RICE UNIVERSITY

Reproductive conflicts in the social wasp, *Eustenogaster fraterna*,
and in the social amoeba, *Dictyostelium discoideum*

By

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A mamma, babbo, Simona
ABSTRACT

My dissertation encompasses three studies of social behavior. Two explore the reproductive conflicts that occur between cooperating individuals. I examine conflict at two levels: within colonies of the social wasp, *Eustenogaster fraterna*, and within chimeric multicellular organisms formed by the social amoeba, *Dictyostelium discoideum*. The third project investigates the occurrence of sexual reproduction in a natural population of *D. discoideum*.

*Eustenogaster fraterna* belongs to the basal eusocial family of stenogastrine wasps. Colonies are small averaging only 2.7 adult females but only one mated female. I examined the reproductive and genetic structure of 18 colonies by genotyping all within-colony individuals and assessing the ovarian development of females. My results show that 85% of females were potential reproducers. Adult females were not related as full sisters. Some brood could not be assigned to any of the adult females, suggesting that subordinate females could inherit the nest and replace the dominant female.

*D. discoideum* live as free-living, single cells, but when starved they aggregate to form a multicellular fruiting body. Genetically distinct clones of *D. discoideum* co-aggregate to form genetic chimeras. 20% of cells form the stalk of the fruiting body and die, while others become reproductive spores. One clone can exploit the other by contributing less than its proportional share to the sterile stalk. I investigated whether cheating is a strategy dependent on the relative proportion of the clones aggregating. In a
chimera, a rare clone could benefit by avoiding forming the stalk because it is less related to the reproductive part. My results do not support the frequency-dependence strategy.

I investigated the occurrence of sexual reproduction in a natural population of \textit{D. discoideum}, using the approach of estimating linkage disequilibrium in the population at one site. My results show that recombination does occur and is due to sexual reproduction not to parasyxuality.

In sum, conflicts within societies can be mitigated. \textit{E. fratetna} workers gain insurance advantages by obtaining indirect genetic benefits or inheritance of work force. \textit{D. discoideum} clones gain cheating advantages either when rare or common in the aggregation. Recombination yields novel genotypes; this might increase the complexity of interactions between co-aggregating clones.
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CHAPTER 1

INTRODUCTION
My dissertation research explores the potential for reproductive conflicts in two
distinct social systems, the wasp Eustenogaster fraterna, and the social amoeba,
Dictyostelium discoideum. My study also investigates the occurrence of sexual
reproduction in D. discoideum, which for a long time has been an obscure aspect of its
life cycle.

The dualism of cooperation and conflicts has long been the leading theme in the
study of animal societies. Cooperation in reproduction is of particular interest, especially
when individuals within a society unequally share reproductive benefits. Altruists pay
costs by not gaining direct reproductive benefits and, in some cases, they totally give up
reproduction. Cheaters unfairly exploit reproductive benefits, without paying the costs
(Wilson, 1975; Velicer 2000; Strassmann 2000a).

Kin selection and mutualism are two theories that explore the evolution and
maintenance of cooperation in societies. Kin selection theory (Hamilton 1964) explains
cooperation between related organisms. Altruists can gain indirect reproductive benefits,
passing on their genes to the next generation by helping reproduction of related
individuals. Altruists gain indirect benefits if they pass on more genes by helping than by
reproducing directly. Mutualism theory (Michener 1974) explores cooperation between
unrelated individuals. Individuals adopt the strategy of forming a group if they benefit
more by cooperating than by being alone. In this case, conflicts of interest over
reproduction are likely to arise because each individual behaves selfishly, trying to gain
most of the reproductive benefits.
Social insects have been widely studied to investigate such cooperation and conflict. Because individuals within a colony are genetically different even if related, conflicts of interests over reproduction are expected to arise. Although social insects provide a wealth of insights into social evolution, these complex systems have a limit with regard to gaining a deeper understanding of the genetic mechanisms underlying cooperation and conflict because it is hard to study conflict at the gene level. Thus, more progress might be made studying cooperation and conflict in simpler organisms. Simple multicellular organisms can be investigated in this regard. Multicellular organisms that descend from a single cell, through mitotic divisions, are not of special interest for social questions because they are expected to be nearly free of internal conflicts, since genetic differences only arise by rare mutations. In contrast, when multicellular organisms form by aggregation of genetically distinct clones, genetic chimeras originate and conflict of interests may arise (Grosberg and Strathmann 1998).

Even though the social wasp, E. fraterna, and the social amoeba, D. discoideum, are two organisms at very different places in the tree of life, they both share the common feature of having simple societies and totipotent individuals. Therefore, E. fraterna and D. discoideum are apt species to investigate for origins of conflicts of interest over reproduction.

My first project investigates the reproductive and genetic structure of the wasp E. fraterna. E. fraterna belongs to the basal eusocial family of wasps, the Stenogastrinae. Stenogastrine wasps form small colonies with low numbers of females; that differ between species (Turillazzi 1989, 1996; Samuel 1987). Although adult females are not morphologically differentiated into castes, only one female reproduces and prevents the
other nestmates from laying eggs. Subordinate females retain the ability of reproducing and can replace the dominant female if she dies (Turillazzi 1989, 1996; Field and Foster 1999). As females reach maturity, they can opt to stay in the natal nest, join another colony as subordinates, or attempt to replace the dominant female (Yamane et al. 1983; Turillazzi 1989). Therefore, adult females could become helpers either in related or in unrelated colonies (Field et al. 1998). *E. fraterna* is interesting to investigate how the helping strategy of a female can shape the reproductive structure of the colony, particularly because both dominant and subordinate females perform highly risky tasks out of the nest (Francescato et al. 2002). Therefore, adult mortality is high and the brood survival may be at risk. Helping can be favored by relatedness, if a female stays on the natal nest and gains indirect reproductive benefits (Hamilton 1964). However, helping could also be a strategy favored because helpers can become reproducers later in their life, either in related or in unrelated colonies. Indirect benefits are likely to come from insurance advantages (Queller 1989, 1994a, 1996). If the longevity of the mother is likely to be shorter than the developmental time of the brood, a helper can gain benefits by rearing the brood. In this way, the reproductive investments are not lost and the helper can rear either related brood and or future unrelated workers that will help her. The helper also gains the opportunity to start her own reproduction. By investigating relatedness between colony members and by assessing the ovarian development of adult females, I show that adult females are potential reproducers, even though only the dominant female is mated. I also demonstrate that some of the brood could not be assigned to any of the adults present on nests. Therefore, females can gain insurance
advantages by becoming helpers since the mothers of the brood often die before the brood reaches maturity.

My second project explores cheating behavior in *Dictyostelium discoideum* chimeras. Specifically, I investigate whether cheating is a strategy dependent on the relative abundance of two clones forming a chimera. This social amoeba has been exploited in the study of cell communication and development (Kessin 2001) and recently it has become a model system to investigate social questions (Strassmann et al. 2000). These slime molds live in the forest soil as free-living single cells, reproducing vegetatively (Bonner 1967; Landolt & Stephenson 1990; Raper 1984). However, as they starve, amoebae cooperate and aggregate into a multicellular structure that undergoes differentiation into a fruiting body. At this stage in its life cycle, *D. discoideum* starts to exhibit social features. Cells within the fruiting body have different fates: 20% die, giving up reproduction and forming the stalk, while 80% become fertile spores (Raper 1984). This unequal share of reproduction within the multicellular organism is analogous to the reproductive division of labor within social insect colonies, where workers and the reproductive female gain differential reproductive benefits.

The aggregation of cells is the stage of crucial interest for studies of social questions, because different clones can aggregate and share reproduction unequally (Strassmann et al. 2000). The resulting multicellular organism is a chimera, and competition over reproduction is expected to arise with one clone cheating and exploiting the other by contributing less to the sterile stalk. Frequency dependent cheating could occur when clones recognize the presence of a foreign clone, assess that they are in a chimera, and alter their developmental program to contribute less to the stalk than they
might otherwise. I investigated whether cheating in chimeras is a frequency-dependent strategy. Knowing the initial proportion of clones in a mixture, I determined the relative allocation of the two clones into spores. I did not find any evidence for frequency-dependence exploitation, since the allocation of clones into spores was proportional to their initial abundance. My results suggest that, although a rare clone is not able to cheat by increasing its relative contribution to spore production, it might still gain fitness benefits. By joining a foreign clone, the rare clone increases the chance to form a big multicellular organism, which may have higher probability to better survive than a small one (Foster et al. in press).

My third study investigates the occurrence of sexual reproduction under natural conditions in *D. discoideum*. Genetic recombination in this slime mold is likely to occur during the macrocyst stage or during parasexuality. Macrocysts are induced under conditions of both starvation and submersion, and they form when two cells join and form a giant cell. Then the giant cell attracts and engulfs all the other cells around, which will then become endocytes. Only the two cells forming the giant cell are likely to recombine, while endocytes die (Raper 1964). Previously, sexual reproduction had been studied in macrocysts under laboratory conditions (Francis and Eisenberg, 1993; Francis, 1998), but there was little clear evidence of recombination. The other stage in which genetic recombination may occur is when two amoebae temporally form diploids and then yield haploid progeny through mitotic divisions. This parasexual process occurs at very low frequencies under laboratory conditions and has been little investigated (Katz and Sussman, 1972; Newell, 1982). I assessed linkage disequilibrium in a natural population of *D. discoideum* and investigated whether loci on the same and on different
chromosomes recombine. My results show that linkage disequilibrium is somewhat broken down by recombination, and that sexual reproduction is the form of reproduction in this natural population of *D. discoideum*.

In sum, this work shows different perspectives of reproductive strategies in two social systems characterized by rudimentary social development. In the wasp I analyzed the perspective of the helper. I demonstrate that the reproductive strategy of subordinate females could shape the structure of the colony. Helpers may remain on colonies where they do not reproduce, waiting to inherit the colony. They could also adopt the strategy of not reproducing without inheriting the colony if they gain life insurance benefits.

On the contrary, in *D. discoideum* chimeras I investigated the perspective of the cheater. My study does not support the hypothesis that cheating is a strategy dependent on the initial relative proportions of clones forming a chimera. Instead, the allocation of a clone into the reproductive spores was positively related to its initial percentage in cell mixtures. This result could be due to the inability of clones to assess the relative abundance of clones.

My work also gives an important contribution to our understanding of whether or not *D. discoideum* amoebae recombine in the wild. It shows that genetic recombination occurs under natural conditions in *D. discoideum*, and in particular, it demonstrates that sexual reproduction, not paraxuality, is the process of recombination. The knowledge that sexual reproduction is not lost in *D. discoideum* is important for molecular biologists, because it means it may be possible to combine mutations from different lines.
CHAPTER 2

COLONY STRUCTURE IN THE PRIMITIVELY SOCIAL

STENOASTRINE WASP, EUSTENOGASTER FRATERNAl
ABSTRACT

The origin of sociality is best studied in groups with rudimentary social development, like the stenogastrine wasps. An example of the earliest phase of sociality is *Eustenogaster fraterna*, which has very small colonies averaging only 2.7 adult females but only one, mated reproductive female. Only 15% of the females had no ovarian development at all, indicating the potential for future reproduction of most females. Microsatellite genotyping showed that adult females were related to each other but not generally as full sisters ($r = 0.427 \pm 0.131, 95\%$ confidence interval). Relatedness of unmated females to female brood is low ($r = 0.210 \pm 0.171$), significantly lower than that of mated females to female brood ($r = 0.374 \pm 0.266$). Mated females are also more related to the male brood ($r = 0.871 \pm 0.168$) than are unmated females ($r = 0.588 \pm 0.339$), suggesting that unmated helpers do not generally produce sons or achieve exceptionally high indirect fitness. In all, 7 of 15 of nests had some brood that could not be assigned to existing adult females, indicating the potential for colony inheritance by subordinate females. Clearly, these small colonies just past the threshold of sociality do not have predominantly mother/daughter groups, which argues against an important role for exceptionally high relatedness in the origin of eusociality.

