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Kin Discrimination in Dictyostelium Social Amoebae

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SYMPOSIUM ARTICLE

Kin discrimination in *Dictyostelium* social amoebae[1]

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ABSTRACT

Evolved cooperation is stable only when the benefactor is compensated, either directly or through its relatives. Social amoebae cooperate by forming a mobile multicellular body in which about 20% of participants ultimately dies to form a stalk. This benefits the remaining individuals that become hardy spores at the top of the stalk, together making up the fruiting body. In studied species with stalked migration, *P. violaceum, D. purpureum,* and *D. giganteum,* sorting based on clone identity occurs in laboratory mixes, maintaining high relatedness within the fruiting bodies. *D. discoideum* has unstalked migration where cell fate is not fixed until the slug forms a fruiting body. Laboratory mixes show some degree of both spatial and genotype-based sorting, yet most laboratory fruiting bodies remain chimeric. However, wild fruiting bodies are made up mostly of clonemates. A genetic mechanism for sorting is likely to be cell adhesion genes *tgrB1* and *tgrC1,* which bind to each other. They are highly variable, as expected for a kin discrimination gene. It is a puzzle that these genes do not cause stronger discrimination between mixed wild clones, but laboratory conditions or strong sorting early in the social stage diminished by later slug fusion could be explanations.

Keywords

Dictyostelium; recognition; kin selection; social amoeba; kin discrimination; altruism; cell adhesion; eusociality; cooperation; *tgrB1* and *tgrC1*;

Recognition of self from non-self is fundamental (Boehm 2006). Recognition of kin has a different intellectual history but is essentially the same topic. Here we discuss kin discrimination and not kin recognition because recognition is detected only when it results in measurable behavioral differences. Kin discrimination is defined as the identification and preferential treatment of relatives over non-relatives (Fletcher and Michener 1987; Tsutsui 2004; Strassmann et al. 2011). The focus on kin discrimination was a natural

outgrowth of Hamilton's inclusive fitness theory because it emphasizes the importance in social organisms of reproductive success achieved by rearing relatives other than progeny (Hamilton 1964; Holmes 2004).

Kin discrimination is often dependent on environmental context. If one's young and only one's own young are in a specific place, say a tree hollow, then actual recognition past that of a specific place itself is less necessary for aid to be appropriately directed. If, on the other hand, one's baby is in a crèche of many, then the individual will benefit from traits that uniquely identify it, as would be true of young in a herd of zebras, or a cave of Mexican free-tailed bats (Fletcher and Michener 1987). Other kin discrimination systems can be based on signals of genetic similarity.

Being recognized as kin can mean substantial rewards, so it is no surprise that all kin recognition systems have their cheaters, within and between species (Ghoul et al. 2014; Stevens 2015). Some cheaters take advantage of place-based systems, laying their eggs in the nests of others, as some cowbirds and cuckoos do (Davies 2010). Other cheaters mimic the recognition signals of individuals, as blue butterfly larvae do to their ant hosts (Nash et al. 2008). To understand how kin recognition systems can be defeated, it is necessary to understand their underlying mechanisms and development, approaches that are particularly feasible in the social amoeba *Dictyostelium.*

In the haploid eukaryote amoeba *Dictyostelium*, the social life cycle is as follows. Free-living amoebae aggregate when they starve to a signal of cAMP. The aggregate then forms a multicellular slug, which moves towards light. Ultimately the slug forms a fruiting body in which about 20% of cells die to form a stalk, which benefits the spores that flow to the top, making them more likely to be dispersed. Without kin discrimination or some other means of ending up with clonemates, a cheater clone could join an aggregation of a foreign clone and take advantage of death of the other clone as it formed the stalk. If they did this regularly, the social stage would fail to persist through evolution.

Aggregation is a very different means of arriving at multicellularity than the single-cell bottleneck most multicellular organisms go through. A single cell bottleneck means that sterile somatic cells aid reproductive cells that are clonemates, so the benefit of one is the benefit of all. It is no wonder that under these circumstances life can form the kinds of elaborate multicellular beings that make up animals, for example. It has been speculated that the similarly ancient Dictyostelia have formed only modest morphological specializations because the cellular members of the multicellular body retain selfish interests (Kessin 2001; Strassmann and Queller 2011). In the laboratory, it is clear that there are many ways that bias cells towards becoming stalk, from having recently divided to being in poor condition, or being late to join the aggregation (Strassmann and Queller 2011). Furthermore, it is clear that some wild clones are more likely to become spore and prevail over other wild clones collected at the same location (Fortunato et al. 2003a). Nevertheless, there is a strong cooperative element to the social stage, one that is maintained by fruiting bodies being largely clonal. This is achieved by either active kin discrimination or because no other clones are nearby. Here we discuss both processes.

