Non-selected incompatibilities and their effects on aggregative multicellularity in the social amoeba *Dictyostelium discoideum*

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ABSTRACT

Selection for cooperation or conflict in multicellular organisms that develop from a mixture of cells has been widely appreciated, but unselected effects of mixing could also have important fitness consequences to the resulting chimeras. We formalized this idea as the 'developmental incompatibility hypothesis' and empirically tested it in the social amoeba *Dictyostelium discoideum*. We mixed *D. discoideum* that evolved in isolation for generations and accumulated mutations that have not been tested against each other by selection. To quantify the effect of developmental incompatibilities in experimental lines that evolved in the presumed absence of kin recognition, we measured the developmental traits of slug migration and spore production. Our results show no evidence for incompatibilities in coordinated movement towards light in the social amoeba. We found support for developmental incompatibilities from our spore production result. The effect was especially clear for a small proportion of fruiting bodies that developed after migration. For slugs that did not migrate, mixing could be harmful if they are smaller or if, the stalks of such fruiting bodies are shorter which would hamper dispersal. Our results indicate that the detrimental fitness consequences of developmental incompatibilities may be context-dependent.

Keywords: multicellularity, incompatibilities, experimental evolution, *Dictyostelium discoideum*

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ADDITIONAL INFORMATION

AUTHOR CONTRIBUTIONS

I. J. , D. C. Q. and J. E. S. conceived of the study. I. J. collected experimental data. I. J., T. J. S., D.C.Q., and J. E. S. participated in the statistical analyses. All authors contributed to writing and editing the manuscript.

ACKNOWLEDGMENTS

We are thankful to the members of the Queller-Strassmann Lab for their feedback on this study. We thank Tyler. J. Larsen for the *D. discoideum* experimental evolution lines and insightful comments on the manuscript.

DATA AVAILABILITY

All images used for this study are archived in a Github repository. Data used for statistical analyses is available in the in the submitted repository. The accompanying R project includes script to reproduce the graphs in results section.

FUNDING

This work was supported by the National Science Foundation under grant numbers NSF DEB-1753743 and DEB-2237266. I.J. was additionally supported by a Ph.D. fellowship from the McDonnell International Scholars Academy, WUSTL.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

Introduction

Many familiar life forms have evolved from groups of smaller, formerly free-living biological entities (Gardner and Grafen, 2009; Szathmáry and Maynard Smith, 1995; Bourke, 2013; Bell and Mooers, 1997; Corning and Szathmáry, 2015; Orr, 2000). The process of groups evolving a higher-level entity upon which natural selection can act is called a major transition. The evolution of multicellularity is one such major transition where single-celled ancestors evolved to take advantages of group living. Early multicellular life set the stage for a new type biological individual, allowing natural selection to operate at levels previously absent (Michod, 2005, 2007; Queller and Strassmann, 2009). How and why multicellular organisms developed and subsequently evolved, diversified, and increased in complexity, is an important question for understanding major transitions.

Multicellularity originated independently at least 20 times across all major domains of life (Grosberg and Strathmann, 2007). Eukaryotic multicellular organisms largely reproduce by undergoing single-cell bottlenecks. For example, sexually reproducing organisms produce gametes that combine into a unicellular zygote. Asexual plants and animals, such as the Amazon molly *Poecilia formosa* (Turner et al., 1980), several weevils (Suomalainen and Saura, 1973), and many angiosperms also reproduce by single-cell propagules (Bicknell and Koltunow, 2004).

Single-cell bottlenecks are thought to be a requirement for complex multicellularity (Wolpert and Szathmáry, 2002; Fisher et al., 2020; Howe et al., 2024). In many organisms, alternative routes to multicellularity also exist where the offspring is likely to develop from more than one genotype. This type of reproduction may involve specialized structures, such as the fungal conidia or gemmae found in algae, mosses, and ferns (Hughes, 1971), or simply budding (Galliot, 2012) or fission. An extreme form of such chimeric multicellularity is perhaps best demonstrated by aggregation of individual cells that reside as neighbors (Jahan et al., 2022), known as aggregative multicellularity. The diversity of routes to simple multicellular development raises a fundamental question for the study of organismal complexity: Why haven't other modes of multicellularity like aggregation equally contributed to complex development?

The accepted explanation for the correlation between single-cell bottlenecks and complexity is kin selection (Hamilton, 1963; Axelrod and Hamilton, 1981; Bonner, 1988; Fisher et al., 2013). Development from a single-cell bottleneck results in a multicellular body with cells that are essentially genetically identical, minimizing genetic conflict and maximizing cooperation between cells within a clonal organism. When an exploiter mutant arises within the multicellular body during development, it might gain an initial advantage. But after a single-cell bottleneck, the mutant will exist in bodies with all mutant cells, and therefore unable to exploit further. A single-celled propagule thus reestablishes genetic uniformity for the germline, such that exploiters can exploit only in the lifespan of a single organism.

Wolpert and Szathmáry (2002) propose an alternative (but not mutually exclusive) explanation for why complex multicellular organisms develop from a single cell. They suggest that the development of complex multicellularity is not only a question of kin selection controlling conflict between cells, but also an issue of how an organism develops. For example, the authors suggest cells evolving in isolation could fail to generate repeatable developmental patterns:

> *"... patterning processes require signaling between and within cells, leading ultimately to gene activation or inactivation. Such a process can lead to reliable patterns of cell activities only if all the cells have the same set of genes and obey the same rules."*

They add that this would hinder the evolution of novelty:

> *"... practically impossible to have several-to-many asexual, partly differentiated, cell lineages mutating in all sorts of directions in genetic space and yet keep up the ability to evolve into viable novel forms."*

Wolpert and Szathmary's hypothesis remains to be tested empirically despite its important implications for the evolution of multicellular development. The lack of empirical tests of the hypothesis can be attributed to two related issues. The first is an issue of vagueness. Wolpert and Szathmary provide a vague description of the hypothesis and are not explicit about testable predictions. Second, it is difficult to choose a suitable system for experimental manipulation and testing the validity of the hypothesis.