**Key words:** eusocial; life insurance; microsatellite; relatedness; usurpation, Vespidae
INTRODUCTION

The costs and benefits of cooperation are particularly amenable for study in rudimentarily social species where all females retain the options of helping or reproducing. Helping may be favored because helpers are related to the brood they rear and therefore gain sufficient indirect fitness benefits (Hamilton 1964a, b). Or helping may be favored because helpers become reproducers later in their life, by taking over the breeding position. In the Hymenoptera, indirect benefits are likely to come from life insurance advantages (Strassmann and Queller, 1989; Queller, 1989, 1994a, 1996; Gadagkar 1990; Queller and Strassmann 1998; Field et al.1999). The life insurance hypothesis is based on the relative longevity of adult females and their brood. If the mother of the brood dies before the brood has matured, a related female that takes over their care may increase her own genetic representation in the next generation by rearing these half-developed young. However, if the inheriting female is unrelated to the brood, it will only benefit her if those brood eventually help rear her brood.

The stenogastrine wasps of SE Asia (Fig. 2.1) are particularly suitable for studies of nascent sociality. Not only do they have very small colonies with apparently totipotent females, but they also often nest in aggregations, dense enough to present females with a variety of reproductive options (Samuel 1987; Turillazzi 1996; Field & Foster 1999). Furthermore, very long brood development times and high adult mortality rates (Samuel 1987; Field et al. 2000) characterize them. Stenogastrine wasps have very small colonies, with adult colony members typically numbering under 5 in Parischnogaster mellyi and
*Parischnogaster striatula* (Turillazzi 1996), as many as 7-8 in *Liostenogaster flavolineata* (Samuel 1987) and 13 in *P. alternata* (Turillazzi 1989, 1996). Females are not morphologically differentiated into castes. A single wasp typically dominates reproduction in a colony and prevents other nestmates from laying eggs (Turillazzi 1989; 1996; Sumner et al. 2002). As a result, the social structure in stenogastrine wasps is characterized by one egg-layer who spends most of her time on the nest and non-laying females who help with brood care, but who are reproductively competent should a vacancy at the top arise (Field and Foster 1999).

In stenogastrine wasps, helpers can potentially reproduce and replace the dominant individual (Turillazzi 1991; Field and Foster 1999). Therefore, helping behavior may be adopted while waiting to inherit the nest. Adults could be helpers either in related or in unrelated colonies (Field et al. 1998). Once stenogastrine females reach maturity, they can opt to stay in the natal colony, or join another colony as a subordinate or even as a dominant if they succeed in defeating the other females (Yamane et al. 1983; Turillazzi 1989). Relatedness among these nestmates is typically low. In *Liostenogaster flavolineata* low relatedness ($r = 0.22 \pm 0.10$ and $r = 0.52 \pm 0.05$ in another study) was reported and attributed to high usurpation rates by unrelated females (Strassmann et al. 1994; Field et al. 2000). In *L. flavolineata*, helpers are critical for ensuring that immature brood become adults because of the combination of high adult mortality and exceptionally long egg-to-adult developmental times (Field et al 2000), on average more than 100 days (Samuel 1987).

We studied *Eustenogaster fraterna*. This species builds small mud nests with a single entrance hole underneath (Fig. 2.2), and typically has colonies with fewer than five
females (Turillazzi and Gerace 1992; Francescato et al. 2002). This species is particularly interesting because subordinates spend less time caring for brood than do dominants (Francescato et al. 2002). The subordinates do help by foraging, sometimes feeding larvae, and guarding the nest against parasitoids, predators, and usurpers (Francescato et al. 2002). Dominant and subordinate females did not differ in the time they spend away from the nest, the most risky behavior of all (Francescato et al. 2002). If inheritance of the nest is an important factor in driving the decision of a female to become a helper, then we expect colonies often to contain brood of missing females that may or may not be related to the remaining females. We use DNA microsatellite markers and assessments of ovarian development to elucidate the reproductive and genetic structure of colonies of *Eustenogaster fraterna*. 
FIGURE 2.1. Geographic distribution of stenogastrine wasps (Turillazzi 1991). Stenogastrine wasps live in the South-East Asia, ranging from India to Indonesia and New Guinea. They live at various altitudes, from the sea level to high altitudes (1600 mt).
FIGURE 2.2. *Eustenogaster fraterna* builds small mud nests with a single entrance hole underneath. Typically *E. fraterna* has colonies with fewer than five females.
MATERIAL AND METHODS

In February 1999 we collected 18 colonies of *E. fraterna* in the tropical rain forest at Bukit Fraser, Pahang State, Malaysia, at an altitude of 1000-1500 m. We collected in the evening (18:30 to 19:30) and at night (20:30 to 21:00). At neither time did we observe any flying wasps, and the dying light available for evening collections facilitated careful collection of colonies that were difficult to collect. Excessive disturbance during collection could cause the wasps to drop, even at night. Our collecting techniques ensured that we did not lose any wasps present at the nests. Colonies were refrigerated at 4 degrees and the next morning adults, eggs, larvae, and pupae were transferred to plastic tubes containing 100% ethanol. We replaced the ethanol within two days following collection and then again one week later.

*Ovarian analysis*

In order to identify the reproducers in each colony, we dissected all individuals and evaluated their ovaries (Table 2.1). We counted the number of mature and nearly mature eggs and we measured the length of the longest oocyte. A mature egg is at least as long as the smallest egg laid (1.6 mm) and has a clear chorionic membrane. A nearly mature egg is 80% or more of the length of a mature egg, but does not fit the definition of a mature egg. We also determined whether the wasps were mated, by checking the status of the spermatheca. We were unable to assess insemination status from 3 females and for 4 additional females we could not assess insemination or ovarian status because we lost
the abdomen. These problems affected 7 colonies, three with a single queen (Table 2.1). We measured headwidths to determine whether reproducers were larger than non-reproducers. We used a graticule-equipped Wild microscope at 25x for the dissections and morphological measurements.

**Genetic analysis**

I extracted DNA from the thorax tissue of adults, and a similarly located piece from larvae and pupae, using a BIO-RAD CHELEX-100 extraction (Hillis et al., 1996). For genotyping, I used the polymerase chain reaction (PCR) to amplify five microsatellite loci, identified from *Eustenogaster fraterana* (Table 2.2; Zhu et al., 2000). The number of alleles ranged between 7 and 14, with observed heterozygosities of 0.47-0.82 (see Strassmann et al., 1996, for detail on the procedures for the amplification of microsatellites and resolution of PCR products).

The genetic data were generally not adequate for constructing detailed pedigree connections because of the small numbers of adults and brood, the absence of many mothers, lack of information as to whether females mate multiply, and the modest number of microsatellite loci. Therefore, I focused on two modes of analysis that were more robust. I estimated average relatednesses among categories of individuals, and I determined how often I could exclude all adult females as mothers, as an indicator that the mother(s) had died.

I estimated genetic relatedness using Relatedness 5.0.5 (Queller and Goodnight, 1989), weighting nests equally. In the analysis, I excluded individuals with genotypes for fewer than three microsatellite loci. This only affected brood. I jackknifed over colonies
in order to estimate 95% confidence intervals and these are the values reported with all relatedness estimates. I compared relatednesses with unpaired tests, because pairing the nests would reduce the sample size too much (Queller, 1994b). I classified brood as male if they had only one allele at all loci, indicating they were haploid. The chance that a female would be homozygous at all loci was only $5.33 \times 10^{-4}$ so I do not think I assigned any brood incorrectly to the male class.

**Brood assignment**

I determined which brood could be excluded as progeny of all possible mothers in their colony. I excluded female brood if they did not share at least one allele at every locus with a potential mother, where potential mothers are mated females only. If mothers were missing abdomens or spermathecae, I assumed they could have been mated and treated them as potential mothers, so my analysis is conservative with respect to the number of offspring excluded. I assigned sons to possible mothers by requiring that they share an allele at all loci with their mother, who did not have to be mated. I counted missing loci as possibly matching since I could not determine otherwise. This also has the conservative effect of increasing the number of brood assigned to present females.
RESULTS

Nests of *E. fraterna* were small, with cell number ranging from 9 to 23 (average = 15.6 ± S.D. 3.9, N = 18, Table 2.1). Most occupied cells contained eggs (6.7 ± S.D. 3.4, N=18). Fourteen nests also contained larvae (2.4 ± S.D. 2.2, N = 14) and 5 nests contained pupae (0.9 ± S.D. 2.0, N = 5). Seventeen nests had empty cells (5.8 ± S.D. 3.6, N = 17). Twelve nests had male brood (2.1 ± S.D. 1.2, N = 12).

*Colony characteristics*

Nests had few adults on them (Fig. 2.3, Table 2.1). Overall, they averaged 2.7 ± S.D. 1.7 adult females. Two of the larger nests (4 and 6 females) each had a single adult male. The total number of females present on the nests was lower in the evening collection (2.2 ± S.D. 1.5, N = 13) than in the night collection (4.0 ± S.D. 1.6, N = 5; Mann-Whitney U test, N = 18; p<0.05). However, the number of mated females did not differ between evening (0.9 ± S.D. 0.6, N = 10) and night collections (0.6 ± 0.5, N = 5; Mann-Whitney U test, p>0.4). The number of unmated females alone did not differ between evening (1.8 ± S.D. 1.5, N = 9) and night collections (3.3 ± SD 1.5, N = 4) either (Mann Whitney U p>0.1). This means that any effect of evening collecting would have been slight, possibly less than the risk of collecting difficult nests with lights in total darkness.
FIGURE 2.3. Cells per nest and females per nest of *E. fraterna.*
Reproductive structure

In my sample 37% of females had mature eggs, and 24% of females were mated (Figure 2.4, Table 2.1). Among mated adults, 10 of 12 (83.3%) had mature eggs, while only 8 out of 29 (27.6%) non-mated females had mature eggs in their ovaries (G-squared p<0.001). These unmated egg layers do not form a distinct class of females because other females had ovaries nearly developed to that level (Fig. 2.4). The difference in length of the longest oocyte present in ovaries of egg-layers and non-egg-layers was significant (t-test, t = 7.004, p<0.0001, Table 2.1). Most nests had more than one female with mature eggs in her ovaries, though only one of them was mated in any given colony. In one colony two females of three were mated, but only one of them had mature eggs in her ovaries and this is the only case where there was more than one mated female in a colony.

Three colonies lacked a mated egg layer. Two of these had only a single female, in one case with undeveloped ovaries, and in the other case with 3 nearly mature eggs. The third colony lacking a mated egg layer had 4 females, one with a mature egg, and all with some ovarian development.

Size did not differ between reproductive females and nonreproductive females. The average headwidths for mated and unmated females was 4.21 ± S.D. 0.133 (N=12) and 4.19±S.D.0.138 (N=29) respectively, not a significant difference (t-test, p>0.7).
FIGURE 2.4. Ovarian development of females assessed as number of mature eggs in their ovaries and length of the longest oocyte or mature egg. The latter measure is good for assessing intermediate and low levels of development, while the former is good for identifying potential egg layers.
Average relatedness among adult females was 0.427 ± 0.131 (12 colonies 43 females, Fig. 2.5). Mated and unmated females were related to each other by 0.531 ± 0.306 (8 nests, 8 and 18 females, in the two classes respectively). Within colonies, adult females were related to female brood by 0.272 ± 0.11 (15 nests, 42 adults and 33 female brood), and to male brood by 0.673 ± 0.211 (12 colonies, 36 adults, 25 male brood).

Mated females were related to female brood by 0.374 ± 0.266 (9 colonies, 10 and 20 females respectively), and to male brood by 0.871 ± 0.168 (7 colonies, 7 and 17 individuals respectively, Fig. 2.5). Unmated females were related to female brood by 0.210 ± 0.171 (10 colonies, 25 and 18 individuals respectively) and to male brood by 0.588 ± 0.339 (8 colonies, 23 and 13 individuals respectively). Mated females were significantly more closely related to female brood than were non-mated females (unpaired t-test, p = 0.003:10 and 10 colonies). Mated females were also significantly more closely related to male brood than were non-mated females (unpaired t-test, p < 0.001; 8 and 7 colonies).

