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# Tradeoffs and social behaviour in the cellular slime moulds

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# **Trade-offs and social behaviour in the cellular slime moulds**

A Thesis

submitted for the degree of

**Doctor of Philosophy**

in the Faculty of Science

by

**Santosh Sathe**



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Bangalore- 560012

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October 2011

## **DECLARATION**

I hereby declare that the work reported in this thesis titled, “Trade-offs and social behaviour in the cellular slime moulds” is the result of investigations carried out by me at the Centre for Ecological Sciences (CES), Indian Institute of Science, Bangalore, under the supervision of Prof. Vidyanand Nanjundiah. I further declare that this work has not formed the basis for an award of any degree, diploma or fellowship in any institution.

October 2011

Santosh Sathe

*Dedicated to Tai and Nana.....*

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I sincerely apologize to all whose names have been missed inadvertently. The defects remaining in this thesis are unintentional and my responsibility alone.

## Publications

1. **Sathe, S.**, et al., 2010. Genetic heterogeneity in wild isolates of cellular slime mold social groups. *Microb Ecol.* 60, 137-48.
2. **Sathe, S.**, Khetan, N. and Nanjundiah, V., 2014. Interspecies and intraspecies interactions in social amoebae. *Journal of Evolutionary Biology.* 27: 349–362.
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5. **Sathe, S.**, et al., 2011. Development of twelve polymorphic microsatellite markers from the social amoeba *Dictyostelium giganteum* suitable for genetic diversity studies on cellular slime moulds (Permanent Genetic Resources added to Molecular Ecology Resources Database 1 August 2010 – 30 September 2010). *Molecular Ecology Resources.* 11, 219-222.
6. **Sathe, S.**, Nanjundiah, V., Trade-offs as a basis for cooperation in a social amoeba (in preparation).

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## SYNOPSIS

By combining laboratory experiments with field work, I have looked at the following aspects of cellular slime mould (CSM) biology: (a) the genetic structure of social groups (fruiting bodies) in the wild and its relation to the role of large mammals as dispersal agents; (b) social behaviour in clonal, intra-species polyclonal and interspecies social groups and (c) fitness-related trade-offs with respect to life history traits as a possible mechanism for coexistence and cooperative behaviour in CSMs. The major findings of this study are as follows: (a) individuals belonging to different strains of a species, different species and genera occur in close proximity, even on a speck of soil (250µm–1mm) or the same dung pat; (b) social groups formed in the wild by *Dictyostelium giganteum* and *D. purpureum* are generally multiclonal; (c) genetically diverse strains can co-aggregate and form chimaeric social groups; (d) in chimaeric social groups, strains differ in their relative sporulation efficiencies; (e) the fact that strains co-exist in spite of this may be attributable in part to trade-offs between various fitness-related traits as can be demonstrated in the case of wild isolates of *D. giganteum* in pair wise mixes.

The Dictyostelids or CSMs are haploid, eukaryotic, soil dwelling social amoebae with an unusual life cycle (Bonner, 1967; Raper, 1984). They exist as single cells in the presence of food (bacteria, yeast, fungal spores). Once the food is exhausted, they enter the social phase of their life cycle. Approximately  $10^2$  to  $10^6$  amoebae aggregate at a common collection point and form a starvation resistant structure called the fruiting body. In many species a fruiting body is made up of an aerial stalk of dead cells and a ball of viable spores on top. In other CSM species (not part of this study), all amoebae in a fruiting body differentiate into spores and the stalk is an extracellular secretion.

The CSM life cycle raises fundamental questions related to the evolution of an extreme form of 'altruism' in the form of reproductive division of labour in social groups. The spore–stalk distinction in the CSMs is analogous to the germ–soma distinction in metazoans, although, the CSMs achieve multicellularity not by repeated divisions of a zygote but via the aggregation of many cells which may or may not be clonally related (Bonner, 1982; Kaushik and Nanjundiah, 2003). Social behaviour in the CSMs offers interesting parallels to what is seen in the social insects

(Gadagkar and Bonner, 1994). The origin and maintenance of 'altruism' has been a long-standing issue in sociobiology. Because of their simple life cycle and experimental tractability, the CSMs are ideal for studying the evolutionary origin and maintenance of social behaviour, in particular of 'altruistic' behaviour. By elevating spores above soil level, stalk cells, protect them from noxious compounds and predators present in soil and also facilitate their passive dispersal. In the course of doing so they die. The death of stalk cells appears to be an extreme form of altruism.

Knowledge of the genetic structure of social groups and populations including patterns of kinship is essential for modelling the evolution of 'altruism'. Thus, it is important to understand the genetic structure of CSM social groups in the wild. For this, social groups (fruiting bodies) of CSMs were isolated from undisturbed forest soil of the Mudumalai forest reserve in South India. Soil and animal dung samples were brought to the laboratory and quasi-natural social groups were generated by inoculating the samples on non-nutrient agar. The fruiting bodies from various CSM species were formed by these isolates. Since soil and dung samples were not perturbed in any way, the fruiting bodies were formed as they would have in nature.

When compared to soil, dung samples contained a higher CSM diversity and more CSM propagules. The presence of CSMs in fresh animal dung makes it likely that they were transported and dispersed over long distances through the gut of these animals. Such dispersal is likely to be preceded by a thorough mixing of spores in the gut. That increases the probability of co-occurrence of different genotypes in a social group. This possibility was confirmed by genetically characterizing spores in social groups of *Dictyostelium giganteum* and *D. purpureum* collected from the wild. Random amplification of polymorphic DNA (RAPD), a simple and reliable molecular technique, was used for genotyping spores within a fruiting body. 17 fruiting bodies (8 from animal dung and 9 from soil) were studied. 15 out of 17 (9 out of 11 of *D. giganteum* and 6 out of 6 *D. purpureum*) were polyclonal; the minimum number of distinct clones in a single fruiting body was 3 to 7 (animal dung) and 1 to 9 (soil). Therefore in *D.giganteum* and *D. purpureum*, chimaeric social groups seem to be the

norm. This suggests that other species of CSMs form intra-species chimaeric social groups in wild, though clonal fruiting bodies occur too.

The next objective of this thesis was to test whether genetic heterogeneity had functional consequences. That is, when different strains come together in an aggregate, do they contribute equally to the reproductive (spore) and non-reproductive (stalk) pathways? Amoebae of different clones (strains) of *D. giganteum* or *D. purpureum* were mixed and developed together and the number of spores formed by each strain was counted. These experiments confirmed that strains of *D. giganteum* or *D. purpureum* can aggregate together and form chimaeric fruiting bodies. The ability to mix (measured as the frequency of chimaerism) depended on the strains used and varied from one mix to another. One strain was often found to 'exploit' the other during sporulation, that is, it formed more spores than its expected share. Despite this, strains are found in very close proximity in the soil, which raises an important question: when one strain is more efficient at sporulating than other, how can the two co-exist stably?

To investigate what might lie behind the stable co-existence of strains, I studied various fitness-related traits in the life cycle of *D. giganteum*. They included the rate of cell division, the time taken to go through multicellular development, the efficiency of slug migration through various depths of soil and the probability of differentiation into a spore. Measurements were carried out on strains taken separately and on their pair wise mixes. Five different *D. giganteum* wild strains (46a3, 46d2, 48.1a1, F5 and F16) were used. All were isolated from the Mudumalai forest (India). 46a3 and 46d2 came from soil within 10 cm of each other, 48.1a1 from soil about 200m away from 46a3; and F5 and F16 from the same fruiting body (Kaushik et al., 2006; Sathe et al., 2010). Members of a pair differed significantly in the measured fitness-related traits. For example, in the case of 48.1a1 and 46d2, 48.a1 grew faster than 46d2 both individually and in a mix. After starvation, 48.1a1 formed fruiting bodies faster than 46d2; a mix of the two developed at the rate of the faster member, implying that the slower one (46d2) gained from the association with 48.1a1. During slug migration, slugs formed by 48.1a1 came up through a higher depth of soil than 46d2 slugs and did so earlier. Chimaeric slugs were like the

more efficient member, 48.1a1, in terms of the maximum depth of soil that was covered, but like the less efficient member, 46d2, in terms of the time taken for slugs to be seen on the soil surface. 48.1a1 seems to have an advantage over 46d2 in all these respects. However, during sporulation in chimaeras, 48.1a1 formed relatively fewer spores than 46d2. Similar trade-offs were seen in all mixes. F5 and F16 displayed an unexpected feature during sporulation; the spore-forming efficiency of either strain depended on its proportion in the initial mix in a frequency-dependent manner that was consistent with a stable equilibrium. Thus, trade-offs between different fitness-related traits contribute to the co-existence of strains.

Next, I studied interactions between members of different CSM species. Several species of CSMs were isolated from the same environment (Sathe et al., 2010); a question of interest was to see if amoebae of different species came together to form a chimaeric multicellular body. Five strains (two *D. purpureum* and three *D. giganteum*) were used in this study. Amoebae of *D. giganteum* and *D. purpureum* co-aggregated. However, there were factors that caused amoebae of the two species to sort out thereafter. The extent of segregation differed between strains, a characteristic that inter-species mixes share with intra-species mixes.

In conclusion, the ability of cellular slime moulds to form multiclonal social groups in the wild suggests that one should look to factors in addition to close relatedness to understand the evolution of CSM social behaviour. The existence of fitness-related trade-offs between different traits indicates that individual-level selection can also contribute to the maintenance of chimaeric social groups.

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# **Chapter 1**

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*Cooperative behaviour in the social amoebae*

## 1.1 Introduction

In his epochal book *On the origin of species by means of natural selection*, Darwin (1859) said, “natural selection cannot possibly produce any modification in a species exclusively for the good of another species...”(page 228). As differential reproductive fitness is the means through which natural selection operates, an extension of the above statement is that the behavior of living organisms should be moulded by evolution so as to enhance their own reproductive fitness, not that of others. Cooperation is a phenomenon in which the behavior of an individual can improve the reproductive fitness of other individuals and in the process may lead to a reduction in its own fitness. Cooperation is vulnerable to the evolution of non-cooperators, namely, individuals that obtain advantages from cooperation without contributing anything to the costs of cooperation. Yet, cooperation between members of the same species or different species is widespread. The evolution of cooperation has been a major evolutionary conundrum ever since Darwin.

Examples of cooperative behaviour can be found across all kingdoms; in particular, in both prokaryotes and eukaryotes (Bonner, 1967; Wilson, 1971; Wilson, 1975; Hölldobler and Wilson, 1990; Crespi, 2001; Gadagkar, 2001; Koenig and Dickinson, 2004). Evolutionary biologists are faced by two questions related to the phenomenon of cooperation. First, if cooperation leads to a decrease in fitness, how could it evolve by natural selection? Next, how could behavior of a type that leads to a decrease in relative fitness be sustained in the long run? These questions pertain respectively to the evolutionary origin and maintenance of cooperation. My work deals mainly with the second issue, namely with the maintenance of fitness-reducing cooperative behavior.

The aim of this chapter is twofold. I begin by giving examples of cooperative behavior with an emphasis on cases of apparent altruism. Next I briefly discuss different theories put forward to explain the evolutionary origin and persistence of such behaviours. The rest of this chapter is devoted to social behavior in the life cycle of the cellular slime moulds (CSMs) and explanations for the evolution of cooperation in the CSMs.

## 1.2 Glossary

Before discussing the evolution of social behaviour, it will be useful to clarify certain terms used in this thesis [also found in Kaushik (2002); West et al (2007); Nanjundiah and Sathe (2011)].

**Actor:** An individual who exhibits the behaviour of interest.

**Recipient:** An individual who is affected by the behaviour of the actor.

**Fitness:** Fitness is measured in terms of number of offspring produced. It has two components: direct fitness and indirect fitness. Direct fitness is counted in terms of the number of offspring produced by the actor. Indirect fitness is measured by counting the number of offspring produced by the recipient of an act *that can be attributed to the act*. Both direct and indirect fitness depend on the existence of a positive correlation between a trait and reproduction, the difference being that in the case of indirect fitness the two refer to distinct individuals. Inclusive fitness is the sum of the direct and indirect fitness components.

**Selfishness:** A behaviour performed by the actor that increases its own direct fitness relative to that of the recipient.

**Cooperation:** Behaviour by the actor which enhances the fitness of the recipient. In the process, the actor may or may not reduce its own direct fitness. A question of interest is: to what extent is the behaviour of the actor likely to have evolved *because* of this beneficial effect on the recipient?

**Altruism:** Behaviour by the actor that improves the direct fitness of the recipient relative to the direct fitness of the actor.

**Cheater:** An actor that improves its own fitness by deriving the advantages of cooperation without undergoing any of the cost.

**Individual selection:** A process by which the behaviour of the actor is favoured by natural selection because it increases the direct fitness of the actor.

**Kin selection:** A process by which the behaviour of an actor is favoured by natural selection via its consequences for indirect fitness; i.e., because it enhances the reproductive fitness of genetic relatives other than offspring.

**Group selection:** A process by which behaviour that reduces the direct fitness of the actor is favoured by natural selection because of its beneficial effect on the group to



which the actor belongs. Group members need not be genetically related; if they are, group selection becomes kin selection.

**Social selection:** Social selection is a process of natural selection where the fitness of an individual depends on its own phenotype as well as the phenotype and therefore genotype of the interacting members too. One can think of individual, kin, group and social selection as examples of natural selection that differ in terms of the level at which selection operates (Williams, 1966).

### 1.3 Examples of cooperative behaviour

Cooperative behaviours (including those that appear altruistic) are found across all the branches of the evolutionary tree; some of them are discussed below.

**Cooperative hunting:** Carnivores such as lions, wolves, and wild dogs form small cooperative groups and hunt prey larger than themselves (Harrington and Paquet, 1983; Stander, 1992; Creel and Creel, 2002). These animals use different tactics to hunt their prey e.g. isolating a prey from the herd and synchronised attack.

**Food sharing:** Vampire bats live in small colonies in dark places. They feed largely on animal blood. If individuals fail to find food and remain hungry for 2-3 days, they die. In such situations, individuals who have found food regurgitate some amount of food and share it with hungry individuals (Wilkinson, 1984).

**Cooperative breeding:** In a cooperatively breeding reproductive system, one or more members of a social group (called as helpers or auxiliaries) forgo their own reproduction and provide care to newborns that are not their offspring. Cooperative breeding is observed in social insects (Wilson, 1975), birds (Stacey and Koenig, 1990; Lundy et al., 1998), and mammals (Wilson, 1975; Moehlman, 1979; Jarvis, 1981).

**Cooperative behaviours in social insects:** Extreme forms of cooperation, to the extent that can be called altruism, are found in the insect orders Hymenoptera (ants, bees, wasps) and Isoptera (termites). These insects live in groups comprising one hundred to twenty million individuals (Wilson, 1971; Hölldobler and Wilson, 1990; Gadagkar, 2001; Hölldobler and Wilson, 2009). They show reproductive division of labour: one or at most a small number of individuals (queens) reproduce. The remaining individuals (workers) do not reproduce but are involved in hive maintenance activities including bringing up the queen's brood. Worker honeybees commit suicide while defending their

hive (Winston, 1987; Caron, 1999). Thus, the behaviour of a worker appears to be altruistic as it increases the fitness of the queen at the cost of its own reproductive fitness.

**Alarm calls:** In response to danger (usually approaching predators), some animals warn fellow group members by making appropriate sounds called alarm calls e.g. vervet monkeys and ground squirrels (Sherman, 1977; Seyfarth et al., 1980; Collier et al., 2010). The individuals that give alarm calls draw the attention of the predator and therefore they are at a higher risk of predation than if they had not given the alarm call.

**Social behaviour in microorganisms:** Microorganisms show complex social behaviours, e.g. division of labour and fruiting body formation by cellular slime moulds and myxobacteria as an aid to dispersal (Bonner, 1967; Raper, 1984; Kessin 2001; Crespi, 2001; Kaiser, 2001); biofilm formation for protection from antibiotics and host defences (Davey and O'Toole, 2000); and siderophore production for chelating iron under iron-limiting environments (West and Buckling, 2003). These behaviours are energetically costly to perform, and in some cases (e.g. fruiting body formation in CSMs) involve the death of individuals.

#### 1.4 Evolutionary explanations for cooperation

It is evident in the above-discussed examples of cooperative behaviours that some individuals in a group do not reproduce at all or reduce their chances of survival and reproduction by performing 'costly' behaviours. The question of interest to us is how can a behaviour whose outcome is a decrease in direct reproductive fitness evolve by natural selection? Several possible explanations for the evolution of cooperation have been proposed that go beyond the superficially implausible one of individual selection via direct fitness effects. As listed earlier, they include group selection (Wynne-Edwards, 1962), kin selection (or inclusive fitness theory; Hamilton, 1964), direct and indirect reciprocity (Trivers, 1971; Axelrod and Hamilton, 1981), advertising hypothesis (Carlisle and Zahavi, 1986) and imposed altruism regulated by punishment (Clutton-Brock and Parker, 1995). Cahan et al (2002) have discussed the role of ecological conditions as a basis for cooperation; the idea is organisms perform cooperative behaviours under harsh environmental conditions. For example, in case of cooperative breeding, the potential loss of breeding sites caused by non-cooperation

has been hypothesised as an explanation. Evolutionary explanations for cooperation are discussed in brief here. In the last part of the chapter, I have taken example of the CSMs for a detailed examination of the explanations.

**Group selection:** Darwin came up with a number of ways in which the reduction in direct reproductive fitness could be made up and lead to the evolution of cooperative, even altruistic, traits (Darwin, 1859; also see Nanjundiah, 2010). The idea of what is now called group selection was put forward by him as a possible solution to the evolution of cooperative behaviour (Darwin, 1871). Darwin reasoned as follows. On the one hand, self-sacrificing behaviour appeared to be altruistic and therefore detrimental to the performer. As he said, “it is extremely doubtful whether the offspring of the more sympathetic and benevolent parents... would be reared in greater number than the children of selfish and treacherous parents of the same tribe. He who was ready to sacrifice his life, as many a savage has been, rather than betray his comrades, would often leave no offspring to inherit his noble nature” (p.163). On the other hand, Darwin hypothesised; a group consisting of altruistic and selfish individuals could have a reproductive advantage over a group formed solely by selfish individuals. In his words “..a tribe including many members who from possessing in a high degree the spirit of patriotism, ...were always ready to give aid to each other and sacrifice themselves for the common good, would be victorious over most other tribes; and this would be natural selection” (p.166). Darwin’s argument was fleshed out by Haldane (1932).

The group selection point of view is that the presence of cooperative individuals in a group gives a selective advantage to the group over a group formed by selfish individuals only, even though within a group, selfish individuals do better than cooperators—in this case, altruists. For example, in the case of alarm calls by vervet monkeys, a group containing cooperative individuals (that give alarm calls) could have an advantage over a group made up of non-cooperative individuals (who do not give alarm calls). Although in principle group selection can work, for two reasons it has been considered as a weak evolutionary force for the evolution of cooperative behaviours. One is the paucity of evidence for specifically group-level traits and the other is the requirement of special circumstances to prevent group selection from being overwhelmed by individual selection (Williams, 1966). Cooperative behaviour is

susceptible to exploitation by a selfish mutant that benefits from the cooperative acts of others, but does not pay any of the costs. On the face of it, such a mutant (a 'cheater') can exploit the group and lead to the elimination of co-operators.

Wilson and Wilson (2007) have reviewed the theoretical and empirical evidences supporting group selection. Cooperative behaviour by *Pseudomonas fluorescens* can be used as an example to demonstrate the way group selection works. If grown in an unmixed liquid medium, the wrinkly spreader (WS) strain of *P. fluorescens* colonises the liquid-air interface and avoid the effects of anoxic environments (Rainey and Rainey, 2003). WS cells stay on the surface by producing a cellulosic polymer which forms a mat and floats. Polymer synthesis is costly and therefore mutants ('cheaters') which do not produce polymer have an advantage within a group made up of co-operators and cheaters. However, as the 'cheater' population increases within a group, the entire group sinks to the bottom. Thus, the WS strain has a selective disadvantage within a group but it can be maintained in the population because of between-group selection (Wilson and Wilson, 2007). Other evidences that are claimed to demonstrate the operation of group selection are given in the review by Wilson and Wilson (2007).

**Kin selection:** W. D. Hamilton (1964) proposed a genetic model for the evolution of altruistic behaviour. Hamilton pointed out that the altruistic behaviour can evolve by natural selection if the interacting individuals (the actor and the recipient) are genetic relatives i.e., they share genes by common descent. In mathematical terms, altruism will evolve when  $rb-c > 0$ . Here  $r$  is the coefficient of genetic relatedness between the actor and the recipient,  $b$  is the fitness benefit in terms of direct fitness of the recipient and  $c$  is the cost, also measured in terms of direct fitness, to the actor. Thus altruistic behaviour can evolve if the fitness benefit to the recipient multiplied by genetic relatedness is greater than the cost of the altruistic act. It must be kept in mind that  $b$  and  $c$  are equally important for altruism to evolve; even at low  $r$  altruism will be selected if  $b$  is large and  $c$  is small.

Ever since first proposed, kin selection has remained a popular model. It was widely accepted in social insect studies, partly because Hamilton noted the favourable consequences of haplodiploidy for kin selection. In the insect order Hymenoptera (ants, bees, and wasps), the sex of an individual depends on whether it is haploid or diploid:

haploid individuals are males and diploid individuals are females. Assuming one queen per colony (monogyny) and single mating (monandry), worker bees are more closely related to their sisters ( $r = 0.75$ ) than they are to their sons or daughters ( $r = 0.5$ ). Thus, the 'altruism' of a worker (raising a sister instead of a son or a daughter) can conceivably evolve by kin selection.

Questions have been raised about the applicability of kin selection, especially with regard to the emphasis on haplodiploidy. Several haplodiploid species are not social and several diploid species are social (Wilson, 1975, Hölldobler and Wilson, 1990). Occurrence of more than one queen per colony (polygyny) and multiple mating by queens (polyandry), both known to occur, decreases the average relatedness between workers of the same colony to a level below that of a mother to her offspring (Hacker et al., 2005; Goodisman et al., 2007; Kellner et al., 2007; Thurin et al., 2011; Evison and Hughes, 2011). Further, limited dispersal keeps related members together which can lead to competition for common resources and, thereby, decreased intensity of selection for cooperation (West et al., 2001). Recently, Nowak et al (2010) have reviewed the literature and analysed the supposed limitations of kin selection as a plausible explanation vis-a-vis group selection; their view has been vigorously challenged (Abbot et al., 2011).

**Reciprocal altruism:** Described by Trivers (1971), this theory argues that individuals cooperate with other individuals either in the expectation of return benefits or exclusively with those that have cooperated with them in the past. Reciprocal altruism has been demonstrated in vampire bats; individuals share food with those who have helped them in the past (Wilkinson, 1984).

**Imposed altruism:** This theory suggests that what appears to be altruistic behaviour is in fact the outcome of coercion, not voluntary. A prerequisite is the existence of some asymmetry between individuals that makes coercion possible. A famous instance, that of ant slaves who work for others' nests, was discussed at length by Darwin (1859, page 243-247). *Formica rufescens*, "is absolutely dependent on its slaves; without their aid, the species would certainly become extinct in a single year". This ant "... does not build its own nest, does not determine its own migrations, does not collect food for itself or its young, and cannot even feed itself...". However, our interest is in altruistic behaviour

within social groups of the same species. For example, in meerkats dominant individuals force subordinates to cooperate with them: dominant females suppress reproduction in subordinate females by aggressive attacks (Young et al., 2006).

**Advertising hypothesis:** Carlisle and Zahavi (1986) argued that allofeeding (the act of giving food to other individuals) in cooperatively breeding birds could have evolved because by performing cooperative behaviour "...individuals may increase their social status in the group...". Advertising social status is an indicator of individual's quality and successful advertising can lead to increased reproductive fitness in the long run. As suggested by them (Carlisle and Zahavi, 1986) the advertising hypothesis, which is an extension of the Handicap Principle (Zahavi and Zahavi, 1997), can explain apparent altruism in the absence of kinship or direct reciprocity.

**Summary:** Cooperative behaviours, despite possible associated costs in terms of direct fitness to the actor, are widespread in nature. The evolutionary underpinnings of cooperation continue to be investigated and are far from being understood satisfactorily. Especially in unicellular microorganisms, their evolutionary origin and persistence remains a puzzle. The aim of my thesis is to clarify factors that might lie behind cooperation during the social phase of the life cycle in the single-celled eukaryotic amoebae known as the cellular slime moulds.

### 1.5 Cellular slime moulds

I have used two species of Cellular slime moulds (CSMs), *Dictyostelium giganteum* and *D. purpureum*, in this study. Apart from special features that will be pointed out as and when required, the descriptions of their life cycles that follow hold good for many other species including the most commonly studied species, *D. discoideum* (Bonner, 1967; Raper, 1984; Kessin, 2001; Bonner, 2009). Other CSMs differ a great deal in the way social behaviour is manifested (Bonner, 1967; Raper, 1984; Kaushik and Nanjundiah, 2003).

#### Occurrence

CSMs are eukaryotic amoebae that live in soil on decomposing leaf litter and animal dung where they feed on bacteria, yeasts, and probably other soil microorganisms. They

grow and keep dividing every few hours by binary fission (Bonner, 1967; Raper, 1984; Kaushik, 2002; Sathe et al., 2010). Oskar Brefeld in the late 1860s isolated the first species of CSM (*Dictyostelium mucoroides*) initially from horse dung and subsequently from rabbit dung (cited in Raper, 1984; Kessin, 2001). CSMs have been isolated from soils collected from all over the world (Swanson et al., 1999) and about 120 species have been identified so far.

### **Systematics and Phylogeny**

Traditionally, CSMs are classified under the kingdom Mycetozoa (fungi). However, they are not true fungi because they lack vegetative hyphae or sporangial walls and are not saprophytic (Raper, 1984). They belong to the division Myxomycota and class Acrasiomycetes (Raper, 1984). Acrasiomycetes are further divided into two sub-classes, Acrasids and Dictyostelids; all the species used in this study are Dictyostelids. Based on their gross morphological characters, CSMs have been classified into three distinct genera: *Acytostelium* (fruiting bodies with acellular stalk), *Dictyostelium* (mostly unbranched fruiting bodies with cellular stalk) and *Polysphondylium* (branched fruiting bodies with cellular stalks).

Although morphology-based classification and the resulting nomenclature are still in use, they are likely to be overridden by phylogenetic classification based on molecular markers. The latter show several inconsistencies in the traditional morphology-based classification. Species that were supposed to belong to the different genera *Dictyostelium* and *Polysphondylium* are at times found in the same clade and at times in different clades (Schaap et al., 2006; Mehdiabadi et al., 2009; Romeralo et al., 2010; Romeralo et al., 2011a). The phylogenetic position of the CSMs remains to be fully clarified. Some reports indicate that they diverged from the line that leads to *Homo sapiens* after yeast (Loomis and Smith, 1990; Loomis and Smith, 1995) whereas other reports indicate that they diverged from the line that leads to *Homo sapiens* before yeast (McCarroll et al., 1983; Baldauf and Doolittle, 1997; Eichinger et al., 2005).

## 1.6 Survival strategies

CSM amoebae face various stresses in their natural habitats. Osmotic imbalances in soil can be harmful to these amoebae and hence a stress factor. CSMs have evolved a vacuolar system, which can actively pump the water out and avoid the cell lysis because of the change in osmolarity (Kessin 2001). Nematodes, which are commonly present in soil, can compete with CSMs for bacteria and they predate on CSM amoebae as well. *Dictyostelium discoideum* and *D. purpureum* amoebae avoid nematodes by actively repelling them (Kessin et al., 1996). Although nematodes can consume and digest CSM amoebae, they fail to eat multicellular aggregates (slugs) and cannot digest spores. Since CSM amoebae are voracious predators (each *D. discoideum* amoeba feeds on ~ 1000 bacteria before it divides) they frequently encounter starvation. CSM amoebae avoid starvation-dependent death by following various strategies for survival.

**Microcyst:** In response to nutrient starvation and high osmolarity, amoebae in some species of CSMs form a double-layered structure around them called a microcyst (Raper, 1984; Kessin, 2001; Budniak and O'Day, 2011). Although the microcyst form can protect a solitary amoeba, dispersal of a microcyst is likely to be limited.

**Macrocyt:** Under special environmental conditions that include starvation, high moisture, darkness, and low phosphate concentration, some species of CSMs undergo sexual reproduction and form dormant structures called macrocyts (Blaskovics and Raper, 1957; Nickerson and Raper, 1973a; Saga and Yanagisawa, 1982; Raper, 1984; Urushihara, 1992; O'Day and Keszei, 2011). Different mating types have been identified in CSMs; *D. giganteum* is believed to be heterothallic while *D. purpureum* and *D. discoideum* have both homothallic and heterothallic strains (Erdös et al., 1975; Lewis and O'Day, 1979; Bloomfield et al., 2010). Macrocyt formation starts with the fusion of amoebae of appropriate mating types to form a binucleate giant cell. The giant cell of *D. discoideum* attracts neighbouring amoebae by secreting cAMP and engulfs them. Engulfed cells (endocytes) initially appear distinct but further development leads to the formation of a homogeneous cell mass covered by three layers now called a macrocyt. Macrocyt formation is a true sexual reproduction stage as evident from recombination and meiosis observed in both *D. giganteum* and *D. discoideum* (Erdös et al., 1975; Wallace and Raper, 1979; Mehdiabadi et al., 2010; Flowers et al., 2010). Macrocyts

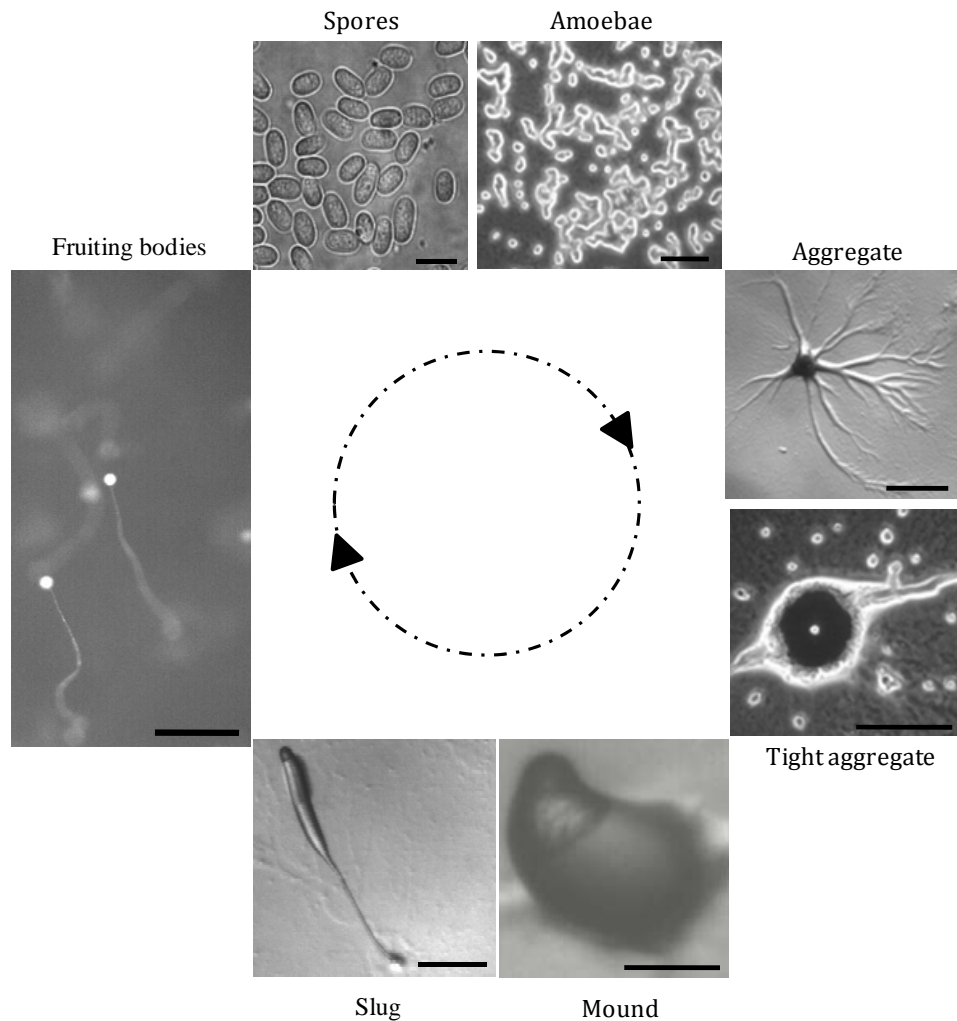


remain dormant until conditions turn favorable for their germination. The germination efficiency of macrocysts is very low under laboratory conditions (Blaskovics and Raper, 1957; Wallace and Raper, 1979; Nickerson and Raper, 1973b). Germination starts with lysis of the different walls of a macrocyst followed by the release of haploid amoebae.

**Aspidocyte:** *D. discoideum* amoebae in the presence of detergents and heavy metals form a resistant cell called an aspidocyte (Serafimidis et al., 2007). These cells lack a cell wall and are morphologically different from a microcyst or macrocyst. Once the stress is removed aspidocytes revert back to an amoeboid state within a few minutes. It has been hypothesized that these cells are an adaptation for a quick reversible response to such stresses (Serafimidis et al., 2007).

**Asexual life cycle (fruiting body formation):** The asexual life cycle is induced when the food supply at a given locale gets over and the density of amoebae is reached above a certain threshold (Konijn, 1968; Jain et al., 1992). *D. discoideum* amoebae secrete factors called prestarvation factor (PSF) and conditioned medium factor (CMF) which allows amoebae to monitor the ratio of their density to bacterial density (Clarke and Gomer, 1995; Jain et al., 1992). Important stages during fruiting body formation in *D. giganteum* are aggregation, mound, slug, and culmination (Fig. 1.1). The life cycle of *D. discoideum* is discussed below (Fig. 1.3) and that of *D. purpureum* described in chapter 2 (Fig. 2.8).

**Aggregation:** Upon starvation a small number of cells within a population acts as a collecting centre and begin to secrete a small molecular weight compound (earlier called as acrasin) in a pulsatile manner, leading to the aggregation of cells (Bonner, 1949). The acrasin in *D. giganteum*, *D. purpureum* and *D. discoideum* is 3', 5' cyclic adenosine monophosphate, cAMP (Konijn et al., 1967; Raper, 1984). Extracellular cAMP is detected by amoebae with the help of G-protein coupled cAMP receptors (cARs) present on the cell surface (Saxe et al., 1991; Saxe et al., 1993; Louis et al., 1994; Firtel, 1995; Parent and Devreotes, 1996). cAMP binding to the neighbouring cells leads to movement of these cells towards the cAMP source and formation of the aggregate (Figs. 1.1, 1.3).

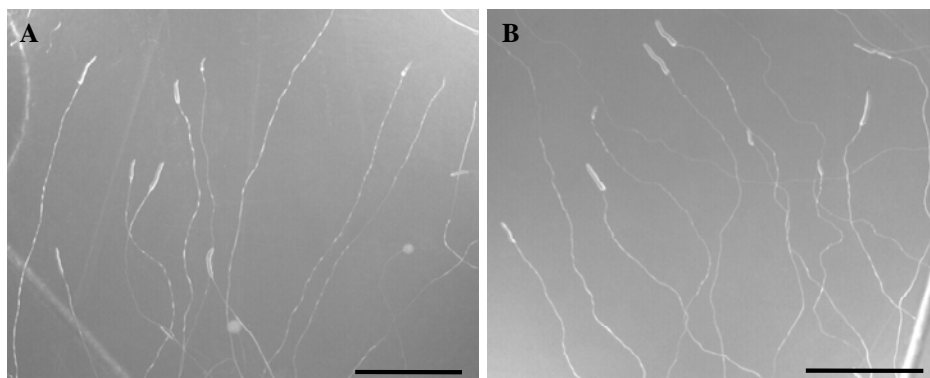


**Fig 1.1: Asexual life cycle of *D. giganteum*, strain 46a3.** After 4-5 hrs starved amoebae form an aggregate. The aggregate becomes compacted and is covered by a slime sheath. After about eight hrs, it becomes a mound. Motile slugs are formed after 10-12 hrs (note stalked slug migration). Slugs develop into fruiting bodies that are unbranched and white colored. Under standard developmental conditions in the laboratory, *D. giganteum* cells take ~ 16 hrs to complete asexual development. The scale bars represent 10 μm (in spores and amoebae) and 250 μm in all other structures.

**The mound:** An aggregate is transformed into a hemispherical mound surrounded by a cellulose slime sheath (Wilkins and Williams, 1995). In the mound, cell adhesion mechanisms are developed; both sheath and cell adhesion is required for further development (Farnsworth and Loomis, 1975; Noegel et al., 1986; Knecht et al., 1987; Brar and Siu, 1993; Kessin et al., 1996; Ponte et al., 1998; Kessin, 2001). In *D. discoideum* early signs of cell differentiation are apparent in the mound stage. Two major cell types are formed: presumptive spore cells (psp) and presumptive stalk cell (pst); these are

the future spore and stalk cells respectively. Initially, prespore cells are scattered all over the mound; later prestalk and prespore cells get segregated in different regions in the multicellular structure (Matsukuma and Durston, 1979; Siu and Kamboj, 1990; Nicol et al., 1999). As the mound develops, it forms a tip, which acts like a classical Spemann organizer for inducing or stabilizing subsequent development (Bonner 1952; Rubin and Robertson 1975). In *D. giganteum* and *D. purpureum* mound develops into a migratory structure called the slug. In *D. discoideum*, mounds form a structure called first finger which depending on environmental conditions can fall on the substratum and form the slug or an intermediate 'Mexican hat' form (Fig. 1.3) (Bonner et al., 1950; Newell et al., 1969).

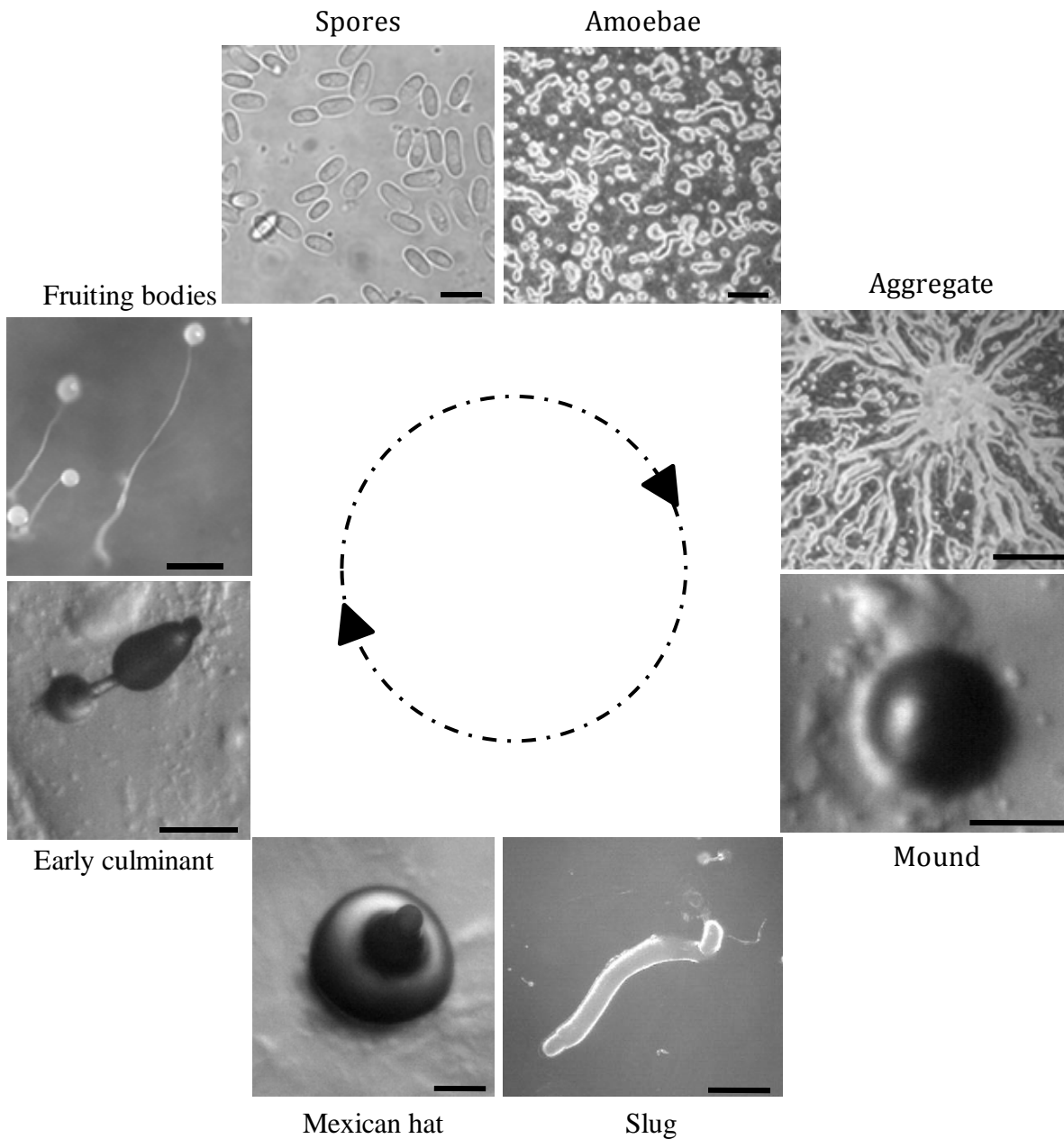
**The slug formation:** The slug is a remarkable structure in the asexual life cycle of *Dictyostelium*. Although the slug is just a group of cells covered by a slime sheath, it can migrate towards light (phototaxis; Fig. 1.2) and respond to temperature gradients (Bonner et al., 1950; Raper, 1984; this study). Slugs show a clear differentiation of cell types: in *D. discoideum* prestalk cells are in the anterior ~20% of the slug and the posterior ~80% is occupied by the prespore cells (Raper, 1940; Bonner, 1952). *D. giganteum* and *D. purpureum* slugs show stalked slug migration i.e., the slugs are oriented vertically, rooted at one place and while migrating leave the stalk behind (Fig 1.1, 1.2). On the contrary, *D. discoideum* slugs show non-stalked migration i.e., the slugs remain horizontal on the soil surface and migrate without concomitant stalk formation (Fig. 1.3). It is possible that stalked migration is more useful while negotiating soil particles in the soil (Bonner, 1982).



**Figure 1.2: Phototaxis in wild isolates of CSMs.** (A) *D. giganteum*, (B) *D. purpureum*. The scale bars represent 500  $\mu\text{m}$  (Fig. 1.2 is reproduced from chapter 2).

**Culmination:** Migrating slug develops into to a fruiting body by a process called culmination. In *D. giganteum* and *D. purpureum* one end of the stalk is anchored in the soil and the other end is extended simultaneously during the slug migration. Finally, slug is converted into a fruiting body made up of dead stalk and viable spores on top (Whittingham and Raper, 1960; Raper, 1984; Kaushik, 2002; this study). Studies in the laboratory on *D. giganteum* and *D. purpureum* indicate that ~ 50% of the cells in a fruiting body die and form the stalk (Kaushik, 2002; kaushik, 2006; this study). In *D. discoideum* ~20% cells form the stalk and ~ 80% form the spores (Kessin, 2001). The death of stalk cells in *D. discoideum* shows some, but not all, criteria of apoptotic cell death as seen in other multicellular organisms (Cornillon et al., 1994; Olie et al., 1998; Kawli et al., 2002). Spores are dormant structures; under favourable conditions such as the presence of food and moisture, they germinate and release amoebae that start feeding on the available food, and the life cycle begins anew (Raper 1984).

The details of culmination process are known in *D. discoideum*. Culmination starts in the presence of low humidity, increased temperature, and removal of ammonia from the surrounding air (Raper, 1940; Bonner and Shaw, 1957; Gross, 1994). During culmination, first a horizontal slug is transformed into a vertical structure. Prestalk cells secrete an extracellular matrix to form the stalk tube and then enter the stalk tube, become highly vacuolated, and die (Raper and Fennell, 1952; Jermyn et al., 1996). Simultaneously, the prespore mass that is covered by a slime sheath is gradually pulled up (Sternfeld, 1998; Mujumdar et al., 2009).



**Fig 1.3: Asexual life cycle of *D. discoideum*, strain NC-4.** Different structures formed in the life cycle are shown here. Freshly starved amoebae after ~ eight hrs form an aggregate (note elongated amoebae and streams). The aggregate develops into a hemispherical structure called the mound, which forms a motile slug after 14-16 hrs. After a brief period of migration the slug develop into a Mexican hat, which starts culminating into a fruiting body (early culminant and fruiting bodies are shown). The fruiting body is made up of the dead stalk and a ball of viable spores (yellow colored) on top. Under standard developmental conditions in the laboratory, *D. discoideum* cells take ~ 24 hrs to complete asexual development. The scale bar represents 10  $\mu\text{m}$  (in spores and amoebae) and 250  $\mu\text{m}$  in all other structures.

### 1.7 'Altruistic' behaviour in CSMs

Fruiting body formation in CSMs is believed to be an adaptation for successful spore dispersal (Bonner, 1967; Bonner, 1982). Because the stalk is an upraised structure formed on the soil surface, the cells that die and form it undoubtedly help in the dispersal of the cells that survive and form spores (Bonner 1982; Huss, 1989). The death of some cells for the survival of the others can be considered as a form of altruistic behaviour.

Stalk cell formation in CSMs have raised two important questions: (a) How does a group of amoebae, which can be genetically clonal and grown in identical environmental conditions, come to adopt a stalk or spore fate? (b) If stalk formation is a genetically coded trait then how do the genes that code for 'altruistic' behaviour survive even though the cells that bear them die? The first question is related to the development (proximate causes) and the second question is related to the evolution (ultimate causes) of social behavior. These are discussed below.

#### **Mechanistic (proximate) explanations for 'altruistic' behaviour in CSMs**

Cell fate determination in *D. discoideum* has attracted a lot of attention in the past and different models have been suggested for its origin (Bonner, 1957; Meinhardt, 1983; Nanjundiah and Saran, 1992; Nanjundiah, 1997). According to one school of thought, the fate of a cell depends on the early history of a cell i.e. even before aggregation, amoebae are biased to form either the stalk or spore cells. According to another view, cell fate is determined based on the position of a cell in a group and differentiation into stalk or spore occurs in response to a gradient created by an inducer (morphogen) and the post-aggregation interactions between the cells. As it will be clear later, both the views are important and the two mechanisms seem to work in concert.

**Pre-aggregation bias and cell fate:** It has been shown that the pre-aggregation amoebae are phenotypically heterogeneous and already have a tendency to become either a stalk cell or a spore cell (Bonner, 1959; Takeuchi, 1969; Bonner et al., 1971; Nanjundiah and Saran, 1992). Phenotypic variation between amoebae could arise stochastically (under identical environmental conditions) or cells can become heterogeneous because of their different pre-aggregation growth history. Pre-

aggregation heterogeneities in the vegetative amoebae and their tendencies to form the stalk or spore are summarized in Table 1.1.

**Table 1.1.** Pre-aggregation biases and the tendency of cells to sort either in the prestalk or the prespore area.

Pre -aggregation phenotypic heterogeneity in	When mixed, tendency to sort in		Reference (s)
	Psp/spores	Pst/ stalk	
Size	Relatively less dense amoebae of a population	Denser amoebae of a population	(Takeuchi, 1969; Bonner et al., 1971)
	Heavy cells	Lighter cells	(Maeda and Maeda, 1974) and Maeda, 1974)
Nutritional status	Cells grown in the presence of 86 mM glucose (G <sup>+</sup> )	Cells grown in the absence of glucose (G <sup>-</sup> )	(Leach et al., 1973)
	Cells grown in the absence of glucose (G <sup>-</sup> )	Cells grown on bacteria	(Leach et al., 1973; Thompson and Kay, 2000)
Cell cycle phase at starvation	Cells in mid G2 to M phase of cell cycle	Cells in S and early G2 phase of cell cycle	(McDonald and Durston, 1984; Weijer et al., 1984)
	Cells in exponential phase	Cells in stationary phase	(Thompson and Kay, 2000a)
Calcium content	Relatively low calcium	Relatively high calcium	(Azhar et al., 2001)
Intracellular pH	Relatively low pH	Relatively high pH	(Inouye, 1985; Inouye, 1988)

Experiments reported below suggest that the pre-aggregation bias is not the only mechanism of cell fate determination. Raper (1940) showed that if the *D. discoideum* slug is cut into two parts one consisting of only the anterior prestalk region (cells here are destined to form stalk) and the other only posterior prespore region and allowed both the parts to develop independently, both fragments can form near-normal looking

fruiting bodies. In a mix of G<sup>+</sup> cells with G<sup>-</sup> cells, G<sup>-</sup> cells are biased to form stalk cells. However if G<sup>-</sup> cells are mixed with cell grown on bacteria they are biased to form spores (Leach et al., 1973; Thompson and Kay, 2000a; Thompson and Kay, 2000b). These results indicate that a cell biased to form a stalk can be made to form a spore; it all depends on the phenotype of the interacting amoebae and interactions between them.

**Position-dependent cell fate:** It has been speculated that a linear gradient of morphogen concentration is present in the slugs (a morphogen is a chemical signal although produced endogenously in a localized manner it diffuses to form a gradient conveying the positional information). An amoeba differentiates into stalk or spore cell depending on its position within the gradient. In *D. discoideum*, the following substances are thought to be candidate morphogens.

**Cyclic adenosine monophosphate (cAMP):** cAMP is required for the aggregation (Konijn et al., 1967; Bonner et al., 1969). In monolayer assays (*in vitro*) cAMP induces stalk cell differentiation (Bonner, 1970) and is required for prespore differentiation (Wang et al., 1988). cAMP forms a gradient with higher cAMP levels in the front than in the back (Bonner, 1949; Brenner, 1977). The observation that a *D. discoideum* mutant that fails to make cAMP show normal development suggests that there must be morphogens other than cAMP that control morphogenesis (Wang and Kuspa, 1997).

**Adenosine:** Adenosine is produced by cells in the tip and it blocks both generation of secondary tips and differentiation of prespore cells near the tip region (Armant et al., 1980; Weijer and Durston, 1985; Schaap and Wang, 1986; Wang et al., 1988).

**Differentiation inducing factor (DIF):** DIF is a common term for a group of low molecular weight compounds produced at the slug stage. In *in vitro* assays, DIFs cause the differentiation of stalk cells and inhibit spore cell formation (Town et al., 1976; Brookman et al., 1982; Morris et al., 1987; Morris et al., 1988; Kay, 1997). The *D. discoideum* mutants blocked in the biosynthesis of DIF-1 (most active among known DIFs) still form a mature stalk; however, the basal disc (a subtype of stalk cell) is defective (Thompson and Kay, 2000b; Saito et al., 2008; Yamada et al., 2011) Surprisingly, DIF forms a gradient across the slugs which is reverse to that expected.



Prespore cells contain twice the activity of DIF as prestalk cells (Brookman et al., 1987; Kay and Thompson, 2001). A possible reason for this is that DIF-1 dechlorinase, the enzyme that metabolizes DIF-1, shows higher activity in the prestalk region of the slug than the prespore region (Insall et al., 1992; Kay et al., 1993).

**Ammonia:** Ammonia is produced in the tip and it inhibits fruiting body formation by inhibiting the prestalk to stalk transition (Davies et al., 1993; Gross et al., 1994; Feit et al., 2001). Ammonia also controls spacing between the fruiting bodies (Feit and Sollitto, 1987). Ammonia is known to inhibit DIF-induced stalk cell differentiation (Wang and Schaap, 1989).

**An unknown signal from the tip:** The slug tip behaves like a classical organizer (a group of cells which function autonomously and define a developmental axis) in *D. discoideum*; when the existing tip is removed, a new tip arises spontaneously (Raper, 1940). In the presence of a tip, newer tips are not formed. (Rubin and Robertson, 1975; Lokeshwar and Nanjundiah, 1983). These observations suggest that the tip secretes a signal (possibly adenosine) which maintains the polarity and inhibits the generation of new tips (Schaap and Wang, 1986).

**Prespore-inducing factor:** *D. discoideum* amoebae secrete a 106-kDa glycoprotein called as  $\Psi$  factor. In monolayer cultures,  $\Psi$  factor causes amoebae to differentiate into prespore cells; whether  $\Psi$  factor forms spatial gradient in the slugs is not known (Kawata et al., 2004).

Since several molecules have been shown to act as morphogens, cell fate determination based on position was a widely accepted view. However, pattern formation based purely on position has been challenged by several experimental observations. For example prestalk and prespore differentiate much early during the development (they appear at scattered positions in the aggregates) suggesting prestalk-prespore pattern can be formed without positional information (Tasaka et al., 1983; Noce and Takeuchi, 1985; Thompson et al., 2004). In conclusion, it looks like in *D. discoideum* the fate of cell is determined based on pre-aggregation heterogeneities,

intercellular interactions and possibly morphogens too play important role (Kawli and Kaushik, 2001).

### **Evolutionary explanations for altruistic behaviour in CSMs**

Evolutionary explanations for 'altruistic' behaviour of stalk cells in the CSMs have been discussed by many authors (Bonner, 1982; Armstrong, 1984; Nanjundiah, 1985; DeAngelo et al., 1990; Matsuda and Harada, 1990; Atzmony et al., 1997; Kaushik and Nanjundiah, 2003). Their arguments are summarised below. Before doing so, I wish to point out that the possibility of reciprocal altruism has not been considered by anyone. It is difficult to see how meaningful reciprocity can be sustained from one generation to the next when individual identity is not preserved.

**Group selection:** The group selection point of view is that the death of few stalk cells seems to be of obvious benefit to the group within which it is expressed i.e., 'altruistic' behaviour of stalk cell has evolved as a group-level adaptation. That is to say, the ecology of the CSMs was, and is, such that the members of a species are dispersed in the form of heterogeneous groups (here, aggregations). Heterogeneity can derive from one or both of two circumstances. It can be that some individuals are predisposed genetically to behave altruistically whereas others are not. Alternatively, an individual of the same genotype can behave altruistically or selfishly with different given probabilities. The productivity of a group has to do with the successful reproduction of the average member of the group. For a given group size, this depends on (among other things) the number of spores formed and on the likelihood that they disperse successfully. A simple trade-off argument shows that optimal group productivity is attained when (a) some members of the group function as altruists, and (b) the relative allocation of amoebae to the spore (selfish) and stalk (altruist) pathways is a constant (Nanjundiah, 1985). The argument works best for clonal groups, in which case it forms a special case of kin selection (see below). Even then, there are problems having to do with the fact that a group is at risk of being exploited by a constitutively selfish individual, a cheater.

In the case of non-clonal groups, there are three possibilities. Groups might be

subject to frequent decline, including extinction (if they contain a larger proportion of cheaters than they can sustain). Alternatively, groups in the wild could harbour a small, but on the average fixed, proportion of cheaters. Matapurkar and Watve (1997) have shown that under suitable assumptions concerning dispersal, the frequency of cheaters can be maintained at a low but stable level. The third possibility is that the altruist is a 'partial altruist', meaning one that can sometimes behave selfishly. In the last case, as we will see, the reasoning once again slides into that of kin-selection. The group selection point of view makes one prediction; in fruiting bodies, the relative proportions of stalk and spore cells will be invariant over a large range of total cell numbers. Where it has been tested (in the case of *D. discoideum*), this prediction appears to be true in the case of aggregations involving 100 or more cells (Bonner, 1967; Nanjundiah and Bhogle, 1995). There is one observation that could be used in support of both group and kin selection models. In laboratory experiments on *D. discoideum*, it has been found that an essential factor, necessary for an amoeba to differentiate into a spore, is provided by another amoeba that differentiates into a stalk (Anjard et al., 1997; Anjard et al., 1998).

**Kin selection:** If all amoebae in an aggregate have the same genes, the ability to have some amoebae give up their lives so that others are benefited could be selected by kin selection. Here kin selection must be thought as acting differently from the way it does in a sexually reproducing population. Instead of the likelihood that two alleles, chosen at random from different individuals, are identical by descent, we have to take into account the likelihood that two individuals picked from a group at random are identical by descent, i.e., are members of the same clone. Then kin selection refers to the possibility that in polyclonal aggregates, natural selection will favour the spread of a gene whose presence makes an amoeba carrying that gene to express a trait that improves the reproductive success of its clonal group over that of other clonal groups, without necessarily improving the reproductive success of the individual amoeba itself. This assumes that the amoeba can recognize clone-mates and direct its behaviour appropriately towards them. In *D. discoideum*, the genes involved in cell adhesion systems have been shown to play role in self/non-self discrimination (Queller et al., 2003; Benabentos et al., 2009; Hirose et al., 2011).

Matsuda and Harada (1990) showed that in a polyclonal group, clonal

reproductive success would be maximized when each clone contributed a fixed number of cells to the spore pathway and the remaining cells to the stalk pathway. In other words, the reproductive success of a clone would be highest when there was a suitable distribution of selfish and altruistic behaviours between the members of the clone. However, if the total number of cells belonging to that clone fell below the relevant fixed number, no stalk cells would be made – i.e., the members would behave selfishly. A subsidiary prediction was that the intensity of altruistic behaviour – as measured by the relative fraction of spore cells overall – would drop with the number of clones that constituted a group. Experiments on *D. giganteum* have falsified both predictions (Kaushik et al., 2006).

Several studies have been carried out on the genetic diversity in close proximity as well as within social groups formed in the wild. The data indicates that the CSM groups in the wild can be polyclonal (Strassmann et al., 2000; Kaushik, 2002; Fortunato et al., 2003; Kaushik and Nanjundiah, 2003; Kaushik et al., 2006; Sathe et al., 2010) or clonal (Gilbert et al., 2007; Gilbert et al., 2009). The important question is whether there are important differences in behaviour between clonal and polyclonal groups. Work from the Strassmann-Queller laboratory on *D. discoideum* and *D. purpureum* has claimed that such differences do exist (Foster et al., 2002; Mehdiabadi, et al., 2006; Ostrowski et al., 2008). Work in our laboratory (on *D. giganteum* and *D. purpureum*) indicates otherwise (Kaushik, 2002; Kaushik et al., 2006). The discrepancy awaits resolution.

As with group selection, kin selection models become unstable if an obligate cheater appears. Such situations have been observed in laboratory strains of *D. discoideum*; here individuals carrying certain mutations differentiate into spores with higher probabilities (Ennis et al., 2000; Santorelli et al., 2008; Khare et al., 2009).

**Individual-level selection:** The essence of this model is that for an amoeba, not joining a group is on an average always a lower-fitness option compared to joining a group, even if its subsequent fate in the group is to die and form a stalk cell. Whether in fact it forms part of the stalk or the spore mass depends on its pre-aggregation phenotype, which can be thought of as a set of semi-independent ‘qualities’ (Atzmony et al., 1997), and on post-aggregation interactions that are influenced by the same qualities. The individual selection model is indifferent to the level of genetic relatedness within a

group and claims that the developmental consequences that follow after aggregation are in a sense mechanical, i.e., they are consequences of cellular phenotypes and their interactions only. According to it, the same proximal factors (cellular properties and cell-cell interactions) that guide behaviour in clonal groups determine the nature of the outcome in polyclonal groups. Observations that support the model are that (a) isolated starved amoebae of *D. mucoroides*, *D. giganteum*, and *D. discoideum* invariably die (Gregg, 1971; Sathe S., personal observation); (b) pre-aggregation amoebae vary in their phenotypes in ways that correlate with their post-aggregation fates; and that because of this, (c) when an amoeba joins an aggregate, it runs a risk of dying but also has a chance of successfully sporulating. A prediction based on the model, namely that a stalk-inducing lipophilic chemical, DIF, would be made selectively by presumptive spore cells, and the prespore cells themselves would be relatively resistant to it, was subsequently confirmed (Thompson and Kay, 2000a). Conceptually, the individual-selection argument scores over the others in one respect: by making pre-aggregation phenotypes central to the issue, it does away with the problem of 'cheaters' (meaning that the success or failure of a prospective cheater depends, as with any other amoeba, on a whole range of pre-aggregation fitness-related traits that it possesses). An important aspect of CSM development that the individual-level selection model cannot account for, at least not automatically, is the relative constancy of cell type proportions – something that a group selection argument explains easily. Partly in order to get over this difficulty, it has been suggested that in evolutionary past of the CSMs, selection must have operated sometimes in clonal groups and at other times in polyclonal groups (Kawli and Kaushik, 2001).

**Imposed altruism:** It is possible that some amoebae in a group (prespore cells) induce the stalk cell pathway in other members of the group (prestalk cells). In that case, the behaviour of stalk cells can be considered as imposed altruism. The following observations can be used in favour of this hypothesis: in *D. discoideum*, prespore cells make more differentiation-inducing factor than prestalk cells (Brookman et al., 1987; Kay and Thompson, 2001) and prestalk cells actively degrade it (Insall et al., 1992; Kay et al., 1993).