We present a formalized non-adaptive hypothesis of why aggregations may be detrimental for the evolution of complex multicellularity, and name it the "developmental incompatibility hypothesis". Though Wolpert and Szathmary's hypothesis may be more complex than our interpretation, we take the developmental incompatibility hypothesis to include at least the following ideas and develop two predictions that we can test. First, aggregative development puts new mutations into many new cellular combinations with no common history of selection, such that new and untested combinations of mutations are likely to be detrimental. If so, we predict that mixing of cells from different lineages will usually be detrimental. Second, even if a new advantageous mutation would be beneficial when fixed, to achieve fixation it must be advantageous across many cell combinations. Even if some combinations were beneficial, evolution would be impeded if it had to pass through any stage with disadvantageous combinations.

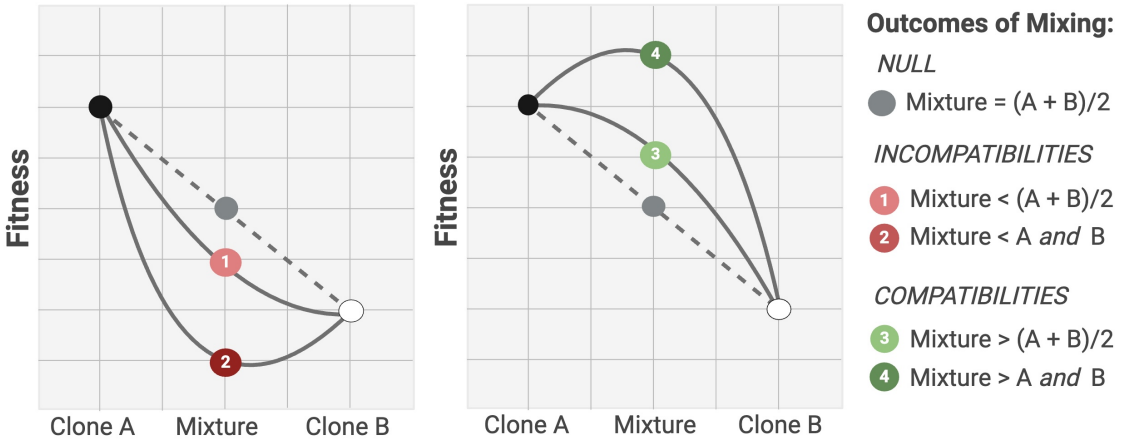

Figure 1. Illustration showing the possible fitness outcomes of mixing between two cell lineages. The gray dotted line represents the Null hypothesis (additivity). The panel on the left shows the two predictions of developmental incompatibilities hypothesis. The panel on the right shows possible beneficial fitness consequences of mixing, also known as the phenomenon of social heterosis.

Specifically, we predict that this could occur when intermediate mixtures – group where only some cells have a beneficial mutation – would have lower fitness than when it is present in all cells or none, that is when mixing produces transgressively lower fitness. In contrast, with a single-cell bottleneck, each organism is genetically uniform and the mutation will fix if it is beneficial all cells in an organisms have it.

To make this logic more concrete, consider how evolution might work on human bodies if they were formed by aggregation of diverse cells. A new mutation might find itself first in the liver, but in the next generation it might find itself in the hypothalamus of one individual, the adenoids of another, and the germ cells of a third. To rise to higher frequencies it would have to be advantageous, on average, across all these contexts. If the frequency did increase, it would find itself in still more localities and combinations of localities. The complexity increases much more if there are other segregating mutations.

In Figure 1, we present a schematic diagram of possible outcomes when cells from distinct lineages are mixed together. When two cell lineages A and B with significant genetic differences are put together, the resultant mixture can have a range of expected values. The null hypothesis (Figure 1, gray dotted line) predicts that mixing is neither a beneficial nor detrimental to multicellularity, such that the fitness of the mixture is the average of the fitness of the two lineages A and B (Figure 1, gray circle). This would not impede evolution from the low-fitness B to the high-fitness A. The developmental incompatibility hypothesis predicts that mixing will have detrimental fitness consequences in the resulting multicellular group (Figure 1, line 1) on average compared to the null expectation. In some extreme cases (Figure 1, line 2), the effect of incompatibilities may result in large fitness costs for the mixture causing it to have the lowest fitness compared to constituent lineages. An outcome of mixing where the mixture has transgressively lower fitness than either parent lineage would suggest that a clone starting with a lower fitness (as is the case for Clone B) would not be able to evolve to the higher-fitness A state because it would require passing through a lower fitness stage. Under this scenario, intermediate cell combinations would hinder the evolution of increased complexity, as Wolpert and Szathmary argued.

In contrast to the developmental incompatibility hypothesis, mixing could sometimes result in improved fitness in the mixtures compared to the expected null (Figure 1, line 3 and 4). Under some conditions, for example social heterosis (Nonacs and Kapheim, 2007), mixing may generate combinations that are complementary in nature such that one lineage compensates for genetic defects in the other and results in higher or even transgressively higher fitness in the mixture. In cases of social heterosis, mixing between disparate lineages is predicted to be favored by natural selection, other things being equal (Nonacs, 2017; Ebrahimi and Nonacs, 2021).

To generate developmental incompatibilities, we require a biological system that can be modified to (1) develop from mixtures of genetically different cells (chimeric development), (2) provide developmental mutations for mixing that have not been previously tested by selection, and (3) exclude kin recognition as a cause of differences in mixtures. The social amoeba *Dictyostelium discoideum* can satisfy these criteria and is an ideal system for this experiment. *D. discoideum* is one of the best-studied organisms that develops by aggregation. Individual *D. discoideum* amoebae divide and grow independently in the presence of abundant edible bacteria but under starvation they aggregate and undergo multicellular development (Figure 2). At one stage in the development, the multicellular aggregate forms a slug that shows coordinated movement towards light. Migrating towards light can assist *D.*