Six of 31 female brood (19%) and 6 of 25 (24%) male brood could not have been the progeny of any of the adults we collected (Table 2.3).
FIGURE 2.5. Genetic relatedness and 95% confidence intervals among listed categories.
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Nests 11 - 15 collected at night

'mated

*one female missing abdomen

**one female missing spermatheca

*** two females missing spermatheca
**TABLE 2.2.** DNA microsatellite primers and their frequencies in this population

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TABLE 2.3. Numbers of full sister groups among adult females, proportion of adult females that could be sisters or daughters of the mated egg layer, and the proportion of brood that could be assigned to these adults. Female brood could be assigned only to mated females while male brood could be assigned to any adult female. Data are based on analysis of microsatellites (see methods).

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DISCUSSION

Temporary helping behavior grades into true eusociality when helpers have low probabilities of ever becoming reproducers (Sherman et al. 1995). Stenogastrine wasps stand on the threshold of eusociality, at a point where the selective factors favoring helping over reproducing can be analyzed. The haplodiploid hypothesis (Hamilton 1964a,b, 1972) offered the possibility of explaining eusociality purely on relatedness differences (see Bourke and Franks 1995 p.77-106 for an excellent review). However, this hypothesis has not been supported well by empirical studies (reviewed in Queller and Strassmann 1998) even though its initial condition of single mating by queens holds widely across the social Hymenoptera (Strassmann 2001). Multiple queens and frequent queen turnover are hallmarks of primitively social Hymenoptera and reduce relatedness below that of full-sister frequently enough that the relatedness advantage of haplodiploidy often cannot apply (Queller and Strassmann 1998). *E. fraterna* is no exception. The key relatedness is that of unmated helpers to the brood they are rearing since these are the altruists that keep the colony going. Genetic relatedness of unmated females to female brood was 0.210 ± 0.171 (Fig. 2.5), well below what would be found on colonies with single, once-mated queens, but significantly above zero. I could not tell if relatedness was lowered because of multiple mating by females. There was too much genetic variability and too few individuals per colony to assess mate number and my attempts to genotype the contents of spermathecae failed, probably because they had been stored in ethanol, not frozen.
Unmated females could obtain direct fitness advantages by producing males, but my data suggest that this is not a major factor in *E. fraterna*. Relatedness of the mated females to male brood was very high (0.871 ± 0.168) and significantly above relatedness of unmated females to males (Fig. 2.5).

 Helpers may remain in colonies where they do not reproduce because they may inherit the nest and its workforce, a direct fitness advantage. High rates of egg layer replacement are a likely explanation for the unattributed brood (Table 2.3). About a fifth of male and female brood could not be attributed to any adult female currently associated with the nest, and must have been offspring of uncollected, presumably dead, females, related or unrelated to the current females. This, together with the extremely small size of *E. fraterna* colonies (Fig. 2.3, Table 2.3), makes inheritance probabilities for individual subordinate females appreciable.

 High adult mortality also leads to life insurance benefits even for helpers that do not inherit, as long as they are related to the brood (Strassmann and Queller 1989; Queller 1989, 1994, 1996; Gadagkar 1990). If a solitary adult is likely to die before she can raise some of her brood to independence, her effort is wasted. By comparison, a helper who dies early does not waste her contributions because she has completed the investments of other females or has her own investments completed by others. The turnover data combined with significant levels of relatedness among adults and brood suggest that life insurance advantages are significant in *E. fraterna*.

 *Eustenogaster calyptrata* is a closely related species studied by Hansell (1987) who observed frequent attempts at usurpation, which were sometimes successful. Usurpations were not followed by brood cannibalism, but instead the brood were adopted
and cared for. *E. calyptodoma*, with only one or two females, has even fewer adults per colony than *E. fraterna*, but their social systems seem quite similar. One difference is the lack of partially developed ovaries in *E. calyptodoma* females associated with nests, though the unaffiliated categories "squatters and vagrants" had intermediate ovarian development (Hansell, 1987).

In another species, *Liostenogaster flavolineata*, with somewhat larger and clumped colonies, Field et al. (1999) evaluated the reproductive options of females. They found that females went through a time window early in their adult life when they were likely to leave their natal nest and attempt to join another nest. However, neither rank nor group size influenced whether females were likely to leave their natal nest. They experimentally demonstrated that even females functioning as helpers can become queens, so poor condition is not likely to influence whether they help at home or float to a new nest they may be able to usurp (Field and Foster, 1999). The chance of becoming a reproductive is higher with smaller groups (Shreeves and Field 2002). Though groups are small, reproduction is not contemporaneously shared: reproductive skew is high (Sumner et al. 2002). Earlier work had clearly demonstrated for this species that maturing brood were viewed as potential helpers, even by non-relatives, since nests with older brood were more likely to be adopted when their adults were removed than were nests with younger brood (Field et al. 1998). Though *L. flavolineata* differs from *E. fraterna* in its larger group sizes, clumped nests, and longer brood development times, it is likely that similar selective factors operate in both species, favoring both moving among nests, and adopting unrelated brood as future caretakers of one's own brood.
The haplodiploid advantage works best when adults, particularly queens, are long-lived, so that daughters nearly always end up helping their mothers. This commonly occurs with advanced social insects because queens have evolved longer lifespans based on a history of social life that has removed external sources of mortality (Alexander et al. 1991; Keller and Genoud, 1997; Keller, 1998). However, these advanced social species are not the best ones to reveal how sociality evolved. In species where queens and workers have not yet evolved divergent lifespans, we expect queen mortality to be higher, and this will often prevent the 3/4 relatedness advantage from being realized. However, high mortality selects for sociality in two other ways, through inheritance, and through life insurance. These advantages appear to be important in *E. fraterna*, and may generally be what has favored the emergence of true sociality in species with extended parental care.
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CHAPTER 3

EXPLOITATION BEHAVIOR AS A FREQUENCY-DEPENDENT STRATEGY IN 

*DICYOSTELIUM DISCOIDEUM* CHIMERAS
ABSTRACT

This project explores cheating behavior in *Dictyostelium discoideum* chimeras. The multicellular stage of *D. discoideum* life cycle can be a chimera, if it forms by aggregation of genetically distinct clones. Within chimeras, genetic conflicts of interest are likely to arise, with one clone cheating by unfairly gaining reproductive benefits and exploiting the other clone. The cheater clone contributes less than its proportional share to the stalk. I investigated whether cheating behavior is a strategy dependent on the initial relative abundance of two clones aggregating. A rare clone is expected to contribute less to the stalk formation, because of its low relatedness to the overall group. My results did not support the frequency-dependence hypothesis, showing cells of the rare and of the common clone are equally likely to allocate into the reproductive spores. It is possible that clones are unable to detect the presence of foreign clones, or are able to assess the presence of a foreign clone but not to sense their relative abundance. Although rare clones do not gain high reproductive benefits, they still can gain fitness advantages by aggregating in a chimera. The strategy of joining a foreign clone when forming a group protects them from the high risk of dying when starvation occurs.

Key words: *Dictyostelium discoideum*, chimeras, cheating
INTRODUCTION

Cooperation in reproduction is an intriguing aspect of animal societies, particularly when division of labor occurs. This kind of cooperation implies an unequal partitioning of reproduction between altruistic and selfish individuals, which ends with an unequal share of benefits. While selfish individuals gain direct reproductive benefits, altruists get fewer reproductive benefits and in some extreme cases, they lose the ability to reproduce. In this case, altruists devote their lives to help the dominant individual. Two theories can explain the evolution and maintenance of cooperation in reproduction within societies.

Kin selection theory (Hamilton 1964ab; Hamilton 1972) explains helping behavior when individuals are related. In this case, the individual that gains few reproductive benefits, or gives up reproduction, can still pass on its genes to the next generation through the individual that reproduces. The loss of direct reproduction can be outweighed by the benefits of indirect reproduction, explaining why some individuals totally give up the possibility of reproduction.

Mutualism is a theory that explores cooperation between non-kin (Michener 1974). If being in the group makes one much more successful than being alone, then groups of non-relatives can form (Williams 1966) and individuals cooperate in order to increase their own benefits. But a cooperating group may be vulnerable to cheaters who reap group benefits without paying the costs. Any given individual may either cooperate or cheat. Within cooperating groups, selfish cheaters may do better than real cooperators
because they gain the benefits of cooperation without paying the costs. If this is not controlled by others, cheating can easily spread. Who gains reproductive benefits and who does not can not be predetermined when the group forms or the victims would not join groups without relatives. Cheating is expected to be frequency-dependent because when it becomes too common, there are too few cooperators left to victimize. These conditions mean sociality based on mutualism is likely to be more limited than that based on kin selection.

Examples of reproductive cooperation between kin and between non-kin are widespread in nature. Social insects represent an important example (Hamilton 1964). They have societies formed by related (Crozier and Pamilo 1996; Queller and Strassmann 1998) or by unrelated individuals (Bernalsoni and Strassmann 1999; Queller et al. 2000). Although individuals cooperate, genetic conflict of interests over reproduction arises among colony mates. Queen-worker conflicts arise over the female-male sex ratio within the colony. Due to the haplodiploid genetic system of social insects, the queen is equally related to her son and daughters by a relatedness degree of 0.5, while a worker is related to her full sisters by 0.75 and to her brothers by 0.25. Therefore, while the queen benefits by investing equally in female-male production (sex ratio 1:1), the workers gain benefits by rearing more females than males, favoring a sex ratio of 3:1 (Trivers and Hare 1976).

Multicellular organisms provide further examples of cooperation between related and between unrelated individuals (Gadakar and Bonner 1994; Armstrong 1984; Kessin 2001). Multicellular organisms are generally clonal, having all the cells derived from division of a single cell. Therefore, cells are expected to be highly cooperative and free of conflicts. Despite this high level of cooperation, conflicts over reproduction may arise
within multicellular organisms. Within the same multicellular organism the coexistence of genetically distinct cells can arise, by a mutational process, as in cancer cells. Some multicellular individuals are formed by an aggregation of genetically different cells (chimeras). The coexistence of genetically distinct cells within the same multicellular organism can lead to competition over dominance in reproduction (Buss 1999). For example, in the colonial tunicate *Botryllus schlosseri* one of the clone conquers the reproductive part of chimeras, acting like a cheater (Pancer 1995), and exploits the other by relegating it to the non-reproductive part. Therefore, chimeras allow the investigation of the evolutionary question of how cooperation and conflicts can coexist within societies.

Relatedness, or the lack of it, can be a key feature explaining the spread of cheaters within chimeras. In a chimera, the indirect benefits of helping decrease, because altruists are less likely to be genetically related to the reproductive part that gains benefits. On the contrary, when related individuals aggregate, altruists still gain indirect benefits. If individuals can assess relatedness, it should affect the behavioral strategy that they will play. If a clone is rare, it benefits by avoiding allocation of cells into the non-reproductive part. If two unrelated clones are mixed at 20% and 80%, then the average relatedness of the rare type to its whole group is 0.2 and the average relatedness of the common type is 0.8. Therefore, when rare, a clone should cheat more. The strategy that a rare individual plays against a common one may be different from the strategy adopted when each individual is rare. For an individual a strategy that is stable to cheating could be to behave as a cheater when rare and as an altruist when common.
The social amoeba, *Dictyostelium discoideum*, allows investigation of cheating behavior within chimeras and whether or not cheating is a strategy that might be affected by relatedness, because it forms its multicellular stage by aggregation then can include non-relatives.

