the greatest number of macrocysts are produced when mating partner X is very rare but the fewest number of macrocysts are produced when its partner is very rare. This figure most closely resembles the proposed differential contribution to peripheral cells from the literature. Partner X would gain a reproductive advantage by contributing disproportionately less to the cannibalized peripheral cells.

Physical contact is required for macrocyst production

When plated alone, NC4, V12, WS205 and IR1 each were unable to produce macrocysts, consistent with their classification as self-incompatible strains. From the diffusion chambers, we found no evidence of induced macrocyst production without the possibility of sexual cell fusion. We set up four diffusion chambers each with the following combinations: two NC4 and one V12,

two V12 and one NC4, and one NC4, one V12 and one with both NC4 and V12. We set up two diffusion chambers each with the following combinations: three NC4 and three V12. While macrocysts were produced in all four of the small plates inoculated with both NC4 and V12 clones, no other cultures produced macrocysts. We did the same experiment with WS205 and IR1 and again found that macrocysts were produced in the small plates inoculated with both WS205 and IR1, but not in any other plates.

In clones we collected from wild populations, partners contribute to reproduction relative to their own frequency, regardless of mating type

All 24 wild clones showed no evidence of macrocyst production when plated alone, but produced macrocysts at all other pairwise population frequencies (Fig. 4.4). We found a significant quadratic relationship between the initial frequency of a given partner and macrocyst production in each of the three mating type pairings (Type I x Type II: $F_{2,30}$=9.84, p<0.0001; Type I x Type III: $F_{2,30}$=14.28, p<0.0001; Type II x Type III: $F_{2,30}$=8.80, p=0.001). Though evidence of a significant quadratic effect fits our first alternative hypothesis in which peripheral cells are contributed exactly evenly, the shallowness of the curve fits our null hypothesis in which peripheral cells are contributed relative to their frequency in the population.

Disproportionate contribution to macrocyst production is rare, but clone-specific

When paired with their respective partners, macrocysts were produced at all population frequencies of NC4 and V12 and WS205 and IR1, respectively. Both between NC4 and V12 and between WS205 and IR1, we found a significant linear relationship between macrocyst production and the initial frequency of NC4 or WS205, respectively (NC4xV12: $F_{1,19}$=29.40,

$p<0.0001$; WS205xIR1: $F_{1,7}=414.98$, $p<0.0001$, Fig. 4.5). However, the best-fit regression curve indicated that the direction of the effect differed between the two pairings, with increased frequency of the Type I clone correlating with increased macrocyst production in one pair but a decreased macrocyst production in the other. We found that an increased frequency of NC4 had a significant positive linear effect on macrocyst production, while increasing the frequency of WS205 had a significant negative linear effect on macrocyst production. These results most closely resemble our hypothesis that one mating type cheats another during macrocyst production (Fig. 4.3C) but they go in opposite directions with respect to mating type.

Because we found clone-specific linear relationships in crosses between NC4 and V12 and WS205 and IR1, respectively, we also calculated best-fit linear regressions for each of the wild clone pairings (Fig. A3.1). We found significant linear relationships between only two Type I x Type III North Carolina pairs (Type I NC60.2 x Type III NC75.2: $p=0.05$; Type I NC105.1 x Type III NC61.1: $p=0.007$). The rest showed no significant linear or quadratic relationships, similar to what we would have expected if contribution to macrocyst production followed our null hypothesis (Fig. 4.3A).

Discussion

Dictyostelium discoideum offers an unusual and interesting model for investigating differential investment during reproduction. Like many other systems, nutrients to the reproductive zygote are provided by the parents, although the mechanism in *Dictyostelium* is unique. Differential contribution to these nutrients is common in nature, with primarily maternal investment dominating. Until now, however, it was unclear in *D. discoideum* if nutritional contribution to the zygote was uniparental or biparental. In this study, we show not only that

sexual investment in *D. discoideum* is biparental, but also that it is dependent on the frequency of a given partner in the population rather than its mating type.

Evidence suggesting that one partner disproportionately contributed to macrocyst production by providing more of the cannibalized peripheral cells was introduced by O'Day and Lewis (1975) and independently verified with the same clone pair in the same year by MacHac and Bonner (1975). Since then, the possibility of differential macrocyst induction by *D. discoideum* mating types has persisted in the literature. Nonetheless, because these prior studies primarily focused on a single pair of clones, representing only two of the three *D. discoideum* mating types, we expanded our investigation to include not only all three mating types, but also multiple representatives of each of these three mating types. We tested eight independent sets of wild *D. discoideum* clones, each containing representatives of all three mating types, and found no evidence for an ability by any of the mating types to induce macrocyst production in others (Fig. 4.4). Instead, we found an overall quadratic relationship between frequency of partner and macrocyst production where more macrocysts were produced when both partners were equal and fewer at the more uneven frequencies. A quadratic effect suggests that these findings are similar to what we predicted in Fig. 3B, in which we hypothesized that if each partner contributes the same number of sacrificed peripheral cells during the formation of macrocysts, macrocyst production will be limited by the number of cells of the rarer type. However, the shallowness of the curve suggests that we cannot rule out our null hypothesis (Fig. 4.3A), in which the number of cells each mating type contributes to macrocyst production is directly proportional to the number of cells of each mating type in the population. This is further supported by looking at the relationship between partner frequency and macrocyst production at the level of the individual clone pair. In 22 of the 24 pairings, we found nonsignificant relationships between

frequency of partner and macrocyst production, with the other two showing linear relationships. Since there were no individual pair quadratic effects, even though there are collective ones, the power must be fairly low for the individual effects, quadratic or linear, a pattern most similar to our prediction in Fig. 4.3B.

Though the individual experiments provide some support for our null hypothesis, we cannot dismiss the significant quadratic effect at the mating type level. We predict that this effect is likely due to population structure, such that when compatible mating types no longer come in contact, zygote production ceases. Though spores were mixed initially, once amoebae hatched from these spores and subsequently divided as they consumed the provided bacteria, patches of identical individuals are likely to occur. Evidence for this type of structured growth in *D. discoideum* has been shown in asexual development (Buttery et al. 2012; smith et al. 2016). In the beginning of our experiments, when the density of cells was at its highest, cells of differing mating types were more likely to come into contact. Once many of the cells committed to taking part in macrocyst production and overall cell densities became lower, partner accessibility may become a limiting factor. This may be why we found fewer macrocysts at the more unequal frequencies than at 50:50. Cells of the high frequency type would often be surrounded by their own clones and would be unable to produce further macrocysts, even though compatible mating partners were likely still present in the population.

In our experiments, macrocyst production never fully exhausted the available cell population regardless of partner ratios. In every pairing that produced macrocysts, we observed free living amoebae that seemingly avoided or were excluded from participating in the sexual process. In addition to possible effects of population structure, avoiding aggregation could be a strategy to avoid contributing to the peripheral cells if another option is possible. In the asexual

life cycle, non-aggregating cells that do not participate in fruiting body formation can colonize remaining nutrients in the environment (Dubravcic et al. 2014; Tarnita et al. 2015). This observation was important for our understanding of altruism in *D. discoideum*, as clones that were labeled "losers" for producing relatively fewer spores when mixed with other genotypes, could in reality be following an alternative strategy of producing more non-aggregating cells. In our experiments, non-aggregating cells had no advantage over aggregating cells as the subsequent lab environment was unsuitable for continued growth. In nature, however, nutrients can reestablish and failure to participate in macrocyst formation may not be an evolutionary dead end.

Evidence that cells are likely to be phagocytized relative to their frequency in the population, rather than their mating type identity, provides further insight into how the zygote giant cell feeds. As described earlier, mating in *D. discoideum* begins with the production of the giant cell, a fusion product of two cells that differ in mating type. This giant cell then produces large quantities of the chemoattractant, cAMP, attracting surrounding cells. In *D. discoideum*, giant cells have been shown to preferentially phagocytize cells of their own species over cells from other slime mold species (Lewis and O'Day 1986). However, it was unclear if they preferentially consume some *D. discoideum* cells more than others. In wild clones, this does not appear to be the case. Instead, our results suggest that the giant cell acts as more of an opportunistic feeder, consuming whatever conspecific amoebae are attracted to it. Since our pairwise mating design guaranteed that giant cells would be equally related to all of their potential "victims", we cannot draw conclusions on whether giant cells attract unrelated *D. discoideum* cells more or less than cells identical to the two that fused originally.