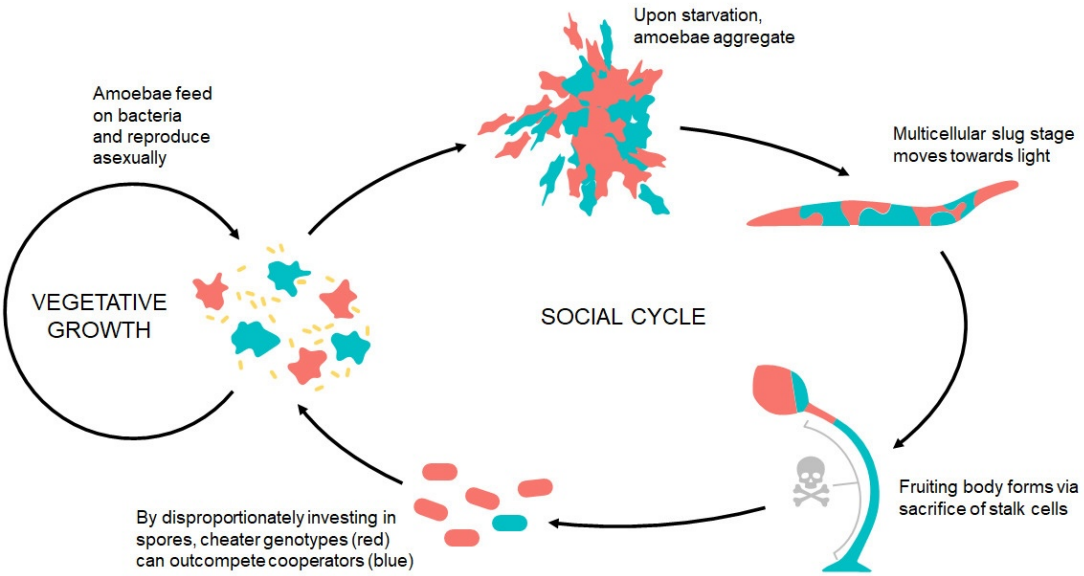

Figure 2. Simplified life cycle of *D. discoideum* illustrating potential for cheating. Credits Tyler Larsen, CC BY-SA 4.0, via Wikimedia Commons

discoideum in moving towards the soil surface and likely increases the chance of dispersal by insects, other small invertebrates, and birds (Huss, 1989; Castillo et al., 2005; smith et al., 2014). The slug culminates into a fruiting body with some cells becoming spores held atop a slender stalk made up of dead cells. Because of the unique lifecycle, *D. discoideum* has been extensively used to study chimeric development (Mehdiabadi et al., 2006; Khare and Shaulsky, 2006; Khare et al., 2009; Sathe et al., 2010; Castillo et al., 2011; Foster, 2010). Genetic chimeras in *D. discoideum* – aggregations comprised of cells of two or more different strains – can be developed in the laboratory and thus have allowed researchers to study the adaptive strategies to deal with grouping with non-relatives (Strassmann et al., 2000; Foster et al., 2002; Fortunato et al., 2003). Genotypes in chimeras could invest less in dead stalk production while maximizing increasing the fitness benefits of producing more spores (Santorelli et al., 2008; Khare and Shaulsky, 2010; Buttery et al., 2010; Noh et al., 2018). Chimeric slugs move shorter distances than clonal slugs when started with the same number of cells (Foster et al., 2002). This could result from competition among the genotypes to avoid the pre-stalk region located in the front of the slug by moving towards the posterior pre-spore region. Most known cases of costs of chimeric development in *Dictyostelium discoideum* have been studied by mixing natural strains that that have been under selection over a long evolutionary timescale, and arguably could have evolved a kin recognition system.

At least in the early stages of aggregation, a matching pair of cell surface receptors encoded by the *tgrB1* and *tgrC1* alleles is necessary and sufficient for attractive self-recognition in *D. discoideum* (Hirose et al., 2011; Ho et al., 2013; Hirose et al., 2017). Amoebas can selectively bind to other cells with compatible *tgr* alleles such that a mismatch can cause poor cell-cell adhesion, and even assorting into individual clones later on (Ostrowski et al., 2008; Benabentos et al., 2009; Hirose et al., 2011; Ho et al., 2013; Hirose et al., 2017), although sorting may largely disappear at later stages, perhaps due to slug fusion, Since incompatibilities in the *tgr* allele system may be an evolved discrimination system, and we need to eliminate them to detect the unselected incompatibilities of the developmental incompatibility hypothesis.

This can be achieved by using genetically different clones recently derived from a common ancestor. When lineages selected from the same ancestor are evolved without the multicellular stage, the descendants are not expected to recognize each other as foreign when mixed. In a recent experimental evolution study (Larsen et al., 2023), we created such lineages of *D. discoideum* clones. Amoebas were transferred on nutrient plates before the onset of starvation for 30 transfers (more than 200-300 cell divisions). With abundant nutrients, these *D. discoideum* lines did not form multicellular groups and would therefore have experienced no selection for novel kin recognition variants. These evolved lines of *D. discoideum* showed average reduced social function in terms of slug movement distance and cheating capability (Larsen et al., 2023). Thus, in the absence of direct selection for these traits, indirect and presumably deleterious genetic changes occurred, via drift or antagonistic pleiotropy with traits under selection. The lines also evolved increased spore production. In sum, we have experimental lines that likely have the same kin discrimination genotypes but differ genetically in social traits that have never been selectively tested in combination.

Here we employ a controlled experimental approach, using ancestral clones of *D. discoideum* and evolved lines

from the same ancestor to observe the fitness characteristics of social hybrids or chimeras in the presumed absence of kin recognition. By mixing these genetically distinct lineages with their ancestors and among themselves, we tested the validity of the developmental incompatibilities hypothesis using two key traits in *Dictyostelium discoideum* that require coordinated development: slug migration, and spore production.

