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A cost to chimerism in *Dictyostelium discoideum* on natural substrates

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ABSTRACT

Most multicellular organisms go through a single cell bottleneck in development, a process that ensures subsequent clonality of the cells within the individual. Selection for clonality among cells could reduce costly intra-organismal conflicts that would occur in mixtures of unrelated cells (chimeras). In *Dictyostelium discoideum*, the usually solitary amoebae aggregate with nearby cells when starving to form a motile, multicellular slug that may be clonal or chimeric. This slug migrates to the soil surface and forms a ball of spores held aloft by a stalk of dead cells. Previous work on *D. discoideum* has shown that uniclonal slugs migrate further than chimeric slugs of the same size across agar, indicating a functional cost to chimerism. Here we test whether this cost to chimerism results in a fitness cost under more natural conditions. First, we examine migration of slugs across decaying leaves or soil. Second, we examine migration up through layers of these substrates, which most closely reflects the natural migration of *D. discoideum* slugs to the soil surface. In most trials, chimeras performed worse than single clones. Our results indicate that chimerism in *D. discoideum* has a real fitness cost in the wild, likely to be compensated only by the larger size chimeras can attain in nature.

Keywords: chimera, conflict, cooperation, social amoebae.

INTRODUCTION

Multicellular organisms typically develop from a single cell, so that within an organism all cells are genetically identical (Bonner, 1974; Dawkins, 1982; Maynard Smith, 1988; Maynard Smith and Szathmáry, 1995; Grosberg and Strathmann, 1998; Michod, 1999). In species in which genetically distinct cells do mix, chimeras typically form only among closely related cells (Buss, 1982; Grosberg and Strathmann, 1998). The avoidance of low relatedness among cells predicts a reduction in costly intra-organismal conflicts. Reproductive conflict may result from competition among cells for access to the reproductive tissues (Dawkins, 1982; Maynard Smith, 1988; Maynard Smith and Szathmáry,

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1995; Grosberg and Strathmann, 1998; Michod, 1999; Pál and Papp, 2000). In addition, developmental conflict may occur if unrelated cells are unable to synchronize and communicate effectively (Wolpert and Szathmáry, 2002). Testing whether low relatedness causes costly within-organism conflict, however, requires one of the rare organisms in which unrelated cell lines fuse into single bodies (Maynard Smith, 1988; Strassmann *et al.*, 2000; Wolpert and Szathmáry, 2002). Here we use *Dictyostelium discoideum*, a social amoeba that readily forms chimeras with unrelated cells (Strassmann *et al.*, 2000), to explore the costs of chimerism under natural conditions.

Dictyoselium discoideum is a predatory soil amoeba that is unicellular when its bacterial prey is abundant, but which aggregates when starved to form a multicellular slug (Raper, 1984; Kessin, 2001). This mobile slug moves towards heat and light over and through the forest floor (Raper, 1984; Kessin, 2001). Upon reaching its destination, the slug differentiates into a fruiting body consisting of a stalk of about 20% dead cells that holds aloft the other 80% in a sorus of reproductive spores. The stalk raises the spores from the soil surface promoting their dispersal by passing organisms (Bonner, 1982; Huss, 1989). Genetic analysis of soil samples has shown that distinct clones frequently occur in very small volumes (0.2 g) of soil, indicating that clones regularly mix in the wild to form chimeras (Fortunato *et al.*, 2003). Furthermore, there is evidence of reproductive competition among clones. In chimeras of two equally proportioned clones, one clone is often over-represented in the spores as compared to the stalk (Strassmann *et al.*, 2000).

Uniclonal slugs of *D. discoideum* migrate further than chimeric slugs of the same size (Foster *et al.*, 2002). This supports the hypothesis that there is a functional cost to chimerism. Why then, would *D. discoideum* form chimeras? In *D. discoideum*, there is also a special benefit to chimerism because mixing with unrelated cells forms larger slugs, and larger slugs will move further (Foster *et al.*, 2002). This size benefit outweighs the cost of chimerism observed in same sized slugs (Foster *et al.*, 2002).