Though we present here robust evidence against differential investment between the mating types among wild *D. discoideum* clones, we also showed that disproportionate contribution to macrocyst production can happen between two clones. Significant linear relationships between four sets of clones, including the originally discussed NC4 and V12, suggest that though not universal, uneven investment may occur during the sexual cycle. Interestingly, the direction of unfairness that we found between Type I NC4 and Type II V12 is opposite of what was previously observed. Instead of finding evidence that NC4 cheats V12, we found that when V12 was rare, more macrocysts were produced than when NC4 was rare. This suggests that in our conditions, V12 gained the reproductive advantage. This pattern was consistent across all five strains of this clone pair. This surprising find could indicate a hint of plasticity in the inducing trait. That unknown, and therefore uncontrollable, environmental factors impact how clones interact during the sexual cycle.

Our data clearly show that varying the availability of compatible partners impacts macrocyst production, but our understanding of sexual compatibility in *D. discoideum* remains incomplete. Early studies proposed that disproportionate contribution to macrocyst production, comparable to what we observed in just a few clone pairs, was induced by a diffusible hormone that could even make otherwise self-incompatible clones undergo homothallic mating (Lewis and O'Day 1975; MacHac and Bonner 1975). Since we were unable to induce macrocyst production in this way, we conclude that both clones are required to produce macrocysts, likely due to an inability to self. This agrees with other studies that were also unable to recreate this induced selfing (Erdos et al. 1973b; Wallace 1977; Bozzone and Bonner 1982). Required heterothallic mating supports our hypothesis that the linear patterns reflect cheating. The cheater can gain a reproductive advantage if more macrocysts are produced when it is rare by

contributing the same number of cells as its partner to the reproductive zygote, but at a relatively lower cost by contributing disproportionately fewer cells to be cannibalized.

Overall, our findings contribute further evidence that mating type-specific differential investment during sex is unlikely in microbial eukaryotes. Our results complement previous findings that reproduction in *D. discoideum* is isogamous, involving gametes identical in size and form (Douglas et al., 2016). They also fit with the assumption that evolved differences between sexes are correlated with vegetative complexity (Knowlton 1974; Bell 1978). Though *D. discoideum* aggregates into a multicellular structure during its social and sexual cycles, most of its life is spent as a unicellular amoeba. In addition to being indistinguishable in appearance, the three *D. discoideum* sexes are also indistinguishable in their investment to nutrient provisioning during macrocyst production. This differs from what would be expected if the peripheral cell contribution was more analogous to yolk production or other primarily maternal investments. In general, the cost of mating (i.e. sacrificed peripheral cells) is distributed fairly between two mating partners in *D. discoideum*. However, we also provide evidence for cheating between individual pairs. This suggests that, though not dictated by mating type, social conflict similar to that described in asexual fruiting body formation is also a factor during macrocyst production.

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Figure 4.1. Alternative strategies for contributions to cannibalized peripheral cells in *Dictyostelium discoideum*. Shown are illustrations of populations of cells before macrocyst production followed by these same populations after macrocyst production. At the center of each macrocyst is a zygote formed from the fusion of one gray cell and one white cell. Here we only show scenarios where one partner is rare, represented by gray cells and the other is common, represented by white cells. In A), peripheral cells are contributed by each partner relative to its frequency in the population. In B), each partner contributes exactly the same number of peripheral cells as its mate in each macrocyst. In C), one partner induces the other to contribute disproportionately more peripheral cells, while it contributes few to no peripheral cells. In this case, the gray cells represent cells of a mating type that induces overcontribution of peripheral cells by its partner, while the white cells represent cells of a mating type that responds to this induction.

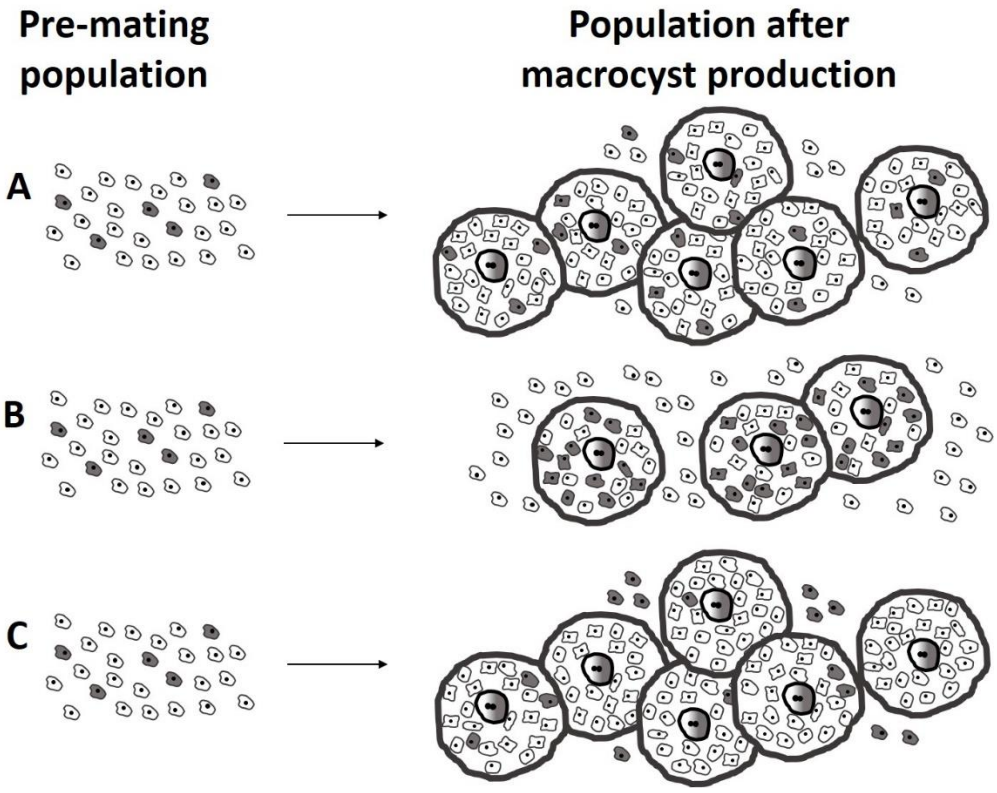


Figure 4.2. An example of a diffusion chamber between NC4 and V12 with the combinations of clones to be tested and the expected outcomes for each combination. Based on the literature, two chambers of NC4 should induce macrocyst production in V12.

Diffusion Chamber Combinations

Plate A	Plate B	Plate C	Expected Outcome
NC4	NC4	V12	Macrocysts in Plate C
V12	V12	NC4	No macrocysts
NC4	NC4	NC4	No macrocysts
V12	V12	V12	No macrocysts
NC4	V12	NC4 + V12	Macrocysts in Plate C

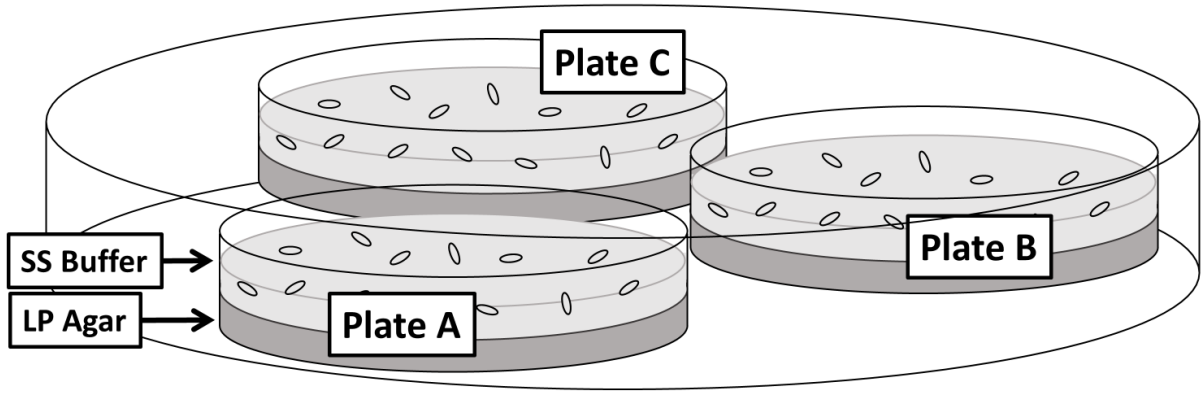


Figure 4.3. Predicted outcomes of different hypotheses. Macrocyst production may reflect A) proportional contribution to peripheral cells such that a given partner contributes a number of cells relative to their frequency in the population (proportional fairness), B) equal contribution to peripheral cells such that each partner contributes the same number of cells (absolute fairness), or C) differential contribution to peripheral cells such that one partner contributes disproportionately fewer cells (cheating).