In the rest of this review, we focus on kin discrimination and not on cheating or competition to become spore and not stalk, which has been covered elsewhere (Ennis et al. 2000; Strassmann and Queller 2011; Ostrowski et al. 2015). *D. discoideum* is the best-studied species of social amoeba (Kessin 2001). It has long been a model for development, chemotaxis, and more recently pathogenesis (Steinert and Heuner 2005). It has a

sequenced genome, a solitary stage as a haploid predatory amoeba, a social stage where amoebae aggregate into a slug of tens to hundreds of thousands of cells, and a sterile caste of stalk cells in the fruiting body (Kessin 2001; Eichinger et al. 2005). It has a dedicated center, dictybase.org. It is unusual in the Dictyostelia because it has stalkless migration in the social stage, while most other species have stalked migration as dead cells form a sturdy stalk as the slug moves (Schaap et al. 2006; Gilbert et al. 2012a). Most studies of kin discrimination have focused on this species, but understanding species level discrimination and discrimination in other species puts the work on *D. discoideum* in context.

SPECIES LEVEL DISCRIMINATION IN *DICTYOSTELIUM*

Since different species can sometimes co-aggregate in the social process, resulting in formerly independent amoebae becoming dead stalk cells, a fundamental kind of recognition involves avoiding other species. Unless both species benefit, there would be no advantage to forming chimeric slugs. A number of early studies have indicated that different species of *Dictyostelium* can mostly sort into species-specific fruiting bodies (Bonner and Adams 1958; Filosa 1962; Nicol and Garrod 1978). The focus of the early studies was on developmental patterning more than on evolutionary outcome, so they did not always result in precise counts of final spore number of each species. In 1979 Sternfeld showed that when *D. discoideum, D. mucoroides, D. purpureum,* and *Polysphondylium violaceum* are mixed, they largely sort into different fruiting bodies (Sternfeld 1979). He ascribed this pattern to differential cell adhesion and also noted that mixtures of *D. discoideum* with *D. purpureum* produced chimeric fruiting bodies. More recent work supports this result and includes other species (Sathe et al. 2014). A study of *D. discoideum – D. purpureum* mixtures found that fruiting bodies always looked like one clone or the other (Jack et al. 2008). Fruiting bodies that look like *D. discoideum* contained about 15% spores from *D. purpureum,* while fruiting bodies that looked like *D. purpureum* contained only 6% spores from the other species.

Co-aggregation might be hard to avoid since multiple species in the same subgenus as *D.* discoideum use the same chemoattractants in the social stage (Kessin 2001; Schaap et al. 2006). It might be a way to achieve larger slug size which in turn results in enhanced slug movement, though larger size increases vulnerability to exploitation. Since different species regularly co-occur in soil, further study into this phenomenon would be interesting. Also, the mechanistic relationship between species discrimination and kin discrimination has not been explored.

KIN DISCRIMINATION IN DICTYOSTELIDS WITH STALKED MIGRATION

Three species of dictyostelids with stalked migration, *Polysphondyleum violaceum, D. purpureum,* and *D. giganteum* have been studied for kin. Stalked migration means that formerly independent amoebae form stalk cells right from the first movement of the slug. Because death can occur so early in the social stage, there may be a special evolutionary incentive to sort by genotype. By contrast, in *D. disocideum* this ultimate sacrifice for the group is delayed until after the slug has moved to a location where it will form a fruiting

body. In all three of these species, considerable sorting occurs (Kaushik et al. 2006; Mehdiabadi et al. 2006; Sathe et al. 2010; Kalla et al. 2011).

To estimate kin discrimination in *P. violaceum,* we compared sorting among fruiting bodies when genetically distinct clones were mixed to values obtained when the only differences were the fluorescent label(Kalla et al. 2011). We performed 13 reciprocal pairwise mixes with a fluorescent label (Cell Tracker) on one clone at a time. We mixed five pairs from within phylogenetic group B, four pairs from within groups C to F, and four pairs between groups B and the others at a density of 2000 cells per mm^2. In no cases did the controls segregate based on the fluorescent label. Sorting was high and consistent between groups, and present in all but three within-group mixes. Average relatedness of the pairwise mixes was 0.8±0.05 (SE) (Kalla et al. 2011).