However, this demonstration of the costs and benefits of chimerism was for horizontal migration on agar plates, and this raises two questions. First, although the ability to migrate horizontally is presumably useful, it still warrants direct demonstration. Vertical migration up to the soil surface has a more obvious function because this puts spores in the position for dispersal by surface organisms. Second, agar substrates can affect the observed phenotype of *D. discoideum*. A good example of this comes from studies of the csA gene, which codes for a cell adhesion glycoprotein in *D. discoideum* (Queller *et al.*, 2003). This protein accumulates during aggregation and is specific for EDTA-resistant cell-to-cell adhesion. However, experiments with a csA-null knockout mutant on agar suggest that it is not required for successful aggregation (Harloff *et al.*, 1989). In contrast, when tested on soil, the reduced adhesion of the csA-null cells showed them unable to aggregate normally (Ponte *et al.*, 1998). This raises the question of whether the cost of chimerism found in *D. discoideum* (Foster *et al.*, 2002) is a true fitness detriment in the wild, motivating the experiments reported here.

We examined migration of uniclonal and chimeric slugs of *D. discoideum* under natural conditions. In our first set of experiments, we examined migration of slugs across substrates of decaying leaves or soil. In our second set of experiments, we examined slug mobility up through layers of these substrates. This latter experiment closely reflects the natural migration of slugs up to the soil surface (Raper, 1984; Kessin, 2001).

MATERIALS AND METHODS

We used genetically distinct wild clones of *D. discoideum*, as determined by genotyping at five microsatellite loci, which were isolated from soil collected at Mountain Lake Biological Station, Virginia (Fortunato *et al.*, 2003) (Table 1). In each experiment, we obtained amoebae by plating out spores of single clones with the bacterium *Klebsiella aerogenes* as prey, on SM/5 agar (2 g glucose, 2 g peptone, 2 g yeast, 1 g K_2HPO_4, 1.9 g KH_2PO_4, 0.2 g $MgSO_4$, 20 g agar per litre of ddH_2O). After 36 h, before starvation and aggregation, we harvested the amoebae by washing the plates with KK_2 buffer solution (1.0 g K_2HPO_4, 1.9 g KH_2PO_4 in 1 litre of ddH_2O: pH 6.1) and counted cell numbers.

The basis of all experiments was to compare slugs formed from cells of a single clone to slugs formed from a mixture of two clones (chimera). Each clonal and chimeric trial developed in a separate plate or beaker, to which we added the same total number of cells. All experimental plates and beakers used nutrient-free agar as a base for the substrates, insuring that bacterial prey do not multiply, inducing immediate amoebae starvation. In some experiments, we autoclaved soil and leaf litter and added it onto the agar. The soil and leaf litter came from a pine oak forest in the arboretum in Houston, Texas, where we have previously isolated *D. discoideum*.

Horizontal mobility

Trial 1a: migration across 9 cm plates

We placed starved amoebae at one end of 9 cm Petri plates. The plates were stacked over each other, separated by dark cards. We then wrapped the tower of plates with black

Table 1. Clones used in the experiments

	Trial				
	1A	1B	2A	2B	2C
V55C2			×	×	
V56A1					×
V56A2		×	×		
V64D1				×	
V72A2			×	×	
V77B	×	×	×		×
V78C			×	×	
V301B1	×	×	×	×	×
V301B2			×	×	×
V305B3		×			
V319B3	×				
V324B1	×				×
V326D1	×	×	×	×	
V327A1	×				×
V330D2	×	×	×	×	
V336B1	×				×
V342B2					×

paper. At the level of each plate, at the opposite side from the amoebae, a 1 mm pinhole created a unidirectional light source towards which the slugs would migrate. We used a haphazard order in stacking the plates. In this way, we compared the distance migrated towards light by uniclonal and two-clone chimeric slugs across two natural substrates: soil and decayed leaves. We used eight clones previously collected from the field (V327A1, V319B3, V336B1, V77B, V301B1, V330D2, V324B1, V326D1; Fortunato *et al.*, 2003; Table 1), which we compared to every possible pairwise combination (28 two-clone chimeras in total).

