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ORIGINAL ARTICLE

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Social behaviour in genetically heterogeneous groups of *Dictyostelium giganteum*

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Abstract The Dictyostelid or cellular slime moulds (CSMs) are soil amoebae with an asexual life cycle involving social behaviour and division of labour. The most obvious distinction is between 'germ line' or pre-spore cells, which survive, and 'somatic' or pre-stalk cells, which eventually die. A plausible hypothesis to explain the apparent altruism of pre-stalk cells is that it is directed at clonal relatives. We have tested this hypothesis by comparing indices of altruistic behaviour between clonal and chimeric (genetically heterogeneous) social groups. The groups were generated by mixing amoebae belonging to distinguishable strains of *Dictyostelium giganteum*. The amoebae of one strain do not aggregate at all when mixed with any of three other strains and aggregate poorly with a fourth. Among the latter, co-aggregation occurs but is followed by varying extents of sorting out. At times, two strains form separate fruiting bodies; in other cases, they remain together but are clustered in clonal groups within a single chimeric structure. Our expectation was that the allocation of cells to the stalk pathway would be higher, and to the spore pathway lower, in clonal social groups than in chimeras. The expectation was not always fulfilled. In addition, three strains could be arrayed in a linear rank order in terms of the relative efficiencies of spore-formation in binary mixtures; but when all three were mixed, they were equally efficient. More than overall genetic similarity, cell fate in a chimera seems to result from complex non-linear interactions based on epigenetic differences.

Keywords Social behaviour · *Dictyostelium* · Kin selection · Altruism

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Introduction

The cellular slime moulds (CSMs) are soil amoebae that can lead both unicellular (or solitary) and multicellular (or social) lives. Single amoebae feed on bacteria, grow and divide by mitosis. When starvation sets in, they stop dividing, aggregate and form an integrated multicellular unit, the slug, which exhibits division of labour. Eventually, some amoebae differentiate into dormant spores and form a coalescent mass, the sorus. The remaining amoebae form a dead cellular stalk and support the sorus; the whole is called a fruiting body (Bonner 1967). The relative proportion of spore and stalk cells in a fruiting body is fairly constant over an enormous range of total cell numbers (Bonner and Slifkin 1949). The two cell types are comparable to the metazoan germ line and soma (Bonner 1982). Sporulation can also be thought of as 'selfish' behaviour on the part of an amoeba and stalk formation as 'altruistic' behaviour, a terminology that suggests parallels between cell differentiation in the CSMs and caste differentiation in social insects (Gadagkar and Bonner 1994). As in the social insects, in the CSMs also one can address the importance of genetic relatedness for social behaviour by comparing clonal and non-clonal groups.

The present study reports mixing experiments carried out with wild isolates of *Dictyostelium giganteum*, a common species in Indian soils. Our aim was to address two questions bearing on the evolution of social behaviour: (1) Does the relative allocation of amoebae to the spore and stalk pathways differ between clonal groups and groups of unrelated individuals? (2) Is the outcome significantly influenced by the level of genetic similarity within the group?

Methods

Isolation of strains

D. giganteum was isolated using standard methods (Raper 1984) from two hectares, numbered 46 and 50, in a 1,000×500 m (50-ha) rectangular plot of undisturbed moist deciduous forest soil in the Mudumalai wildlife reserve, Tamil Nadu, South India. Two to three sori were picked from each primary culture plate, subcultured and cloned by serial dilution. Clones were maintained on phosphate-buffered agar (PBA; 2% agar made up of KK2 buffer, where KK2 refers to 0.66 g KH_2PO_4 and 0.20 g K_2HPO_4 in 300 ml H_2O and at pH 6.4). Five clones of *D. giganteum*, which were confirmed as genetically distinct strains by random amplification of polymorphic DNA (RAPD; see below), were used for further work. The strains were maintained routinely at 22°C in association with *Klebsiella aerogenes* on PBA plates. Strain numbers 46a3, 46c6 and 46d2 were picked from hectare number 46, from soil located at the vertices of a right-angled triangle. 46a3 was found at a distance of 7.1 cm from both 46c6 and 46d2, and the latter were obtained from points 10 cm apart. 50c1 and 50d8 were found in hectare number 50, at a distance of 10 cm from each other and approximately 400 m distant from the 46th-hectare strains (Kaushik 2002).