Methods

Chimeric Mixes:

Our study involved two kinds of mixtures: pairwise and complex. First, we made pairwise mixes of clonal ancestors with one of their evolved lineages. We used 10 *D. discoideum* ancestors (QS6, QS9, QS11, QS18, QS69, QS70, QS159, QS161, QS395, QS859) that had been experimentally evolved in triplicate to generate 30 evolved lines in total (Larsen et al., 2023). We made pairwise mixes of the ancestral clones (A) with each of their own derived lines (E1, E2, E3) to generate a total of 30 mixed lines (A+E1, A+E2, A+E3; we call these "lines" for consistency but they are really mixtures without a common history). Therefore, we had a total of 70 experimental lines from 10 *D. discoideum* clones. We had 3 technical replicate plates for each of the 70 experimental lines resulting in a total of 210 plates for 3 Treatments – Ancestor, Evolved, and Mixed (shown in Table 1).

For generating a complex mixture, we mixed the three evolved lineages derived from a common ancestor with each other for 3 out of 10 strains (QS6, QS9, QS18). There should be twice as many differences between two evolved lines as between ancestor and evolved, generating greater power in detecting developmental incompatibilities, should they occur. Mixed lines from complex mixtures did not include the ancestors (as shown in Table 2).

We repeated the experiment with complex mixtures on 3 different days to enhance power resulting in a total of 90 plates for the analyses (30 plates $\times$ 3 days). On a particular day, we had two replicate plates for every 'Unmixed' evolved line (E1 or E2 or E3) and four replicate plates for every 'Mixed' treatment (equal proportions of E1 and E2 and E3) resulting in a total of 10 plates for every strain.

Slug Migration Assay

To obtain spores to inoculate the slug migration assays, we first took glycerol stocks of *D. discoideum* spores stored at 80 ℃ and streaked each frozen sample on solid SM/5 agar plates (Fey et al., 2007). After development, we collected spores and plated 2×10^5 of them onto a fresh SM/5 plate along with 200µl *K. pneumoniae* food bacteria resuspended in KK2 buffer (OD600=1.5). This round of growth was to remove freezer effects. On day 4 we harvested spores from developed fruiting bodies, and initiated the slug migration assay on non-nutrient agar plates (13 cm diameter), in three replicates for each ancestral clone, their evolved lineages, and mixtures.

On every plate, we marked a 10 cm secant line (henceforth inoculation line) on which we loaded spore samples (Figure 3). For pairwise mixtures, we loaded sample consisting of 10^7 spores in 50µL of *K. pneumoniae* (OD600=50.0) suspended in KK2 buffer. The 'Mixed' treatment for pairwise mixtures were made of 50% of Evolved (E1 or E2 or E3) and 50% Ancestor (A) spores in the total 10^7 spore suspension. For samples of complex mixtures, we loaded 9×10^6 spores suspended in 50 µL of *K. pneumoniae* food bacteria (OD600=50.0) on the inoculation line as in pairwise mixtures. We repeated the experiment on 3 different days and collected data from a total 90 plates.

After loading the suspension along the starting line of the agar plates, we allowed the sample to dry and wrapped the plates individually in aluminum foil. We made a small pinhole opposite the starting line on each wrapped plate. For the duration of the experiment, light enters only through this small pinhole towards which slugs migrate from the other end of the plate. We then left the wrapped plates undisturbed for 8 days with the pinhole side of the plate facing a light source in the laboratory. Because the plates had no nutrients, bacteria and amoebas grew only at the starting line, but slugs could migrate off the starting line towards the light. We unwrapped the plates at the end of the 8 days, and allowed fruiting bodies to finish developing fully under direct light.

Image Analysis

We used a Canon EOS 5D Mark III camera to photograph each plate. We put each plate on a laboratory bench with the camera mounted at a fixed distance. We obtained slug migration distances using the software Fiji and ImageJ (Bourne and Bourne, 2010; Schindelin et al., 2012; Rueden et al., 2017). We processed each image by first scaling it and then overlaying a $1\text{cm} \times 1\text{cm}$ grid. We recorded the distance of each fruiting body from the starting line. For pairwise mixes, we assigned distance=0 cm for fruiting bodies that developed directly on the inoculation line, and refer to those as 'non-migrators'. Fruiting bodies that traveled beyond the inoculation site are referred to as 'migrators' (as shown in Figure 3).

We observed a few fruiting bodies traveled in the opposite direction to light. To account for such slugs that may contribute to a reduced average migration distance of slugs on a plate, we measured the distances of all fruiting bodies from the inoculation line on a plate in the follow-up experiment. Our measurements for complex mixtures therefore account for positive or negative movement towards light for slugs on a plate.

Spore Production Assay

At the end of the slug migration assay for pairwise mixtures, we quantified the number of spores produced on a plate by slugs that migrated towards light as well as those that did not leave the inoculation site. We collected

Clones	Treatments					
	Ancestor		*Evolved*		*Mixed*	
	Line	**Plates**	**Line**	**Plates**	**Line**	**Plates**
QS6, QS9, QS11, QS18, QS69, QS70, QS159, QS161, QS395, QS859	A	A1	E1	E1-1	A + E1	A + E1-1
				E1-2		A + E1-2
				E1-3		A + E1-3
		A2	E2	E2-1	A + E2	A + E2-1
				E2-2		A + E2-2
				E2-3		A + E2-3
		A3	E3	E3-1	A + E3	A + E3-1
				E3-2		A + E3-2
				E3-3		A + E3-3

Table 1. Experimental design showing experimental lines derived from 10 *D. discoideum* ancestral clones and their pairwise mixes resulting in three Treatments. The three treatments were compared for the developmental traits of slug migration and spore production.

Clones	Treatments			
	Unmixed		*Mixed*	
	Line	**Plates**	**Line**	**Plates**
QS6, QS9, QS18	E1	E1-1	E1 + E2 + E3 (M)	M1 M2 M3 M4
		E1-2		
	E2	E2-1		
		E2-2		
	E3	E3-1		
		E3-2		

Table 2. Experimental design showing experimentally evolved lines from *D. discoideum* clones and their complex mixture. The two resulting Treatments of unmixed evolved lines of a clone in isolation, and a mixture of all evolved lines of a particular clone, were compared for slug migration distances.