## Summary

Cooperative behaviour, and altruistic behaviour in particular, poses an interesting challenge to explanations based on evolution by natural selection. Cellular slime mould amoebae aggregate and form multicellular fruiting bodies made up of a column of dead stalk cells holding a ball of viable spores on top. The death of stalk cells appears to be a form of altruistic behaviour. Thus, cellular slime mould amoebae offer a good study system for exploring the evolutionary forces that lead to the emergence of cooperation.

## Scope of this thesis

The knowledge of the genetic structure of social groups is essential for understanding the role of genetic relatedness in the evolution of social behaviour. Therefore, experiments were designed to monitor genetic heterogeneity within social groups of *D. giganteum* and *D. purpureum* formed under natural conditions. A majority of social groups formed by *D. giganteum* and *D. purpureum* were polyclonal (see chapter 2). In polyclonal social groups, amoebae belonging to different genotypes can compete to form spores. This was observed when two genotypes belonging to the same CSM species were mixed and allowed to form chimaeric social groups (see chapter 3). Despite such competition and the tendency of certain genotypes to form spores, multi-clonal social groups are frequently observed in the wild. In order to address this puzzle, several fitness-related traits distributed over the entire life cycle of cellular slime moulds were studied. The results indicate that fitness-related trade-offs exist in the life cycle of *D. giganteum*; conceivably they can contribute to the maintenance of polyclonal social groups in nature (see chapter 3). In chapter 4, I discuss inter-species interactions in the CSMs. All the observations and question for future are summarized in chapter 5.

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## Chapter 2

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### *Large Mammals as Dispersal Agents and Genetic Heterogeneity in Wild Isolates\**

\*A major part of this study has been published as

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## 2.1 Introduction

Spatial distributions of microorganisms and their ecological importance have been discussed for a long time (O'Malley, 2008). It has been proposed that populations of macroorganisms (large animals) are mostly viscous, and spatial distribution is mainly determined by patterns of dispersal. On the contrary, in case of microorganisms it has been suggested that dispersal is rampant (that is, "everything is everywhere") and spatial distribution is determined based on adaptations to local environments (O'Malley, 2008). In the case of social organisms such as cellular slime moulds (CSMs), dispersal patterns have a bearing on whether the social groups formed by them are made up of genetic relatives (clones) or unrelated individuals. The genetic structure of social groups, therefore, is essential for understanding the importance of genetic relatedness in the evolution of social behaviour. This study was carried out to address these two related issues: (a) spatial distribution and modes of dispersal of CSMs and (b) genetic heterogeneity in social groups of CSMs found under natural conditions.

Although CSMs have been studied for close to 150 years, our knowledge about their ecology and social behaviour is rather poor. We still lack clear answers to several questions. What are the different habitats occupied by CSMs? What is their biogeographical pattern, in other words, how are different species of CSMs distributed across the globe? Are there 'specialists' (adapted to limited environments) and 'generalist' (adapted to all kinds of environments) among CSMs? How do different CSMs interact with each other and with other organisms present in the wild? CSMs build social groups in response to starvation. It is believed that the formation of a fruiting body in CSMs has evolved as an adaptation for dispersal from a nutrient-poor environment. How is dispersal achieved? Are propagules densely distributed - or are they sparse, so that social groups in the following generation are made up of clones?

Multicellularity in CSMs is achieved by the aggregation of free living amoebae. This peculiar way of becoming multicellular can lead to co-aggregation of genetically unrelated amoebae. It is important to know if this actually happens in the wild, as in

most species of CSMs, formation of a fruiting body involves division of labour; stalk cells sacrifice their lives in an 'altruistic' manner and support a mass of live spores on top. It has been hypothesised that relatedness by common descent could be a factor behind the existence of 'altruistic' traits (Hamilton, 1964). Relatively simple and experimentally tractable organisms such as CSMs are ideally suited for validating this hypothesis. In this study, I have attempted to address this hypothesis by genotyping spores from CSM fruiting bodies isolated from the wild. I have also made an attempt to address other interesting questions which deal with the population structure of CSMs. Among them are: (a) How many CSM species are present in a small locale? (b) How are they related with each other? (c) If there is more than one species in a small area, how do they interact with each other? (d) Assuming that these species compete with each other for common resources, can they co-exist stably? Questions b to d are mentioned here only briefly, they are explored in detail in chapters 3 and 4. What follows is a discussion of these fundamental issues in CSM ecology and sociobiology.

## **2.2 Cellular slime moulds in nature**

The first CSM species known to us, *Dictyostelium mucoroides*, was isolated by Oskar Brefeld in the late 1860s initially from horse dung and subsequently from rabbit dung (cited in Raper, 1984; Kessin, 2001). At one time, CSMs were considered to be coprophilous organisms, associated with herbivore dung. This is because earlier investigators like Nadson (1884), Olive (1901; 1902), Potts (1902) and Pinoy (1903) found CSMs primarily in animal dung (cited in Raper 1984). Olive (1975) reported CSMs from the dung of horse, cow, muskrat and rabbit. Opinion changed gradually as Krzemieniewski in 1927 and Harper in 1929 isolated CSMs from soil samples collected from Poland and New York city respectively (cited in Raper, 1984). Raper (1935) isolated *D. discoideum* from decomposing leaf litter collected in a hardwood forest of the North Carolina. These findings motivated many others to look for CSMs from other habitats. Singh (1946) found *Dictyostelium* species in soil collected from various parts of Britain. Cohen (1953), Cavender and Raper (1965a, 1965b) isolated many species of CSMs from temperate deciduous forest soils. CSMs

have been isolated from soils from all parts of the world including environments that vary from cold temperate to tropical (Swanson et al., 1999). CSMs have also been reported from unusual environments like soil collected from dark caves (Landolt et al., 2006) or animal dung like fecal pellets of crickets and bats (Waddell, 1982; Stephenson et al., 2007). Bat and cricket fecal samples that yielded CSMs were also collected from dark caves (Waddell, 1982; Stephenson et al., 2007). CSMs have been isolated from the gut and dung of arthropods (Huss, 1989), nematodes (Kessin et al., 1996), amphibians (Stephenson and Landolt, 1992; Gilbert et al., 2007), birds (Suthers, 1985; Stephenson and Landolt, 1992) and small mammals (Stephenson and Landolt, 1992). CSMs are not restricted only to soil or animal dung; even plant structures such as moribund fruits, flowers and leaves have been found to contain them (Olive, 1960). From these studies, it is fair to conclude that CSMs can be recovered from most environments that are moist, aerobic and can support bacterial growth.

### 2.3 Cellular slime moulds from India

Agnihotrudu (1956) found a number of CSM species in cultivated and uncultivated soils as well as in rhizosphere soils from South India. Rai and Tewari (1961; 1963a; 1963b) isolated *D. mucoroides*, *D. sphaerocephalum*, *Polysphondylium violaceum* and an aberrant form of *P. violaceum* from soil on the campus of Lucknow University (26° 55' N, 80° 59' E) and from Pallia, Kheri district (27° 60' to 28° 60' N, 80° 34' to 81° 30' E). Cavender and Lakhanpal (1986) isolated *D. giganteum*, *D. purpureum*, *D. mucoroides*, *P. violaceum*, *P. pallidum*, *D. polycephalum*, *D. aureo-stipes*, *D. macrocephalum*, *D. tenue* and *D. vinaceo-fuscum* from soils collected from tropical evergreen and deciduous forests. The following species have been isolated from soils collected from the Mudumalai dry deciduous forest: *D. purpureum*, *D. giganteum*, *D. rosarium*, *D. discoideum*, *D. macrocephalum*, *D. polycephalum*, *D. minutum*, *D. mucoroides*, *D. tenue*, *Dictyostelium* species which resembled *D. bifurcatum*, *P. violaceum* and *P. pallidum* (Kaushik et al., 2006; Sathe et al., 2010).

## 2.4 Dispersal vectors

CSM amoebae are voracious eaters that quickly consume local food sources resulting in nutrient starvation. In order to avoid starvation and possible death, amoebae must travel towards other food sources where they can continue their growth and cell division. Although amoebae are motile, their movement is very slow (Singh, 1946); for example, *D. discoideum* amoebae move at a speed of 20-30  $\mu\text{m}/\text{min}$  for nearly 100 seconds in response to cAMP (Hofer et al., 1995). In the absence of any signal, *D. discoideum* and *P. pallidum* amoebae on an agar surface move with an average speed of 7  $\mu\text{m}/\text{min}$  for up to 10 hr (Li et al., 2008). With such a slow speed amoebae probably can get to a nearby ( $\sim 5$  mm) food source but cannot go much further than that. Also the speed of amoebal movement is likely to be slow in soil because of its rough texture as compared to agar. The strategy adapted by CSMs to tide over starvation is to undergo sporulation and passive dispersal.

CSMs under nutrient starvation form a fruiting body made up of spores and a stalk. Spores are dormant and can tolerate unfavorable conditions for long durations of time (Bonner, 1967; this study). Also, slugs, formed prior to the fruiting body, are motile and can aid in dispersal. Fruiting bodies generally develop above the soil surface and spores are sticky, they can get away from a nutrient poor environment with the help of suitable agents of dispersal.

**Water:** O'Dell (1979) found four species of CSMs of the genera *Dictyostelium* and *Polysphondylium* from lake-bottom samples, showing that they can survive under water and are possibly dispersed by moving water. Human agricultural activities such as irrigation could spread CSMs. In wet environments amoebae of heterothallic strains are known to fuse and form giant cells ( $\sim 50$   $\mu\text{m}$ ) known as macrocysts (Erdös et al., 1973; Kessin 2001). Macrocysts are resistant to high moisture and starvation and can conceivably be transported by water.

**Air:** since CSM spores are embedded in a mucopolysaccharide (slime) sheath, spores are sticky. This makes individual spore dispersal by air unlikely. However, in summer the soil becomes dry and spores attached to dust particles could be dispersed by wind (Cavender, 1973). Also, the entire spore mass could be spread by wind and carried elsewhere.

**Birds:** CSMs can be dispersed by several birds. Suthers (1985) found 11 species of CSMs in fecal samples of migratory birds. In a series of feeding experiments, Suthers demonstrated that, amoebae, spores and macrocysts survive in the gut of birds up to 10 days. Stephenson and Landolt (1992) isolated CSMs from three types of birds: Wood thrush (*Hylocichla mustelina* Gmelin), Carolina wren (*Thryothorus ludovicianus* Latham) and Slate-coloured junco (*Junco hyemalis* Linnaeus). These are ground feeding bird species and likely to encounter CSMs during feeding. Since birds fly over large distances, they can disperse CSMs to sites that can be quite far from each other.

**Arthropods and Annelids:** Huss (1989) isolated *D. giganteum*, *D. purpureum*, *D. mucoroides*, *D. minutum*, *D. aureo-stipes*, *P. tenuissimum*, *P. violaceum* and *P. pallidum* from the gut of earthworms (*Aporrectodea caliginosa* and *Octolasion tyrtaeum*) and pillbugs (*Armadillidium nasatum* and *A. vulgare*). Huss later force-fed these arthropods and annelids with CSM spores and vegetative amoebae and showed that spores and (to some extent) amoebae can survive the gut environments. Stephenson et al. (2007) isolated five species of dictyostelids (belonging to *Dictyostelium* and *Polysphondylium*) from the body surface of a cave cricket (*Ceuthophilus gracilipes gracilipes*), and a single species (*D. sphaerocephalum*) from a fecal sample of a cricket. These dispersal agents can pick CSMs actively (by feeding on them) or passively (spores are sticky and stick to body parts) and disperse them.

**Small and large mammals:** Waddell (1982) isolated 8 species of CSMs from bat guano collected from dark caves; one isolate (*D. caveatum*) exhibited a remarkable predatory behaviour on other species of the CSMs. Stephenson and Landolt (1992) found CSMs in the dung of five small mammals; a brown bat (*Eptesicus fuscus*), an eastern woodrat (*Neotoma floridana*), a white-footed deer mouse (*Peromyscus leucopus*), a pine vole (*Pitymys pinetorum*), and an eastern chipmunk (*Tamias striatus*). Gilbert et al. (2007) recovered CSM fruiting bodies from the dung of whitetail deer (herbivore). In this study I report the isolation of several CSM species from the dung of a variety of large mammals.

**Other agents:** Kessin et al. (1996) showed that spores of *D. discoideum* could be dispersed by the nematode *Caenorhabditis elegans*. Although this worm can digest

amoebae, aggregates, slugs and spores seem to be protected. CSMs have also been isolated from an amphibian (the red-backed salamander *Plethodon cinereus*) by Stephenson and Landolt (1992) and from fecal pellets of a salamander (name not mentioned) by Gilbert et al. (2007). Salamanders eat small insects present in leaf litter (Jaeger, 1972) and probably during foraging ingest CSMs.

### **2.5 Diversity in the wild and genetic heterogeneity within social groups**

Our understanding of CSM diversity in the wild and genetic heterogeneity within social groups comes from bringing CSM samples to the laboratory and examining population structure by standard isolation and DNA genotyping methods. Samples are analysed in the laboratory because CSM fruiting bodies have rarely been observed in their natural habitats with the exception of observations by L Olive (cited in Fortunato, 2003) and Gilbert et al (2007).

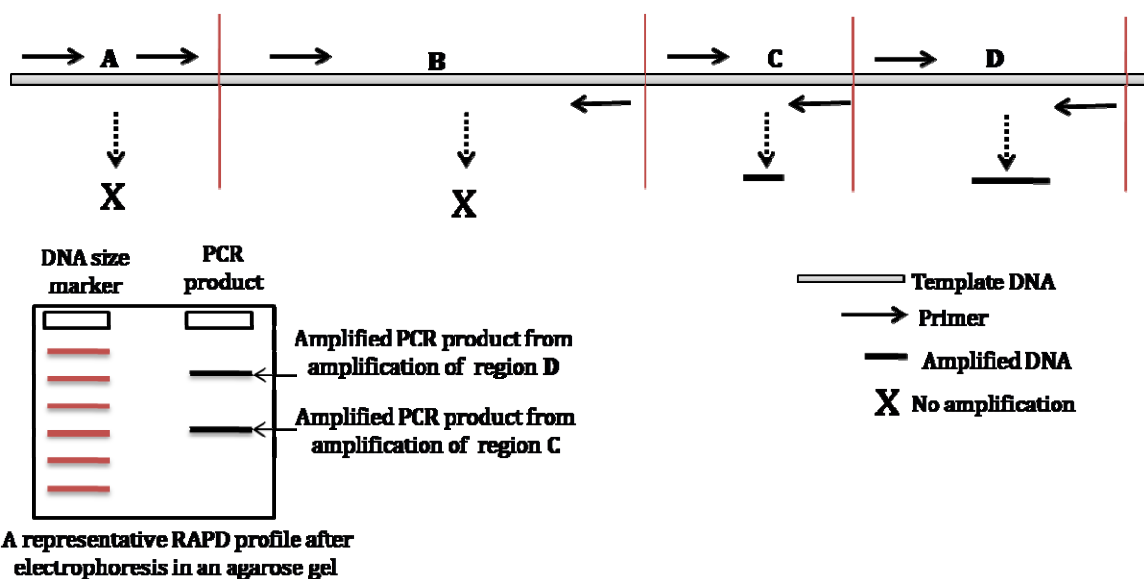
Earlier studies have addressed the question of genetic diversity of CSMs, and in some cases also from the same social group. Agnihotrudu (1956) found *D. mucoroides*, *D. discoideum*, *D. giganteum*, *D. purpureum*, *D. minutum*, *P. violaceum* and *P. pallidum* in soils; in many instances he observed 2 to 3 species of CSMs from the same sample (10 gm soil). Rhizosphere samples (5 gm soil) of the pigeon pea (*Cajanus cajan*) in one instance yielded 4 species and that of the peanut (*Arachis hypogea*), 5 species of CSMs. Rai and Tewari (1961) found *D. mucoroides* and *P. violaceum* co-occurring in several soil samples collected from different areas around Lucknow University campus, India. These reports indicate a finding of different species of the CSMs; however, the question of whether different strains (genotypes) of one species co-occur was not addressed. Filosa (1962), while working on *D. mucoroides* (isolated from giraffe dung collected from a zoo) that had been maintained for 8 years in the laboratory, found that the cultures contained four variants. Clonally isolated spores from these cultures exhibited heterogeneity; some formed 'wild-type' fruiting bodies, while others (variants of 'wild-type') did not and were stalk-less. Variants which were incapable of forming normal fruiting bodies in isolation could do so in the presence of 'wild-type' amoebae. Buss (1982) isolated stalked ('wild-type') and a stalk-less aberrant variant of *D. mucoroides* from

chimaeric fruiting bodies isolated from small soil area (1mm). Francis and Eisenberg (1993) while looking at the genetic structure of *D. discoideum* isolated from North Carolina soil found genetically distinguishable strains over an area of 50 x 100 m, while some samples collected at scattered points ~ 1500 m away from each other. Ketcham and Eisenberg (1989) found different *P. pallidum* strains occurring close to each other (~ 1cm) in North American hardwood forest soil; these strains belonged to different mating types and had differences in growth rates. Fortunato et al. (2003) collected soil (mean sample weight was 0.2 gm) from Virginia, USA with a 6-mm diameter plastic straw and found that several haplotypes (clones) of *D. discoideum* co-occurred in their samples. Mixing experiments performed in the laboratory showed that wild isolates of *D. discoideum* can form chimaeric fruiting bodies (Strassmann et al., 2000; Fortunato et al., 2003). Kaushik and Nanjundiah (2003) isolated CSMs from dry deciduous forest soil and reported at least 9 different genotypes within a single spore mass in a *D. giganteum* fruiting body that had formed under quasi-natural conditions. Genetically distinct strains of *D. giganteum* were isolated from close by (~10 cm) distance in soil; these strains formed chimaeric fruiting bodies under laboratory environment (Kaushik, 2002; Kaushik et al., 2006). From these studies, the inference is that there is much genetic diversity within CSM groups in the wild. Contrary to this, Gilbert et al. (2007) isolated fruiting bodies from white tail deer pellets in North America and found that most of them consisted of a single clone. Gilbert et al. (2009) reported a dense, 12m clonal patch of *D. discoideum* in a cattle pasture located in a Texas Gulf Coast prairie indicating that clonal social groups also exist in nature.

### **A method for estimating genetic heterogeneity**

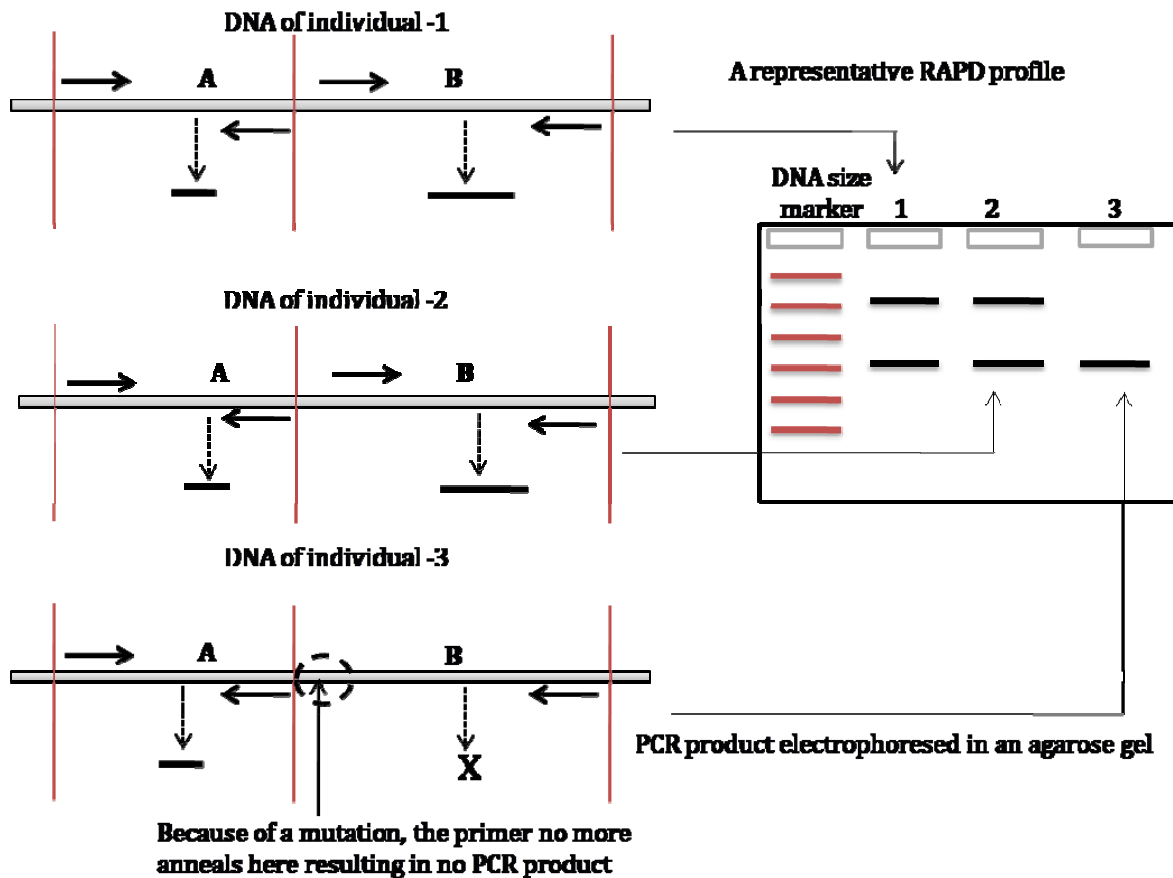
Random amplification of polymorphic DNA (RAPD) is a simple and reliable technique frequently used for genetic variation analysis (Williams et al., 1990). RAPD involves the use of primers with random sequences (usually 10 nucleotides long) which anneal specifically to complementary regions on a template DNA. Polymorphism can be inferred when a DNA sequence amplified in one individual is not amplified in another. This could happen because of mutations at primer

annealing regions on a template DNA or insertions/deletions among primer binding sites. A schematic representation of the technique is shown in Fig. 2.1. RAPD is a popular method for assessing genetic heterogeneity between DNA samples as it requires no prior knowledge of the DNA sequence. Low cost, simplicity of the technique and no involvement of radioactivity mean that numerous markers can be developed quickly and safely. RAPD based genetic variation can be easily analysed by agarose gel electrophoresis (see Fig. 2.2).



**Figure 2.1: A schematic representation of the Random Amplification of Polymorphic DNA (RAPD) method.** Primers (oligomers usually 10 nucleotides long, shown as black arrows) anneal on a template DNA (gray bar) at the specific complementary regions. If a primer binds at locus A (same orientation 5' to 3') or at locus B (in opposite orientation but away from each other) template DNA remains unamplified. However, if the primers anneal at locus C and/or D (in opposite directions, close to each other on the template DNA) they will amplify the template DNA at locus C and D. Amplified DNA from locus C yields a smaller sized PCR product compare to locus D and migrates faster when electrophoresed on an agarose gel.





**Figure 2.2:** A schematic representation of the principle of RAPD. DNA from three individuals (spores) is subjected to RAPD-PCR using a single primer. If the template DNAs are the same (e.g. DNAs from individuals 1 and 2) the primer anneals at similar regions in both individuals, which leads to amplification of similar-sized PCR products. If the template DNA is different (e.g. individuals 1 and 3), the primer annealing sites are likely to be different or even absent in one of them. Amplification of different DNA regions produces different sized PCR products; the absence of a primer binding site leads no amplification (as shown in the case of individual 3). The differences in amplification status between individuals can be visualized by electrophoresis in an agarose gel.

RAPD has been successfully used for studying various phenomena in bacterial plant and animal systems. Reiter et al. (1992) constructed a genetic map of *Arabidopsis thaliana* based on RAPD polymorphism and Lery et al. (2003) used RAPD for characterization and authentication of insect cell lines. Okatani et al. (2000) detected species-specific RAPD patterns of *Erysipelothrix* and used them for

epidemiological analysis. Inglis et al. (2001) used RAPD to analyse genetic variation within Brazilian isolates of *Cercospora caricis*. RAPD has been shown to be a useful tool in epidemiological studies and for finding genetic differences in bacterial isolates (Lam et al., 1995; Ozbey et al., 2004), and in identifying markers linked to disease resistance genes in tomato (Martin et al., 1991) and barley (Barua et al., 1993).

Although RAPD markers have many advantages over other DNA markers (viz., Restriction Fragment Length Polymorphism, Amplified Fragment Length Polymorphism, Isozymes and Microsatellites) the reproducibility of the method has been questioned. Campbell et al. (2000) evaluated its usefulness for typing *Pseudomonas aeruginosa* and found a reproducibility of 98.5%. As McClelland and Welsh (1994) pointed out, in almost all cases inadequate preparation of DNA leads to non-reproducible results. As shown below, the RAPD data generated in this study was highly reproducible. Special precautions were taken to ensure the reliability of the data, these included: (a) use of only high quality, purified DNA samples for analysis; (b) initial testing of reproducibility of amplification for the selected primers on at least two different PCR-machines (I-Cycler, BioRAD, USA and thermocycler PTC-200, MJ Research, USA); and (c) generation of data on all the samples for a minimum of two times, followed by scoring of only reproducible well-resolved/amplified RAPD fragments. Also, because CSMs are haploid, the issue of relative dominance between alleles does not arise and band intensity differences (which can be a problem in diploid organisms because of competitive amplification) do not affect the outcome.

## 2.6 Materials and methods

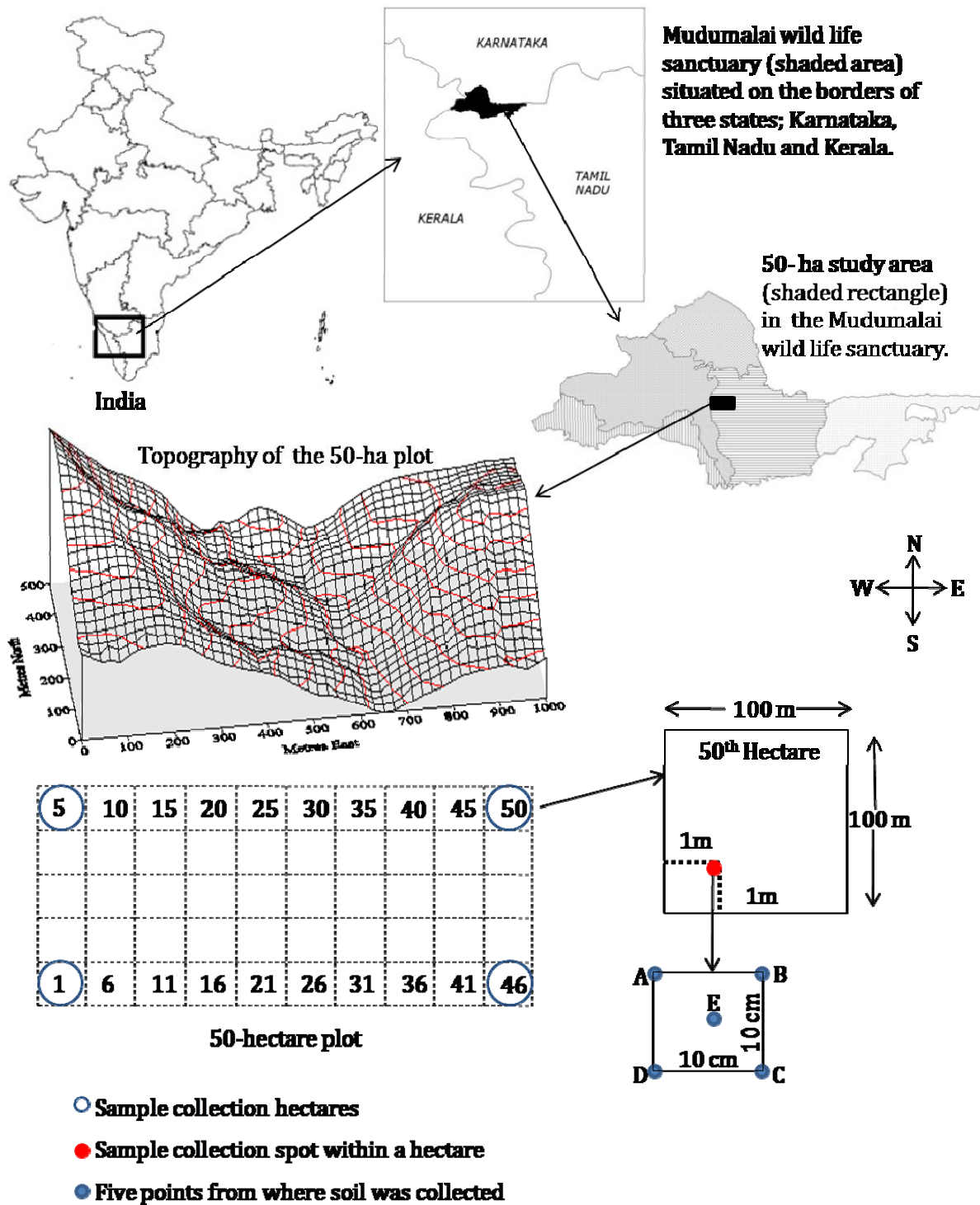
### 2.6.1 Sample collection

Soil and animal dung were collected twice (between 10:00 AM - 01:00 PM on 3<sup>rd</sup> January 2008 and 7<sup>th</sup> October 2008) from grassy areas near the Teppekadu-Mudumalai road, Teppekadu-Kargudi road and the 50-ha study plot in the Mudumalai wild-life sanctuary, South India. This sanctuary is located between 11°32' - 11°43' N and 76°22' - 76°45' E in the Nilgiri range of the Western Ghats at an altitude of 850 - 1,250 m. It covers an area of about 320 km<sup>2</sup> and consists mainly of dry deciduous forest. Some part of the sanctuary is also covered by moist deciduous and dry thorn forests. The topography of the sampling area is shown in Fig. 2.3. Fresh dung samples were lifted with sterile forceps and immediately transferred to wide-mouthed sterile plastic tubes (2 X 10 cm), sealed with Parafilm tape, and transported the same day to the laboratory in Bangalore about 300 km away. Soil contamination was avoided by picking samples only from the top and middle portions of animal dung pellets. Yak (*Bos grunniens*) dung was collected on 25<sup>th</sup> July 2008 by V. Nanjundiah from animals grazing in the Changla pass in the dry high-altitude desert of Ladakh, north India (33°95' N, 77°85' E; altitude 5,300 m). It was placed in a sterile plastic bag and brought to the laboratory after a week.

Soil samples were collected from the southern part of each of the 1<sup>st</sup>, 5<sup>th</sup>, 46<sup>th</sup> and 50<sup>th</sup> hectares from the 50-ha plot (see Fig. 2.3; hectares are shown by blue circles and sample collection area is shown by red dots, these points lie 1 meter away from the southern and western boundaries of the hectare). Five soil samples were collected from each hectare (four corners and centre of a 10 x 10 cm square). Points A, B, C, D, and E shown as blue dots in Fig. 2.3 indicate the place of soil collection in each hectare. The smallest particle of soil that could be picked (approximately 250-1000 µm) was collected with sterile forceps and immediately transferred on SM/10, phosphate buffered agar (PBA) or PBA plates preinoculated with *Klebsiella aerogenes*. A large mass of soil (approximately 5gm) was collected from the same points mentioned above and transferred to wide-mouthed sterile

plastic tubes. All plates and bottles were sealed immediately with Parafilm tape and brought to the laboratory without disturbing the soil particles.

At the time of sample collection the 50<sup>th</sup> hectare soil was relatively dry (moisture content 7% at top soil layer, 17% at 5 cm depth), black, loosely packed, and covered with grass (*Themeda triandra*). The last rain had fallen 20 days before sampling; air and soil (at 5 cm depth) temperatures were 22°C and 20°C respectively. Soil samples were collected from the base of a *Tectona grandis* tree which was covered with termites and appeared dead as no leaves or bark could be observed. The 46<sup>th</sup> hectare soil was relatively dry (moisture content 10% top layer, 13% at 5 cm depth), ash colored, tightly packed, and covered with shrub (*Lantana camara*). The soil (at 5 cm depth) and air temperatures were 22.5°C and 26°C respectively.



**Figure 2.3:** A schematic representation (a modified version from Kaushik, 2002) of the area from where soil and animal dung was collected.

### 2.6.2 Isolation of CSMs from animal dung and soil

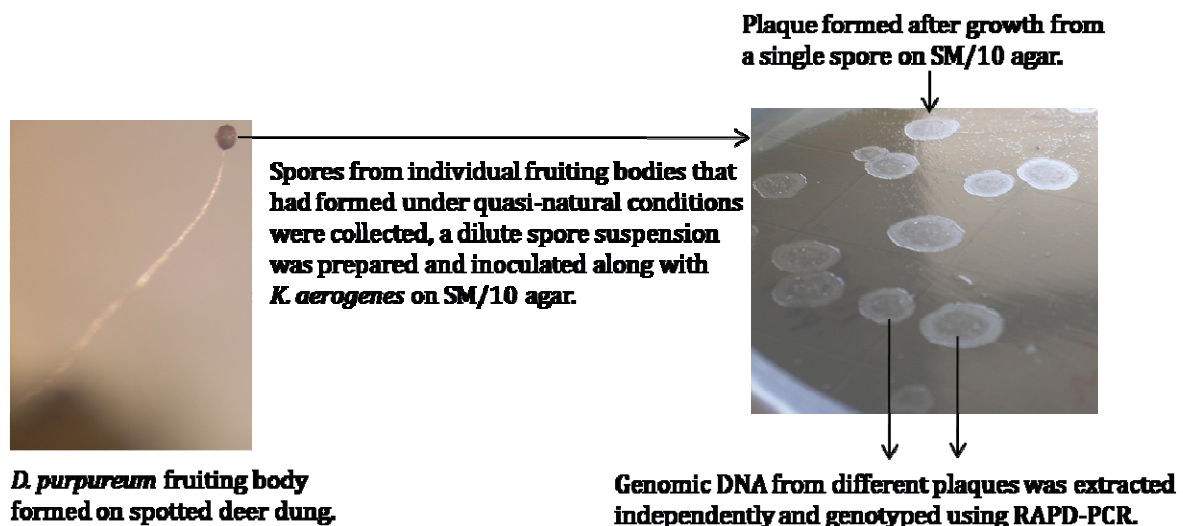
All growth media and buffer components used for CSM isolations unless otherwise stated were from Himedia, India or Sigma chemical company, U.S.A. To isolate CSMs from the collected soil and dung samples, three types of media were used. These were: (1) 2% phosphate-buffered, non-nutrient agar (PBA;  $\text{KH}_2\text{PO}_4$  2.25 gm,  $\text{K}_2\text{HPO}_4$  0.67 gm, agar 20 gm,  $\text{H}_2\text{O}$  1,000 ml, pH 6.4); (2) SM/10 nutrient agar (a 10% dilution of the nutrients in standard SM agar; Sussman, 1987); (3) PBA pre-spread with *Klebsiella aerogenes*. Non-nutrient PBA and SM/10 were used to see if CSMs could be isolated without any *externally* added food source, thereby mimicking the natural situation.

All soil and dung samples collected from the Mudumalai area were processed within 24 hrs of collection. Yak dung was analysed after a week as it took time to bring a sample to the laboratory. Dung samples (30 mg-1 gm) were carefully transferred to 60-cm<sup>2</sup> plastic plates containing media as mentioned above. Plates were immediately sealed with Parafilm and incubated in the dark in a moist incubator at 22°C. In other experiments when the aim was only to look for the presence of CSMs, not to study genetic heterogeneity, samples were analysed by a different method. In these experiments, a small portion of a dung sample (approximately 500 mg) was shaken thoroughly in 1 ml sterile phosphate buffer, 100 µl of this mixture was mixed with a thick suspension of *K. aerogenes* and inoculated on a PBA plate. Soil samples were treated in the same fashion as the dung samples. For CSM diversity measurements (types of CSMs present), 1 gm of soil was diluted in sterile water by gentle mixing and 100 µl of the mixture was inoculated on a PBA plate along with a thick suspension of *K. aerogenes*. Plates were monitored intermittently from the second day onwards. CSM fruiting bodies that had formed were observed using a stereomicroscope (ZEISS, Stemi 2000-C) and photographed directly using a CCD camera. For identification, spore masses were picked up with a sterile needle, suspended in 50 µl sterile  $\text{KK}_2$  buffer and plated on SM/10 medium at low density. The resulting clones were subcultured on fresh PBA plates with *K. aerogenes*. The preliminary identifications reported here (Table 2.2, 2.3) were based

on morphological criteria described by (Raper, 1984). Here, I wish to thank Dr. James Cavender (Ohio University) for his help in CSM identifications.

### 2.6.3 Estimating genetic heterogeneity within fruiting bodies

In order to estimate genetic heterogeneity within CSM social groups in nature, fruiting bodies were allowed to develop in conditions more-or-less similar to wild. Soil or animal dung samples were transferred carefully to non-nutrient (PBA) agar or, in some cases, to agar that contained only bacterial nutrients (SM/10 agar). Fruiting bodies that developed on these samples were picked up individually with a fine needle and suspended in sterile distilled water. A dilute suspension of spores (~ 1 spore/ $\mu\text{l}$ ) was prepared, 50  $\mu\text{l}$  of spore suspension was mixed with 500  $\mu\text{l}$  of thick suspension of *K. aerogenes* and inoculated on SM/10 agar. Plates were incubated at 22°C in the dark until well-separated plaques were seen; typically this took 2-3 days (Fig. 2.4). 15 to 25 single plaques were picked individually with sterile micropipette tips, suspended in 100  $\mu\text{l}$  sterile water, and stored at 4°C until use. From each plaque (which is likely to have formed from a single spore) DNA was isolated and used for RAPD analysis.



**Figure 2.4:** A schematic representation of isolation of individual spores from fruiting bodies that had developed on non-nutrient agar.

#### **2.6.4 DNA isolation**

DNA was isolated according to published protocols with minor modifications (Pilcher et al., 2007). Vegetative amoebae were harvested from growth medium (SM agar) in cold  $\text{KK}_2$  buffer, spun down three times at 300g for 3 min at 4°C to remove the remaining bacteria and re-suspended at a density of  $10^7$  cells/ml in cold nuclear buffer (25mM tris, 5mM magnesium acetate, 0.5 mM EDTA, 5% sucrose, pH 7.6). Amoebae were lysed using 2% NP40 (non-ionic detergent, United States Biochemical) and nuclei were separated by centrifugation at 5,000g for 10 min at 4°C. Nuclei were then suspended in a lysis buffer that was preheated to 70°C (4% SDS, 20mM EDTA, 10mM NaCl), incubated at 70°C for 5 min, and then at 50°C for 1 hr. The samples were incubated at 37°C with RNase (1µl of 10 µg/ml) and then with proteinase K (1µl of 20 mg/ml) for 1 hr each. Proteins were removed by adding an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) solution followed by chloroform: isoamyl alcohol (24:1) solution. During these stages the supernatant was removed by centrifugation at 12,000g for 15 min, carefully collected in new Eppendorf tubes and DNA was precipitated by adding double volumes of ice-cold ethanol, 0.7 mM ammonium acetate and incubating tubes at -20°C for 1 hr. The DNA pellet was collected by centrifugation at 12,000g for 10 min at 4°C and then rinsed twice in 70% ethanol, dried briefly at 37°C and dissolved in 50µl sterile water. The isolated DNA was further purified using the PhytoPure resin provided in a plant DNA extraction kit (Amersham Inc. USA) and then passed through a GFX-DNA purification Kit (GE healthcare). DNA purity and concentration were assessed after electrophoresis on a 0.8% agarose gel and measuring absorbance in a spectrophotometer (NanoDrop, Thermo Scientific, USA) at 260nm and 280nm. Use of purified high-quality DNA was found essential for reproducibility of RAPD-based DNA variation data.

#### **2.6.5 Random Amplification of Polymorphic DNA**

Purified genomic DNA samples were amplified using 10 bp long random primers (Operon Technologies., U.S.A.). The primer sequences are shown in Table 2.1. Each PCR reaction was carried out in a 15 µl reaction volume that contained 20



ng DNA as a template, 0.16  $\mu$ M of primer, 1 U Taq DNA polymerase (Amplitaq Gold, Applied Biosystems), 1x Taq buffer (with 1.5 mM  $MgCl_2$ ), and 150  $\mu$ M of each deoxynucleoside triphosphates (dNTPs) and 1x additive which was developed in Dr. Aggarwal's laboratory at Centre for Cellular and Molecular Biology, Hyderabad, India. The RAPD amplification profile initially standardized and used consistently was: an initial DNA denaturation step of 95°C for 3 min, followed by 36 three-step cycles of denaturation at 94°C for 45 sec, primer annealing at 36°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 5 min. Amplified RAPD products were resolved on a 1% agarose gel in 0.5x TBE buffer (Tris borate EDTA buffer: Tris-HCL base 5.4 g, boric acid 2.75 g, 0.5M EDTA 2 ml/L, pH 8.0) using a standard electrophoresis apparatus. Electrophoresis was carried out at constant voltage (~3-4 V/ cm gel length). Gels were stained with ethidium bromide and amplified DNA bands photographed under UV light.

**Table 2.1:** RAPD primers used in this study.

Serial number	Primer name	Nucleotide sequence (5'-3')
1	OPAV-01	TGAGGGGGAA
2	OPAV-16	GACAAGGACC
3	OPAV-18	TTGCTCACGG
4	OPAV-20	TCATGCGCAC
5	OPBG-06	GTGGATCGTC
6	OPBG-09	GGCTCTGGGT
7	OPBG-15	ACGGGAGAGA
8	OPBD-04	TCGGGTGTTG
9	OPBD-08	CATACGGGCT
10	OPBD-20	AGGCGGCACA
11	OPAD-02	CTGAACCGCT

### 2.6.6 Isolation of bacteria

Bacteria were isolated from the same soil samples from which CSMs were isolated. 1 gm soil was suspended in 9 ml sterile distilled water and mixed for 10-15

min in a shaker at 120 strokes/ min. This gentle agitation was found to be necessary for appropriate mixing of soil. The soil suspension was serially diluted by mixing 1 ml soil suspension and 9 ml sterile water. 100  $\mu$ l of  $10^3$  and  $10^5$  fold diluted soil suspensions were inoculated on low-strength nutrient agar (NA/10: peptone 0.5 gm/L, yeast extract 0.2 gm/L, NaCl 0.5 gm/L, agar 15 gm/L, pH 7.0–7.2), and lysogeny-broth agar (Bertani, 1951; enzymatic hydrolysate of casein 10 gm/L, yeast extract 5 gm/L, NaCl 5 gm/L, agar 20 gm/L). Low-strength nutrient medium was used to avoid growth of fungi. Plates were incubated at 37°C for 24-72 hrs and observed after every 12 hrs. Bacterial colonies were counted manually on a colony counter (LAPIZ, Bacteriological digital colony counter, India). Single, well isolated colonies were picked with a sterile nichrome wire-loop and streak-plated on to fresh nutrient agar plates. Single colonies formed thereafter were picked up and used for further analysis.

#### **2.6.7 Identification of bacterial isolates**

A preliminary characterisation of the bacterial isolates was based on their colony morphology. Morphological characters such as appearance (circular, irregular, convex, flat, etc.), size (tiny, big), pigment production (positive/negative, color) and motility (swarming growth) were studied. Morphological characterization alone was found to be useful to differentiate the bacterial isolates. Detailed biochemical characterisation for identification of bacterial species is in progress.

#### **2.6.8 Statistical analysis**

The RAPD- based genetic variation analysis indicates a minimum number of distinct clones in a spore mass. Since analysis involved only a minor fraction of total spores from the fruiting bodies it is possible that some clones were left undetected. The following approach has been used to estimate (theoretically) the number of undetected clones. It was necessary to assume that all the clones contribute equally to the spore mass and that the clones analysed were picked randomly. Let us say that the spore mass has **S** cells (total spores) belonging to **C** clones (distinct genotypes). Each clone will have **S/C** cells because it is assumed that all the clones

are represented equally. The question is, if a sample of  $n$  spores from such a fruiting body had  $m$  clones, how many clones may have been left undetected (i.e.  $C-m$ )? The maximum value of  $n$  in this analysis was 15. However, the total cells ( $S$ ) in a fruiting body are likely to be a few thousand at least. The analysis shown below helped to estimate the total number of clones. We term the detected clones ( $m$ ) as Class I and the undetected ( $C-m$ ) ones as Class II. Since these two form complementary classes their relative proportions can be treated as probabilities of occurrences of a given clone. The probability that a spore belongs to Class I is  $p = m/C$  and the probability that it belongs to Class II is  $q = (C-m)/C$ , where  $p+q = 1$ . Since the values of  $n$  and  $m$  are known one can calculate the values of  $p$  and  $q$  and estimate  $C$  as explained below.

The probability that none of  $n$  spores picked at random are from Class II is  $(1-q)^n$ , implying that the probability that at least one of those  $n$  spores belongs to Class II is  $1-(1-q)^n$ . Since in fact none of the  $n$  spores does belong to Class II, it can be said that  $[1-(1-q)^n]$  is likely to be  $\leq 1/n$ . This can be used to estimate  $q$ , and therefore  $C$ . This will be a conservative estimate in the sense that  $C$ , and therefore  $q$ , could be much larger and still lead to an absence of Class II spores from the sample of  $n$ . A better estimate will result if we reason that a Class II spore ought to have been picked up unless the probability of its being represented at least once in the sample of  $n$  had been lower than some pre-assigned threshold. We have taken the threshold to be as high as 0.95 and solved the equation  $1-(1-q)^n = 0.95$  for each sample size  $n$  to get  $q$ ,  $m/C$  and  $C$  (it turns out that  $C$  is essentially the same as  $m$  up to an assumed threshold of 0.5).

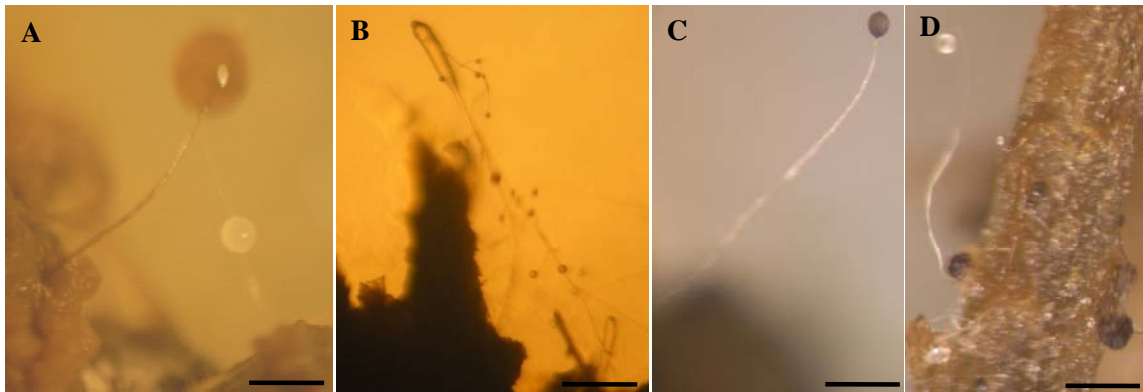
## 2.7 Results

### 2.7.1 Presence of CSM in large mammal dung

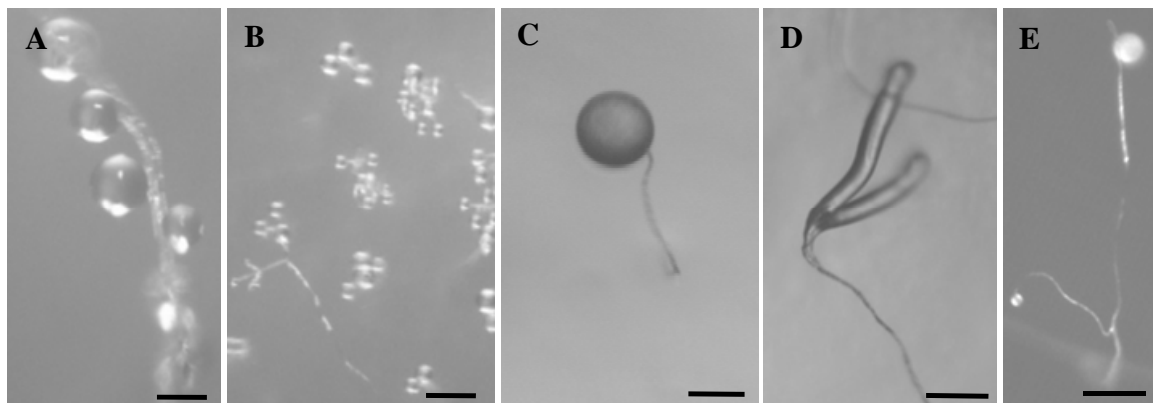
Dung samples inoculated on plates were observed after 2-8 days of incubation for the presence of CSMs. All plates (nutrient and non-nutrient) supported CSM development. The CSM fruiting bodies were visible within 2-3 days on plates that had been pre-spread with bacteria or where the dung had been deposited on nutrient agar; it took a day longer on non-nutrient PBA plates. The tiger scat samples were exceptional in that large fruiting bodies were visible within 24hrs on both nutrient and non-nutrient media. As compared to other dung samples, the elephant dung samples took much longer (6-8 days) to show CSM fruiting bodies. Dung samples inoculated on PBA (non-nutrient) plates also showed presence of CSM fruiting bodies. In these cases, presumably, the dung had sufficient nutrients for endogenous bacteria to grow and, in turn, to allow CSM amoebae to grow and form fruiting bodies. Most of the dung samples had up to 2 genera and 5 species of CSMs. In some cases, CSM fruiting bodies belonging to two different genera (viz. *Polysphondylium* and *Dictyostelium*) developed from neighboring regions (3-4 mm apart) from the same sample (Fig. 2.5A and 2.5B). In one instance, *P. violaceum* and *D. giganteum* fruiting bodies developed so close that the mature fruiting bodies were entangled with each other (Fig. 2.5B). All CSM genera/species reported here were identified using criteria described earlier (Raper, 1984); however, a DNA-based phylogeny (Schaap et al., 2006) suggests inconsistencies in the traditional classification as some species are paraphyletic.

*D. giganteum* was most common (found in almost all the dung samples) followed by *D. purpureum*. *D. discoideum*, *D. minutum*, *D. macrocephalum*, *D. rosarium*, *D. polycephalum*, *P. pallidum* and *P. violaceum* were also seen. One Dictyostelid species observed had an interesting phenotype, it formed stalked slugs which after a brief period of migration bifurcated and formed two slugs, each of which differentiated into a fruiting body (Fig. 2.6D and 2.6E). Since this species resembled *D. bifurcatum* it was called as *Dsp (bifurcating)*. Many fungi, bacteria, and nematodes were also observed, as were myxobacterial fruiting bodies

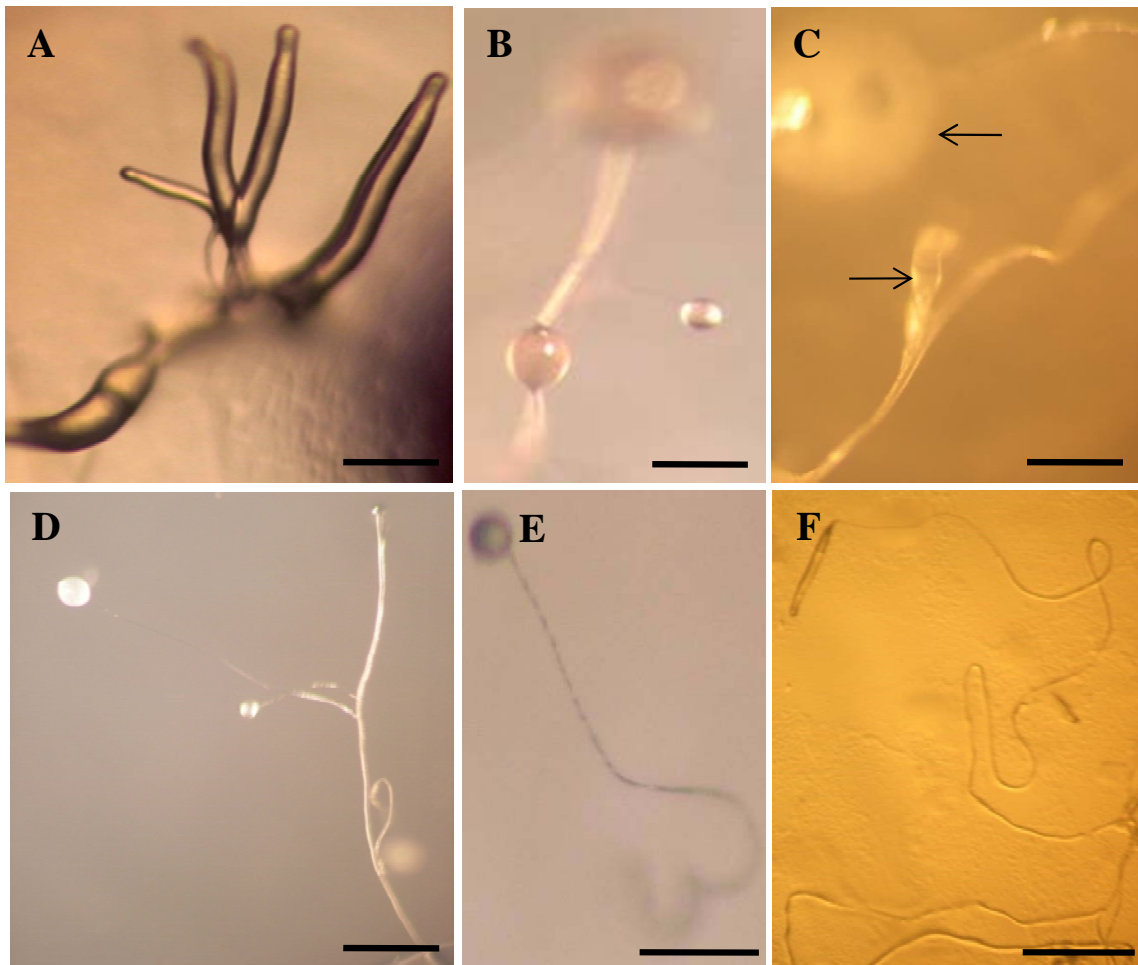
(occasionally). Presumably, the tiger scat sample was enriched with a large number of CSM propagules because big fruiting bodies were seen within 24 hrs of plating. Spotted deer samples yielded mainly *D. purpureum* fruiting bodies that were exceptionally long (0.5-1.5 cm) with large sori (diameter, ~ 150  $\mu$ m). Elephant dung yielded a small number of fruiting bodies belonging to *D. giganteum*, *D. purpureum* and *Dsp (bifurcating)*; these fruiting bodies were relatively smaller than those that developed on tiger scat or spotted deer dung pellets. Variants of the 'standard' phenotypes of CSMs were observed in the gaur, porcupine and yak dung samples. They were seen only on plates containing the primary isolates and included phenotypes like aggregates with many tips, fruiting bodies with spiral-shaped stalks and fruiting bodies with multiple, purple-colored spore heads (Fig. 2.7). Such variants and some other 'aberrant' forms of CSMs have been described previously (Rai and Tewari, 1963a; Raper, 1984). One CSM isolate from yak dung was unusually long-stalked, remained migratory for many days on non-nutrient agar and seldom culminated in fruiting bodies (Fig. 2.7F). Unfortunately, some of the aberrant forms observed on preliminary isolation plates could not be purified and subcultured and they are no more available for further study. Fruiting bodies formed on dung samples were larger and appeared more vigorous than those that were observed in the soil isolates (both on nutrient and non-nutrient agar). Based on the number of fruiting bodies and the time taken for the appearance of fruiting bodies after samples were incubated my impression was that the dung samples contained a higher density of propagules as compared to soil.



**Figure 2.5: CSM fruiting bodies developed on animal dung.** Animal dung samples were inoculated on non-nutrient agar (no addition of *external* food source or any other treatment was involved). (A) *D. purpureum* and *Dictyostelium* species from tiger scat. (B) *D. giganteum* and *P. violaceum* from gaur dung. (C) *D. purpureum* from spotted deer pellet. (D) *D. giganteum* from elephant dung. Note two different species of CSMs (Fig. 2.5A) and two different genera of CSMs (Fig. 2.5B) were seen developing close to each other on animal dung. The scale bars represent 1 mm.



**Figure 2.6: CSM isolates from animal dung.** A small portion of dung (~ 500 mg) was suspended in sterile water, mixed by gentle shaking and a small amount of the suspension was inoculated on PBA plates along with *K. aerogenes*. The fruiting bodies developed after 3 to 4 days. Shown here are the fruiting bodies of different species of CSMs: (A) *D. rosarium*, (B) *D. polycephalum*, and (C) *D. minutum*. (D) and (E) are the bifurcating slug and fruiting body respectively of *Dsp* (*bifurcating*). The scale bars represent 250  $\mu\text{m}$ .



**Figure 2.7: Variants of “standard” phenotypes of CSMs.** (A) multiple slugs from a common aggregate. (B) Unusual branching pattern with purple colored sori (morphology neither of *D. purpureum* nor of *P. violaceum*). (C) A mature fruiting body of the *Dictyostelium* species with a branch showing developing slug. (D) Fruiting body with unusual branching pattern. (E) Fruiting body with curly stalk. (F) Unusually long migrating slugs isolated from yak dung, this particular isolate remained migratory for up to a week and only rarely formed fruiting bodies with small spore mass. The scale bars represent 500  $\mu\text{m}$ .

### 2.7.2 CSMs from soil

Soil samples that were mixed in sterile water, diluted and plated on SM/10 and PBA pre-inoculated with *K. aerogenes* showed CSM aggregations and fruiting bodies within 6-8 days post-inoculation. Similar to dung samples, *D. giganteum* and *D. purpureum* were commonly observed. *D. mucoroides*, *D. minutum*, *P. pallidum*, *P. violaceum*, *Dsp (bifurcating)*, *D. discoideum*, *D. polycephalum*, *D. rosarium*, *D. macrocephalum* and *D. tenue* were also seen. *D. mucoroides* and *D. tenue* were found only in the soil samples and not in the animal dung.

In another set of experiments, small speck of soil (250-1000  $\mu\text{m}$ ) was inoculated on non-nutrient PBA and SM/10 agar. Interestingly, these small specks of soil also yielded fruiting bodies; in some cases it showed two or more species of CSMs. The fruiting bodies that had developed on these tiny particles of soil were less vigorous (small size) compared to those formed on animal dung. Apart from CSMs, many fungi, bacteria, nematodes, myxobacterial fruiting bodies (occasionally) were also seen.

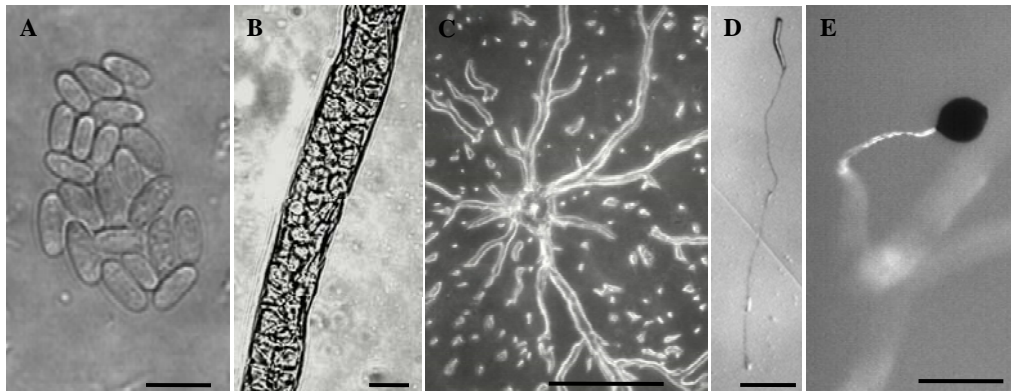
CSMs isolated from the soil samples and animal dung were characterized in detail with phenotypes being compared with published records and named accordingly. The CSM isolates and their descriptions are mentioned in Table 2.2, 2.3 and Figs. 2.8, 2.9, 2.9a, 2.10 and 2.11. Comparisons with previous CSM isolates from India are shown in Table 2.5. *Dsp (bifurcating)* (Fig. 2.6 D, E) and the unidentified species isolated from yak dung (Fig. 2.7 F) are the two potentially new species found in this study. *D. aureo-stipes*, *D. sphaerocephalum*, *D. vinaceo-fuscum* and *A. subglobosum* were reported earlier by others, but not found in this study.