RAPD analysis

The genetic structure of all five strains was evaluated by polymerase chain reaction amplification of random DNA primers (RAPD-PCR). Eight primers, each 10 nucleotides long, were used (OPA 01, OPA 03, OPA 07, OPA 10, OPA 11, OPA 13, OPA 17 and OPA 18, Operon Technologies Inc., USA). Nuclear DNA was extracted from amoebae as described (Francis and Eisenberg 1993). PCR reactions were carried out in a 25-μl volume containing 50 ng DNA as template, 0.16 μM of primer, 1 U *Taq* DNA polymerase and 200 μM of each dNTP (Sambrook et al. 1989). The amplified fragments were fractionated on a 0.8% agarose gel in 0.5× Tris borate EDTA buffer at pH 8.0 (5.4 g Tris base, 2.75 g boric acid and 2 ml of 0.5M EDTA in a total volume of 1 l) using standard electrophoresis apparatus. Gels were stained with ethidium bromide and the DNA bands were analysed after comparing all RAPD patterns. The presence of a band in a lane was scored as 1 and its absence as 0; in this way, a unique binary data string represented a strain. Strings were compared using a matching coefficient (Legendre and Legendre 1998). For a given primer, the genetic similarity, S, between strains A and B was defined as $S(A, B)=x/y$, where x is the number of bands common to the strains and y is the number of distinct bands generated by that primer over all strains. Similarity values were calculated for each primer, and the value of S averaged over all primers was taken as the overall measure of similarity between two strains. If, instead of adopting this procedure, we took S (for a given primer) to be the ratio of the number of common bands to the total number of bands obtained with that primer and that pair of strains, the results were not affected to any perceptible degree.

Mixing experiments

We used five strains of *D. giganteum*, three from the 46th hectare (46a3, 46d2 and 46c6) and two from the 50th hectare (50c1 and 50d8). Amoebae were starved by washing them off growth plates (that still retained an abundant supply of food, *K. aerogenes* bacteria) using ice-cold KK2, spun down twice at 300×*g* for 3 min, resuspended in buffer at a density of 2×10^6/ml and, while being shaken at 140 rpm, stained for 45 min at 22°C (5 μM of cell tracker blue, cell tracker green or cell tracker orange; Molecular Probes, Inc., USA). After this, they were washed twice in ice-cold KK2. Each component of the final mix was resuspended at a density of 2×10^6 cells/100 μl, mixed with the other component in a 1:1 ratio (or, when three strains were used, in a 1:1:1 ratio) and briefly vortexed. Two hundred microlitres of the mixture were spread evenly on 10-cm PBA plates. The plates were incubated in the dark at 22°C, and observations were made as required.

Scoring of genotypes: spore counts

Plates were scored after approximately 3 days, by which time fruiting bodies had formed. Sori were selected randomly under a dissecting microscope. Individual spore masses were transferred with a moistened needle to different 0.5-ml centrifuge tubes containing 30 μl of KK2, and the suspension was vortexed vigorously. About 200 spores were picked from each of 10–35 sori. A drop was pipetted onto a haemocytometer or glass slide, and the spores belonging to each strain were counted. Sometimes an entire spore mass was transferred to a glass slide, lightly disturbed with a needle to separate the spores and observed in a Leica fluorescence microscope with a 60× objective and appropriate filters. The relative contribution of strains to spore formation was estimated using two methods. In one, an entire spore mass from one fruiting body was picked and the spores were thoroughly mixed as before. The spore suspension was transferred to a glass slide, and a large number of spores of each genotype were scored. Each experiment was repeated thrice. Independently, all the spores on a plate were collected, and the overall, as well as individual, spore-forming efficiencies (SFEs; see below) were estimated by fluorescence-activated cell sorting (FACS). The number of spores belonging to different strains was monitored in a FACStar instrument using the CELL Quest software (Becton-Dickinson, USA). In each sample, the output from 10,000 cells was analysed at a flow rate of 12 μl/min as described previously (Saran et al. 1994).