We examined kin discrimination in *D. purpureum* using similar techniques to *P. violaceum,* mixing cells of clones pairwise with a fluorescent marker (Mehdiabadi et al. 2006). We found that 12 of 14 mixes showed strong sorting. Genetic relatedness in fruiting bodies was 0.81 overall and there was no sorting based simply on the fluorescent marker in the controls.

Another group found that in both *D. purpureum* and *D. giganteum* chimeras occurred but were variable and depended on the precise clones studied (Kaushik et al. 2006; Sathe et al. 2014).

LABORATORY KIN DISCRIMINATION IN *DICTYOSTELIUM DISCOIDEUM*

The clearest evidence for kin discrimination in *D. discoideum* would be clonal fruiting bodies. But clonality could arise either as the result of active discriminatory mechanisms that favor clonemates and exclude non-clonemates or because different clones rarely co-occur in nature, so aggregation areas will only contain clonemates. To answer this question, we first took wild-collected clones of *D. discoideum* that could be distinguished by microsatellite loci and mixed the clones. Our early studies examined the slugs right before stalk formation and invariably found evidence of both clones in the slugs (Strassmann et al. 2000; Fortunato et al. 2003a). Another kind of evidence for clonal segregation out of mixtures could be increased numbers of fruiting bodies as amoebae aggregate, then segregate, as has been found in the other species discussed above. However, we found that mixtures of two or more *D. discoideum* clones did not produce any more fruiting bodies than did pure cultures of single clones (Foster et al. 2002).

From these initial studies that suggested rather free mixing, we moved on to more precise observations of spores. It is unlikely but not impossible that final sorting between slug and fruiting body could occur, so in subsequent studies we looked at the spores themselves. In one study, we mixed equal numbers of cells from all pairwise mixtures of seven clones, then genotyped spores from each of 16 fruiting bodies (Fortunato et al. 2003a). We found both clones to be present in all of the fruiting bodies. In this study we used two densities of amoebae on non-nutrient plates, 3.4×10^6 and 6.8×10^6 cells per plate. These are the equivalent of 599 cells/mm^2 and 1198 cells/mm^2, since our Petri plates are actually 8.5cm in diameter.

In another study we mixed 8 clones at two densities and quantified genetic relatedness among the spores in the same fruiting bodies (Saxer et al. 2010). In this study we used clones we collected at Bald Knob VA. We mixed these clones equally on non-nutrient plates at densities of 500 spores/Petri plate and 50,000 spores/Petri plate. This is equivalent to 0.088 spores/mm^2 in the less dense treatment and 8.81 spores/mm^2 in the more dense treatment. We predicted higher relatedness in fruiting bodies from the less dense treatment and lower relatedness in the more dense treatment. We collected 66 (dense) and 71 (less dense) fruiting bodies and plated them out clonally, then genotyped 4 plaques from each to measure relatedness in the two treatments. If the 8 clones mixed completely, relatedness within fruiting bodies would be 0.125. In fact, relatedness within fruiting bodies was 0.82 ± 0.08 (95% confidence interval) in the less dense treatment and 0.31±0.10 (95% confidence interval) in the more dense treatment, both elevated above the null value of no sorting, but we could not differentiate between passive sorting due to density and active sorting due to genotype.

To look at sorting due to spatial structure, we first looked at sorting when clones grow out from a central location (Buttery *et al.* 2012). We put labeled and unlabeled amoebae of the same clone in the center of an agar plate with bacteria. The clones were genetically identical, only differing in the fluorescent label. As they grew out from the center, they sorted into patches of labeled or unlabeled clones by totally passive drift processes. This sorting was more pronounced on low food plates, leading to high relatedness over 0.6 (Buttery *et al.* 2012). The low food plates are more likely to mimic what is found in nature, making this a powerful force for sorting just because of drift the the growing edge of a plaque. A further study on passive sorting found similar patterns of high relatedness when spores were plated out at different densities. The less dense the spores, the more likely they were to develop clonal fruiting bodies even though they only differed in carriage of a fluorescent marker (smith et al. 2016).