We placed a total of 3.6×10^6 cells (3.6×10^6 cells of one clone or 1.8×10^6 cells of each of two clones) at one end of three types of 9 cm Petri dishes: agar (control), soil and leaves. The plates with natural substrates had a strip of soil or leaves added to the surface of the agar that formed a barrier about 10 mm wide and 3 mm high, standardized by weight (soil ~1 g, leaf fragments ~0.3 g) between the cells and the light source. One plate of each type was prepared for each of the eight single clones and 28 chimeric pairs (total plates = 108).

After 8 days, we removed the plates and evaluated slug motility by dividing them into 10 equal sections perpendicular to the light source (Foster *et al.*, 2002). On each plate, we then counted the developed fruiting bodies in each section and calculated mean distance (number of 10 mm sections) travelled by their original slugs. Slugs reaching the end could not progress further and as the plates were 90 mm in diameter, this was the maximum distance obtainable.

Trial 1b: migration across 13.5 cm plates

The second trial was a repeat of the first using six clones (V56A2, V77B, V301B1, V305B3, V326D1, V330D2) and six chimeras (V56A2 × V77B, V77B × V301B1, V301B3 × V326D1, V56A2 × V326D1, V301B1 × V305B3, V305B3 × V330D2; total plates = 36). To investigate a longer distance migration, we used larger plates (13.5 cm) and more substrate (soil ~5 g, leaves ~1.5 g), which we spread out over the majority of the plate (80 × 125 mm), leaving 10 mm of clear space at each end of the race path. We also used fewer cells per clone; pure lines had 1.43×10^6 cells and chimeras had two clones of 7.1×10^5 cells each.

Statistical analysis

We used two-way analyses of variance (ANOVA) to compare the mean distances travelled by chimeras versus pure clones as well as the effects of substrate type on the results. We used one-tailed tests when testing for the cost of chimerism because we have a clear theoretical one-way prediction, as well as because previous studies support this prediction (Foster *et al.*, 2002).

Vertical mobility

Trial 2a: migration up through 1 cm and 3 cm substrate

The second set of experiments evaluated vertical migration of slugs to the surface of substrates. Since we could only observe the results of vertical migration for those slugs that reached the surface, our measure was of a threshold and not an average distance. No difference between treatments is expected if this threshold is too easy to reach (all slugs reach the surface), or too difficult (no slugs reach the surface). Thus, we set up three trials and varied conditions in later trials in response to the results of early trials. In the first trial, we compared slug migration upwards through soil or leaf litter of eight single clones

(V55C2, V56A2, V72A2, V77B, V78C, V301B1, V326D1, V330D2) to eight chimeric pairs (V55C2 × V56A2, V56A2 × V72A2, V72A2 × V77B, V77B × V78C, V78C × V301B1, V301B1 × V301B2, V301B2 × V326D1, V326D1 × V330D2; total = 85 beakers). We added either 2.0×10^7 cells of one clone or a mixture of two clones (1.0×10^7 cells per clone) on top of non-nutrient agar at the bottom of glass beakers. We tested two depths of soil and leaves (1 cm and 3 cm). We sprayed 1 ml of ddH_2O into each beaker, and sealed each with Parafilm to maintain humidity. We wrapped each beaker with black paper allowing only overhead light to enter. After leaving the beakers for 10 days, we counted the total number of fruiting bodies on the surface of the soil or leaves. For all clones and chimeras, we had strong growth on agar controls, showing that all strains could develop.

Trial 2b: migration up through 1 cm and 2 cm substrate

Trial 2a revealed that few slugs were able to make it up through 3 cm of substrate. Hence, Trial 2b tested depths of 1 cm and 2 cm. In addition, we increased ddH_2O to 8 ml, since the substrates dried out in the first trial. We used the same clones as in Trial 2a except that V64D1 and V301B2 replaced V56A2 and V77B, thus creating eight new chimeric mixtures (V55C2 × V64D1, V64D1 × V72A2, V72A2 × V78C, V78C × V301B1, V301B1 × V301B2, V301B2 × V326D1, V326D1 × V330D2, V330D2 × V55C2; total = 57 beakers; Table 1). Seven beakers in this trial were overgrown with fungus, and we left those samples out of the analysis.