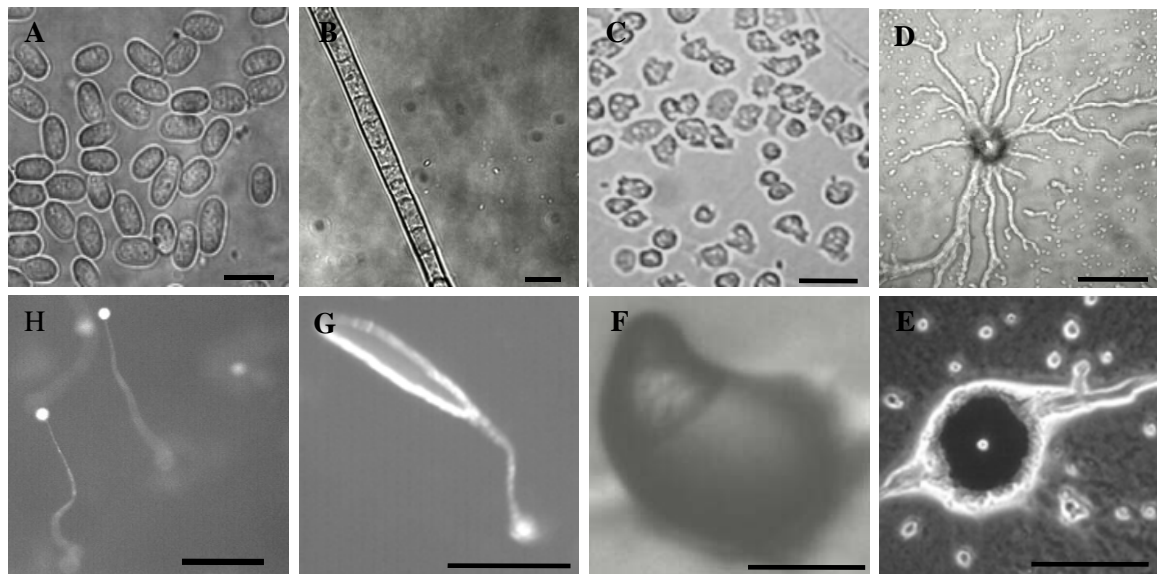
**Table 2.2:** CSMs found in large mammal dung and soil collected from the dry deciduous forest in India. CSM identifications were based on fruiting body morphology, sori pigmentation, stalked versus non-stalked slug migration and spore and stalk cell morphology. The guidelines used for CSM identification were from Raper (1984).

CSM species	Species description based on this study
<i>D. purpureum</i>	Aggregates are large (2-5 mm diameter), stalked slug migration, strong phototaxis (Fig. 2.9a). The fruiting bodies develop solitary with long stalk (3-15 mm long, 15-25 $\mu\text{m}$ thick); sori are spherical (85-180 $\mu\text{m}$ in diameter), purple colored and show no branching; spores are capsule shaped [ $6.14 \pm 0.88\mu\text{m} \times 2.70 \pm 0.55\mu\text{m}$ (mean $\pm$ sd, n = 160 spores)]; see Fig. 2.8].
<i>D. giganteum</i>	Aggregates are large (1-2 mm diameter); slugs are highly phototactic, and show stalked migration. Fruiting bodies are solitary, unbranched, have cellular stalk (0.5-1.5 mm long, 4-5 $\mu\text{m}$ thick); sori are spherical (50-150 $\mu\text{m}$ in diameter), white; spores are oval shaped [ $6.05 \pm 0.74\mu\text{m} \times 3.64 \pm 0.55\mu\text{m}$ (mean $\pm$ sd, n = 180 spores)]. For the pictorial descriptions see Fig. 2.9 and 2.9a).
<i>D. rosarium</i>	Erect or semi-erect fruiting bodies, long stalk with white, multiple sessile sori giving beaded appearance. Number of sori per fruiting body $8 \pm 2$ (mean $\pm$ s.d., range 5 to 10), see Fig. 2.10.
<i>D. polycephalum</i>	Fruiting bodies usually develop as a cluster, erect, small (up to 500 $\mu\text{m}$ in height) bearing white, round, 3-9 sori per fruiting body, see Fig. 2.6B.
<i>Dsp (bifurcating)</i>	The slugs show stalked migration. After a brief period of migration each slug bifurcates into two, each develops into a fruiting body. Fruiting bodies are semi-erect and colorless (Fig. 2.6D and 2.6E).
<i>D. discoideum</i>	Unbranched, solitary fruiting bodies with globose, creamy to yellow colored sori. Presence of basal disc and stalk-less slug migration.
<i>D. macrocephalum</i>	Solitary, erect fruiting bodies with spherical, white sori.
<i>D. minutum</i>	Fruiting bodies are small ( $\sim 500 \mu\text{m}$ ), erect and white colored, see Fig. 2.6C.
<i>P. violaceum</i>	Fruiting bodies are branched; can be distinguished easily from others because of their conspicuous violate colored sori, see Fig. 2.11.
<i>P. pallidum</i>	Fruiting bodies are solitary, erect and branched. Sori are colorless and develop as multiple clusters, 4 to 5 sori in one cluster.

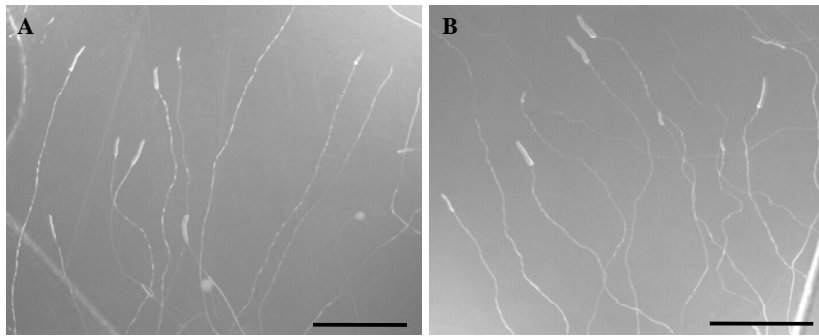
*D. tenue* and *D. mucoroides*, the other two CSM species reported in this study, were isolated and described by a previous graduate student from our laboratory hence they are not mentioned here (Kaushik, 2002).



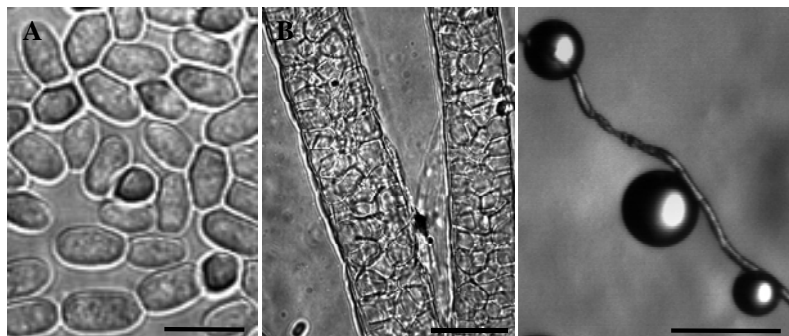
**Figure 2.8: *D. purpureum* isolated from spotted deer dung.** (A) Spores, (B) Stalk, (C) Aggregate, (D) Slug and (E) Fruiting body. Morphological criteria of *D. purpureum* developmental structures showed here matches with earlier descriptions of the species (Raper, 1984; Whittingham and Raper, 1960). The scale bars represent 10  $\mu\text{m}$  (A, B) and 500  $\mu\text{m}$  (C, D, E).



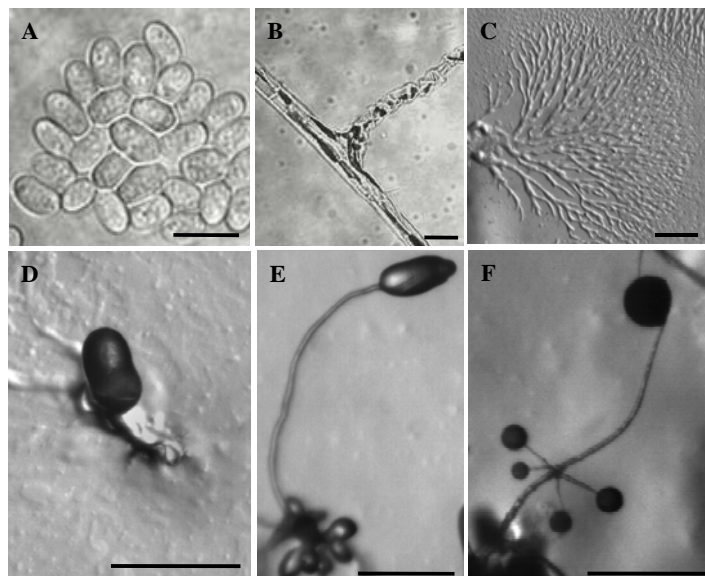
**Figure 2.9: *D. giganteum* isolated from elephant dung.** (A) Spores, (B) Stalk, (C) Amoebae, (D) Aggregate, (E) Tight aggregate, (F) Mound, (G) Slug and (H) Fruiting bodies. The scale bars represent 10  $\mu\text{m}$  (A, B, C) and 500  $\mu\text{m}$  (D-H).



**Figure 2.9a: Phototaxis in wild isolates of CSMs.** (A) *D. giganteum*, (B) *D. purpureum* (Fig. is reproduced from chapter 1) The scale bars represent 500  $\mu\text{m}$ .



**Figure 2.10: *D. rosarium* isolated from yak dung.** (A) Spores, (B) Stalk and (C) Fruiting body. The scale bars represent 10  $\mu\text{m}$  (A, B) and 500  $\mu\text{m}$  (C).



**Figure 2.11: *P. violaceum* isolated from gaur dung.** (A) Spores, (B) Stalk, (C) Aggregate, (D) Mound, (E) Culminant and (F) Fruiting body. The scale bars represent 10  $\mu\text{m}$  (A, B) and 500  $\mu\text{m}$  (C-F).

**Table 2.3:** CSMs obtained from animal dung and soil.

Source	No. of independent samples analysed	Cellular slime moulds isolated
Spotted deer: <i>Axis axis</i>	3	<i>Dpu, Dg, Dsp (bifurcating), Pv</i>
Tiger: <i>Panthera tigris</i>	1	<i>Dpu, Dg, Dd</i>
Elephant: <i>Elephas maximus</i>	2	<i>Dpu, Dg, Dsp (bifurcating), Dr</i>
Wild dog: <i>Cuon alpinus</i>	2	<i>Dpu, Dg</i>
Sambar: <i>Cervus unicolor</i>	2	<i>Dpu, Dg, Dma</i>
Porcupine: <i>Hystrix indica</i>	2	<i>Dg, Dpo</i>
Yak: <i>Bos grunniens</i>	1	<i>Dg, Dsp (bifurcating), Dd, Dr, Dmi</i>
Gaur (Bison): <i>Bos gaurus</i>	2	<i>Dg, Dsp (bifurcating), Dr, Pv, Pp</i>
Panther: <i>Panthera pardus</i> <sup>#</sup>	Many (count not kept)	Diverse, not identified
Hyena: <i>Crocuta crocuta</i> <sup>#</sup>	Many (count not kept)	Diverse, not identified
Barking deer: <i>Muntiacus muntjac</i> <sup>#</sup>	Many (count not kept)	Diverse, not identified
Soil (from different areas in the Mudumalai forest range)	10	<i>Dpu, Dg, Dsp (bifurcating), Dr, Dd, Dma, Dpo, Dmi, Dmu, Dt, Pv, Pp</i>

<sup>#</sup>Data collected by Sonia Kaushik, an earlier graduate student from our laboratory.

*Dpu* = *Dictyostelium purpureum* Olive, *Dg* = *D. giganteum* Singh, *Dd* = *D. discoideum* Raper, *Dr* = *D. rosarium* Raper and Cavender, *Dma* = *D. macrocephalum* Hagiwara, *Dpo* = *D. polycephalum* Raper, *Dmi* = *D. minutum* Raper, *Dmu* = *D. mucoroides* Brefeld, *Dt* = *D. tenue* Cavender, Raper, and Norberg, *Pv* = *Polysphondylium violaceum* Brefeld, *Pp* = *P. pallidum* Olive. *Dsp (bifurcating)* refers *Dictyostelium* species in which aggregates form aerial slugs that split up while migrating (see Fig. 2.6D and 2.6E).

**Table 2.4:** Home ranges (rough linear extent), and feeding habits of animals.

<b>Animals</b>	<b>Home range of animal (rough linear extent) Km</b>	<b>Feeding habits</b>
Spotted deer	4.5	Primarily grazer, browser (in dry season) also eats fallen fruits, and leaves.
Tiger	15	Carnivore.
Elephant	32	Depending on season and habitat, either grazer or browser.
Wild dog	4.5-8	Carnivore.
Sambar	14	Depending on season and habitat, either grazer or browser.
Porcupine	15	Tubers, roots, fruit.
Yak	50	Mainly grazer, also known to eat mosses and lichens.
Gaur	20	Mainly browser, some reports of eating tree-bark and grasses.
Panther	15	Carnivore.
Hyena	5-10	Scavenger (carnivore).
Barking deer	5	Fruits, buds, freshly sprouted leaves, seeds, young grass.

**Table 2.5:** Comparison of CSMs isolated from soil and animal dung in different parts of India.

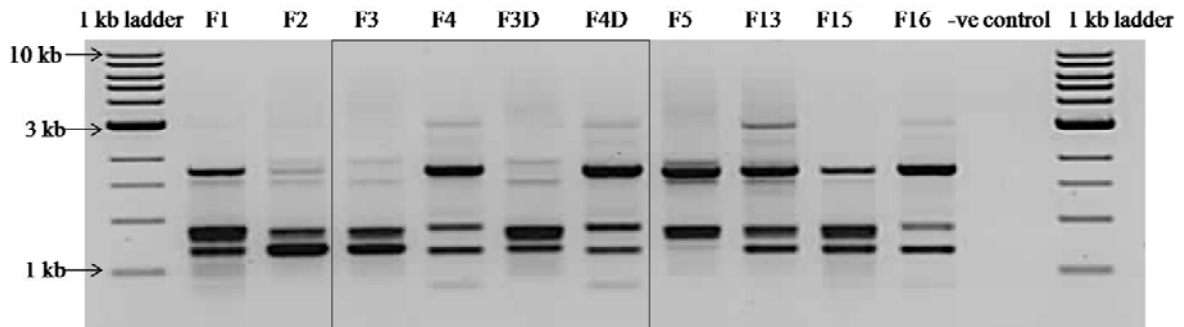
Sample source	Cellular slime mould isolates	Reference
Cultivated, uncultivated and rhizosphere soil from South India	<i>D. purpureum</i> , <i>D. giganteum</i> , <i>D. mucoroides</i> , <i>D. minutum</i> , <i>D. discoideum</i> , <i>P. pallidum</i> and <i>P. violaceum</i> .	(Agnihotrudu, 1956)
Soil from West-Central Himalaya and tropical forests in peninsular India	<i>D. purpureum</i> , <i>D. giganteum</i> , <i>D. mucoroides</i> , <i>D. minutum</i> , <i>P. pallidum</i> and <i>P. violaceum</i> . <i>D. aureo-stipes</i> , <i>D. sphaerocephalum</i> , <i>D. tenue</i> , <i>D. polycephalum</i> , <i>D. vinaceo-fuscum</i> , <i>A. subglobosum</i> .	(Cavender and Lakhanpal, 1986)
Soil from Lucknow	<i>D. mucoroides</i> , <i>D. sphaerocephalum</i> , <i>P. violaceum</i> .	(Rai and Tewari, 1961; Rai and Tewari, 1963b)
Soil (from areas in Mudumalai forest)	<i>D. purpureum</i> , <i>D. giganteum</i> , <i>D. mucoroides</i> , <i>D. minutum</i> , <i>P. pallidum</i> , <i>P. violaceum</i> , <i>Dsp (bifurcating)</i> , <i>D. discoideum</i> , <i>D. rosarium</i> , <i>D. polycephalum</i> , <i>D. macrocephalum</i> , <i>D. tenue</i> .	This study (Sathe et al., 2010)
Animal dung	<i>D. purpureum</i> , <i>D. giganteum</i> , <i>D. minutum</i> , <i>P. pallidum</i> , <i>P. violaceum</i> , <i>Dsp (bifurcating)</i> , <i>D. discoideum</i> , <i>D. polycephalum</i> , <i>D. rosarium</i> , <i>D. macrocephalum</i> .	This study (Sathe et al., 2010)

*D. aureo-stipes*, *D. sphaerocephalum*, *D. vinaceo-fuscum* and *Acytostelium subglobosum* were reported by Cavender and Lakhanpal; I could not recover them in this study. *Dsp (bifurcating)* and some other aberrant forms (see Fig. 2.7) were not isolated in any of the earlier studies. All the CSM isolates were identified using traditionally accepted criteria and named accordingly (Raper, 1984). However, it is worth mentioning that there are inconsistencies in the classification of CSMs; for example, molecular phylogeny (based on small subunit ribosomal RNA and  $\alpha$ -tubulin DNA sequences) shows that “*Dictyostelium*” and “*Polysphondylium*” species are sometimes found in the same major clade and sometimes in different clades (Schaap et al., 2006).

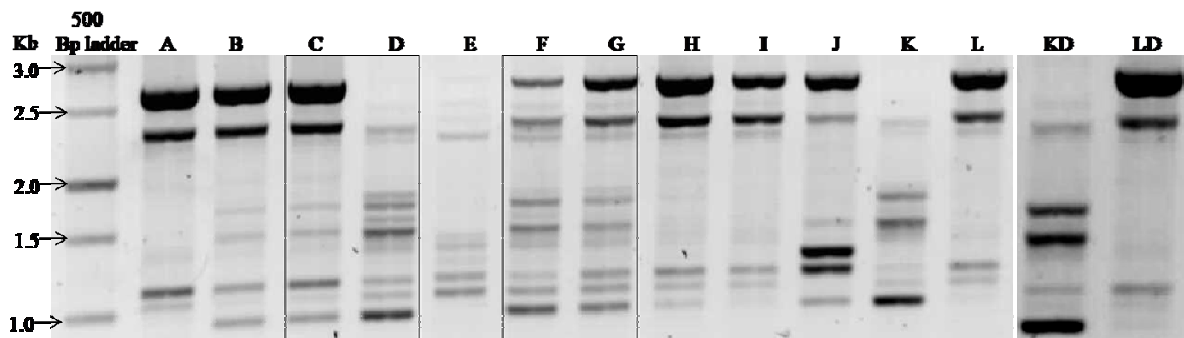
### 2.7.3 Genetic heterogeneity in CSM social groups

Since social group formation in CSMs involves aggregation of free living amoebae, can amoebae of different genotypes (strains) co-aggregate to form chimaeric social groups in the wild? To answer this question first step was to collect CSM fruiting bodies that had developed in the wild. Several locations in the Mudumalai forest (viz. undisturbed forest soil, leaf litter and animal dung) were inspected with a magnifying glass for the presence of CSM fruiting bodies. Unfortunately, no fruiting bodies were seen developing under wild conditions. This made it essential to bring soil and animal dung samples to the laboratory and rear CSM fruiting bodies under quasi-natural environment. This approach did work, many CSM fruiting bodies were seen developing on soil and animal dung. *Only those fruiting bodies which had developed on plates containing non-nutrient agar or nutrient for bacteria were used for the genetic heterogeneity analysis. D. giganteum and D. purpureum* fruiting bodies (because of their common occurrence and ease of identification) were analysed extensively; fruiting bodies of other species remain to be analysed.

The results of the experiments in which chimaerism in fruiting bodies of *D. giganteum* and *D. purpureum* was studied are summarised in Table 2.6. Typical RAPD profiles of spores from *D. giganteum* and *D. purpureum* fruiting bodies are shown in Figs. 2.12 and 2.13 respectively. A total of 17 fruiting bodies isolated from soil specks (9) and animal dung (8) were used for genetic heterogeneity analysis. Of the 17 fruiting bodies formed under quasi-natural conditions, 15 were multiclonal (9 out of 11 *D. giganteum* and 6 out of 6 *D. purpureum*). The minimum number of distinct genotypes detected in a single fruiting body was three to seven (animal dung) and one to nine (soil). The statistical approach too yielded numbers that were more or less the same as the actual counts (Table 2.6).



**Figure 2.12: Genetic heterogeneity within a *D. giganteum* fruiting body isolated from spotted deer dung.** Lanes marked F1, F2, F3, F4, F5, F13, F15, and F16 show RAPD profiles of different clones derived from spores from a single fruiting body using the OPAD-4 primer. F3D and F4D represent independently isolated DNA samples from the same clone as F3 and F4, respectively. F3 and F4 have different RAPD amplification pattern pointing heterogeneity in spores from a fruiting body. Note the amplification patterns between lanes F1 and F15 appear similar suggesting them to be genetically similar.



**Figure 2.13: Genetic diversity within a *D. purpureum* fruiting body isolated from wild dog scat.** Lanes marked A to L represent RAPD profiles of different clones derived from spores from a single fruiting body using the OPAV-20 primer. The patterns in lanes C and D were considered different; the patterns in lanes F and G were considered similar. Small intensity differences (as seen in F and G) were not considered as different. The lanes marked KD and LD indicate a repeated RAPD profile of clones K and L starting from DNA isolation to agarose gel electrophoresis. Other Similar/dissimilar patterns are also visible in Figs. 2.12 and 2.13.



**Table 2.6:** Chimaerism in the fruiting bodies of *D. giganteum* and *D. purpureum*.

Fruiting body (species and designation)	Source of isolation	Total RAPD primers used (informative)	No. of spores monitored (n)	No. of clones found (m)	Estimated total number of clones (C)
<i>D. purpureum</i> (SD-1)	Spotted deer (non-nutrient agar).	7 (5)	8	4	6
<i>D. purpureum</i> (SD-3)		2 (2)	12	5	6
<i>D. purpureum</i> (SD-4)		7 (5)	12	6	8
<i>D. purpureum</i> (SD-5)		8 (8)	10	5	7
<i>D. giganteum</i> (SD-8)		2 (2)	13	5	6
<i>D. purpureum</i> (WD-1)	Wild dog (non-nutrient agar).	4 (4)	12	7	9
<i>D. giganteum</i> (E)	Elephant (non-nutrient agar).	6 (3)	10	3	4
<i>D. giganteum</i> (F)		7 (7)	8	4	6
<i>D. giganteum</i> (18)	Soil speck from 50 <sup>th</sup> ha. SM/ 10 agar, (No exogenous bacteria).	3 (3)	10	5	7
<i>D. giganteum</i> (20)		3 (3)	7	4	6
<i>D. giganteum</i> (21)		4 (3)	9	5	7
<i>D. giganteum</i> (23)		5 (5)	15	7	9
<i>D. giganteum</i> (24)		6 (0)	4	1	2
<i>D. purpureum</i> (B)	Soil speck from 46 <sup>th</sup> ha. SM/ 10 agar, (no exogenous bacteria).	4 (4)	9	3	4
<i>D. giganteum</i> (D)		5 (5)	7	4	6
<i>D. giganteum</i> (5B-1)	Soil speck from 5 <sup>th</sup> ha. (non-nutrient agar).	7 (0)	5	1	2
<i>D. giganteum</i>	IISc campus soil on PBA plate, <i>K. aerogenes</i> added.	4 (4)	10	9	12

Hectare numbers refer to subsections in the 50-hectare study plot in the Mudumalai. The spores within the fruiting bodies were genotyped using RAPD-PCR. "Informative" means to a primer that yielded at least two different DNA band patterns from the spores that were analysed. The last *D. giganteum* fruiting body is an outlier in several aspects. It was isolated from soil collected from Indian Institute of science campus; soil sample was carefully transferred to an agar plate and subsequently moistened by gently adding 50 $\mu$ l of *K. aerogenes* suspension. The last column indicates estimates of the total number of clones in a fruiting body which have been calculated using information mentioned in 4<sup>th</sup> and 5<sup>th</sup> column (values of **n** and **m**) and certain assumption mentioned in methods.

#### 2.7.4 Bacteria from Mudumalai forest soil

A dilute soil suspension was inoculated on a low-strength nutrient agar (NA/10) and lysogeny-broth agar. Plates were observed for the presence of bacteria after every 24 hrs and final bacterial counts were recorded after 72 hrs. The bacterial colonies appeared on these plates were morphologically distinct. For example, some were pigmented while some had white powdery appearance. Colonies had either a smooth or rough edges, some were highly mucoid. There were noticeable differences between colony sizes. One isolate (50G2) was highly motile, if inoculated on a fresh plate, within 2-3 days of incubation it formed a thick mucoid growth all over the plate. The soil sample collected from the 50<sup>th</sup> hectare had higher bacterial counts (colony-forming units on nutrient agar =  $3.27 \times 10^6 \pm 4.9 \times 10^5$ ; mean  $\pm$  s.d, n = 3) than the soil collected from 46<sup>th</sup> hectare (colony-forming units on nutrient agar =  $1.94 \times 10^6 \pm 1.9 \times 10^5$ ; mean  $\pm$  s.d, n = 3). The bacterial counts mentioned here must be a lower limit of bacterial density in the soil; this is because nutrient agar, presumably, does not support the growth of all soil bacteria, many unculturable bacteria as well as those failed to grow on nutrient agar must remain undetected. In any case, my aim was to isolate as many as possible different bacteria and it was done. Many fungal and Actinomyces colonies were also seen. Morphologically different, well-isolated colonies from both NA/10 and lysogeny-broth agar were streak-plated onto fresh nutrient plates and further characterized as mentioned in the methods. Four bacterial isolates were identified as *Micrococcus lylae* 50F2, *Bacillus cereus* 50G2 and *B. megaterium* 46A2 and 46B. The numbers (for ex., 50F2) followed by the bacterial species name were chosen by us to indicate the strains. The remaining isolates were grouped into two broad categories: Gram negative rods and Gram negative coccobacilli. Their identification is still pending and will be reported later. Bacterial isolation and identification was carried out with the help of Dr. Kavitha B. (Department of Microbiology, Maharani's Science College for Women, Bangalore, India).

## 2.8 Discussion

### 2.8.1 Large mammals as CSM dispersal agents

Although CSMs were considered coprophilous organisms, they have never been reported in large mammal dung. A preliminary study by Kaushik (2002) and this study (Sathe et al., 2010) show that CSMs are regular inhabitants of large mammal dung. In this study, CSMs were isolated from the dung of a variety of obligate herbivores as well as carnivores (see Table 2.3). The reasons mentioned below makes a case for large mammals being CSM dispersal vectors: (i) The dung samples (especially of spotted deer and yak) were collected immediately after defecation; other dung samples too were fresh although in those cases the possibility that the CSMs were deposited in dung via an arthropod vector or air currents, and were not present in the animal gut, cannot be completely ruled out. (ii) A single dung pellet yielded several distinct clones (belonging to different genera and species) this again helps us to rule out the possibility of a passive transfer of CSMs to dung by an insect vector or air currents. (iii) In a number of earlier studies CSMs have been commonly found in animal dung; at one time, they were considered to be coprophilous (associated with animal dung) organisms. Animal dung has ample nutrients to support bacterial growth which in turn can support CSM growth; Brefeld and Olive routinely used animal dung or dung-decoction as a growth medium for CSMs (cited in Raper 1984). The activities of coprophagous insects such as dung-beetles can disperse CSMs from one dung pat to several other places. Confirmatory experiments that these large mammals carry CSMs in their gut are needed. For this, dung samples must be collected without any contamination, preferably, directly into sterile collection vials. This was done by Stephenson and Landolt (1992); they found that spores, and possibly, amoebae, can be dispersed after passage through the gut of many vertebrates including small mammals.

How do these large mammals pick up CSMs? Soil is a rich source of CSMs (Raper, 1984; this study). Interestingly, it is also seen that plant structures such as moribund fruits, flowers also contain CSMs (Olive, 1960; Olive, 1975). Therefore, obligate herbivores (viz. elephant, spotted deer, gaur, yak, sambar, porcupine, and

barking deer) can pick up soil along with grass during grazing. Gaur and elephant graze as well as browse and so they can pick up CSM propagules while grazing and/or browsing. The presence of CSMs in the scat of obligatory carnivores (For example, tiger, wild dog, and panther) was rather unanticipated. It is possible that they ingest CSMs in the same way as the herbivores do i.e., from plant structures or grass (carnivores such as cats and dogs are occasionally seen eating grass) or, it is possible that they get CSMs indirectly, say by feeding on herbivores. The areas over which these animals move vary from about 20 km<sup>2</sup> (spotted deer) to approximately 1000 km<sup>2</sup> (elephant). Therefore, it is safe to conclude that in addition to small mammals [as shown by Stephenson and Landolt (1992)], large mammals too can be agents of long-distance dispersal of CSMs. Dispersal by air, water, insects or nematodes would likely involve small numbers of spores at a time. On the other hand, given how large animals graze, one might expect a great many CSM groups to be dispersed en masse by them-both to the same place (within one dung pat) and after being thoroughly mixed in the digestive tract. In terms of the distance of dispersal, only migratory birds can compete with them (Suthers, 1985); however range of dispersal and total amount of CSMs transferred taken together large mammals appears to be efficient dispersal agents.

It would be interesting to see if the various dispersal agents considered together lead to uniform spore dispersal at all distances or it may be that a small number of CSM propagules are dispersed over short distance (e.g., via soil dwelling arthropods) or they are dispersed in a large number over very long distances (e.g., via large animals). This mode of CSM dispersal (via large mammal dung after vigorous mixing in the gut environment) has important bearing on the way social groups are formed by CSMs in the wild. In social organisms, clonal groups may have a selective advantage over multiclonal groups. However, because CSM propagules get thoroughly mixed in the animal gut before dispersal, multiclonal groups may be unavoidable (unless clonal groups segregate from each other).

### 2.8.2 Genetic diversity in fruiting bodies

This study (Sathe et al., 2010), along with previous preliminary observations (mentioned in Kaushik and Nanjundiah, 2003), explicitly demonstrate the widespread occurrence of chimaeric social groups in both undisturbed forest soil and animal dung. This observation may be debated on two grounds: (a) the fruiting bodies were reared in the laboratory (quasi-natural) environment and/or (b) the use of RAPD to analyse the genetic heterogeneity. I have taken many precautions that adequately address these issues. CSM fruiting bodies being tiny and delicate make their isolation from wild difficult, if not impossible. This made it necessary for us to collect dung samples from the field and raise CSMs under laboratory environment. The CSM fruiting bodies analysed in this study were developed on non-nutrient agar without disturbing the samples and no *external* food source was added. These conditions can be considered as good substitutes for their natural development. Similarly, rigorous precautions taken during RAPD analysis (see methods) made the RAPD data highly reliable and reproducible [for example, comparison between F3 and F3D, and F4 and F4D (Fig. 2.12), K and KD, and L and LD (Fig. 2.13)]. Only those DNA amplicons that were well resolved with no ambiguity were considered during analysis and small intensity differences were ignored (for example, in Fig. 2.12, F1 and F15 were considered as the same pattern). All these measures helped to conclude that the social groups formed by two CSM species (*D. giganteum* and *D. purpureum*) in wild are made up of more than one genotype.

I have studied genetic heterogeneity only in spores, since stalk cells are dead their DNA could not be analysed. This could mean that the absence of polymorphism among a sample of spores is no proof of clonality - stalk cells could be chimaeric or may belong to genotypes other than those represented in the spores. The number of distinct clones detected in a fruiting body (Table 2.6) should be considered to be the lower limit of the total clones present. This is because of the following reasons: (1) small sample sizes (maximum number of spores analysed from a fruiting body was only 15, and the actual number of spores in a single fruiting body are likely to be at

least a few thousand), (2) limited number of RAPD primers used which reduces the ability to detect different genotypes and, (3) stalk cells being dead had to be ignored. Also, the statistical analysis involved unrealistic assumption that the different clones are represented in equal proportions in a chimaeric fruiting body, this may not be realistic. This means that in Table 2.6, the values of **C** are likely to be underestimates: genotypes that were represented in small numbers as spores would have been missed out. It is known that some genotypes, even if they represent a minor fraction of the population, can exert a significant effect on the functioning of the social group. This has been shown in several laboratory studies on wild isolates as well as on artificially generated mutants. The 'stalkless' forms of *D. mucoroides* isolated by Filosa (1962) and Buss (1982) could form both stalk and spores in the presence of the 'wild-type'. Similarly, a *D. discoideum* mutant (NP-160) which fails to aggregate by itself can aggregate and form normal fruiting bodies in the presence of a very small proportion (probably a single cell) of wild-type cells (Huang et al., 1997).

In this study, I have studied the genetic heterogeneity in fruiting bodies of *D. giganteum* and *D. purpureum*; fruiting bodies of other species have not investigated. However, considering the way large mammals disperse CSMs (after thoroughly mixing in the gut) it is possible to speculate that the other CSM species too can form chimaeric social groups. Other studies, mostly on *D. discoideum*, show some similarities and some differences with the observations reported here. Francis and Eisenberg (1993) found several strains of *D. discoideum* (these strains had unique restriction fragment length polymorphism patterns), they possibly form chimaeric groups in the wild. The studies by Filosa (1962) and Buss (1982) indicate that a stalkless variant of *D. mucoroides* can form chimaeric, normal 'wild-type' fruiting bodies in a mix with 'wild-type' *D. mucoroides*. Fortunato et al., (2003) isolated many haplotypes of *D. discoideum* from small soil samples (0.2 gm); these haplotypes mixed freely in laboratory conditions and formed chimaeric fruiting bodies (Strassmann et al., 2000) indicating that multiclonal group structures in CSMs can exist, although a study by Gilbert et al. (2007) indicates that most social groups formed by *D.*

*discoideum* in the wild are clonal. The reasons for these differences are not clear. I have studied *D. giganteum* and *D. purpureum* in South India; whereas others have studied *D. discoideum* in North America. The species studied and their geographical locations could partly explain the differences. The careful inference one can draw is that the CSM amoebae can form both clonal and multiclonal social groups. Therefore the evolution of social behaviour might have involved selection on both clonal and multi-clonal situations (Kawli and Kaushik, 2001).

The highest altitude at which CSMs were recorded so far is by Hagiwara, who found *D. brefeldianum* (also known as *D. mucoroides* Raper) at an altitude of 4680m in the Himalayas (Hagiwara, 1990). The CSMs isolated from yak dung shows that they can be found in habitats at much higher altitudes (up to 5,300m). Most CSM isolates had 'standard' phenotypes, i.e., they were comparable with earlier descriptions of 'wild-types' (Raper, 1984). However, some variants of 'standard' phenotypes were also seen (see Fig. 2.7). The origin and the selective advantages of such variants are not immediately clear. Rai and Tewari (1963a) isolated aberrant forms of *P. violaceum* from Indian soil which they speculated could be adapted for special modes of dispersal. Huffman and Olive (1963) found a variant of *D. mucoroides* Brefeld in decomposing wood of *Citrus sinensis* collected from Turrialba, Costa Rica. The aggregation pattern of this variant was comparable with *D. polycephalum* but the mature fruiting body was branched, like that of *polysphondylium*. They concluded that this variant could be a significant phylogenetic link in the Dictyosteliaceae. To follow a thought on similar lines it could be that a variant observed in this study (an isolate with a purple-color fruiting body and irregular branching; see Fig. 2.7B) looks like an intermediate phenotype between *D. purpureum* and *P. violaceum*.

In Filosa's study reported earlier (Filosa, 1962), the spores were functionally heterogeneous. If plated clonally, they formed phenotypically different structures ('variants') after starvation, but a mix of 'variant' and wild-type amoebae formed chimaeric fruiting bodies with the 'wild-type' phenotype. Bonner (1967) has pointed out that in naturally-occurring chimaeras different phenotypes may complement

each other in a functional sense and this could be one explanation for the co-existence of different genotypes with different phenotypes in nature.

The asexual life cycles of the myxobacteria (a prokaryote) and CSMs (an eukaryote) show strong convergent evolution: both are soil dwelling microorganisms, live a solitary life in presence of food, aggregate upon starvation and form social groups in which a proportion of cells form viable spores and others sacrifice themselves in an 'altruistic' manner to form stalk. Similar to what we find in the case of CSMs, natural isolates of *Myxococcus xanthus* show a great deal of genetic diversity with as many as 22 distinct genotypes being found in 78 samples collected from a small 16cm x 16cm plot (Vos and Velicer, 2006). However, the genetic heterogeneity in groups of social organisms need not always be high, a study on the colonial coral *Acropora millepora* found that just 2-5% of 984 colonies sampled from two different locations were chimaeras (Puill-Stephan et al., 2009).

Upon starvation, CSMs form fruiting bodies with varying morphology. They differ in sori color, branching pattern, and the presence of cellular vs. acellular stalk, to name a few (Raper, 1984). Why do CSMs form so many different kinds of fruiting bodies? In other words, what is the adaptive significance of having so much variation? Assuming that each fruiting body morphology is correlated with fitness (differential dispersal, protection), how do so many species of CSMs, all with different phenotypes, co-exist together? Bonner (2009) views these differences in fruiting body phenotypes as being 'neutral', i.e., all the phenotypes are equally adapted to the local environment. This idea of phenotypes being neutral, if true, could partly explain the species co-existence in CSMs. Horn (1971) found food preference in CSMs, i.e., different species of CSMs feed preferentially on particular types of bacteria. He concluded that in the absence of competition for food, CSM species can co-exist stably. Ketcham and Eisenberg (1989) found *P. pallidum* isolates in a small area which had different growth rates suggesting that reduced competition for food alone cannot explain the species coexistence. In their words, "competition has not only failed to eliminate different species from the community but has failed to eliminate clonal diversity from within species". It would be of great



interest to see if the CSMs isolated in this study have preferences for specific bacteria as food sources. I have co-isolated bacteria and CSMs from the same environment. Using these bacteria as food source, it can be tested if CSMs prefer some bacteria as food over others.

Three major findings of this study [(1) large mammals as CSM dispersal vectors, (2) the high level of within-group genetic diversity and, (3) presence of CSM propagules belonging to different genotypes of the same species or even different species on a 250-1000  $\mu\text{m}$  speck of soil or small dung samples] extends our knowledge of the ecology and social behaviour of the CSMs. It makes us aware about the spatial distribution, dispersal and genetic heterogeneity in social groups formed in the wild. Although these observations have taught us many lessons they have also raised several questions. Is the genetic polymorphism seen within a spore mass neutral polymorphism? In other words, are there functional differences between the genotypes represented in a chimaeric spore mass? These genotypes could differ from each other with respect to the rates of spore germination and growth, post-aggregation development, relative sporulation efficiencies, etc. These issues are explored in chapter 3.

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## **Chapter 3**

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*Trade-offs as a basis for maintenance of cooperation in Dictyostelium giganteum*

### 3.1 Introduction

This study addresses an important question in cellular slime mould (CSM) ecology, namely, what are the factors behind stable persistence of genetic heterogeneity in social groups of CSMs in wild? Genetically different strains of *Dictyostelium giganteum* (along with several species of CSMs) were isolated from undisturbed forest soil and animal dung collected from the Mudumalai wild life sanctuary, South India (Kaushik et al., 2006; Sathe et al., 2010; this study, chapter 3). *D. giganteum* strains were found to live together in close proximity, in fact, some of them were from the same social group (fruiting body) that had formed under wild conditions (Sathe et al., 2010). The experiments performed in our laboratory show that these strains can develop together and form chimaeric (mixed) aggregates and fruiting bodies. In a chimaera, one strain often 'exploits' the other during spore formation, i.e., it forms relatively more spores than the other. Despite these differences in relative sporulation efficiencies, these *D. giganteum* strains were found to coexist in the wild. This observation had raised an important issue, namely, what might account for the coexistence of different strains of a species even when, in a mixture, one is more efficient at sporulating than the other? In order to look for an answer, I have compared several fitness-related traits in the life cycle of *D. giganteum* and found that there are trade-offs between different life cycle components of fitness, and such trade-offs may explain the co-existence of these strains. Here, the word trade-off has been used in the following sense: when a given strain of *D. giganteum* does 'better' in one trait (for example, in growth, it doubles relatively faster) but does 'poorly' in another trait (for example, in sporulation, it sporulates with a relatively lower efficiency) then there exists a trade-off between two fitness-related traits (growth and sporulation). Implications of these results are discussed in the light of evolutionary models for cooperative behaviour in CSMs.

#### **Co-occurrence, chimaerism and differential success in cellular slime moulds**

A brief discussion of the following points is relevant to this study: (a) Several strains (genetically different clones of a species) of CSMs co-occur in natural habitats. (b) Different strains can form chimaeric (mixed) social groups. (c) In a chimaera, strains

can form a disproportionate number of spores. (d) Despite these differences in relative sporulation efficiencies in chimaeras, the strains appear to coexist in wild. First, I will briefly review the available literature to establish some of these claims followed by a detailed description of the experiments performed to validate the hypothesis that trade-offs with regard to different fitness-related traits could be a factor behind the stable persistence of CSM genetic heterogeneity in the wild.

The co-occurrence of different strains of CSMs in the wild is well established (reviewed in chapter 2 of this thesis). Francis and Eisenberg (1993) found genetically distinguishable strains of *D. discoideum* in soil samples collected from North Carolina. Ketcham and Eisenberg (1989) found different mating types of *Polysphondylium pallidum* in North American hardwood forest soil. Several strains of *D. discoideum* (Fortunato et al., 2003), *D. giganteum* and *D. purpureum* (Kaushik et al., 2006; Sathe et al., 2010) were isolated from small soil samples. These results suggest extensive CSM diversity within a small area. On the other hand, Gilbert et al (2009) found only one clone of *D. discoideum* in a 12 m soil patch in a cattle pasture located in a Texas Gulf Coast prairie. Therefore CSMs can also exist as clonal groups.

Although different CSM strains, all with ‘wild-type’ phenotype, have been found in close proximity (i.e., within a range of aggregation) do they co-aggregate and form chimaeric social groups? Genetically distinct clones of *D. discoideum* can form chimaeric fruiting bodies in laboratory conditions (Strassmann et al., 2000; Fortunato et al., 2003). Studies conducted on different strains of *D. giganteum* and *D. purpureum* indicate intra-species chimaeric fruiting body formation both in laboratory as well as in the wild (Kaushik, 2002; Kaushik and Nanjundiah, 2003; Kaushik et al., 2006; Sathe et al., 2010; this study). In chimaeric social groups different strains can form more spores than their expected proportion leading to asymmetric (‘unfair’) allocations to spore and stalk cells (Strassmann et al., 2000; Fortunato et al., 2003a; Kaushik et al 2006; this study).



### Scope of this study

The aim of this study was to understand the factors responsible for stable coexistence between naturally occurring strains of *D. giganteum*. Whether these findings can be extended to other species of CSMs, and to what extent they help us understand cooperative behaviour, is a matter of discussion.

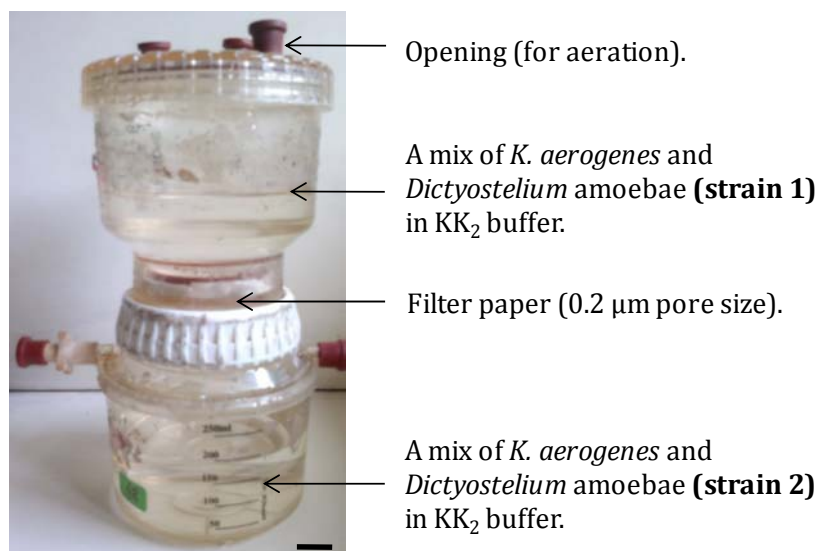
### 3.2 Materials and methods

**3.2.1 *D. giganteum* strains:** All the experiments mentioned below were carried out using compatible *D. giganteum* strains (i.e., different genotypes) isolated from natural habitats. Strains 46a3, 46d2, and 48.1a1 were isolated from soil collected from the 50-hectare plot of undisturbed forest in the Mudumalai nature reserve, South India. They were isolated by Sonia Kaushik (an earlier graduate student from our laboratory) and have been described in previously published work (Kaushik, 2002; Kaushik et al., 2006). Strains F5 and F16 were isolated from the same spore mass from a fruiting body that had developed on elephant dung collected from the Mudumalai forest range (Sathe et al., 2010; this study). The strains used were chosen to provide a pair of members isolated far apart from each other, a pair isolated in close proximity, and a pair from the same fruiting body. Strain 48.1a1 was found 200m away from strains 46a3 and 46d2, and may or may not co-aggregate with 46a3 or 46d2 in the wild. Strains 46a3 and 46d2 were found close by (approximately 10 cm from each other) and could form chimaeric groups in nature (kaushik et al., 2006; this study). F5 and F16 provided a pair of strains that exhibits natural chimaerism. *D. giganteum* strains used in this study mix freely and form chimaeric social groups (Kaushik et al., 2006, Sathe et al., 2010; this study), however, it must be noted that not all wild isolates of *D. giganteum* form chimaeras readily (kaushik et al., 2006). Clonal stocks were prepared as spores in a 25% glycerol solution and stored at -80°C. Old cultures were discarded and new cultures started from the stocks regularly. After every few experiments, the genetic purity of the strains was confirmed using DNA markers described earlier (Kaushik et al., 2006; Sathe et al., 2010).

The experiments were performed on vegetative amoebae which were derived as follows:  $1.5 \times 10^4$  spores of a given *D. giganteum* strain along with 500  $\mu$ l thick suspension of *Klebsiella aerogenes* was inoculated on a 60 cm<sup>2</sup> SM plate. The SM composition was as mentioned in Sussman (1987): glucose 1%, peptone 1%, yeast extract 0.1%, MgSO<sub>4</sub> 0.1%, KH<sub>2</sub>PO<sub>4</sub> 0.225%, K<sub>2</sub>HPO<sub>4</sub> 0.067%, pH 6.4, Agar 2%. Plates were incubated at 22°C in the dark for 36-40 hrs to obtain a confluent growth of amoebae with no signs of starvation. Amoebae were then gently scraped off using a glass spreader and collected in 35-40 ml cold KK<sub>2</sub> buffer (KH<sub>2</sub>PO<sub>4</sub> 2.25g, K<sub>2</sub>HPO<sub>4</sub> 0.67g, H<sub>2</sub>O 1000 ml, pH 6.4) and residual bacteria were removed by three rounds of differential centrifugation at 300g for 3 min at 4°C. Amoebae were re-suspended in cold KK<sub>2</sub> buffer at a density of  $1 \times 10^7$  amoebae/ml and used for the experiments. The following experiments were carried out with the strains either alone or as a mix of two.

**3.2.2 Growth:** Doubling times of exponentially growing amoebae were estimated on solid agar plates and in shaken suspension. About 1000 amoebae of a *D. giganteum* strain were suspended in 200  $\mu$ l KK<sub>2</sub> buffer to which 200  $\mu$ l of amoebal food (*K. aerogenes* suspension, optical density at 600nm = 1) was added, mixed thoroughly and spread evenly on 2% agar made up in KK<sub>2</sub> buffer. 15-20 plates were inoculated in this way and incubated in the dark in a moist chamber at 22°C. After every 3-4 hrs, amoebae were washed off from two different plates with cold KK<sub>2</sub> buffer and counted using a haemocytometer. It was confirmed that the amoebae left behind after washing were insignificant in number. Liquid-phase growth was monitored in 250 ml flasks. A 2 ml *K. aerogenes* suspension (optical density at 600nm = 1) and  $0.7 - 1 \times 10^7$  amoebae were suspended in 100 ml KK<sub>2</sub> buffer. The flasks were shaken in a gyratory shaker at 150 rpm at 22°C. After every 4-5 hrs, a sample was collected from the flasks and amoebae were counted using a haemocytometer. When strains were grown together, individual growth rates were estimated by shaking them in a common growth medium as follows: a thick suspension of *K. aerogenes* (2 ml) and 50 ml KK<sub>2</sub> buffer were pipetted separately into either sides of a cellulose acetate filter (0.2  $\mu$ m pore size, Sartorius stedim biotech, Germany) that divided a growth

chamber into two halves. Amoebae of different stains were inoculated in these chambers (one strain in each chamber at a density of  $1 \times 10^5$  amoebae/ml), and the entire device was incubated at 22°C on a reciprocal shaker (150 strokes/min). It was confirmed that neither bacteria nor CSM amoebae were exchanged through filter papers. This method was suitable to check the influence of different strains on each other's growth without mixing amoebae between strains. Although direct cell-to-cell contact was not possible, small molecules influencing growth produced by these strains could easily be exchanged. A pictorial representation of this device is shown in Fig. 3.1.



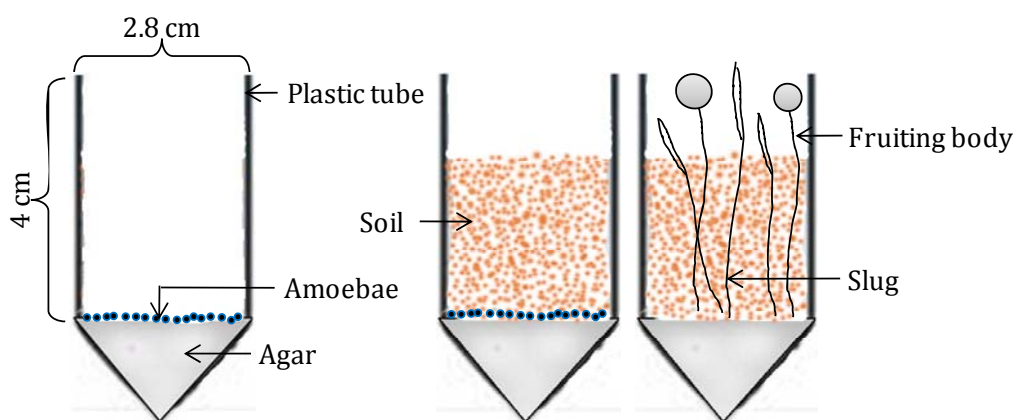
**Figure 3.1: Device (membrane filter holder, Tarsons, India) used to monitor growth in mixed cultures.** It consists of two chambers (each 250 ml) separated by a filter paper holder. After cultures were inoculated into each chamber, the entire device was shaken in a horizontal shaker so that the liquid medium, but not the cells, can be exchanged freely across the filter paper barrier. Scale bar represents 1 cm.

*D. giganteum* growth was also monitored on five different species of bacteria isolated from the Mudumalai forest (from a portion of the same soil sample used for CSM isolations, see chapter 2). These bacterial isolates were: *Micrococcus lylae* 50F2, *Bacillus cereus* 50G2 and *B. megaterium* 46A2 and 46B and two other unidentified bacterial species. In these experiments,  $1 \times 10^5$  freshly starved amoebae of a *D. giganteum* strain were suspended in 200  $\mu\text{l}$   $\text{KK}_2$  buffer to which 200  $\mu\text{l}$  thick

bacterial suspension (one bacterial species at a time) were inoculated on 20 cm<sup>2</sup> KK<sub>2</sub> agar plates. In each experiment, the number of amoebae and the amount of bacteria inoculated were kept constant. Plates were incubated in the dark at 22°C. In these experiments doubling times were not checked instead the time taken by amoebae to consume all the provided bacteria was monitored. It is assumed that if amoebae of a strain 1 consumes the supplied amount of bacteria before amoebae of strain 2 does, the difference could be due to faster growth rate of strain 1. Although qualitative, this method was suitable for comparing growth rates between strains and also helpful in establishing preferences (if any) of the different strains of *D. giganteum* for particular types of bacteria as a food source.

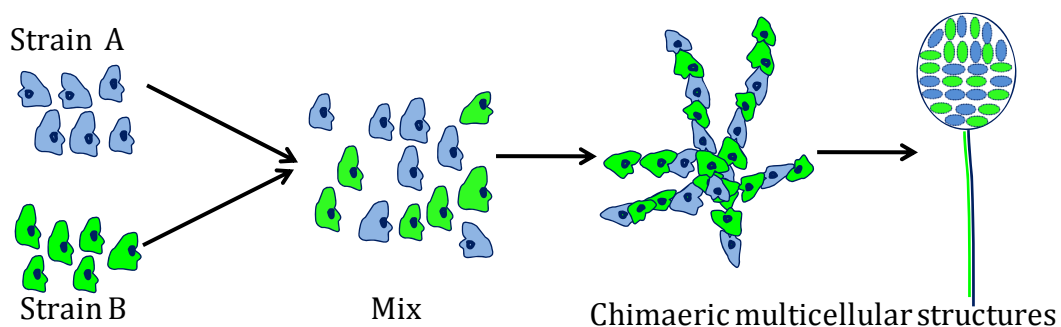
**3.2.3 Developmental rate:** Post-starvation development rate (the time required for freshly starved amoebae to form fruiting bodies) was studied on Millipore filter papers (Type HA, 0.45 µm pore size, Millipore, U.K.) and on KK<sub>2</sub> agar (2% agar in KK<sub>2</sub> buffer). Millipore filter papers were placed on 4-5 sheets of ordinary blotting paper and kept moist by adding KK<sub>2</sub> buffer. Freshly starved amoebae were spread on these filter papers or on KK<sub>2</sub> agar plates at a density of 1 × 10<sup>5</sup> amoebae/cm<sup>2</sup>. The data from previous studies (Kaushik et. al., 2002) as well as this study suggests that this density is appropriate for *D. giganteum* development. Plates were incubated in a dark, moist chamber at 22°C and observed after every 2 hrs using a stereomicroscope (Zeiss) and the developmental stages were recorded. Observations were terminated once fruiting body formation was over. For observing the development of mixed cultures, amoebae of two strains were mixed in a 1:1 ratio [combined plating density (1 × 10<sup>5</sup> amoebae/cm<sup>2</sup>) was kept constant] and allowed to develop together. In order to verify that the strains had co-aggregated, amoebae were differentially labelled using fluorescent dyes before mixing (a protocol for labeling amoebae is described below). The different multicellular structures from intermediate developmental stages (aggregates, slugs) were picked with a needle and observed under a fluorescent microscope (Leica DM-IRB) to see if they contained amoebae of the two strains.

**3.2.4 Slug migration through soil:** These experiments were carried out in plastic tubes (2.8 cm diameter, depth as required per experiment, Fig. 3.2). Each tube was filled with 5 ml KK<sub>2</sub> agar to form a thick (2 cm), moist, solid substratum for the development of amoebae. Freshly starved amoebae (either of a single strain or a 1:1 mix of two strains at a density of  $1 \times 10^6$  amoebae/cm<sup>2</sup>) were spread evenly on the agar surface of the tubes and allowed to settle down by drying the tubes in a laminar air flow for ~10 min. Soil was collected from a garden and sieved through a fine mesh (to obtain a particle size < 1mm), autoclaved at 121°C at 15 lbs pressure for 30 minutes and cooled down to 20-22°C before use. A measured amount of soil was then spread gently over a layer of amoebae to the desired depth. At the time of application the moisture content of the soil was 4-5%, but it may have changed over time as the tubes were incubated in a dark moist chamber at 22°C. The experiments performed earlier by Bonner and Lamont (2005) showed that the size of soil particle does not affect the migration of *P. pallidum*, *D. discoideum* or *D. rosarium* slugs through soil; however, the moisture content in the soil was found to have a significant effect on *Dictyostelium* development (Ponte et al., 1998). The tubes were incubated in the dark at 22°C and observed under direct light using a 5X objective under a dissection microscope after every 12-15 hrs for up to 10 days, after which no more new fruiting bodies appeared on the surface. The time taken by slugs to migrate and emerge above the soil surface and, finally, the number of emerged fruiting bodies from the different depths were also monitored.



**Figure 3.2:** A pictorial description of experimental design to study the slug migration through soil (for description, see the text above).

**3.2.5 Sporulation efficiency in mixtures:** Sporulation efficiency (the ratio of number of spores formed to number of amoebae plated) was estimated in the following way. Amoebae were grown and harvested as mentioned earlier and resuspended in cold  $\text{KK}_2$  buffer at a density of  $1 \times 10^7$  amoebae/ml. Amoebae of different strains in a mix were differentiated by labeling them with a fluorescent dye (either cell tracker blue or cell tracker green) at a final concentration of  $5 \mu\text{M}$ . Staining was performed in the dark for 45 minutes at  $22^\circ\text{C}$  with constant shaking at 140 strokes/min. Amoebae in control experiments were treated with DMSO (the solvent used for preparing stock solutions of the fluorescent dyes). After staining, amoebae were washed three times in ice-cold  $\text{KK}_2$  buffer to remove any residual dye from the suspension. During the washes, amoebae were centrifuged at 300g for 3 min at  $4^\circ\text{C}$ . After every round of centrifugation, the supernatant was discarded and amoebae were resuspended in ice-cold  $\text{KK}_2$  buffer. Finally, amoebae were suspended in  $\text{KK}_2$  buffer at a density of  $1 \times 10^7$  amoebae/ml. Amoebae of either a single strain or a combination of two (in various ratios) were spread evenly at a density of  $1 \times 10^5$  amoebae/ $\text{cm}^2$  on  $\text{KK}_2$  agar plates and incubated in the dark at  $22^\circ\text{C}$ . In another set of experiments, the sporulation efficiency was measured on a natural substrate such as soil. In these experiments, amoebae of two strains (a 1:1 mix) were spread evenly at a total density of  $1 \times 10^6$  amoebae/ $\text{cm}^2$  on  $\text{KK}_2$  agar plates after which sterile soil was sprinkled over the amoebae to achieve different depths. The plates were incubated in a dark, moist chamber at  $22^\circ\text{C}$ . A typical mixing experiment is sketched in Fig. 3.3.



**Figure 3.3: A schematic representation of mixing experiment.** Amoebae of two strains (shown in blue/green) were mixed at the required ratio and allowed to co-develop on  $\text{KK}_2$  agar plates or soil. In a situation where amoebae of two strains mix freely, they form chimaeric aggregates and fruiting bodies as shown.

Absolute and relative sporulation efficiencies were calculated in two ways. (a) Spores from all the fruiting bodies which had developed on a KK<sub>2</sub> agar plate were harvested in 2 ml cold KK<sub>2</sub> buffer, and total numbers as well as relative proportions of spores from each strain were counted (this method could not be used when sporulation efficiencies were measured on soil). (b) 2-3 days after plating, individual fruiting bodies were picked randomly from agar or soil plates with the help of a needle, transferred to a water drop on a glass slide, covered with a coverslip and observed under a 60X objective of fluorescence microscope (Leica) with appropriate filters. In some cases, fruiting bodies were transferred to 10 µl water in an Eppendorf vial, the spores mixed thoroughly, mounted on a glass slide and then observed under a microscope. Stained and unstained spores were counted and the proportions of spores belonging to different strains were calculated. The following controls were used: (a) stained and unstained amoebae were developed independently; (b) to ensure that the fluorescent dye or the staining protocol per se did not affect the proportion of amoebae that differentiated into spores, stained and unstained amoebae of the same strain were mixed in a 1:1 ratio ('self mixing') and the number of stained and unstained spores were counted; (c) in the case of inter-strain mixes, the strain that was stained was switched (for example, in a mix of strains A and B, stained strain A was mixed with unstained strain B, and vice-versa); (d) it was verified that aggregates formed after mixing two strains contained stained and unstained cells in the same ratio that they were mixed in.

To check if the homogeneously mixed amoebae of different strains physically segregate in chimaeric structures, slugs and fruiting bodies were picked, carefully mounted on a glass slide and observed under a microscope. The extent of chimaerism in fruiting bodies was estimated in two ways: (a) the number of fruiting bodies that had both stained and unstained spores relative to the total number of fruiting bodies formed in a mix was counted; (b) stained and unstained spores were counted *within* a fruiting body (independently from 30-50 fruiting bodies per plate in each experiment).