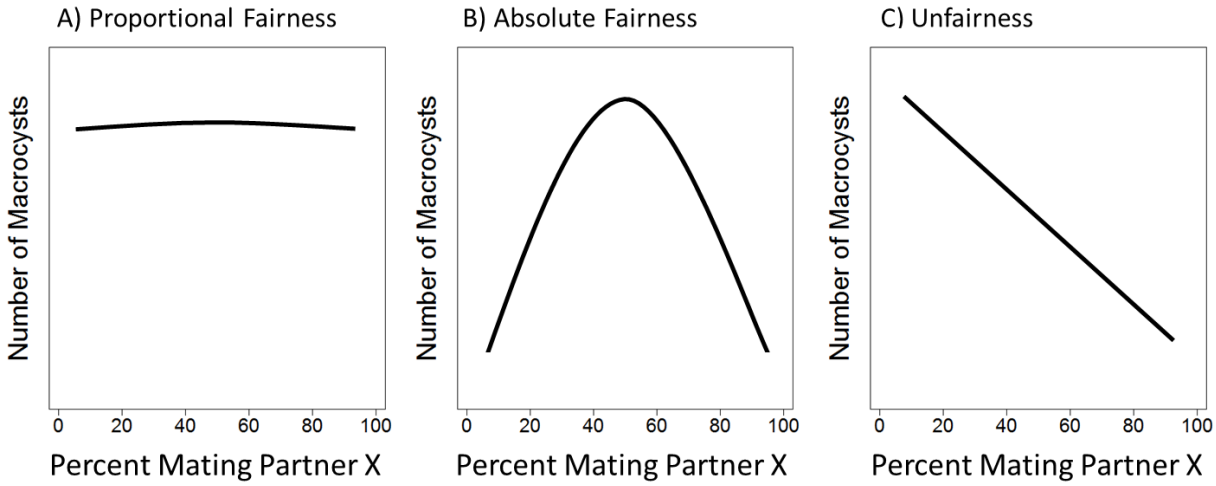


Figure 4.4. Fewer macrocysts are formed when either mating type in a pairing is very rare. Symbols represent macrocyst production between individual clone pairs. Lines represent best-fit regression curve for each mating type overall.

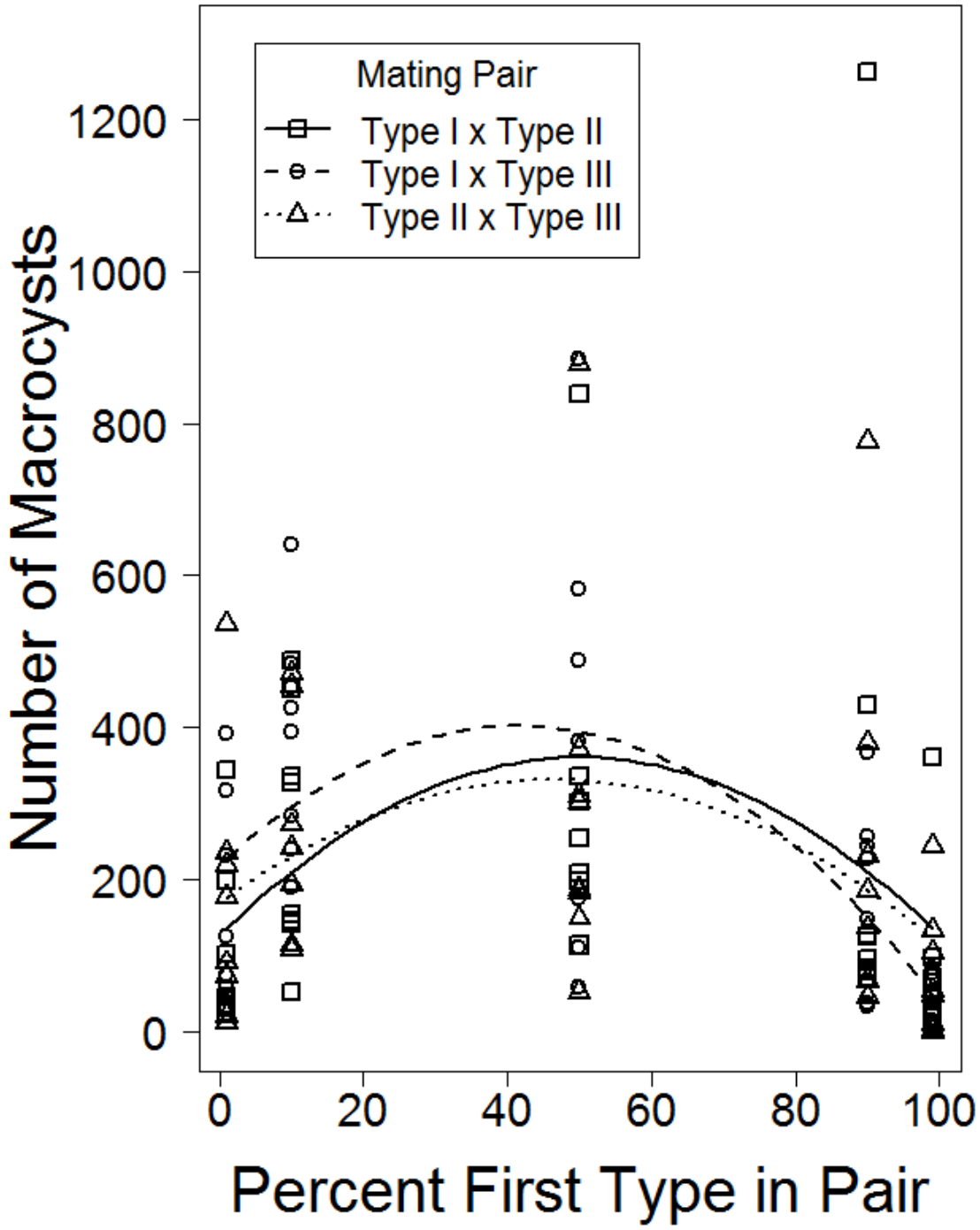
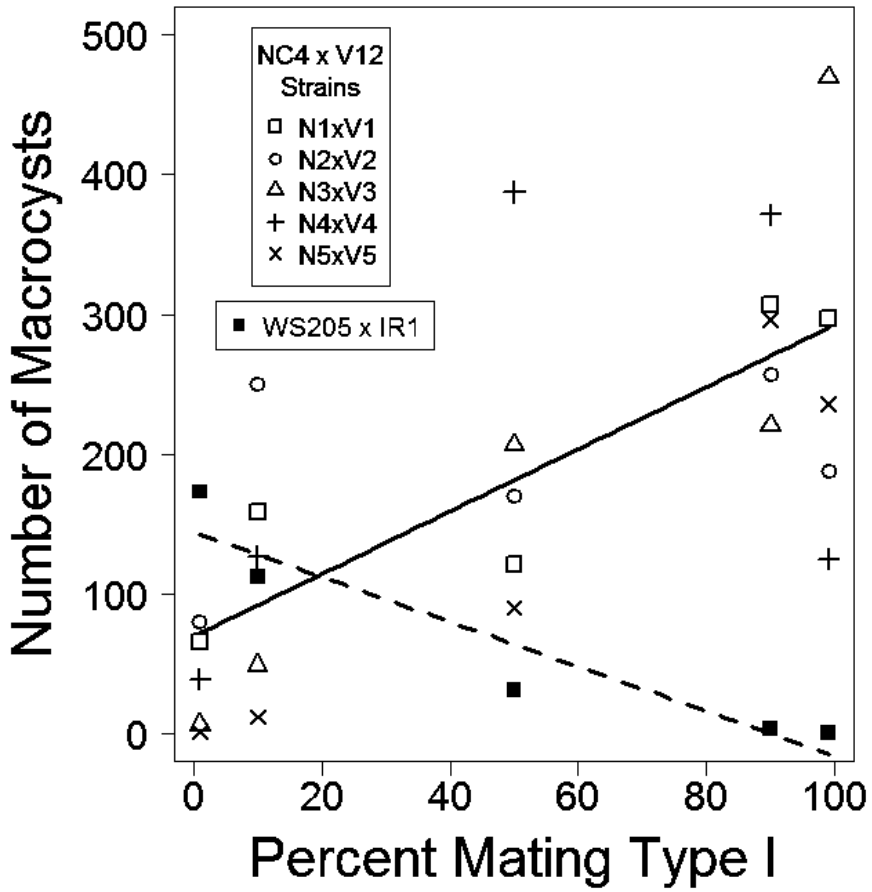


Figure 4.5. Type I WS205 induces macrocyst production in Type II IR1, and Type II V12 induces macrocyst production in Type I NC4. Figure shows the number of macrocysts produced at five starting frequencies of either WS205 or NC4 (both mating type I) (1%, 10%, 50%, 90% and 99%) with the reciprocal frequency of IR1 or V12, respectively. Symbols represent macrocyst production between the five strains of clone pair NC4 and V12 and the one strain of clone pair WS205 and IR1. Best-fit regression line is solid for overall NC4 x V12 and dashed for WS205 x IR1.