Spore-forming efficiency

Spore-forming efficiency is defined as the ratio of the number of spores formed to amoebae plated. Freshly starved amoebae were shaken in KK2 for 5–6 h (to make sure that any residual cell divisions had been completed), and 4×10^6 amoebae were spread evenly on 10-cm-diameter PBA plates. About 3 days later, after fruiting bodies had appeared, plates were washed thoroughly with cold KK2 and the spores were counted using a haemocytometer. A negligible number of spores were left behind on the plates (there were practically no amoebae). To measure the SFE in mixtures, freshly starved amoebae belonging to two strains were stained differentially as described earlier and then mixed in a 1:1

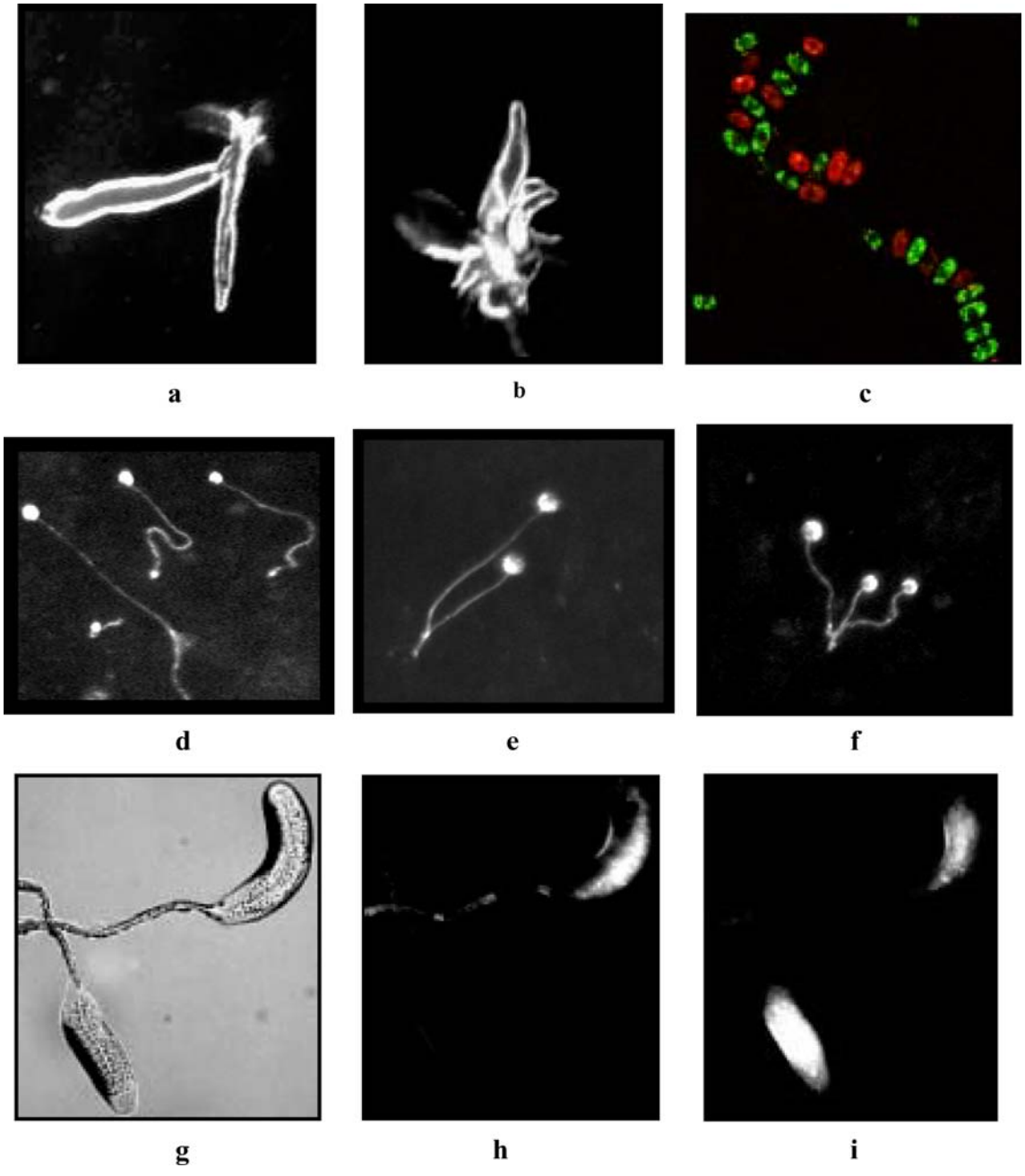

Fig. 1 (See electronic version for colours.) **a**, **b** Instances of more than one slug emerging from chimeric aggregates formed by 46d2 and 50c1. As judged by examining spore masses, in this case, about one fifth of the aggregates gave rise to chimeric fruiting bodies. **c** In a chimeric fruiting body, spores of 50c1 (*red*) can be distinguished from those of 46d2 (*green*) **d** Chimeric fruiting bodies formed by 46a3 and 46d2. As a rule, all 46th-hectare strains co-aggregate and one fruiting body emerges from an aggregate. **e**, **f** In contrast, when 50c1 and 46d2 co-aggregate, many fruiting bodies emerge from the same aggregate. **g**–**i** As seen with the help of different filters, a single aggregate made up of 50c1 (stained with cell tracker red) and 46c6 (stained with cell tracker green) can give rise to both chimeric and pure slugs. **g** Bright-field picture of the two slugs, **h** shows that the slug on the top is partially stained *red* and **i** shows that the upper slug is *partially green*, whereas the lower slug is *almost entirely green*. (Scales: spore lengths, 5–8 μm; fruiting bodies, 0.8–1.4 mm; spore masses, 80–120 μm)

ratio. Two hundred microlitres of the suspension containing 4×10^6 cells was spread on 10-cm-diameter PBA plates. In each such experiment, the SFE of the component genotypes was also computed.

Results