To look at sorting due to genotype, we took advantage of a strain of the lab clone Ax4 labeled with green fluorescent protein (GFP) and mixed 15 other clones with it, including its own ancestral clone, NC4 (Ostrowski et al. 2008). A key feature of this study (like the *D. purpureum* and *P. violaceum* ones) is that a control for spatial structure is included. This control is a mix at the same density with labeled and unlabeled Ax4. This indicates the level of sorting without active recognition; it was very low. We assessed genetic diversity with 12 DNA microsatellite loci. We found that the variance between fruiting bodies in fluorescence increased with genetic distance between Ax4 and the clone with which it was mixed(Ostrowski et al. 2008). This indicates that some level of sorting into clone groups not based on spatial structure occurred, though it was far from complete. The pattern was not dependent on any feature of Ax4 since we corroborated the result by showing that variance was higher among the genetically dissimilar fruiting bodies of chimeric QS32 and QS38 compared to the mix of QS32 with similar QS33 (Ostrowski et al. 2008).

In sum, these studies have shown that when different clones of *D. discoideum* are plated together, the resulting fruiting bodies are chimeric, but there is some active sorting. We have not fully explored the impact of spatial structure in the laboratory, but indications are that it can cause considerable sorting (smith *et al.* submitted). Furthermore, it is clear that drift can cause sorting as cells grow out from a central location (Buttery et al. 2012).

Other investigations not covered here show that the chimeras suffer various kinds of costs including retarded slug migration and exploitation of one clone by another for access

to spore tissues over stalk (Strassmann et al. 2000; Foster et al. 2002; Castillo et al. 2005; Buttery et al. 2009; Jack et al. 2015).

FIELD KIN DISCRIMINATION IN *DICTYOSTELIUM DISCOIDEUM*

Even though clones sort imperfectly in the laboratory, results in the wild could be different either because of different encounter rates, or because the substrate changes interactions such as adhesion (Queller et al. 2003). In this section we discuss the distribution of genetically distinct clones in nature, chimerism in wild fruiting bodies, and chimerism in laboratory clones cultured on natural soil.

Genetically distinct clones in nature encounter each other at some frequency. We have found multiple clones even in small soil samples of a fifth of a gram or less at our study site at Mountain Lake Biological Station (Fortunato et al. 2003b). We took paired samples in 6mm diameter drinking straws along a 25m transect and isolated 46 different haplotypes from 102 samples. Within each 0.2g sample, there were from 0 to 6 different clones. Genetic relatedness within a soil sample was 0.52±0.014 (SE).

The ideal solution to understanding field interactions among clones would be to find wild fruiting bodies, something we succeeded in doing in naturally occurring samples of deer scat. On these small spherical balls, we were able to isolate and genotype fruiting bodies either in nature, or from fruiting bodies that emerged after the scat was brought undisturbed to the laboratory and placed carefully on a non-nutrient agar plate (Gilbert et al. 2007). In these wild fruiting bodies, relatedness was 0.86 measured by whole fruiting bodies. 77% of fruiting bodies were completely clonal (88 fruiting bodies studied). These are higher levels of clonality than would be predicted if clones in the small soil samples mentioned above mixed freely.

We designed another study in as natural a set up as possible to see if there was evidence for active sorting when amoebae were very dense and allowed to develop on soil (Gilbert et al. 2012b). We chose clones that naturally co-occurred in an earlier study (Fortunato et al. 2003b). We set up 18 pairs, 9 where each clone was from the same tiny soil sample and 9 where the clones were collected 20 m apart. We plated 10^7 spores with bacterial slurry on non-nutrient agar, covered it with autoclaved, moistened soil from the study site to 1.5 mm depth, allowed fruiting bodies to form, and then collected and genotyped 16 of them from each small beaker. This starting density is 1762 spores/mm^2 and would increase several fold as the spores hatched and ate the bacteria, making any spatial sorting unlikely, so all sorting should be due to active processes. We found from studying 1047 fruiting bodies that only 25% were clonal, a much lower number than we found in the fruiting bodies collected naturally from dung (Gilbert et al. 2007). But we did not find complete mixing either. However, the increase in relatedness due to active sorting in this study was only 0.049. This value is based on the starting frequency, 0.50, the overall frequency of each clone on the plate (to take into account differences in spore hatching and growth), and then the fraction of each clone in each fruiting body. Since 0.049 is only a modest increase due to sorting, these results indicate that active sorting alone cannot explain high relatedness found in wild fruiting bodies. This study points to micro-scale

population structure as important for maintaining high relatedness in wild fruiting bodies. Generally, *D. discoideum* spores and amoebae occur in proximity to clone-mates, likely to have been generated as recent ancestors from binary fission processes. This means that in the social process relatedness in fruiting bodies will be high because it is usually but not always clonemates that aggregate.