CHAPTER 5

CONCLUSIONS

The goal of this dissertation was to examine the social and selective pressures influencing the evolution and maintenance of sex and mating types in the social amoeba *Dictyostelium discoideum.* I first searched for hidden genetic population structure that could muddle our ability to interpret variation in social and sexual interactions. I then characterized key aspects of the *D. discoideum* mating types to investigate selective pressures on gamete size and mating type number. Finally, I looked for evidence of differential sexual investment in *D. discoideum* to explore the roles of each mating type during reproduction.

I used phylogeny building and other population genetics techniques to analyze variation in ribosomal and mitochondrial DNA sequences from a large collection of *D. discoideum* clones isolated from the wild. I found that the majority of *D. discoideum* clones form a single, monophyletic group, reinforcing the usefulness of morphological characteristics as species identification markers. However, I also found pronounced genetic differentiation between geographic populations and eight isolates that may represent one or more cryptic tropical species. This evidence for population structure among *D. discoideum* isolates contributes to the long-standing debate of whether and to what extent population structure exists in microbial populations (Finlay 2002; Fenchel and Finlay 2004; Foissner 2006, 2008; Smith and Wilkinson 2007). It also strongly highlights the need to identify genetic variation between clones before investigating how they interact.

High relatedness plays a key role in promoting and maintaining cooperation in nature, especially when the cost to cooperation is high, such as during fruiting body formation in *D. discoideum* (Fletcher & Michener 1987; Gilbert et al. 2007; Hamilton 1964). It has been shown in *D. discoideum* that unrelated clones will mix to form chimeric multicellular structures, and in many cases, one clone benefits disproportionately more from these interactions (Strassmann et

al. 2000; Foster et al. 2002; Buttery et al. 2009; Jack et al. 2008). However, genetic relatedness is a factor in how these unrelated clones will interact, with more closely related individuals more likely to interact (Ostrowski et al. 2008; Mehdiabadi et al. 2009). Taking into account genetic relatedness and the now no-longer hidden population structure in *D. discoideum* is important to interpreting how and why social and sexual interactions evolve and are maintained.

I uncovered further evidence for population structure by geographic location when I investigated the evolution of mating types in *D. discoideum* and discovered unique mating type distributions at each of three wild populations. Investigating these mating type frequencies and also gamete size, I also identified two different selective pressures acting on the mating types that resolve the discrepancy between theory on how mating type numbers evolve and how many mating types we actually see in *D. discoideum*. Theory predicts that the number of mating types should tend towards infinity due to balancing selection, or remain at two due to alternative selective pressures, such as disruptive selection or drift (Iwasa and Sasaki 1987; Hurst 1996; Billiard et al. 2011). The mating system in *D. discoideum* deviates from this theory with only three mating types, clearly more than two but fewer than many. Prior to this study, it was known that these three mating types differed at the genetic level (Bloomfield et al. 2010), but physical differences between the gametes, an indication of differing selective pressures on mating types, were unknown. I found that gametes did not differ in size between the three mating types, confirming my prediction that balancing selection acting on gametes may have allowed for the evolution of multiple mating types.

Investigating mating type distributions in wild populations also supported my prediction that balancing selection is acting on the mating types in *D. discoideum*. I compared these distributions to the distributions of more neutral microsatellite markers to look at the levels and

types of selection acting on the mating types. Overall, I found significant genetic differentiation at the microsatellite loci, reinforcing our current understanding of population structure in wild *D. discoideum*, but comparatively no evidence of differentiation at the mating type locus. This evidence that mating types are maintained at more even frequencies than neutral alleles, paired with the previous evidence of gamete similarity, shows that balancing selection has likely allowed for the evolution of multiple mating types. However, drift is also acting on these populations, based on the evidence discussed previously of mating type distribution variation across population, suggesting that the rare sex advantage experienced due to balancing selection is relatively weak.

Though gamete size and sex ratio are two commonly studied indicators of selective pressures acting on mating systems, differential investment is also a crucial factor in the evolution of sex and sexual roles. I investigated *D. discoideum* for evidence of differential sexual investment by assessing the relative cost of sex for each partner during macrocyst formation. Sex is costly in *D. discoideum*. Upon formation, the diploid zygote attracts and cannibalizes hundreds of surrounding amoebae. Previous literature suggests that this sacrifice is made disproportionately more by one partner than the other (O'Day and Lewis, 1975; MacHac and Bonner, 1975; Lewis and O'Day 1976; Lewis and O'Day 1979). In *D. discoideum*, this unequal contribution was made by cells of V12 (a mating type II clone) when paired with cells of NC4 (a mating type I clone). This information was used to conclude that Type I induces Type II.

Contrary to this conclusion however, I found that overall, there is no pattern of dominance at the mating type level. Instead, peripheral cells appear to be sacrificed relative to their frequency in the population, regardless of mating type. This fits with my previous findings that reproduction in *D. discoideum* is isogamous in reference to gamete size and form. However,

contrary to what I expected, I did find significantly fewer macrocysts produced when partner frequency was uneven compared to 50:50. We predict this is likely due to population structure that arises during vegetative growth such that patches of identical individuals occur (Buttery et al. 2012; smith et al. 2016). When one type is rare, partner availability is likely limiting as cells surrounded by their clones do not form zygotes. While at the mating type level, a clone's contribution to macrocyst production is relative to its abundance, I did find clone-specific patterns similar to what was found between NC4 and V12. This suggests that, though rare, disproportionate contribution to macrocyst production does occur in some contexts.

Overall, this dissertation further characterizes the sexual system of the social amoeba *D. discoideum* and provides important information on the phylogenetic and geographic relationships between clones. This information is important for understanding how and why interactions between individuals vary. *Dictyostelium discoideum* continues to be an important model organism for the study of social and sexual interactions. We provide evidence that cheating is not limited to the asexual social cycle and that the sexual cycle offers an alternative system for studying questions about altruism and cooperation.

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APPENDIX 1

Supplementary tables for Chapter 1

Table A1.1. *Dictyostelium discoideum* and *D. citrinum* strains used in this study. Concatenated sequence numbers based on combined ribosomal and mitochondrial DNA sequence. *Dictyostelium citrinum* strains are designated by the term 'citr'.