We worked with five strains of *D. giganteum*, three from the 46th hectare (46a3, 46d2 and 46c6) and two from the 50th hectare (50c1 and 50d8). Each strain had a distinct genotype as verified by the band patterns obtained after carrying out RAPD with eight primers (not shown). In all, at least 16 DNA bands were obtained with each primer. For a given strain, the band patterns were highly reproducible. The mean coefficient of variation in the similarity index *S*, measured among and between the five strains taken pairwise, was 9% (range, 0–19%). The staining procedure did not affect developmental rates, fruiting body sizes or any other visible features (not shown). The time taken from starvation to fruiting body completion was about 24 h.

Aggregation and fruiting in mixtures: qualitative description

When labelled and unlabelled cells belonged to the same strain, they co-aggregated freely, remained intimately intermixed all through development and participated equally in spore formation. This was inferred by viewing aggregates over entire plates (not shown). Amoebae from different strains co-aggregated to varying extents. 50d8 was an exception; it aggregated only with 50c1. With inter-strain mixes, co-aggregation did not guarantee that the amoebae remained together subsequently. In these cases, although the cells aggregated into one mass, varying extents of sorting out preceded slug or fruiting body formation, so much so that some fruiting bodies contained spores of a single genotype (50c1 + 46d2; Fig. 1). (We never observed a chimera in which one strain contributed exclusively to spores and another exclusively to the stalk.) The relative proportions of chimeric and pure fruiting bodies depended on where the strains originated from; chimeric fruiting bodies were markedly more prevalent in intra-hectare mixtures when compared to the inter-hectare ones. The number of fruiting bodies on a plate did not differ appreciably between experiments—that is, it appeared to be a consequence of the cell density on a plate, not of the genotypic composition of the cells. Although the conditions we used disfavour sexual development (Raper 1984), macrocysts were sometimes seen in these experiments. However, their numbers were so small that relative spore counts did not have to be modified.