Trial 2c: migration up through 1.75 cm substrate

The third experiment tested only one depth of substrate (1.75 cm) based on the results of the two previous runs and used 4 ml of ddH_2O. Eight clones were used: V56A1, V77B, V301B1, V301B2, V324B1, V327A1, V336B1 and V342B2, with eight chimeric mixtures (V56A1 × V77B, V77B × V301B1, V301B1 × V301B2, V301B2 × V324B1, V324B1 × V327A1, V327A1 × V336B1, V336B1 × V324B2, V324B2 × V56A1; total = 48 beakers).

Statistical analysis

The data were analysed using a paired *t*-test that compared the number of fruiting bodies at the surface for each chimera (A/B chimera) with the average number of the two single clones when alone – that is, the number of AB chimera versus (number of A + number of B)/2. We used this test because it eliminates the effects of high variance in the number of fruiting bodies at the surface among the eight different clones.

RESULTS

Horizontal mobility

We found a cost to chimerism in both horizontal mobility trials across all substrates (Trial 1a, $n = 108$ plates, two-way ANOVA: $P < 0.0001$; Trial 1b, $n = 36$ plates, two-way ANOVA: $P < 0.0001$) (Fig. 1). The substrate type did not have a significant effect in the first trial (Trial 1a, $n = 108$ plates, two-way ANOVA: $P = 0.1113$). Yet, there was an effect on slug performance in the second trial (Trial 1b, $n = 36$, two-way ANOVA: $P = 0.0044$); slugs migrating only on agar moved much farther than did slugs on either natural substrate.

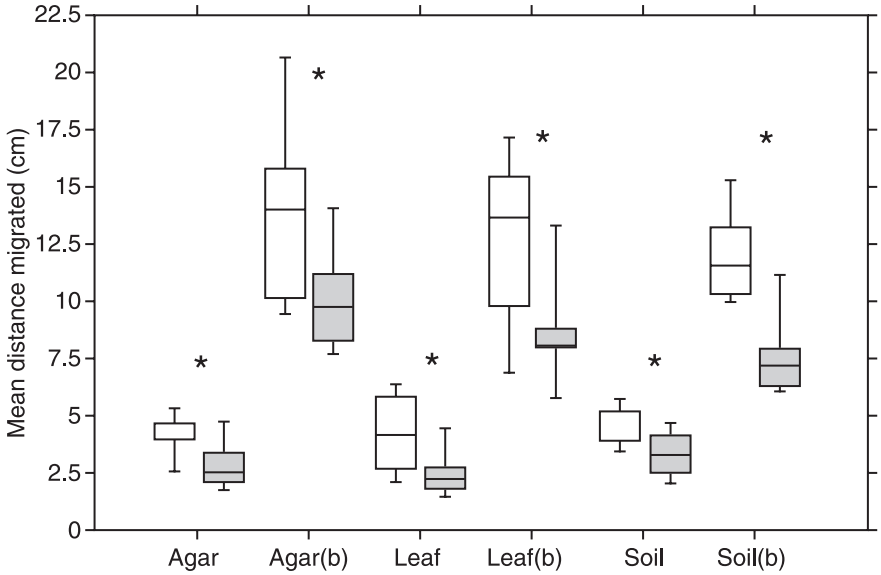

Fig. 1. Migration of uniclonal and chimeric slugs horizontally across natural substrates. Trial 1a showed that uniclonal slugs travelled consistently farther regardless of substrate ($n = 108$ plates, unpaired one-tailed t-test: $P < 0.0001$). Trial 1b showed that the reduction in mobility also occurred over a larger terrain ($n = 36$ plates, unpaired one-tailed t-test: $P = 0.0003$). Trial b results are denoted by (b). All differences are statistically significant (see text). White columns are pure clones, while grey columns are chimeras. *$P < 0.0001$ (see text for details).

Vertical mobility

Our vertical experiment tested various successive depths. Overall, fewer chimeric slugs reached the substrate surface than their respective uniclonal slugs. Eight of our nine treatments showed that pure clones had a propensity for better vertical migration than chimeras. Out of these eight, four were significant. Interestingly, we observed that the most significant results occurred at intermediate depths. In our experiment, the depth of 1.75 cm proved to be quite advantageous for pure clones.