Concatenated Sequence	Haplotype		Clone Name	Location Isolated	GPS Coordinates	Collected By
	Ribosomal	Mitochondrial				
1	A	A	CRII6C	Costa Rica	10°03'52" N, 83°58'12" W[a]	John Landolt
2	B	A	OT3A	Mexico	19°12'51" N, 98°06'38" W	John Landolt
3	C	A	QS43	La Malintzi Park, Mexico	19°12.850' N, 98°6.467' W	John Landolt
4	D	B	AC4	Mexico	N/A	James Cavender
5	E	C	S6B	Costa Rica	10°55'40" N, 85°28' W	John Landolt
6	F	D	ZA3A	Costa Rica	N/A	Steve Alexander
7	G	E	QS42	Monteverde, Costa Rica	10°18' N, 84°26' W[a]	John Landolt
8	H	-	CF3B	Mexico	19°49'41" N, 97°12'05" W	John Landolt
9	I	-	QS94	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
10	J	F	QS114	Bloomington, IN	39°13.227' N, 86°21.534' W	J Strassmann, D Queller
10	J	F	QS117	Linden, TX	33˚03.710' N, 94˚16.414' W	J Strassmann, D Queller
10	J	F	QS36	Land Btw Lakes, KY	36°59.856' N, 88°13.132' W	J Strassmann, D Queller
11	K	F	QS1	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
11	K	F	QS95	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
12	L	G	42A	Virginia	37°22'32" N, 80°31'20" W	John Landolt
13	M	H	DCB5A	N. Carolina	35°28'32" N, 83°25'38" W	John Landolt
14	M	I	GC1A	Tennessee	35°36'31" N, 83°48' W	John Landolt
15	N	J	BM5A	N. Carolina	35°35'17" N, 83°03'54" W	John Landolt
16	O	K	C5A	Virginia	37°22'32" N, 80°31'20" W	John Landolt
17	P	L	IR1	Wisconsin	43°47' N, 88°47' W[a]	Rob Insall
18	P	M	QS96	Mt.Greylock, MA	42°38.200' N, 73°10.367' W	J Strassmann, D Queller
18	P	M	QS97	Mt.Greylock, MA	42°38.200' N, 73°10.367' W	J Strassmann, D Queller
18	P	M	QS98	Mt.Greylock, MA	42°38.200' N, 73°10.367' W	J Strassmann, D Queller
18	P	M	QS118	Linden, TX	33˚03.710' N, 94˚16.414' W	J Strassmann, D Queller
18	P	M	QS99	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
18	P	M	QS12	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
19	P	N	DCB10C1	N. Carolina	35°28'32" N, 83°25'38" W	John Landolt
20	P	I	QS119	Houston, TX	29° 46' N, 95° 27' W	J Strassmann, D Queller

21	P	O	QS120	Mt.Greylock, MA	42°38.200’ N, 73°10.367’ W	J Strassmann, D Queller
21	P	O	QS121	Mt.Greylock, MA	42°38.200’ N, 73°10.367’ W	J Strassmann, D Queller
22	Q	P	QS40	Mt.Greylock, MA	42°38.200’ N, 73°10.367’ W	J Strassmann, D Queller
22	Q	P	QS122	Mt.Greylock, MA	42°38.200’ N, 73°10.367’ W	J Strassmann, D Queller
22	Q	P	QS123	Mt.Greylock, MA	42°38.200’ N, 73°10.367’ W	J Strassmann, D Queller
22	Q	P	QS124	Mt.Greylock, MA	42°38.200’ N, 73°10.367’ W	J Strassmann, D Queller
23	R	Q	QS111	Bloomington, IN	39°13.227' N, 86°21.534' W	J Strassmann, D Queller
24	S	R	SA2	Tennessee	35°44’26” N, 83°13’11” W	John Landolt
25	T	S	QS47	St. Louis, MO	38°46’08” N, 90°11’7” W	J Strassmann, D Queller
25	T	S	QS113	Effingham, IL	39°5.467’ N, 88°34.833’ W	J Strassmann, D Queller
26	T	T	QS107	St. Louis, MO	38°46’08” N, 90°11’7” W	J Strassmann, D Queller
27	T	U	QS106	St. Louis, MO	38°46’08” N, 90°11’7” W	J Strassmann, D Queller
28	U	V	QS6	Mt. Lake, VA	37°21’ N, 80° 31’ W	J Strassmann, D Queller
29	U	W	QS125	Mt. Lake, VA	37°21’ N, 80° 31’ W	J Strassmann, D Queller
30	U	X	QS35	Mt. Lake, VA	37°21’ N, 80° 31’ W	J Strassmann, D Queller
31	U	Y	QS14	Mt. Lake, VA	37°21’ N, 80° 31’ W	J Strassmann, D Queller
32	V	Z	B10	Virginia	37°22’32” N, 80°31’20” W	John Landolt
33	V	AA	QS44	Mt. Fuji, Japan	35°25’ N, 138°41’ E	J Strassmann, D Queller
34	V	Y	QS127	Houston, TX	29° 46’ N, 95° 27’ W	J Strassmann, D Queller
34	V	Y	QS128	Houston, TX	29° 46’ N, 95° 27’ W	J Strassmann, D Queller
34	V	Y	NC28.1	Little Butts Gap, NC	35°46’ N, 82°20’ W	J Strassmann, D Queller
34	V	Y	NC39.1	Little Butts Gap, NC	35°46’ N, 82°20’ W	J Strassmann, D Queller
34	V	Y	QS116	Mt. Lake, VA	37°21’ N, 80° 31’ W	J Strassmann, D Queller
34	V	Y	QS34	Bloomington, IN	39°13.227' N, 86°21.534' W	J Strassmann, D Queller
34	V	Y	QS131	Mt. Lake, VA	37°21’ N, 80° 31’ W	J Strassmann, D Queller
34	V	Y	QS17	Mt. Lake, VA	37°21’ N, 80° 31’ W	J Strassmann, D Queller
35	V	BB	QS132	Mt. Lake, VA	37°21’ N, 80° 31’ W	J Strassmann, D Queller
35	V	BB	QS18	Mt. Lake, VA	37°21’ N, 80° 31’ W	J Strassmann, D Queller
36	V	CC	QS81	Houston, TX	29° 46’ N, 95° 27’ W	J Strassmann, D Queller
36	V	CC	QS73	Houston, TX	29° 46’ N, 95° 27’ W	J Strassmann, D Queller
36	V	CC	QS31	Houston, TX	29° 46’ N, 95° 27’ W	J Strassmann, D Queller
36	V	CC	QS115	Bloomington, IN	39˚13.227' N, 86˚20.534' W	J Strassmann, D Queller
36	V	CC	QS32	Pasadena, TX	29°35’ N, 95°4’ W	J Strassmann, D Queller

36	V	CC	QS33	Webster, TX	29°32' N, 95° 9' W	J Strassmann, D Queller
37	W	DD	QS9	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
38	X	EE	QS23	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
39	X	FF	QS11	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
40	X	Y	QS102	Monteverde, Costa Rica	10°18' N, 84°26' W[a]	J Strassmann, D Queller
40	X	Y	QS38	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
40	X	Y	QS2	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
40	X	Y	QS135	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
41	X	GG	QS15	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
42	X	CC	QS8	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
42	X	CC	QS136	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
43	X	-	QS108	Forest City, AK	34˚50.146' N, 91˚28.060' W	J Strassmann, D Queller
43	X	HH	QS21	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
43	X	HH	QS22	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
44	Y	II	WS205	Wisconsin	43°47' N, 88°47' W[a]	Richard Kessin
45	Z	JJ	NC85.2	Little Butts Gap, NC	35°46' N, 82°20' W	J Strassmann, D Queller
46	AA	KK	QS4	Mt. Lake, VA	37°21' N, 80° 31' W	J Strassmann, D Queller
47	AA	LL	QS137	Linville Falls, NC	35°57.197 N, 81°56.516 W	J Strassmann, D Queller
47	AA	LL	QS48	Linville Falls, NC	35°57.197 N, 81°56.516 W	J Strassmann, D Queller
47	AA	LL	NC63.2	Little Butts Gap, NC	35°46' N, 82°20' W	J Strassmann, D Queller
47	AA	LL	NC69.1	Little Butts Gap, NC	35°46' N, 82°20' W	J Strassmann, D Queller
47	AA	LL	NC98.1	Little Butts Gap, NC	35°46' N, 82°20' W	J Strassmann, D Queller
47	AA	LL	QS39	Indian Gap, TN	35°36.606' N, 83°26.821' W	J Strassmann, D Queller
48	AA	MM	NC47.2	Little Butts Gap, NC	35°46' N, 82°20' W	J Strassmann, D Queller
49	AA	NN	NC4	Little Butts Gap, NC	35°46.317' N, 82°20.533' W	Kenneth Raper
50	BB	-	Disc X00601	unknown	N/A	N/A
51	CC	OO	Disc NW 001852778	Little Butts Gap, NC	35°46.317' N, 82°20.533' W	Kenneth Raper
citr 1	-	-	WS584	Wisconsin	43°47' N, 88°47' W[a]	Kenneth Raper
citr 2	-	-	WS526	Wisconsin	43°47' N, 88°47' W[a]	Kenneth Raper
citr 3	-	-	V34	Virginia	N/A	J Strassmann, D Queller
citr 4	-	-	Citr DQ340385	unknown	N/A	N/A

[a]GPS coordinates for this isolate approximated using Google Earth.

Table A1.2. PCR primer pairs for amplification of 8 kb DNA sequence data.