### 3.3 Results

**3.3.1 Growth:** The time taken by an individual amoeba to divide into two during the exponential phase of growth is an important life-history trait. Any advantage in growth rate must contribute significantly to the fitness of an amoeba. Growth rates of *D. giganteum* strains were monitored to check if they divide at significantly different rates. Growth rates were analysed using *K. aerogenes* and other wild bacterial isolates as food source and they were performed on solid plates as well as in liquid suspensions. All the strains of *D. giganteum* grew significantly more slowly in shaken liquid suspension than on agar plates. Strains 46a3 and 48.1a1 grew faster than 46d2 on *K. aerogenes* both on plates as well as in suspension cultures (Table 3.1). The difference between the growth rate of F5 and F16 on plates or in suspension was statistically insignificant (Table 3.1). These trends in growth rate differences persisted when pairs of strains were grown as mixtures in suspension cultures, i.e., 46a3 and 48.1a1 grew faster than 46d2 and F5 and F16 grew at similar rates. The difference in growth rates of 46a3 and 46d2 continued when five different species of bacteria isolated from the Mudumalai soil were used as food sources. When similar number of 46a3 and 48.1a1 amoebae and fixed amount of bacteria were inoculated together, 46a3 amoebae consumed the provided bacteria 6-8 hrs earlier than 46d2, implying that their amoebae grew faster than 46d2. F5 and F16 consumed all wild bacteria at comparable rates. Amoebae of strain 46d2 failed to consume an unidentified bacterial species (50E). This may indicate that 46d2 had differential food preferences, something that needs to be confirmed. All other strains of *D. giganteum* used in this study were able to utilize all the bacteria provided as food sources. To summarise these findings symbolically: in terms of growth, 46a3 and 48.1a1 > 46d2, F5 = F16 (where '>' and '=' indicates faster and comparable growth rates respectively).

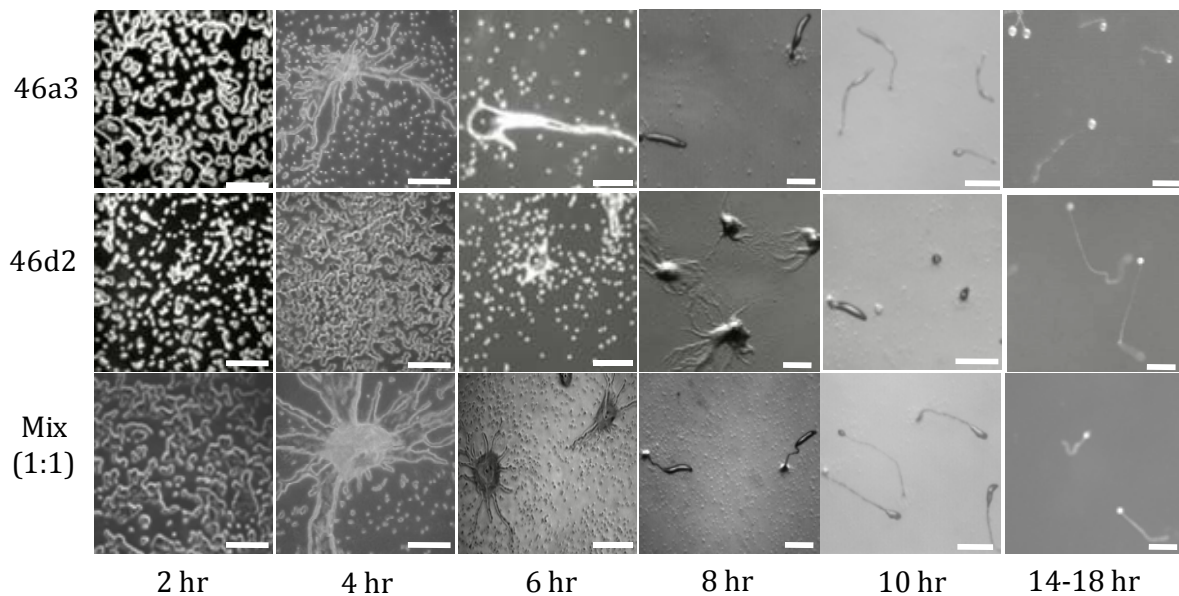


**Table 3.1:** Growth rates of *D. giganteum* strains. 46a3 and 48.1a1 divided significantly faster than 46d2 under all conditions tested, whereas, F5 and F16 grew about equally fast, comparable to the rate of 46a3.

Strains	Doubling time (hrs; mean $\pm$ sd)	Remarks
<b>On plates</b>		
46a3	3.45 $\pm$ 0.36	46a3 grew faster than 46d2 (n = 7, t-test, p = 0.003).
46d2	4.27 $\pm$ 0.53	
48.1a1	3.51 $\pm$ 0.10	48.1a1 grew faster than 46d2 (n = 4, t-test, p = 0.004).
F5	3.43 $\pm$ 0.40	F5 and F16 grew at a similar rate (n = 7, t-test, p = 0.543).
F16	3.31 $\pm$ 0.36	
<b>In suspension</b>		
46a3	8.33 $\pm$ 0.57	46a3 grew faster than 46d2 (n = 5, t-test, p = 0.002).
46d2	10.60 $\pm$ 1.03	
48.1a1	8.06 $\pm$ 0.97	48.1a1 grew faster than 46d2 (n = 5, t-test, p = 0.002).
F5	7.10 $\pm$ 0.98	F5 and F16 grew at a similar rate (n = 4, t-test, p = 0.974).
F16	7.08 $\pm$ 1.12	
<b>In mixed cultures (1:1)</b>		
46a3	8.17 $\pm$ 0.82	46a3 grew faster than 46d2 (n = 6, t-test, p = 0.002).
46d2	10.07 $\pm$ 1.14	
48.1a1	8.51 $\pm$ 0.93	48.1a1 grew faster than 46d2 (n = 4, t-test, p = 0.018).
46d2	10.13 $\pm$ 1.02	
F5	8.88 $\pm$ 1.31	F5 and F16 grew at a similar rate (n = 3, t-test, p = 0.958).
F16	8.81 $\pm$ 0.85	

**3.3.2 Post-starvation developmental rate:** After a local food source is depleted, the faster development is completed the sooner can spores disperse and a second round of growth start. Faster development also means relatively lesser exposure to potential predators present in the soil (e.g. nematodes, Kessin et al., 1996) or adverse conditions. Developmental rate (the time taken by freshly starved amoebae to form fruiting bodies) of a single strain or a 1:1 mix of two different strains was checked on KK<sub>2</sub> agar plates and on moist filter papers.

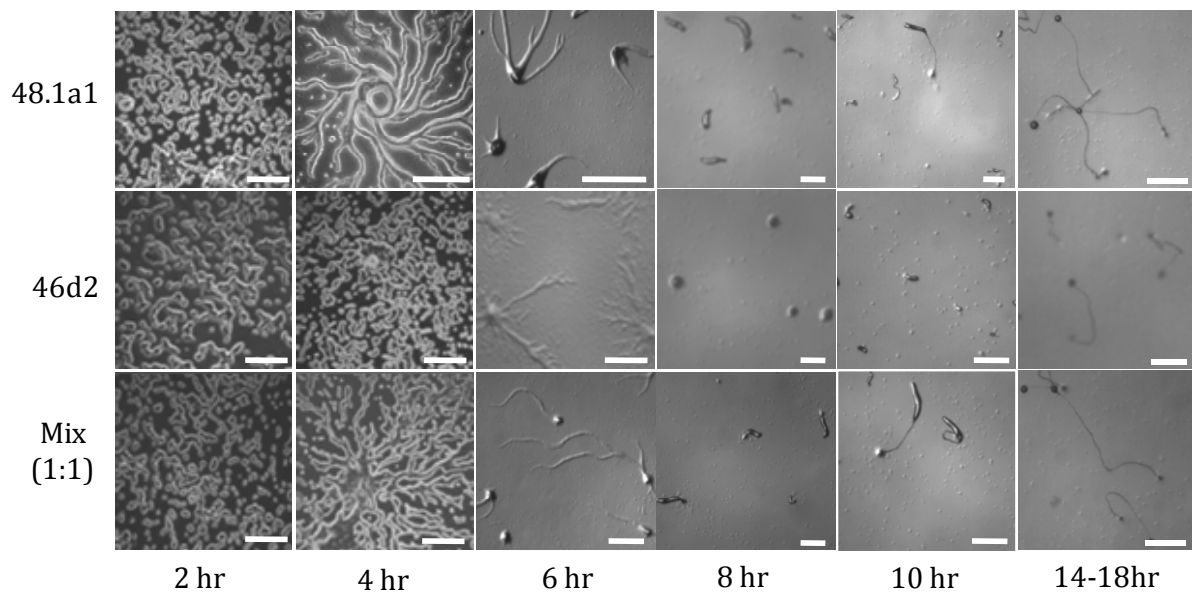
Amoebae of strain 46a3 developed significantly faster than 46d2. The difference in developmental rate was visible within the first 4-6 hrs of plating; by then 46a3 was in mid-to-late aggregation whereas 46d2 was just initiating aggregation. The migrating, stalked-slugs of 46a3 were seen 7-8 hrs after plating, but took 3-4 hrs longer to appear in 46d2. The difference in development rate persisted till the end of development; 46a3 formed fruiting bodies 3-4 hrs before 46d2. A mix (1:1) of 46a3 and 46d2 developed at the same rate as the faster member (46a3). An examination of intermediate stages during development (mounds and slugs) showed that both 46a3 and 46d2 amoebae had co-aggregated, there was no physical separation of amoebae, i.e., amoebae mixed in a “salt-and-pepper” fashion and remained so in the slugs and fruiting bodies. These results were reproducible over 5 independent replicates. A typical case is shown in Fig. 3.4.



**Figure 3.4: Developmental rate of 46a3, 46d2 and a 1:1 mix of the two.** 46a3 developed faster than 46d2. A mix (1:1) of the two developed like faster component, i.e. 46a3. Scale bar represents 250  $\mu\text{m}$ .

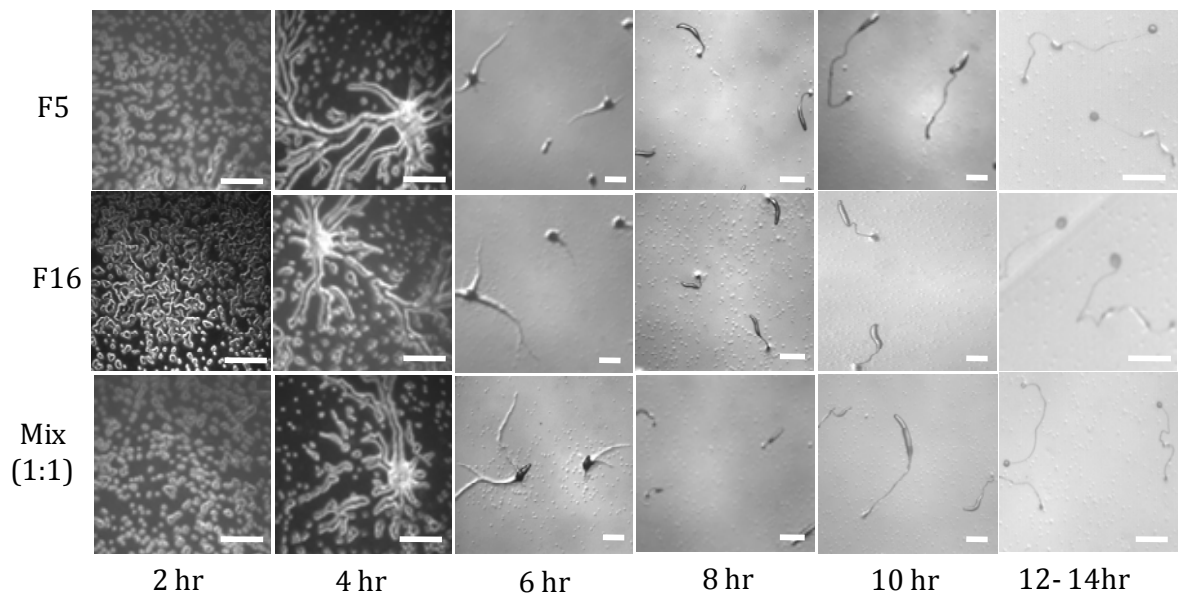
In the case of 48.1a1, 46d2 and a 1:1 mix of the two, the results were as follows: 48.1a1 developed significantly faster than 46d2. After 3-4 hrs of plating 48.1a1 had already formed aggregates with long streams; however, 46d2 took 7-8 hrs to form the aggregates. After 10 hrs of development, 48.1a1 cells had formed

slugs whereas 46d2 cells were still at the mound stage. A 1:1 mix of 48.1a1 and 46d2 developed at the same rate as the faster member of the mix, namely, 48.1a1. The difference in rate of development continued till fruiting body formation was over; 48.1a1 and a 1:1 mix of the two formed fruiting bodies 3-4 hrs before 46d2. These observations were reproducible over 4 independent replicates; a typical example is shown in Fig. 3.5.



**Figure 3.5: Developmental rate of 48.1a1, 46d2 and a 1:1 mix of 48.1a1+ 46d2.** 48.1a1 developed faster than 46d2. A mix of the two developed like faster component, 48.1a1. Scale bars represent 250  $\mu\text{m}$ .

The developmental rate of F5, F16 and their 1:1 mix was comparable; they formed aggregates by 3-4 hrs, slugs at 7-8 hrs and by 12-14 hrs fruiting body formation was over (see Fig. 3.6). Interestingly, F5, F16 or a mix of the two were faster by 2-3 hrs than 46a3, 46d2 or 48.1.a1. The results were reproducible over 4 independent experiments and a typical case is shown in Fig. 3.6.



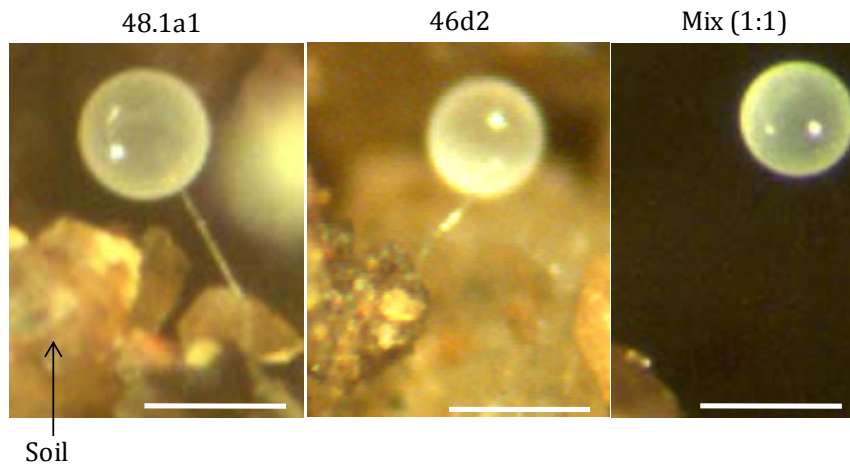
**Figure 3.6: Developmental rate of F5, F16 and a 1:1 mix of F5 + F16.** F5, F16 and a 1:1 mix developed at comparable speeds. Scale bars represent 250  $\mu\text{m}$ .

In terms of speed of development, a symbolic summary of these results would be as follows:  $46a3 > 46d2$  and a mix of  $46a3 + 46d2 = 46a3$ ;  $48.1a1 > 46d2$  and a mix of  $48.1a1 + 46d2 = 48.1a1$ ;  $F5 = F16$  and a mix of  $F5 + F16 = \text{either } F5 \text{ or } F16$  (where ' $>$ ' and ' $=$ ' indicates faster development and development at a comparable tempo respectively).

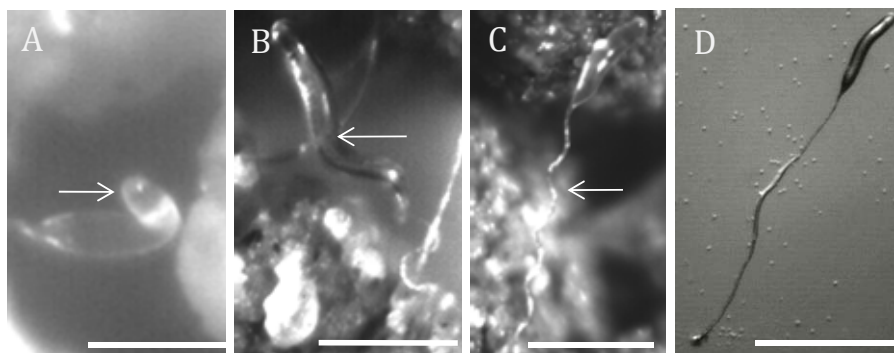
**3.3.3 Slug migration through soil:** These experiments were conducted to measure the ability of slugs that had formed within soil to reach the surface. Upon starvation, *Dictyostelium* amoebae come together and form fruiting bodies above the soil surface. The depth of soil through which a slug can burrow and the time it takes to do so decides how many and how fast can their spores disperse. Spore dispersal to a favourable location will lead to spore germination and start of a new life cycle. Therefore, a *Dictyostelium* strain which can migrate more efficiently through soil could have a selective advantage over a strain which is not so efficient at migration through soil.

All five strains used in this study and their 1:1 mixes (3 different combinations as reported above) could migrate through soil. A typical outcome is shown in Fig. 3.7. In general, the deeper the soil layer, the longer the time taken by

slugs to emerge at the surface. Even after emergence, slugs showed stalked migration for 5-6 hrs and then formed fruiting bodies. The morphology of slugs that migrated through soil was very different from those that developed only on KK<sub>2</sub> agar. The anterior portion of the slugs that migrated through soil were bent and these slugs also had stalks which were curved at several places (Fig. 3.8).



**Figure 3.7: Fruiting bodies formed above 1.5 cm soil depth by 48.1a1, 46d2 and a 1:1 mix of the two. Scale bar represents 250  $\mu$ m.**



**Figure 3.8: Morphology of the slugs formed by 48.1a1 above 1.5 cm soil layer.** (A) A slug making its way through soil and in a process its anterior portion gets curved. (B) and (C) are curved slug and stalk respectively (see arrows). (D) A slug developed on only KK<sub>2</sub> agar (no soil). Scale bar represents 250  $\mu$ m.

The slugs formed by all *Dictyostelium* strains used in this study migrated through soil with different efficiencies. There were significant differences with respect to the following: (a) depth of soil through which slugs migrated; (b) time

required for slugs to migrate and emerge above the soil surface; (c) total number of slugs and fruiting bodies that emerged above the surface.

Slugs formed by amoebae of strains 46a3, 46d2 or their mix were relatively weak at migration through soil; they could emerge from a soil layer that was 1.5 cm thick, but could not do so through a 2 cm layer (Table 3.2). With respect to the time required to migrate through a 1.5 cm deep soil layer and the number of fruiting bodies visible above soil after migration, both strains showed similar results. At a 1 cm soil depth, the number of fruiting bodies per tube were as follows:  $47.0 \pm 13.2$  for 46a3,  $45.0 \pm 12.0$  for 46d2 and  $47.5 \pm 18.3$  for their mix (results are mean  $\pm$  sd; n = 3). A pair wise comparison of the three means shows that they are not significantly different from each other (t-test,  $p > 0.05$ ).

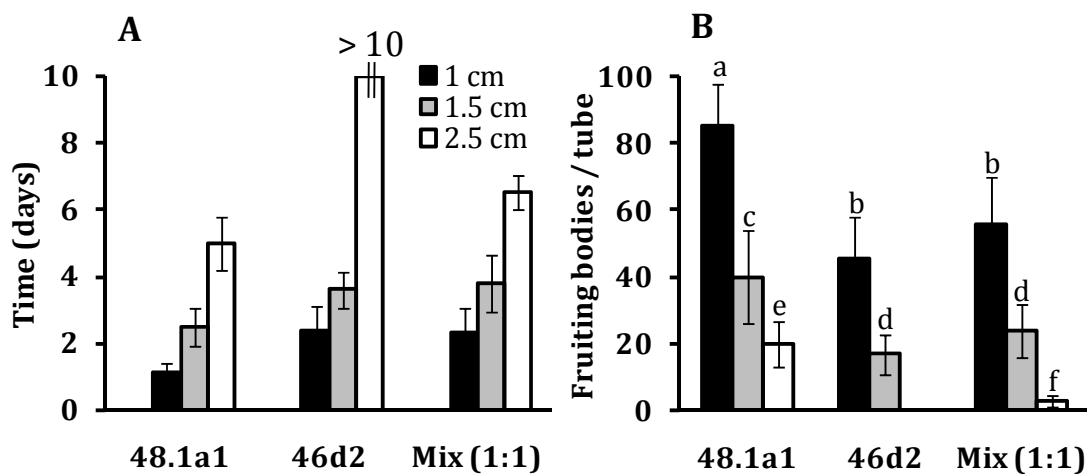
**Table 3.2:** Slug migration through different depths of soil.

Strains	Depth of soil through which slugs can migrate (cm)					
	1	1.5	2	2.5	3	4
46a3	+	+	-	-	-	-
46d2	+	+	-	-	-	-
46a3 + 46d2 (1:1)	+	+	-	-	-	-
48.1a1	+	+	+	+	-	-
46d2	+	+	-	-	-	-
48.1a1 + 46d2 (1:1)	+	+	+	+	-	-
F5	+	+	+	+	+	-
F16	+	+	+	+	+	-
F5 + F16 (1:1)	+	+	+	+	+	-

+, successful migration; -, not successful (up to 10 days)

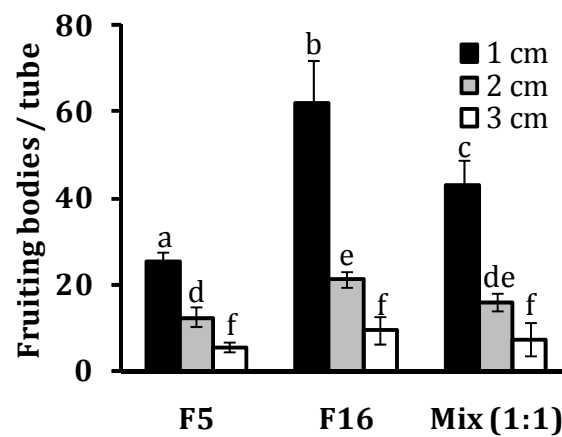
48.1a1 and 46d2 differed significantly with respect to the ability of their slugs to migrate through soil. 48.1a1 could migrate through a 2.5 cm layer of soil, but

could not do so through a 3 cm soil layer. Strain 46d2 only managed to migrate out of a 1.5 cm layer of soil. A mix (1:1) of 48.1a1 and 46d2 was able to migrate through a 2.5 cm layer of soil (Table 3.2). It was confirmed that both strains were represented in the fruiting bodies that emerged above soil. In addition to their ability to cover a greater depth, 48.1a1 fruiting bodies also emerged before those of 46d2, implying that their slugs were faster; chimaeric slugs behaved like the slower member of the pair in terms of the time taken. Finally, the number of fruiting bodies formed by 48.1a1 were significantly more than 46d2 whereas a mix of the two formed an intermediate number of fruiting bodies (Figs. 3.9 A and 3.9 B).



**Figure 3.9: Slug migration through soil (48.1a1, 46d2 and their 1:1 mix).** '> 10' indicates that no fruiting bodies were observed upto 10 days. The different letters on top of the histograms refer to statistical significance as assessed by the t-test (for example 'a' indicates significant difference than 'b'). (A) Time taken for fruiting bodies to emerge through various depths of soil. Slugs of 48.1a1 emerged before 46d2 at all soil depths (n = 5 in every case; 1 cm,  $p < 0.001$ ; 1.5 cm,  $p = 0.004$ ; 2.5 cm 46d2 failed to migrate through); similarly, 48.1a1 was more efficient at migration through soil than a mix of 48.1a1 + 46d2 (1:1) (n = 5 in every case; 1 cm,  $p = 0.001$ ; 1.5 cm,  $p = 0.014$ ; 2.5 cm,  $p = 0.025$ ). The difference between 46d2 and the 48.1a1 + 46d2 mix except at 2.5 cm was not significant ( $p > 0.05$ ). (B) No. of fruiting bodies to emerge from different depths of soil. 48.1a1 formed more fruiting bodies than 46d2 at all three soil depths (n = 5 in every case; 1 cm,  $p = 0.001$ ; 1.5 cm,  $p = 0.009$ ; 2.5 cm, 46d2 did not form any fruiting bodies). 48.1a1 formed more fruiting bodies than a mix of 48.1a1 and 46d2 (n = 5 in each case; 1 cm,  $p = 0.010$ ; 1.5 cm,  $p = 0.044$ ; at 2.5 cm,  $p = 0.033$ ). The difference in the number of fruiting bodies formed by 46d2 on its own and in a 1:1 chimaera with 48.1a1 was not statistically significant at 1 and 1.5 cm.

Slugs formed by F5, F16 or their 1:1 mix of the two strains were able to emerge from a 3 cm soil layer, but could not do so through a 4 cm soil layer (Table 3.2). At all soil depths, F16 fruiting bodies developed 24-48 hrs before those of F5 or a mix of the two. In terms of the number of fruiting bodies seen on the soil surface, F16 slugs were better at migrating through soil than those of F5; a 1:1 combination of the two was comparable with either partner by itself (Fig. 3.10).

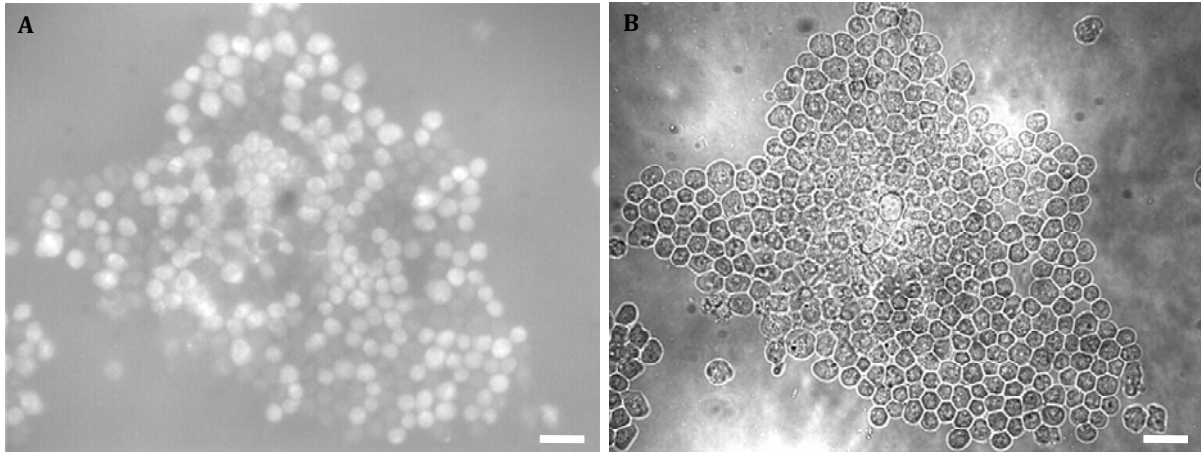


**Figure 3.10: Number of fruiting bodies to emerge from different depths of soil (F5, F16 and their 1:1 mix).** Means with different letters (indicated on top of the histograms) are significantly different. F16 formed more fruiting bodies than F5 at most soil depths tested; three depths are shown here (n = 5 in every case and significance by t-test; 1 cm,  $p \ll 0.001$ ; 2 cm,  $p = 0.029$ , 3 cm,  $p = 0.121$ ). The number of fruiting bodies formed by a 1:1 mix of F5 + F16 was comparable with that formed by either partner except at 1 cm where the mix formed fewer fruiting bodies than F16 ( $p = 0.006$ ) and more fruiting bodies than F5 ( $p = 0.003$ ). Two-tailed t-tests were performed assuming unequal variances.

**3.3.4 Sporulation efficiency:** Spore formation is the most obvious way in which an amoeba is assured of a reproductive future. The higher the probability of sporulation, the higher the relative frequency of a cell's genotype in the next generation. The sporulation efficiency of amoebae was checked individually and in binary mixtures by counting the total number of spores formed relative to the no. amoebae plated. The development of stained and unstained amoebae was similar,



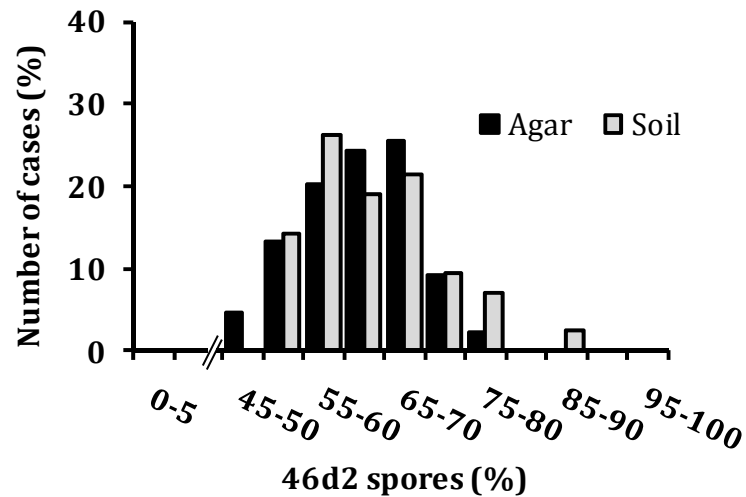
and, amoebae in an aggregate remained in the same ratio as that when mixed (Fig. 3.11).



**Figure 3.11: A chimaeric aggregate formed by a mix of 46a3 and 46d2 amoebae.** 46a3 amoebae were stained with cell tracker blue and mixed with 46d2 unstained amoebae in a 1:1 ratio and plated on 2%  $\text{KK}_2$  agar. Amoebae of both the strains aggregated and formed a chimaeric (mixed) aggregate in which the ratio of two strains was 1:1. A and B are fluorescent and bright field images respectively. Scale bars represent 10  $\mu\text{m}$ .

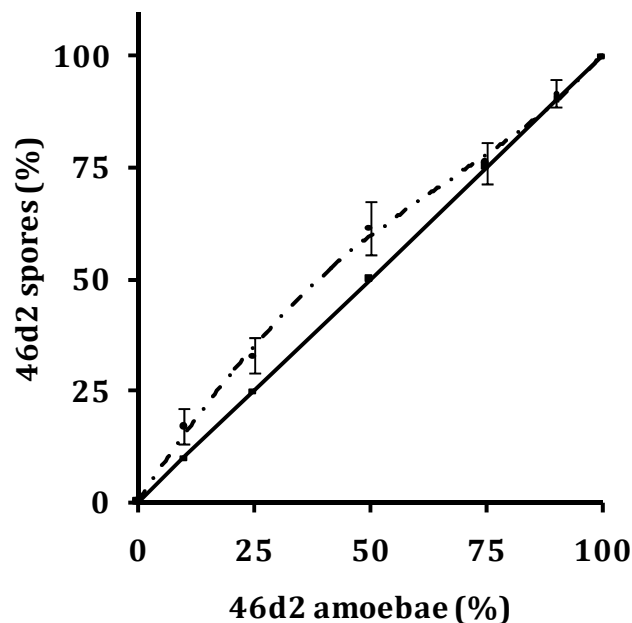
When amoebae of strains 46a3 and 46d2 were mixed in a 1:1 ratio and allowed to develop together on a substrate (soil or agar), all the fruiting bodies formed were chimaeric, in other words, they had spores belonging to both strains. On agar, the representation of 46d2 in the spore population was higher than that of 46a3 ( $62.1 \pm 6.1\%$ , mean  $\pm$  sd;  $n = 4$ ; t-test,  $p = 0.007$ ). Although most of the fruiting bodies formed by a mix had more spores of 46d2 than 46a3, a minority of fruiting bodies – about one in twenty – contained fewer spores of 46d2 than 46a3 (Fig. 3.12). When a similar mixing experiment was carried out on soil, once again fruiting bodies had a larger proportion of 46d2 spores than of 46a3 ( $62.0 \pm 6.9\%$ ; mean  $\pm$  sd;  $n = 3$ ; t-test,  $p = 0.040$ ). However, in this case none of the fruiting bodies examined showed the opposite trend, i.e., contained fewer spores of 46d2 than 46a3 (Fig. 3.12). The ratio of numbers of spores formed to amoebae plated was similar for each strain measured separately and in a 1:1 mixture:  $45.5 \pm 7.7\%$  for 46a3,  $47.9 \pm 10.4\%$  for 46d2 and  $46.2 \pm 13.0\%$  for 46a3 + 46d2 mix (results are mean  $\pm$  sd;  $n = 10$  in every

case, t-test,  $p > 0.05$ ). There was no obvious sorting out of the strains, whether in aggregates or spore masses.



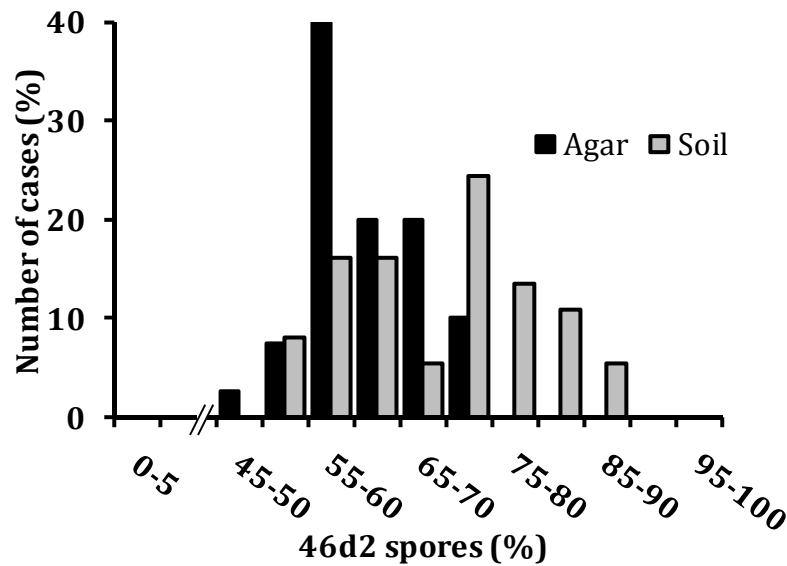
**Figure 3.12: Frequency distribution of 46d2 spores in chimaeric fruiting bodies formed by 46a3 + 46d2 1:1 mix.** On average, 300 spores from each of 30–50 sori were counted in each experiment. Spores were counted from individual fruiting bodies as well as en masse from aggregation plates, for convenience, the latter measurement was carried out only when cells were developed on agar. On average, 46d2 formed more spores than 46a3, however, about 5% of the fruiting bodies developed on agar showed a majority of 46a3 spores. All the fruiting bodies formed on soil (1 cm depth) had a larger proportion of 46d2 spores than 46a3.

In order to see if the sporulation efficiency of a strain was dependent on the initial mixing ratio, freshly starved amoebae of the two strains were mixed in 5 different ratios (10:90, 25:75, 50:50, 75:25 and 90:10). It was seen that 46d2 continued to be better at sporulation than 46a3 at initial frequencies of  $< 75\%$ , and no better or worse than it at higher frequencies (Fig. 3.13).



**Figure 3.13: Proportions of 46d2 amoebae (X axis) and spores (Y axis) in 46a3 + 46d2 mixtures.** Results are mean  $\pm$  sd;  $n = 4$  in every case; in each experiment 30-50 fruiting bodies and  $\sim 300$  spores from each fruiting body were counted. Continuous line indicates expected outcome if strains do not interact. 46d2 formed more spores than 46a3 in almost all mixing ratios (10:90,  $p = 0.013$ ); 25:75,  $p = 0.007$ ; 50:50,  $P = 0.008$ ) except when it was mixed with 46a3 at a ratio of 75:25 and 90:10 where it formed similar numbers of spores ( $p > 0.5$ ). Statistical significance was assessed by t-test.

All fruiting bodies formed by 48.1a1 + 46d2 (1:1) mix were chimaeric. In the presence of 48.1a1, 46d2 formed significantly more spores than expected both on agar ( $61.5 \pm 5.2\%$  mean  $\pm$  sd;  $n = 4$ , t-test,  $p = 0.005$ ) and on soil ( $68.2 \pm 8.9\%$  mean  $\pm$  sd;  $n = 3$ , t-test,  $p = 0.025$ ). In contrast to development on agar (black bars), every fruiting body that emerged in the soil experiments (grey bars) had more 46d2 than 48.1a1 spores (Fig. 3.14). Differences in total spores formed/total amoebae plated were similar: 48.1a1 =  $51.01 \pm 9.1\%$ ,  $n = 5$ ; 46d2 =  $47.9 \pm 10.4\%$ ,  $n = 10$  and 48.1a1 + 46d2 mix (1:1) =  $48.75 \pm 8.5\%$ ,  $n = 5$ . The difference between the means was statistically insignificant (t-test,  $p > 0.05$ ).

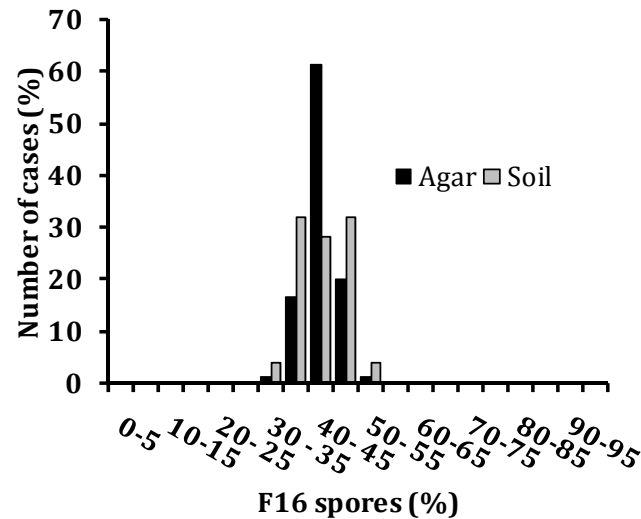


**Figure 3.14: Frequency distribution of 46d2 spores in chimaeric fruiting bodies formed by 46d2 + 48.1a1 mix (1:1).** 46d2 did significantly better than 48.1a1, both on agar and under a 1 cm layer of soil. As with the 48.1a1 + 46d2 combination, the disparity between the strains was more marked in the case of development on soil than on agar. On average, 200 spores from each of 15–20 sori were counted in each experiment on soil ( $n = 3$ ) and 300 spores from each of 30–50 sori were counted in each experiment on agar ( $n = 4$ ).

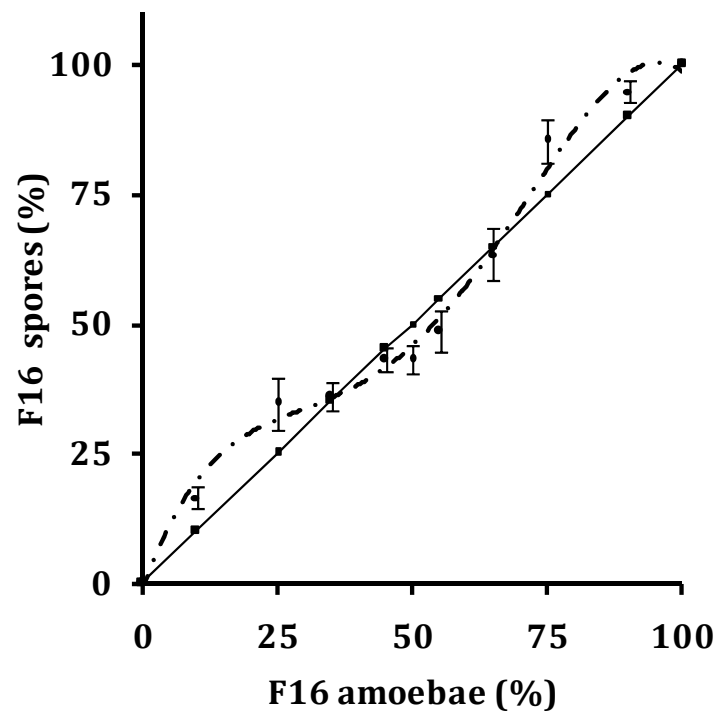
In the case of F5 + F16 1:1 mixes on  $KK_2$  agar and on soil, all the fruiting bodies were chimaeric and no gross sorting out in the slugs or fruiting bodies could be detected. F16 invariably contributed lesser spores than F5 when they were developed on agar ( $43.0 \pm 2.8\%$ ; mean  $\pm$  sd;  $n = 3$ ; t-test,  $p = 0.015$ ). When F5 and F16 amoebae were developed below 1 cm soil the fruiting bodies formed on the surface had fewer F16 spores than F5 spores ( $42.5 \pm 4.0\%$ , mean  $\pm$  sd;  $n = 3$ ; t-test,  $p = 0.035$ ). In 2–3% of the fruiting bodies (both on agar and soil), the trend was reversed, with F16 contributing more spores than F5 (Fig. 3.15).

When proportions of F5 and F16 amoebae in the initial mix were varied a new outcome emerged, namely an efficiency of spore formation that varied with frequency. The ability of F5 amoebae to form relatively more spores than F16 in F5 + F16 mixtures was true within a small range of initial frequencies close to 50%. F16 formed more spores than F5 outside 50:50 mixing ratio, i.e., at initial mixing ratio of 1–40% and 65–100% (F16: F5), F16 formed more spores than F5. This implies the

existence of two initial mixing ratios at which the strains can exist in equilibrium, one of them stable (~40% F16 and 60% F5) and the other unstable (~68% F16 and 32% F5) (Fig. 3.16).



**Figure 3.15: Frequency distribution of F16 spores in the chimaeric fruiting bodies formed by F5 + F16 mix (1:1).** F16 formed significantly fewer spores than F5. In each experiment, approximately 400-500 spores from each of 25-30 sori formed on agar and 10-15 sori emerged on a 1cm soil layer were counted.



**Figure 3.16: Proportions of F16 amoebae (X axis) and spores (Y axis) in chimaeric fruiting bodies formed by F5 + F16 mixtures.** Continuous line indicates expected outcome if strains do not interact; broken line indicates curve fitted to experimental data points. Results are mean  $\pm$  sd;  $n = 4$  in all cases; approximately 300 spores from each of 30 – 50 sori counted in each experiment. The data were fitted to non-linear polynomials of varying degrees using the on-line software available at [http://phet.colorado.edu/sims/curve-fitting/curve-fitting\\_en.html](http://phet.colorado.edu/sims/curve-fitting/curve-fitting_en.html). A chi-squared goodness of fit test shows that a quartic (shown;  $\chi^2 = 0.62$ ) fits the data better than any lower-degree polynomial (linear,  $\chi^2 = 1.22$ , quadratic,  $\chi^2 = 1.25$ , cubic,  $\chi^2 = 1.44$ ). Sporulation efficiencies were measured with initial mixing ratios on either side of the two potential equilibrium points (F16;  $\sim 40\%$  and  $68\%$ ) to confirm their existence.

### 3.4 Discussion

**3.4.1 Caveats:** All the fitness-related traits monitored in this study were on the strains in isolation or in a mixture of two strains. The restriction to pairs of strains was imposed by practical limitations. The genetic heterogeneity of CSM social groups in nature can range from zero (i.e., clonal groups) to at least 9 clones (Kaushik and Nanjundiah, 2003; Gilbert et al., 2007; Gilbert et al., 2009; Sathe et al., 2010). Therefore, in order to understand the factors that contribute to the coexistence of different strains one needs to study the social groups which are formed by as many of them as possible. This is because the 'performance' of a strain changes in presence of other group members. For example, Kaushik et al. (2006) measured relative sporulation efficiencies after mixing three *D. giganteum* strains. The behaviour of a strain (sporulation efficiency) when it was alone was significantly different in presence of two other strains. Saxer et al. (2010) have monitored strain frequency changes after co-culturing 8 strains of *D. discoideum* through many asexual life cycles. Here too, it was not possible to predict the frequency of a strain in the next generation based only on a study of the behaviour of a single strain.

I have studied fitness-related traits spread over the asexual life cycle; *D. giganteum* and other CSM amoebae can go through sexual life cycle (Blaskovics and Raper, 1957; Erdős et al., 1973). It would have been interesting to see if fitness-related trade-offs exist in the sexual phase of the life cycle. Finally, these experiments do not replicate natural conditions entirely as most of them have been performed under laboratory conditions. Although the use of soil substrates to monitor slug migration and relative sporulation efficiency is a good proxy for natural habitats. It is important to study the development of CSMs on natural substrates because the development and behaviour of some strains of *D. discoideum* show differences in development on soil vs agar. Ponte et al (1998) found that the moisture content in the soil has a significant effect on *Dictyostelium* development and some developmental phenotypes which were not visible on agar became obvious when the strains were developed on soil (Queller et al., 2003).

Earlier studies on *D. mucoroides*, *D. discoideum*, *D. giganteum* and *D. purpureum* have shown that genetically different strains of a CSM species co-occur in close proximity, such as in the same soil particle or within the same social group formed in the wild (Buss, 1982; Fortunato et al., 2003; Kaushik et al., 2006; Gilbert et al., 2007; Sathe et al., 2010). With the exception of a strain of *D. mucoroides* (Buss, 1982), these strains had the same 'wild-type' developmental phenotype i.e., they formed "normal" fruiting bodies consisting of stalk and spore cells. When different strains belonging to the same species were mixed as amoebae in 1:1 ratios in pairs, they formed chimaeras in which some of the strains had different sporulation efficiencies (Strassmann et al., 2000; Kaushik et al., 2006, and this study). These observations raised an issue of how such strains can coexist, in other words why the strain that forms spores most efficiently in chimaeras does not eliminate the rest. As has been mentioned, the present study was designed to address this question.

A possible answer for the coexistence of different strains is that relative success in sporulation depends on the number of strains in a chimaeric social group. A strain of *D. giganteum* that forms fewer spores than another when the two are combined, forms the same number of spores as the others when a third strain is present (Kaushik et al., 2006). This was also observed in *D. discoideum* strains (Khare et al., 2009). I have investigated other possible factors that might contribute to the observed co-existence of *D. giganteum* strains.

The present study is based on the hypothesis that in addition to sporulation, the fitness of a strain depends on many other traits and on biotic and abiotic environmental factors. It is essential to study fitness-related traits as a whole. I have examined a number of phenotypic traits besides sporulation which impinge on the fitness of an amoeba: growth rate, tempo of post-starvation development and migration of slugs through soil. These traits were monitored both individually for each strain as well as in mixes of different proportions using natural isolates of *D. giganteum* to see how they contribute to the observed pattern of co-existence in the strains. Growth rate and sporulation certainly contribute to a cell's reproductive fitness; the growth rate of a strain decides how many amoebae are produced in a

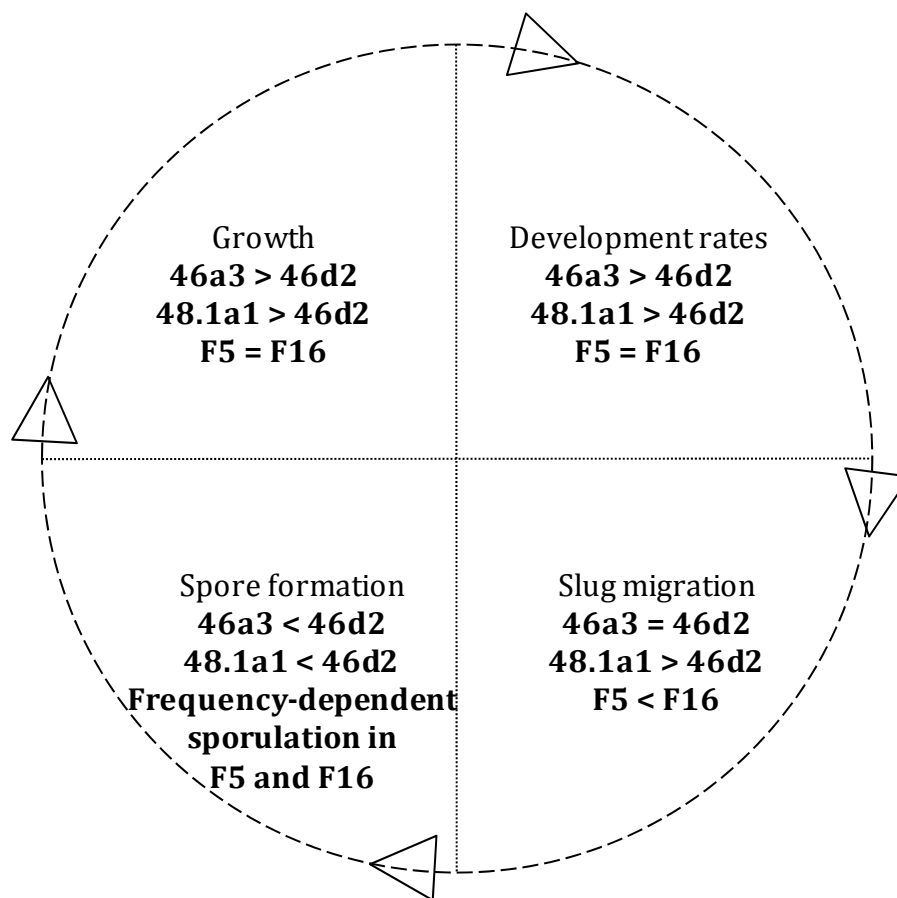


given generation and sporulation decides how many of those amoebae can survive to the next generation. Regarding the rate of development and migration through different depths of soil, it is plausible that the earlier a fruiting body is formed on the soil surface, the sooner its spores can disperse. There are no studies to suggest how long spores wait to be dispersed by dispersal vectors. Therefore it remains a matter of speculation whether a head start of even a few hours can make a difference in the beginning of a new life cycle. In addition, earlier fruiting body formation could mean amoebae are exposed for a shorter duration of time to an unfavorable environment conditions, toxic compounds or predation. Spores are more resistant than amoebae to a host of environmental stresses (Kuserk et al., 1977), and predators like nematodes which can digest amoebae but not spores (Kessin et al., 1996). It would have been ideal to combine measurements of developmental rate and slug migration through soil into a single measure of development, but there is no easy method to monitor early development in a soil environment. Similarly, absolute sporulation efficiencies (number of spores formed/number of amoebae plated) too should have been checked on soil. This is not an impossible task, but requires a reliable way of harvesting spores from the soil; the same problem does not exist in the case of relative sporulation efficiencies, which can be estimated after amoebae develop on soil.

The following general conclusions can be drawn from this study: (i) wild-type strains of *D. giganteum* differ in respect of more than one fitness-related trait (Table 3.3); (ii) in chimaeras, strains can interact in a way that influences traits; (iii) for the same pair of strains the relative influence can be different in respect of different traits, and therefore (iv) trade-offs between different fitness-related traits do exist (Fig. 3.17) and can probably contribute to the co-existence of strains. When these observations are examined pair by pair, a number of interesting features emerge. They are summarised in Table 3.3 and discussed in greater detail below.

### 3.4.2 Trade-offs

**46a3 and 46d2:** 46a3 amoebae grows faster than 46d2 amoebae (Table 3.1), in migration, both perform equally well (Table 3.2). In a mix, the two develop on agar at a rate corresponding to that of the faster member, 46a3 (Fig. 3.4). 46d2 forms relatively more spores than 46a3 (Fig. 3.12, 3.13). Overall, for 46a3 and 46d2 the relative differences in growth and sporulation efficiency represent a trade-off; 46a3 exhibits faster growth rate but has poorer sporulation efficiency, whereas, 46d2 exhibits the opposite characteristics (Fig. 3.17).



**Figure 3.17: Trade-offs in the life cycle of *D. giganteum*.** ">" indicate relatively better than; "=" similar.

**48.1a1 and 46d2:** 48.1a1 amoebae grows faster than 46d2 (Table 3.1), 48.1a1 amoebae also develop faster than 46d2 (Fig. 3.5), and in a mix, both develop like 48.1a1; 46d2 amoebae gets advantage by joining with 48.1a1. 48.1a1 amoebae do significantly better than 46d2 amoebae in terms of migration through different

depths of soil (Table 3.2, Fig. 3.9A, and Fig. 3.9B). When developed alone, both 48.1a1 and 46d2 form equal numbers of spores, however, 46d2 forms significantly more spores than 48.1a1 when mixed in a 1:1 ratio. (Fig. 3.14). Frequency-dependent sporulation in this pair of strains was not monitored. Similar to the 46a3 + 46d2 mix, a trade-off between growth and sporulation efficiency stands out; 48.1a1 grows and develops faster than 46d2, whereas, 46d2 forms more spores than 48.1a1 (Fig. 3.17).

**F5 and F16:** F5 and F16 grow and develop at similar rates (Table 3.1, Fig. 3.6). The depth through which slugs can migrate and emerge above soil is similar for F5, F16 and a mix of the two. However, F16 and a mix of F5+F16 is better than F5 in terms of the time taken by slugs to emerge and the number of total fruiting bodies formed above soil surface (Table 3.2, Fig. 3.10). The individual efficiencies of sporulation is comparable between F5 and F16 individually, but when mixed, F16 does better than F5 when present in a large minority or large majority and worse at intermediate mixing ratios (Fig. 3.16). If judged solely on this basis, the two can be expected to co-exist in a stable equilibrium in which the relative frequencies of the two strains are ~60% (F5) and 40% (F16). However, if for some reason F16 amoebae happen to be present at an initial proportion of greater than ~68%, their advantage in sporulation will eliminate F5 (again, going by this factor only). The only other case of a similar frequency-dependence selection was reported by Buss (1982), on naturally occurring variants of *D. mucoroides*.

The following conclusions follow from these observations. *D. giganteum* amoebae – and, by extension, those of other CSMs – interact during all phases of the life cycle. Such interactions may have a significant effect on the fitness of a strain, as they can cause a strain to do better or worse than when on its own; depending on the strain and the trait (Table 3.3). It is difficult to predict if a strain will do better or worse with respect fitness-related traits after it joins in aggregation with cells of another strain. In the same mix, individuals belonging to one strain can do better than the other (as compared to when they are on their own) with respect to one fitness-related trait and worse in respect of another. Therefore, it is inappropriate to

draw inferences regarding fitness based on measurements of just one trait, most often the sporulation efficiency of a strain relative to another. The fitness of an individual strain needs to be computed over the entire life cycle.

**Table 3.3:** Comparison of clonal and chimaeric groups of *D. giganteum* in respect of traits monitored.

Trait	Strains compared		
	46a3 and 46d2	48.1a1 and 46d2	F5 and F16
Growth (doubling time)	46a3 faster by itself and in combination <sup>†</sup>	48.1a1 faster by itself and in combination <sup>†</sup>	Similar rates, separately and in combination <sup>†</sup>
Development (time to aggregate and fruit)	46a3 faster; chimaeras like 46a3 <sup>a</sup>	48.1a1 faster; chimaeras like 48.1a1 <sup>a</sup>	Similar, both separately and in chimaeras <sup>a</sup>
Migration (ability of slugs to migrate through depths of soil)	Similar, both separately and in chimaeras *	48.1a1 better; chimaeras like 48.1a1 (depth covered) and like 46d2 (time taken)*	F5, F16 and chimaera similar (depth covered); F16 better (time taken and no. of fruiting bodies formed), chimaera resembles F16*
Sporulation (no. of spores formed relative to amoebae plated)	Similar separately; 46d2 better in chimaeras	Similar separately; 46d2 better in chimaera, especially on soil*	Similar separately; frequency-dependent outcome in chimaeras <sup>a</sup>

†, cells grown in shaken liquid suspension while being separated by a permeable barrier (see Methods); a, tested only on agar (not on soil); \*, only 1:1 mix carried out.

### 3.4.3 How does this study help us to understand cooperation in *D. giganteum*?

It is important to distinguish between the evolutionary origin and maintenance of cooperation as it is possible that similar or even entirely different factors underlie these two phenomena. Today, in absence of the knowledge of earlier ecological conditions, our chances of understanding the maintenance of

cooperation are higher than of understanding the evolutionary basis of its origin. If we wish to understand the maintenance of cooperation it is necessary to consider all the parameters that are involved in it. Most of the available literature on the evolution of cooperation concentrates only on the genotype; the tendency to label a genotype as a cheater or as a victim makes this obvious. The phenotype of a cell certainly depends on the genotype, but, in addition, also on the cell's physical and biotic environments – and crucially, on the social environment that is provided by the rest of the group (Sawarkar et al., 2009; Saxer et al., 2010; Nanjundiah and Sathe, 2011). A lot of earlier studies on the development of *D. discoideum* support this point. Several pre-aggregation and post-aggregation biases are known to influence the probability of an amoeba that differentiates into a stalk cell or a spore cell. Besides the genotype, they include nutritional status, cell cycle phase at starvation, cellular calcium content, position in an aggregate, intercellular signals and, plausibly, stochastic effects (reviewed in Kaushik and Nanjundiah, 2003). For example, clonal populations of *D. discoideum* amoebae that are either raised in a glucose-poor medium, are lighter (less dense) in size, harvested early in the cell cycle, contain higher levels of cellular calcium, show a tendency to differentiate into stalk cells when mixed with amoebae that are grown in a glucose-rich medium, relatively denser, are at a late stage of the cell cycle when starved or have lower calcium levels, respectively (Takeuchi, 1969; Leach, et al., 1973; Maeda and Maeda, 1974; McDonald and Durston, 1984; Weijer et al., 1984; Thompson and Kay, 2000; Azhar et al., 2001). Post aggregation biases such as cells in the centre of an aggregate show higher tendency to form spores (Huang et al., 1997). Similarly, non-autonomous factors are equally important influences (Buttery et al., 2010; Mujumdar et al., 2011). Task-related biases in workers belonging to different patriline provide a parallel example from social insects (Hughes et al., 2003). Considering there are so many other factors besides genotype which influence a cell's fate in becoming a spore or part of the stalk, it is inappropriate to label the genotypes as 'selfish', 'altruist', 'cheater', and even 'victim' and 'noble' (Strassmann et al., 2000; Khare and Shaulsky, 2010). The cell which is a cheater in one context could be a victim in another; it

depends on the genotype of a cell of course, but importantly, on several other biases as mentioned above.

It is appropriate to look at the division of labour (stalk vs. spore) in *Dictyostelium* as being based primarily on a competition between amoebae of different phenotypes for their ability to sporulate (Atzmony et al., 1997). Competition could be mediated via interactions between cells and feedbacks. So, in an aggregate, relatively 'stronger' amoebae (for example, having relatively higher levels of stored glycogen) forms spores and relatively 'weaker' ones form the stalk. The terms 'stronger' and 'weaker' are relative and they would depend on several pre- and post-aggregation biases mentioned above (Nanjundiah and Saran, 1992; Atzmony et al., 1997). The very same factors, stochastic (Nanjundiah and Bhogle, 1995) and deterministic (Nanjundiah and Saran, 1992), which decide cell fate in clonal populations would work as well in polyclonal populations. For it to happen the members of a group should be compatible to each other in terms of responding to a common set of cellular and extracellular signals and not that they should have common genes. This implies that they should have co-evolved, as can be expected if they are found in the same microenvironment (Kawli and Kaushik, 2001; Sathe et al., 2010).

Earlier studies suggest when genetically different strains of a species are mixed as amoebae, in several cases one of them forms more spores than the other (Strassmann et al., 2000; Kaushik et al., 2006, this study). There are mutants of *D. discoideum* that sporulate with a better efficiency than their 'wild-type' parent when mixed with it (Ennis et al., 2000; Benabentos et al., 2009). It is known that amoebae belonging to different species can sort out from each other after aggregating together (Raper and Thom, 1941; Bonner and Adams, 1958; Bonner, 1959), as can natural isolates of the same species (Kaushik et al., 2006; Mehdiabadi et al., 2006; Ostrowski et al., 2008; Flowers et al., 2010). These observations suggest that discrimination between amoebae can increase with genetic distance; this phenomenon has been interpreted in terms of association preferences between genetically similar individuals. These could conceivably work as a kin-recognition

mechanism and therefore permit kin selection to operate (Mehdiabadi et al., 2006; Ostrowski et al., 2008). However, social groups can be polyclonal (Kaushik and Nanjundiah, 2003; Sathe et al., 2010, this study see chapter 2), which makes the operation of kin selection more difficult than it would be in a clonal group (but not impossible). The inclusive fitness of an amoeba that differentiates into a stalk cell will be higher when the amoeba is part of a clonal group than when the group consists of many clones. That does not make clonality a prerequisite for the evolutionary origin or maintenance of stalk cell death. The apparent altruism of stalk cells is best viewed as the outcome of social selection – namely of a complex interplay between heterogeneous units that make up a dynamic system (West-Eberhard, 1979; Wolf et al., 1999; Nanjundiah and Sathe, 2011), a conclusion that is extendable to other social groups.

### 3.5 References

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# Chapter 4

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*Chimaerism and Segregation in Inter-species*

*Mixtures of Dictyostelium purpureum*

*and Dictyostelium giganteum*

## 4.1 Introduction

The stable coexistence of different species in a shared environment depends on the type of interactions between their members. Exploitative or parasitic interactions can be harmful for members of one or more of the interacting members. The competitive exclusion principle or Gause's law suggests that the members of two species sharing identical niches and competing for the common resources cannot coexist permanently (Gause, 1934). Several species of cellular slime moulds (CSMs) all with more-or-less similar life cycles live together in the same environment (Agnihotrudu, 1956; Rai and Tewari, 1961; Horn, 1971; Kaushik, 2002; Fortunato et al., 2003; Sathe et al., 2010). This means that individuals of different species of CSMs have an opportunity to interact with each other. In addition, because of the idiosyncratic way in which CSMs achieve multicellularity (by the aggregation of free-living amoebae), they are potential models for studying interactions between members of different species. In the case of CSMs, a question of interest is to see if amoebae of different species can come together to form a chimaeric multicellular body. Different reasons motivate a study of the interactions between members of different CSM species. Many CSM species co-occur in the same natural habitat (reviewed in chapter 2). Many use a common chemical signal for aggregation, cAMP being the most common, the dipeptide glorin and pterin being others (Konijn et al., 1968; Shimomura et al., 1982; Van Haastert et al., 1982). As expected, amoebae of different species can be attracted by each other's centers and form chimaeric aggregates (Raper and Thom, 1941; this study). These observations suggest the possibility of interactions between members of different CSM species. CSMs also offer a good model to study self/non-self discrimination mechanisms in asexual (even if facultative) species.