Aggregation and fruiting in mixtures: quantitative data

When and to what extent can different genotypes be made to participate in the same social group? The frequency of chimerism, defined as the number of fruiting bodies that contained cells of both strains divided by the total number of fruiting bodies, gives part of the answer. This frequency varied between different pairs of strains. Among the 46th-hectare strains, which were isolated from locations within 10 cm of one another, it ranged from 67.5±7.5 to 85.0±2.4% (Table 1). Within the small range of genetic similarities in these experiments (0.64–0.74), the frequency of chimerism was positively correlated with the degree of genetic similarity.

All the 46th isolates formed chimeric fruiting bodies with 50c1; it should be noted that the average genetic similarity within and across hectares for these strains was about the same (0.69; Table 1). However, in this case, the frequency of chimerism varied from 16.8 to 21.5% (overall mean±SD, 18.7±1.4%), which was significantly lower than the frequency of chimerism within the 46th-hectare strains (78.4±9.5%, t=11.08, n=3, p=0.004). In one experiment, 50d8 and 50c1 formed chimeric fruiting bodies but at a low frequency of 18% (data not shown).

Sorting out

When an amoeba belongs to a social group that has both clonal relatives and non-relatives, does it discriminate between the two? We found that when amoebae from two different strains formed a chimeric aggregate, in the beginning the cells were intermingled haphazardly. However, as development proceeded, they tended to segregate into distinct groups, and by the slug stage, they could be seen to occupy more or less contiguous clusters. The pattern of

Table 1 Frequency of chimerism in *D. giganteum*

Genotypes	Frequency of chimerism (%)	Genetic similarity
Within the 46th hectare		
46a3+46c6	67.5±7.5	0.64±0.08
46a3+46d2	82.7±1.6	0.70±0.05
46c6+46d2	85.0±2.4	0.74±0.09
Between the 46th and 50th hectares		
46a3+50c1	16.8±5.6	0.71±0.05
46d2+50c1	17.7±3.7	0.70±0.10
46c6+50c1	21.5±6.1	0.65±0.07
Within the 50th hectare		
50c1+50d8	18.0[a]	0.60±0.14
46th-hectare strains (averaged pairwise)	78.4±9.5	0.69±0.05
50c1 and all 46th-hectare strains	18.7±1.4	0.69±0.03

Exponentially growing amoebae of two strains were washed free of bacteria, mixed in a 1:1 ratio and allowed to develop on plates. The frequency of chimerism is the number of fruiting bodies containing both kinds of spores divided by the total number of fruiting bodies on a plate. Approximately 10–35 fruiting bodies were scored on a plate, and experiments were carried out at least thrice. Values are means±SDs (significance: t test, p<0.05)