Region	Direction	Primer Sequence (5' to 3' direction)
17S	Forward	GCTCGTAGTTGAAGTTTAAG
	Reverse	AGATAATACAAGCTGAACTA
	Forward	CTAAGATATAGTAAGGATTG
	Reverse	ATGATCCATCCGCAGGTTCA
ITS-5.8S	Forward	ACGGTAAAGTTAACGGATCG
	Reverse	ACTCTCACCCAAGTATAACA
	Forward	AAACTGCGATAATTCACTTG
	Reverse	CCGTCTTCACTCGCCGTTAC
26S	Forward	ATTACCCGCTGAACTTAAGC
	Reverse	TCCGAAGATAACCTGTAGAC
	Forward	TCATCAAGAGTGCAAAATGG
	Reverse	ACATCGCCAGTTCTGCTTAC
IGS-5S	Forward	ATTCACAAAGTGTTGGATTG
	Reverse	GCTTACTATGGACAAATGGC
LSU intron	Forward	ACGGATAAAAGGTACGCTAG
	Reverse	TAATTAATACACCAGTGATC
COX-ATPase intergene	Forward	TGGATTTATGCCAATTAAAG
	Reverse	TCCACTTACTTTTACTACTC

APPENDIX 2

Supplementary tables for Chapter 2

Table A2.1. *Dictyostelium discoideum* clones from the four populations used in this study (LF = Linville Falls [35°57.197' N, 81°56.516' W], LBG = Little Butts Gap [35°46' N, 82°20' W], H = Houston [29°46' N, 95°27' W], MLBS = Mountain Lake Biological Station [37°21' N, 80°31' W]) and their associated mating type genes and/or microsatellite allele sizes. X's denote confirmed presence of mating type genes. To confirm types, we required evidence of at least one mating type gene associated with that type (Type 1: matA; Type 2: matB, matC, matD; Type 3: matS, matT). Microsatellite allele sizes are from Smith (2004).

			Confirmed Mating Type Genes						Microsatellite Allele Size (bp)				
Clone Name	**Population**	**Type**	**matA**	**matB**	**matC**	**matD**	**matS**	**matT**	**Dict 5**	**Dict 13**	**Dict 19**	**Dict 23**	**Dict 25**
NC21B1	N. Carolina (LF)	1	X						234	187	158	182	226
NC21C1C	N. Carolina (LF)	2		X		X			-	-	-	-	-
NC21D1	N. Carolina (LF)	1	X						240	187	161	206	253
NC21H1A	N. Carolina (LF)	3					X	X	240	160	176	185	205
NC22J1	N. Carolina (LF)	1	X						-	-	-	-	-
NC26D1	N. Carolina (LF)	1	X						234	187	158	182	226
NC26L1	N. Carolina (LF)	1	X						210	199	161	161	262
NC28A1	N. Carolina (LF)	3						X	-	-	-	-	-
NC28B1	N. Carolina (LF)	1	X						234	187	158	182	226
NC28C1	N. Carolina (LF)	1	X						240	187	158	188	262
NC28D1	N. Carolina (LF)	2		X		X			237	187	173	188	220
NC29B1	N. Carolina (LF)	1	X						294	250	161	188	247
NC29E1	N. Carolina (LF)	1	X						252	265	161	188	247
NC29R1	N. Carolina (LF)	1	X						294	250	161	212	172
NC32B1	N. Carolina (LF)	2		X	X	X			210	238	170	200	259
NC105.1	N. Carolina (LBG)	3					X	X	-	-	-	-	-
NC28.1	N. Carolina (LBG)	1	X						-	-	-	-	-
NC34	N. Carolina (LBG)	2		X	X	X			-	-	-	-	-
NC34.1	N. Carolina (LBG)	3					X	X	-	-	-	-	-
NC39.1	N. Carolina (LBG)	1	X						-	-	-	-	-
NC41.2	N. Carolina (LBG)	1	X						-	-	-	-	-
NC43.1	N. Carolina (LBG)	3					X	X	-	-	-	-	-
NC47.2	N. Carolina (LBG)	-							237	187	158	197	223
NC4B	N. Carolina (LBG)	3					X	X	-	-	-	-	-
NC4C	N. Carolina (LBG)	1		X					-	-	-	-	-

NC52.3	N. Carolina (LBG)	1	X						-	-	-	-	-
NC58.1	N. Carolina (LBG)	-							210	160	173	182	244
NC59.2	N. Carolina (LBG)	-							237	160	173	161	256
NC60.1	N. Carolina (LBG)	-							237	160	173	182	244
NC60.2	N. Carolina (LBG)	-							210	184	173	182	205
NC61.1	N. Carolina (LBG)	-							240	160	161	239	220
NC63.2	N. Carolina (LBG)	3					X	X	240	160	176	185	205
NC66.2	N. Carolina (LBG)	-							234	160	173	182	253
NC67.2	N. Carolina (LBG)	-							237	187	176	230	205
NC69.1	N. Carolina (LBG)	-							213	238	161	173	271
NC70.1	N. Carolina (LBG)	2		X	X	X			-	-	-	-	-
NC74.1	N. Carolina (LBG)	-							231	187	173	194	223
NC75.2	N. Carolina (LBG)	1	X						240	160	161	239	220
NC76.1A	N. Carolina (LBG)	1	X						-	-	-	-	-
NC76.1B	N. Carolina (LBG)	3					X		-	-	-	-	-
NC78.2	N. Carolina (LBG)	1	X						-	-	-	-	-
NC80.1	N. Carolina (LBG)	1	X						-	-	-	-	-
NC85.1	N. Carolina (LBG)	2		X	X	X			-	-	-	-	-
NC85.2	N. Carolina (LBG)	3					X	X	-	-	-	-	-
NC98.1	N. Carolina (LBG)	1	X						-	-	-	-	-
NC99.1	N. Carolina (LBG)	1	X						-	-	-	-	-
H10C	Texas (H)	1	X						-	-	-	-	-
H15B	Texas (H)	3					X	X	-	-	-	-	-
H3	Texas (H)	3					X	X	-	-	-	-	-
H3B	Texas (H)	1	X						-	-	-	-	-
HD12C	Texas (H)	1	X						-	-	-	-	-
HD13A1	Texas (H)	2		X	X	X			255	211	161	158	256
HD1D1	Texas (H)	1	X						255	211	161	158	256
HD20B2b	Texas (H)	3					X	X	-	-	-	-	-
HD24A	Texas (H)	3					X	X	-	-	-	-	-
HD24B1	Texas (H)	2		X	X	X			228	205	176	227	184
HD24C1	Texas (H)	2		X	X	X			234	208	182	167	172
HD24D1	Texas (H)	1	X						225	205	161	158	256
HD25A1	Texas (H)	2		X	X	X			228	205	176	227	184
HD2D1	Texas (H)	1	X						255	211	161	158	256
HD30A1	Texas (H)	3					X	X	282	181	161	230	250
HD31B1	Texas (H)	1	X						225	205	161	158	256
HD31C1	Texas (H)	1	X						255	211	161	158	256
HD32C1	Texas (H)	2		X	X	X			234	208	182	167	172
HD35D1	Texas (H)	1	X						255	211	161	158	256
HD37D1	Texas (H)	1	X						255	211	161	158	256
HD38A1	Texas (H)	-							255	208	161	158	256