## 4.2 Inter-species interactions

**(A) Previous studies:** Vegetative amoebae of different CSM species repel each other, suggesting a possibility of inter-species interactions during growth-phase of the life cycle (Keating and Bonner, 1977), however, this study concentrates on post-starvation interactions. Olive (1902) investigated whether amoebae of different species co-aggregate and form chimaeric multicellular structures. He plated spores

of *Dictyostelium mucoroides* (fruiting bodies with white sori) and *D. purpureum* (purple sori) together with bacteria, allowed them to germinate, grow and form fruiting bodies jointly. No fruiting bodies with intermediate phenotypes were observed; all fruiting bodies had either purple or white sori. However, Olive did not analyse individual spores or stalk cells within fruiting bodies and so may have missed possible cases of weak chimaerism (Olive, 1902). Raper and Thom (1941) carried out inter-species mixing experiments using four CSM species: *D. discoideum*, *D. mucoroides*, *D. purpureum* and *Polysphondylium violaceum*. In one set of experiments, spores of two species (for example, *D. discoideum* and *D. mucoroides* or *D. discoideum* and *D. purpureum* or *D. mucoroides* and *D. purpureum*) were co-inoculated on a common growth medium. The spores of the two species germinated, grew, and after starvation formed common aggregates. It was observed that during the pseudoplasmodium (slug) stage amoebae of different species segregated and formed separate pure fruiting bodies. Similar experiments with spores of *P. violaceum* in a binary mix with either *D. discoideum*, *D. mucoroides* or *D. purpureum* showed no co-aggregation or chimaerism between *Polysphondylium violaceum* and *Dictyostelium* species. In another experiment by Raper and Thom (1941) portions of slugs from any two CSM species (from *D. discoideum*, *D. mucoroides*, *D. purpureum* and *P. violaceum*) were exchanged by grafting. It was observed that cells of two CSM species did not mix; instead they formed separate fruiting bodies. When amoebae of the two species were allowed to form slugs independently, and then those slugs were mechanically crushed together and permitted to reform, *D. discoideum* and *D. purpureum* amoebae formed chimaeric fruiting bodies; a similar experiment with *D. discoideum* and *P. violaceum* showed no such chimaera formation (Raper and Thom, 1941). Bonner and Adams (1958) expanded our understanding on inter-species interactions by showing that amoebae of different CSM species in a mixture segregate from each other during development. Segregation involved emergence of separate fruiting bodies of two species from the same aggregate, formation of double-decker fruiting bodies (one on top of another) or segregation of spores of two species within the same spore mass. Nicol and Garrod (1978) took a different approach to study the compatibility of CSM species. In their study amoebae of any

two CSM species (from *D. discoideum*, *D. purpureum*, *D. mucoroides* and *P. violaceum*) were mixed and suspended in phosphate buffer, and as the amoebae were continuously shaken, their cohesive properties were analysed. Amoebae of all the CSM species used could clump (stick) together with the exception of *P. pallidum* which showed only a weak cohesiveness to *Dictyostelium* cells and almost complete sorting-out in a chimaeric clump (Nicol and Garrod, 1978). Jack et.al (2008) showed that *D. discoideum* and *D. purpureum* can mix together and form chimaeric fruiting bodies.

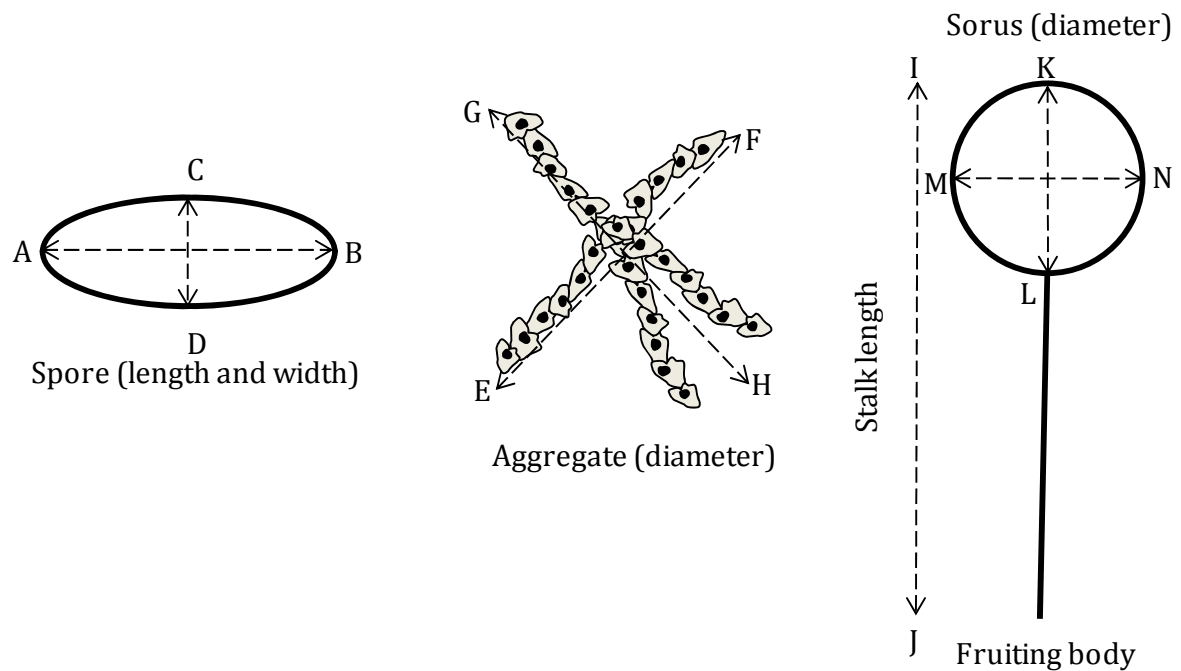
**(B) This study:** I have examined the propensity of *Dictyostelium giganteum* and *D. purpureum* to co-aggregate and the composition of the cells in chimaeric fruiting bodies. This study differs from earlier inter-species mix studies with respect to many points. The two CSM species used in this study are a new pair. They were found to live together under natural conditions (Sathe et al., 2010 and chapter 2) and have similar life cycles. Their ability to form chimaeras was checked under two different scenarios, by co-inoculation of spores with bacteria and by co-developing freshly starved amoebae. I have analysed individual cells within fruiting bodies to assess chimaera formation. This approach is different from the one used in earlier studies where fruiting body morphology was the only criterion used to assess chimaerism something which could be misleading because fruiting bodies displaying weak chimaerism are likely to be ignored. Finally, widely separated and co-occurring strains were used to see if spatial proximity plays a role in the degree of mixing.

For the purpose of this study, chimaerism is defined as the presence of cells from both species in one fruiting body, either in the stalk, spore-mass or both. Even if the stalk of a fruiting body is composed of cells from one species and the spore mass of the other, it is classified as chimaeric. Physical segregation or sorting out is a process in which amoebae of two species aggregate together and later bifurcate into two different fruiting bodies, each made up of only one species. Similarly, if individuals of two species occupy different regions in a fruiting body (within a stalk, spore-mass or both) it too is classified as sorting-out within a fruiting body.

### 4.3 Materials and methods

**4.3.1 *Dictyostelium* strains:** The inter-species mixes were carried out on natural isolates of *Dictyostelium giganteum* and *D. purpureum*. *D. giganteum* strain 8 and *D. purpureum* strains 3 and 5.2 were found on the same spotted deer dung pellet in the Mudumalai forest; *D. giganteum* strains F5 and F16 were isolated from elephant dung ~10 kms away from the spotted deer dung sample (Sathe et al., 2010 and chapter 2). For convenience, *Dictyostelium giganteum* and *Dictyostelium purpureum* are abbreviated as Dg and Dp respectively. Strains were characterized with respect to their morphological and genetic distinctiveness, spore germination rates, doubling times during exponential growth and post-starvation development tempo.

**4.3.2 Morphological and genetic distinctiveness:** Identification of *Dictyostelium* species was carried out using the methods described earlier (chapter 2). Genetic distinctiveness was established on the basis of RAPD polymorphism (for RAPD methods, see chapter 2). Morphological features such as aggregate and sorus diameter, spore size (length and width) and stalk length were measured from photographs taken using appropriate objective lenses (10X or 60X) in a light microscope (Leica). Spore length and width were measured as shown by arrows A–B and C–D respectively in Fig 4.1. The longest diameter of loose aggregates was estimated at different orientations, for example, lengths E–F and G–H in Fig. 4.1 and the result was expressed as the mean of the two diameters. For the stalk length, fruiting bodies developed on agar were photographed and the distance from the top to the bottom of fruiting bodies was estimated as indicated by length I–J in Fig. 4.1. Sorus diameter was measured along two different directions (vertical length K–L and horizontal length M–N in Fig. 4.1) and the means calculated. Here, I wish to thank Neha Khetan (a visiting student from Rashtreeya Vidyalaya College of Engineering, Bangalore) for her help in recording morphological features of *Dictyostelium* and performing some experiments mentioned below.



**Fig 4.1: Morphological features of *Dictyostelium* strains monitored in this study.** For description, see text above. Pictures are not to scale.

**4.3.3 Spore germination:** The method of Cotter and Raper (1966) with suitable modifications was used to compare spore germination rates. Spores of all the five *Dictyostelium* strains were collected independently by harvesting 3–4 day old sori with sterile tips. Spores were suspended in 2 ml  $\text{KK}_2$  buffer ( $\text{KH}_2\text{PO}_4$  2.25 g,  $\text{K}_2\text{HPO}_4$  0.67 g,  $\text{H}_2\text{O}$  1000 ml, pH 6.4) and mixed thoroughly by vortexing for 2–3 min. This spore suspension was then centrifuged at 300g for 3 min at room temperature. The supernatant was discarded and the spores were resuspended in 2 ml  $\text{KK}_2$  buffer. The spore suspension was once again centrifuged and the pellet was resuspended in 2 ml  $\text{KK}_2$  buffer. Spores were appropriately diluted in  $\text{KK}_2$  buffer and counted using a haemocytometer and inoculated at a density of  $5 \times 10^4$  spores/ml in 250 ml sterile flasks containing 50 ml 1% peptone. After spores were inoculated in 1% peptone the spore counts were repeated. The flasks were shaken in a reciprocal shaker at 150 strokes/min at 22°C for 12 hrs. Samples were collected from the flasks and the number of emerged amoebae (as a proxy for germinated spores) and ungerminated spores were counted using a haemocytometer. The results of spore germination were computed as: spore germination = total number of amoebae counted  $\div$  (total



number of amoebae + ungerminated spores). Ideally, spore germinations of wild isolates of *Dictyostelium* should have been monitored in the presence of bacteria on either solid agar media or soil, but the method used here was less tedious and since spores of all the strains could be germinated in the same environment, it was possible to compare germination rates across the strains.

**4.3.4 Doubling times and developmental rates:** Doubling times during exponential growth (in the presence of *K. aerogenes* on agar plates), post-starvation developmental rates, total number of fruiting bodies and total number of spores formed were measured as described in chapter 3.

Media, chemicals, growth and development conditions were as before (chapters 2 and 3).

**4.3.5 Inter-species interactions:** Inter-species interactions were studied under two different conditions: (1) spores of the two CSM species along with bacteria were inoculated together; (2) freshly starved amoebae were mixed and allowed to co-develop. The protocol was as follows.

**(1) Mixing spores of Dg and Dp:** The method of Olive (1902) and Raper and Thom (1941) was modified. It involved mixing spores of two strains, one each of Dp and Dg, in a 1:1 ratio and allowing them to germinate, grow and develop together. Totally six combinations were analysed: Dp 3 + Dg 8, Dp 3 + Dg F5, Dp 3 + Dg F16, Dp 5.2 + Dg 8, Dp 5.2 + Dg F5 and Dp 5.2 + Dg F16. Spores of each of these strains were harvested independently from fruiting bodies that had developed on SM agar and suspended in separate Eppendorf tubes containing 2 ml  $\text{KK}_2$  buffer. Spores were washed thrice with  $\text{KK}_2$  buffer (the method for harvesting and washing spores has been described in the spore germination section above). Spores were counted using a haemocytometer and spread uniformly at a density of 500 spores/cm<sup>2</sup> (i.e. 250 spores of each strain) along with a 400  $\mu\text{l}$  suspension of *K. aerogenes* on  $\text{KK}_2$  agar plates (20cm<sup>2</sup>). The plates were incubated in the dark at 22°C until fruiting bodies had developed, which typically took 4–6 days.

Fruiting bodies were classified under two broad categories by eye: *D. purpureum*-type (Dp-type) and *D. giganteum*-type (Dg-type). These were identified based on morphological criteria. The Dp-type had a purple colored spore mass and the Dg-type was white; other characteristics reinforced the distinction based on colour. These were bigger aggregates, a longer duration of slug migration, and a relatively long and thick stalk in the case of the Dp-type and comparatively smaller aggregates, a smaller duration of slug migration with a short and thin stalk for the Dg-type. There was no situation in which a fruiting body appeared ambiguous and so difficult to type. From each plate ~10 spore-masses (5 each from Dg-type and Dp-type fruiting bodies) were picked individually using sterile micropipette tips, and suspended in 100  $\mu$ l KK<sub>2</sub> buffer. Spores were diluted appropriately in KK<sub>2</sub> buffer and plated on KK<sub>2</sub> agar plates along with *K. aerogenes* to get well isolated plaques. After the plaques had formed they were transferred to fresh KK<sub>2</sub> agar plates (1 plaque/plate) or on to a 30cm wide x 40cm long plastic tray containing KK<sub>2</sub> agar (approximately 200 plaques, well separated from each other) and allowed to form fruiting bodies in the dark at 22°C and their phenotypes noted.

**(2) Mixing freshly starved amoebae of Dg and Dp:** Amoebae of *Dictyostelium* strains were grown independently on SM medium with *K. aerogenes* as a food source. After 34–36 hrs, amoebae were harvested from the growth medium (SM agar), suspended in cold KK<sub>2</sub> buffer and stained with cell tracker blue (methods for harvesting amoebae and staining are described in chapter 3). Amoebae of two species (one of them stained) were mixed in a 1:1 ratio and spread evenly on 2% KK<sub>2</sub> agar plates at a density of  $1 \times 10^5$  amoebae/cm<sup>2</sup>. Plates were incubated in the dark at 22°C. Mixing experiments were performed on KK<sub>2</sub> agar plates and not on a natural substrate such as soil. The following features were monitored in each experiment (independently for Dg, Dp and a 1:1 mix of the two): (a) post-starvation development; (b) total fruiting bodies and total spores formed; (c) the relative proportions of spores belonging to the two species in chimaeric fruiting bodies; (d) frequency of chimaerism and physical segregation of amoebae of the two species in a mix.

In another set of experiments, freshly starved amoebae of two strains of the same species (*D. giganteum* or *D. purpureum*) were mixed in the same way as mentioned above. The combinations used were: Dg 8 + Dg F5, Dg 8 + Dg F16, Dg F5 + Dg F16 and Dp 3 + Dp 5.2. The purpose of carrying out these inter-strain mixes was to see whether the strains used in this study mixed freely with each other any trends could be observed on comparing mixes involving cells of different strains vis-a'-vis cells of different species.

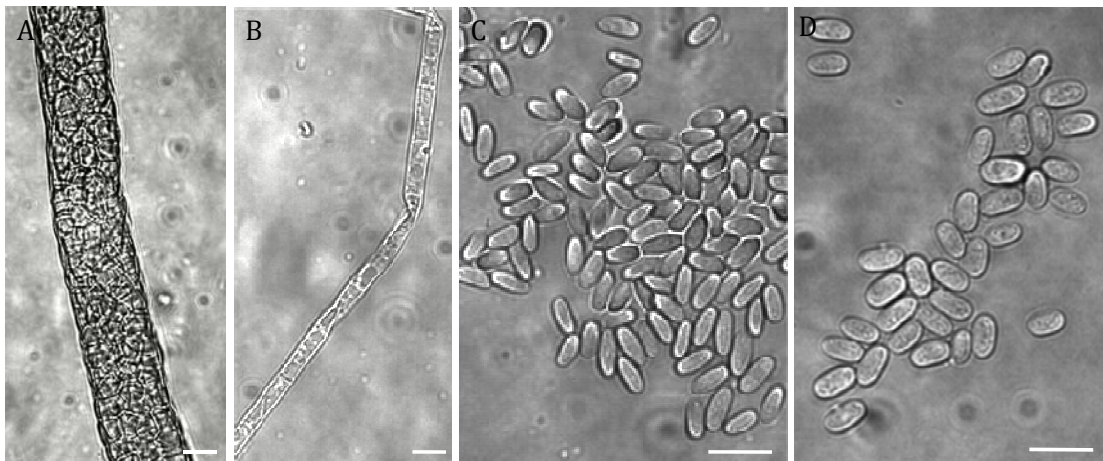
Chimaerism and sorting-out in multicellular structures were monitored in individual fruiting bodies. Dg-type and Dp-type fruiting bodies formed in each mix were picked randomly with a needle and transferred to a water drop on a glass slide. Coverslips were fixed over these preparations and observed under a 60X objective lens in a fluorescence microscope (Leica) with appropriate filters. Stained and unstained spores were counted manually and relative proportions of spores from each species were calculated. Since the amoebae were suitably stained before mixing it was possible to distinguish between the cells that went into chimaeric fruiting bodies. In addition, the stalk and spore cells of Dg and Dp differ sufficiently to make it possible to tell them apart (Fig. 4.2). Frequencies of chimaerism were calculated separately for Dg-type and Dp-type fruiting bodies using the formula: chimaerism in Dg-type = number of fruiting bodies with both Dg and Dp cells ÷ total Dg-type fruiting bodies analysed. Likewise, chimaerism in Dp-type = number of fruiting bodies with both Dg and Dp cells ÷ total Dp-type fruiting bodies analysed. The relative contribution of Dg cells and Dp cells within chimaeric fruiting bodies was also recorded.

## 4.4 Results

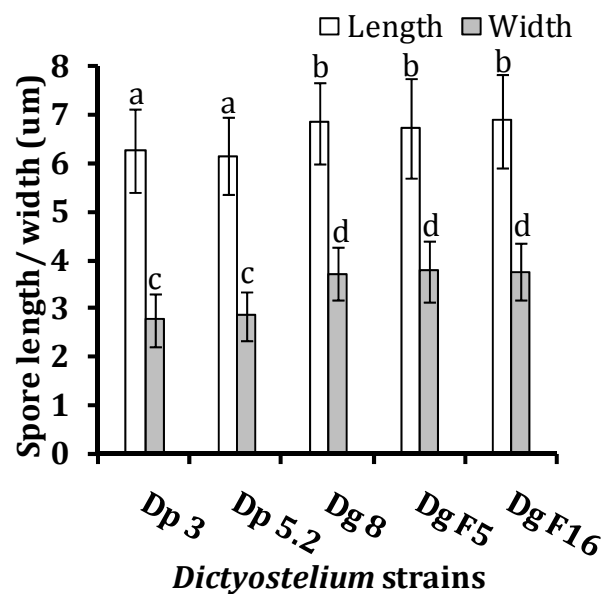
**4.4.1 Morphological and genetic distinctiveness:** The five strains used in this study belonged to two CSM species: *Dictyostelium giganteum* and *D. purpureum* (detailed characterizations shown in Table 2.2, chapter 2). The two strains of Dp used were genetically different, as were the three strains of Dg (see Table 2.6 and Fig. 2.12 in chapter 2). Other morphological characteristics of Dp and Dg were as follows (Table 4.1). Dp aggregates were large (Fig. 4.5A), slugs showed stalked migration and were highly phototactic, fruiting bodies were unbranched, and usually developed a relatively long and thick stalk (Fig. 4.2A). Sori were spherical and developed a dark purple color after 2–3 days of development (Fig. 4.5C). Spores were ellipsoid in shape (Figs. 4.2C, 4.3). Dg aggregates were smaller than Dp aggregates (Fig. 4.5D). Dg fruiting bodies were solitary, unbranched, had a thin stalk (Fig. 4.2B) and a white-colored, spherical spore mass with ellipsoid spores (Fig. 4.2D, Fig. 4.3). Slugs were phototactic and showed aerial migration. The morphological characters of *D. purpureum* and *D. giganteum* described here and earlier by Raper (1984) are more-or-less similar. The differences in sorus colour, spore width, stalk thickness and length of Dg and Dp fruiting bodies were used as phenotypic markers to differentiate two species in a mix (though never any one by itself). There were no significant differences with respect to morphological characters monitored within the two Dp strains (Dp 3 and Dp 5.2) and the three Dg strains (Dg 8, Dg F5 and Dg F16).

Table 4.1: Morphological characters of *D. purpureum* and *D. giganteum*.

Morphological character	<i>D. purpureum</i> 3	<i>D. giganteum</i> 8
Aggregate size (diameter; means $\pm$ sd)	3.9 $\pm$ 1.1 mm; n = 10 aggregates	0.7 $\pm$ 0.1 mm; n = 8 aggregates
Stalk size (range)	3.1 – 15.2 mm long, 15.0 – 25.3 $\mu$ m thick; n = 7 stalks	0.5 – 1.5 mm long, 4.0 – 7.1 $\mu$ m thick; n = 10 stalks
Sorus diameter (means $\pm$ sd)	87.1 $\pm$ 27.0 $\mu$ m, n = 40 sori	41.7 $\pm$ 12.6 $\mu$ m, n = 36 sori
Spore size (means $\pm$ sd)	6.1 $\pm$ 0.9 $\mu$ m long and 2.7 $\pm$ 0.6 $\mu$ m wide; n = 160 spores	6.8 $\pm$ 0.7 $\mu$ m long and 3.7 $\pm$ 0.6 $\mu$ m wide, n = 150 spores

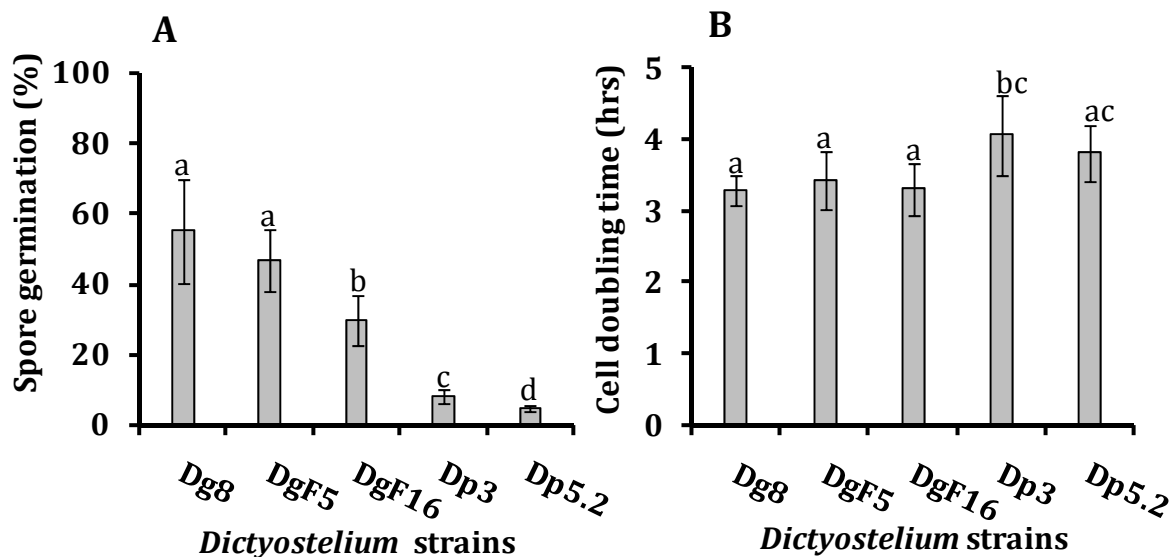


**Figure 4.2: Morphology of the stalk and spore cells in *Dictyostelium*.** Dp formed a thicker stalk (A) than Dg (B). Spores of Dp were shorter and narrower (C) than the spores of Dg (D), for the quantitative data on spore dimensions see Fig. 4.3. Scale bars represent 10  $\mu\text{m}$ .



**Fig. 4.3: Spore length and width in Dp and Dg strains:** Different letters above the bars indicate the differences being statistically significant (for example, 'a' indicates significant difference from 'b'; non-significant differences are indicated by common letters). Significance was assessed by a t-test assuming unequal variances. Dp and Dg indicates *D. purpureum* and *D. giganteum* respectively. The spore length and width (mean  $\pm$  sd) were measured from 190–195 spores from each of the *Dictyostelium* strains. Spores of Dg strains (8, F5 and F16) were significantly longer and wider than the spores of Dp strains (3 and 5.2);  $p < 0.05$ . The differences in spore length and width *within* the Dg strains or Dp strains were not significant ( $p > 0.05$ ).

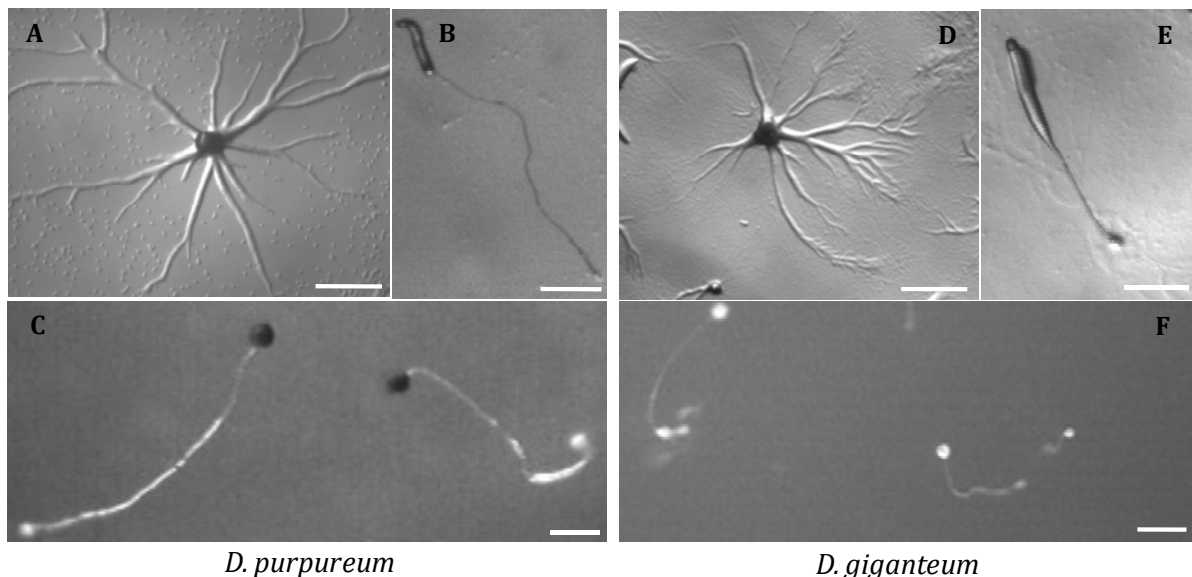
**4.4.2 Spore germination and growth rates:** Spore germination rates were assessed in 1% peptone and growth rates (doubling times during exponential phase) were measured on solid agar plates in the presence of *K. aerogenes*. Spores of all the Dg strains (Dg 8, Dg F5 and Dg F16) germinated significantly faster than the spores of Dp strains (Dp 3 and Dp 5.2). Among Dg strains, Dg F16 spores germinated significantly slower than the spores of Dg F5 and Dg 8; no significant difference between Dg F5 and Dg 8 was observed. Among Dp strains, Dp 3 spores germinated significantly faster than Dp 5.2 spores. (Fig. 4.4A). The doubling times of Dp and Dg strains were similar, with the exception of Dp 3 which was significantly smaller than all Dg strains but comparable with Dp 5.2 (Fig. 4.4 B).



**Fig. 4.4: Spore germination (A) and growth rates (B) of *Dictyostelium* strains.** Different letters above the bars indicate the differences being statistically significant (for example, 'a' indicates significant difference from 'b'; 'a' is comparable with 'ac' but different than 'bc'). Results are means  $\pm$  sd. The number of independent spore germination experiments (n) = 5 each for Dg 8, Dg F5, Dg F16 and 4 each for Dp 3 and Dp 5.2. Similarly, the number of growth experiments (n) = 7 each for Dp 3, Dg F5, Dg F16 and 3 each for Dp 5.2 and Dg 8. Statistical significance was assessed by a t-test assuming unequal variances, p values less than 0.05 were considered statistically significant. The growth rate data shown here for Dg F5 and Dg F16 has also been used in chapter 3 in a different context.

**4.4.3 Post-starvation development:** The time taken by freshly starved amoebae to complete the formation of fruiting bodies was monitored on KK<sub>2</sub> agar plates or moist filter papers and was done for a single strain or a 1:1 mix of two strains or two species.

**(A) Development of *D. purpureum* (Dp).** Freshly starved Dp amoebae formed fruiting bodies in 24–26 hrs (Fig. 4.5 A–C); there was no significant difference between the development rate of Dp 3 and Dp 5.2. Aggregation was visible after 10–12 hrs of plating, and migrating slugs had formed after 18–20 hrs. Following a brief period of migration, long, semi-erect, solitary fruiting bodies were seen at 24–26 hrs.

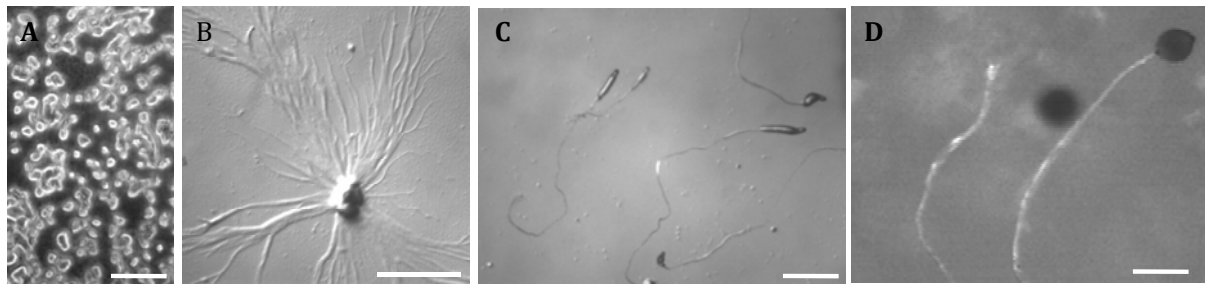


**Figure 4.5: Asexual life cycle of Dp 3 (A–C) and Dg 8 (D–F).** Aggregates (A and D); slugs (B and E) and fruiting bodies (C and F). Scale bars represent 250  $\mu$ m).

**(B) Development of *D. giganteum* (Dg).** All Dg strains (Dg F5, Dg F16 and Dg 8) developed at similar rates and all of them were significantly faster than the Dp strains; fruiting body formation was complete in 14–16 hrs. The developmental rate of Dg F5 and Dg F16 has been described earlier (see Fig. 3.6 in chapter 3). In the case of Dg 8; aggregates were visible after ~4 hrs of plating, after ~8 hrs migrating slugs were spotted and after ~14 hrs fruiting bodies were formed (Fig. 4.5 D–F).

**(C) Development of a mix of different strains of *D. purpureum* (Fig. 4.6 A–D).**

Freshly starved amoebae of Dp 3 and Dp 5.2 were mixed in a 1:1 ratio and developed together. A mix of the two developed like the individual strains by themselves; a clear sign of aggregation with long streams of amoebae was visible after 10–12 hrs of plating and migrating slugs with long stalks were observed after 18–20 hrs. Fruiting-body formation was complete after 24–26 hrs of plating. There were no obvious differences between the development of Dp 3, Dp 5.2 and a 1:1 mix of the two. Although both the strains in a mix developed synchronously, in ~ 40% fruiting bodies all the cells exclusively belonged to only one strain indicating physical segregation of the two strains in a mix.



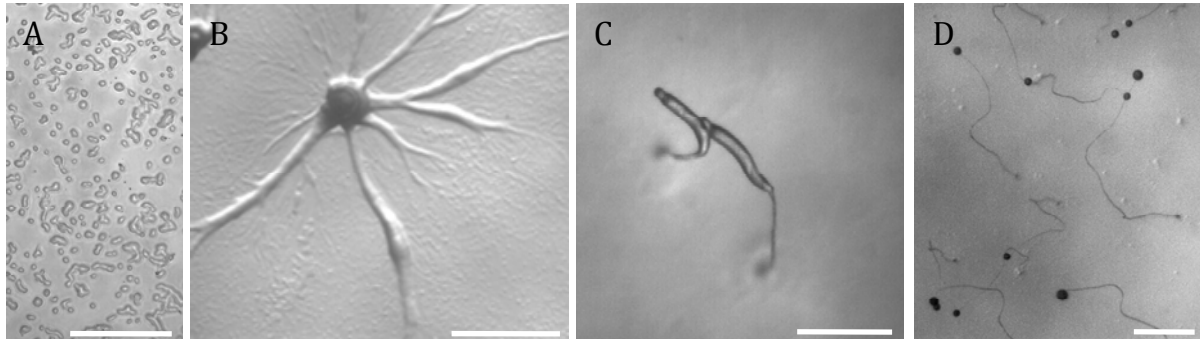
**Figure 4.6: Development of a mix of Dp 3 + Dp 5.2.** (A) Amoebae; (B) Aggregate; (C) Migrating slugs; (D) Fruiting bodies. Scale bars represent 50  $\mu\text{m}$  (A) or 250  $\mu\text{m}$  (B–D).

**(D) Development of a mix of different strains of *D. giganteum* (Dg).**

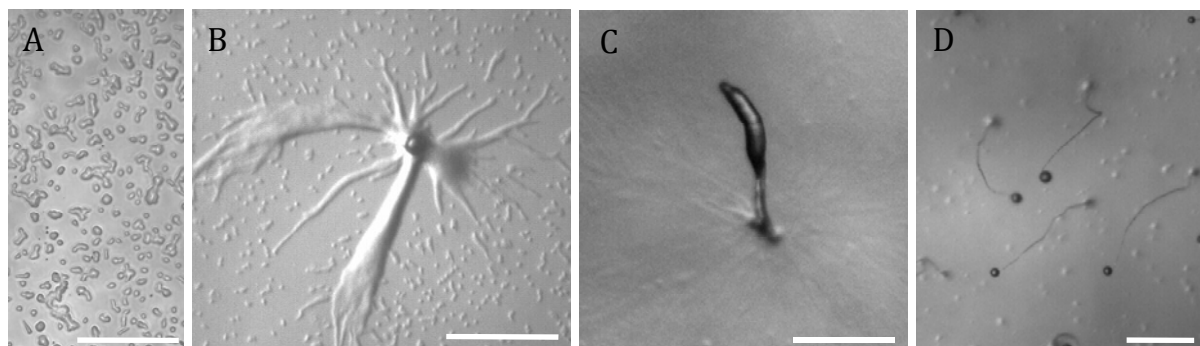
Freshly starved amoebae of different strains of Dg (Dg 8, Dg F5 and Dg F16) were mixed in a 1:1 ratio and developed together. Three combinations (Dg 8 + Dg F5, Dg 8 + Dg F16 and Dg F5 + Dg F16) were studied. The developmental rate of Dg F5, Dg F16 and a 1:1 mix of the two was similar; aggregation was visible after 3–4 hrs of plating, slugs were formed after 7–8 hrs and by 12–14 hrs fruiting body formation was over (see Fig. 3.6 in chapter 3). Developmental rate of a mix of Dg 8 + Dg F5 and Dg 8 + Dg F16 (Figs. 4.7 and 4.8) was similar and the mixtures developed like the individual members. When freshly starved amoebae of the two strains were mixed homogeneously and plated together they started aggregating after ~ 4 hrs and showed tight aggregates after 5–6 hrs (Fig. 4.7B and Fig. 4.8B). The aggregation



process was completed 2–3 hrs later and after ~ 10hrs, migrating slugs were seen. After ~ 14 hrs, fruiting bodies were observed.



**Figure 4.7: Development of a mix of Dg 8 + Dg F5.** (A) Amoebae; (B) Aggregate; (C) Slugs; (D) Fruiting bodies. Scale bars represent 50  $\mu\text{m}$  (A) or 250  $\mu\text{m}$  (B–D).



**Figure 4.8: Development of a mix of Dg 8 + Dg F16.** (A) Amoebae; (B) Aggregate; (C) Slugs; (D) Fruiting bodies. Scale bars represent 50  $\mu\text{m}$  (A) or 250  $\mu\text{m}$  (B–D).

**(E) Development of a mix of cells from different species (Dp and Dg):**

Development involving cells from two species was studied under two different conditions. In the first case, spores of DP and Dg were mixed along with bacteria, grown and developed together. In the second case, freshly starved amoebae of Dg and Dp were mixed and their development was followed.

**(F) Development of a mix of Dp and Dg after their spores were mixed:**

Dp and Dg spores, which were mixed and spread uniformly on non-nutrient agar along with *K. aerogenes*, germinated and the emerged amoebae grew together. After the food was depleted, starved amoebae aggregated and formed fruiting bodies. Two types of fruiting bodies were visible (typing based on morphology, see methods): Dp-type

(purple sori) and Dg-type (white sori). The relative numbers of fruiting bodies of each type were different and varied depending on the strains in a mix (Table 4.2). Overall, Dg-type fruiting bodies were more abundant than Dp-types. In two mixes (viz. a mix of Dp 3 + Dg 8 and Dp 5.2 + Dg 8) Dp-type fruiting bodies were almost absent (Table 4.2). In the case of other mixing combinations, Dp-type fruiting bodies were seen developing along with Dg-types (Table 4.2). When spores were mixed, the development was asynchronous within the plates, such that the tempo of development and sorting-out of cells of the two species from aggregates (if any) could not be monitored in these experiments. However, the corresponding observations were made in detail when amoebae of the two species were mixed (see below). Inter-strain mixtures (mixing of spores of different strains from the same species) were not studied.

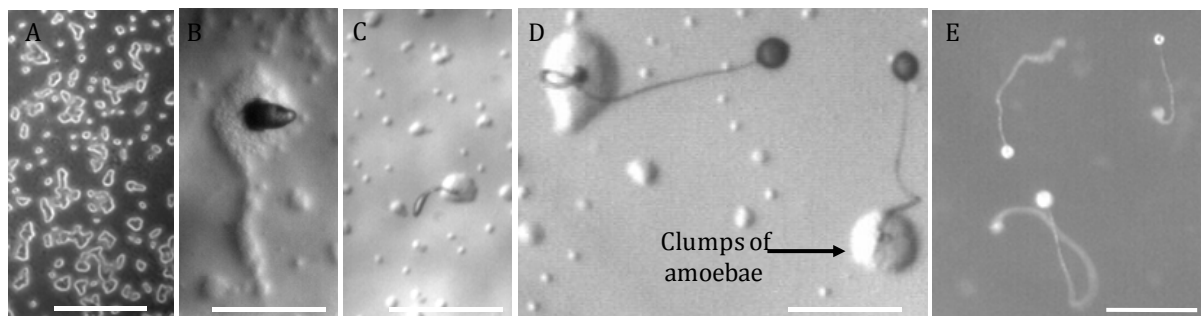
**(G) Development of a mix of Dp and Dg after their amoebae were mixed:**

Freshly starved amoebae of Dp and Dg were mixed in a 1:1 ratio and developed together. The development of a mix was considerably different from that of amoebae of the individual species and depended on the strains involved.

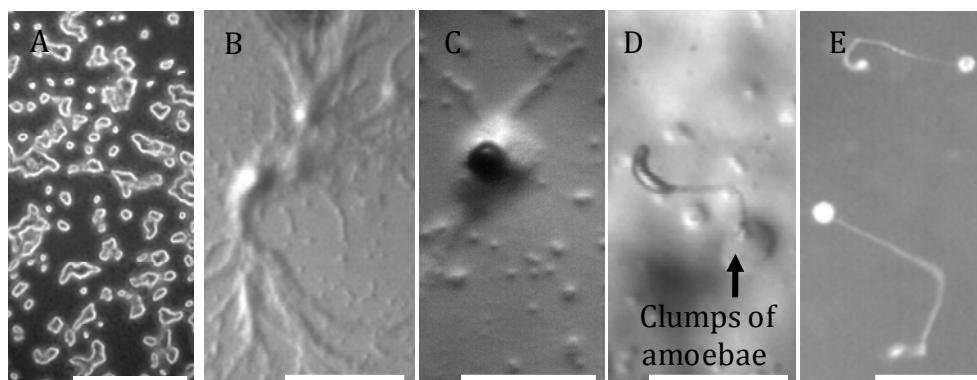
**(H) Dp 3 and Dg 8 (strains found on the same dung pellet):** After 4–5 hrs of plating, cells of both the species began to aggregate together. From these aggregates, after 7–8 hrs, relatively small slugs had emerged, which after 14–16 hrs formed Dg-type fruiting bodies as was confirmed later (Fig. 4.9). At the base of these tiny fruiting bodies, large clumps of amoebae were seen (Fig. 4.9 C–D). After ~ 30 hrs, some of these had become migrating slugs that later differentiated into Dp-type fruiting bodies. Finally, two types of fruiting bodies (Dp-type and Dg-type) had formed; Dp-types were significantly fewer in number than Dg-types (Fig. 4.9 D and E; Table 4.3).

**(I) Dp 5.2 and Dg 8 (strains found on the same dung pellet):** The development of this mixture was similar to the development of a mix of Dp 3 + Dg 8. Freshly starved amoebae of the two species aggregated after 4–5 hrs of plating (Fig. 4.10B) and formed mounds after 7–8 hrs (Fig. 4.10C); from these mounds after 12–14 hrs tiny slugs (Dg-type) had developed. At the base of these slugs large clumps of amoebae

were seen (Fig. 4.10D), some of these clumps later developed into Dp-type fruiting bodies. Once again, Dg-type fruiting bodies were significantly more in number than Dp-types (Fig. 4.10E; Table 4.3).



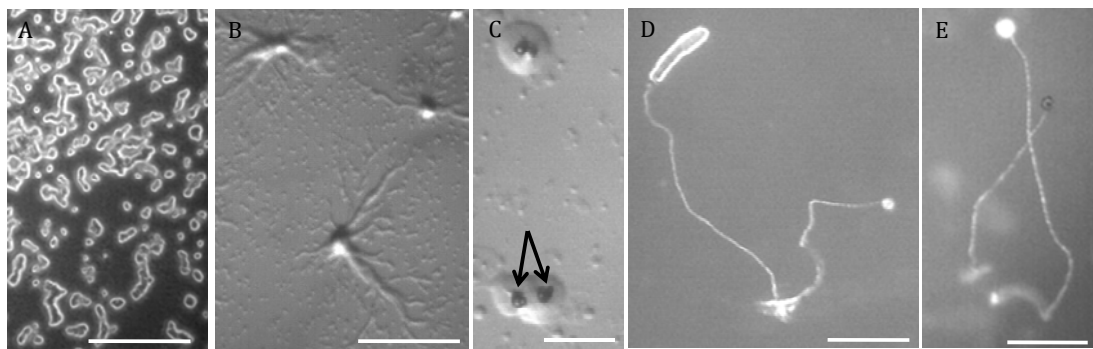
**Figure 4.9: Development of a mix of Dp 3 and Dg 8.** (A) Freshly starved amoebae; (B) Mound; (C) Dg-type slug; (D) Dg-type fruiting bodies and clumps of amoebae at the base of fruiting bodies; (E) Dg-type fruiting bodies (Dp-type fruiting bodies were rarely seen). Scale bars represent 50  $\mu\text{m}$  (A) or 250  $\mu\text{m}$  (B–E).



**Figure 4.10: Development of a mix of Dp 5.2 and Dg 8.** (A) Amoebae; (B) Aggregate; (C) Tipped mound and clumps of amoebae at the base; (D) Stalked-slug of Dg-type and a clump of amoebae at the base (E) Dg-type fruiting bodies. Scale bars represent 50  $\mu\text{m}$  (A) or 250  $\mu\text{m}$  (B–E).

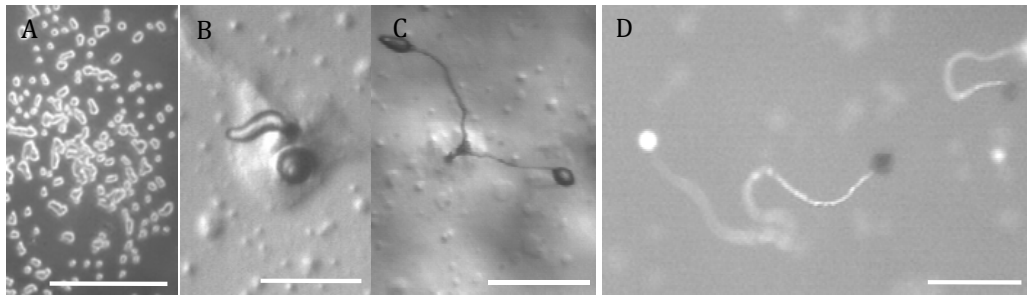
**(J) Dp 3 and Dg F5 (strains found ~ 10 kms away from each other).** Unlike the mix of Dp 3 + Dg 8 or Dp 5.2 + Dg 8 the development of a mix of Dp 3 + Dg F5 was synchronous. That is, the two strains developed together and at roughly the same rate, which was that of the faster member, namely, Dg F5. After 5–6 hrs, loose aggregates were visible which later formed tight aggregates (Fig. 4.11B). A very different behavioural outcome was visible thence: from the same aggregate two separate tips emerged (Fig. 4.11C). After 14–16hrs, both tips had initiated separate

fruiting bodies, one of them of the Dg-type and the other of the Dp-type (Fig. 4.11D). Dp 3 by itself develops to the fruiting body stage within 24–26hrs, but here, in combination with Dg F5, it developed within 16–18 hrs (~8 hrs earlier). The development of Dg F5 amoebae, in the presence of Dp 3, was delayed by 3–4 hrs. Overall, the numbers of Dp-type fruiting bodies were relatively more than those of the Dg-type which is the opposite of what was seen in the Dp 3 + Dg 8 and Dp 5.2 + Dg 8 mixes (Table 4.3).



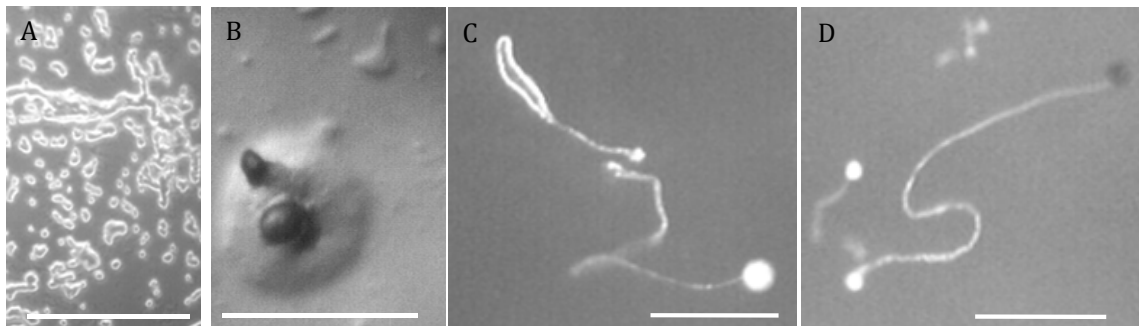
**Figure 4.11: Development of a mix of Dp 3 and Dg F5.** (A) Amoebae; (B) Aggregates; (C) Bifurcated tipped-mounds; (D) Dp-type slug and Dg-type fruiting body coming out from the same aggregate; (E) Fruiting bodies of Dg-type (white sori) and Dp-type (purple sori). Scale bars represent 50  $\mu\text{m}$  (A) or 250  $\mu\text{m}$  (B–E).

**(K) Dp 5.2 and Dg F5 (strains found ~ 10 kms from each other):** Overall, the development of this mixture was like the development of a mix containing cells of Dp 3 and Dg F5. Amoebae of these species developed synchronously, with aggregations being visible by 5–6 hrs and mounds seen after 8–10 hrs. From these mounds, two separate tips emerged, one of which formed the Dg-type fruiting body and the other differentiated into the Dp-type fruiting body (Fig. 4.12 B–D). Dp 5.2 in the presence of Dg F5 formed fruiting bodies 6–8 hrs earlier than by itself. The majority of fruiting bodies were found to be of the Dp-type (Table 4.3).



**Figure 4.12: Development of a mix of Dp 5.2 and Dg F5.** (A) Amoebae; (B) Tipped mounds showing bifurcation of two species; (C) Two culminates (Dg-type and Dp-type) bifurcated from the same aggregate and (D) Fruiting bodies of Dg-type (white sori) and Dp-type (purple sori). Scale bars represent 50  $\mu\text{m}$  (A) or 250  $\mu\text{m}$  (B–D).

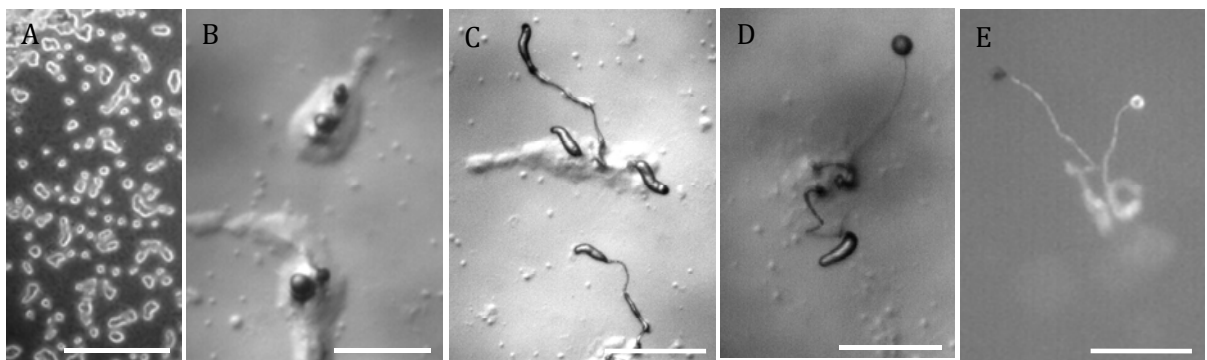
**(L) Dp 3 and Dg F16 (strains found ~ 10 kms away from each other).** Amoebae of these two started aggregating together within 5–6 hrs of plating and formed mounds after 7–8 hrs. Two different tips emerged from the same mound and each of them differentiated into either a Dg-type or a Dp-type fruiting body (Fig. 4.13B and C). This kind of segregation of amoebae of the two species was more prominent (the frequency of bifurcations was high; Table 4.3) in this mix than in the mix of Dp 3 and Dg F5. Finally, both Dp-type and Dg-type fruiting bodies were seen; Dg-types were slightly more frequent than Dp-types (Fig. 4.13D).



**Figure 4.13: Development of a mix of Dp 3 and Dg F16.** (A) Amoebae; (B) Bifurcated tipped-mounds; (C) Dg-type fruiting body and Dp-type slug developing from the same mound; (D) Bifurcated fruiting bodies: Dg-type (white sorus) and Dp-type (purple sorus). Scale bars represent 50  $\mu\text{m}$  (A) or 250  $\mu\text{m}$  (B–D).

**(M) Development of Dp 5.2 and Dg F16 (found ~ 10 kms away from each other).** Dp 5.2 and Dg F16 amoebae developed synchronously and at the rate of the faster member (Dg F16). After ~ 6hrs of plating, amoebae started aggregating and

formed mounds within 8–9 hrs of plating. From each mound two tips emerged with one forming a Dg-type fruiting body and the other developing into a Dp-type. Occasionally three tips emerged from the same mound, with one forming a Dp-type fruiting body and the other two forming Dg-type fruiting bodies. Both types of fruiting bodies were formed within 18 hrs of plating. This means that development of Dp 5.2, in the presence of Dg F16, was accelerated by ~ 8hrs. At the end of development, both Dp-type and Dg-type fruiting bodies were seen, with slightly higher numbers of Dg-type fruiting bodies than Dp-types (Fig. 4.14E).



**Figure 4.14: Development of a mix of Dp 5.2 and Dg F16.** (A) Amoebae; (B) Mounds with two tips; (C) Tri-furcation; three slugs, two Dg-type and one Dp-type, emerged from the same mound; (D) Bifurcated culminates; (E) Fruiting bodies typical of Dg and Dp. Scale bars represent 50  $\mu\text{m}$  (A) or 250  $\mu\text{m}$  (B–E).

**Summary:** A brief summary of development of *D. purpureum*, *D. giganteum*, inter-strain and inter-species mixes is as follows: the Dp strains (Dp 3 and Dp 5.2) developed significantly slower than the Dg strains (Dg 8, Dg F5 and Dg F16). There were no obvious differences in the tempo of development when different strains of Dg or Dp were made to develop in 1:1 pair wise mixes. It was confirmed that the strains in a mix co-aggregated, although the degree of mixing (% chimaerism) was below 100% in some pairs (see below). There were significant differences in the tempo of development when mixtures were formed by cells of different species. Dp-type fruiting bodies formed from a mix of Dp 3 + Dg 8 or Dp 5.2 + Dg 8 were exceptionally slow developing as they took ~15 hrs long to appear. No clear bifurcation of Dg-type and Dp-type was observed and a large majority of fruiting bodies were Dg-type. The course of development was changed in mixes involving Dp

strains 3 or 5.2 with Dg strains F5 or F16; in these mixtures Dp-type fruiting bodies developed faster than Dp strains by themselves and Dg-type fruiting bodies developed slower than Dg strains on their own. When Dp strains 3 or 5.2 were mixed with Dg strains F5 or F16, two clearly distinguishable types of fruiting body were seen, resembling the two species respectively. In these mixes, the number of fruiting bodies of the Dp-type exceeded or was comparable to those of the Dg-type.

**Table 4.2:** Total fruiting bodies formed by Dg and Dp strains after their spores were mixed and inoculated on KK<sub>2</sub> agar plates along with *K. aerogenes*.

Strain (s)	Fruiting bodies /cm <sup>2</sup>	
Dp 3	27.5 ± 9.2*	
Dp 5.2	28.8 ± 10.1*	
Dg 8	95.1 ± 14.7	
Dg F5	62.8 ± 9.8	
Dg F16	65.4 ± 14.2	
	<b>Dg-type</b>	<b>Dp-type</b>
Dp 3 + Dg 8	101.4 ± 16.2	5.6 ± 3.3#
Dp 5.2 + Dg 8	94.2 ± 15.2	2.3 ± 2.0#
Dp 3 + Dg F5	44.8 ± 9.6	45.4 ± 13.9
Dp 5.2 + Dg F5	72.7 ± 14.9	60.5 ± 10.7
Dp 3 + Dg F16	68.3 ± 12.3	29.1 ± 9.3
Dp 5.2 + Dg F16	50.6 ± 7.8	19.9 ± 6.5

“Dp” = *D. purpureum*; “Dg” *D. giganteum*; “+” mixed in equal ratio with; \* Relatively larger fruiting bodies and longer slug migration than Dg strains; “#” Dp-type fruiting bodies were rarely seen. Fruiting bodies were counted in 5 to 6 squares (1cm × 1cm) which were randomly selected from centre of 10 cm plates; those that developed on the boundaries of the plates were avoided. The results are means ± sd from 5 independent experiments, each of these experiments were performed in duplicate.

**Table 4.3:** Fruiting bodies formed in mix of Dg and Dp strains after their amoebae were mixed.

Strains	Fruiting bodies /cm <sup>2</sup>		Fruiting bodies (%)	
	Total	pairs <sup>€</sup>	Dg-type	Dp-type
Dp 3	27.1 ± 9.1	0	0	100
Dp 5.2	31.0 ± 7.2	0	0	100
Dg 8	149.0 ± 40.7	0	100	0
Dp 3+dg 8*	209.7 ± 24.6	0	99	1
Dp 5.2+Dg 8 *	124.5 ± 21.8	0	93	7
Dg F5	90.3 ± 27.1	0	100	0
Dp 3+Dg F5\$	90.1 ± 22.1	39.3 ± 11.0	45	55
Dp 5.2+Dg F5\$	65.3 ± 9.6	>2	20	80
Dg F16	91.2 ± 18.6	0	100	0
Dp 3+dg F16	124.2 ± 32.0	27.8 ± 9.5	60	40
Dp 5.2+dg F16	140.1 ± 37.4	15.6 ± 2.73	70	30

\*' Mainly Dg-type fruiting bodies; '\$' mainly Dp-type fruiting bodies; € indicates a situation where Dg-type and Dp-type fruiting bodies emerged from the same aggregate. Results are mean ± sd; n ≥ 4.

**4.4.4 Chimaerism in inter-species mixtures:** The propensity of Dg and Dp cells to co-aggregate and form mixed fruiting bodies was analysed under two conditions: when spores of the two species were mixed and when freshly starved amoebae of the two were mixed. Six combinations (as mentioned above) were analysed.

Spores of Dp and Dg (which were mixed and spread uniformly on non-nutrient agar along with *K. aerogenes*) co-germinated and the emerged amoebae grew together. After starvation, amoebae of both the species aggregated together and formed two distinct types of fruiting bodies: Dg-type and Dp-type. Freshly starved amoebae of the two species (mixed homogeneously and plated together)



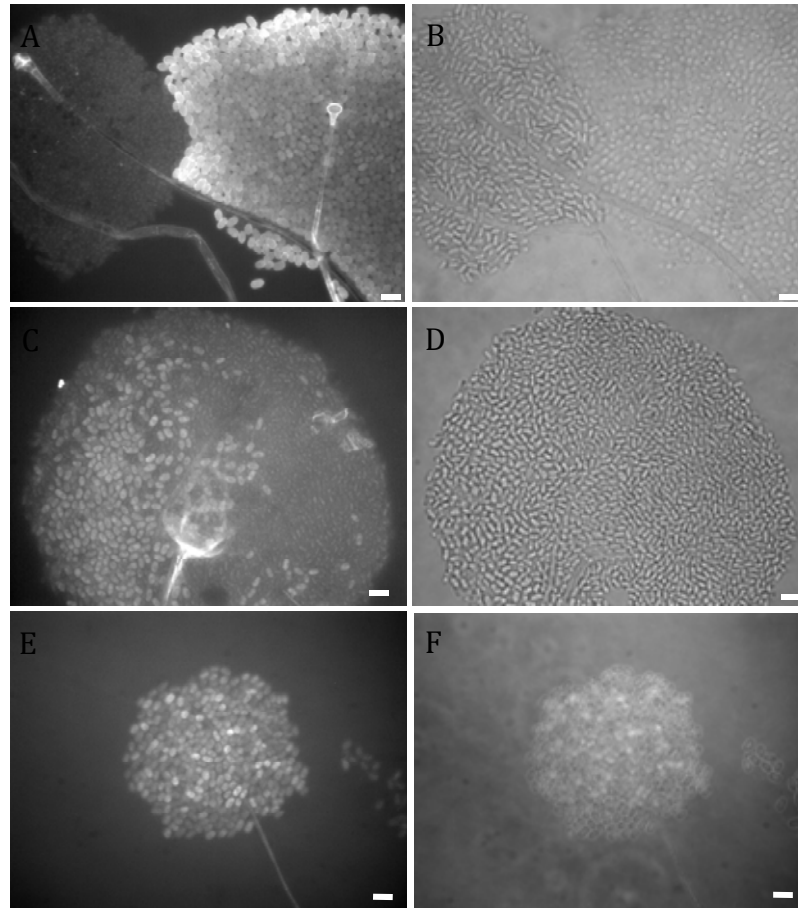
also developed together. Here also, in every mix, two types of fruiting bodies (Dg-type and Dp-type) were seen. The frequency of chimaerism in sori was analysed independently for Dg-type and Dp-type fruiting bodies.

In general, the frequency of mixing (% chimaerism) was dissimilar for the different pairs of Dg + Dp analysed; some pairs mixed relatively freely while others did not mix at all. Even in the same combination of Dg and Dp, different levels of mixing between different fruiting bodies was observed. For example, in a mix of Dp 3 + Dg F16 there were cases where some fruiting bodies were pure (Fig. 4.15 A–B), some were chimaeric, but with cells of the two species sorting-out in different locations within the same spore mass (Fig. 4.15 C–D) and in others, freely mixed cells of the two species were seen, without any sign of segregation (Fig. 4.15 E–F).

