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ORIGINAL ARTICLE

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.

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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,

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. Agnihothrudu [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′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. Fresh dung samples were lifted with sterile forceps and immediately transferred to wide-mouthed sterile plastic tubes (2×10 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° 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 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^2 plastic plates containing 2% phosphate-buffered non-nutrient agar (PBA; KH_2PO_4 2.25 g, K_2HPO_4 0.67 g, agar 20 g, H_20 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 $MgCl_2$), 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**<<**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**.

Appoach 1: The probability that none of **n** spores picked at random belong to class II is $(1 - q)^{\mathbf{n}}$, implying that the probability that at least one of those n spores belongs to class II is $1 - (1 - q)^{\mathbf{n}}$. Because none of our n spores does, we can say that $[1 - (1 - q)^{\mathbf{n}}] \leq 1/n$ 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 $1 - (1 - q)^{\mathbf{n}} = 0.95$ 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).

Appoach 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 $\mathbf{m_{n-1}}$. After sampling **n** spores, the corresponding mean, $\mathbf{m_n}$, would either remain unchanged (in case the **n**th spore too came from a preexisting clone, which would happen with a probability $\mathbf{m_{n-1}/C}$) or increase by 1 (in case the **n**th spore came from a new clone, which would happen with a probability $1 - \mathbf{m_{n-1}/C}$). In short, the difference $(\mathbf{m_n} - \mathbf{m_{n-1}})$ would either be 0 (with a probability $\mathbf{m_{n-1}/C}$) or 1 **(**with a probability $1 - \mathbf{m_{n-1}/C}$). Thus, we may write $\mathbf{m_n} - \mathbf{m_{n-1}} = 0 \times (\mathbf{m_{n-1}/C}) + 1 \times (1 - \mathbf{m_{n-1}/C})$. This results in the recurrence relation $\mathbf{m_n} = \mathbf{m_{n-1}}\,(1 - 1/\mathbf{C}) + 1$, which is easily solved to yield $\mathbf{m_n} = \mathbf{C} \times (1 - K^{\mathbf{n}})$ where K stands for $1 - 1/\mathbf{C}$. Now, if we take the number of clones observed by us, namely **m**, as a measure of the true mean $\mathbf{m_n}$, 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-*

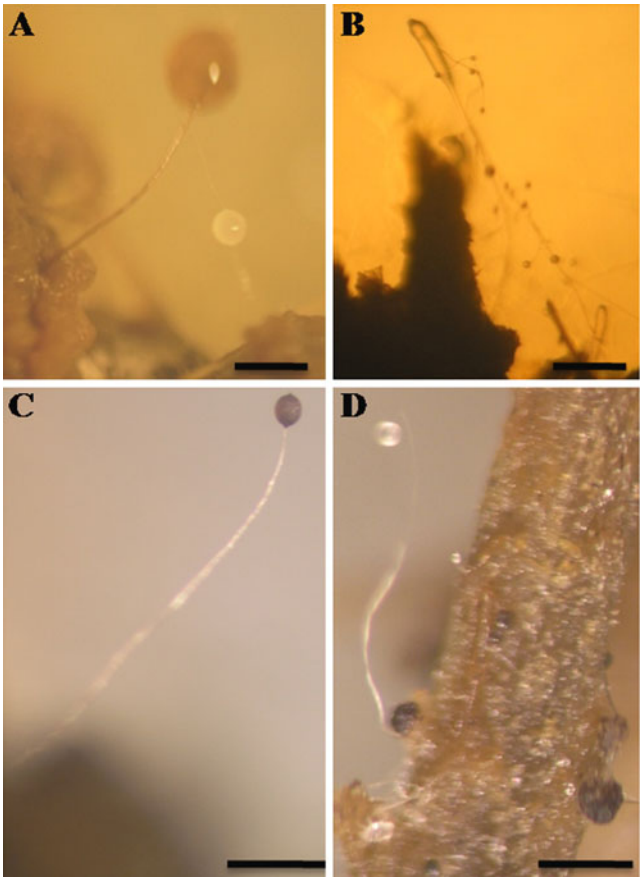

Figure 1 CSM fruiting bodies seen on animal dung samples that were not treated in any way. **A** *Dictyostelium purpureum* and *Dictyostelium* spp. from tiger scat. **B** *Dictyostelium giganteum* and *Polysphondylium violaceum* from gaur dung. **C** *Dictyostelium purpureum* from spotted deer pellet. **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 $\mathbf{m_n}$ curve rises sharply and tends toward infinity—in practical terms, toward the total number of spores—as $\mathbf{m_n}$ 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-*