[a]Single experiment

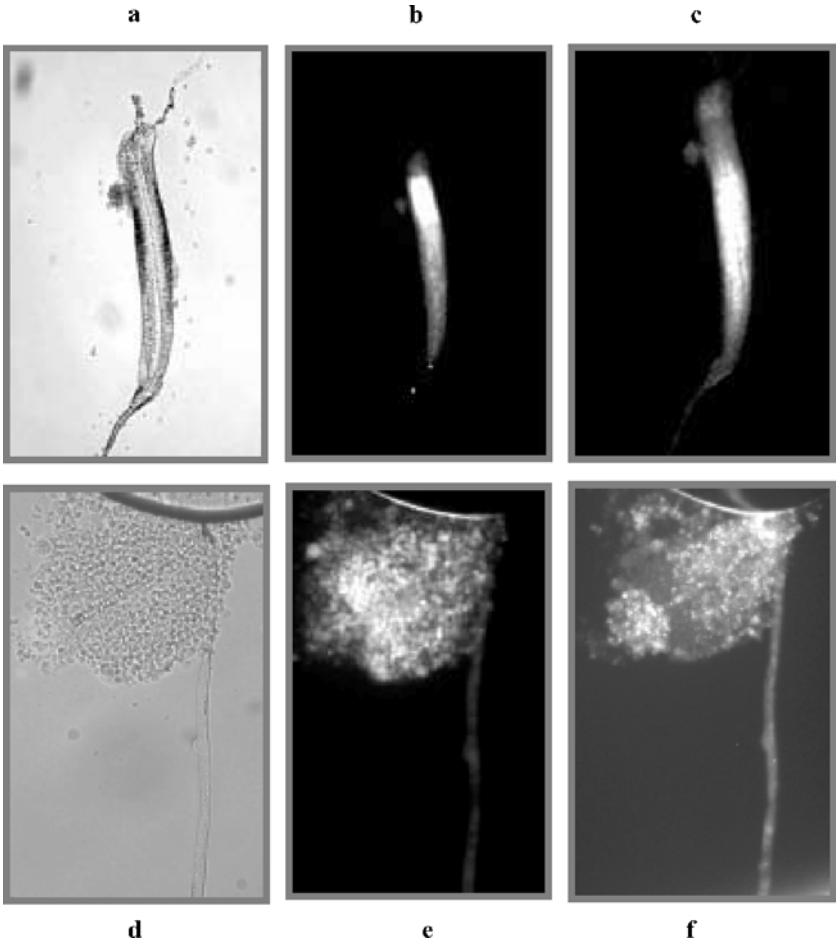

Fig. 2 Sorting out seen in 46d2 and 46a3 mixtures that give rise to chimeric slugs and fruiting bodies. **a**–**c** Chimeric slug and **d**–**f** chimeric fruiting body formed by another aggregate (the spore mass was bent down by a glass slide). **a**, **d** Bright field; **b**, **e** 46d2 visible; **c**, **f** 46a3 visible. Overall, **c** 46a3 amoebae are in the slug's posterior, **b** whereas those of 46d2 are concentrated just behind the anterior boundary. Spores of the same kind seem to be clustered in both (**e**) and (**f**). The length of the slug (excluding stalk) is 0.28 mm and the fruiting body (including stalk) is 0.35 mm long

sorting out was reproducible for a given pair of strains but varied from one pair to another.

When 46a3 and 46d2 were mixed, 46d2 amoebae mostly occupied the 'neck' of the slug (the region immediately behind the tip), and to a lesser extent the posterior (Fig. 2a–c). 46a3 amoebae were found in the posterior and to some extent also in the anteriormost portion. The two strains remained segregated even as spores (Fig. 2d–f). Also in 46c6 and 46d2 mixtures, the two genotypes sorted out. However, in this case, the clusters did not exclude each other as clearly as with 46a3 and 46d2 chimeras (Fig. 3a–c; compare with Fig. 2). In another experiment, all three 46th-hectare strains were mixed in a 1:1:1 ratio. Here too, the strains co-aggregated and sorted out to form contiguous spatial clusters, albeit with overlaps (Fig. 4a–f).

As we have seen, mixtures of the 46th-hectare strains displayed an anterior–posterior pattern of spatial segregation. In striking contrast, when a 46th-hectare strain was mixed with 50c1, there was a left–right pattern of spatial segregation in the slug (Fig. 5a–c; 'left' and 'right' are defined relative to the direction of movement). In a given slug, one strain appeared to contribute more or less exclusively to the left half and the other, to the right half. Between different slugs, there was no clear preference on