HD38B1	Texas (H)	1	X						282	181	161	230	250
HD38C1	Texas (H)	2		X	X	X			234	166	161	161	253
HD40D1	Texas (H)	1	X						225	205	161	158	256
HD41B1	Texas (H)	3					X	X	225	205	161	158	250
HD41C1	Texas (H)	3					X	X	282	181	161	230	250
HD42A1	Texas (H)	3					X	X	282	181	161	230	250
HD43C1	Texas (H)	3					X	X	282	181	161	230	250
HD44A1	Texas (H)	1	X						282	181	161	230	250
HD44B1	Texas (H)	3					X	X	282	181	161	230	250
HD45A1	Texas (H)	1	X						234	166	161	140	250
HD45B1	Texas (H)	1	X						225	205	161	158	256
HD45C1	Texas (H)	2		X					228	205	176	-	184
HD45D1	Texas (H)	3					X	X	234	187	161	197	220
HD47B	Texas (H)	1	X						-	-	-	-	-
HD48B1	Texas (H)	3					X	X	231	187	173	188	220
HD48C1	Texas (H)	3					X	X	-	181	161	230	250
HD48D1	Texas (H)	1	X						225	205	161	158	256
HD49A1	Texas (H)	3					X	X	282	181	161	230	250
HD49B1	Texas (H)	3					X		234	187	161	197	220
HD49C1	Texas (H)	1	X						255	211	161	158	256
HD4A1	Texas (H)	1	X						234	205	161	146	250
HD4B1	Texas (H)	3					X	X	234	205	161	146	250
HD50A1	Texas (H)	1	X						225	205	161	158	256
HD50C1	Texas (H)	3					X	X	234	166	158	185	175
HD54C1	Texas (H)	1	X						-	-	-	-	-
HD5A1	Texas (H)	2		X	X	X			234	205	161	146	250
HD5B1	Texas (H)	1	X						234	205	161	146	250
HD5C1	Texas (H)	3					X	X	234	205	161	146	250
V301B1	Virginia (MLBS)	2		X	X	X			234	163	161	161	253
V301B2	Virginia (MLBS)	2		X	X	X			234	163	161	152	253
V303A1	Virginia (MLBS)	3					X*	X*	234	205	176	179	172
V303A2a	Virginia (MLBS)	2		X	X	X			228	205	176	227	184
V303A2b	Virginia (MLBS)	-							228	166	158	227	184
V303C1a	Virginia (MLBS)	3					X	X	234	205	176	185	172
V303C1b	Virginia (MLBS)	-							234	166	158	185	172
V303D1	Virginia (MLBS)	1	X						234	205	176	185	172
V304A1	Virginia (MLBS)	1				X*			234	205	176	179	172
V304A2b	Virginia (MLBS)	3					X	X	234	163	158	179	172
V304B1	Virginia (MLBS)	1	X						234	163	176	179	172
V304B4	Virginia (MLBS)	-							234	163	158	185	172
V304C1a	Virginia (MLBS)	3					X	X	234	205	176	179	172
V304C1b	Virginia (MLBS)	-							234	166	176	179	172
V304D1	Virginia (MLBS)	3					X*	X*	234	163	158	185	175

V305B1	Virginia (MLBS)	3						X*	234	163	158	185	172
V305B4	Virginia (MLBS)	3					X	X	234	163	161	158	256
V306D1	Virginia (MLBS)	2		X	X	X			-	-	-	-	-
V315B1	Virginia (MLBS)	1	X						255	211	161	158	256
V315D1	Virginia (MLBS)	1	X						228	205	176	227	184
V315D2	Virginia (MLBS)	2		X	X	X			228	205	176	227	184
V316A1	Virginia (MLBS)	3					X	X	264	226	161	158	169
V317A1	Virginia (MLBS)	2				X*			228	205	176	227	184
V317D	Virginia (MLBS)	1	X						228	205	176	227	184
V318A1	Virginia (MLBS)	2			X	X			228	205	176	227	184
V319A	Virginia (MLBS)	3					X	X	264	205	161	158	172
V319B1	Virginia (MLBS)	3					X	X	255	214	161	158	256
V319B3	Virginia (MLBS)	3					X	X	234	163	158	185	175
V319C1	Virginia (MLBS)	1	X						234	163	161	161	253
V319D2	Virginia (MLBS)	3					X	X	234	163	158	185	277
V320C1	Virginia (MLBS)	2				X			234	163	161	161	253
V321B1	Virginia (MLBS)	3						X	234	208	158	167	172
V321C1	Virginia (MLBS)	-							234	166	161	161	253
V321D1	Virginia (MLBS)	1	X						225	205	161	158	259
V322A1a	Virginia (MLBS)	1	X						255	211	161	158	259
V322A1b	Virginia (MLBS)	-							255	166	161	158	175
V322B1	Virginia (MLBS)	1	X						225	205	161	158	259
V322C3a	Virginia (MLBS)	1	X						225	205	161	158	256
V322C3b	Virginia (MLBS)	-							225	205	161	158	172
V322D1a	Virginia (MLBS)	-							225	205	161	167	172
V322D1b	Virginia (MLBS)	-							234	205	182	167	172
V323A1	Virginia (MLBS)	-							234	166	161	140	250
V323C1a	Virginia (MLBS)	3					X	X	255	214	161	158	217
V323C1b	Virginia (MLBS)	-							255	163	161	158	256
V323D1	Virginia (MLBS)	1	X						234	166	161	140	250
V324B1	Virginia (MLBS)	1	X†						234	163	161	140	217
V324B3	Virginia (MLBS)	1	X*						234	163	161	140	250
V324D1	Virginia (MLBS)	1	X						255	211	161	158	256
V324D2	Virginia (MLBS)	-							255	211	158	158	256
V325A1a	Virginia (MLBS)	1	X						255	211	161	158	256
V325A1b	Virginia (MLBS)	-							255	211	161	158	172
V325B4	Virginia (MLBS)	3						X	255	214	161	158	256
V325D1	Virginia (MLBS)	2		X	X	X			234	208	182	167	172
V326A1	Virginia (MLBS)	2		X	X	X			255	214	161	158	256

V326B1	Virginia (MLBS)	-							255	208	161	158	256
V326D1	Virginia (MLBS)	3					X	X	282	178	161	230	250
V327A1	Virginia (MLBS)	2		X	X	X			234	205	182	167	172
V327A2	Virginia (MLBS)	2		X	X	X			234	208	182	167	172
V327B1	Virginia (MLBS)	3					X	X	234	163	158	191	172
V327C1	Virginia (MLBS)	2		X	X				255	211	161	158	256
V327C2	Virginia (MLBS)	1	X						234	208	182	167	172
V327D1	Virginia (MLBS)	-							234	208	182	167	172
V327D2	Virginia (MLBS)	1	X						255	211	158	158	256
V329C1	Virginia (MLBS)	-							264	163	158	158	232
V330A	Virginia (MLBS)	3					X		228	205	176	227	184
V330B1	Virginia (MLBS)	2		X	X	X			234	208	182	167	172
V330B2	Virginia (MLBS)	2		X	X	X			228	205	176	227	184
V330D2	Virginia (MLBS)	1	X*†						279	205	176	140	178
V331B1	Virginia (MLBS)	2		X	X	X			255	208	182	170	172
V331C1	Virginia (MLBS)	1	X						234	214	161	158	256
V331C2	Virginia (MLBS)	1	X						255	214	161	158	256
V331D1	Virginia (MLBS)	2		X	X				234	208	182	167	172
V331D2	Virginia (MLBS)	3					X	X	255	214	161	158	256
V335B1	Virginia (MLBS)	3					X	X	255	214	179	158	256
V335C1	Virginia (MLBS)	1	X						255	208	161	158	172
V335D1	Virginia (MLBS)	-							255	214	161	158	256
V336B1	Virginia (MLBS)	2		X*†	X*†	X*†			228	205	176	227	184
V336D1	Virginia (MLBS)	1	X						228	205	176	227	184
V337C1	Virginia (MLBS)	3					X	X	282	181	161	233	250
V337D1	Virginia (MLBS)	1	X						255	214	161	158	256
V341A2	Virginia (MLBS)	1	X						255	211	161	158	256
V341C2	Virginia (MLBS)	3					X	X	288	205	161	158	250
V341D1	Virginia (MLBS)	-							234	205	176	140	178
V342A2	Virginia (MLBS)	2		X	X	X			234	163	161	161	253
V342B2	Virginia (MLBS)	1	X*†						255	208	161	158	256
V345D1	Virginia (MLBS)	1	X						279	205	176	140	178
V53A	Virginia (MLBS)	2		X	X	X			279	205	176	140	178
V53B	Virginia (MLBS)	2		X	X	X			234	163	161	161	253
V53D1	Virginia (MLBS)	1	X						234	163	161	161	253
V55A1	Virginia (MLBS)	2		X	X	X			228	205	176	227	184
V55A2	Virginia (MLBS)	1	X						228	205	176	227	250
V55A5	Virginia (MLBS)	2			X				255	211	161	158	256
V55C1	Virginia (MLBS)	-							234	208	161	140	253

V55C2	Virginia (MLBS)	3					X	X	234	205	161	140	253
V55D2	Virginia (MLBS)	3					X	X	255	214	161	158	256
V56A1	Virginia (MLBS)	1	X						255	211	161	158	256
V56A2	Virginia (MLBS)	1	X*†						264	163	176	212	178
V56B2	Virginia (MLBS)	2		X	X	X			228	205	176	227	184
V56C1	Virginia (MLBS)	3					X	X	234	205	161	146	250
V64A	Virginia (MLBS)	3					X	X	255	214	161	158	256
V64D1	Virginia (MLBS)	3					X	X	279	229	176	140	178
V64D2	Virginia (MLBS)	1	X						255	214	161	158	256
V72A1	Virginia (MLBS)	3					X	X	234	208	161	233	250
V77A	Virginia (MLBS)	1	X						234	205	161	146	253
V77B	Virginia (MLBS)	1	X						225	205	161	158	256
V78B	Virginia (MLBS)	2		X	X	X			264	163	158	212	229
V78C	Virginia (MLBS)	1	X						234	205	176	179	172

Table A2.2. PCR primer pairs for amplification of mating type genes. Primer design based on the published DNA sequence data from Bloomfield et al. (2011).