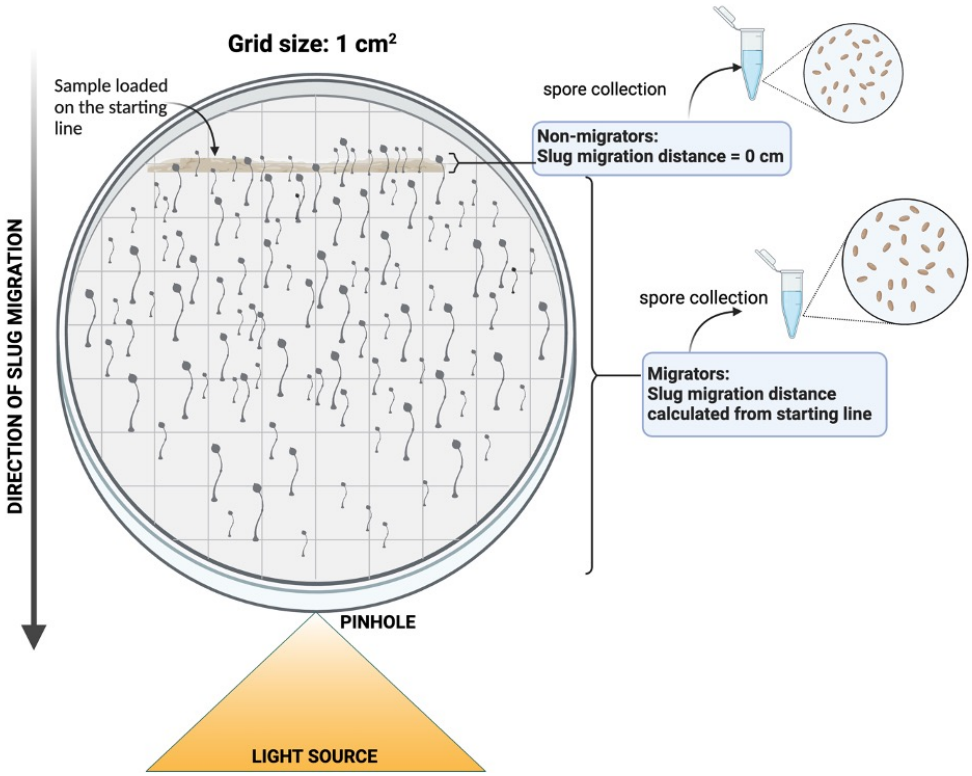

Figure 3. Schematic depiction of an experimental plate at the end of the slug migration assay. Fruiting bodies on a plate were more numerous than shown in the diagram. We refer to fruiting bodies that developed directly on the inoculation site as non-migrators and fruiting bodies that developed beyond the inoculation site as migrators. Distance travelled by migrators towards light source was measured from the inoculation site. We counted spores from both migrator and non-migrator fruiting bodies and added the two to obtain total spores produced on a plate. Created with BioRender.com

non-migrators and migrators for each plate in separate eppendorf tubes (Figure 3) containing 1mL KK2 buffer. We made 1:100 dilutions of the collected spores and counted them using a hemacytometer. We added the number of spores produced by migrators and non-migrators to obtain the total spore production on a plate for each strain across three treatments. We performed a log transformation for use in our analyses. This assay was performed only for pairwise mixtures and not for complex mixtures.

Statistical Analysis

We used R version 4.2.1 (R Core Team, 2022) for all our analyses. We used the *tidyverse* (v2.0.0) suite of packages for data cleaning (Wickham et al., 2019), and the package *fitdistrplus* (v1.1.11) for fitting univariate distributions to our data (Delignette-Muller and Dutang, 2015). For both pairwise mixtures and complex mixtures, we performed linear mixed effects modelling with the *lme4* (v1.1.35.2) package (Bates et al., 2015). Treatment and Clone were fixed effects, and replicate line a random effect for the analysis of both slug migration and spore production data (see Table 1 and Table 2 for details on experimental lines). Pairwise mixtures included three treatments (Ancestor, Mixed, and Evolved) whereas complex mixtures included two treatments (Mixed and Unmixed). We used Akaike Information Criteria (AIC) for model selection which estimates the relative quality of each model based on model quality and parsimony. To assess model fit and assumptions, we used the package *performance* (v0.11.0) (Lüdecke et al., 2021).

We fitted a generalized linear mixed effects model to our proportion data from pairwise mixtures using the package *glmmTMB* (v1.1.9) with a beta-binomial distribution (Brooks et al., 2017). For the analysis of slug migration distances, we used the *robustlmm* (v3.3.1) package (Koller, 2016) to account for outliers without their removal from the data used in our final model. A robust analysis of our final mixed effects model prevents outliers from influencing estimations by inversely weighing them. We reported estimated marginal means averaged at the level of clone using the *emmeans* package (v1.10.1) (Lenth, 2022) for all the statistical models. We performed hypothesis testing using the estimated marginal means and inferred statistical significance of our results after correcting for false discovery rates. All graphs in the results section were plotted with the *ggplot2* (v3.5.1) package (Wickham, 2016) and significance values were layered using the *ggsignif* (v0.6.4) package (Constantin and Patil, 2021).

Results

Slug migration in mixtures is not different from the mean of the unmixed populations

We quantified the outcome of pairwise mixtures between clonal ancestors and their experimentally evolved descendants on slug migration in 10 strains of *D. discoideum*. First, we compared the proportion of migrators (fruiting bodies that travelled beyond the inoculation line) and non-migrators (fruiting bodies that developed at the site of inoculation) for the three treatments: Ancestors, Evolved, and Mixed (50% Ancestor + 50% Evolved). There was no significant difference between the proportion of slugs that migrate across the three treatments (Figure 4).

Next, we measured average slug migration distances for migrators for the three treatments in pairwise mixtures. We found that slugs from mixed lines, on average, traveled farther compared to the evolved lines (Mixed – Evolved=0.349, se = 0.160, z. ratio = 2.178, p. value = 0.0441) but were not significantly different from the ancestors (Ancestor – Mixed =0.342, se = 0.227, z. ratio = 1.509, p. value = 0.1312). Mixed lines travelled intermediate distances between ancestral and evolved lines of *D. discoideum*, with the difference from the expected linear average of ancestors and evolved lines being less than a thousandth of the total (Mid-point estimate = Observed – Expected = 0.00346) as shown in Figure 5. We confirmed this with a post hoc comparison using a planned emmeans contrast M – (A+E)/2, by averaging over the level of clones (SE = 0.16, z.ratio = -0.022 , p = 0.9828).