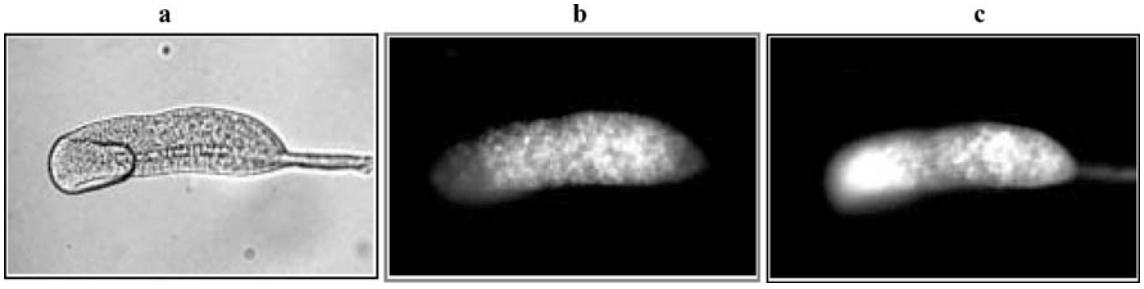

Fig. 3 Spatial segregation of 46c6 and 46d2 cells in chimeric slugs. **a** Bright field; **b**, **c** fluorescence. **c** 46d2 (stained with cell tracker blue) tends to localize more in the anteriormost and posterior regions of the slug, with the exception of the posteriormost margin. **b** 46c6 (stained with cell tracker green) occupies part of the anterior and most of the posterior region. The slug tip has been bent backwards during photography; in this case, the segregation of genotypes is not as distinct as in the case of 46a3 and 46d2 (Fig. 2). These slugs were small, typically 0.20 mm in length excluding the stalk

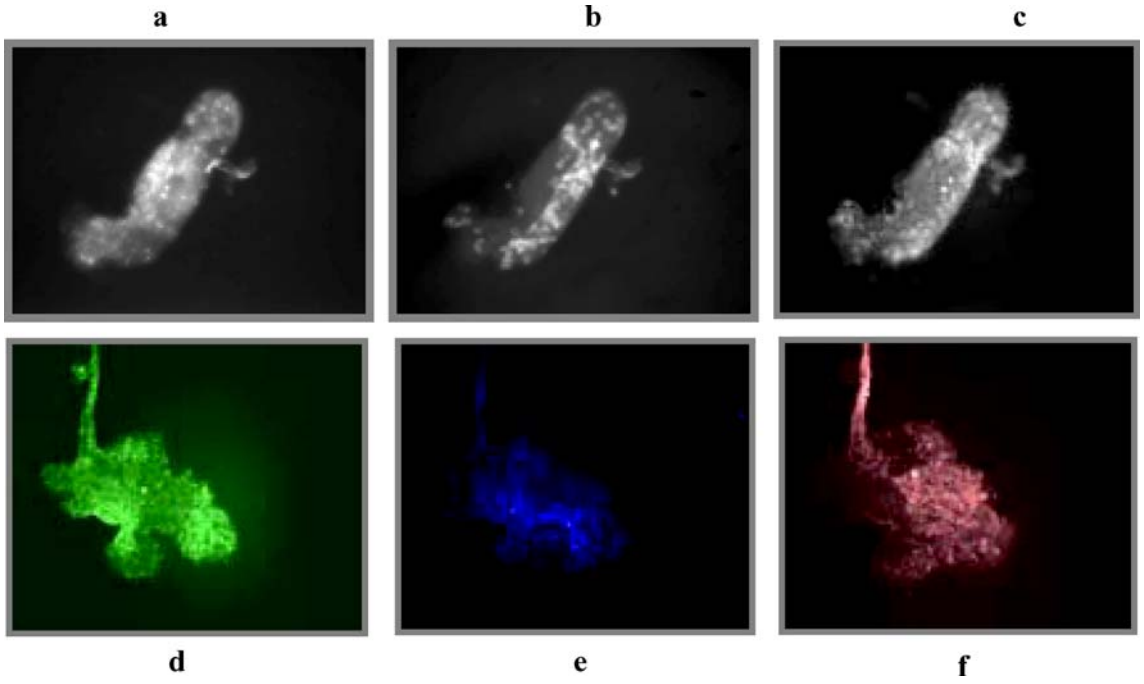

Fig. 4 Sorting out in a mixture of all three 46th-hectare strains. (See electronic version for colours.) **a**–**c** One slug; **d**–**f** one spore head (not from the slug in **a**–**c**). **a**, **d** 46d2 amoebae and spores (stained with cell tracker green); **b**, **e** 46c6 amoebae and spores (stained with cell tracker blue); **c**, **f** 46a3 amoebae and spores (stained with cell tracker orange). The spore mass is slightly misshapen because of squashing. The strains cluster, but their segregation is not complete. Both the slug and the spore mass were about 0.18 mm in their longest dimension (stalk excluded)