*D. discoideum* is a haploid slime mold that lives in the soil of deciduous forests in the temperate zone (Bonner 1967; Landolt & Stephenson 1990; Raper 1984). For a long time, it has been a model system for development and cell signaling (Kessin 2001). Recently, it has been identified as an interesting organism to study social questions because it exhibits fascinating social behaviors (Strassmann 2000b). When food is depleted, *D. discoideum* amoebae cooperate and adopt the strategy of aggregation, a process mediated by release of cyclic adenosine 3’, 5’-phosphate (cAMP) (Barkley 1969; Konijn et al. 1967). Aggregation is a crucial event in the *D. discoideum* life cycle. Amoebae pass from a unicellular to a multicellular stage, which shows all the features of a society. The multicellular stage is initially a slug, which migrates from harsh conditions to better conditions for dispersal (Kessin 2001), (Fig. 3.1). Amoebae within the slug start a process of differentiation into different types of cells (Bonner 1967; Raper 1984) that culminates in a completely transformed multicellular structure: the fruiting body (Raper 1984). Roughly 20% of amoebae form the stalk of the fruiting body and die, while the remaining 80% allocate into the sorus, becoming reproductive spores. Due to the relatively high proportion of cells that sacrifice reproduction and die, genetic conflicts of interest are likely to arise within the multicellular stage.

Genetic studies recently revealed an unknown aspect of the *D. discoideum* life cycle. Amoebae from genetically different clones can aggregate (Fig. 3.2) to form a slug
that undergoes differentiation into stalk and sorus (Strassmann et al. 2000b). This aggregation of genetically distinct clones leads to the formation of chimeras (Strassmann et al. 2000b; Queller et al. in press). In a chimera, the clone that preferentially allocates cells into the sorus is the cheater and gains reproductive benefits. This strategic behavior is supported by the finding that under laboratory conditions, some clones are over represented in the spores and under represented in the stalk, dominating the victim clone (Strassmann et al. 2000b). Cheaters are selfish individuals that unfairly exploit benefits of reproduction, by contributing less than their proportional share to the stalk, without paying the costs (Velicer 2000; Strassmann 2000a). These observations show that in *D. discoideum* chimeras, there is reproductive division of labor, and clones compete over reproduction. Chimeras are also likely to occur in nature, genetically distinct clones are found in close proximity in small (0.2 gm) soil samples (Fortunato et al. in prep). Consequently, we expect that cheating may occur in nature.

In the forest soil, the high density of amoebae (Eisenberg 1976; Cavender 1980; Stephenson and Landolt 1996), and the variability in clonal abundance (Fortunato et al. in prep) leads to the expectation that the frequency of a clone in amoebae aggregation might vary, and therefore potentially affect the cheating strategy. If *D. discoideum* clones can assess their frequency in an aggregation, *D. discoideum* cheaters should be able to spread by exploiting altruists when cheaters are rare and their victims are common.

The aim of my study is to investigate whether or not the initial frequency of a clone in a *D. discoideum* affects its ability to preferentially contribute cells to the fertile spore tissues. I used DNA microsatellite markers to investigate cheating as a frequency dependent strategy in *D. discoideum* chimeras. A game theory model predicts the fate of
cells in a mixture depending on their frequency (Matsuda and Harada 1990; Hudson et al. 2002) and it states that clones below a threshold percentage would not behave altruistically. A rare clone in a mixture is less related to others therefore it should avoid paying the costs of stalk formation to benefit non-relatives. In aggregates formed by unrelated individuals, a cheater that always reproduces can gain a fitness advantage (Matapurkar and Wolve 1997).

The frequency-dependent strategy is subtle and would imply that *D. discoideum* amoebae are able to recognize clone mates versus non-clone mates and to assess their own frequency. The assumption that *D. discoideum* is able to assess its frequency when in mixture is fundamental to this hypothesis. Although *D. discoideum* is able to assess the density of starved cells, through a chemical communication mediated by the conditioned medium factor (CMF) (Gomer 1994a, 1999), there is no information on whether clones can recognize clone-mates.
**FIGURE 3.1.** *Dictyostelium discoideum* life cycle. This figure shows both the vegetative cycle and the developmental cycle. During the vegetative cycle, *D. discoideum* reproduces clonally, dividing by binary fission. Starvation induces *D. discoideum* to start the developmental cycle, during which amoebae aggregate, forming a slug that undergoes differentiation into a fruiting body. Amoebae forming the reproductive spores of the fruiting body reproduce asexually. ([www.unikassel.de/fb19/genetics/projects/devcyc.gif](http://www.unikassel.de/fb19/genetics/projects/devcyc.gif)).
FIGURE 3.2. Genetic chimeras formation in *Dictyostelium discoideum* (www.ruf.rice.edu/~evolve/ study.html. Used with permission of Mary Wu © 2000). Genetic distinct clones can aggregate to form a multicellular slug, which transforms into a fruiting body. Conflicts of interests over reproduction are likely to arise within chimeras.
MATERIALS AND METHODS

In order to investigate whether a rare clone cheats more when rare in the aggregate, I performed pairwise mixing experiments using three different proportions of clones in the cell mix: 20:80, 50:50, 80:20 (Fig. 3.3). I then quantified the relative proportion of the two clones in the sori (Fig. 3.4).

I used 3 clones of *D. discoideum* (98.1, 85.2 and 105.1) collected at Little Butts Gap, North Carolina (Francis and Eisenberg 1993). Clones were stored as spores at 4 °C in silica gel.

Clone preparation:

I first grew clones individually. I ground granules of silica gel and mixed them with 150 µl of *Klebsiella aerogenes* and grew each *D. discoideum* clone on SM/5 plates (Glucose 2g, Oxoid Bacto peptone 2g, Yeast extract 2g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 g, KH<sub>2</sub>PO<sub>4</sub> 1.9g K<sub>2</sub>HPO<sub>4</sub> 1 g, Bacto agar 20g, H<sub>2</sub>O to 1000 ml for 40 Petri dishes). This first step allows me to grow fruiting bodies, which I used to obtain cells for mixing experiments.

Pairwise mixing experiments:

To perform pairwise mixing experiments I used different percentages of starved cells instead of different percentages of spores to avoid the problem of differential growth between clones. Differential growth could lead one clone to reproduce more than another.
To obtain cells, I collected five sori of each *D. discoideum* clone and grew them on 3 SM/5 plates, providing them with an initial quantity of 150 µl of *Klebsiella aerogenes* at stationary stage as food source. These *K. aerogenes* consumed the nutrients in the plate and increased in number allowing plenty of food for the *D. discoideum* amoebae. After 24 hours, I washed plates using KK$_2$ buffer (KH$_2$PO$_4$, K$_2$HPO$_4$) and collected each *D. discoideum* clone in centrifuge tubes. At this stage of the *D. discoideum* life cycle, cells are reproducing vegetatively (Kessin 2001) and are not aggregating. Therefore, I was certain to collect only single cells and no cell aggregations, which would not have given me a good estimate of the number of cells, and which could have cells that already started to differentiate.

I spun each tube in the centrifuge at 2000 rpm for 5 minutes and discarded the supernatant. I resuspended cells with new KK$_2$ buffer, in order to separate them from bacteria. I repeated this procedure several times until all bacteria were washed out. Therefore, cells would begin to starve. I then counted cells of each clone using a hematocytometer. I diluted each clone until I could count a number of cells low enough for easy counting, but still high enough to reduce the variability between different counts. I counted cells from five squares, repeated the counts for each clone, and estimated the mean number of cells. After identifying the clone with the lower density, I brought the density of each one to the same value, by diluting the more concentrated ones. This step allowed me to simplify the estimation of cell percentages in the next step of mixing clones. For each pair of clones, I did three different treatments: 20:80, 50:50, 80:20, where the number on each side of the colon represents the percentage of the two clones. Thus in each pair, each clone is tested as rare, equal and common.
I plated out 100 µl of each pairwise mix on 2 starving plates (50X starving buffer 20 ml, Bacto agar 20 g, H₂O 980 ml; Starving buffer; Na₂HPO₄ 1.78 g, KH₂PO₄ 9.9 g, H₂O 100 ml), equivalent to 4.9-5.6 x10⁶ cells for each experiment. These conditions induce aggregation of cells without further cell division.

Genetic analysis:

As aggregations of cells developed into fruiting bodies, I collected 4 sorocarps from each plate and extracted DNA using 150 µl 5% Bio-Rad-Chlex (Hillis, 1996) and 10 µl Proteinase K (10mg/ml). I ran the DNA extraction in the PTC-100 programmable thermal controller (step1: 56.0 C for 4 hours step 2: 98.0 C for 30 minutes step 3: END).

I then genotyped the DNA extracts to assess the relative abundance of the 2 clones in the sorocarps (Fig. 3.4). I genotyped clones using two primers, Dict13.CAT and Dict19.AAC (Table 3.1). I chose these primers because they had different alleles for the clones I used. These alleles were not so close together to cause overlap of stutter bands, but not so far apart that they amplify differentially (Table 3.2). If DNA bands differ too much in size, the shorter one may amplify better, resulting in differential estimation of two clones. Two other primers I tried in preliminary experiments amplified alleles that were too far apart (Dict25.AAC) or too low in resolution (Dict23.AAC).

I amplified microsatellite loci with a PCR reaction using fluorescent-labeled primers (44 cycles: 95°C for 30s, annealing temperature for 30s, 72°C for 30s, 72°C for 45s). I ran the amplification products on the automatic sequencer, ABI Prism® 3100 (22 cm column) and analyzed alleles of each clone using the program Genotyper 3.7.
Chimeras should show both alleles from the two mixed clones. Since the area of a DNA peak is proportional to the relative abundance of the allele that is amplified it allows estimation of the relative abundance of each clone. Therefore, I used the area of DNA peaks to assess the relative proportion of each clone in the reproductive sori. For each treatment, within the same experiment, I first calculated the total peak area of DNA that was amplified from the sori, and then the proportion of that total area occupied by the allele of each clone.

**Experimental controls:**

I used three different types of experimental controls. The first is the estimation of the percentage of a clone in each experiment by the same PCR method that I also used to assess contribution to spores. I extracted DNA from cells of the same mix I plated out, using 200 μl of 5% Chelex and 10 μl Proteinase K (10mg/ml). This kind of control allows me to check for possible errors in cell counting and for differential amplification of the marker alleles. The second control is the replication of each experiment. I replicated each experiment four times, except for the mix of clones 85.2 and 105.1, which I replicated five times. This control allows me to check for consistency in my results and to check for possible mistakes. The third control is a check of the growth of each clone grown alone in starving conditions. I plated out each clone separately on starving plates, using the same number of cells I used for mixing experiments. Having this control allows me to notice whether clones have developmental problems under these conditions and to make sure that scarcity of one clone in spores of a mixture was not due to inability to develop.
FIGURE 3.3. Procedure for pairwise mixing experiments. Clone growth, cell wash and count and Pairwise mixing experiment procedure.

A → Washing cells → Counting cells → B

Cells growth on SM/5

A

%A 20 50 80

%B 80 50 20

Development control on starving medium

B

Development control on starving medium

Pairwise mixing experiments

Experiments

DNA extract from cells: Controls

Starving medium
**FIGURE 3.4.** Genetic analysis of sori. Sorus collection and assessment of relative proportion of clones within the reproductive sori.

%A %B
20 : 80

50 : 50

80 : 20

Genetic analysis
**TABLE 3.1.** Microsatellite primers used in genotyping. Number of repeats and allele size are for the initial sequence.

<table>
<thead>
<tr>
<th>Locus</th>
<th># of repeats</th>
<th>Allele size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Forward and reverse primers (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dict13.CAT</td>
<td>18</td>
<td>157</td>
<td>49</td>
<td>cccctttttactttttgcac</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ccaacaactataacctcata</td>
</tr>
<tr>
<td>Dict19.AAC</td>
<td>14</td>
<td>173</td>
<td>49</td>
<td>gcttgatggccaatagttc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>tcaaaacctgatccattacc</td>
</tr>
</tbody>
</table>

**TABLE 3.2.** Clone allele sizes. Size (bp) is the allele size run on the automatic sequencer, Abi Prism 3100 (22 cm column). I used primer Dict19.AAC for all three pairs of clones (*), while I used Dict13.CAT only for two pairs: 85.2 and 105.1 (**) and 98.1 and 105.1 (^).