We performed a second experiment with the aim of getting increased power to detect developmental incompatibilities. We mixed three descendants (from the same ancestor) to obtain a more complex mixture that should allow greater statistical power and thus robust inference. We expect any incompatibilities to be greater when more clones are mixed together. Two descendants have twice as many differences as ancestor and descendant. Furthermore, the mixes of three evolved lines include novel mutations absent in pairwise mixes. Our analysis showed no significant differences in slug migration distances between unmixed and mixed evolved lineages of a clonal ancestor (Unmixed - Mixed = -0.00976, se =0.197, z. ratio = -0.050, p. value = 0.9604) as shown in Figure 6. Again, there was no evidence for incompatibilities and the difference was only approximately a thousandth of the total.

Total spores produced by fruiting bodies from mixed lines, on average, is significantly greater than the average of ancestor and evolved lines

We measured the number of spores produced by developed fruiting bodies from pairwise mixes at the end of slug migration assay. We counted spores from both migrator (distance > 0.5 cm) and non-migrator fruiting bodies (distance = 0 to 0.5 cm) for all three treatments: Ancestors, Evolved, and Mixed. By adding the number of spores produced by migrators and non-migrators, we obtained the estimate for total spore production on a plate. We found that mixed lines, on average, produced significantly more total spores at the level of the plate compared to the expected average of ancestral and evolved lines of *D. discoideum* (estimate = -0.143, SE = 0.053, z.ratio = 2.697 , p = 0.0102) as shown in Figure 7.

Migrating fruiting bodies in mixed lines contribute significantly fewer spores to the total spores production than the average of ancestors and evolved lineages

We further estimated whether migration had an effect on spore production by quantifying the proportion of spores produced by migrators with respect to total spore production on a plate. The proportion of spores produced by migrators in mixed lines was significantly less than the expected average of ancestors and mixed lines (Mixed – (Ancestor + Evolved) /2 = -0.0413, se = 0.0109, z. ratio = -3.790, p. value = 0.0002), as shown in Figure 8. Spore production was primarily driven by fruiting bodies that did not migrate.

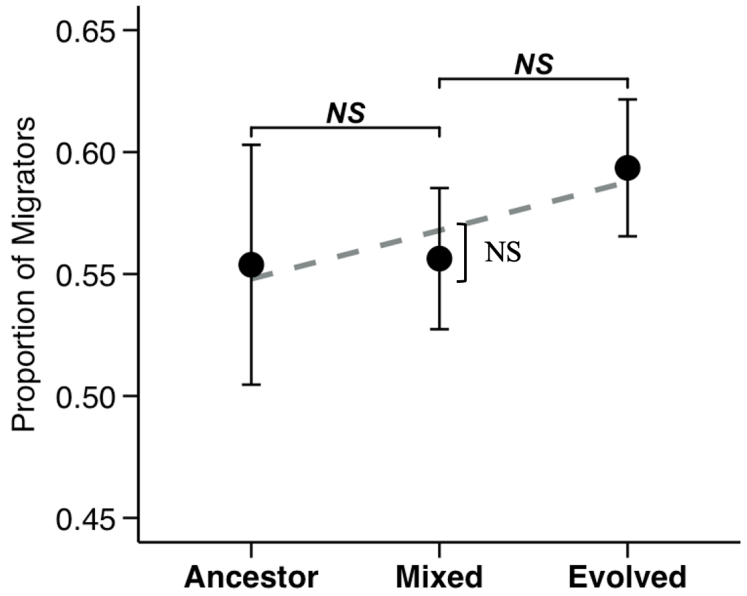

Figure 4. Proportion of migrators and non-migrators across three treatments averaged at the level of clones. Pairwise comparisons of migrators (grey) and non-migrators (white) on a plate indicate no significant difference among the treatments. Mixed fruiting bodies that migrated beyond the inoculation line were not significantly fewer than the expected average of Ancestors and Evolved lines

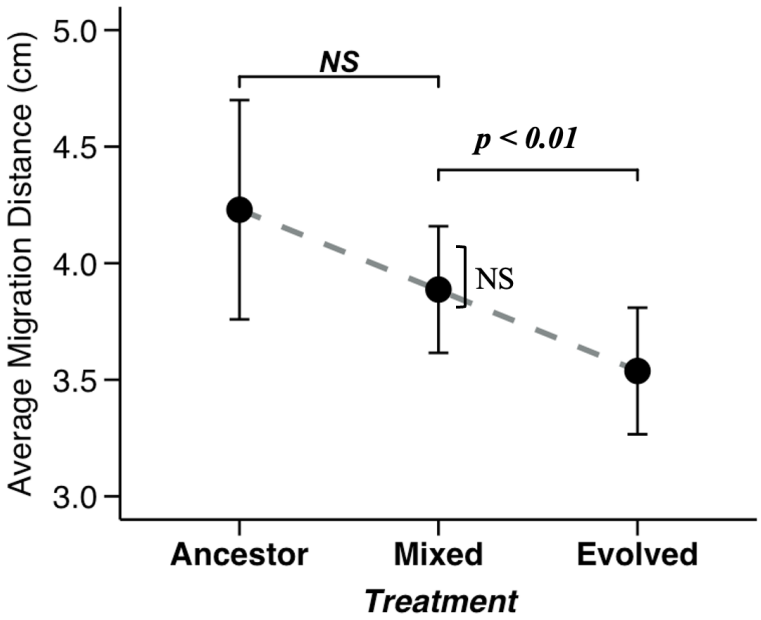

Figure 5. The observed values for slug migration distance across three treatments are depicted with a filled circle. The expected average of ancestor and evolved lines is depicted with a dashed line, against which the observed mixed line is compared. Error bars correspond to 95% confidence intervals. Estimated average slug migration distance of mixed lines is not significantly different than the expected average for a mix of ancestors and their recently diverged evolved lines in *D. discoideum*. The developmental trait of slug migration shows, on average, additive effects in experimental lines that are an equal mix of ancestors and evolved lines