It turned out that most Dg-type chimaeric fruiting bodies had significantly more spores and Dp-type chimaeric fruiting bodies had significantly fewer spores, of Dg than of Dp respectively. In every mix, at least a minority of Dp-type fruiting bodies were found to be chimaeric, whereas, mixing combinations in which not even a single Dg-type chimaeric fruiting body was found were seen (this happened when the mixes involved spores of Dp 5.2, Table 4.4). What follows is a brief description of frequency of chimaerism in all the six combinations made using different strains of Dg and Dp.

**(i) Dp 3 and Dg 8:** In this mix, Dg-type fruiting bodies developed well before and in significantly larger numbers than Dp-types. When spores of these two species were mixed, 7.3% of Dg-type and 31.4% of Dp-type fruiting bodies turned out to be chimaeric (i.e., they had both Dg and Dp cells in them, see Table 4.4). When freshly starved amoebae of these two species were mixed, 19.3% of Dg-type and 28.7% of Dp-type fruiting bodies were chimaeric (Table 4.4). Although chimaeric fruiting bodies were formed, mixing was rather poor (i.e., only a minority of spores belonged to Dg in Dp-type fruiting bodies and vice-versa). The numbers were as follows: in Dp-type chimaeric fruiting bodies Dg spores were rare ( $1.5 \pm 3.4\%$  when mixed as spores and  $9.1 \pm 9.8\%$  when mixed as amoebae), similarly in Dg-type fruiting bodies Dp spores were infrequent ( $4.4 \pm 3.1\%$  when mixed as spores and  $3.6 \pm 5.2\%$  when

mixed as amoebae). For frequency distributions of spores of Dg and Dp in Dg-type and Dp-type chimaeric fruiting bodies see Figs. 4.16 and 4.17.



**Figure 4.15: Different degrees of mixing in Dp and Dg.** No mixing (A–B): The cells of Dp 3 + Dg F16 were mixed homogenously and plated together; cells of the two species segregated and formed separate fruiting bodies. Mixing but sorting-out within a fruiting body (C–D): The cells of Dp 3 + Dg F16 formed chimaeric fruiting bodies; however, cells of the two species occupied different regions in chimaeric fruiting bodies. Relatively free mixing (E–F): The cells of Dp 3 + Dg F16 formed chimaeric fruiting bodies without any sorting out of the cells. A, C and E are fluorescent images and B, D are bright-field images, F was taken as an overlay of fluorescent and bright-field. Scale bar represents 50  $\mu\text{m}$  in every case.

**(ii) Dp 5.2 and Dg 8:** Although the development of this mix was comparable with a mix of Dp 3 and Dg 8, the frequency of chimaerism within Dg-type fruiting bodies was not similar (Table 4.4). When these two species were mixed as spores 0% of Dg-type and 29.4% of Dp-type fruiting bodies were chimaeric; when these two were

mixed as amoebae 28.7% of Dg-type and 27.8% of Dp-type fruiting bodies were chimaeric. In both types of chimaeric fruiting bodies a minority of the spores belonged to the 'non-type' species: 0% and  $7.5 \pm 4.3\%$  spores belonged to Dp in Dg-type fruiting bodies formed after Dp 5.2 was mixed with Dg 8 as spores and amoebae respectively. Similarly,  $13.5 \pm 7.9\%$  and  $15.8 \pm 14.1\%$  spores belonged to Dg in Dp-type fruiting bodies formed after Dp 5.2 and Dg 8 were mixed as spores and amoebae respectively (Table 4.4 and Fig. 4.16 and 4.17).

**(iii) Dp 3 and Dg F5:** When these two were mixed as spores, 5% of Dg-type and 15% of Dp-type fruiting bodies were chimaeric (mix). When these two were mixed as amoebae, 17.0% Dg-type and 17.3% of Dp-type fruiting bodies turned out to be chimaeric. In both Dg-type and Dp-type fruiting bodies a minority of the spores belonged to 'non-type' species:  $12.5 \pm 0\%$  and  $2.7 \pm 1.6\%$  Dp spores in Dg-type fruiting bodies formed after Dp 3 and Dg F5 were mixed as spores and amoebae respectively. Similarly,  $0.5 \pm 0.7\%$  and  $2.9 \pm 1.5\%$  Dg spores in Dp-type fruiting bodies formed after Dp 3 and Dg F5 were mixed as spores and amoebae respectively (Table 4.4, Fig. 4.16 and 4.17).

**(iv) DP 5.2 and Dg F5:** The development of this mix was synchronous and a majority of the fruiting bodies were Dp-type. When spores of Dp 5.2 and Dg were mixed and developed together, 0% of Dg-type and 15.0% of Dp-type fruiting bodies were chimaeric; when these two were mixed as amoebae 29.4% of Dg-type and 17.6% of Dp-type fruiting bodies were chimaeric. This shows that there were differences in the degree of mixing depending on whether these two were mixed as spores or amoebae. Once again, in chimaeric fruiting bodies a minority of the spores were contributed by the 'non-type' species: 0% and  $8.6 \pm 2.6\%$  spores were of Dp 5.2 in Dg-type fruiting bodies formed after Dp 5.2 and Dg F5 were mixed as spores and amoebae respectively. Similarly,  $11.8 \pm 4.0\%$  and  $10.5 \pm 2.4\%$  Dg spores in Dp-type fruiting bodies formed after Dp 5.2 and Dg F5 were mixed as spores and amoebae respectively (Table 4.4, Fig. 4.16 and 4.17).

**Table 4.4:** Chimaerism in Dg-type and Dp-type fruiting bodies formed after cells of Dg and Dp were mixed as spores (along with bacteria) and as freshly starved amoebae (without bacteria).

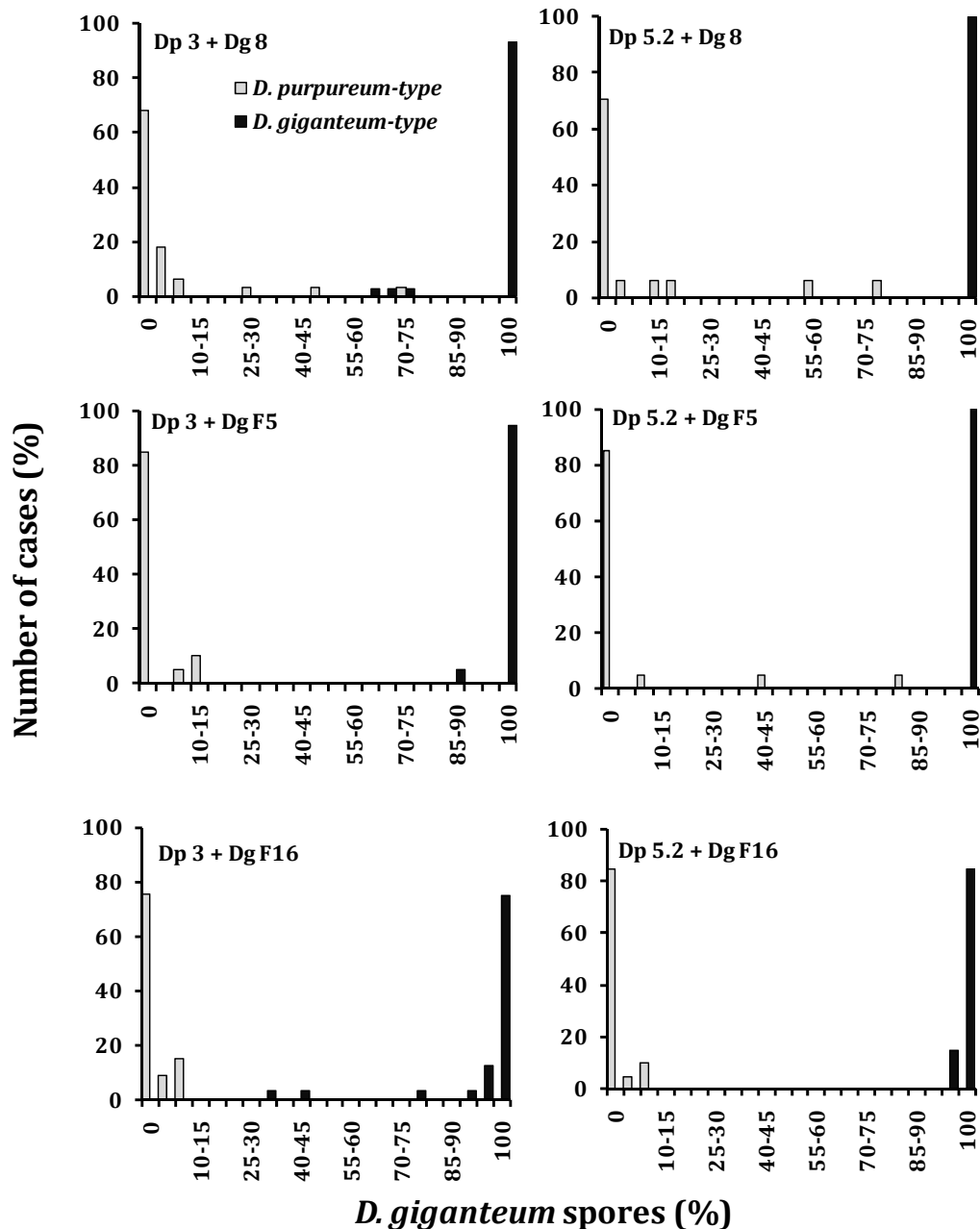
Strains	Strains mixed as	Dg-type fruiting bodies				Dp spores in Dg-type chimaeric fruiting bodies (%)	Dp-type fruiting bodies				Dg spores in Dp-type chimaeric fruiting bodies (%)
		P	C	T	C (%)		P	C	T	C (%)	
Dp 3+	Spores	38	3	41	7.3	1.5 ± 3.4	24	11	35	31.4	4.4 ± 3.1
Dg 8	Amoebae	442	106	548	19.3	9.1 ± 9.8	57	23	80	28.7	3.6 ± 5.2
Dp 5.2+	Spores	20	0	20	0.0	0	15	5	17	29.4	13.5 ± 7.9
Dg 8	Amoebae	55	22	77	28.7	7.5 ± 4.3	57	22	79	27.8	15.8 ± 14.1
Dp 3+	Spores	19	1	20	5.0	12.5 ± 0*	17	3	20	15.0	0.5 ± 0.7
Dg F5	Amoebae	34	7	41	17.0	2.7 ± 1.6	115	24	139	17.3	2.9 ± 1.5
Dp 5.2+	Spores	20	0	20	0.0	0	17	3	20	15.0	11.8 ± 4.0
Dg F5	Amoebae	24	10	34	29.4	8.6 ± 2.6	103	22	125	17.6	10.5 ± 2.4
Dp 3+	Spores	24	8	32	25.0	2.4 ± 2.3	25	8	33	24.2	1.7 ± 0.5
Dg F16	Amoebae	22	5	27	18.5	5.5 ± 0.8	46	11	57	19.3	6.1 ± 0.6
Dp 5.2+	Spores	17	3	20	15.0	3.1 ± 0.2	17	3	20	15.0	6.2 ± 4.1
Dg F16	Amoebae	29	6	35	17.1	3.0 ± 0.9	56	3	59	5.1	9.2 ± 1.0

'Dg' *D. giganteum*; 'Dp' = *D. purpureum*; 'P' = pure i.e., in these fruiting bodies all spores belonged exclusively to one or the other species; 'C' = chimaeric fruiting bodies; 'T' = total fruiting bodies \* only one fruiting body was chimaeric. The results are means ± sd; number of independent experiment (n) ≥ 4.

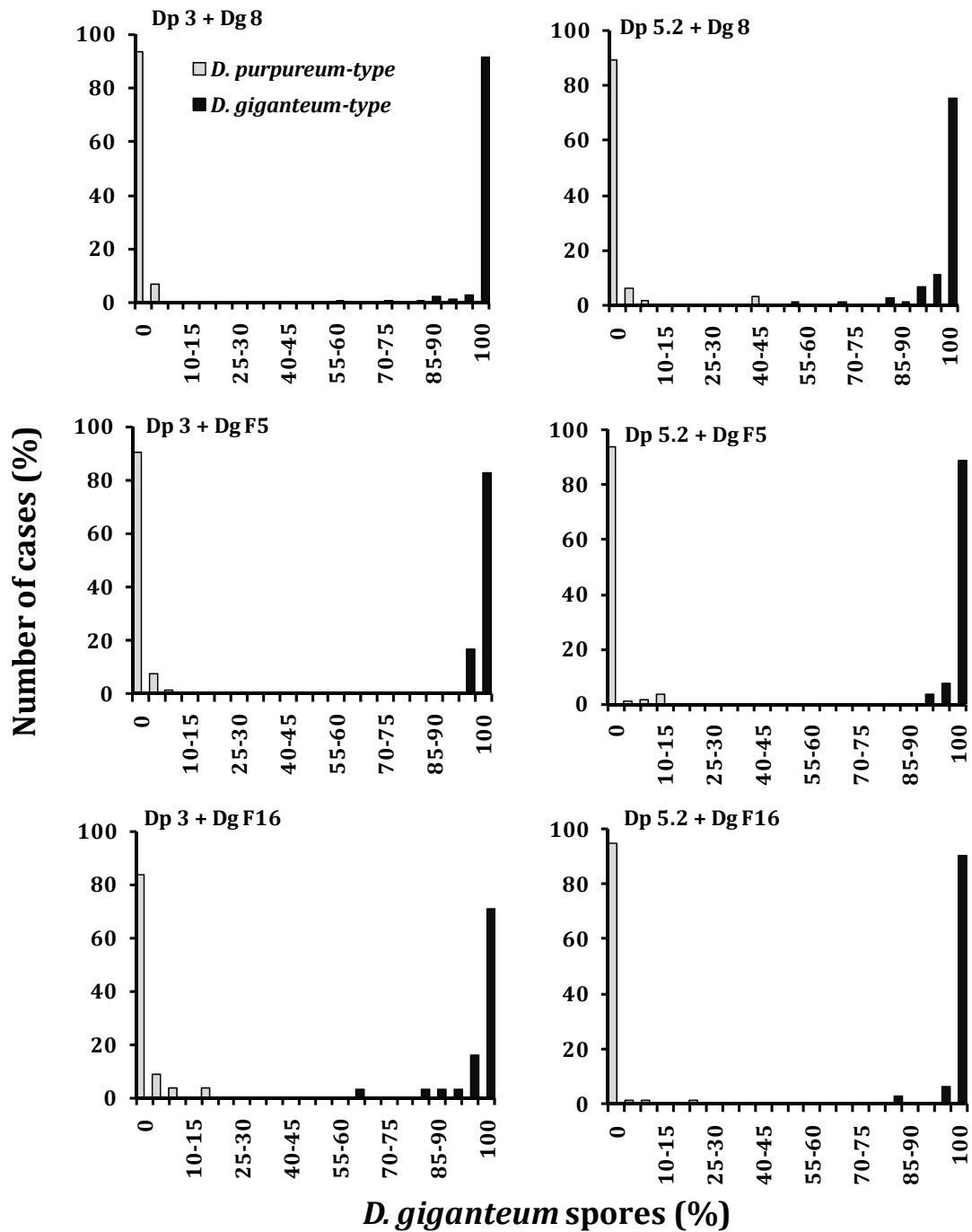
**(v) Dp 3 and Dg F16:** When spores of these two species were mixed 25.0% of Dg-type and 24.2% of Dp-type fruiting bodies were chimaeric and when amoebae of these two were mixed 18.5% of Dg-type and 19.3% of Dp-type fruiting bodies were chimaeric. In chimaeric fruiting bodies a minority of the spores belonged to the 'non-type' species: 2.4 ± 2.3% and 5.5 ± 0.8% Dp spores in Dg-type fruiting bodies formed after Dp 3 and Dg F16 were mixed as spores and amoebae respectively. Similarly, 1.7 ± 0.5% and 6.1 ± 0.6% Dg spores in Dp-type fruiting bodies formed after Dp 3 and Dg F16 were mixed as spores and amoebae respectively (Table 4.4, Fig. 4.16 and 4.17).

**(vi) Dp 5.2 and Dg F16:** When Dp 5.2 and Dg F16 were mixed as spores, 15.0% of Dg-type and 15.0% of Dp-type fruiting bodies were chimaeric; when these two were mixed as amoebae, 17.1% of Dg-type and 5.1% of Dp-type fruiting bodies were chimaeric. The spore contributions in chimaeric fruiting bodies were: 3.1 ± 0.2% and 3.0 ± 0.9% Dp spores in Dg-type fruiting bodies formed after Dp 5.2 and Dg F16

were mixed as spores and amoebae respectively. Similarly,  $6.2 \pm 4.1\%$  and  $9.2 \pm 1.0\%$  Dg spores in Dp-type fruiting bodies formed after Dp 5.2 and Dg F16 were mixed as spores and amoebae respectively (Table 4.4, Fig. 4.16 and 4.17).

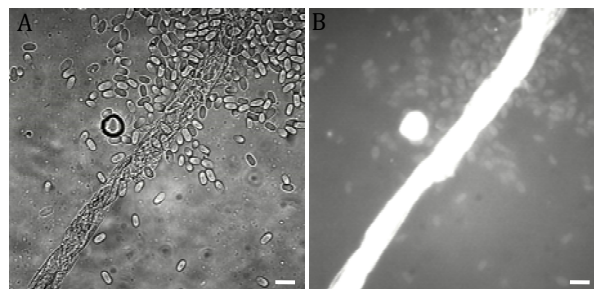


**Figure 4.16: Frequency distributions of Dg spores in Dg-type and Dp-type fruiting bodies.** Dg and Dp spores were mixed and plated on  $KK_2$  agar along with *K. aerogenes*. Six combinations, as shown on top of each histogram, were made. Class '0' and '100' indicates fruiting bodies in which all spores belonged exclusively to one or the other species. Other classes were made with intervals of 5% starting from 0–5 and ending with 95–100.

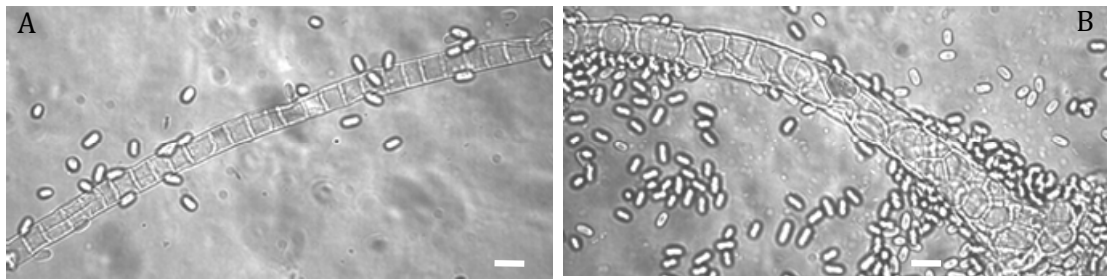


**Figure 4.17: Frequency distributions of Dg spores in Dg-type and Dp-type fruiting bodies.** Dg and Dp amoebae (six combinations as mentioned on top of each histogram) were mixed and plated on KK<sub>2</sub> agar; Dg spores were counted in both Dg-type and Dp-type fruiting bodies formed from each mix. Class '0' and '100' indicates fruiting bodies in which all spores belonged exclusively to one or the other species. Other classes were made with intervals of 5% starting from 0–5 and ending with 95–100.

Quantitative data pertaining to the contribution of cells from two species in chimaeric fruiting bodies (Table 4.4) were obtained by monitoring spore cells within fruiting bodies. Chimaerism in the stalk could not be quantified because of several practical limitations: stalk cells had bright auto-fluorescence and separating single cells from stalks is very difficult. This is why only qualitative data could be obtained about the stalk. Chimaerism in the stalk was rather curious. In some Dg-type fruiting bodies formed after mixing freshly starved amoebae of Dp 3 + Dg 8 or Dp 5.2 + Dg 8 the entire spore mass contained only Dg spores and the entire stalk was formed by Dp cells (Fig. 4.18); no cases of the opposite sort were seen, where spores were from Dp and the stalk from Dg. Sometimes a fruiting body appeared to be of Dg-type and had only Dg spores, but the stalk was found to be chimaeric with Dp cells being present only in the lower part of the stalk (Fig. 4.19). The frequency of occurrence of such fruiting bodies was less than 2% and they were seen only when the mixes involved Dp 3 or Dp 5.2 with Dg 8.



**Figure 4.18: An extreme case of chimaerism and segregation.** In this Dg-type fruiting body almost all the cells in stalk were contributed by Dp 3 whereas all spores were contributed by Dg 8. Since Dp 3 amoebae were stained before mixing with Dg 8 the identity of the cells that went into stalks was easy to confirm. As mentioned earlier stalk cells exhibited strong auto-fluorescence, that is, even unstained cells appeared bright. To rule out a possibility of auto fluorescence, stalk cell morphology was also looked at (Dp forms thick stalk cells). Fig. 4.18A and Fig. 4.18B are bright field and fluorescent images respectively of one such chimaeric fruiting body. The scale bar represents 25  $\mu\text{m}$ .



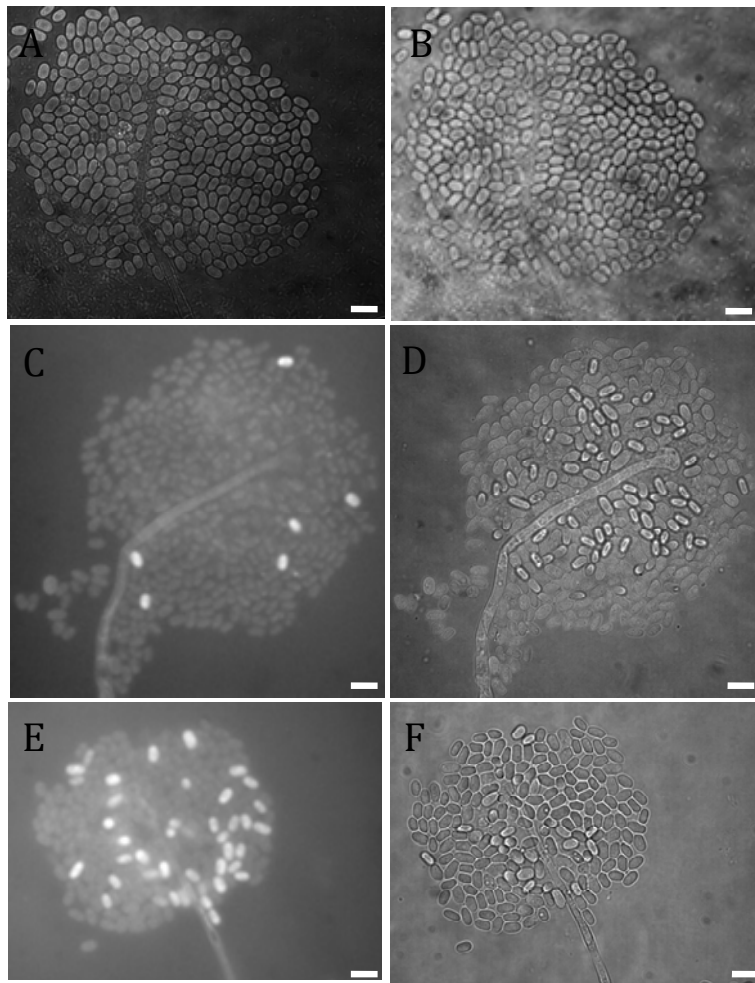
**Figure 4.19: Chimaeric stalk.** Fig. 4.19A shows the upper portion (towards spore mass) and it was made up of Dg 8 cells, Fig. 4.19B shows the base of chimaeric stalk and is made up of Dp 3 cells. Since the stalk was very long different parts of the stalk had to be photographed independently, two such parts are shown here. Spores which were picked along with stalk are also visible. Scale bar is 25  $\mu\text{m}$ .

**Summary:** A brief summary of chimaerism when different species were mixed is as follows. In each mix involving spores or amoebae of Dp and Dg, a minority of the fruiting bodies were chimaeric. The maximum percent chimaerism was found to be 31.4% when spores of Dp 3 were mixed with spores of Dg 8. In general, the frequency of chimaerism was lesser when spores of two species were mixed than when mixing involved amoebae; although there were some exceptions (Table 4.4).

**4.4.5 Chimaerism in inter-strain mixtures:** These experiments were performed to see if different strains of the same species mix freely and form chimaeric fruiting bodies. Only freshly starved amoebae were mixed; mixing experiments involving spores were not carried out.

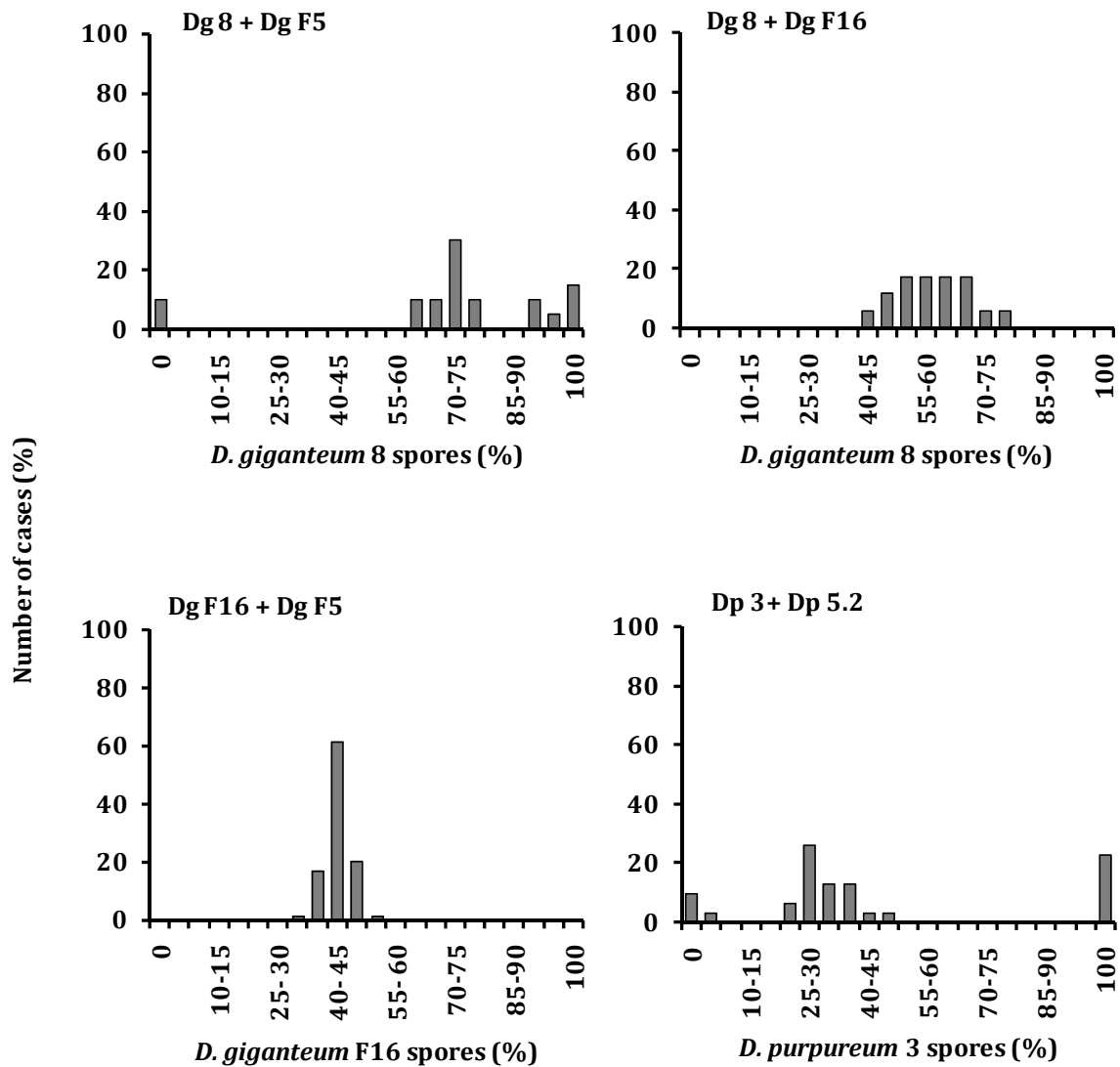
**(i) Dg 8 and Dg F5:** Freshly starved amoebae of these two strains co-aggregated and formed chimaeric fruiting bodies; the frequency of chimaerism was  $75.2 \pm 6.6\%$ . Within such chimaeric fruiting bodies, the majority of spores belonged to Dg 8 ( $76.5 \pm 9.3\%$  spores; mean  $\pm$  s.d;  $n = 3$ ; t-test,  $p = 0.002$ ). Similar to what was seen when amoebae of different species were mixed, here too, the degree of chimaerism between different fruiting bodies varied:  $\sim 25\%$  fruiting bodies were pure (Fig 4.20 A–B, Fig. 4.21),  $\sim 25\%$  fruiting bodies were weak chimaeras, i.e., in these fruiting bodies a minority of spores were of Dg F5 (Fig 4.20 C–D, Fig. 4.21), and the remaining 50% of the fruiting bodies were chimaeric with 60–80% spores belonging to either one of the genotype (Fig 4.20 E–F, Fig. 4.21).





**Figure 4.20: Different degree of mixing in Dg 8 + Dg F5 mix.** Freshly starved amoebae of Dg 8 were mixed with Dg F5 and resulting fruiting bodies were analysed for chimaerism in spore masses. ~ 25% fruiting bodies formed from this mix were pure, i.e., these fruiting bodies had either Dg 8 or Dg F5 spores (see Fig. 4.20 A-B). ~ 75% fruiting bodies were chimaeric, i.e., they had spores which belonged to both Dg 8 and Dg F5. However, in these chimaeric fruiting bodies ~ 25% fruiting bodies had a minority of cells belonging to Dg F5 (5.20 C-D). In the remaining 75% fruiting bodies the proportions of spores belonging to the two strains were relatively less skewed (4.20 C-D). There were fruiting bodies where cells of a genotype remained together and formed small islands of spores. A, C and E are fluorescent images and B, D and F are respective bright-field images. Scale bar represents 10  $\mu$ m.

**(ii) Dg 8 and Dg F16:** A 1:1 mix of these two developed synchronously and all the fruiting bodies formed were chimaeric (% chimaerism  $100 \pm 0$ ); within such chimaeric fruiting bodies Dg 8 formed significantly more spores than Dg F16 (Dg 8 spores:  $60.7 \pm 5.8\%$ ; mean  $\pm$  sd n = 3; t-test, p = 0.035 ).



**Figure 4.21: Frequency distributions of spores in inter-strain mixtures of Dg and Dp.** Class '0' and '100' indicates fruiting bodies in which all spores belonged exclusively to one or the other species. Other classes were made with intervals of 5% starting from 0–5 and ending with 95–100.

**(iii) Dg F5 and Dg F16:** Mixing results for this pair have already been discussed in chapter 3. Briefly, when freshly starved amoebae of Dg F5 and Dg F16 were mixed the frequency of chimaerism was  $100 \pm 0\%$ ; the contribution of Dg F16 spores in chimaeric fruiting bodies was significantly less than Dg F5 (Dg F16 spores:  $43.0 \pm 2.8\%$ ; mean  $\pm$  sd;  $n = 3$ ; t-test,  $p = 0.015$ ).

**(iv) Dp 3 and Dp 5.2:** When freshly starved amoebae of Dp 3 were mixed with Dp 5.2,  $60.0 \pm 14.1\%$  fruiting bodies were chimaeric and within such chimaeric fruiting bodies a majority of spores were contributed by Dg 5.2 (Dg 5.2 spores:  $66.8 \pm 8.2\%$  mean  $\pm$  sd;  $n = 3$ ; t-test,  $p = 0.024$ ). In all the inter-strain mixes the contribution of strains to stalk cells could not be analysed because there were no differences in the stalk morphology of these two strains and stalk cells had strong auto-fluorescence.

#### 4.5 Discussion

Inter-species interactions in CSMs were first studied by Olive (1902). The experiments performed by Olive (1902) involved planting spores of two CSM species (one with white spore-mass and another with purple spore-mass, presumably *D. mucoroides* and *D. purpureum* respectively) on a solid medium along with a heap of bacteria as the food source. Two distinct types of fruiting bodies, resembling either *D. mucoroides* or *D. purpureum* were seen developing side by side; no fruiting bodies with intermediate phenotypes were observed. From these observations, Olive (1902) concluded that the CSM species do not form chimaeric fruiting bodies. However, the absence of fruiting bodies with intermediate phenotypes is not a reliable criterion to rule out weak mixing. A better way to detect a low level of mixing is to collect cells from fruiting bodies, plate them at a low density with bacteria and record the phenotypes of fruiting bodies formed thereafter.

This was done earlier (Raper and Thom, 1941) and in this study. Raper and Thom (1941) repeated the experiments of Olive and also extended the range of the inter-species interaction studies by using four CSM species: *D. discoideum*, *D. purpureum*, *D. mucoroides* and *P. violaceum*. In their experiments spores of two CSM species were planted together along with *Escherichia coli* or *Serratia marcescens*. *P. violaceum* in a binary mix with any of the three *Dictyostelium* species resulted in the formation of separate centers and aggregates. However, if the mixtures involved amoebae of any two *Dictyostelium* species, co-aggregation was seen but after aggregation cells drew apart and formed separate fruiting bodies. When plated clonally along with bacteria spores collected from these fruiting bodies formed

fruiting bodies typical of one species, implying these CSM species did not mix. In this study, the experiments were repeated with *D. purpureum* and *D. giganteum*; the frequency of chimaeric fruiting body formation was strikingly higher than in the earlier studies (Olive, 1902; Raper and Thom, 1941).

When spores of the two CSM species were mixed and plated with bacteria, two distinct types of fruiting bodies developing side by side were observed; no fruiting bodies with intermediate phenotypes were seen. However, when spores from these fruiting bodies were collected and clonally plated, a considerable number of chimaeric fruiting bodies were seen (Table 4.4). This indicates that just the external appearance of a fruiting body is not a reliable criterion to infer mixing within fruiting bodies. With the exception of Dp 5.2 spores mixed with Dg 8 spores and Dp 5.2 spores mixed with Dg F5 spores all pairs formed chimaeric fruiting bodies, although the total chimaeric fruiting bodies formed by any pair never crossed a limit of 32% (Table 4.4). A majority of the chimaeric fruiting bodies were weak chimeras, i.e., only a small proportion of the spores came from the 'non-type'.

The observations of this study show similarities with findings of Jack et al. (2008). In their experiments amoebae of *D. purpureum* and *D. discoideum* were inoculated on non-nutrient agar at exceptionally high density ( $2 \times 10^6$  amoebae of each species/cm<sup>2</sup>). 50% of the *D. discoideum*-like and 22% of the *D. purpureum*-like fruiting bodies that formed were chimaeric, though a major fraction of the spores in a fruiting body were either of *D. purpureum* or *D. discoideum* ( $85 \pm 3\%$  *D. discoideum* spores in *D. discoideum*-like fruiting bodies and  $94 \pm 2\%$  *D. purpureum* spores in *D. purpureum*-like fruiting bodies). In this study, when amoebae of *D. giganteum* and *D. purpureum* were mixed at a somewhat lower density ( $1 \times 10^5$  amoebae of each/cm<sup>2</sup>), 20.0% of the Dg-type and 19.4% of the Dp-type fruiting bodies formed after a mix of the two were chimaeric; only a small portion of spores in each type of fruiting body belonged to 'non-type' spores (Table 4.4).

Even though *D. giganteum* and *D. purpureum* cells were mixed in a 1:1 ratio, almost in every mix Dg-type fruiting bodies were more common than Dp-type (Table 4.2 and 4.3). Similar observations were made by Raper and Thom (1941), in their

experiments, *D. purpureum* fruiting bodies predominated in a mix with *D. discoideum*. This could be because *D. giganteum* spores germinate faster than *D. purpureum* spores (Fig. 4.4A) or *D. giganteum* amoebae double more quickly than *D. purpureum* amoebae (Fig. 4.4B). However, differential spore germination or doubling time differences do not exist if freshly starved amoebae of the two are mixed. Even in the experiments where the two species were mixed as starved amoebae, Dg-type fruiting bodies were seen more often than Dp-type. Efforts were made to achieve a balanced number of fruiting bodies by inoculating Dp spores ~ 12 hrs before Dg spores were added to the plates. But here too, Dg-type fruiting bodies predominated. *D. purpureum* fruiting bodies were bigger than *D. giganteum* fruiting bodies when both were plated separately. However, in a mix, Dp-type fruiting bodies were not as big as when they were alone, thus, ruling out the possibility of size differences being a reason for their low numbers. In Dp 5.2 + Dg 8 and Dp 3 + Dg 8 mixes Dp-type fruiting bodies were almost absent (Tables 4.2 and 4.3). When I looked for 'missing' *D. purpureum* amoebae I found a large number of unaggregated amoebae, besides which they were present in the stalks of Dg-type fruiting bodies (Figs. 4.18, 4.19).

In general, the frequency of chimaerism in Dg-type fruiting bodies was higher when *D. giganteum* and *D. purpureum* were mixed as amoebae than when they were mixed as spores (Table 4.4). Such differences were not apparent in Dp-type fruiting bodies (Table 4.4). In every Dg + Dp mix, at least a few Dp-type fruiting bodies turned out to be chimaeric, no Dg-type fruiting bodies were chimaeric when mixes involved Dp 5.2 spores + Dg 8 spores or Dp 5.2 spores + Dg F5 spores (Table 4.4). The differences in chimaerism in Dp-type and Dg-type fruiting bodies could be because the surface properties of *D. purpureum* cells are such that it makes them 'stickier' and hence they can stick even to non-self cells and therefore could not exclude *D. giganteum* amoebae entirely; however, this speculation remains to be tested. When *D. purpureum* amoebae were spread on agar plates they immediately adhered to the agar surface (within 30-45 seconds). However, *D. giganteum* amoebae took much longer to stick (4-5 minutes), implying that *D. purpureum* amoebae are more adhesive.

The high degree of chimaerism between these two CSM species was unexpected because of several reasons: a molecular phylogeny shows *D. giganteum* and *D. purpureum* are present in different sub-clades in a same major group (group 4 or Dictyostelid) and these two species are separated by 13 more species (Schaap et al., 2006). Despite their phylogenetic divergence several reasons could account for partial mixing of these two species. Both *D. purpureum* and *D. giganteum* amoebae use cAMP as aggregation signal (Bonner, 1967; Raper, 1984); this could be a reason why amoebae of these two species are attracted to each other's centre. Although these two species seem to have significantly different development tempo (when alone, *D. purpureum* amoebae aggregate significantly slower than *D. giganteum* amoebae), in a mix, they develop with the speed of the faster member of a pair (Fig. 4.5 and Figs. 4.5–4.14). This means that the differences in development rates would not affect the ability of the two to join the same aggregate. Fruiting bodies formed after mixing *D. purpureum* and *D. giganteum* cells were carefully looked for chimaerism; spores were collected from the fruiting bodies, plated at very low density, and the phenotypes of fruiting bodies formed thereafter were recorded, all this ensured that even a low level of chimaerism was not missed out.

Gregg (1956) raised antibodies against vegetative amoebae of different species of CSMs by injecting them in rabbits. The resulting antibodies could specifically bind to the respective vegetative amoebae. However, the antibodies failed to differentiate between aggregation-stage amoebae (Gregg, 1956). This could partly explain the results of Raper and Thom (1941) where CSM species were found to mix only in the later stages of development. Nicol and Garrod (1978) showed that the aggregation-stage amoebae of each of the four CSM species (*D. discoideum*, *D. mucoroides*, *D. purpureum* and *P. violaceum*) can stick to each other in a liquid environment. From this observation they concluded that CSM species fail to form mixed fruiting bodies on agar surfaces not because of a lack of mutual cohesiveness, but due to different chemotactic mechanisms. Shaffer (1957a; 1957b) has pointed out the role of both cell surface stickiness and different aggregation signals in co-aggregation and chimaera formation in CSMs.

Several interesting parallels emerge when cell-cell interactions between members of different strains of the same species are compared to those between cells belonging to different species. Bonner and Adams (1958) reported that certain strains of *D. mucoroides* if mixed as amoebae did not form chimaeric fruiting bodies; similar behaviour was exhibited when inter-strain mixes were performed using strains of *P. violaceum*, *D. giganteum*, *D. discoideum* and *D. purpureum* (Bonner and Adams, 1958; Kaushik et al., 2006; Ostrowski et al., 2008; Mehdiabadi et al., 2009). Interestingly, when *D. mucoroides* cells were mixed with *D. discoideum* cells they formed chimaeric fruiting bodies (Bonner and Adams, 1958). Inter-strain mixes like inter-species mixes showed differences in percent chimaerism (Fig. 4.21) and physical segregation within fruiting bodies (Fig. 4. 15 and 4. 20). This means the parameters which determine the compatibility of cells of different strains or different species could be similar and the quantitative differences may have played a role in their divergence.

The degree of mixing when cells belong to different species is rather weak; either the chimaeras are formed at very low frequency or within chimaeras a minority of cells belongs to 'non-type' species. This suggests that the general tendency in *D. giganteum* and *D. purpureum* amoebae seems to be to segregate and form separate fruiting bodies, in those cases where chimaerism was observed could be the cases where for some reason *D. giganteum* and *D. purpureum* amoebae failed to segregate.

There are several advantages of fruiting body formation in CSMs: (a) if amoebae aggregate and form fruiting bodies they survive for relatively longer durations (Bonner, 1967), but if amoebae remain unaggregated they die as the time progresses (Gregg, 1971); (b) amoebae in the slugs or fruiting bodies are protected from predators (nematodes) present in the soil (Kessin et al., 1996); (c) larger (actually longer) slugs move faster and for longer durations (Inouye and Takeuchi, 1979; Foster et al., 2002); (d) fruiting body formation aids in spore dispersal (Bonner, 1967; Bonner, 1982; Suthers, 1985; Huss, 1989). It is possible that in a situation where numbers of self amoebae (amoebae belonging to the same species)

are below a critical density to permit pure fruiting body formation, CSM amoebae form chimaeric fruiting bodies and benefit from all the advantages of fruiting body formations.

Gause (1934) hypothesized that two species sharing a similar resource cannot coexist in the same environment. The observation that several species of CSMs occupy a similar niche raised important question; how can different species of CSMs, all with similar life cycle coexist in the same environment? An interesting hypothesis suggested by Bonner (2009) is worth mentioning: different species of CSMs have been considered as neutral phenotypes, i.e., all species are equally adapted to the local environment and this may lead to coexistence of several species. Horn (1952) found that different species of CSMs have food preferences, in other words, the available resources are portioned among different species, and this too could lead to the species coexistence. The observations from this study suggest that CSM species can co-exist in the same environment by preferentially forming fruiting bodies only with self members.

**Summary:** Although amoebae of *D. giganteum* and *D. purpureum* are able to co-aggregate, there are factors that lead to segregation (sorting-out) between cells of the two genotypes thereafter. This agrees with the earlier findings of Olive, Raper and Thom, Bonner and Adams, and others. The interesting point is that the extent of segregation appears to differ between strains, a feature that inter-species mixes share with intra-species mixes of two genotypes suggesting there is a continuum of cell-cell interactions possible between CSM amoebae, ranging from members of different strains of the same species to those between cells belonging to different species. Quantitative trait differences may have been responsible for species divergence in these soil amoebae. Although *D. giganteum*, *D. purpureum* and several other *Dictyostelium* species occupy similar niches the ability to recognize non-self individuals and preferentially co aggregate with self individuals may be involved in co-existence in CSMs.



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# **Chapter 5**

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*Summary and questions for future*

In this thesis I have made an attempt to understand the evolutionary factors that are likely to be involved in the maintenance of social behaviour in the cellular slime moulds (CSMs). The major findings of my study are as follows.

Several species of CSMs including *D. giganteum* and *D. purpureum* were found together in what appears to be the same niche. Indeed CSM propagules belonging to different genotypes of the same species or even different species can be isolated from a small speck of soil (250-1000  $\mu\text{m}$ ) or small dung samples. Amoebae of these two species can co-aggregate and form inter-species chimaeras. However, after aggregation amoebae of the two species physically segregate and form separate fruiting bodies or they occupy different regions in a chimaeric fruiting body. The extent of segregation appears to differ depending on the strains of *D. giganteum* and *D. purpureum* that participate in forming chimaeric social groups. CSMs occur in the dung of large mammals, indicating that the animals probably act as dispersal agents. If this mode of dispersal operates, it would involve thorough mixing in the animal gut. In that case CSM fruiting bodies in the wild can be expected to contain spores of more than one genotype i.e. be chimaeras, just as I have found for the fruiting bodies of *D. giganteum* and *D. purpureum*.

Laboratory experiments using pair-wise mixes of wild isolates showed that in chimaeric social groups some strains form more spores than expected, leading to asymmetric ('unfair') allocations to spore and stalk cells. Despite the reproductive asymmetry, polyclonal groups are maintained in the wild (as shown by this study). A reason could be that wild-type strains differ in respect of more than one fitness-related trait, for example growth, development rate, slug migration through soil, and sporulation efficiency. If so, trade-offs between different traits exist could contribute to the stable co-existence of different strains. I have demonstrated that trade-offs do exist.

The observations from this study open up several questions for future investigation. What accounts for the stable coexistence of so many CSM species, all with different phenotypes, in the same niche? As a corollary of that, which of the

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phenotypic differences that are manifest are in fact adaptations to different microenvironments present in the soil and which are neutral? For instance, different CSM species might prefer different bacteria as their food source. This hypothesis can be tested using the CSMs used by me and bacteria isolated from Mudumalai soil (see chapter 2). A related issue is that of self/non-self discrimination, whose intensity appears to range smoothly from strong discrimination between members of different species and discrimination of varying strengths between members of the same species. What are the factors behind this?

One strain in a chimaeric social group can do better than another with respect to several fitness-related traits. Most obviously, it can form proportionately more spores than the other. What proximate mechanisms are involved in this? To what extent do the mechanisms involve genetic predispositions as reflected in, for example, intracellular calcium, and production of morphogens (e.g., DIF-like substances); and to what extent do they depend on the dynamics of intercellular signaling? I have hypothesized that trade-offs between different fitness-related traits can account for stable co-existence of polyclonal groups in the wild. However, fitness-related traits were studied only in binary mixtures. It would be interesting to generate polyclonal social groups containing more than two strains and study the behaviours exhibited by these groups. Exploring these questions will help us to learn more about the complex social behaviours displayed by these fascinating organisms.

# Publications

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# Genetic Heterogeneity in Wild Isolates of Cellular Slime Mold Social Groups

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**Abstract** This study addresses the issues of spatial distribution, dispersal, and genetic heterogeneity in social groups of the cellular slime molds (CSMs). The CSMs are soil amoebae with an unusual life cycle that consists of alternating solitary and social phases. Because the social phase involves division of labor with what appears to be an extreme form of “altruism”, the CSMs raise interesting evolutionary questions regarding the origin and maintenance of sociality. Knowledge of the genetic structure of social groups in the wild is necessary for answering these questions. We confirm that CSMs are widespread in undisturbed forest soil from South India. They are dispersed over long distances via the dung of a variety of large mammals. Consistent with this mode of dispersal, most social groups in the two species examined for detailed study, *Dictyostelium giganteum* and *Dictyostelium purpureum*, are multi-clonal.

## Introduction

The existence and implication of spatial structuring in microbial populations is a theme of long-standing interest in ecology [36]. At one extreme, there is the hypothesis that—as with large animals—populations tend to be more or less viscous, and spatial structure is determined by patterns of dispersal. At the other extreme, there is the view that dispersal is rampant (“everything is everywhere”) and what persists is determined by adaptations to local conditions. In the case of social organisms, an important aspect of spatial structure is whether or not groups consist of close relatives (conceivably clones). This is because kinship plays a crucial role in some models for the evolution of social behavior [11, 23]. Both spatial structure (which is related to dispersal) and kinship bear on the evolution of the life cycle in the social amoebae, also known as the Dictyostelid or cellular slime molds [6].

The cellular slime molds (CSMs) are found in soils all over the world in environments that vary from cold temperate to tropical [53]. As far as CSMs from India go, Agnihothrudu [1] described finding a number of species in cultivated and uncultivated soils as well as in rhizosphere soils from South India (location unspecified). Rai and Tewari [42, 44] isolated *Dictyostelium mucoroides*, *Dictyostelium sphaerocephalum*, and *Polysphondylium violaceum* from soil on the campus of Lucknow University (26°55' N, 80°59' E). Cavender and Lakhanpal [9] were able to recover *Dictyostelium giganteum*, *Dictyostelium purpureum*, *Dictyostelium mucoroides*, *Polysphondylium violaceum*, *Polysphondylium pallidum*, *Dictyostelium polycephalum*, *Dictyostelium aureo-stipes*, *Dictyostelium macrocephalum*, *Dictyostelium tenue*, and *Dictyostelium vinaceo-fuscum* from tropical evergreen and deciduous forest soils. All CSMs feed on bacteria and possibly yeasts and, in the absence of special conditions,

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propagate asexually, that is clonally. The defining feature of their life cycle is that once they exhaust the local food supply, they enter a cooperative social phase: anywhere from  $10^2$  to  $10^6$  amoebae aggregate and form a motile multicellular mass that exhibits division of labor and subsequently differentiates into a fruiting body (<http://www.dictybase.org/Bonner%20paper.pdf>) [6]. Some or all of the cells that form the fruiting body are starvation-resistant spores that are elevated approximately 1 mm to 1 cm above the soil surface on top of a stalk. In the advanced CSM species, the stalk is cellular and consists of dead amoebae. Thus, CSM social behavior involves an extreme form of cooperation: it appears that these amoebae forego the prospect of their own reproduction in the process of enhancing the reproductive potential of those that differentiate into spores [4]. Following dispersal to a favorable environment, spore can germinate, whereupon the emergent amoebae begin a new life cycle. Spores can be dispersed by a variety of agencies including arthropods—cave crickets [25, 49], nematodes [29], amphibians [18, 48], birds [48, 52], bats [48, 55], and small mammals [48]; in fact, the first CSM to be discovered was isolated from horse dung [7, cited in 35]. Air and water are also possible but unproven agents of dispersal.

Earlier studies that addressed the issue of genetic diversity of CSMs from the same neighborhood, and in some cases also from the same fruiting body, came up with different findings. Agnihotrudu [1] discovered several CSM species in the rhizosphere associated with two plants: four species in one case (pigeon pea, *Cajanus cajan*) and five species in another (peanut, *Arachis hypogea*). Cavender (unpublished) was able to isolate seven to eight species from individual 20-g soil samples from certain forests in Ohio. These findings indicate a substantial degree of co-occurrence of different species of CSMs, which is an interesting phenomenon in its own right (the question of different strains of the same species was not addressed). Filosa [14] monitored CSM cultures that had been maintained for eight years in the laboratory (they were originally isolated from giraffe dung from a zoo). When sub-cloned, spores from fruiting bodies showed clear evidence of distinctiveness; also, they gave rise to amoebae that complemented each other's development. Buss [8] was able to isolate morphologically distinct forms of *Dictyostelium mucoroides* from the same soil microhabitat; among them, there was a variant that formed only spores when forced to develop by itself but joined in constructing a fruiting body when combined with a normal form. In studies on North American hardwood forest soil, Ketcham and Eisenberg [30] found diverse *Polysphondylium pallidum* types that belonged to different mating types and had varying growth rates, all within a spatial scale of 1 cm. Fortunato et al. [16] collected soil samples from Virginia, USA with a 6-mm diameter plastic straw and saw that many haplotypes (clones) of *Dictyostelium discoideum* co-occurred, making it plausible

that they gave rise to polyclonal social groups in nature. On the other hand, in the study that comes closest to ours, Gilbert et al. [18] isolated fruiting bodies from whitetail deer pellets in North America and found that most were clonal. Recently, Gilbert et al. [19] have reported a large clonal swathe of *Dictyostelium discoideum* in a cattle pasture located in a Texas Gulf Coast prairie. A preliminary study by Kaushik and Nanjundiah [27] reported the presence of at least ten different genotypes within a single spore mass in a *Dictyostelium giganteum* fruiting body formed under quasi-natural conditions.

Sociality appears to have evolved in the CSMs as an adaptation for promoting dispersal from a nutrient-poor environment [5]. The evolutionary basis of social behavior, especially when some of the individuals that cooperate behave “altruistically,” continues to intrigue biologists. It has been hypothesized that relatedness by common descent—implying, for an asexual species, membership of the same clone—could be a possible explanation for the existence of “altruistic” traits [15, 21, 23]. Relatively simple and experimentally tractable organisms such as the CSMs are ideally suited for testing the hypothesis. In the case of the CSMs, the issue is, do the spores in a fruiting body share a common genetic interest? The present work is concerned with (a) the presence of CSMs from undisturbed forest soil and animal dung samples in South India and (b) the extent of genetic heterogeneity in fruiting bodies formed under quasi-natural conditions.

## Methods

### Sample Collection

Fresh animal dung was collected from grassy areas within or abutting a mainly dry deciduous forest in South India. Most collections were from a 50-ha study plot in the Mudumalai wildlife sanctuary, located between  $11^{\circ}32'N$ – $11^{\circ}43'N$  and  $76^{\circ}22'E$ – $76^{\circ}45'E$  in the Nilgiri range of the Western Ghats at an altitude of 850–1,250 m. Fresh dung samples were lifted with sterile forceps and immediately transferred to wide-mouthed sterile plastic tubes ( $2 \times 10$  cm), sealed with paraffin tape, and carried on the same day to the laboratory in Bangalore about 300 km away. Soil contamination was avoided by picking samples only from the top and middle portions of animal dung pellets. Yak (*Bos grunniens*) dung was also collected following a chance encounter with a grazing herd near the Changla pass in the dry high-altitude desert of Ladakh, North India ( $33.95^{\circ}N$ ,  $77.85^{\circ}E$ ; altitude 5,300 m). It was placed in a sterile plastic bag and brought to the laboratory after a week. Soil samples were collected from the 50-ha plot and handled further as described earlier [28]. Both well-dispersed soil samples (obtained after shaking the soil



in sterile buffer) and the smallest soil particles that could be picked up (ranging in size from 250 µm to 1 mm) were used. Because large soil fragments tend to crumble, we took care to pick up and transfer tiny particles that remained whole.

#### Isolation of CSMs

Except for yak dung, all other samples were processed within 24 h of collection. Dung samples (approximately 2 cm or smaller and weighing 30 mg–1 g) or soil particles (250 µm to 1 mm) were transferred carefully to 60-cm<sup>2</sup> plastic plates containing 2% phosphate-buffered non-nutrient agar (PBA; KH<sub>2</sub>PO<sub>4</sub> 2.25 g, K<sub>2</sub>HPO<sub>4</sub> 0.67 g, agar 20 g, H<sub>2</sub>O 1,000 ml, pH6.4), SM/10 nutrient agar [51] or PBA pre-spread with *Klebsiella aerogenes*. Only the last set of plates contained exogenously added amoebal food (i.e., bacteria). Plates were sealed with Parafilm and stored in the dark in a humid chamber at 22°C. In other experiments (not among the ones listed in Table 2), the aim was solely to look for the presence of CSMs, not to monitor the genetic structure of groups. In those cases, a portion of a soil or dung sample (approximately 500 mg) was shaken thoroughly in 1 ml sterile phosphate buffer, and 100 µl of the mixture was inoculated on a PBA plate along with a thick suspension of *Klebsiella aerogenes*. Plates were monitored intermittently from the second day onwards. The CSM fruiting bodies that had formed were observed using an inverted microscope (Leica DM-IRB) or a stereomicroscope (ZEISS Stemi 2000-C) and photographed directly. For identification, spore masses were picked up with a sterile needle, plated on growth medium at low density, and the resulting clones subcultured on fresh PBA plates with *Klebsiella aerogenes*. The tentative identifications reported here are based on published keys [45].

#### Estimating Genetic Heterogeneity

Spores from individual fruiting bodies that had developed from soil or animal dung samples that had been transferred to non-nutrient (PBA) agar or, in some cases, to agar that contained only bacterial nutrients (SM/10 agar) were picked up with a fine needle and suspended in sterile distilled water. Fruiting bodies that had formed on the agar itself were never used (there were hardly any at the time of observation). A dilute suspension of spores was mixed with *Klebsiella aerogenes* and inoculated on 20-cm SM/10 agar plates and incubated at 22°C in the dark. Well-separated plaques were seen on plates after 2 to 3 days. Single plaques were picked individually with sterile micropipette tips, suspended in 100 µl sterile water, and stored at 4°C until use. In this way, 15 to 25 sub-clones generated from spores belonging to one fruiting body were collected and their DNA analyzed.

#### DNA Isolation

DNA was isolated from vegetative amoebae according to published protocols [39]. Cells were lysed using 2% NP40 and nuclei were separated by centrifugation at 8,000 rpm for 10 min at 4°C. Nuclei were then suspended in a lysis buffer that was preheated to 70°C, incubated at 70°C for 5 min, and then at 50°C for 1 h. RNase and proteinase K were added for 1 h each. Proteins were extracted with phenol-chloroform and DNA precipitated from the aqueous phase with a double volume of ice-cold ethanol. The pellet was rinsed twice in 70% ethanol, dried briefly, and then dissolved in 50 µl sterile water. DNA purity and approximate amount was assessed after electrophoresis on a 0.8% agarose gel. All the isolated DNA samples were further purified using the PhytoPure resin provided in a plant DNA extraction kit (Amersham Inc., USA). Use of purified high-quality DNA was found essential for reproducibility of random amplification of polymorphic DNA (RAPD)-based DNA variation data.

#### Random Amplification of Polymorphic DNA

RAPD analysis was done using a modified method that has been described previously [28]. Purified genomic DNA samples were amplified using decamer arbitrary primers (Operon Technologies, USA). Each PCR reaction was carried out in a 15 µl reaction volume that contained 20 ng DNA as a template, 0.16 µM of primer, 1 U Taq DNA polymerase, 1× Taq buffer (having standard 1.5 mM MgCl<sub>2</sub>), and 150 µM of each dNTP and 1× additive which was developed in the laboratory. The RAPD amplification profile comprised an initial denaturation step of 95°C for 3 min, followed by 36 three-step cycles of denaturation at 94°C for 45 s, primer annealing at 36°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 5 min. Amplified RAPD products were resolved on a 1% agarose gel in 0.5× TBE buffer (standard Tris borate EDTA buffer, pH8.0; Tris–HCL base 5.4 g, boric acid 2.75 g, 0.5 M EDTA 2 ml/l) using a standard electrophoresis apparatus. Electrophoresis was carried out at constant voltage (~3–4 V/cm gel length). Gels were stained with ethidium bromide and amplified DNA bands photographed under UV light. Special precautions were taken to ensure the reliability of the RAPD data. These included: (a) use of only high-quality, purified DNA samples for analysis; (b) initial testing of reproducibility of amplification for the selected primers on at least two PCR machines (I-Cycler, BioRAD, USA or thermocycler PTC-200, MJ Research, USA); and (c) generation of RAPD data for all the samples for a minimum of two times, followed by scoring of only reproducible, well-resolved/amplified RAPD fragments. All chemicals used were of standard laboratory grade (from Sigma) and agar (HIMEDIA).

## Statistical Analysis

The RAPD data give a lower limit to the clonal diversity within a fruiting body. We have used two different approaches to estimate the total number of clones in it (Table 2; see “Discussion” for remarks on the validity of the approaches). Both approaches assume that clones are equally represented in the spore mass and that spores are sampled at random. Suppose the spore mass consists of  $S$  cells made up of  $C$  clones, each having  $S/C$  cells; a sample of  $n$  spores yields  $m$  clones ( $n \ll S$ ; the largest value of  $n$  is 15, while  $S$  is likely to be a few thousand at least). Given just this, can we say anything about how many clones may have been missed out (i.e.,  $C - m$ )? Yes, if we are allowed certain additional assumptions. For the sake of simplicity, we refer to the  $m$  clones that were detected as belonging to class I and the undetected  $C - m$  clones to (a hypothetical) class II. The assumption of equal representation of all clones and the existence of two complementary classes makes it possible to treat the relative frequencies of the two clones as probabilities. The probability that a spore belongs to class I is  $p = m/C$  and the probability that it belongs to class II is  $q = (C - m)/C$ , where  $p + q = 1$ . We can obtain rough estimates for  $p$  and  $q$  (and so for  $C$ ) from our knowledge of  $n$  and  $m$ .

- Approach 1: The probability that none of  $n$  spores picked at random belong to class II is  $(1 - q)^n$ , implying that the probability that at least one of those  $n$  spores belongs to class II is  $1 - (1 - q)^n$ . Because none of our  $n$  spores does, we can say that  $[1 - (1 - q)^n] \leq 1/n$  and use the equality to estimate  $q$ , and therefore  $C$ . This will be a conservative estimate in the sense that  $C$ , and therefore  $q$ , could be much larger and still lead to an absence of class II spores from the sample of  $n$ . A better estimate will result if we reason that a class II spore ought to have been picked up unless the probability of its being represented at least once in the sample of  $n$  had been lower than some pre-assigned threshold. We have taken the threshold to be as high as 0.95 and solved the equation  $1 - (1 - q)^n = 0.95$  for each sample size  $n$  to get  $q$ ,  $m/C$  and  $C$  (it turns out that  $C$  is essentially the same as  $m$  up to an assumed threshold of 0.5).
- Approach 2: What follows is a simplified version of an elegant stochastic formulation kindly provided by an anonymous referee. Suppose we had repeatedly drawn independent samples of spores from the same spore mass and calculated the mean number of clones contained in them.