<table>
<thead>
<tr>
<th>Locus</th>
<th>85.2</th>
<th>98.1</th>
<th>105.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dict13.CAT</td>
<td>187**</td>
<td>187^</td>
<td>184**^</td>
</tr>
<tr>
<td>Dict19.AAC</td>
<td>161*</td>
<td>158*</td>
<td>173*</td>
</tr>
</tbody>
</table>
RESULTS

Figure 3.5 shows the proportion of clones in the reproductive sori, depending on their initial frequencies in aggregates. This analysis allows me to assess whether any clone shows consistent cheating and also if cheating depends on the initial frequency of a clone in the cell mixture. For each replicate of the experiments, I first illustrate the proportion of a clone in the sori as a function of the initial frequencies of that clone as estimated by cell counts, 20%-50%-80% (Fig. 3.5, shown on left). A line consistently below the 0-1 diagonal might indicate that one clone cheats, while if it is consistently above the diagonal it suggests cheating by the other clone. These results could be biased by a differential amplification of alleles. To correct for this possible variable, I then show the relative proportion of each clone in the spores versus the percentage of the clone estimated analyzing the cell mixture by PCR (Fig. 3.5, shown on right). For each replicate, the two methods give very similar results, suggesting that differential amplification is not a problem.

The results of my experiments indicate that clone 85.2 might show cheating when forming chimeras with clone 105.1. All the repeats of the experiments, except for repeat 1, consistently show a line above the diagonal one (Fig. 3.5). The inconsistent result of the first repeat might be due to errors when performing the experiment. Clone 98.1 loses its competition when in chimeras with clone 85.2, allocating fewer cells in the spores. The situation is less clear when clone 98.1 aggregates with clone 105.1. In one replicate (3) clone 98.1 clearly shows cheating, but this result is not confirmed by any of the other
replicates, which show that clone 98.1 allocate less into spores than its proportional initial frequency. Figure 3.6 shows results for each experiment with all replicates combined.
FIGURE 3.5. Relative representation of each clone in spores. Each graph shows the proportion of the first clone in the Pairwise mixing experiment versus theoretical proportions 20%, 50%, 80%. For each experiment, I also represent the proportion of the same clone versus its relative proportion into the initial cell mixture.
FIGURE 3.6. Relative representation of each clone in spores, estimated by PCR, versus its proportion 20-, 50-, 80- (determined by cell counts), and versus its initial proportion in cell mixtures (estimated by PCR). Each graph shows a combination of all replicates within the same experiment. In the legend, each different symbol corresponds to a different replicate.
An effect of frequency or relatedness would be clear if the line connecting the points crosses the diagonal. For example, if it starts out above the diagonal and ends up below it, then it is evident that the rare clone is always getting more cells into the spores than is expected, and if the line crosses the diagonal in the opposite manner, it indicates a common-clone advantage. But this rule will not necessarily pick up all interesting frequency effects, particularly if the spore line is always above or always below the diagonal. For that reason, I transformed the peak area data in a way that converts the measure from one of total PCR signal from each clone, to a measure of per capita signal from each clone (Fig. 3.7). The peak areas of the 50:50 mixtures are already suitable, but the 20:80 and 80:20 mixtures need converting. In each case, I multiplied the peak area of the 20% clone by 4. This compensates for the fact that the 20% clone has 1/4 of the cells put into the mixture as the 80% clone. Therefore even if there is differential amplification, the two peaks should be in the same ratio as the two peaks from the 50:50 mixture. The peak areas are then converted into relative area of clone 1 over the total, as before. After this transformation to per capita signal, the three points should lie on a flat line of zero slope if there is no frequency effect. That is, the per capita signal should be the same, no matter what the frequency. If, however, the slope is negative, it means the per capita signal increases for whichever clone at 20% frequency. And if the slope is positive, it means the per capita signal decreases for whichever clone is at 20%.

I plotted the transformed values versus the proportion of the clone in the mixture, estimated by cell counts. Comparing both analyses, of spores and cell mixes estimated by PCR, allows me to assess whether the observed pattern is consistent with a frequency effect and whether there is discrepancy between the analysis of spores and of cell mix.
The line showing the cell mix is expected to be straight, while the line representing the proportion of the clone into spores can change. If clones do not adopt a strategy that varies with their frequency (relatedness) in the mixture, as predicted by the null hypothesis, then the regression line should be straight (Fig. 3.8). A negative slope indicates the advantage of the rare clone, while a positive slope shows the advantage of the common clone.

My results do not show any evidence of cheating behavior dependent on the initial relative proportion of the clones aggregating (Fig. 3.9, 3.10); none of the regression lines had slope different from zero (Table 3.4). In table 3.3, I report all the p values for regression analysis of each pairwise mixing experiment and for each replicate. Figure 3.10 shows results for the same transformed data, with combined replicates. Regression p values for this analysis are on Table 3.4.

The analysis of chimeras formed by clones 85.2 and clone 105.1 does not conform to an advantage of the rare clone (Fig. 3.10, show on right). Although the negative slope of the regression line might look consistent with a relatedness-dependent strategy of clone 85.2 when mixed with clone 105.1 the regression line is not significant (Table 3.4).

The analysis of clone 98.1, when in chimeras with clone 85.2, yields a flat line (Fig. 3.10, show on right), which indicates that the initial frequency of the clone does not have an effect on its strategy. The regression analysis is not significant (p = 0.18 and p = 0.41 for cell mix and spores, respectively; Table 3.4). Replicate 1 for this experiment would suggest that the frequency of clone 98.1 affects its allocation into spores (Fig. 3.9t), but this effect is not confirmed by the other replicates.
When clone 98.1 forms mixtures with clone 105.1 the regression line has a positive slope (Fig. 3.10, show on right), that might suggest an advantage of the clone. Replicate 2 shows a significantly positive slope \( p = 0.025 \); Fig. 3.9n; Table 3.3), but results from all the other replicates do not confirm this (Fig. 3.9 l, p and r). Over all, this experiment does not have any significant evidence of frequency affecting spore allocation.
**FIGURE 3.7.** Transformation of peak area data. In order to normalize the abundance of each clone within treatments, I multiplied times 4 the peak area data of the clone that started at 20% in the cell mixture. I then estimated the total amount of signal amplified in the sori, and calculated the proportion of signal of each clone. The blue line shows the peak area of the clone that before transformation was proportionally lower. The red line shows the peak area of the clone that started at a relative higher proportion.
FIGURE 3.8. Expected regression line after transformation of peak area. This transformation allows representing whether the frequency of a clone in the mixture has an effect in its allocation into spores. If there is no effect, the line should be straight. A negative slope indicates that the rare clone gains reproductive benefits, by allocating cells into spores. A positive slope shows that the common clone in a chimera gains reproductive advantages.

No effect

Rare clone advantage

Common clone advantage
FIGURE 3.9. Hypothesized frequency of a clone in the pairwise mixing experiments. Table 3.5 indicates regression p values for each graph.

Experiment 85.2 + 105.1, replicate 1

A

B

Experiment 85.2 + 105.1, replicate 2

C

D
Experiment 85.2+ 105.1, replicate 3

**E**
Proportion clone 85.2 in cell mix, by cell counts

**F**
Proportion clone 85.2 in cell mix, by cell counts

Experiment 85.2+ 105.1, replicate 4

**G**
Proportion clone 85.2 in cell mix, by cell counts
67.505 - .173*X; R^2 = .995

**H**
Proportion clone 85.2 in cell mix, by cell counts
89.5 - .389*X; R^2 = .999
Experiment 85.2+105.1, replicate 5

**I**

![Graph](Image)

Proportion clone 85.2
in cell mix, by cell counts

**J**

![Graph](Image)

Proportion clone 85.2
in spores by PCR
Experiment 98.1+ 105.1, replicate 1

K

L

Proportion clone 98.1 in cell mix, by cell counts

Experiment 98.1+ 105.1, replicate 2

M

N

Proportion clone 98.1 in cell mix, by cell counts
Experiment 98.1+ 105.1, replicate 3

O

P

Experiment 98.1+ 105.1, replicate 4

Q

R
Experiment 98.1+85.2, replicate 1

**S**

Cell: 98.1+85.2, 1

Proportion clone 98.1 in cell mix, by cell counts

**T**

Cell: 98.1+85.2, 1

Proportion clone 98.1 in spores by PCR

Experiment 98.1+85.2, replicate 2

**U**

Cell: 98.1+85.2, 2

Proportion clone 98.1 in cell mix, by cell counts

**V**

Cell: 98.1+85.2, 2

Proportion clone 98.1 in spores by PCR
Experiment 98.1+85.2, replicate 3

**W**

Cell: 98.1+85.2, 3

Normalized proportion 98.1 in cell mix, by cell counts

Proportion clone 98.1 in cell mix, by cell counts

**X**

Cell: 98.1+85.2, 3

Normalized proportion 98.1 in spores by PCR

Proportion clone 98.1 in cell mix, by cell counts

Experiment 98.1+85.2, replicate 4

**Y**

Cell: 98.1+85.2, 4

Normalized proportion 98.1 in cell mix, by cell counts

Proportion clone 98.1 in cell mix, by cell counts

**Z**

Cell: 98.1+85.2, 4

Normalized proportion 98.1 in spores by PCR

Proportion clone 98.1 in cell mix, by cell counts
**TABLE 3.3.** Regression p values for the hypothesized frequency of a clone in each pairwise mixing experiment; transformed data. The clone analyzed is the first one of the cell mix. P values refer to the analysis of one clone in the spores and to the analysis of the same clone in the initial cell mix.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Replicate</th>
<th>P values for spores</th>
<th>P values for cell mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>85.2+105.1</td>
<td>1</td>
<td>0.354</td>
<td>0.341</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.424</td>
<td>0.257</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.195</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.218</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.179</td>
<td>0.421</td>
</tr>
<tr>
<td>98.1+105.1</td>
<td>1</td>
<td>0.418</td>
<td>0.399</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.025</td>
<td>0.765</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.516</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.151</td>
<td>0.317</td>
</tr>
<tr>
<td>98.1+85.2</td>
<td>1</td>
<td>0.368</td>
<td>0.579</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.093</td>
<td>0.308</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.296</td>
<td>0.486</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.245</td>
<td>0.181</td>
</tr>
</tbody>
</table>
FIGURE 3.10. Hypothesized frequency of a clone in the pairwise mixing experiments. For each graph, replicates are combined. Across experiments, each replicate number has the same symbol. Table 3.6 indicates regression p values for each graph.
**TABLE 3.4.** Regression p values for the hypothesized frequency of a clone in each pairwise mixing experiment; transformed data. The clone analyzed is the first one of the cell mix. P values refer to the analysis of one clone in the initial cell mix and to the analysis of the same clone in the spores. For each experiment replicates are combined. If there is no effect of initial frequency on spores allocation, the slope of the regression line does not significantly differ from zero.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>P values for cell mix versus cell mix, by cell counts</th>
<th>P values for spores versus cell mix, by cell counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>85.2+105.1</td>
<td>0.155</td>
<td>0.377</td>
</tr>
<tr>
<td>98.1+105.1</td>
<td>0.293</td>
<td>0.604</td>
</tr>
<tr>
<td>98.1+85.2</td>
<td>0.183</td>
<td>0.412</td>
</tr>
</tbody>
</table>
DISCUSSION

My results do not support the hypothesis that the initial relatedness of a clone in a *D. discoideum* chimera can affect its allocation into spores (Fig. 3.8, Table 3.6). The initial concentration of a clone in a mixture did not affect its allocation into reproductive spores. In fact, in the experiments, the proportion of a clone in spores was positively related to its initial frequency. The high number of replicates showed clearly that within chimeras individual cells of the common and rare clones were equally likely to get into spores.