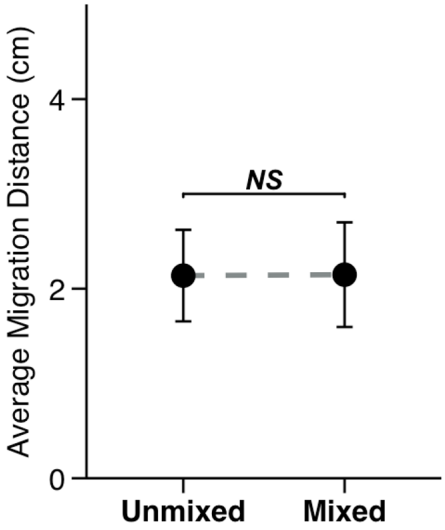

Figure 6. Complex mixes of evolved lineages of a clonal ancestor are not statistically different from evolved lines in isolation for the developmental trait of average slug migration distance. Data is averaged at the level of clones (QS6, QS9, QS18) and the error bars show 95% confidence intervals. p-values shown in the graph were obtained by Post hoc comparison using emmeans. We used Z-tests, corrected for false discovery rates.

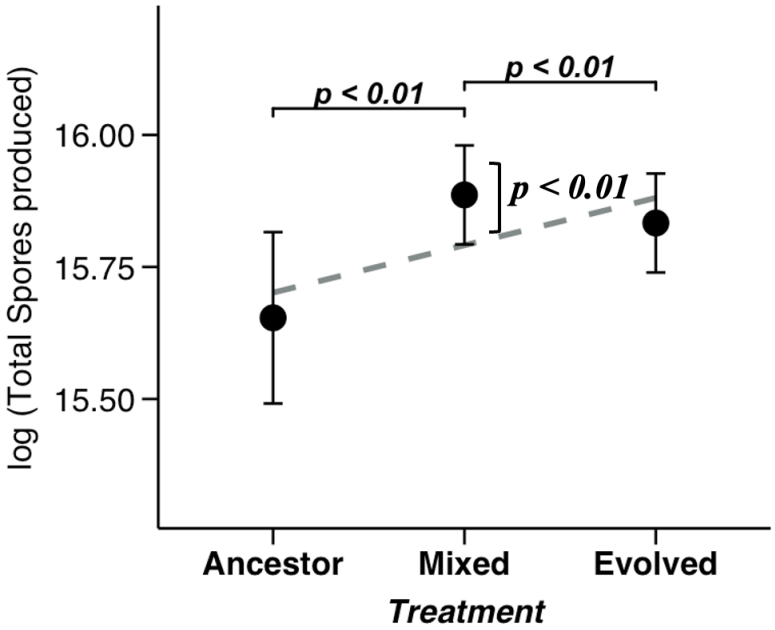

Figure 7. The log transformed estimate of total spores produced by mixed lines on average is significantly greater than the expected average when ancestors and their evolved counterparts are mixed in equal proportions in *D. discoideum*. The expected average of ancestor and evolved lines is depicted with a dashed line, and the observed mixed line value is depicted with a circle. Error bars correspond to 95% confidence intervals. We used Z-tests, corrected for multiple comparisons using false discovery rates method. p-values shown in the graph were obtained by using planned contrasts in emmeans.

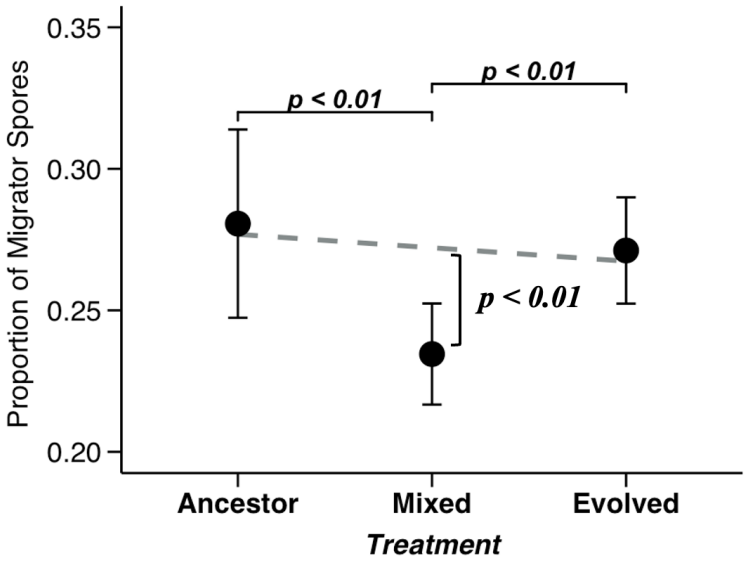

Figure 8. Slugs that migrated towards light in mixed lines on average show significantly lower contribution towards total spores produced than both ancestors and their evolved counterparts in *D. discoideum*. The expected average of ancestor and evolved lines is depicted with a dashed line, and the observed mixed line value is depicted with a circle. p-values shown in the graph were obtained by Post hoc pairwise comparisons of the ratio of migrator spores to total spores produced on a plate. We used Z-tests, corrected for false discovery rates

Discussion

Complex multicellular life more commonly arises in lineages with single-celled bottlenecks (Grosberg and Strathmann, 2007; Fisher et al., 2020; Howe et al., 2024). This may be because of reduced conflict among relatives or because of an inherent reduced ability of genetically different cell mixtures to coordinate development, and evolve complex adaptations. An alternative explanation is that mixing of genetically distinct cells with mutations that are of a novel, untried nature, are expected to have a detrimental effect on the developing multicellular organism, and can thus be called developmental incompatibilities. The presence of developmental incompatibilities in aggregative multicellularity could help explain why aggregative organisms are relatively rare and less complex compared to clonally developing multicellular organisms (Wolpert and Szathmáry, 2002).