KIN DISCRIMINATION GENES *TGRB1* AND *TGRC1*

From the earliest references on sorting in Dictyostelids, cell adhesion genes have been suspected to be important (Bonner and Adams 1958; Sternfeld 1979). More recently attention has focused on *lag* genes, *lagC1* and *lagB1,* recently renamed *tgrC1* and *tgrB1* (Benabentos et al. 2009; Hirose et al. 2011). These genes are co-expressed at 8 to 12 hours in the social stage. They are physically adjacent to each other, so likely to be inherited together. Knocking out either one causes arrest at the aggregation stage (Benabentos et al. 2009). When either one is knocked out and mixed with wild type of the other, clumps of same-type cells form in the slug stages. Furthermore, two clones that were similar in sequence for these two genes but not overall genetically similar, did not segregate at levels predicted by overall genetic similarity (Ostrowski et al. 2008). Compelling evidence for the likelihood that these cell adhesion genes could function in recognition also comes from their highly polymorphic nature more polymorphic than nearly all other genes (Benabentos et al. 2009).

These initial studies indicating that *tgrC1* and *tgrB1* are highly polymorphic, temporally co-express, are expressed in development on the cell surface, and show clumping with like cells in chimeras might seem to be enough to establish their importance in recognition. However recent work has taken the story even farther. In an insightful study, Hirose and colleagues replaced the native sequences in the lab strain, *Ax4,* with sequence from wild strains QS4, QS31, QS38, and QS45 (Hirose et al. 2011). The resulting clones thus differed only in *tgrB1* or *tgrC1* alleles. As predicted, when their *tgr* alleles did not match, clumping of different cell types occurred. When they matched, they segregated. They further made merodiploid lines containing more than one allele at each locus. These mixed without trouble, indicating the system is one that favors binding with self rather than excluding non-self (Hirose et al. 2011).

Subsequent studies of this system have shown that discrimination is strongest early in development, so even carriers of rare alleles can ultimately join aggregates and produce spores (Ho and Shaulsky 2015a).

Enticing as these genetic studies are, they fail to answer two big questions. The first one is how do we explain low levels of genetic kin sorting in all the previous studies that used wild clones. Might it be due to actions of genes other than the *tgr* genes? A developmental answer might be that early sorting is not maintained to the final stage of fruiting body formation since discrimination diminishes in the slug stage. This could be tested. Hirose *et al.* (2011) shows an image of several differentiated fruiting bodies in the supplement, but does not offer a quantitative assessment of the level of sorting.

The second big question is how variation is maintained in spite of Crozier's paradox, which states that common recognition genes should be favored, but as they are, they function less and less effectively as recognition genes. This is because as they become common, they are less good markers for kinship because everyone has the same allele. It is possible that recognition genes function early but not later in social development and rare or mismatched cells eventually join into the slugs and become spores (Fortunato et al. 2003a; Ho and Shaulsky 2015b). This might preserve the efficacy of the recognition system. Alternatively, the *tgr* genes might have other functions that select for variability. A protein kinase A suppression mutant, stcA^{ins}, modifies developmental but not recognition functions of *tgrC1,* making this a possibly fruitful approach to resolving Crozier's paradox (Wang and Shaulsky 2015).

CONCLUSIONS

For costly social interactions to evolve, there must be a way for the genes that cause the beneficial actions to prosper. In the social amoebae, the beneficial action is to die as a stalk cell and help the spores disperse. Kin discrimination in this system has been extensively studied. It is clear that wild fruiting bodies are mostly but not entirely clonal, but more studies on this point would be welcome, since the conclusions so far are only from a small sample at Mountain Lake Biological Station. It appears that distribution and growth processes are important contributors to this pattern. To the extent there is active sorting, we appear to know the genes, cell adhesion genes *tgrB1* and *tgrC1.* But in isogenetic backgrounds, these genes appear to confer more sorting on their bearers than is observed in natural clones.

The reasons that fruiting bodies in the laboratory are more chimeric than either measured chimerism in the wild, or *tgr* genes would predict could be due to imperfect working of a recognition mechanism at high densities, or on unnatural substrates. Or it could be that this is a mechanism that is secondary to passive spatial sorting. It is clear that chimerism is common enough in nature for there to be a suite of evolved competitive interactions, but the full story is yet to come (Strassmann and Queller 2011; Ostrowski et al. 2015).

Future research on this system will include other functions of these genes and their modifiers, a better understanding of exactly how discrimination works in the wild, and how these genes and discrimination in general have evolved. Other future studies will look at these questions in other species of dictyostelids in which sorting in wild clones seems stronger. All told, it is an excellent system for understanding protist recognition and discrimination.

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