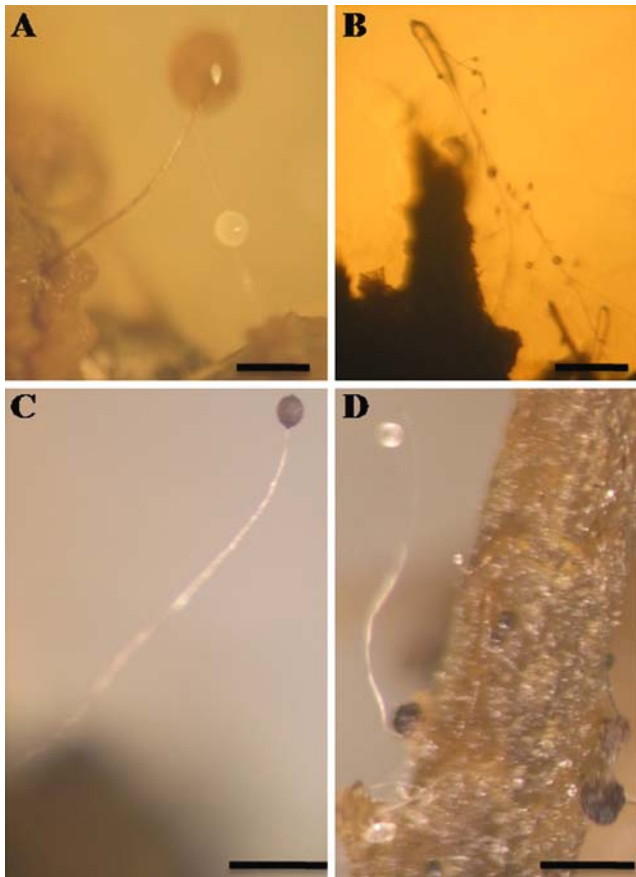
Based on a sample of  $(n - 1)$  spores, all belonging to class I, let us say the mean had been  $m_{n-1}$ . After sampling  $n$  spores, the corresponding mean,  $m_n$ , would either remain unchanged (in case the  $n$ th spore too came from a preexisting clone, which would happen with a probability  $m_{n-1}/C$ ) or increase by 1 (in case the  $n$ th spore came from a new clone, which would happen with a probability  $1 - m_{n-1}/C$ ). In short, the difference  $(m_n - m_{n-1})$  would either be 0 (with a probability  $m_{n-1}/C$ ) or 1 (with a probability  $1 - m_{n-1}/C$ ). Thus, we may write  $m_n - m_{n-1} = 0 \times (m_{n-1}/C) + 1 \times (1 - m_{n-1}/C)$ . This results in the recurrence relation  $m_n = m_{n-1} (1 - 1/C) + 1$ , which is easily solved to yield  $m_n = C \times (1 - K^n)$  where  $K$  stands for  $1 - 1/C$ . Now, if we take the number of clones observed by us, namely  $m$ , as a measure of the true mean  $m_n$ , the equation can be used (though not in closed form) to derive  $C$  from  $m$ .

## Results

## CSMs from Large Mammal Dung

Dung was distributed in the form of patches consisting of many dung pats. Plated samples were monitored after 2 to 8 days of incubation for the presence of fruiting bodies. The ones that were seen earliest came from plates that had been pre-spread with bacteria or where the dung had been deposited on nutrient agar. However, we could also see fruiting bodies develop on non-nutrient (PBA agar) plates. Typically, it took 2–3 days for a fruiting body to be seen on nutrient plates and a day longer on non-nutrient plates. The tiger scat samples were exceptional in that fruiting bodies were visible the very next day (within 24 h) on all three incubation media. In comparison, the elephant dung samples took much longer, about 6–8 days. Presumably, in those cases where no bacteria were added, the dung contained enough nutrients for endogenous bacteria to grow and, in turn, to allow CSM amoebae to grow and form aggregations. Many dung samples yielded up to two genera and five species of CSMs (“genera” in the commonly used nomenclature, a DNA-based phylogeny [47], suggests inconsistencies in the traditional classification). At times, CSM fruiting bodies belonging to two different genera developed from neighboring regions (3–4 mm apart) from the same sample (Fig. 1a).

*Dictyostelium giganteum* and *Dictyostelium purpureum* were commonly seen; *Dictyostelium discoideum*, *Dictyos-*



**Figure 1** CSM fruiting bodies seen on animal dung samples that were not treated in any way. **A** *Dictyostelium purpureum* and *Dictyostelium* spp. from tiger scat. **B** *Dictyostelium giganteum* and *Polysphondylium violaceum* from gaur dung. **C** *Dictyostelium purpureum* from spotted deer pellet. **D** *Dictyostelium giganteum* from elephant dung. The scale bar represents 1 mm

*telium minutum*, *Dictyostelium macrocephalum*, *Dictyostelium rosarium*, *Dictyostelium polycephalum*, *Polysphondylium pallidum*, and *Polysphondylium violaceum* were also observed. An interesting species that showed up in many isolates showed bifurcating slugs and fruiting bodies. It remains to be identified and for the present will be referred to as *Dsp* (*bifurcating*). Many fungi and nematodes were also observed, as were occasional myxobacterial fruiting bodies. The tiger scat sample must have contained a large number of CSM propagules because large fruiting bodies were seen within 24 h. Spotted deer samples yielded mainly *Dictyostelium purpureum* fruiting bodies that were exceptionally long (0.5–1.5 cm). Elephant dung yielded a small number of fruiting bodies belonging to *Dictyostelium giganteum*, *Dictyostelium purpureum*, *Dsp* (*bifurcating*), as well as one more unidentified *Dictyostelium* species. Variants of “standard” phenotypes were observed in the gaur and porcupine dung samples, but only on plates containing the primary isolates. They included aggregates with many tips, fruiting bodies with spiral-shaped stalks, and unusually long-

stalked fruiting bodies (and are not described further in the present study). These observations are presented in Figs. 1 and 2 and Table 1. Fruiting bodies that developed on dung were distinctly larger and looked more vigorous than those that were seen in the soil isolates (both on nutrient and non-nutrient agar and without any added bacteria). Based on the number of fruiting bodies seen, our preliminary impression was that the dung samples contained a higher density of propagules. *Dictyostelium mucorides* and *Dictyostelium tenue* were found only in soil samples, not on dung.

#### Genetic Heterogeneity

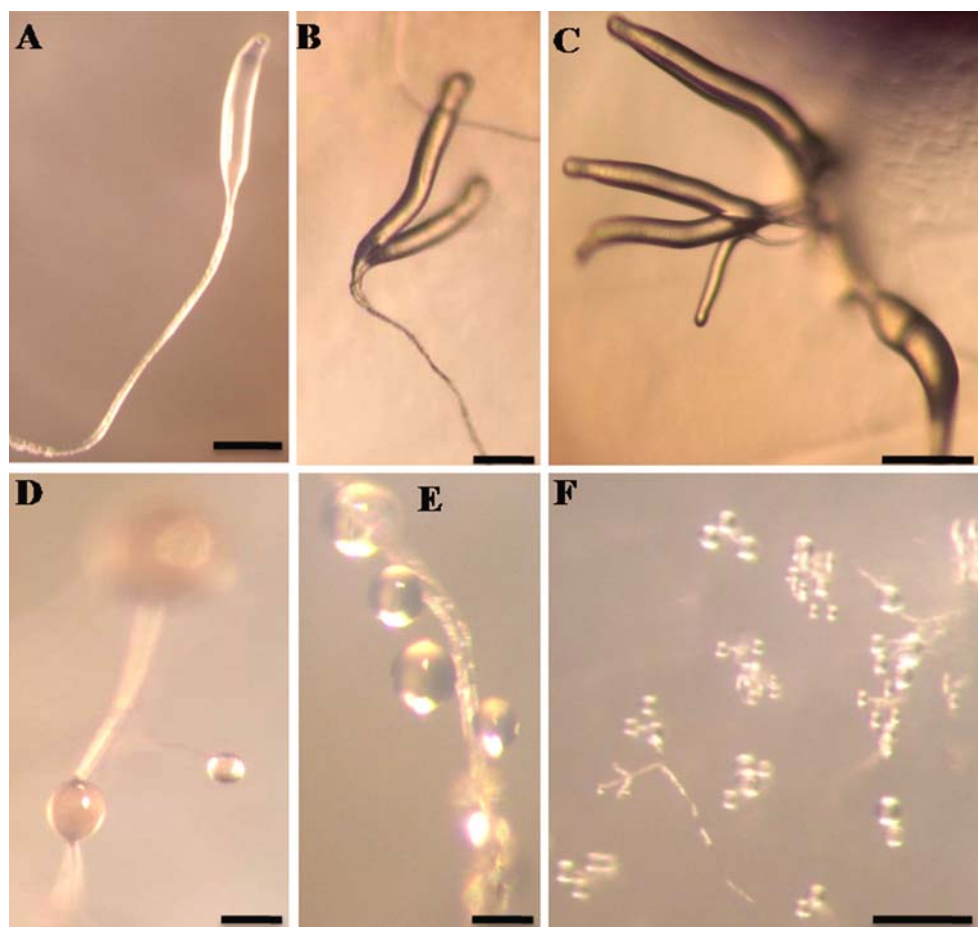
RAPD has been successfully used for answering various issues in bacterial, plant, and animal study systems and has been shown to be a useful tool for monitoring genetic differences [32, 38]. Although RAPD markers have many advantages over others, the reliability of the method has been questioned. Thanks to the special precautions that we took while preparing genomic DNA, and during subsequent analysis, the RAPD banding patterns were highly reproducible. Also, because CSMs are haploid, the issue of relative dominance between alleles does not arise: band intensity differences (which can be a problem in diploid organisms because of competitive amplification) do not affect our results. Our interest is restricted to the issue of clonal identity and is easily addressed.

The results of experiments in which we looked for chimerism in fruiting bodies of *Dictyostelium giganteum* and *Dictyostelium purpureum* are summarized in Table 2 (other species remain to be tested). Nine out of 11 *Dictyostelium giganteum* and six out of six *Dictyostelium purpureum* fruiting bodies that were examined, meaning 15 out of 17 fruiting bodies in all, were chimeras (eight of those fruiting bodies were from animal dung and nine from soil). The minimum number of distinct genotypes in a single fruiting body was three to seven (animal dung) and one to nine (soil). Apart from the last fruiting body in Table 2, both approaches yielded numbers that were more or less the same as the actual counts. The exception arises, that too only on using approach 2, because the actual number of clones is likely to be much larger than those observed when the observed number is almost the same as the number of spores sampled, but not when it is much smaller (mathematically, the  $C$  versus  $m_n$  curve rises sharply and tends toward infinity—in practical terms, toward the total number of spores—as  $m_n$  approaches its maximum possible value, which is  $n$ ).

#### Comparison with Previous Isolates from India

This is shown in Table 3. *Dsp* (*bifurcating*) and the unidentified strains are the two potentially new species found in this study; on the other hand, *Dictyostelium aureo-*

**Figure 2** CSM multicellular stages obtained after generating clonal subcultures from animal dung isolates. **A** Slug of *Dictyostelium giganteum* with a long stalk. **B** Bifurcating slug of *Dictyostelium* species. **C** Many slugs emerging from one aggregate. **D** CSM species with branched fruiting body and purple sori. *Dictyostelium rosarium* (**E**) and *Dictyostelium polycephalum* (**F**) fruiting bodies. The scale bar represents 1 mm



*stipes*, *Dictyostelium sphaerocephalum*, *Dictyostelium vinaceo-fuscum*, and *Acytostelium subglobosum* were found earlier by others but not by us.

## Discussion

### Large Mammals as CSM Dispersal Agents

The yak dung isolate shows that CSMs can be found in habitats at altitudes up to 5300m. To our knowledge this is highest reported so far (Hagiwara [20] found *D. brefeldianum*, since known to be the same as *D. mucoroides* Raper, at an altitude of 4680m, also in the Himalayas). In Mudumalai, CSMs were obtained from the dung of obligate herbivores as well as carnivores. The following reasons make us believe that the mammals must have transported CSM propagules. (1) The dung samples were collected soon after deposition, though (except for the spotted deer and yak samples) we cannot completely rule out the possibility that the CSMs followed quickly but later—say via an arthropod vector—and were not present in the animal gut. (2) CSMs have been found in animal dung previously; indeed, at one time, these organisms were

considered to be coprophilous. By making collections from fecal samples that were directly expelled into collection vials, Stephenson and Landolt [48] found that spores, and less likely, amoebae, can be dispersed after passage through the gut of many vertebrates including small mammals. (3) The fact that a single dung pellet contains different clones (and species) is difficult to reconcile with their presence being on account of passive transfer by an insect. (4) Lastly, even if that were to be the case, it would not affect our major conclusion, namely, that polyclonal social groups co-occur commonly in nature.

It is easy to understand how a grazing animal can pick up CSMs along with grass and soil, but we do not know whether a carnivore ingests them in the same way (cats and dogs are known to eat grass on occasion) or gets them indirectly by feeding on herbivores. Gaur and elephant browse as well as graze, but in terms of dispersal mechanisms, it would be of interest if one could show that they had picked up propagules from fruit or other plant structures which can contain CSMs ([35] and SS, unpublished). The areas over which these animals move vary from about 20 km<sup>2</sup> (spotted deer) to approximately 1,000 km<sup>2</sup> (elephant). The inference is that in addition to small mammals [48], large mammals too can be agents of long-distance dispersal of CSMs (though in terms

**Table 1** CSMs obtained from animal dung and soil

Source	Home range of animal (rough linear extent) (km)	Feeding habits	No. of independent samples analyzed	Cellular slime molds isolated
Spotted deer (Chital): <i>Axis axis</i>	4.5	Primarily grazer, browser (in dry season) also eats fruits, flowers, and fallen leaves	3	<i>Dpu, Dg, Dsp (bifurcating), Pv</i>
Tiger: <i>Panthera tigris</i>	15	Carnivore	1	<i>Dpu, Dg, Dd</i>
Elephant: <i>Elephas maximus</i>	32	Depending on season and habitat, either grazer or browser	2	<i>Dpu, Dg, Dsp (bifurcating), Dr</i>
Wild dog: <i>Cuon alpinus</i>	4.5–8	Carnivore	2	<i>Dpu, Dg</i>
Sambar: <i>Cervus unicolor</i>	14	Depending on season and habitat, either grazer or browser	2	<i>Dpu, Dg, Dma</i>
Porcupine: <i>Hystrix indica</i>	15	Tubers, roots, fruit	2	<i>Dg, Dpo</i>
Yak: <i>Bos grunniens</i>	50	Mainly grazer, also known to eat mosses and lichens	1	<i>Dg, Dsp (bifurcating), Dd, Dr, Dmi</i>
Gaur (Bison): <i>Bos gaurus</i>	20	Mainly browser, some reports of eating tree bark and grasses	2	<i>Dg, Dsp (bifurcating), Dr, Pv, Pp</i>
Panther: <i>Panthera pardus</i>	15	Carnivore	Many (count not kept)	Diverse, not identified
Hyena: <i>Crocuta crocuta</i>	5–10	Scavenger (carnivore)	Many (count not kept)	Diverse, not identified
Barking deer: <i>Muntiacus muntjac</i>	5	Fruits, buds, freshly sprouted leaves, seeds, young grass	Many (count not kept)	Diverse, not identified
Soil (from different areas in the Mudumalai forest range)	–	–	10	<i>Dpu, Dg, Dsp (bifurcating), Dr, Dd, Dma, Dpo, Dmi, Dmu, Dt, Pv, Pp</i>

*Dsp (bifurcating)* refers to an unnamed species, tentatively classed as *Dictyostelium*, in which aggregates forms aerial slugs that split up while moving (see Fig. 2b)

*Dpu* *D. purpureum* Olive, *Dg* *D. giganteum* Singh, *Dd* *D. discoideum* Raper, *Dr* *D. rosarium* Raper and Cavender, *Dma* *D. macrocephalum* Hagiwara, *Dpo* *D. polycephalum* Raper, *Dmi* *D. minutum* Raper, *Dmu* *D. mucoroides* Brefeld, *Dt* *D. tenue* Cavender, Raper, and Norberg, *Pv* *P. violaceum* Brefeld, *Pp* *P. pallidum* Olive

of the range of dispersal, they cannot compete with migratory birds [52]). Dispersal by air, water, insects, or nematodes would likely involve small numbers of spores at a time. On the other hand, given how large animals graze, one might expect a great many CSM groups to be dispersed en masse by them—both to the same place (within one dung pat) and after being thoroughly mixed in the digestive tract. Even if there is a selective advantage associated with forming clonal social groups, the mode of dispersal may decide whether the next generation is also spent in a clonal group or, unavoidably, in a multi-clonal group (unless kin groups segregate from each other). It would be interesting to see if the various agencies taken together lead to a roughly uniform probability of spore dispersal at all distances or whether dispersal involves a small number of propagules over very short distances (e.g., via insects or nematodes) and much larger numbers over very long distances (e.g., via large animals).

#### Genetic Diversity in Fruiting Bodies

The data generated in this study point to the widespread occurrence of multi-clonal groups in quasi-natural CSM

social groups derived from both undisturbed forest soil and animal dung samples, confirming the finding from a preliminary study [27]. These findings may be debated on two grounds, i.e., the status of analyzed fruiting bodies being developed in vitro rather than being in situ isolates and/or the use of RAPD as an indicator of the genetic makeup of CSMs. We have taken precautions that amply address these issues. Practical considerations made it necessary to collect the dung samples from the field and return to the laboratory. Although directly derived from field samples, none of the fruiting bodies analyzed in the study were actually formed under natural conditions. However, the way in which the samples were handled—in particular, when they were directly transferred to agar after collection without disturbing them and without added bacteria—would have favored their formation just as in nature (under food starvation). We are confident that at least those conditions can be considered to act as excellent proxies for the natural environment.

Similarly, RAPD analysis was done with care to ensure the reproducibility of data (see “Methods”). In our hands, RAPD worked as a simple and reliable means of assaying genetic differences; in general, band patterns with a given

**Table 2** Majority of the fruiting bodies formed under quasi-natural conditions (15 of the 17 tested) are multi-clonal

Fruiting body (species and designation)	Source	No. of RAPD primers used	No. of spores monitored ( <b>n</b> )	No. of clones found ( <b>m</b> )	Estimated total number of clones ( <b>C</b> )	
					Approach 1	Approach 2
<i>D. purpureum</i> (SD-1)	Spotted deer (non-nutrient agar)	7 (5 informative)	8	4	6	5
<i>D. purpureum</i> (SD-3)		2 (2 informative)	12	5	6	5
<i>D. purpureum</i> (SD-4)		7 (5 informative)	12	6	8	7
<i>D. purpureum</i> (SD-5)		8 (8 informative)	10	5	7	6
<i>D. giganteum</i> (SD-8)		2 (2 informative)	13	5	6	5
<i>D. purpureum</i> (WD-1)	Wild dog (non-nutrient agar)	4 (4 informative)	12	7	9	9
<i>D. giganteum</i> (E)	Elephant (non-nutrient agar)	6 (3 informative)	10	3	4	3
<i>D. giganteum</i> (F)		7 (7 informative)	8	4	6	4
<i>D. giganteum</i> (18)	Soil speck from 50th ha. SM/ 10	3 (3 informative)	10	5	7	6
<i>D. giganteum</i> (20)	agar (no exogenous bacteria)	3 (3 informative)	7	4	6	5
<i>D. giganteum</i> (21)		4 (3 informative)	9	5	7	6
<i>D. giganteum</i> (23)		5 (5 informative)	15	7	9	8
<i>D. giganteum</i> (24)		6 (0 informative)	4	1	2	1
<i>D. purpureum</i> (B)	Soil speck from 46th ha. SM/10	4 (4 informative)	9	3	4	3
<i>D. giganteum</i> (D)	agar (no exogenous bacteria)	5 (5 informative)	7	4	6	5
<i>D. giganteum</i> (5B-1)	Soil speck from 5th ha. (non-nutrient agar)	7 (0 informative)	5	1	2	1
<i>D. giganteum</i>	IISc campus soil on PBA plate, <i>Klebsiella aerogenes</i> added	4 (4 informative)	10	9	12	28

Hectare numbers refer to the 50-ha study plot. The fruiting bodies were genotyped using RAPD-PCR. “Informative” refers to a primer that yielded at least two different DNA band patterns from the clones that were analyzed. The last two columns contain independent estimates of the total number of clones in a fruiting body (see “Methods”) rounded off to the nearest whole number. When more than one value of **C** will do, the smallest has been chosen. The last fruiting body in the list pertains to an experiment in which soil was carefully transferred to an agar plate and subsequently moistened by gently adding 50  $\mu$ l of bacterial suspension

primer and DNA sample were reproducible (see Fig. 3, especially the comparison between F3 and F3D and F4 and F4D). Furthermore, as a final measure of caution, we were conservative in our comparisons and counted as different only those DNA amplicons that were well resolved and there was no ambiguity (for example, in Fig. 3, we classify F1 and F15 as the same pattern). All these measures ensured the reproducibility and reliability of the genetic diversity data that explicitly demonstrate the extensive chimerism in the CSMs.

The mere absence of polymorphism among a set of spores is no proof of clonality, and the observed numbers of distinct genotypes in a fruiting body (Table 2) can only provide lower limits. Apart from the small sample sizes and limited number of primers used being factors, we could examine only those clones that were found in the spore population (the genotypes that gave rise to stalk cells may or may not have been the same; in any case, they remain unaccounted for). On top of that, the statistical estimates that we carried out involved—purely for convenience—the drastic assumption that the number of spores belonging to each clone in a chimera is the same. This means that in Table 2, the values of **C** too are likely underestimates:

clones that were represented by small numbers of spores would have been missed out, however significant their presence for the group. It is known that a minority population of one genotype can exert a significant effect on the functioning of the social group. The observations of Filosa [14], Buss [8], and Ketcham and Eisenberg [30] are relevant to the situation in the wild and have been mentioned earlier. Bonner [4] lists several examples of such synergistic interaction in the older CSM literature. Though not pertaining to naturally occurring isolates, a spectacular example is the aggregation and subsequent sporulation of mutant cells that are unable to aggregate (and therefore develop further) on their own, but do so when wild-type cells added in a ratio of less than one part in 1,000 [24].

#### Implications for the Evolution of Social Behavior

The finding that *Dictyostelium giganteum* and *Dictyostelium purpureum* groups derived from animal dung are multi-clonal suggests that other species can form multi-clonal social groups too and is in accord with the speculations pertaining to mode of dispersal and genetic heterogeneity made above. Most fruiting body phenotypes were “normal,”

**Table 3** Comparison of CSMs isolated from soil and animal dung in different parts of India

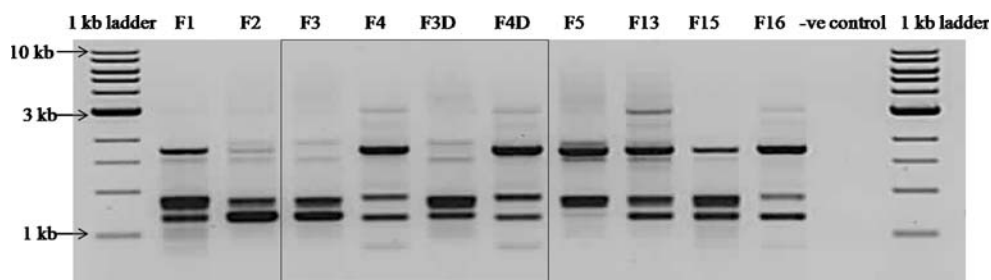
Sample source	Cellular slime mold isolate	Reference
Cultivated, uncultivated and rhizosphere soil from South India	<i>D. purpureum</i> , <i>D. giganteum</i> , <i>D. mucoroides</i> , <i>D. minutum</i> , <i>D. discoideum</i> , <i>P. pallidum</i> , and <i>P. violaceum</i>	[1]
Soil from West Central Himalaya and tropical forests in peninsular India	<i>D. purpureum</i> , <i>D. giganteum</i> , <i>D. mucoroides</i> , <i>D. minutum</i> , <i>P. pallidum</i> , and <i>P. violaceum</i> . <i>D. aureo-stipes</i> , <i>D. sphaerocephalum</i> , <i>D. polycephalum</i> , <i>D. tenue</i> , <i>D. vinaceo-fuscum</i> , <i>A. subglobosum</i>	[9]
Soil from Lucknow	<i>D. mucoroides</i> , <i>P. violaceum</i> , <i>D. sphaerocephalum</i>	[42, 44]
Soil (from areas in Mudumalai forest)	<i>D. purpureum</i> , <i>D. giganteum</i> , <i>D. mucoroides</i> , <i>D. minutum</i> , <i>P. pallidum</i> , <i>P. violaceum</i> , <i>Dsp (bifurcating)</i> , <i>D. discoideum</i> , <i>D. polycephalum</i> , <i>D. rosarium</i> , <i>D. macrocephalum</i> , <i>D. tenue</i>	This study
Animal dung	<i>D. purpureum</i> , <i>D. giganteum</i> , <i>D. minutum</i> , <i>P. pallidum</i> , <i>P. violaceum</i> , <i>Dsp (bifurcating)</i> , <i>D. discoideum</i> , <i>D. polycephalum</i> , <i>D. rosarium</i> , <i>D. macrocephalum</i>	This study

*D. aureo-stipes*, *D. sphaerocephalum*, *D. vinaceo-fuscum*, and *A. subglobosum* were seen by Cavender and Lakhnupal and not by us. *Dsp (bifurcating)* was not reported in any of the earlier studies. The species and genus names will have to be treated as traditionally accepted but subject to change in the future. A molecular phylogeny shows that both “Dictyostelium” and “Polysphondylium” species are sometimes found in the same major clade (groups) and sometimes in different clades [46]

that is, as expected on the basis of descriptions of studied wild types [45]. However, some appeared unusual even when they belonged to a recognizable species. The extent and role of the variant phenotypes seen in these isolates needs proper quantification. Rai and Tewari [43] have speculated that phenotypically aberrant CSMs may be adapted for special modes of dispersal. In Filosa’s [14] study referred to earlier, the spores were functionally heterogeneous in the sense that they gave rise to variant structures after aggregation. Interestingly, when variant and wild-type amoebae were mixed, the chimeric fruiting bodies that were formed had the wild-type phenotype. Bonner [4] has pointed out that in naturally occurring chimeras, different phenotypes may be able to complement each other in a functional sense. He goes on to add that the aggregation of different genotypes in an aggregate resembles the coming together of diverse nuclei in the heterokaryon of Ascomycetes, which, as Haldane [22] hypothesized, could be a primitive analogue of sexuality. This could be one explanation for the co-existence in nature of different genotypes with different phenotypes.

The high incidence of genetic heterogeneity within social groups found by us pertains to *Dictyostelium giganteum* and *Dictyostelium purpureum* in South India, whereas much of the literature is concerned with *Dictyostelium discoideum* in North America. This may account for some of the differences in what we see. One study on *Dictyostelium discoideum* [16] raised the likelihood of extensive multi-clonality under natural conditions, whereas others [18, 19] indicate that clonal social groups may be the norm. The prudent inference to draw would be that the natural aggregation–sporulation–dispersal–germination life cycle of the CSMs can involve living as members of both clonal and multi-clonal groups. Therefore, the evolutionary forces that molded their cooperative life style must have involved both situations [26].

Besides reporting on the presence or absence of clonal or polyclonal CSM social groups in nature, laboratory-based studies have monitored cellular behavior within groups and tried to estimate the extent to which cooperative behavior is correlated with cell-to-cell differences in genotype or phenotype. Specifically, an attempt has been to correlate some property of an amoeba with its propensity to



**Figure 3** Genetic diversity within a fruiting body: Lanes marked *F1*, *F2*, *F3*, *F4*, *F5*, *F13*, *F15*, and *F16* show RAPD profiles of different clones derived from spores from a single fruiting body using the OPAD-4 primer. *F3D* and *F4D* are from independently isolated DNA

samples from the same clone as *F3* and *F4*, respectively. Note the identity in patterns between lanes *F1* and *F15* and the obvious differences between all others

differentiate into a stalk or spore cell. Developmental studies, again mainly with *Dictyostelium discoideum*, have shown that pre-aggregation amoebae that are of the same genotype and are raised under the same conditions differ in several aspects that correlate with post-aggregation fate [34]. For example, amoebae that are raised in a glucose-poor medium, are harvested early in the cell cycle, or contain enhanced levels of cellular calcium show a tendency to differentiate into stalk cells when mixed with amoebae that are grown in a glucose-rich medium, are at a late stage of the cell cycle when starved, or have lower calcium levels, respectively. A point to note is that in all such experiments, it is the phenotypic status of an amoeba relative to other amoebae that seems to matter. One interpretation of these observations is that there are inter-individual differences in traits (“qualities”) related to fitness. All amoebae compete to become spores, and natural selection working at the level of the individual cell has led to high-quality amoebae winning against low-quality amoebae [2]. The quality of a cell depends on its genotype and prior history; besides that, it can have a stochastic component. When mixing experiments are carried out with cells from different clones, there are genotype-associated effects that bias the spore-forming tendency of a cell.

When pairs of *Dictyostelium discoideum* strains isolated from the wild are mixed as amoebae, more often than not, one of them contributes disproportionately to forming spores [50], and a similar observation has been made in *Dictyostelium giganteum* [28]. There are mutants of *Dictyostelium discoideum* that sporulate with a better efficiency than their wild-type parent when mixed with it [12]. It has been known for a long time that amoebae belonging to different species can sort out from each other after aggregating together [3], as can natural isolates of the same species [28]. Such findings, and observations suggesting that discrimination can increase with genetic distance, have been interpreted in terms of association preferences between genetically similar individuals. These could conceivably work as a kin recognition mechanism and therefore permit kin selection to operate [33, 37, 41]. The high level of within-group genetic diversity reported in this study makes the operation of kin selection more difficult than it would be in a clonal group (but not impossible). At the same time, potentially confounding factors should be kept in mind before using kin selection as the sole explanation for the apparent altruism displayed by those amoebae that die.

For one thing, almost all published studies concentrate on the efficiency of sporulation. Besides non-obvious fitness benefits that might be derived from stalk cells, they ignore other components of fitness in the life cycle [8]. Then there is the striking observation that the outcome of mixing three strains of *Dictyostelium giganteum* at a time is not predictable in any simple way from the outcome of

pairwise mixes. Thus, one can order genotypes in a linear hierarchy of sporulation efficiencies [17, 28], but when three genotypes are mixed, the hierarchy may be evened out. Such findings point to the existence of complex interactions between cells that depend on factors beyond the extents to which they are related [28] and reinforce the need to be cautious in interpreting data. For instance, when a strain that does relatively better at sporulation when mixed with a wild type (or another strain) is termed a “cheater,” one should bear in mind that the terminology refers to an outcome that depends on the specific context. A recent study with *Dictyostelium discoideum* makes the point effectively. In terms of relative sporulation efficiencies in pairwise mixes, a mutant can counteract a previously isolated *Dictyostelium discoideum* mutant that outcompetes the wild type; but the new mutant does no better than the wild type when mixed with it [31]. Lastly, as Haldane [21] pointed out long ago, ecological factors—specifically how groups disperse and are reconstituted in each life cycle—can be critical for the natural selection of “altruistic” behavior. Recent laboratory experiments with mixtures of synthetic *Escherichia coli* strains show this nicely: cells belonging to a genotype that does worse than another within a group can persist, even increase in frequency, if they contribute sufficiently to the productivity of the group as a whole and propagules disperse at very low density [10]. Thus, when it comes to groups, the manner in which natural selection acts depends strongly on the internal structure and dynamics of the group [46]. Finally, the fact remains—as Ketcham and Eisenberg [30] state with regard to CSMs—that “competition has not only failed to eliminate different species from the community but has failed to eliminate clonal diversity from within species”. Therefore, they go on, “Previous explanations of species coexistence need reevaluation”. To this we might add, so do explanations for the coexistence of different genotypes belonging to the same species.

The asexual life cycles of the myxobacteria and the CSMs represent a remarkable example of convergent evolution: both are soil microorganisms, have a unicellular feeding phase during which cell numbers increase, aggregate after starvation, and form fruiting bodies in which viable spores are held up by a stalk. In both groups, sporulation is accompanied by the death of other cells. When different strains of *Myxococcus xanthus* are mixed, they form chimeric fruiting bodies in which one genotype can form a disproportionate number of spores relative to the other [13]. Similar to what we find in the case of CSMs, natural isolates of *Myxococcus xanthus* show a great deal of genetic diversity: as many as 22 distinct genotypes were found in 78 samples collected from a small 16×16-cm plot [54]. However, the level of genetic diversity in groups of social organisms need not always be high: a study on the colonial coral *Acropora millepora* found that



just 2–5% of 984 colonies sampled from two different locations were chimeras [40].

### What Does This Say About CSM Ecology?

When taken along with older observations, the findings reported here extend our knowledge of the ecology of the CSMs, specifically with regard to the spatial structure of natural populations and how it relates to dispersal and genetic relatedness. Because large mammals can ingest food from a substantial area, clonal groups of spores can become mixed and get deposited in the same dung pat in sufficient proximity to give rise to polyclonal groups in the next generation. As we have seen, a 250  $\mu\text{m}$ –1 mm speck of soil can contain propagules belonging to different genotypes of the same species or even different species. Thus, in the case of the cellular slime molds, “everything is everywhere” [36] over fairly large spatial extents: the ranges covered by large mammals, not to mention birds [52], are vast in relation to typical aggregation territory sizes of  $\sim$ 1 mm. On top of that, individuals belonging to different genotypes go through their life cycles as members of the same social group. The proximal factors that mediate their co-existence and how they impinge on the evolution of social behavior in this group of microorganisms remains to be fully understood.

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PERSPECTIVE

## Social selection and the evolution of cooperative groups: The example of the cellular slime moulds†

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In social selection the phenotype of an individual depends on its own genotype as well as on the phenotypes, and so genotypes, of other individuals. This makes it impossible to associate an invariant phenotype with a genotype: the social context is crucial. Descriptions of metazoan development, which often is viewed as the acme of cooperative social behaviour, ignore or downplay this fact. The implicit justification for doing so is based on a group-selectionist point of view. Namely, embryos are clones, therefore all cells have the same evolutionary interest, and the visible differences between cells result from a common strategy. The reasoning is flawed, because phenotypic heterogeneity within groups can result from contingent choices made by cells from a flexible repertoire as in multicellular development. What makes that possible is phenotypic plasticity, namely the ability of a genotype to exhibit different phenotypes. However, co-operative social behaviour with division of labour requires that different phenotypes interact appropriately, not that they belong to the same genotype, or have overlapping genetic interests. We sketch a possible route to the evolution of social groups that involves many steps: (a) individuals that happen to be in spatial proximity benefit simply by virtue of their number; (b) traits that are already present act as preadaptations and improve the efficiency of the group; and (c) new adaptations evolve under selection in the social context—that is, *via* interactions between individuals—and further strengthen group behaviour. The Dictyostelid or cellular slime mould amoebae (CSMs) become multicellular in an unusual way, by the aggregation of free-living cells. In nature the resulting group can be genetically homogeneous (clonal) or heterogeneous (polyclonal); in either case its development, which displays strong cooperation between cells (to the extent of so-called altruism) is not affected. This makes the CSMs exemplars for the study of social behaviour.

### Introduction

Understanding the structure and functioning of cooperative groups from an evolutionary point of view requires that we pay heed to the role played by the social environment of an

individual. The social environment is determined by other individuals, which means that it can evolve along with the group. All members of the group constitute the social environment of any one of them, and a trait can be both the target of selection (when viewed in a member of the group) and its agent (when it influences the strength of selection on other members).

This leads to the concept of social selection, in which individual phenotype, and so individual fitness, is meaningful only in a given social context. Therefore the phenotype is ‘non-autonomous’: it depends on traits exhibited by other

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### Insight, innovation, integration

An analysis of cooperative behaviour in the cellular slime mould amoebae (CSMs) offers insights into the evolution of social groups (at one end) and multicellular development (at the other). In the CSMs one can verify experimentally that the phenotype of an amoeba depends both on its own genotype and on the phenotypes of other amoebae: every individual in a

group forms part of every other individual's environment. Thus the notion of individual phenotype is meaningful only within a clearly defined social context. Rather than shared genes, appropriately interacting phenotypes are the essential requirement for group life. These insights may be useful for understanding abnormal development as well.

individuals, whether those traits are similar or dissimilar to the one being monitored in the focal individual.<sup>1,2</sup> For non-autonomy to be meaningful, the individual phenotype must be flexible or plastic, *i.e.*, capable of being influenced by the environment. An extreme case of plasticity is when different individuals of the same genotype give rise to different phenotypes in the same environment. Individual and group behaviour in the cellular slime moulds (CSMs) illustrate these concepts nicely, and also provide useful insights into the evolutionary basis of social behaviour in groups, including multicellular embryos—generally.

We first lay the groundwork by explaining our use of ‘social selection’ and ‘superorganism’; ‘non-autonomy’, ‘niche construction’ and ‘phenotypic plasticity’, terms that are relevant in the context of social selection, are introduced next. They are linked as follows: natural selection can act on phenotypes that depend on interactions with other phenotypes and so cannot be considered in isolation (social selection); social selection can take place in tightly organised groups of the same species (superorganism); the ultimate superorganism is the multicellular embryo; differentiation during multicellular development depends on reciprocal phenotype-moulding interactions between different cell types (non autonomy, niche construction); and behind it all is the capacity of single genotypes to exhibit different phenotypes (phenotypic plasticity). This will turn out to be somewhat discursive but should help the reader to appreciate that significant concepts have not always been used consistently, but for a good reason: it can help not to push definitions too hard and to retain a certain degree of elasticity in usage.

The second part sketches a plausible route for the evolution of group-level traits that begins with a purely physical advantage of increased size. Preadaptations reinforce those advantages by bringing into play similar traits in all members of the group. Next, group effects are reinforced *via* social selection for newly evolved reciprocal interactions between group members leading to division of labour. The examples that we list in support under these heads are mostly drawn from the social insects (the Volvocales are another well-studied case, not discussed here).<sup>3,4</sup> The third and longest part of the paper draws attention to the fact that observations made on the development (or, equivalently, social behaviour) of the Dictyostelid amoebae or cellular slime moulds (CSMs) support the evolutionary route just sketched. Traces of the steps listed above can be seen in present-day CSM species, and there is considerable evidence for the importance of reciprocal interactions between cells. We end the paper by raising the question whether the concept of social selection can be useful for the study of not only normal, but also pathological multicellular development.

### Social selection

Even a cursory survey of the literature shows that ‘social selection’ has meant different things to different people. The concept, though not the term, originates from Darwin’s pointing out that behavioural interactions between individuals of the same sex or different sexes (male-male competition, female choice) could lead to selection and phenotypic

evolution. Chance and Russell discussed social selection (again without using the term) in the context of allaesthetic behaviour, meaning ‘properties of animals (structures, colours, postures, movements, vocalisations, *etc.*) which produce behavioural effects on other animals’.<sup>5</sup> Wynne-Edwards<sup>6</sup> used ‘social selection’ to stand for an extension of sexual selection in which competition could operate within groups for any resource, not necessarily males. In a later work he made the important point that social competition resulted ‘in selection, . . . not so much for particular genes as for good all-round genotypes’, which meant that social selection had ‘a strong tendency to preserve genetic variance in the population’ (and preserve, as the context makes clear, phenotypic variation).<sup>7</sup>

Crook<sup>8</sup> distinguished between four kinds of social selection based on (a) inter-individual competition for resources essential for reproduction, (b) competition for mates, (c) competition for resources required in the course of parental care, and finally, (d) cooperation towards establishing a social unit that would benefit the offspring of the co-operators and possibly their kin. According to him ‘social selection’ could accommodate conventional individual-level selection with no direct interactions (except possibly with offspring), sexual selection in the sense of Darwin and selection based on shared interests, whether or not the interests were identical. West-Eberhard<sup>9</sup> discussed all this and more in a classic analysis. She drew attention to three features of social selection that are relevant for us: non-heritable variation, that can be significant for the maintenance of group life; facultative (as opposed to genetic) ‘switches’, that can lead to alternative behaviours in a social group, and, mutually exclusive tasks performed by the members of a group, which can follow from the above. Wolf *et al.*<sup>10</sup> extended the quantitative treatment of interacting phenotypes begun by Moore *et al.*<sup>1</sup> and used ‘social selection’ to mean ‘a process distinct from other forms of natural selection’ which is likely to occur ‘whenever individual fitness varies as a result of interactions with conspecifics’. For Nesse<sup>11</sup> social selection was a ‘subtype of natural selection in which choices made by other individuals influence [an individual’s] fitness and change gene frequencies’: selection was followed by evolution, implying that the phenotypes (*i.e.*, different behaviours) in question were associated with different genotypes.

A slight rewording of Nesse’s formulation provides what in our opinion is a broad and practical definition of social selection: it is a form of natural selection in which phenotypes of other individuals influence an individual’s fitness. *Ipsa facto*, social selection operates in groups, because the character of a group is defined by the extent to which its members interact. The interactions can be direct, mediated *via* another member of the group, or *via* the physical or (other) biotic environment. In the absence of any influence of one individual on another, ‘group’ would be just another name for a collection of individuals who, for all practical purposes, lead a solitary existence.

So social selection acts within groups, involves phenotypic interactions and has consequences for fitness. ‘Fitness’ can apply to the individual *vis-à-vis* the rest of the group or to the group in relation to other groups. Other qualifications can be

**Table 1** Examples of the means, not mutually exclusive, through which social selection can occur in variously constituted groups.

Is inter-individual social selection mediated <i>via</i>	Group consists of						
	Predator and prey	Potential mates	Parasite and host	Two species (symbiosis/mutualism)	Cellular slime moulds	Social insect colony	Metazoan embryo
interacting phenotypes?	Yes	Yes	Yes	Yes	Yes	Yes	Yes
shared reproductive interests?	No	Yes	No	Yes	Possible, not necessary	Possible, not necessary	Yes
different phenotypes?	Yes	Yes	Yes	Yes	Possible	Possible	Possible
different genotypes?	Yes	Yes	Yes	Yes	Possible	Possible	No

made as applicable in specific situations. Our emphasis is on phenotypic interactions between individuals within groups, whether or not they are accompanied by a ‘convergence of interests among group members’.<sup>9</sup> Table 1 outlines the circumstances in which social selection can operate and the nature of the relevant group. Except for brief allusions, the rest of this paper does not deal with sexual selection or interactions between members of different species, though both are important topics, the latter particularly so for behaviour and multicellular development.<sup>12</sup>

### The superorganism

Darwin spent a considerable portion of *The Origin of Species* wrestling with the problem of how the neuter state might have evolved in social insect workers: because they did not reproduce, even if selection acted on them, there could be no direct consequences for evolution.<sup>13</sup> He got around the difficulty by comparing the queen (with her mate(s)) and the neuters to the germ line and soma: ‘natural selection, by acting on the fertile parents, could form a species which should regularly produce neuters’. Weismann concurred that ‘the whole colony [of a social insect] behaves as a single animal; the state is selected, not the single individuals; and the various forms behave exactly like the parts of one individual in the course of ordinary selection’.<sup>14</sup>

Despite these precedents, Wheeler is frequently credited with being the first person explicitly to draw an analogy between a social insect (ant) colony and a multicellular organism.<sup>15</sup> He too compared the various sterile castes to somatic cells and the reproductive caste(s) to germ cells and subsequently coined the word ‘super-organism’ for social insects generally, because their colonies were akin to ‘a living whole bent on preserving its moving equilibrium and its integrity’.<sup>16</sup> Fisher echoed Weismann in emphasising the unitary structure of a social insect colony but pointed out that whenever the queen was heterozygous at a locus, genetic variation between workers could be expected, and that could result in within-colony polymorphism and selection.<sup>17</sup> Emerson<sup>18</sup> expanded on the notion of a ‘biological individual’ as ‘an organized biological unit relatively independent of other units’ and proposed that entities ranging from genes to cells to multicellular organisms to societies (including those of social insects) and interspecific groups should be thought of as individuals, each at its appropriate level of organisation.

In the sources quoted above there is no attempt to restrict ‘superorganism’ to the highly eusocial insects. It is only recently that Hölldobler and Wilson have attempted to categorise superorganisms into different grades: ‘The social

organizations, however, vary greatly among the social insect species, and we can recognize different evolutionary grades of superorganismic organization . . . A “primitive” (less derived) grade is represented by several ponerine species, where members of the colony have full reproductive potential and there is considerable interindividual reproductive competition within each colony. Highly advanced grades are represented . . . by the leafcutter ant genera *Atta* and *Acromyrmex* and the *Oecophylla* weaver ants, where the queen caste is the sole reproductive, and the hundreds of thousands of sterile workers occur as morphological subcastes.’ They continue (questionably) with ‘These societies exhibit the ultimate superorganism states, where interindividual conflict within the colony is minimal or nonexistent’.<sup>19</sup> Colony-level selection as the defining element of what constitutes a superorganism is a relatively recent development and has saddled the concept with unnecessary controversy. For us, *superorganism* will simply mean a group of individual organisms or cells that resembles a multicellular organism—that is, functions like an integral social or biological unit (taken from <http://www.bugsinthenews.com/Definition%20of%20Superorganism.htm>), with the added proviso that reproductive division of labour may or may not exist.

### Multicellular development and social selection

Typically, metazoan development begins with a fertilised egg, the zygote, and ends with the differentiation of germ cells and highly diverse somatic tissue; both are needed to ensure that the genes of the parents are successfully transmitted. On its way to becoming an adult, the zygote goes through a large number of mitotic divisions accompanied by complex morphogenetic movements and tissue-specific patterns of gene expression.<sup>20</sup> If we ignore some well-recognised exceptions (*e.g.* the vertebrate immune system and somatic mutations that are neutral or affect gene functions that are not essential for normal development), the cells comprising the adult have the same genotype—for all practical purposes the adult is a clone. Therefore the genetic interest of any cell coincides with that of the whole organism (this may not be true among germ cells: being meiotic products, they need not be genetically identical). Because multicellular development is viewed as the acme of social behaviour, it has been an implicit belief that clonality must be the sole reason behind the extreme division of labour seen in embryos. However, the argument is incorrect. Everything else being equal, shared genes should improve the probability that selection can favour a trait in one individual that appears to benefit another individual (‘kin selection’), more so when the sharing extends to all genes. But the crucial point is that for two units to exhibit concerted

cooperative behaviour, their phenotypes must *covary* in an appropriate fashion,<sup>10,21</sup> which does not require shared genes. Selection between interacting phenotypes is an obvious outcome to be expected when the individuals differ genetically. However, such selection can also act in genetically homogeneous groups, though ‘we will...not see its operation in most modern contexts, because conflicts between units of selection are evident only when a chimera is formed’.<sup>22</sup>

There is abundant evidence for phenotype-phenotype interactions and feedbacks in multicellular development. The clearest instances come from what developmental biologists call regulative development.<sup>20</sup> In a regulative embryo the removal of a part of the embryo—sometimes as much as one-half—results in the reorganisation of the remaining portion, which goes on to form a normally-proportioned whole. The most dramatic demonstration of this was by Driesch, who showed that a single amphibian blastomere at the two-cell stage, that normally gave rise to a left or right half-embryo, would develop into an entire embryo if the other blastomere was removed.<sup>20</sup> The capacity of tissues to regenerate, and of body parts to maintain their relative proportions (analogous to regulation), are further evidences for the existence of intercellular communication, competition and feedback.<sup>23–25</sup> Thus the requirements for social selection to act are present in embryos.

Not surprisingly, multicellular organisation has been compared to a society. Schleiden said so much before Darwin (whose comparison was in the opposite direction), apparently without referring to social insects. In 1858 Virchow made Schleiden’s analogy explicit: ‘[Schleiden]... declared that each cell has an individual existence, and that the life of an organism comes from the way in which the cells work together’ and ‘Rudolph Virchow took Schleiden’s observation a step further. He declared that “the composition of the major organism, the so-called individual, must be likened to a kind of social arrangement or society, in which a number of separate existences are dependent upon one another, in such a way, however, that each element possesses its own peculiar activity and carries out its own task by its own powers” (both quotations from Bloom<sup>26</sup>).

### Levels of selection

We are compelled to confront the issue of the level at which natural selection acts when we think of social selection acting in groups—for example in multicellular social groups that can be called superorganisms. It might appear that the preceding discussion has conflated selection at two levels. Concepts such as coordination and efficiency seem to call for a group-level view of selection; on the other hand competition and dominance are traits that reflect phenotypic differences between individuals within a group. Intuitively, to think of embryos as cohesive social groups with a unitary individuality seems self-evident; with colonial organisms that are constituted by the coming together of single cells, both points of view appear valid; whereas with social groups that are built by multicellular organisms (*e.g.*, social insect colonies), it appears strange to ignore the distinct individuality of each organism.

At the same time, the deep similarities between multicellular organisms and social groups cannot be ignored. As mentioned, Emerson was one of the many who drew an analogy between division of labour in social insects and somatic cell differentiation in multicellular development, with reproductives being compared to the germ line.<sup>18</sup> He extended the analogy by bringing in other features: regeneration, the restoration of missing components (regulation), interactions between units and dominance, all of which are seen in social insect groups and embryos. These points have been advanced repeatedly by Bonner, with metabolic coordination, integration by means of mutual communication and specialised reproduction as the common underlying themes.<sup>27,37,38</sup>

These commonalities risk being obscured by the assertion that the embryo is a constitutive structure (a group of genetically identical cells) whereas a social organism is a facultative entity (a group of genetically more or less diverse individuals). The assertion is valid but irrelevant. Ever since Price’s<sup>21</sup> demonstration that trait frequency change in evolution can be partitioned into changes based on within-group selection and between-group covariance, we have known that individual selection and group selection, involving kin or otherwise, can be treated under a common rubric. To be sure, Price’s formulation has no bearing on the utility of one or the other description. In studying a trait and the factors that impinge on it, there may be more to be gained by focussing on the individual than the group, or the other way round; it depends on the situation. Also, in certain circumstances, both views may need to be adopted simultaneously (consider the example of a stray lioness attempting to join an existing pride). The virtue of Price’s formulation is that it separates the two central players in the evolutionary origin and maintenance of the group, namely (direct or indirect) competition within groups and between groups. In any case, as said above certain traits in social groups stand out as having a ‘group’ character, however much one wishes to adopt an individual-level viewpoint. Group longevity, which affects the success of the group and its constituents, could be an example. Longevity depends in part on metabolic efficiency, which is a collective property of the networks of food and energy transfer in the group.<sup>28</sup>

### Phenotypic plasticity, niche construction and non-autonomy

It is a common observation that in a given natural environment, the majority of a species is made up of individuals whose adult phenotypes are, for all practical purposes, the same. (It is noteworthy that the best-known exceptions involve phenotypic traits that are directly concerned with reproduction: inter-sexual dimorphism and intra-sexual polymorphisms.) They constitute the ‘wild-type’, the systematist’s ideal holotype. However, as we know today, the wild-type hides within itself considerable genetic variation which is cryptic or masked (‘buffered’). Buffering is adaptive and takes place thanks to the manner in which the coordinated functioning of different genes—broadly speaking, the developmental process—has evolved. C. H. Waddington, who devoted considerable attention to cryptic genetic variation and its consequences, referred to the buffering of the possible phenotypic consequences of genetic variation as the *canalisation* of development.<sup>29</sup>

The concept of canalisation is related to that of norm of reaction and bears on the phenomenon of niche construction. ‘Norm of reaction’ implies that depending on the environment, the same genotype can express different phenotypes.<sup>30</sup> The path taken by developmental canalisation depends on the environment; therefore, instead of ‘the’ phenotype of an individual one should say ‘the range of phenotypes found normally in an individual of the same genotype’.

Canalisation and the norm of reaction point to contrasting aspects of genetic flexibility. Canalisation shows that different genotypes can lead to the same phenotype in similar environments, whereas the norm of reaction shows phenotypic plasticity: the same genotype can lead to different phenotypes in different environments. Multicellular development displays both features. The first aspect of genetic flexibility is exhibited, as already discussed, by the masking of genetic differences between different ‘wild-type’ individuals. Cell-type differentiation provides a striking example of the second aspect. Here the different environments may be dependent on positions within the embryo (*e.g.*, inside-outside differences in the mammalian blastula), parental influences (*e.g.*, *via* the egg cytoplasm, a maternal product, or within the maternal body) or phenotypes of other cells; different temporal environments, meaning different developmental histories, may also play a role. Phenotypes can differ even in the absence of a proximate cause. The genotypes and environments may be the same, but the developmental histories of the individuals in question may have been different (as Novick and Weiner showed of *Escherichia coli* and the lac operon).<sup>31</sup> Finally, stochastic effects—‘noise’—can determine which of two alternative states of gene expression is actually chosen, and therefore which phenotype is expressed.<sup>32,33</sup>

Phenotypic plasticity can be manifested in yet another way and that is in the phenomenon known as niche construction. ‘Niche construction’ originally referred to the ability of organisms to modify their environment—to have a hand in constructing their own niche and so to influence how selection acts on them (beaver dams and earthworm burrows are examples often cited).<sup>34</sup> It is a pervasive feature of the life cycle of many organisms and has been discussed as a likely major force in evolution.<sup>35</sup> Gilbert and Epel point out that niche construction is a useful concept in a rather different situation, that of interacting phenotypes in multicellular development.<sup>12</sup> As they argue, niche construction is another name for the phenomenon of embryonic induction (they go on to point out that it also applies to inter-organism interactions of developmental importance).

Embryonic induction is said to take place when signalling between two sets of cells that are in contact, and have each attained the appropriate competences, leads to differentiation. If the interaction fails to take place when it should, the course of differentiation is altered. An example from Gilbert and Epel is the ‘complex dialogue’ of reciprocal interactions that takes place in mammalian development between the neural tissue of the presumptive retina and the ectoderm of the presumptive lens, leading each to differentiate into its final state. Developmental geneticists inferred the existence of intercellular interactions from the observation that many mutations showed cell non-autonomous effects: the aberrant phenotype was manifested in

a cell which was itself genetically wild-type.<sup>36</sup> In the sense used by us, niche construction in embryonic development leads to non-autonomous differentiation; the fact that it does so implies that the cellular genotype can exhibit a range of phenotypes. The language and the conclusion apply equally well to individual phenotypes in social groups.

## The steps to group living and social integration

We proceed to sketch a possible evolutionary route to social integration that can eventually become so strong that individuals become ‘trapped into group life, and group living may become virtually “obligatory” for them’.<sup>9</sup> Our approach follows Bonner in attempting to ‘extend the principles of development to include the development of the whole association.’<sup>37</sup> He states a number of important general principles while discussing the evolutionary basis of division of labour, whether in groups of cells within an organism or groups of organisms.<sup>38,86,98,104</sup> They may be summarised as follows. One, the functioning of living organisms involves ‘respecting physical laws as well as biological processes’; two, ‘the main cause of the appearance of division of labour ... is natural selection for efficiency’; three, ‘some aspects of division of labour can be explained by properties of similar units that exist in a group’; four, division of labour can come about from ‘somatic, physiological differences’ among individuals of the same genotype (here Bonner is referring to dominance hierarchies); and five, division of labour can be explained ‘by ... sensitivity to the [physical] environment as well as to the activity of [conspecifics]’ (this in the context of worker ants). We expand on his argument.

The phenomenon of more than one potential or actual unit of reproduction functioning as part of a larger whole (that is composed of similar or dissimilar units) is common in biology. The unit can be a gene or DNA sequence, a chromosome, an intracellular organelle, a cell, a tissue (organ) or an entire organism—a microbe, plant or animal. In many cases the whole displays ‘emergent’ properties, that is, modes of functioning that are not seen, or sometimes not possible, in its constituent units. When that happens, one should attempt to account for the property in question (“X”) in terms of the properties that the units display when examined separately; and only when that seems not to work, to look for other explanations.

Thus one should begin by seeing whether X can be explained as a straightforward consequence of the fact that the group consists of more than one unit. Perhaps X resembles what chemists call a colligative property, namely one that depends merely on number—for example, the elevation in the boiling point of a solution as the concentration of the solute is increased (except that in biology, the nature of the ‘solute’ cannot be ignored). Simply by being part of a crowd, so to speak, an individual may gain advantages. For example, the probability of capture by a predator could decrease. Or, because a predator must consider the risk of hurting itself in a physical collision (as with bird or fish flocks), numbers could act as a deterrent. In both situations the extent of protection should go up with group size. In the process, the group may develop a spatial structure that derives from nothing more

than each member acting independently in trying to do the same thing (e.g., staying as far from the periphery as possible).<sup>39</sup>

The well-known 'square root of n' rule—more precisely, the Central Limit Theorem of statistics<sup>40</sup>—may be behind apparent group-level adaptations that are an automatic consequence of group size. For example, given some distribution in the efficiency with which an individual performs a task, the performance of the same task by a group of similar individuals is expected to become ever more reliable as their number increases. Therefore, if (for example) an estimate of the amount of forage to be gathered in the future is important for running a beehive efficiently, it helps to increase the number of foragers. It has been proposed that this principle may have been one of the factors behind the evolution of sociality in bees.<sup>42,43</sup>

In an environment that varies over space and/or time, the appropriate measure of long-term fitness (of the individual or the group as the case may be) is the geometric mean, which depends on both the arithmetic mean and the variance—in fact it decreases with increasing variance,<sup>44</sup> the essence of the argument goes back all the way to Daniel Bernoulli.<sup>41</sup> Thus, given that variance decreases with size as discussed above, the commonly observed tendency of size increase in evolution<sup>4</sup> may be a consequence of selection for the increased reliability that is provided by a larger size. A different advantage provided by number may be traced to the fact the ratio of volume to surface, and therefore a measure of how successfully a system can be maintained in a stable condition in the face of the tendency to equilibrate with the environment, increases as a function of the size (meaning typical linear extent) of the system. This too could have been a factor behind the evolution of multicellularity.

When a 'colligative' explanation does not suffice, one looks for an explanation at a different level. Now, over and above the properties of the individual units, one takes into account the interactions that occur between them. Here it is useful to make a distinction between two situations. Interactions could be based on traits that already existed in the solitary state; or, a trait in question may have been absent in single cells but evolved subsequent to the evolution of group living. The first situation involves a preadaptation which may or may not have served an adaptive role previously; if it did not, one would call it an exaptation.<sup>11</sup> A preadaptation that is relevant to group living is when an individual's traits vary in a stage-specific manner over the life cycle. For example, in both primitive<sup>45</sup> and advanced<sup>46</sup> social insects there is the phenomenon of age-polyethism, defined as a 'statistical shift in the activities of workers over time'<sup>47</sup>; rather than absolute age, what counts is the relative age within a cohort. And when, as is likely, a randomly assembled group happens to contain individuals of different ages, the outcome can be adaptive division of labour.<sup>48</sup> Another possibility can be envisaged when the development of the traits of interest depends on a small number of inputs, in the extreme case just one. Then the individuals in whom the inputs happened to differ (even by chance), would exhibit different traits.<sup>49</sup>

Thus group living would have been reinforced whenever the stability or the efficiency of the group was enhanced by the

expression of traits that were already present. However, if the units that made up a group had been living by themselves earlier, and if the initial selective advantage of group life was slight, solitary living and group living would have co-existed. The outcome can be compared to the equilibrium that is reached in a reversible chemical reaction of the sort  $A \leftrightarrow B$ , except that in our case A stands for solitary existence and B for group existence. The equilibrium situation reflects the relative *a priori* probabilities of the two states (and not, as in a chemical reaction, the probabilities of transitions between them). The coexistence of A and B would be favoured by spatial or temporal fluctuations in environmental conditions, or by trade-offs between the advantages of group life (e.g., improved chances of survival) and its disadvantages (e.g., lowered rate of reproduction). Alternatively, A and B could represent evolutionarily stable phenotypic compositions (ESCs) of genotypes.<sup>50</sup>

A decisive step in the evolution of social behaviour must have been taken when 'truly social' properties emerged, based on traits that did not pre-exist but originated *de novo*. It is evident that the traits in question must have involved interactions between individuals. They would depend on signals from one individual that influenced the behaviour of a second individual in a way that fed back on the signaller. If the interests of the two individuals overlap little, signalling is selected or not depending on its consequences for the signaller and recipient, assessed independently. However, if the interests overlap significantly (say because the traits complement each other), the situation would automatically lend itself to selection at the level of the group; signalling between potential mates is an example. As far as any member of the group is concerned, all other members form part of its environment and specify the constraints to which it has to adapt; each member of the group is simultaneously actor and responder. If an adaptive outcome does evolve, it will be identified as a specifically group-level trait that improves the reliable maintenance of social behaviour while benefiting the long-term reproductive interest of the individual. A well-studied example of reciprocal feedbacks in group behaviour is the allocation of workers to different behavioural castes in a eusocial insect colony in which the relative proportions of different castes are restored by the group after they have been altered experimentally.<sup>51</sup> As far as we are aware, the necessary manipulations have not been carried out on primitively eusocial insects. Still, the observations provide striking evidence of interactions between phenotypes and feedbacks in social groups.