Mating Type	**Gene**	**Direction**	**Primer Sequence (5' to 3' direction)**
Type I	matA	Forward Reverse	CACACTAAACATGGACCCAC CCCCTAAATCTTTACCAAGTCA
Type II	matC	Forward Reverse	GGGTACAAATATTACAGTGAG CCCCTTTAAAAATGTATTCATAT
	matB	Forward Reverse	CCCCGAATAAACATTTTAATGA GCGAACTCAATTACTATGGG
	matD (partial)	Forward Reverse	CCCATAGTAATTGAGTTCGC GGGCACTGTTATCTTGTTAAT
Type III	matS	Forward Reverse	CGATCAGTTGGAAAACATTAC GGATAGCCAAAAAACTAGTTT
	matT (partial)	Forward Reverse	CGAAAACAGTCAAAAGTCAA CATTATATTGCATTTCAGTGG

Table A2.3. Standardized chi-square residuals for each population. Standardized residuals greater than 2 indicate significantly more individuals than expected of that mating type in the population and standardized residuals less than -2 indicate fewer than expected. Asterisks denote significance.

Population	**Standardized Residuals**		
	Type I	**Type II**	**Type III**
Texas	*1.84*	*-2.45**	*0.61*
North Carolina	*3.18**	*-2.12**	*-1.06*
Virginia	*1.14*	*-0.91*	*-0.23*
Overall	*3.24**	*-2.92**	*-0.32*

APPENDIX 3

Supplementary tables and figures for Chapter 3

Table A3.1. List of *Dictyostelium discoideum* clones used in the experiments to assay differential contribution to macrocyst production. Dicty Stock Center identification numbers are in parentheses.

Clone Name	Population	Mating Type	Experiment Group
HD13B1	Texas	1	A
HD45C1	Texas	2	A
HD48B1	Texas	3	A
HD35D1	Texas	1	B
HD38C1	Texas	2	B
HD45D1	Texas	3	B
HD48D1	Texas	1	C
HD32C1	Texas	2	C
HD49B1	Texas	3	C
17S 10.1	Virginia	1	D
14S 6.1	Virginia	2	D
19S 8.2	Virginia	3	D
V315B1	Virginia	1	E
V331B1	Virginia	2	E
V341C2	Virginia	3	E
NC59.2	North Carolina	1	F
NC34.1	North Carolina	2	F
NC63.2	North Carolina	3	F
NC60.2	North Carolina	1	G
NC66.2	North Carolina	2	G
NC75.2	North Carolina	3	G
NC105.1	North Carolina	1	H
NC58.1	North Carolina	2	H
NC61.1	North Carolina	3	H
WS205	Wisconsin	1	I
IR1	Wisconsin	2	I
NC4 (DBS0302497)	North Carolina	1	N1
NC4 (DBS0235761)	North Carolina	1	N2
NC4 (DBS0235763)	North Carolina	1	N3
NC4 (DBS0235759)	North Carolina	1	N4
NC4 (DBS0235762)	North Carolina	1	N5
V12 (DBS0302498)	Virginia	2	V1
V12 (DBS0235789)	Virginia	2	V2
V12 (DBS0235788)	Virginia	2	V3
V12 (DBS0235787)	Virginia	2	V4
V12 (DBS0235786)	Virginia	2	V5

Table A3.2a. Test for mating compatibility (macrocyst production) between Houston, TX clones.

		Mating Type II		Mating Type III		
		HD5A1	HD13A1	HD48C1	HD50C1	HD41C1
Type I	HD40D1	No	No	No	Yes	No
	HD49C1	No	No	No	No	No
	HD24D1	No	No	No	No	Yes
Type II	HD5A1	-	-	No	No	No
	HD13A1	-	-	No	No	No

Table A3.2b. Test for mating compatibility (macrocyst production) between Texas clones.

		Mating Type II				Mating Type III		
		HD1D1	HD32C1	HD45C1	HD38C1	HD45D1	HD49B1	HD48B1
Type I	HD45B1	No	Yes	Yes	-	Yes	Yes	Yes
	HD31B1	No	Yes	Yes	-	Yes	Yes	Yes
	HD48D1	No	No	No	-	No	No	Yes
	HD35D1	-	-	-	Yes	-	-	Yes
Type II	HD1D1	-	-	-	-	Yes	No	No
	HD32C1	-	-	-	-	Yes	Yes	Yes
	HD45C1	-	-	-	-	Yes	Yes	No
	HD38C1	-	-	-	-	-	-	Yes

Table A3.3. Test for mating compatibility (macrocyst production) between Virginia clones.

		Mating Type II				Mating Type III				
		V330C	V331B1	V327A2	V55B1	V331D2	V341C2	V335B1	V303C1	V319B1
Type I	V331C1	Yes	No	Yes	No	Yes	Yes	Yes	No	No
	V335C1	No	No	No	No	Yes	Yes	No	No	No
	V315B1	No	Yes	Yes	No	No	Yes	Yes	No	No
	V317D1	No	No	No	No	Yes	Yes	Yes	No	No
Type II	V330C	-	-	-	-	Yes	Yes	No	No	No
	V331B1	-	-	-	-	No	Yes	No	No	No
	V327A2	-	-	-	-	Yes	Yes	No	No	No
	V55B1	-	-	-	-	No	No	No	No	No

Table A3.4a. Test for mating compatibility (macrocyst production) between North Carolina clones.

		Mating Type II			Mating Type III	
		NC32B1	NC28D1	NC34	NC85.2	NC34.1
Type1	NC29E1	No	No	No	No	No
	NC26L1	No	No	No	No	No
	NC21D1	No	No	No	No	No
	NC98.1	No	No	No	No	No
	NC28E1	No	Yes	No	No	No
	NC22J1	No	No	No	No	No
	NC105.1	No	No	No	No	No
Type II	NC32B1	-	-	-	No	No
	NC28D1	-	-	-	Yes	No
	NC34	-	-	-	Yes	No

Table A3.4b. Test for mating compatibility (macrocyst production) between North Carolina clones.

		Mating Type II			Mating Type III		
		NC66.2	NC34.1	NC58.1	NC75.2	NC63.2	NC61.1
Type I	NC60.2	Yes	Yes	Yes	Yes	Yes	Yes
	NC59.2	Yes	Yes	Yes	Yes	Yes	Yes
	NC105.1	Yes	Yes	Yes	Yes	Yes	Yes
Type II	NC66.2				Yes	Yes	Yes
	NC34.1				Yes	Yes	Yes
	NC58.1				Yes	Yes	Yes

Table A3.5. Test for mating compatibility (macrocyst production) between different clones of NC4 and V12 and their unique strain ID (beginning with "DBS").

		Mating Type II						
		V12 DBS0235786	V12-M2 DBS0235788	V12-H DBS0235787	V12-M2 DBS0302498	V12 DBS0235785	V12 DBS0235784	V12 DBS0235789
Mating Type I	NC4 DBS0302497	Yes	Yes	Yes	Yes	Yes	No	Yes
	NC4 DBS0235760	No	No	No	No	No	Yes	No
	NC4 DBS0235761	Yes	Yes	Yes	Yes	Yes	No	Yes
	NC4 DBS0235764	Yes	No	No	No	No	No	No
	NC4 DBS0235763	Yes	Yes	Yes	Yes	Yes	No	No
	NC4 DBS0235759	Yes	Yes	Yes	Yes	No	No	Yes
	NC4 DBS0304666	No	No	No	No	No	Yes	No
	NC4 DBS0235762	Yes	Yes	Yes	Yes	Yes	No	Yes

Figure A3.1. Differential investment to macrocyst production is rare. Symbols represent macrocyst production between individual clone pairs. Lines are best-fit regression curves for individual clone pairs. Dashed lines show nonsignificant relationships, solid lines show significant relationships.