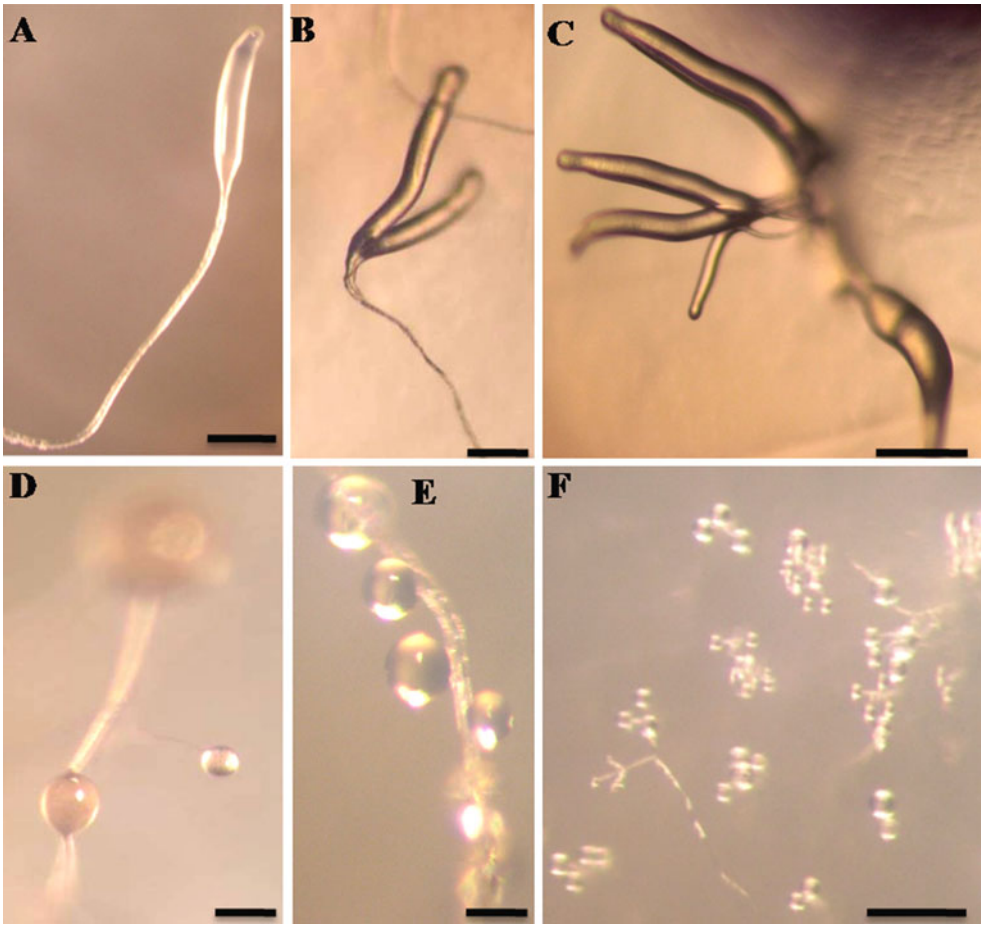

Figure 2 CSM multicellular stages obtained after generating clonal subcultures from animal dung isolates. **A** Slug of *Dictyostelium giganteum* with a long stalk. **B** Bifurcating slug of *Dictyostelium* species. **C** Many slugs emerging from one aggregate. **D** CSM species with branched fruiting 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^2 (spotted deer) to approximately 1,000 km^2 (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): *Axis axis*	4.5	Primarily grazer, browser (in dry season) also eats fruits, flowers, and fallen leaves	3	*Dpu*, *Dg*, *Dsp* (*bifurcating*), *Pv*
Tiger: *Panthera tigris*	15	Carnivore	1	*Dpu*, *Dg*, *Dd*
Elephant: *Elephas maximus*	32	Depending on season and habitat, either grazer or browser	2	*Dpu*, *Dg*, *Dsp* (*bifurcating*), *Dr*
Wild dog: *Cuon alpinus*	4.5–8	Carnivore	2	*Dpu*, *Dg*
Sambar: *Cervus unicolor*	14	Depending on season and habitat, either grazer or browser	2	*Dpu*, *Dg*, *Dma*
Porcupine: *Hystrix indica*	15	Tubers, roots, fruit	2	*Dg*, *Dpo*
Yak: *Bos grunniens*	50	Mainly grazer, also known to eat mosses and lichens	1	*Dg*, *Dsp* (*bifurcating*), *Dd*, *Dr*, *Dmi*
Gaur (Bison): *Bos gaurus*	20	Mainly browser, some reports of eating tree bark and grasses	2	*Dg*, *Dsp* (*bifurcating*), *Dr*, *Pv*, *Pp*
Panther: *Panthera pardus*	15	Carnivore	Many (count not kept)	Diverse, not identified
Hyena: *Crocuta crocuta*	5–10	Scavenger (carnivore)	Many (count not kept)	Diverse, not identified
Barking deer: *Muntiacus muntjac*	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	*Dpu*, *Dg*, *Dsp (bifurcating)*, *Dr*, *Dd*, *Dma*, *Dpo*, *Dmi*, *Dmu*, *Dt*, *Pv*, *Pp*