Assuming that what we have called truly social properties exist, how should we go about looking for them, keeping in mind the fact that what we see may be the result of many generations of evolutionary modification? A good strategy would be to compare homogeneous and heterogeneous groups where the components that make up the heterogeneous group exhibit clear differences in traits when they form groups by themselves. For the strategy to work, the differences should reflect stable properties of the members of either group. Ideally, this means something correlated with the genotype. Then, each homogeneous group will be genetically uniform and the heterogeneous group will be genetically mosaic. Under these circumstances, if the phenotype of a member of the



heterogeneous group is influenced by the presence of other individuals, the influence can easily be detected. In the case of the cellular slime mould (CSM) or Dictyostelid amoebae, manipulations of this sort are straightforward and a huge amount of experimental data is available. In the next section, after a brief introduction to the CSM life cycle, we draw attention to diverse findings that can be interpreted within the framework of social selection.

## Social selection and the cellular slime moulds

**1. The life cycle: aggregation-driven multicellularity as a facultative response to stress.** The Dictyostelid or cellular slime moulds (CSMs) achieve multicellularity in an unusual manner, namely by aggregation (see the books by Raper, Kessin and Bonner<sup>52–54a,b</sup> for subtleties and details). These amoebae are ubiquitous in the soil or on animal dung. As single cells, they feed on bacteria and yeasts (or on synthetic media in the laboratory), grow and divide by mitosis. After food runs out, either the amoeba dies or one of many defensive reactions ensue and enable it to withstand the stress of starvation. An amoeba can encyst itself or, along with other cells, mount a collective response. Amoebae that are spread over a wide area (typically ~ one to a few mm<sup>2</sup>; the dimension of an amoeba is ~ 10 µm) can attract each other, come together at common collection points and collapse into aggregations. They do so by moving up spatial gradients of a chemical attractant released by some, if not all, of the cells (chemotaxis; the numbers can vary enormously, with a normal range of ~ 10<sup>2</sup>–10<sup>6</sup>), ref. 55–57 and <http://www.dictybase.org/Bonner%20paper.pdf>. Having formed the equivalent of an embryo in this unusual fashion, two outcomes are possible. The aggregate can proceed to differentiate in the manner of any metazoan embryo and form a terminal ‘adult’ structure, known as a fruiting body—this is during the ‘standard’ life cycle that is studied in the laboratory. Or, when cells of opposite mating types are present, the aggregate can get converted into a giant cell known as the macrocyst, which is the intermediate phase in an alternative, sexual, life cycle. The process is intricate (e.g., in addition to nuclear fusion and meiosis it includes cannibalism) and favoured by special circumstances (e.g., flooding by water).<sup>58</sup> CSM species, and even different genotypes within the same species, differ in their propensities to form microcysts, macrocysts and fruiting bodies; and more than one structure can be seen in the same culture plate. It would be of the utmost interest to know the relative probabilities of these developmental strategies under various conditions; the point of interest is that there is a great deal of developmental flexibility in the response to starvation. From now on, we will deal mostly with the life cycle that involves the fruiting body.

Post-aggregation development involves a constant number of cells (in the laboratory, after one has taken into account stragglers in the cell division cycle that may divide soon after the food supply is removed). Aggregation is followed by complex morphogenetic movements both within the mass and by the mass as a whole, giving rise finally to a terminally differentiated ‘adult’ structure known as the fruiting body. Within the fruiting body, some cells make up a ball of

stress-resistant spores and other cells form a stalk of dead cells that supports the spore mass at its end. This arrangement, with spores raised above the substrate, is believed to aid passive dispersal. If dispersal occurs to an environment where food is available, each spore can germinate and the amoeba that emerges can feed, grow, divide and set off a new multicellular stage after the food runs out.

Spores can be compared to germ cells and the stalk can be thought of as the equivalent of somatic tissue. However, in contrast to the cells of a metazoan embryo, the CSM amoebae that form a social group need not be genetically identical. Under natural conditions they can constitute a clone,<sup>59</sup> belong to different genotypes within the same species,<sup>60–64</sup> or, rarely, belong to different species, ref. 65 and unpublished work. When an aggregate is genetically heterogeneous, phenotypic heterogeneity can be a reflection of diverging reproductive interests between the amoebae in a group. Even if the aggregate consists of a clone of cells, individual reproductive interests need not coincide: phenotypic differences between cells can be expected to be translated into reproductive differences (as indeed they are). Here, though the situation appears to lend itself to a kin-selection based explanation, one must keep in mind that what may be at work is individual-level selection and physiological dominance.<sup>66</sup> The presence of different genotypes in a group makes the likelihood of phenotypic differences more likely, and also easier to verify.

CSM development occurs within a specified range of the physical environment, and its outcome can differ significantly within that range. In *Dictyostelium discoideum*, a decrease in humidity speeds up development,<sup>67</sup> whereas a decrease in temperature slows it down<sup>67</sup> and increases the distance over which amoebae can be attracted.<sup>68</sup> Starved cultures develop faster, and aggregates are smaller, in light than in the dark.<sup>67</sup> These effects of light are marked in the case of *Polysphondylium pallidum*, in which aggregation cannot commence in the dark.<sup>69</sup> The nature of the substrate on which development occurs also has a significant effect on development (for example, whether it is relatively smooth or granular), ref. 70 and unpublished work.

Interestingly, there can be significant differences between supposedly wild-type strains in the biochemical details of development. A prominent instance involves variations in the kinetics of production and release of two proteins that modulate the strength of the cAMP signal between its release by one *D. discoideum* cell and reception by another, namely a secreted cAMP phosphodiesterase and its inhibitor.<sup>71,72</sup> The implication is that the norm of reaction for the species as a whole—with its members being of different ‘wild-type’ genotypes—extends over a broader range than the norm for a single individual. Not unexpectedly, mutants show a stronger sensitivity to environmental conditions than the wild-type does, which points to a component of Genotype × Environment interaction in overall phenotypic variation. For example, some mutants of *D. discoideum* are unable to aggregate on growth medium—that is, when they are plated with bacteria on glucose-peptone agar—but develop normally when plated without food on deionised agar.<sup>73,74</sup>

## 2. Advantages to group living as a fall-out of increased size.

Spatial clustering, which was conceivably the precursor to aggregation by chemotaxis, can arise simply as a consequence of large numbers. This was shown in a simulation by Houchmandzadeh<sup>75</sup> who found that within a certain range of the relevant parameters, a combination of events involving the birth, uncorrelated random dispersal and death could lead to strong clustering. The result was confirmed by observations on clonal growth of *D. discoideum* amoebae on a surface.<sup>76</sup> The speed of migration of CSM aggregates (slugs) increases with their size (or more accurately, length).<sup>77,78</sup> The implication is that the larger the number of amoebae in a group, the better the chances for any cell to disperse to a more favourable environment. Size can favour dispersal in another way, which is by raising the spore mass to a greater height above the soil. Kessin *et al.* showed that differentiation into spores permitted amoebae of *D. discoideum* to survive after being eaten by a soil nematode.<sup>79</sup> It may be that aggregation *per se*, via an increase in size, also acts as a defence against nematode attack. Nanjundiah and Bhogle<sup>80</sup> found that the reliability of differentiation, measured as the variance in spore or stalk cell proportion relative to the mean, decreased as the size of the aggregate increased, though only a part of the decrease could be attributed to cell number *per se*.

## 3. Phenotypic differences among individuals of the same genotype; division of labour

(a) *Spontaneous or 'random' variation.* Cell to cell differences in behaviour can be observed in clonal populations of *D. discoideum* right from the onset of starvation. Starved cells develop a number of competencies in the course of aggregation. One, they synthesise and release the chemoattractant, cyclic AMP, in a pulsatile fashion;<sup>81</sup> two, they sense, amplify and relay an external cAMP stimulus;<sup>82,83</sup> three, they sense and respond to an external source of cAMP by moving towards it;<sup>84</sup> and four, they develop an intercellular cell adhesion system.<sup>85</sup> These competencies arise in all cells roughly in reverse order, albeit with some overlap and not synchronously.<sup>86,87</sup> Their appearance can be speeded up and reinforced by providing external pulses of cAMP.<sup>87–89</sup> The onset of competence (d) is at about the same time or somewhat after (b). Superficially, the situation appears to be different in *P. violaceum*, where a case can be made for a specialised 'founder cell' becoming the centre of an aggregate. But here too, if a founder is removed, another cell replaces it, though after an appreciable amount of time;<sup>57</sup> the essential difference between the two species seems to be one of heterochrony.<sup>90</sup> Early variation may not be without consequence: in *D. discoideum* a positive correlation has been found between the probabilities of a cell initiating aggregation and ending up as a spore.<sup>91</sup> The simplest explanation of the visible heterogeneities that accompany early aggregation—some cells at the centre of aggregation, ramifying streams and single cells moving towards a stream or directly towards the centre—is that they are due to random physiological differences between cell and cell reflected as cell-to-cell variations in the onset of developmental competences.

(b) *Factors that accentuate spontaneous variation.* In *D. discoideum*, at the time that they begin aggregation, the

amoebae already have stalk-forming or spore-forming tendencies; the evidence is mostly indirect. When a suitable external bias is imposed, of a type that can exist under natural conditions (for example differences with respect to the energy content of the food;<sup>92,93</sup> or variations in time of starvation relative to the phase of the cell division cycle,<sup>94,95</sup> the bias makes it more or less likely that an amoeba differentiates into a spore).<sup>66,95–99</sup> Even in the absence of any external bias, spontaneously occurring cell-to-cell variations in cellular calcium enable a correlation to be made between 'low calcium' and spore-forming tendency and 'high calcium' and stalk-forming tendency.<sup>100</sup> Interestingly, 'high' and 'low' refer to relative, not absolute differences. Data on other species are meagre, but it is known that some of them (*e.g.*, *Polysphondylium* species) do not show a clear distinction between presumptive stalk and spore cells. *D. mucoroides* makes prespore cells, of which some get converted to stalk cells as the slug migrates.<sup>101a</sup> In *Acytostelium leptosomum*, the stalk is an extracellular structure made by all (presumptive spore) cells.<sup>101b</sup> Therefore the question of different cell types does not arise—unless, as may be the case in *D. discoideum*,<sup>98,102</sup> spores can exhibit functional differences.

(c) *Interactions between cells belonging to different phenotypes.* Right from the onset of aggregation, interactions between cells are central to the entire process of development. However, the features of development discussed above in (a) and (b) can be accounted for either as outcomes of stochastic processes or as arising from inter-individual variations based on differences in pre-aggregation environments. Now we take up examples of interactions based on phenotypic differences that have already built up. They show the role of intercellular interactions most clearly; their outcome is that the phenotype of one cell depends on that of another.

The single most striking piece of evidence in favour of intercellular interactions is that the ratio of differentiated cell types is approximately invariant with respect to total cell number over two to three orders of magnitude—what has been called 'the supreme problem of differentiation' in the cellular slime moulds.<sup>54a</sup> More to the point, the relative proportions of stalk to spore cells can re-adjust if the developing mass is fragmented, even if most cells in the fragment belong to a single (presumptive) cell type<sup>103</sup> (again pointing to relative differences in phenotype rather than absolute phenotype as the essential element that leads to functional differences). The actual proportions of the two differentiated cell types range from about 20:80 in *D. discoideum* to 50:50 in *D. giganteum* (for wild type strains studied under standard laboratory conditions).<sup>67,104,105</sup>

It turns out that in order to account for the magnitude of the fluctuations in cell type proportions (mentioned earlier), one has to invoke, over and above a purely stochastic basis for cell type determination, reciprocal negative feedbacks between the presumptive cell types and an ability on the part of a cell to sense the number of cells in its social group.<sup>80</sup>

Group integrity within the aggregate is maintained by intercellular adhesion and cell-to-cell signalling. Though all cells take part in these, there are significant differences between the two presumptive cell types in traits related to adhesion and

cAMP signalling.<sup>106</sup> The bulk of the motive force for the movement of the slug is provided by cells in its front.<sup>107</sup> Besides constituting a minority (~20%) of the total number of cells, these anterior cells die as they become part of the stalk. In an interesting partial overlap with *Acytostelium*, where the stalk is wholly extracellular, *Dictyostelium* cells first secrete an extracellular stalk tube, then form the stalk proper by moving into the tube, dying, and synthesising a cellulose wall.<sup>53</sup> Eventually the slug stops moving and begins to erect itself while terminal cell differentiation into stalk and spore is taking place. Spore differentiation requires that presumptive stalk cells signal by means of a small peptide SDF-2.<sup>108,109</sup> The cells that will eventually sporulate climb up the stalk, and their ascent is helped by two ‘cups’ of cells that cradle the presumptive spore mass from above and below. Of these, the lower cup appears to provide mechanical support while the upper cup actively lifts up the presumptive spore mass.<sup>110–111</sup> Neither lower cup cells nor upper cup cells sporulate; they are believed to die while remaining amoeboid in morphology.<sup>112</sup>

**4. Intercellular interactions and phenotypic heterogeneity accompanied by genetic heterogeneity.** Because of the manner in which the CSMs become multicellular (*i.e.*, by aggregation of spatially separated amoebae), it is possible that the amoebae that come together belong to different genotypes. In accordance with this expectation, fruiting bodies formed under natural conditions (or under laboratory conditions that may mimic what happens in nature) can be genetic chimeras.<sup>63,64</sup> The presence of multiple genotypes in the same aggregate or same fruiting body carries with it implications for the evolution and maintenance of social behaviour in the CSMs, because the stable coexistence of different genotypes requires that stringent conditions be fulfilled.<sup>113</sup> For example, everything else being equal, if an amoeba has a lower probability of sporulation than one belonging to another genotype, the frequency of its own genotype should fall steadily from one generation to the next. Genetic heterogeneity within groups is easily achieved by mixing cells belonging to two or more clones, and most of what we have learnt about behaviour in mixed genotype groups comes from laboratory experiments. As will be seen, the consequences of mixing experiments are extraordinarily diverse. But they carry a common lesson: phenotypic plasticity is a pervasive feature of the division of labour displayed by CSM social groups. The phenotype of a cell, and therefore of the group, is strongly influenced by intercellular interactions. This makes it plausible that plasticity, or developmental flexibility, must have been central to the evolution of sociality in the CSMs.

(a) *Synergism between naturally occurring strains that differ in their developmental phenotypes.* Starting from the spores in a natural isolate of *Dictyostelium mucoroides*, Filosa managed to obtain four clones of amoebae that, when observed separately, displayed distinct behaviours: one developed normally and the remaining three gave rise to aberrant-looking fruiting bodies.<sup>114</sup> One of the aberrant forms, MV, formed spores at a low efficiency and did not form a wild-type stalk. A wild-type + MV mix developed like the wild-type and, after many cycles of growth and development, attained a stable

equilibrium in which the relative proportions were ~90% wild-type: 10% MV.

Buss obtained two *D. mucoroides* strains in close proximity in the soil.<sup>60</sup> When studied in isolation, both displayed stable but distinct phenotypes: one went through normal development whereas the other did not. After starved amoebae of the second strain aggregated, all of them differentiated into spores that remained on the surface; the aggregate did not form a stalk. The indications are that the strains were genetically distinct, though they may have been related (there is no evidence bearing on the issue). When mixed, as must have been happening in nature, the two strains developed in concert and formed chimaeric fruiting bodies. However, the two components differentiated in a way that depended on the composition of the mix. When the ratio of ‘stalkless’ to normal amoebae was very low, ‘stalkless’ amoebae were over-represented in the spore population; and when the ratio was very high, the ‘stalkless’ strain formed fewer spores than expected. In other words, there was a frequency dependence of just the sort required for a stable equilibrium to be attained—as turned out to be the case. Thus, everything else being equal, the two would be expected to co-exist stably in nature. In this case, the nature of the interaction is such that it serves little purpose to use words such as parasite, cheater or altruist as descriptors of the phenotype of either strain. Depending on the circumstances, any of these labels can be attached to any of them; it is the dynamics of their interaction that is relevant.

Kawakami and Hagiwara found that *P. pallidum* strains that belonged to complementary mating types also differed in aspects, including fruiting body morphology, in their asexual life cycles,<sup>115</sup> indicating a more usual type of ‘synergism’—mutually beneficial interactions between a pair of individuals belonging to different phenotypes. Moreover, it is an unusual example of genotypes switching roles, by giving rise to sexual dimorphism (in one context) and cooperating within a social group (in another context). This is reminiscent of a school of fish in which an individual of one sex changes into another sex in response to a change in the social structure of the population—for example, when the dominant position falls vacant, except that role-switching in fish is a form of temporal polyethism (for an evolutionary perspective on sequentially hermaphroditic fish, see ref. 116). A curious example of what could be cross-species synergism between a CSM and a fungus was found by Ellison and Buss (though only a benefit to the CSM was established).<sup>117</sup> They isolated normal looking fruiting bodies of *D. mucoroides* along with the fungus *Mucor hiemalis* from soil samples. After purifying the two separately, they noted that when allowed to develop by themselves, the amoebae of *D. mucoroides* aggregated and differentiated straightaway into a ball of spores; intermediate developmental stages were absent. However, when their environment included fungal hyphae, or if they were provided access to a diffusible substance released by the fungus, the amoebae went through normal development.

(b) *Synergism between wild-type and mutant phenotypes.* Sussman and co-workers isolated many spontaneously occurring and artificially generated (*e.g.* after UV treatment) developmental

mutants of *D. discoideum*. Among them, some were unable to complete development whereas others went through development and produced abnormal-looking fruiting bodies.<sup>73,118,119</sup> Either a mixture of cells derived from two mutant strains or a wild-type-mutant combination was studied to see whether the presence of one genotype influenced the other. In many cases the outcome was normal development. For example: a combination of the wild-type and an aggregateless ( $\text{agg}^-$ ) mutant, when plated at a low density at which the wild-type was unable to aggregate (below 100 amoebae  $\text{mm}^{-2}$ ), formed normal aggregations, and it appeared that the presence of the wild-type induced the  $\text{agg}^-$  cells, not just to participate as responders to the wild-type, but also to form aggregation centres too. However none of the spores isolated from chimaeric fruiting bodies formed by  $\text{agg}^-$  and wild-type amoebae were of the  $\text{agg}^-$  type.<sup>120</sup> Similarly, many pairs of developmentally aberrant mutants were able to complement each other's defects when mixed and go through normal development.<sup>73,118,120,121</sup>

Similar experiments were carried out by Weber and Raper,<sup>74</sup> who found that two  $\text{agg}^-$  mutants of *D. discoideum* aggregated and formed normal fruiting bodies when they were put into contact with wild-type *D. discoideum* amoebae or even amoebae of other species such as *D. purpureum* and *D. mucoroides*. A plausible explanation of the outcome comes from the observations of Darmon *et al.* that the developmental defect in many  $\text{agg}^-$  mutants of *D. discoideum* could be cured by periodic stimulation of starved cells with extracellular cAMP.<sup>88</sup> Ennis *et al.* isolated a *D. discoideum* mutant that exhibited aberrant developmental morphology and poor differentiation.<sup>122</sup> When mixed with its parental wild-type strain, the mutant participated in normal development and was more efficient than the wild-type at forming spores: its proportions kept increasing with each developmental cycle. Huang *et al.* showed that a very small proportion of wild-type cells (probably a single cell) was sufficient to initiate development in an otherwise  $\text{agg}^-$  mutant.<sup>91</sup> Clark found a low incidence of synergism when pairs of developmentally defective mutants of *Polysphondylium violaceum* were mixed.<sup>123</sup> On the other hand, Rafaeli found that the wild-type and mutant or two different mutants (for example, *Stumpy* and *Fruity*) formed chimaeric, normal fruiting bodies.<sup>124</sup>

Sussman<sup>120</sup> and Kahn<sup>125</sup> made an extraordinary observation pertaining to synergism in their experiments with *D. discoideum* and *D. purpureum* respectively. They reported that a single round of joint development between an  $\text{agg}^-$  mutant and the wild-type (in the course of which mutant cells participated in normal development and differentiated into stalk and spore cells) was sufficient to transform the mutant's phenotype. Thereafter, mutant cells were competent to aggregate and differentiate by themselves. A parallel finding was made by Kaushik.<sup>126</sup> She mixed spores of three strains of *D. giganteum* (46a3gig, 46d2gig and 46c6gig) in pairs as well as in three-way mixtures and grew them in the presence of bacteria for approximately 24 generations. After that, spores from the resulting fruiting bodies were spread with bacteria on nutrient agar plates at a very low density. When they germinated they cleared the bacterial lawn around them forming plaques. Interestingly, many of the plaques when transferred to other

plates and allowed to grow and develop further, could not do so. Some of the plaques did not proceed beyond loose aggregations and a few of them did not aggregate at all.

(c) *Antagonistic interactions.* Examples are known in which cells of the same CSM strain affect each other negatively. Amoebae of *D. discoideum* repel each other during growth, as do *D. purpureum* amoebae. *P. violaceum* amoebae do not repel each other but move away from a diffusible product released by *D. discoideum*,<sup>127</sup> which could affect the distribution of the two species in areas of co-occurrence. During the normal post-aggregation development of *D. discoideum*, presumptive spore and stalk cells—which at an early stage of development are better termed 'high quality' and 'low quality' amoebae respectively<sup>66</sup>—interact in a remarkable manner that points to phenotype-dependent developmental plasticity as the basis of division of labour. *D. discoideum* cells synthesise and release a family of variously modified membrane-permeable chlorinated phenolic compounds that are lethal and are generically known as DIF, meaning 'differentiation-inducing factor'. The name derives from their ability to induce amoebae to die and differentiate to a stalk cell-like condition. DIF-1 is the most potent of them. As predicted<sup>66</sup> and subsequently verified,<sup>128,129</sup> DIF-1 is made predominantly by high-quality amoebae at the back of the slug. Not only that, it is broken down by low-quality amoebae in the front of the slug<sup>129</sup>; this happens even when the amoebae belong to the same clone.<sup>66,128–130</sup>

There are also cases from mixed genotype cultures where cells of one genotype repress the development of the other. 50d8, a wild-type strain of *D. giganteum*, aggregates and forms normal fruiting bodies when by itself, but does so only rarely when mixed with amoebae of the (also wild-type) strains 46a3, 46d2 or 46c6. The inhibitory effect appears to depend on cell-cell contact, because 50d8 develops normally when separated from any of the others by a filter paper barrier.<sup>104</sup>

Mujumdar *et al.* (unpublished work) found that when a minority of *trishanku* mutant cells are mixed with wild-type *D. discoideum*, aggregation streams break up and fruiting bodies are smaller than normal—phenotypes characteristic of the mutant.<sup>131</sup>

The antagonism between strains can be mediated *via* bacteria, which are their normal food. Following growth and starvation, amoebae of the *D. discoideum* mutant *Agg*<sup>-</sup>208 aggregated on a 'minimal agar' medium that had been repeatedly washed with distilled water, but did not do so even after the food was exhausted if they were allowed to remain on growth plates; another mutant, *Agg*<sup>-</sup>206, did not aggregate under either condition. Co-development of these two mutants on minimal agar led to the partial or complete loss of *Agg*<sup>-</sup>208's ability to aggregate.<sup>73</sup> Weber and Raper found that two developmentally aberrant mutants of *D. discoideum*, *agg*<sup>-</sup>1 and *agg*<sup>-</sup>2, aggregated on non-nutrient agar but when grown on plates containing *Escherichia coli*, did not do so even after food depletion.<sup>74</sup>

(d) *Complex interactions between wild-type strains.* Experiments with *D. mucoroides*,<sup>60</sup> *D. discoideum*<sup>64,132</sup> and *D. giganteum*<sup>104</sup> have shown that in mixtures of different strains of a species, the efficiency with one of the members

of a pair sporulates (meaning the ratio of the number of spores formed to the starting number of amoebae) is different from its sporulation efficiency when by itself. In some of these cases, both strains were wild-type and in other cases one or the other member of the pair was a mutant. After a number of different strains are mixed pair wise, it turns out that the strains can be arranged in a linear transitive hierarchy of relative sporulation efficiencies, akin to a pecking order or dominance hierarchy.<sup>104,133</sup> In the case of *D. giganteum*, the presence of a third strain in the mixture shows that the underlying interactions are in fact non-linear—the strain that is predicted to be lowest in the hierarchy can do as well as the other two.<sup>104</sup> In *D. discoideum* too, it turns out that the quantitative outcome pertaining to relative sporulation efficiency in pair wise mixes is not predictable from the outcome of mixing the same strains separately with a third strain. Khare *et al.* worked with three stains, wild-type, *chtC* ('cheater') and *recA* ('cheater resister').<sup>134</sup> In wild type + *recA* or *recA* + *chtC* mixtures, the contributions of the two components to the spore population are approximately the same; but in a 1:1 wild type + *chtC* mixture, *chtC* forms more spores than the wild type. However, in a three-way mixture *chtC* forms fewer spores than expected. Finally, a loss of function mutation in *hdaB*, one of the four genes known to code for histone deacetylase, does not lead to any obvious change in the development of *D. discoideum*. However, when mixed with the wild-type in a 1:1 ratio, mutant cells form fewer spores than the wild type.<sup>135</sup> Santorelli *et al.* have reported a complementary case, in which a *D. discoideum* mutant forms more spores than the wild-type when mixed with it in a 1:1 ratio, but develops normally when by itself.<sup>136</sup>

## Discussion

Thanks to the unusual route adopted by them to achieve multicellularity, the CSMs display traces of all the steps that can plausibly be envisaged as having taken place in the transition from unicellular life to full-blown group behaviour, whether in groups of cells (as in embryos), or in groups of metazoan individuals. Still, the CSMs that we study today must have an ancient evolutionary history. Therefore, in a strict sense, today we can expect to identify only the factors that lie behind the maintenance of traits, not the factors that were responsible for their origin. However, one can argue on grounds of plausibility that there is likely to be an overlap between the factors that led to a trait evolving in the first place and the factors that are responsible for its maintenance. Precisely because of the nature of the CSM life cycle, we can perceive traces of those steps more clearly than we might hope to in organisms that achieve multicellularity *via* continued divisions of a zygote. In this respect, the CSMs offer advantages for studying the evolution of social behaviour similar to those provided by primitively eusocial insects.<sup>137</sup> One might say that they too are 'superorganisms' in which many individuals live in stable groups with division of labour. There are other organisms in which spatially separated multicellular units form by aggregation, including myxobacteria,<sup>138</sup> myxomycetes<sup>139</sup> and the ciliate *Sorogena stoianovitchae*,<sup>140</sup> but in hardly any of them do we have experimental evidence in

comparable detail to that in the case of the cellular slime moulds—including knowledge of whether aggregations can be genetically heterogeneous (as in the CSMs) or not. In the colonial ascidian *Botryllus schlosseri*, individuals can fuse and give rise to chimaeras containing more than one genotype. Rinkevich and Shapira found that chimaeras made up of four genotypes had advantages over ones that contained two or three genotypes—they grew more rapidly and survived longer<sup>141</sup> (a complication in interpreting their findings is that group size may have influenced the outcome; it went up with the number of clones).

Equally, because they often exist in multi-clonal groups, the CSMs are a useful guide to understanding multicellular development from an evolutionary point of view. On the face of it this appears unlikely—it tends to be taken for granted that all features of development in a clonal embryo can be explained on the basis that what is 'good for the whole' is *ipso facto* 'good' for any cell. But there are many reasons for saying that the study of development in a polyclonal group is meaningful for understanding cell behaviour in clonal groups. To begin with, if our interest is in the evolution of group behaviour, what matters is that the group is made up of individuals with different phenotypes and therefore intrinsically different capacities to reproduce (as members of the group); and different phenotypes are just as likely in genetically homogeneous groups as within heterogeneous groups. Second, it has been argued that the reason why only some cells differentiate into germ cells is because by virtue of their phenotypes they out-compete the others and, in a sense, compel them to contribute to somatic tissue,<sup>60,142</sup> a line of argument that has also been used to explain the evolution of differentiation in the CSMs.<sup>66</sup> Third, the inevitability of somatic mutations implies that so-called clonal development actually involves groups whose members may not be genetically identical.<sup>143</sup> Thus CSM development, and by extension that of intra- and inter-specific chimaeric embryos,<sup>144,145</sup> may have much to tell us about the evolution of development in general. The life cycle of Dictyostelid amoebae has long been viewed as an example of multicellular development that throws up interesting questions when viewed from an evolutionary perspective. It is time to turn tables and, as integrative biologists, ask what new insights into normal and abnormal development we can gain by thinking about the evolution of cooperative behaviour in the cellular slime moulds. The same point applies with regard to social behaviour in larger animals. The fact that a starved amoeba can differentiate into a spore or stalk cell depending on the phenotypes of other amoebae implies plasticity of a high order. This is mirrored in the functional plasticity displayed by primitively eusocial or facultatively social insects. Workers can take over a queen role when the opportunity presents itself;<sup>146</sup> distinct behavioural biases that impinge on social roles can be found in largely solitary species<sup>147</sup> and the biases can be overridden by social competition.<sup>148</sup>

On the basis of studying the evidence for socially selected traits in CSM groups, two broad generalisations may be made. The first is that whenever social selection operates on a phenotype, it is impossible to draw a distinction between 'cell-autonomous' and 'cell-non-autonomous' effects of the

underlying genes. The second is that it is inappropriate to label genotypes by words such as ‘selfish’, ‘altruist’, ‘cheater’ and so on (‘noble’ has joined the list recently). That is because at best such words can convey a verbal description of traits expressed in very specific, and therefore restricted, social settings. In general, they convey nothing about how cells of the same genotype might behave in groups containing some other set of genotypes. Both comments are in the same spirit as that pertaining to the phenotype of a single individual. Namely, it is inappropriate to associate a phenotype with a gene (or allele). Rather, the phenotype results from a complex interplay between allelic activity and the rest of the genome, that too in a specified environment. When every individual is part of every other individual’s environment, the phenotype of any one depends on the phenotypes of the rest. In a literal sense, the phenotype of the individual is a social construct.

Moore *et al.*<sup>1</sup> and Frank<sup>2</sup> have discussed the principles of social selection with the help of a general method for partitioning phenotypic variance that, as mentioned earlier, was pioneered by Price.<sup>21</sup> The method awaits application to genetically heterogeneous groups, especially when their behaviour is pathological. Evidence is accumulating that many cancers can be described as the consequences of social networks that malfunction.<sup>149</sup> Among the attendant factors are phenotypic heterogeneities between the members of a cellular group and, just as in ordinary development, reciprocal interactions between cells and their cellular and extracellular environments (*i.e.*, cellular non-autonomy).<sup>150–152</sup> But the outcome is to derail the normally stable structure of the group. An analogy has been made recently between groups of cancer cells and species.<sup>153</sup> It would be worthwhile to examine whether pathological development, including of the sort that leads to cancer, can be analysed usefully within the Price framework as an example of social selection that has gone awry.

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## Interspecies and intraspecies interactions in social amoebae

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### Keywords:

chimaera;  
cooperation;  
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guild;  
microbial diversity;  
niche overlap;  
reproductive skew;  
social behaviour;  
sorting out.

### Abstract

The stable co-existence of individuals of different genotypes and reproductive division of labour within heterogeneous groups are issues of fundamental interest from the viewpoint of evolution. Cellular slime moulds are convenient organisms in which to address both issues. Strains of a species co-occur, as do different species; social groups are often genetically heterogeneous. Intra- and interspecies 1 : 1 mixes of wild isolates of *Dictyostelium giganteum* and *D. purpureum* form chimaeric aggregates, following which they segregate to varying extents. Intraspecies aggregates develop in concert and give rise to chimaeric fruiting bodies that usually contain more spores (reproductives) of one component than the other. Reproductive skew and variance in the proportion of reproductives are positively correlated. Interspecies aggregates exhibit almost complete sorting; most spores in a fruiting body come from a single species. Between strains, somatic compatibility correlates weakly with sexual compatibility. It is highest within clones, lower between strains of a species and lowest between strains of different species. Trade-offs among fitness-related traits (between compatible strains), sorting out (between incompatible strains) and avoidance (between species) appear to lie behind coexistence.

### Introduction

The basis of co-existence in a species whose members differ in fitness-related traits is a long-standing issue in evolutionary biology (Futuyma, 1998). The situation is especially baffling when it comes to species in whose life cycles the sexual phase is rare, intermittent or absent (Rainey *et al.*, 2000). The cellular slime mould or social amoebae present us with a glaring example. Groups can consist of distinct strains of a species (in nature; Sathe *et al.*, 2010) or even different species (in the laboratory and by inference also in nature; Raper & Thom, 1941; Bonner & Adams, 1958; Jack *et al.*, 2008; this study). Crucially, members of a group differ in the efficiencies with which they form reproductives (spores). This raises the evolutionary question: 'What

makes it possible for different strains of a species, or different species, to co-exist in the long run?'

Many factors can lie behind the co-occurrence of different species in a shared environment. The species can occupy different niches or the niches can overlap. In addition, the species can exhibit commensalism, symbiosis or exploitation (Tokeshi, 1999). If two species compete for the same resources, it is believed that they cannot co-exist stably ('Gause's principle'; Gause, 1934). In practice, the application of this principle requires several caveats (Hardin, 1960). Hutchinson (1961) claimed that the high diversity of phytoplankton and the limited range of resources on which they survive refuted it. The existence of stable polymorphisms within an asexual sympatric species whose members differ in fitness-related traits (Rainey *et al.*, 2000) is also a possible refutation. Bonner (2009, 2013) has put forward a radical hypothesis to account for the co-existence of diverse forms in microorganisms: they could be neutral phenotypes, that is, their morphologies could be more the result of drift than selection. Among the organisms used by Bonner as illustrations are

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# Social Selection in the Cellular Slime Moulds

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**Abstract** Starvation triggers a complex series of intercellular interactions in the cellular slime mould amoebae. As a result the amoebae aggregate, form a coherent multicellular structure with division of labour and, eventually, differentiate into a fruiting body made up of a stalk and a spore mass. Whether an amoeba dies and forms part of the stalk or becomes a stress-resistant spore depends both on pre-existing biases and on post-starvation signalling between amoebae. Mutual communication permits one amoeba to influence the phenotype, and therefore affect the fitness, of another. The implication is that social selection has been a major factor in the evolution of cooperative behaviour in these amoebae.

## 1 Introduction

This article discusses the potential for social selection during the cellular slime mould (CSM) life cycle. Social selection is natural selection in the context of social behaviour. With two exceptions the term 'social behaviour' is used as commonly understood: it is "the suite of interactions that occur between two or more individual[s]..., usually of the same species, when they form...aggregations, cooperate... or simply communicate across space".<sup>1</sup> The exceptions pertain to

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<sup>1</sup> <http://www.britannica.com/EBchecked/topic/550897/social-behaviour-animal>

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1 **Development of twelve polymorphic microsatellite markers from the social amoeba**  
2 ***Dictyostelium giganteum* suitable for genetic diversity studies on cellular slime moulds.**

3

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11 **Keywords:** Cellular slime moulds, *Dictyostelium giganteum*, microsatellite markers, social  
12 behaviour, kin selection.

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26 **Running title:** Microsatellite markers of amoeba *D. giganteum*

27 *The cellular slime moulds (CSMs) constitute an experimentally tractable system for*  
28 *studying the evolutionary basis of sociality. However, except in Dictyostelium*  
29 *discoideum, studies on kinship evaluation in CSMs have been hampered due to non-*  
30 *availability of suitable genetic markers. Here we describe twelve microsatellite markers*  
31 *developed from an SSR-enriched genomic DNA library of D. giganteum. New markers*  
32 *resulted in two to nine alleles per marker when tested on 57 strains of D. giganteum and*  
33 *six other related CSM species. Polymorphic nature and broad cross-species*  
34 *transferability of these markers demonstrate their potential for studying genetic diversity,*  
35 *species relationships and kinship in CSMs.*

36

37 The cellular slime mould amoebae upon starvation aggregate and form a fruiting body  
38 made up of dead stalk cells and a mass of viable spores on top (Bonner 2009). The  
39 formation of dead stalk cells has been looked upon as ‘altruistic’ behaviour. One way of  
40 accounting for the evolutionary basis of the ‘altruism’ displayed by stalk cells is to ascribe  
41 it to shared genetic interest between them and spores (‘kin selection’; Hamilton 1964).  
42 Studies on social behaviour demand knowledge of the genetic structure of groups and  
43 populations including patterns of kinship. Our knowledge of genetic variation in CSM  
44 social groups has suffered due to the non-availability of reliable genetic and/or DNA  
45 markers. The situation warrants efforts to develop new efficient markers like ‘PCR-based  
46 microsatellites’ that can facilitate detailed studies on population dynamics of CSMs.

47

48 Microsatellites or simple sequence repeats (SSRs) are hypervariable repetitive DNA  
49 stretches (2 to 6 bps long), which are frequently found in the genome. Their abundance in  
50 the genome, variability, co-dominance and simplicity in use, make microsatellites attractive  
51 for genetic studies on: characterization of biodiversity, understanding population structure,

52 find species relationships and to estimate intra-/inter-group genetic diversity. Here we  
53 describe new microsatellite markers from *D. giganteum* suitable for genetic studies even in  
54 other CSMs.

55

56 A total of 57 CSM strains, 42 belonging to *D. giganteum* and another 15 belonging to six  
57 related species were used in the study. A majority of these strains were isolated from the  
58 Mudumalai Wildlife Sanctuary, India (Kaushik *et al.* 2006; Sathe *et al.* 2010). DNA was  
59 extracted from vegetative amoebae as described earlier (Sathe *et al.* 2010). For  
60 development of microsatellite markers, a partial small-insert genomic library enriched for  
61 SSR repeats was constructed from genomic DNA of *D. giganteum* (strain-46d2) as  
62 described by Aggarwal *et al.* (2004). Briefly, it involved: digestion of total genomic DNA  
63 with *EcoRI* and *AluI* restriction endonucleases (New England Biolabs); agarose gel-elution  
64 of 0.5-1.5 kb fragments followed by ligation with *MluI* adaptors; Selection of SSR-rich  
65 adaptor-ligated DNA fragments by hybridization to biotinylated oligonucleotides  
66 [(GA)<sub>15</sub>,(CA)<sub>15</sub>,(AGA)<sub>10</sub>, CAA<sub>10</sub>] and separation with streptavidin-conjugated magnetic  
67 beads (Dynabeads, DYNAL). The selected fragments were cloned into vector pTZ57-R/T  
68 (Fermentas) using *Escherichia coli* DH10B competent cells. Plasmids were isolated from  
69 ~300 random clones using standard protocols (Sambrook *et al.* 1989). Insert DNA was  
70 amplified/sequenced for both strands using universal M13 primers and BigDye®  
71 Terminator v3.1 Cycle Sequencing Kit on ABI-prism3700 automated DNA sequencer  
72 (Applied Biosystems). Sequences containing >20 bp long SSR-repeat cores were used for  
73 marker development. Primer-pairs were designed from the flanking sequences using  
74 GENETOOL\_ver1.0 (<http://www.doubletwist.com>). All primers and oligos were custom  
75 synthesized (<http://www.bioserve.co.in>).

76

77 PCR conditions were standardized and working primer-pairs were tested for their  
78 suitability as genetic markers using DNA samples of 57 CSMs. All PCR amplifications  
79 were performed on PTC-200 thermal-cycler (MJ Research Inc.) in 15  $\mu$ l reaction volumes  
80 that comprised 20 ng template DNA, 2 pM of each primer, 1U AmpliTaqGold DNA  
81 polymerase (Applied Biosystems), 1x PCR buffer-1, 100  $\mu$ M of each dNTP and 1.5 mM  
82  $MgCl_2$ . PCR conditions comprised an initial denaturation at 94°C for 10 min, followed by  
83 35 cycles of 3-step PCR amplification (denaturation at 94°C for 30s, annealing for 30s at  
84 primer specific annealing temperature (Table-1) and extension at 72°C for 45s) followed by  
85 a final extension at 72°C for 30 min. PCR products were resolved on ABI 3700/3730  
86 automated DNA sequencer, and precisely sized using GENESCAN and GENOTYPER  
87 software using GS500LIZ ladder as the internal size standard (Applied Biosystems, UK) as  
88 per the manufacturer's instructions. Further, haplotype diversity ( $H$ ) instead of  
89 heterozygosity, was calculated as a suitable measure of genetic diversity at each locus  
90 following 'Shane's simple guide to F-statistics'  
91 (<http://www.library.auckland.ac.nz/subjects/bio/pdfs/733Pop-g-stats2.pdf>) for haploid data.

92

93 Sequencing of ~300 randomly selected recombinant clones yielded 237 good sequences  
94 (300 to 700 bp long); 144 of these were SSR-positive. Most of the microsatellites were  
95 compound repeats; and interestingly, 98 % were TNRs (trinucleotide repeats; TTG and  
96 CAA being the most common), despite the use of equal number of DNRs for hybridization/  
97 enrichment. The observation, though preliminary, may suggest that *D. giganteum* genome  
98 is rich in TNRs, something that needs further validation.

99

100 A total of 12 primer pairs (out of 25 designed/tested) could be validated as useful  
101 polymorphic microsatellite markers. Characteristics of the 12 markers and their

102 amplification status in 42 test isolates of *D. giganteum* are enumerated in table-1. In  
103 general, no null or multiple alleles were observed for *D. giganteum* strains. Further testing  
104 of the markers on 15 strains of six related CSM species, revealed their broad cross-species  
105 transferability and suitability for studies on population structure and kinship even in related  
106 species (table-2). Here it may be mentioned that, in few cases of cross-species typing,  
107 either no amplification or multiple alleles were evident. In our opinion, while the first  
108 situation may suggest presence of 'null alleles' generally expected in related species, the  
109 other situation was apparently due to samples being contaminated (from mixed cultures) as  
110 multiple alleles at most loci were restricted to only three strains, albeit other possibilities  
111 (like duplicated loci) can not be discounted.

112

113 These markers were also found useful for differentiating CSM strains both at intra-/inter-  
114 species level robustly resolving their phylogenetic relationship (data not shown), which  
115 was largely comparable with earlier CSM phylogenies (Schaap *et al.* 2006). Thus, we  
116 describe 12 new *D. giganteum* specific microsatellites and demonstrate them to be highly  
117 polymorphic which can be potentially used for studying various aspects of CSM biology in  
118 a number of species.

119

120

## 121 **Acknowledgment**

122

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125 thank *Dictyostelium* stock centre, Columbia University, New York for providing *D.*  
126 *discoideum* strains.

127

128

129 **References:**130 Aggarwal RK, Velavan T, Udaykumar D, *et al.* (2004) Development and characterization131 of novel microsatellite markers from the olive ridley sea turtle (*Lepidochelys olivacea*).132 *Molecular Ecology Notes*, **4**: 77-79.133 Bonner J (2009) *The social amoebae* Princeton Univ. Press, Princeton.134 Hamilton WD (1964) The genetical evolution of social behaviour. I and II. *J Theor Biol* **7**:

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**Table 1: Characteristics of the new microsatellite markers developed for *Dictyostelium giganteum***

Locus	Repeat motif	Primer sequence (5' -3')	Tag*	T <sub>a</sub> (°C)	Alleles/Polymorphism in <i>D. giganteum</i> (n= 42)				GenBank Accession No
					Status (no. of samples amplified)	Size range # (FA)	N <sub>a</sub>	Haplotype Diversity (H)	
<i>Dicty.gig_1</i>	(TTG) <sub>8</sub>	F: TGTGGATGTTGAGCTGTTGAATTG R: CTCAAAGACAAGCCCCAACTATTATTACA	FAM	52	P (42)	116, 119 (119)	2	0.22	GU904568
<i>Dicty.gig_2</i>	(CAA) <sub>8</sub>	F: AACCACAAAATACTAATAATCAACAACCAC R: TGAGGTAATATTGAGGGATGAGGATG	HEX	52	P (42)	172, 175 (172)	2	0.22	GU904573
<i>Dicty.gig_3</i>	(GTTGGT) <sub>6</sub>	F: GCTGGTGCACGAGTTTGAGTTTGA R: ATGGTGGTAGTGAAAAATTTAGGTGATGA	FAM	52	P (42)	204-237 (204)	4	0.54	GU904569
<i>Dicty.gig_8</i>	(TTG) <sub>13</sub>	F: TAGGTGGTGGTGGCATCAATTGT R: GCAAATCAACAAAAGAAGCACAAGC	HEX	55	P (41)	243-282 (253)	4	0.66	GU904555
<i>Dicty.gig_9</i>	(ACA) <sub>12</sub>	F: CGTGCCGATATTGATTCAGGTC R: TGTTCCTTTGGTGATGATTTATGAG	FAM	55	P (42)	110-129 (129)	7	0.73	GU904556
<i>Dicty.gig_16</i>	(TTG) <sub>9</sub> C (TGT) <sub>8</sub>	F: TGAGGTGAGTTTGAAATATAGGTGGT R: CCATCAGATTCCTTTTCAGGTTTCAG	FAM	55	P (42)	196, 215 (215)	2	0.22	GU904559
<i>Dicty.gig_17</i>	(TTG) <sub>17</sub>	F: ACCTGATGATGATGCTGAATTTGTAC R: TGCAGCTAGTGGTCATATTGGTTTTA	HEX	55	P (42)	200-226 (226)	4	0.65	GU904560
<i>Dicty.gig_19</i>	(CAA) <sub>7</sub>	F: CAACCTCAATTAAGAAGAAGCAGTG R: TAGGCTTCATTCCAATCTTACCAT	HEX	55	P (42)	128-143 (143)	4	0.44	GU904562
<i>Dicty.gig_20</i>	(TTG) <sub>19</sub>	F: TGGTGATGACAAAAGTACTAGGTGAAG R: ACCAAATACTCCAAATAATACTGCATC	FAM	55	P (42)	128-226 (226)	6	0.63	GU904563
<i>Dicty.gig_21</i>	(ACA) <sub>8</sub> G (CAA) <sub>9</sub>	F: ATTCCATCAGATTCCTTTTCAGGTTTCAG R: TGAGGTGAGTTTGAAATATAGGTGGT	HEX	55	P (42)	190, 208 (208)	2	0.50	GU904564
<i>Dicty.gig_22</i>	(TGT) <sub>12</sub>	F: TGGAACGTACAGAGATTGGTGGAT R: CACCATTACAAGATCCACATAGTCC	FAM	55	P (42)	115-180 (164)	6	0.64	GU904565
<i>Dicty.gig_25</i>	(CAA) <sub>9</sub>	F: GCTGGTAATCAACATCCTCTTTCTC R: ACTTGATGGGTAATGCTTGATGA	HEX	55	P (42)	122-224 (128)	4	0.60	GU904567

F: forward primer; R: reverse primer; \*: Fluorescence tag at the 5'-end of the forward primer; T<sub>a</sub>: annealing temperature; P: polymorphic; M: monomorphic; #: allele size in base pairs; N<sub>a</sub>: Number of alleles observed; FA: most frequent allele in the tested strains of *D. giganteum*.

**Table 2: Cross-amplification of the *D. giganteum* specific microsatellites in six other CSM species.**

Locus	<i>D. macrocephalum</i> (n =4)			<i>D. discoideum</i> (n =4)			<i>D. purpureum</i> (n =3)			<i>D. mucoroides</i> (n =2)			<i>D. rosarium</i> (n=1)	<i>Polysphondylium pallidum</i> (n=1)	Frequency of -null alleles (if any) <sup>\$</sup>
	Status	Size range #	Na	Status	Size range #	Na	Status	Size range #	Na	Status	Size range #	N <sub>a</sub>	Size	Size	
<i>Dicty.gig_1</i>	P	113 - 119	3*	P	116, 119	2*	M	115, 110	2*	P	88 - 149	4*	119	119	-nil-
<i>Dicty.gig_2</i>	P	158, 172	2	P	158, 172	2	P	112 -172	3*	M	158	1	85,158 *	158	-nil-
<i>Dicty.gig_3</i>	P	196, 204	2	P	196, 204	2	M	204	1	M	196	1	204	204	-nil-
<i>Dicty.gig_8</i>	P	246 - 267	3*	M	252	2	M	252	1	M	-na-	0	-na-	-na-	0.26
<i>Dicty.gig_9</i>	P	106 - 128	4*	P	112 - 128	4*	M	114, 128	2*	M	128	1	128	128	0.06
<i>Dicty.gig_16</i>	P	195, 214	2	P	195, 214	2*	P	195, 214	2*	M	176	1	214	214	-nil-
<i>Dicty.gig_17</i>	P	199 - 226	3	P	202 - 226	2*	M	199, 226	2*	P	157, 227	2	157, 227*	227	-nil-
<i>Dicty.gig_19</i>	P	122 - 142	4*	P	124 - 142	4*	P	127 - 142	3*	P	127, 142	2*	142	142	-nil-
<i>Dicty.gig_20</i>	P	121 - 184	3	M	184	1	P	128, 184	2*	M	-na-	0	-na-	-na-	0.26
<i>Dicty.gig_21</i>	P	190, 208	2	M	208	1	P	190 - 208	2*	M	-na-	0	-na-	-na-	0.26
<i>Dicty.gig_22</i>	P	161 - 167	3	P	164, 167	2*	P	110 - 164	3*	M	164	1	164	164	0.06
<i>Dicty.gig_25</i>	P	122 - 128*	3	P	122 - 128	2	M	122, 128	2	M	122, 128	2*	128	128	-nil-

M: Monomorphic, P: Polymorphic; #: Observed alleles (size in base pairs); N<sub>a</sub>: Number of alleles; n: Number of samples analyzed;

\*: Indicates more than one allele in one of the test sample; -na-: no amplification (possibly null allele). \$: proportion of samples of related CSM species wherein no amplification was seen, which is considered to be probably due to null alleles

## PERMANENT GENETIC RESOURCES NOTE

# Permanent Genetic Resources added to Molecular Ecology Resources Database 1 August 2010 – 30 September 2010

MOLECULAR ECOLOGY RESOURCES PRIMER DEVELOPMENT CONSORTIUM, RAMESH K. AGGARWAL,<sup>1</sup> JOEL ALLAINGUILLAUME,<sup>2</sup> M. M. BAJAY,<sup>3</sup> SANTAN BARTHWAL,<sup>4</sup> P. BERTOLINO,<sup>5, 6, 7</sup> PRITI CHAUHAN,<sup>4</sup> SOFIA CONSUEGRA,<sup>2</sup> ADAM CROXFORD,<sup>2</sup> DESIRÉ L. DALTON,<sup>8, 9</sup> E. DEN BELDER,<sup>10</sup> E. DÍAZ-FERGUSON,<sup>11</sup> M. R. DOUGLAS,<sup>12</sup> MICHAEL DREES,<sup>13</sup> J. ELDERSON,<sup>10</sup> G. D. ESSELINK,<sup>10</sup> J. F. FERNÁNDEZ-MANJARRÉS,<sup>5, 6, 7</sup> N. FRASCARIA-LACOSTE,<sup>5, 6, 7</sup> STEFFI GÄBLER-SCHWARZ,<sup>14</sup> CARLOS GARCIA DE LEANIZ,<sup>15</sup> H. S. GINWAL,<sup>4</sup> MICHAEL A. D. GOODISMAN,<sup>16</sup> BAOLING GUO,<sup>17</sup> M. B. HAMILTON,<sup>18</sup> PAUL K. HAYES,<sup>19</sup> YAN HONG,<sup>20</sup> TADASHI KAJITA,<sup>21</sup> STEVEN T. KALINOWSKI,<sup>22</sup> LAURENT KELLER,<sup>23</sup> BEN F. KOOP,<sup>24</sup> ANTOINETTE KOTZÉ,<sup>8, 9</sup> ALBERT LALREMRUATA,<sup>1</sup> FLORIAN LEESE,<sup>25</sup> CHUNHONG LI,<sup>20</sup> W. Y. LIEW,<sup>26</sup> S. MARTINELLI,<sup>27</sup> EMILY A. MATTHEWS,<sup>16</sup> LINDA K. MEDLIN,<sup>28, 29</sup> AMBER M. MESSMER,<sup>24</sup> ELISABETH I. MEYER,<sup>13</sup> M. MONTEIRO,<sup>3</sup> G. R. MOYER,<sup>30</sup> R. JOHN NELSON,<sup>31</sup> THUY T. T. NGUYEN,<sup>32, 33, 34</sup> C. OMOTO,<sup>35</sup> JUNYA ONO,<sup>21</sup> V. A. C. PAVINATO,<sup>35</sup> MORGAN PEARCY,<sup>23</sup> J. B. PINHEIRO,<sup>3</sup> L. D. POWER,<sup>18</sup> ANITA RAWAT,<sup>4</sup> THORSTEN B. H. REUSCH,<sup>36</sup> DAN SANDERSON,<sup>24</sup> J. SANNIER,<sup>5, 6, 7</sup> **SANTOSH SATHE**,<sup>37</sup> C. K. SHERIDAN,<sup>18</sup> M. J. M. SMULDERS,<sup>10</sup> A. SUKGANAH,<sup>26</sup> KOJI TAKAYAMA,<sup>21, 38</sup> MARIKO TAMURA,<sup>21</sup> YOICHI TATEISHI,<sup>39</sup> DELPHINE VANHAECKE,<sup>2</sup> NINH V. VU,<sup>22</sup> R. WICKNESWARI,<sup>26</sup> A. S. WILLIAMS,<sup>30</sup> G. M. WIMP,<sup>18</sup> VOLKER WITTE,<sup>40</sup> and M. I. ZUCCHI<sup>41</sup>

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## Abstract

This article documents the addition of 229 microsatellite marker loci to the Molecular Ecology Resources Database. Loci were developed for the following species: *Acacia auriculiformis* × *Acacia mangium* hybrid, *Alabama argillacea*, *Anoplopoma fimbria*, *Aplochiton zebra*, *Brevicoryne brassicae*, *Bruguiera gymnorhiza*, *Bucorvus leadbeateri*, *Delphacodes detecta*, *Tumidagena minuta*, *Dictyostelium giganteum*, *Echinogammarus berilloni*, *Epimedium sagittatum*, *Fraxinus excelsior*, *Labeo chrysophekadion*, *Oncorhynchus clarki lewisi*, *Paratrechina longicornis*, *Phaeocystis antarctica*, *Pinus roxburghii* and *Potamilus capax*. These loci were cross-tested on the following species: *Acacia peregrinalis*, *Acacia crassicarpa*, *Bruguiera cylindrica*, *Delphacodes detecta*, *Tumidagena minuta*, *Dictyostelium macrocephalum*, *Dictyostelium discoideum*, *Dictyostelium purpureum*, *Dictyostelium mucoroides*, *Dictyostelium rosarium*, *Polysphondylium pallidum*, *Epimedium brevicornum*, *Epimedium koreanum*, *Epimedium pubescens*, *Epimedium wushanense* and *Fraxinus angustifolia*.

This article documents the addition of 229 microsatellite marker loci to the Molecular Ecology Resources Database. Table 1 contains information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and

GenBank. The authors responsible for each set of loci are listed in the final column. A full description of the development protocol for the loci presented here can be found in the Molecular Ecology Resources Database (<http://tomato.biol.trinity.edu/>).

**Table 1** Information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
<i>Acacia auriculiformis</i> × <i>Acacia mangium</i> hybrid	20	<i>Acacia peregrinalis</i> , <i>Acacia crassicarpa</i>	44734–44741, 44812–44823	HQ110862–HQ110881	Sukganah, A; Liew, W.Y.; Wickneswari, R.
<i>Alabama argillacea</i>	10	n/a	44502–44508, 44510–44512	GF102184–GF102193	Pavinato, V.A.C.; Bajay, M.M.; Martinelli, S.; Monteiro, M.; Pinheiro, J.B.; Zucchi, M.I.; Omoto, C.
<i>Anoplopoma fimbria</i>	13	n/a	44824–44836	GO616605.1, GO616986.1, GO617191.1, GO618107.1, GO618227.1, GO618807.1, GO618865.1, GO619216.1, GO620444.1, GO620529.1, GO629344.1, GO638529.1, GO646855.1	Messmer, Amber M.; Sanderson, Dan; Nelson, R. John; Koop, Ben F.
<i>Aplochiton zebra</i>	13	n/a	44587–44599	HM997136–HM997140, HM997142–HM997148, HQ003931	Vanhaecke, Delphine; Croxford, Adam; Allainguillaume, Joel; Garcia de Leaniz, Carlos; Consuegra, Sofia

Table 1 Continued

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
<i>Brevicoryne brassicae</i>	9	n/a	44548–44556	FN820283–FN820291	Esselink, GD; den Belder, E; Elderson, J; Smulders, MJM
<i>Bruguiera gymnorrhiza</i>	14	<i>B. cylindrica</i>	44644–44654, 44656–44658	AB571659–AB571669, AB571671–AB571673	Takayama, Koji; Tamura, Mariko; Ono, Junya; Tateishi, Yoichi; Kajita, Tadashi
<i>Bucorvus leadbeateri</i>	12	n/a	44565–44567, 44569–44577	HM590197–HM590203, HM590206–HM590210	Dalton, Desiré L; Kotzé, Antoinette
<i>Delphacodes detecta</i> , <i>Tumidagena minuta</i>	10, 7	<i>Delphacodes detecta</i> , <i>Tumidagena minuta</i>	44673–44693	HM626384–HM626400	Sheridan, C. K.; Douglas, M. R.; Power, L. D.; Wimp, G. M.; Hamilton, M. B.
<i>Dictyostelium giganteum</i>	12	<i>Dictyostelium macrocephalum</i> , <i>Dictyostelium discoideum</i> , <i>Dictyostelium purpureum</i> , <i>Dictyostelium mucoroides</i> , <i>Dictyostelium rosarium</i> , <i>Polysphondylium pallidum</i>	44709, 44710, 44712–44721	GU904555, GU904556, GU904559, GU904560, GU904562–GU904565, GU904567–GU904569, GU904573	Sathe, Santosh; Lalremruata, Albert; Aggarwal, Ramesh K.
<i>Echinogammarus berilloni</i>	11	n/a	44600–44610	HQ185684–HQ185694	Drees, Michael; Reusch, Thorsten B. H.; Meyer, Elisabeth I.
<i>Epimedium sagittatum</i>	8	<i>Epimedium brevicornum</i> , <i>Epimedium koreanum</i> , <i>Epimedium pubescens</i> , <i>Epimedium wushanense</i>	44557–44564	HM623765–HM623772	Li, Chunhong; Guo, Baoling; Hong, Yan
<i>Fraxinus excelsior</i>	15	<i>Fraxinus angustifolia</i>	44694–44708	FR635387, FR636736, FR637753, FR638723, FR639294, FR639485, FR639792, FR640915, FR642190, FR644535, FR644953, FR645030, FR645771, FR645842, FR646655	Sannier, J.; Bertolino, P.; Frascaria-Lacoste, N.; Fernández-Manjarrés, J. F.
<i>Labeo chrysophekadion</i>	9	n/a	44578–44586	HM641012–HM641020, AJ291680, AJ507524	Nguyen, Thuy T. T.
<i>Oncorhynchus clarki lewisi</i>	12	n/a	44536–44547	HM153812–HM153823	Vu, Ninh V.; Kalinowski, Steven T.
<i>Paratrechina longicornis</i>	15	n/a	44611–44625	HM210893–HM210895, HM210900, HM210909, HM210910, HM210912, HM210913, HM210915–HM210917, HM210919–HM210921, HM210929, HM210934, HM210935, HM210937, HM357722	Matthews, Emily A.; Percy, Morgan; Witte, Volker; Keller, Laurent; Goodisman, Michael A. D.
<i>Phaeocystis antarctica</i>	8	n/a	44636–44643	HQ132752–HQ135759	Gäbler-Schwarz, Steffi; Leese, Florian; Hayes, Paul K.; Medlin, Linda K.

Table 1 Continued

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
<i>Pinus roxburghii</i>	19	n/a	44793–44811	See text for details.	Chauhan, Priti; Ginwal, H.S.; Rawat, Anita; Barthwal, Santan
<i>Potamilus capax</i>	12	n/a	44661–44672	HM991151, HM991153–HM991163	Díaz-Ferguson, E.; Williams, A.S.; Moyer, G.R.