We investigated whether mixing experimentally evolved lineages (with ancestors, and among themselves) with mutations that have not been tested against each other by selection would result in detrimental effects in an aggregative social amoeba. We measured developmental incompatibilities by quantifying coordinated development in terms of slug migration and fruiting body production of *Dictyostelium discoideum*. By assembling into a slug, the *D. discoideum* amoebae travel through soil and reach the surface, where they can complete their social life and disperse as spores, each capable of becoming a new amoeba. Slug migration, the coordinated movement towards light, over long distances is an adaptation for the survival of a group of amoebae. Additionally, amoebae not only had to coordinate their movement over large distances but also successfully develop into a fruiting body with spores.

We observed no difference in average slug migration distances in *D. discoideum* mixed lines compared to the expected average of ancestors and their evolved descendants (Figure 4 and 5). We further found no indication of a reduction in slug migration distances upon mixing of lineages evolved in the lab from common ancestor (Figure 6). These results fail to provide any support for the hypothesis that previously unselected combinations of genotypes would be deleterious.

Spore production in mixed lineages differed from the expected average of ancestors and evolved lineages, which is a requirement for the incompatibility hypothesis (Figure 7). However, we observed a significant increase in spore production in the chimeras which, on the surface, is contrary to the prediction of the developmental incompatibility hypothesis. Spores are often used as a measure of fecundity (Kuzdzal-Fick et al., 2023; Scott et al., 2022; Buttery et al., 2009), and therefore our spore production result would appear to represent an improvement in fitness upon mixing. However, an increase in spore production may not necessarily result in improved fitness if it is generated at the cost of stalk formation or by producing smaller spores. Support for this possibility comes from the experiment that generated these lines (Larsen et al., 2023). Because there was no social stage during the evolution, we expected most aspects of social fitness to usually change negatively, and the ability to cheat did decline. However, spore production increased during the experiment, which is consistent with an increase representing a decline in fitness.

The ability to migrate in *D. discoideum* comes with the cost of spore production in mixed lineages. When compared for the proportion of spores produced after migration with respect to total spore production, mixes in *D. discoideum* migrators produced fewer spores than expected from the average of the lineages grown alone (Figure 8). Therefore, our result for total spore production was primarily driven by fruiting bodies that did not migrate towards light. Our results indicate that the costs and benefits of mixing may be dependent on whether amoebas should stay in place where food is abundant or risk producing fewer spores while migrate towards light.

A similar study with social bacteria that cooperatively form multicellular structures reported incompatibilities in merging when isolated lineages were mixed (Rendueles et al., 2015). Authors showed that M. xanthus develops colony merging incompatibilities that can evolve *de novo* due to different mutations being fixed in isolated lineages. However, whether the observed colony merging incompatibilities were unselected incompatibilities, as we define in this study, in unclear. *M. xanthus* were selected to function together in swarms and allowed to form fruiting bodies. The experimental evolution thus selected for *de novo* kin discrimination.

Previous studies in *D. discoideum* that investigated the fitness consequences of mixing, have created chimeras by mixing naturally isolated wild genotypes (such as QS6, QS9) with each other, and compared them to clonal isolates (Foster et al., 2002; Castillo et al., 2005; Jack et al., 2011, 2015; Kuzdzal-Fick et al., 2023). For example, Foster et al. (2002) showed that slug migration distances are lower in chimera compared to clones when compared between slugs of the same size. The lower migration distances of the chimeric slugs could have a mechanistic explanation – poor cell adhesion between amoebae from distinct wild strains. An adaptive explanation for poor cell adhesion in slugs on the basis of the tgr alleles system would be kin discrimination, which is a potential way of preventing cheaters from joining an aggregate (Ostrowski et al., 2008, 2015; Benabentos et al., 2009). Moreover, in the later stages of development the tgr genes may not even be relevant to kin discrimination (Gilbert et al., 2012; Noh et al., 2018, 2020). In any case, a mismatch between allotypes of the *tgr* recognition system is an evolved incompatibility that has been tested by natural selection on distinct wild clones of *D. discoideum*.

Our study avoids the complications with kin recognition systems by mixing experimental lines that were evolved from clonal ancestors in the absence of an aggregative stage. We expect the experimental lines to have the same

tgr alleles between ancestors and their evolved descendants. Of course, it is not impossible for *tgr* loci to have evolved during the evolution, but it seems highly unlikely that this was common. The relatively short duration of our experimental evolution, and the large population sizes, preclude genetic drift causing changes in *tgrB1* and *tgrC1* across multiple lines. And there was no selection for changes in the *tgr* loci because there was no multicellular development during the experimental evolution of these lines (Larsen et al., 2023). As expected in the absence of direct selection, cheating ability declined (Larsen et al., 2023). There was therefore also no selection for avoiding variants who could be cheaters. Taken together with the fact that *D. discoideum* has one of the lowest mutation rates measured in eukaryotes (Kucukyildirim et al., 2020), we are confident that the ancestors and evolved lines used in this study did not change significantly for the supposed kin recognition loci.

Our definition of non-selected incompatibilities is a generalization of the concept of Bateson-Dobzhansky-Muller incompatibilities, which cause isolated populations to be increasingly reproductively isolated and become separate species (Orr, 1996; Dobzhansky, 1934; Bateson, 2009). As one population evolves through natural selection, newly emerged genes are tested against existing genes in the population and selected to be compatible with the background. Novel genes that cause mismatches within a population are weeded out, but new genes in one population are never tested with genes that may have newly emerged in another isolated population. Therefore, mismatches between populations gradually accumulate and only become visible to natural selection when the populations later come into contact.

Similarly, interactions of a novel, untried nature can have unexpected effects on the resulting multicellular organisms. The emergence of a new, non-intermediate trait may result from a combination of two or more traits not previously associated with a single organism resulting in unexpected outcomes in the hybrid organism. By making a conceptual parallel between the origins of multicellular organisms and the origin of species through the evolution of non-adaptive incompatibilities, our study has added to the new avenues for research into direct and indirect factors influencing complex development. The study of multicellularity can greatly benefit from considering non-adaptive incompatibilities and how a shared selection history may influence the evolution of multicellular development.

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