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 (**n**)	No. of clones found (**m**)	Estimated total number of clones (**C**)	
					Approach 1	Approach 2
D. purpureum (SD-1)	Spotted deer (non-nutrient agar)	7 (5 informative)	8	4	6	5
D. purpureum (SD-3)		2 (2 informative)	12	5	6	5
D. purpureum (SD-4)		7 (5 informative)	12	6	8	7
D. purpureum (SD-5)		8 (8 informative)	10	5	7	6
D. giganteum (SD-8)		2 (2 informative)	13	5	6	5
D. purpureum (WD-1)	Wild dog (non-nutrient agar)	4 (4 informative)	12	7	9	9
D. giganteum (E)	Elephant (non-nutrient agar)	6 (3 informative)	10	3	4	3
D. giganteum (F)		7 (7 informative)	8	4	6	4
D. giganteum (18)	Soil speck from 50th ha. SM/ 10 agar (no exogenous bacteria)	3 (3 informative)	10	5	7	6
D. giganteum (20)		3 (3 informative)	7	4	6	5
D. giganteum (21)		4 (3 informative)	9	5	7	6
D. giganteum (23)		5 (5 informative)	15	7	9	8
D. giganteum (24)		6 (0 informative)	4	1	2	1
D. purpureum (B)	Soil speck from 46th ha. SM/10 agar (no exogenous bacteria)	4 (4 informative)	9	3	4	3
D. giganteum (D)		5 (5 informative)	7	4	6	5
D. giganteum (5B-1)	Soil speck from 5th ha. (non-nutrient agar)	7 (0 informative)	5	1	2	1
D. giganteum	IISc campus soil on PBA plate, *Klebsiella aerogenes* 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 μl 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	*D. purpureum*, *D. giganteum*, *D. mucoroides*, *D. minutum*, *D. discoideum*, *P. pallidum*, and *P. violaceum*	[1]
Soil from West Central Himalaya and tropical forests in peninsular India	*D. purpureum*, *D. giganteum*, *D. mucoroides*, *D. minutum*, *P. pallidum*, and *P. violaceum*. *D. aureo-stipes*, *D. sphaerocephalum*, *D. polycephalum*, *D. tenue*, *D. vinaceo-fuscum*, *A. subglobosum*	[9]
Soil from Lucknow	*D. mucoroides*, *P. violaceum*, *D. sphaerocephalum*	[42, 44]
Soil (from areas in Mudumalai forest)	*D. purpureum*, *D. giganteum*, *D. mucoroides*, *D. minutum*, *P. pallidum*, *P. violaceum*, *Dsp (bifurcating)*, *D. discoideum*, *D. polycephalum*, *D. rosarium, D. macrocephalum*, *D. tenue*	This study
Animal dung	*D. purpureum*, *D. giganteum*, D. *minutum*, *P. pallidum*, *P. violaceum, Dsp* (*bifurcating*), *D. discoideum*, *D. polycephalum*, *D. rosarium*, *D. macrocephalum*	This study

D. aureo-stipes, *D. sphaerocephalum*, *D. vinaceo-fuscum*, and *A. subglobosum* were seen by Cavender and Lakhanpal 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

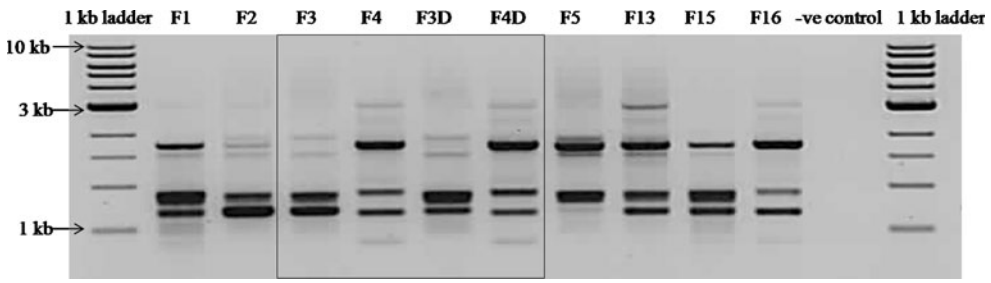

Figure 3 Genetic diversity within a fruiting body: Lanes marked *F1*, *F2*, *F3*, *F4*, *F5*, *F13*, *F15*, and *F16* show RAPD profiles of different clones derived from spores from a single fruiting body using the OPAD-4 primer. F3D and F4D are from independently isolated DNA samples from the same clone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