Since I did not find evidence for a cheating strategy dependent on the initial relative proportion of clones, it is possible that clones are not able to detect the presence of foreign clones. All clones I used for my experiments developed normally under starving conditions even without a partner. They also formed chimeras, suggesting that they did not develop recognition systems to exclude foreign clones. This observation confirms what already observed in a previous study (Strassmann 2000b). In chimeras formed by unequal proportions of two clones, a kin recognition system might allow the common clone to exclude the rare one from allocating cells into the reproductive spores, as happens in marine invertebrates (Feldgarden and Yund 1992). On the other hand, the rare clone might strategically attempt to hide its kin status in order to achieve higher fitness benefits within the chimera. In social insect colonies formed by different genetic lineages, this strategy of modifying recognition signals allows workers of the less
represented lineages to gain more help from workers of the common genetic one (Reeve 1998).

It could also be possible that although clones are able to determine their presence within a chimera, they are not necessarily able to assess their relative abundance. On the contrary, if *D. discoideum* amoebae are able to assess their relative proportion, it could be that the frequency I used to perform pairwise mixing experiments may be above a threshold value. Under these conditions, it could be hard for a clone to distinguish foreign clones. Decreasing the proportion of a rare clone, until it reaches a very low proportion within the aggregate, such as 1%, could induce the clone to adopt the strategy of the cheater, allocating cells into the reproductive spores.

It is possible that if *D. discoideum* clones are able to recognize the presence of foreign clones in a chimera they adopt the strategy to alter the developmental program of the other clone, by contributing less to the stalk than they might otherwise. The intercellular signal differentiation inducing factor or DIF, a chlorinated alkyl phenone, is a possible candidate in this process. DIF promotes the stalk cell pathway during the first stages of cell differentiation within the slug, by inducing genes characteristic of stalk cells and repressing those expressed in spore cells (Kay and Thompson 2001). Ennis et al. (2000) postulated that another mechanism of cheating is based on the manipulation of the pathway that determines the differential fate of cells. According to their study, the cheater would not degrade a protein that induces, at high levels, the prespore pathway. Cheating behavior is particularly expected if clones are able to recognize that they are in minority thus allocating less than they should into the stalk (Matsuda and Harada 1990).

Under natural conditions, aggregations of unrelated cells could be favored because amoebae achieve higher fitness by reaching an optimal group size. Aggregation
is a survival strategy for amoebae when starved of bacteria. If a clone is not abundant, it might benefit by aggregating with a foreign clone instead of forming a small aggregation. Aggregations of small size might not be successful in reaching better locations, resulting in high fitness costs (Gomer 1994b). One of the benefits of being in a chimera is the increased slug movement of a bigger slug (Foster et al. in press.). As a consequence, a rare clone that chooses to form a chimera could achieve higher fitness than being alone, despite the reproductive costs. A rare clone could still reach a different patch of soil to fruit, while it would not have this opportunity by itself, since alone it could only form a small aggregation. Cells that fail to aggregate are destined to die, because D. discoideum does not form microcysts. Therefore, clones mutually cooperate in forming chimeras because both achieve higher fitness by aggregating. Under these conditions, cheating is likely to arise where one clone behaves selfishly, exploiting the other.

In order to have a deeper comprehension of cheating as frequency-dependent strategy in D. discoideum chimeras, it could be useful to repeat pairwise mixing experiments. It might be important to change one of the steps I used in the methodology. To favor aggregation of clones and formation of chimeras, I spread cell mixes all over the surface of small petri dishes (60*15 mm). It could be interesting to evaluate the affect of cell density on the interactions of co-aggregating genetically distinct clones. Density affects the aggregation process of D. discoideum (Gomer 1999). I also suggest repeating the experiments using more clones, so as to increase the probability of detecting frequency-dependent cheating. It also could be interesting to investigate whether cheating occurs when a clone is extremely rare in the aggregate, as 1%. The methodology I used does not allow detecting very low proportion of a clone, therefore it could be possible to use other techniques, like cell-tracker dyes, to quantify the allocation into spores of the clone that starts at 1% in the initial cell mix.
ACKNOWLEDGEMENTS

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CHAPTER 4

OCCURRENCE OF SEXUAL REPRODUCTION IN A NATURAL POPULATION OF *DICTYOSTELIUM DISCOIDEUM*
ABSTRACT

This research investigates the occurrence of sexual reproduction in a natural population of the social amoeba, Dictyostelium discoideum. Although multicellular development in the slime mold D. discoideum has been intensively studied and is well understood, their sexual reproduction is still enigmatic. Indeed, whether sexual recombination occurs at all has been the subject of much debate. To address whether sexual reproduction occurs in natural populations of D. discoideum, I estimated linkage disequilibrium (D') of six tightly linked and eight other loci. The average D' for all loci (0.60 ± s.e. 0.02) indicates that asexual reproduction has a strong influence in shaping the genome. However, the D' of three pairs of tightly linked loci differed significantly from the other D' of those same six loci (p = 0.0199), suggesting that recombination does occur between loci that are not tightly linked. To assess whether recombination is due to sexual reproduction or to parasexual reproduction, for each locus, I compared the mean D' of the loci that are on the same chromosome, which would rarely recombine under parasexuality, with the mean D' of the loci on different chromosomes, excluding tightly linked loci. This difference was not significant (paired t-test, t = -1.571, p = 0.155), but loci on the same chromosomes had lower mean D' (average D' = 0.75) than loci on different chromosomes (average D' = 0.858). These results suggest that although sexual reproduction in nature might be obscured by the high degree of asexual reproduction, sexual reproduction in fact does occur.
**Key words:** linkage disequilibrium, *Dictyostelium discoideum*, sexual reproduction, paragexual reproduction.
INTRODUCTION

*D. discoideum* has been used as a model organism to investigate signaling, differentiation and other cellular processes, since it has a large, motile eukaryotic cell and also undergoes multicellular development (Kessin 2001). *D. discoideum* is a cellular slime mold that lives in the soil of deciduous forests in the temperate zone (Bonner 1967; Landolt & Stephenson 1990; Raper 1984). These amoebae spend most of their life cycle as haploid cells reproducing by binary fission. However, when conditions are such that amoebae starve and detect starvation in others, they can adopt one of two strategies to survive: form fruiting bodies or macrocysts. One of the possible strategies is aggregation. If only starvation occurs, amoebae release a chemo-attractant, cAMP (cyclic adenosine 3', 5' phosphate) (Konijn et al. 1967) and aggregate to form a multicellular structure known as a slug, which can migrate to reach optimal locations where they then differentiate into a fruiting body (Kessin 2001). After reaching a new location, the slug transforms into a fruiting body characterized by a stalk and a sorus (Raper 1984). What makes the fruiting body interesting for evolutionary questions is the differential fate of the cells constituting the stalk and the sorus. The stalk cells vacuolate and die, while the sorus cells become the reproductive spores and, after dispersal and germination, yield a new generation of amoebae. Then, amoebae start a new life cycle.

The alternative survival strategy leads to the formation of macrocysts, a mechanism amoebae rely on under conditions of both starvation and submersion (Kessin 2001). This is the stage in the *D. discoideum* life cycle in which sexual reproduction may
occur. Macrocysts (Fig. 4.1, 4.2) form when two cells of the same or of complementary mating types (Higuchi et al. 1995; Urushihara 1996) join and form a giant cell, analogous to a zygote. By release of cAMP, the giant cell attracts and engulfs other cells, which become endocytes (Kessin 2001; O'Day 1979). Endocytes die while the giant cell eventually undergoes meiotic recombination and may yield recombinant progeny. Although sexual reproduction has been observed in Dictyostelium mucoroides (MacInnes and Bonner 1974) and in Dictyostelium giganteum (Erdos et al. 1975), whether it occurs under natural conditions in Dictyostelium discoideum is unknown.

The macrocyst is not the only stage in the D. discoideum life cycle in which recombination might occur. Gene exchange in D. discoideum might also occur during the formation of a diploid stage, an event that occurs at low frequencies (10^{-5} - 10^{-6}) under laboratory conditions (Katz and Sussman 1972; Newell 1982). This process of recombination, known as parasyxuality, or mitotic recombination, takes place when two amoebae of the same mating type fuse and then go through mitotic divisions. They get back to the haploid state by losing chromosomes one at a time in a little-understood process (Robson and Williams 1979; Francis and Eisenberg 1993). Parasyxuality has been used as tool to investigate the genetic basis of D. discoideum development (Williams and Newell 1976; Coukell 1975) and to map the chromosomes (Wallace and Newell 1982; Welker and Williams 1982). This form of reproduction has also been investigated in imperfect fungi, though only under laboratory conditions, as an alternative to sexual recombination (Taylor et al. 1999).

One of the important differences between the two forms of reproduction is in the degree of genetic exchange. During sexual reproduction, there is recombination both
within and between chromosomes, while during parasexuality recombination occurs
between chromosomes, but extremely rarely within chromosomes (Taylor 1999).

Earlier studies on recombination during macrocyst formation in *D. discoideum* led
to controversial conclusions. In one of the studies, Francis and Eisenberg (1993)
investigated the genetic structure of a natural population of *D. discoideum* to determine
whether genetic exchange occurs within the population. They found little disequilibrium,
which implies that there is gene flow in the population. In contrast with these findings,
Francis and Eisenberg also obtained two other results. First, the haplotype of some
clones they collected had not experienced any change in years, when compared to clones
previously analyzed from the same site. This suggests lack of recombination in nature.
Second, the genetic analysis of progeny germinated from macrocysts induced under
laboratory conditions yielded no recombinants, meaning that meiotic recombination was
absent. Therefore, Francis and Eisenberg concluded that sexual recombination probably
did not occur in the population. Despite the lack of recombination in laboratory
conditions, the authors did not exclude the possibility that genetic exchange occurs in
nature during the formation of diploids.

The lack of recombinants, as observed by Francis and Eisenberg (1993), could be
the result of a genetic conflict of interests between clones forming the giant cell. In fact,
a macrocyst is analogous to a chimera composed of multiple cell lineages, in which
genetic conflict of interest over reproduction can occur and cheating can arise.
Therefore, one clone might cheat by attracting and eating the other clone, instead of
fusing. In this scenario, one of the two clones has the same fate as endocytes. This
behavior could be a strategy that the cheater adopts to ensure its own reproduction.
In the other study, Francis (1998) found different results analyzing the genetic exchange between two strains of *D. discoideum*. Analyzing the macrocyst progeny germinated under laboratory conditions, he observed a high rate of recombination among linked genes. This confirmed a previous study done on the same strains (Wallace and Raper 1979).

These studies reveal that macrocysts reared under laboratory conditions may not be appropriate to investigate sexual reproduction because of their long development time and low germination rate (O'Day 1979; Higuchi et al. 1995). Even though good conditions for macrocyst germination have never been worked out for the laboratory (Raper 1984; Francis and Eisenberg 1993; Francis 1998), macrocyst formation and sexual reproduction could be common in the wild. Due to complications in the previous studies (Francis and Eisenberg 1993; Francis 1998) approaching the problem through the study of macrocysts, I investigated the question of recombination in *D. discoideum* using a different system. I used linkage disequilibrium to explore the occurrence of recombination in a natural population of *D. discoideum*. I also investigated whether recombination really occurs during the sexual stage or if it is due to the exchange of chromosomes during the diploid phase through the parasexual process. Such genetic study of the population allows me to skip the analysis of the macrocyst, yet gives reliable information about the form of reproduction and the system of gene recombination.

Since sexual reproduction in nature can be cryptic if obscured by the degree of asexual reproduction (Kumar et al. 1999), it can be very difficult to determine the form of reproduction by direct observation alone. Therefore, genetic markers are useful tools for this kind of investigation (Burt et al. 1996). The most frequently used are allozymes,
restriction fragment length polymorphisms (RFLP's) and immunological markers (Tibayrenc 1991). Estimating linkage disequilibrium is another important tool for understanding population history (Zhao et al. 1999) and mating behavior (Lewontin 1988; Hedrick 1987) because the degree of linkage disequilibrium mirrors the occurrence of sexual or asexual reproduction. Linkage disequilibrium is a non-random association between alleles at two different loci (Futuyma 1998; Hudson 2001). This association can either be between physically linked alleles, or between unlinked loci, which are on different chromosomes. Therefore, the more appropriate term to indicate this broader association between loci is gametic disequilibrium (Hedrick 1987). In a population that reproduces asexually, gametic disequilibrium is high due to mitotic reproduction of genomes. Under asexual reproduction, the population has a clonal structure, as in Salmonella spp (Maynard Smith 1993) and in Escherichia coli (Whittam et al. 1983; Maynard Smith 1993; Clark and Zheng 1997; Lenski 1993; Guttman et al. 1994). In contrast, in a population with high recombination, gametic disequilibrium is low due to gene reshuffling during the meiotic process, except between tightly linked genes (Tibayrenc, 1991; Futuyma, 1998).