In Trial 2a, clones did significantly better than chimeras in 3 cm of leaves ($n = 17$ beakers, d.f. = 8, paired one-tailed t-test: $P = 0.013$) (Fig. 2). The other treatments of the first trial also suggested a cost to chimerism, but were not significant ($n = 17$ beakers each, d.f. = 8, paired one-tailed t-tests: soil at 1 cm, $P = 0.082$; soil at 3 cm, $P = 0.097$; leaves at 1 cm, $P = 0.231$).

The cost in Trial 2b was significant in the 1 cm soil treatment ($n = 16$ beakers, d.f. = 7, for 1 cm soil, paired one-tailed t-test: $P = 0.021$) (Fig. 2). The other treatments were not significant ($n = 16$ beakers, d.f. = 7, paired one-tailed t-tests: leaves at 1 cm, $P = 0.607$; soil at 2 cm, $P = 0.064$) (Fig. 2). One substrate had partial fungal contamination, so we used only clean samples in an unpaired test that was not significant ($n = 9$ beakers, d.f. = 1, unpaired t-test: leaves at 2 cm, $P = 0.280$).

In Trial 2c, both treatments demonstrated decreased chimera mobility compared with the uniclonal samples (soil at 1.75 cm, $n = 16$ beakers, d.f. = 7, paired one-tailed t-test:

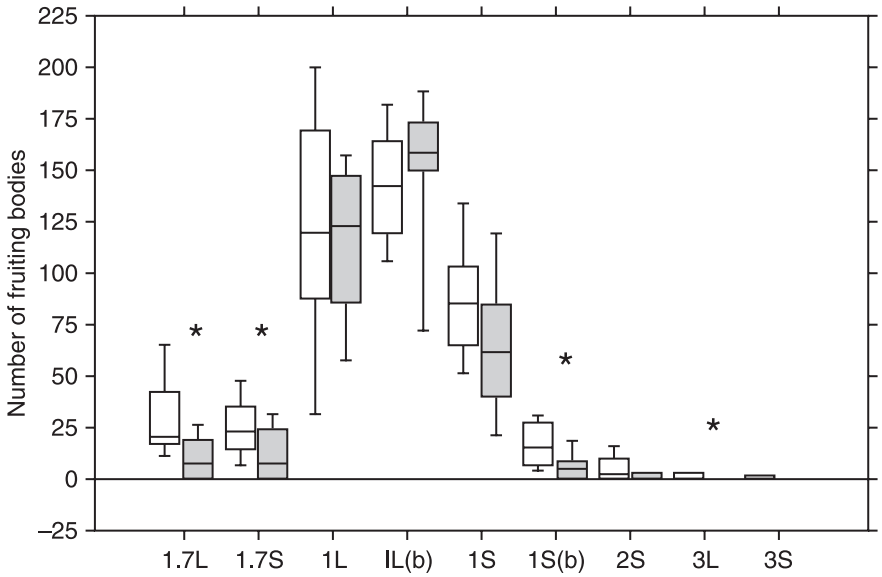

Fig. 2. Number of fruiting bodies at the surface after vertical migration of uniclonal and chimeric slugs through differing depths of natural substrates. White columns are pure clones, while grey columns are chimeras. Treatments are designated by the depth in centimetres and S (soil) or L (leaves) for substrate type. 1S and 1L were each done twice, so a (b) is used to designate Trial 2b instead of 2a. Asterisks indicate $P < 0.05$ for the hypothesis that uniclonal treatments have more fruiting bodies (see text for details).

$P = 0.025$; leaves at 1.75 cm, $n = 9$ beakers, d.f. = 7, paired one-tailed t-test: $P = 0.024$) (Fig. 2).

DISCUSSION

Chimeras were significantly less mobile than uniclonal slugs when travelling horizontally across natural substrates (Table 2, Fig. 1). Established for agar substrates (Foster *et al.*, 2002), it is equally true for natural substrates. Indeed, our first trial did not show any effect of substrate. However, it employed only a small strip of natural substrate, so we reasoned that a larger surface of soil or leaf litter might be necessary to show any possible effects on migration. Our second trial addressed this by adding a longer and wider substrate surface. Our suspicion was correct – when we added a more extensive substrate layer, slugs on the natural substrates moved less far than those on agar. This is not surprising, as results from the csA experiment showed that aggregation is easier for *D. discoideum* on smooth agar than on soil (Ponte *et al.*, 1998). Although substrates can have a strong effect on aggregation, slug mobility appears more robust, perhaps pointing to a benefit of aggregation; multicellular slugs are less susceptible to ill effects caused by difficult substrates than are individual cells.