Linkage disequilibrium is a useful tool to assess the form of reproduction in organisms with two forms of reproduction during their life cycle. These organisms commonly reproduce asexually when conditions are stable, but they rely on sexual reproduction when the environment changes under selective pressures. Examples include microbial populations (Maynard Smith 1993; Clark and Zheng 1997; Guttman 1994; Kumar et al. 1999) as well as some fungi: the human pathogen Candida albicans (Gräser
et al. 1996), the plant pathogen Magnaporthe grisea (Kumar et al. 1999) and D. discoideum amoebae (Kessin 2001).

The aim of my study is to assess the level of linkage disequilibrium in a natural population of Dictyostelium discoideum. In this study, I use the term linkage disequilibrium to indicate association between either linked or unlinked loci. A genetic investigation allows me to determine whether recombination in this species occurs in nature and to investigate whether gene exchange is due to sexual or to parasexual reproduction.

If D. discoideum reproduces clonally, there should be high linkage disequilibrium due to the lack of gene reshuffling. If D. discoideum reproduces sexually, or through parasexual reproduction, there should be lower linkage disequilibrium due to gene reshuffling during the meiotic process. Since it is not possible to know exactly how much linkage disequilibrium is expected if recombination occurs, an approach is to look at differences in linkage disequilibrium. Loci immediately next to each other should almost never recombine even with sexual reproduction. So, these loci give us a good estimate of what linkage disequilibrium should be with no sexual recombination. If other unlinked loci have no recombination, then all D' estimates (measure of linkage disequilibrium) should be similar to those of the tightly linked ones. If there is recombination, these other D' s should be lower.

If parasexuality is the cause of allelic reshuffling and linkage disequilibrium breakdown, loci that are paired on different chromosomes should recombine at higher rates than those on the same chromosome. On the contrary, sexual reproduction causes
both intra- and inter-chromosomal recombination, although the latter is low for tightly linked loci. This allows distinguishing between sexual and parasexual recombination.

My study shows that recombination occurs, both between loci on the same chromosome and between loci on different chromosomes. Therefore, this research demonstrates that, although asexual reproduction is the main form of reproduction in *D. discoideum*, sexual reproduction does occur in a natural population of *D. discoideum*. This finding is important for molecular biologists, and their understanding of the effect of multiple mutations. It suggests that efforts to get sexual reproduction under laboratory conditions may not be futile. Sexual reproduction achieved in the lab would make it easier to put multiple mutations together in the same individual to test their combined effects.
FIGURE 4.1. * Dictyostelium discoideum* sexual cycle: macrocyst formation (Lewis and O'Day 1996). This figure represents amoebae of two mating types, in the specific case NC-4 (mating type A1) and V-12 (mating type A2), which fuse forming the giant cell. Cells forming the giant cell attract and engulf other amoebae, and start the formation of a primary and secondary wall that incorporate all the cells. Only cells within the giant recombine. All the other amoebae become endocytes that die.
FIGURE 4.2. Macrocyst. Macrocysts have this characteristic aspect, of several globes, all together underneath a sheath. *D. discoideum* macrocysts range from 35-90 \( \mu \text{m} \). (Photo by Monica Landi,)
MATERIALS AND METHODS

I grew and genotyped 67 clones from a natural population of *D. discoideum* collected in a deciduous wooded area near Mountain Lake Biological Station, Virginia. The clones had been previously collected and isolated from two adjacent soil samples collected each meter along a transect of 25 meters (Fortunato *et al.*, in prep). The top layer of dry leaves was removed, and two plastic straws 6 mm in diameter were plunged approximately 5 mm into the soil. Clones were then isolated and genotyped (for details see Fortunato *et al.* in prep). For each soil sample, I analyzed one individual of each haplotype, based on five microsatellite loci.

Clone growth:

Clones were stored at −80 °C in SM medium (Glucose 10 g, Oxoid Bacto peptone 10 g, Yeast extract 10 g, MgSO₄·7H₂O 1 g, KH₂PO₄ 1.9g K₂HPO₄ 1 g, H₂O to 1000 ml). I grew each clone on SM/5 plates (Glucose 2g, Oxoid Bacto peptone 2g, Yeast extract 2g, MgSO₄·7H₂O 0.2 g, KH₂PO₄ 1.9g K₂HPO₄ 1g, Bacto agar 20g, H₂O to 1000 ml for 40 Petri dishes), adding 150 µl of bacteria *Klebsiella aerogenes* as the food source. When clones aggregated and formed slugs, due to bacteria depletion, I collected a slug from each plate and extracted DNA using 150 µl of 5% Bio-Rad-Chelex (Hillis, 1995).
Locus selection:

To design primers, we first searched for trinucleotide microsatellite repeats in the *D. discoideum* genome database, using the web site www.sanger.ac.uk/Projects. Then, we chose those that were tightly linked (within several hundred nucleotides). Therefore, each pair of primers allowed me to amplify loci that were physically linked. Three pairs of microsatellite loci that I was able to amplify were located on chromosome 2. The other two pairs and the unpaired microsatellite loci were located on chromosome 6 of the *D. discoideum* genome (Table 4.1).

Once contigs were found, we used the program MacVector to design primers.

When I genotyped clones, I also used five primers already designed and used in a previous study to amplify the same population I worked on (Fortunato et al., in prep.), (Table 4.2).

Genetic analysis:

I amplified 16 microsatellite loci for each DNA extract, using fluorescently labeled primers in touchdown PCR reactions (Step 1: 90°C for 3’. Step 2: 90 °C for 30s. Step 3: highest annealing temperature for 30s, -0.5 °C/cycle. Step 4: 72°C for 30s. Step 5: 20 Times to step 2. Step 6: 90 °C for 30s. Step 7: lowest annealing temperature for 30 s. Step 8: 72°C for 30s. Step 9: 10 times to step 6. Step 10: 72°C for 10’. Step 11: 4°C. END). Ten microsatellite loci were in tightly linked pairs and allowed me to amplify loci that were physically linked, while the others were unpaired. I analyzed the products of amplification in the automatic sequencer, AbiPrism® 3100 Genetic Analyzer, running
samples in the 50-cm column. I analyzed allele sizes using the program Gene Scan 3.7 and Genotyper 3.6.

**Linkage Disequilibrium analysis:**

When I genotyped clones, I could not use two of the loci for linkage disequilibrium analysis (15715AAT, 16325CAC), because they yielded little allelic variation. This reduced the number of useful linked pairs to three.

To estimate the degree of linkage disequilibrium, I ran the program Arlequin version 2000.

The measure used to estimate linkage disequilibrium between two alleles:

\[ D_{ij} = x_{ij} - p_i q_j \]

where \( x_{ij} \) is the observed frequency of the haplotype \( A_iB_j \), \( p_i \) and \( q_j \) are the frequencies of the alleles \( A_i \) and \( B_j \). This measure is problematic for the analysis, because it is highly dependent on allele frequencies. A correction dividing \( D_{ij} \) by the maximum \( D \) possible (\( D' \) max) for those frequencies (Lewontin's \( D' \) 1964) improves the performance of this measure:

\[ D'_{ij} = \frac{D_{ij}}{D_{\text{max}}} \]
In order to have a single value that combines all the $D^\prime$ for a pair of loci, I used Hedrick's $D^\prime$, which runs from 0 for random association to 1 (Hedrick 1987). The mathematical expression to calculate Hedrick's $D^\prime$:

$$D^\prime = \sum_i \sum_j p_i q_j \| D_{ij}^\prime \|$$

where $p_i$ and $q_j$ are the frequencies of the alleles $A_i$ and $B_j$.

This measure of linkage disequilibrium between two loci takes into consideration that each locus can have more than two alleles and it is relatively independent of allele frequencies. The independence from allele frequency is particularly important for comparisons between loci with different allele frequencies.

To run statistical tests, I transformed the $D^\prime$ values, using the arcsin of the square root of each $D^\prime$. I then back-transformed mean values to plot $D^\prime$ into graphs.
TABLE 4.1. LOCI GENOTYPED. Chr.: Chromosome. Size (bp): expected allele size. T.a.: Annealing temperature. The database source for all these primers was the Sanger web site (www.sanger.ac.uk/Projects). Loci with the same five-digit number are tightly linked.

<table>
<thead>
<tr>
<th>Chr</th>
<th>Locus</th>
<th>Size (bp)</th>
<th>Primers sequence (5'-3')</th>
<th>T a (° C)</th>
</tr>
</thead>
</table>
| 6   | 14431 AAT | 120       | F: GCAAATCAAATATCACCTAATTTC  
R: AATGTTATTGTGGTTATTGTTATTG | 49.7      |
| 6   | 14431 AAC | 194       | F: CCACAACTTCAAAACACCC     
R: CAACACTTTATCGGATGATTG | 47.4      |
| 6   | 15200 CAT | 136       | F: CACTTGGCTTCTCTGTTCTATCTTC 
R: TTTTCGATGTTGGTGTTGTTATACC | 56.0      |
| 2   | 15715 AAC | 120       | F: GGTTACATAATCGTTTGTTTTTGG 
R: TGTAACTGTGGTTTCTGTGCTG | 57.1      |
| 2   | 15715 AAT | 149       | F: TTTGTTTACAAATATCAACACCC  
R: TACAAATACACATGCAAATCC | 50.6      |
| 6   | 15945 AAC | 235       | F: AAACAACTCCTCCAATTTCAAAG  
R: CTCATTTAATTCTTAAATCTTACC | 50.4      |
| 6   | 15945 AAT | 224       | F: AACCCAGAGATTTTTATGGATG 
R: AGATTCTTTTGATGCTTCAAC | 49.4      |
| 2   | 16020 AAC | 203       | F: TTGGGCATTACATCTCTC 
R: CTCCTTTTGTTGTTGTCATTG | 49.7      |
| 2   | 16020 AAG | 221       | F: GCTAAAAATAGATATACAAATG  
R: CATATCTTTAATTGTTTCTGTTG | 47.7      |
| 2   | 16325 AAG | 171       | F: TTTCCACCATCTTTATCTCCAAC  
R: CACTTTTGTATCATCATGTCATC | 53.7      |
| 2   | 16325 CAC | 206       | F: CCATTAATTTCTCAACCAGAGATG  
R: TTATCCTACTACCACCCGCCCACCG | 55.3      |
**TABLE 4.2.** LOCI PREVIOUSLY GENOTYPED. Chr.: Chromosome. Size (bp): expected allele size. T.a.: Annealing temperature. Chromosomes numbers for all but the first locus are uncertain. Sequences come from libraries enriched in the chromosome listed.