The more important point is that the effect of chimerism on slowing migration was still present, showing that even on difficult substrates there is a significant migration disadvantage of mixing with unrelated clones. The remaining question is whether horizontal migration matters for fitness. It seems highly likely that it does. Slugs use resources to migrate and

Table 2. Summary of main experimental conditions and results ('Yes' means the result was statistically significant: see text and figures)

Trial	Migration	Condition	Water added?	Soil result significant?	Leaf result significant?
1a	Horizontal	9 cm plate	N/A	Yes	Yes
1b	Horizontal	13.5 cm plate	N/A	Yes	Yes
2a	Vertical	3 cm depth	1 ml	No	No
2a	Vertical	1 cm depth	1 ml	No	Yes
2b	Vertical	1 cm depth	8 ml	Yes	No
2c	Vertical	1.75 cm depth	4 ml	Yes	Yes

migration is a highly ordered response to specific cues such as light and ammonia, both suggesting that it is often adaptive. Slugs that are able to migrate farther would have a greater ability to find optimal fruiting sites. However, to address this concern, we also tested vertical migration through soil or leaves, where failure to migrate far enough carries the more obvious cost of either no spore production or perhaps production of spores that remain buried in the substrate.

Chimeras were either the same or worse than single clones in their ability to migrate vertically to reach the soil surface (Table 2, Fig. 2). The lack of difference between chimeras and clones in some trials is presumably due to the deepest substrates preventing most slugs from making the surface, whereas the shallow ones allow all slugs to reach the surface. If conditions are sufficiently easy or difficult, either all or none of the slugs will reach the surface, irrespective of chimerism. However, the cost manifests under intermediate conditions, and chimeras never performed better than single clones. In nature, migrating *D. discoideum* slugs face a highly variable range of soil depths and moistures (Raper, 1984; Kessin, 2001), so that the cost is likely to affect them some of the time. On average, therefore, chimerism is likely to carry a real fitness cost in nature.

If this inherent cost of chimerism exists, why does *D. discoideum* still form chimeras? One possible explanation is that mixing allows amoebae to form larger aggregates, a particular advantage if cell numbers are limiting (Foster *et al.*, 2002). In the wild, clones growing in close proximity have two possible scenarios: to combine with a neighbour and increase the total cell number, or to preferentially sort and form their own aggregates without gaining any cells. Foster *et al.* (2002) showed that chimeras migrate farther than their clonal counterparts when the mixes have twice as many cells. Therefore, forming a chimera and creating a larger aggregation is preferable to sorting clonally but having fewer cells to form slugs.

The advantage of gaining cells implies that cell density should be an important variable influencing the cost of chimerism. In our vertical experiments, 10^7 cells had a critical depth of 1.75 cm. At this depth, we were able to tease apart the difference between the pure clones and their chimeras. At depths more shallow than that, we could see that chimerism was not relevant to our measured fruiting success. Depths greater than 1.75 cm prohibited chimeras and pure clones alike. For other cell densities, the critical depth will no doubt vary. At a much higher cell density, the larger slugs might all succeed at 1.75 cm, but the disadvantage of chimeric slugs may still apply at 3 cm. The opposite extreme should also hold. At much lower densities, the smaller slugs may all fail at 1.75 cm, and the disadvantage of chimerism would only apply at shallower depths.

In summary, the occurrence of a fitness reduction in chimeras of *D. discoideum* in our realistic environment supports the idea that there is an intrinsic cost when genetically distinct cells mix. We have shown that the effect of environment on selection on chimerism will be substantial in *D. discoideum*. The intrinsic cost should conditionally translate into a real fitness cost in nature, depending on the circumstances such as depth and cell density. However, the circumstances where the cost is manifest are also circumstances where large slug size is important, so that joining in chimeras may still be better than going it alone.

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