<table>
<thead>
<tr>
<th>Chr</th>
<th>Locus</th>
<th>Size</th>
<th>T. a.</th>
<th>Primers sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Dict5.AAC</td>
<td>240</td>
<td>46</td>
<td>F: GATAGAGAAACTGACAACCTGGG&lt;br&gt;R: GGTGGAGCTTTATTTGTCTACC</td>
</tr>
<tr>
<td>1?</td>
<td>Dict13.CAT</td>
<td>157</td>
<td>49</td>
<td>F: CCCCTTTTACTTTTGAC&lt;br&gt;R: CCAACAAACTATAACCTCATC</td>
</tr>
<tr>
<td>1?</td>
<td>Dict19.AAC</td>
<td>173</td>
<td>48</td>
<td>F: GCTTGAATTTGCCAATAGTTC&lt;br&gt;R: TCAAAACCTGATCCATTACC</td>
</tr>
<tr>
<td>1?</td>
<td>Dict23.AAC</td>
<td>240</td>
<td>49</td>
<td>F: TCATTCAACACCACCAACATC&lt;br&gt;R: AGTAAACAGATGGGCAGGGTTATTAC</td>
</tr>
<tr>
<td>2?</td>
<td>Dict25.AAC</td>
<td>210</td>
<td>49</td>
<td>F: AGAGCCACTCATTATCTATTTCC&lt;br&gt;R: CACAACACTATCAGAAACTG</td>
</tr>
</tbody>
</table>

Database source:
- Genbank
- Dicty-genome project
RESULTS

The average $D^\prime$ for all locus pairs was 0.60 ± s.e. 0.02, suggesting a very strong influence of asexual propagation. However, loci significantly differed in their $D^\prime$ values (ANOVA, df = 13, $p = 0.0088$, Fig. 4.3; Table 4.3). This analysis tells me if I need to correct for locus variation in the subsequent tests.

I did a regression analysis to test whether loci with higher heterozygosity, therefore with more variation, have higher $D^\prime$ values. This result might indicate the influence of a mutational process on the linkage disequilibrium because mutation plays an important role in generating both high variability and linkage disequilibrium. I found a significant regression ($p = 0.014$; Fig. 4.4). This result suggests that mutation might determine association between loci.
FIGURE 4.3. Average D' s of loci ± s. e. Loci differed in their (p = 0.0088). Locus; 1= 15945 AAT; 2= 15945 AAC; 3= 16325 AAG; 4= 15715 AAC; 5= 14431 AAT; 6= 14431 AAC; 7= 16020 AAC; 8= 16020 AAG; 9= 15200 CAT; 10= DICT5.AAC; 11= DICT13.CAT; 12= DICT19.AAC; 13= DICT23.AAC; 14= DICT25.AAC
FIGURE 4.4. Regression plot (p = 0.014). D' values versus expected heterozygosity. Loci with higher variability also have higher linkage disequilibrium, suggesting that mutation might play a role in generating associations between alleles.
TABLE 4.3. Average D' and s. e. for all loci used in the analysis

<table>
<thead>
<tr>
<th>Locus</th>
<th>Average D' ± s. e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>15945aat</td>
<td>0.535 ± 0.043</td>
</tr>
<tr>
<td>15945aac</td>
<td>0.502 ± 0.034</td>
</tr>
<tr>
<td>16325aag</td>
<td>0.545 ± 0.04</td>
</tr>
<tr>
<td>15715aac</td>
<td>0.622 ± 0.062</td>
</tr>
<tr>
<td>14431aat</td>
<td>0.542 ± 0.02</td>
</tr>
<tr>
<td>14431aac</td>
<td>0.618 ± 0.028</td>
</tr>
<tr>
<td>16020aac</td>
<td>0.532 ± 0.02</td>
</tr>
<tr>
<td>16020aag</td>
<td>0.628 ± 0.031</td>
</tr>
<tr>
<td>15200cat</td>
<td>0.580 ± 0.07</td>
</tr>
<tr>
<td>DICT5.AAC</td>
<td>0.655 ± 0.041</td>
</tr>
<tr>
<td>DICT13.CAT</td>
<td>0.647 ± 0.026</td>
</tr>
<tr>
<td>DICT19.AAC</td>
<td>0.595 ± 0.047</td>
</tr>
<tr>
<td>DICT23.AAC</td>
<td>0.746 ± 0.033</td>
</tr>
<tr>
<td>DICT25.AAC</td>
<td>0.669 ± 0.035</td>
</tr>
</tbody>
</table>
The difference between the D' of the three pairs of tightly linked loci (mean 0.73 ± s. e. 0.04, n = 3) and the D' of all the other loci (mean 0.60 ± s. e. 0.02, n = 88) was not significant (unpaired t-test, d.f. = 89, t = 1.217, p = 0.227), although it was in the predicted direction for recombination (Fig. 4.5). Since we know there will be little recombination between the linked loci, this result appears to suggest that there is also little recombination among other loci. However, because one set of D' s had only 3 values and because loci varied in their average D', this result could be misleading. This is particularly true because the six loci involved in the tightly linked D' s had low average D' s.

To control for variation in D' between loci, I repeated the comparison using D' s from only the six loci that were in tightly linked pairs. I was able to detect a small but significant influence of recombination by comparing D' for three pairs of loci that were very tightly linked (mean 0.73 ± s. e. 0.04, n = 3) to other D' s of those same six loci (mean 0.52 ± s. e. 0.02, n = 36), (unpaired t-test, d.f.= 37, t = 2.434, p = 0.0199). If all reproduction is asexual, these two D' s should be the same. In fact, the tightly linked loci have significantly higher D' (Fig. 4.6), indicating that the other loci had experienced some recombination.
**FIGURE 4.5.** Average $D'$ and s. e. for tightly linked loci and all the other loci (t-test, $p = 0.227$). Tightly linked loci did not differ significantly from all the other loci, in their $D'$. This result suggests that loci do not recombine.
FIGURE 4.6. Average $D'$ and standard error for tightly linked loci and other loci paired to those same six loci (t-test, $p = 0.0199$). Tightly linked loci had significant higher $D'$, meaning that their alleles were more highly associated than the other loci. This result shows that compared to linked loci that do not experience recombination, the other associations between loci have been broken down.
To assess whether recombination is due to sexual reproduction or to parahsexual reproduction, I compared all the D's of loci on the same chromosomes to all the D's of loci on different chromosomes. Loci with uncertain chromosome assignments were excluded. Both sexual and parahsexual processes cause recombination between chromosomes, but only sexual reproduction also causes considerable recombination within chromosomes. The mean D' of loci on different chromosomes was greater (average D' = 0.858 ± s. e. 0.05) than the mean D' of loci on the same chromosome (average D' = 0.75 ± s. e. 0.06), although the difference was not significant (unpaired t-test, df = 31, t = 1.423, p = 0.165) (Fig. 4.7). The lack of difference could be due to variation between loci, so I repeated the test keeping control of this variation. I compared for each locus, the mean D' of the loci that are on the same chromosome, excluding the three tightly linked pairs of loci, with the mean D' of the loci on different chromosomes. The difference was not significant (paired t-test, df = 8, t = -1.571, p = 0.155). In fact, the difference was in the opposite direction predicted by parahsexuality. If recombination is parahsexual, recombination between chromosomes, but not within chromosomes, is expected. On the contrary, if gene reshuffling is due to sexual recombination, recombination within and between chromosomes are both expected. Therefore, these results indicate that loci experience both intra and inter chromosomal recombination. These results suggest that sexual reproduction is the process that determines gene reshuffling in D. discoideum.
FIGURE 4.7. Average $D'$ of loci on the same chromosome and on different chromosomes. Loci on different chromosomes have $D'$ (average $D' = 0.858$) no different than loci on the same chromosome (average $D' = 0.75$), meaning that recombination reshuffles loci on the same chromosome. This result indicates recombination is sexual rather than parasexual.
DISCUSSION

My study shows that sexual recombination does occur in a natural population of *D. discoideum*. The use of *D. discoideum* clones to estimate linkage disequilibrium, instead of using macrocysts, allowed me to approach the question of occurrence of sexual reproduction from a different perspective than previously adopted. Previous empirical studies suggested that little or no recombination occurs in *D. discoideum*. The main limit to these studies under laboratory conditions was the low germination rate of macrocysts, barely reaching 2% (Francis and Eisenberg 1993; Francis *et al.* 1998) and 0.1-0.5% (Francis 1997). Since these studies were performed under laboratory conditions, they did not reveal whether recombination does occur in nature.

The high average of linkage disequilibrium observed in my analysis (average 0.60 ± s. e. 0.02) leads to a preliminary conclusion that in nature asexual reproduction occurs at high frequency, obscuring the degree of recombination. Loci significantly differed in their D' (ANOVA, p < 0.0001, Fig. 4.3). Locus variation is an important variable to keep under control in the analysis of association between loci, because allele heterozygosity could affect the outcome of linkage disequilibrium. If loci involved in a pair happen to be highly variable their degree of linkage disequilibrium would be highly affected by locus characteristic and little by their linkage. Locus variation can be explained by mutational rate because each new allele is in complete linkage disequilibrium. The correlation between D' and locus expected heterozygosity was significant (p = 0.042, Fig. 4.4). This result can give information about the impact of mutations on linkage
disequilibrium. Loci that vary more are likely to experience high mutation. Since
mutations can cause linkage disequilibrium (Futuyma 1998) these loci may have higher
D'. Variation among loci is important information to evaluate the recombination through
estimation of linkage disequilibrium.

Comparing the D's of the six tightly linked loci to the D' of the other loci allowed
me to further investigate linkage disequilibrium, keeping control of locus variation. The
significant difference (Fig. 4.6) indicates that linked loci have higher linkage
disequilibrium, with tightly linked loci than they do with other loci. Therefore, while
linked loci do not experience recombination, the other loci must.

Sexual reproduction occurs in this natural population of D. discoideum. Gene
reshuffling during sexual reproduction causes recombination both between and within
chromosomes. In contrast, parasaexual recombination causes reshuffling between
chromosomes and only very low recombination within the same chromosome. Francis
and Eisenberg (1998) suggested that recombination in nature might occur through mitotic
recombination and not through meiosis during sexual reproduction. The degree of
linkage disequilibrium lowering observed could be due to the occurrence of parasaexual
recombination in nature, instead of sexual reproduction. The methods I used allowed me
to elucidate whether recombination is due to sexual reproduction or to parasaexuality.
First, I compared the mean D's of all the possible locus pairs on the same chromosome
(mean D' = 0.75) to the mean D' of all the possible locus pairs located on different
chromosomes (mean D' = 0.858), (Fig. 4.7). Then, I repeated the analysis controlling for
locus variation. I compared for each locus, the mean degree of linkage disequilibrium
between loci on their same chromosome and loci on different chromosomes (paired t-test,
p = 0.155). These results show that recombination reshuffles both intra- and inter-chromosomal associations between loci which is more consistent with sexual reproduction than parasexuality. According to Francis (1998) *D. discoideum* clones recombine at a rate of 0.1% per Kilobase. Therefore, two of the three pairs of tightly linked loci I amplified (15945 and 16020) are likely to recombine at the same rate.

The population I analyzed was collected sampling small soil cores along a 25 mt transect. Because the variability of clones encountered in such small amounts of soil (Fortunato *et al.* in prep.) is high, it is therefore likely that amoebae have the opportunity to recombine in nature when proper conditions arise. During the vegetative stage of the *D. discoideum* life cycle, amoebae divide and move around, increasing the chance of encountering other clones. Also, the presence of arthropods in the soil facilitates spore dispersal (Huss 1989), increasing the opportunity for clones to mix and recombine.

Recombination might confer benefits to *D. discoideum* clones. As is the case for some fungi (Gräser *et al.* 1996; Kumar *et al.*, 1999), sexual reproduction yields genetic variation in the *D. discoideum* life. The generation of new genotypes is an adaptation to environments that rapidly change, as the forest soil does. Environmental factors that might induce clones to adopt the strategy of recombination are not only physical pressures, such as submersion and food deprivation. Cheaters, which are individuals that unfairly reap the benefits of reproduction (Velicer 2000), could stimulate the same behavior, because cheaters act as parasites. In a population where cheaters are likely to spread, as probably happens in this natural population of *D. discoideum* (Fortunato *et al.* in prep.), clones that recombine are less likely to be exploited by cheaters. New
genotypes confer to the new generation of amoebae a greater chance to avoid being victims of cheaters' exploitation.

Knowledge of the occurrence of recombination in *D. discoideum* is important also for molecular biologists. Sexual reproduction can combine mutations from different clones, therefore knowing that *D. discoideum* undergoes recombination in the lab it would be very useful. Our study shows that sexual reproduction has not been lost from this species, so it may be worth it to keep trying to get it to work in the lab.
ACKNOWLEDGEMENTS:

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