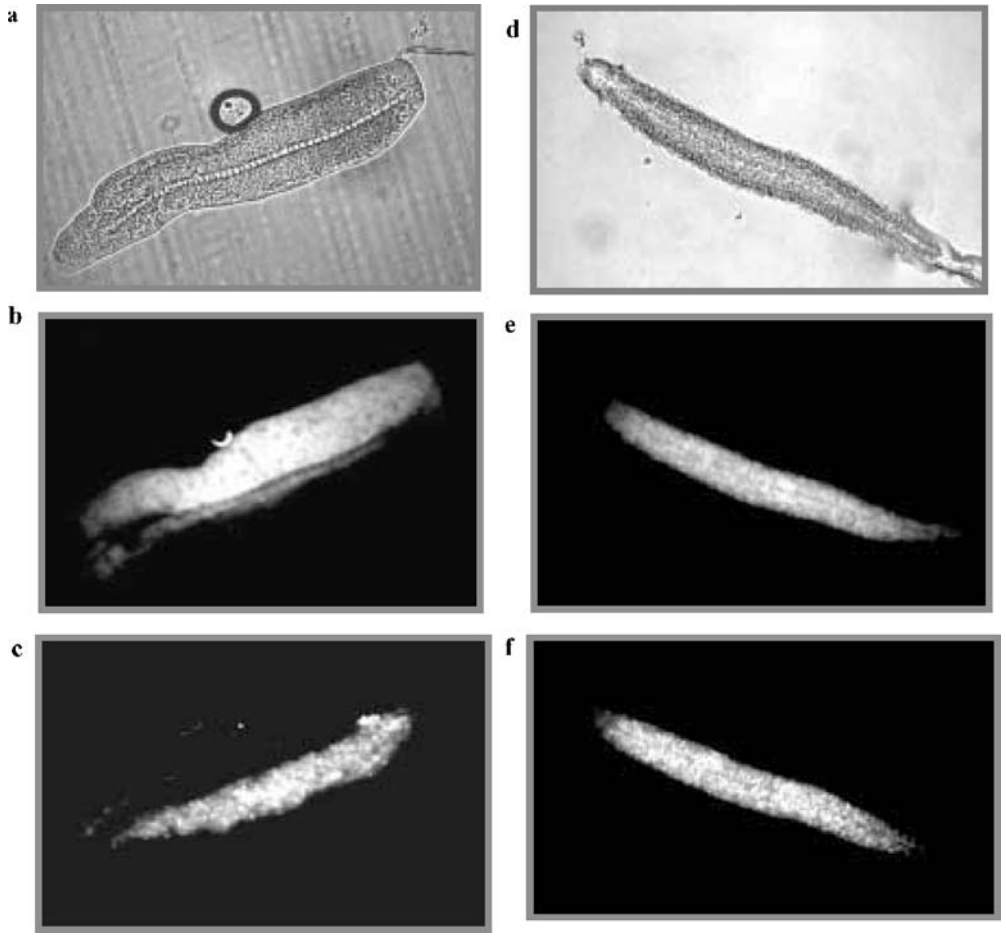

Fig. 5 'Left–right' segregation of 50c1 and 46a3 amoebae in slugs. **a**, **d** Bright-field images and the others are fluorescence pictures. **a**–**c** One slug, **b**–**f** another slug. Amoebae belonging to 46a3 (stained with cell tracker green) occupied the *'right' half* of the slug (**b**) and the ones belonging to 50c1 (stained with cell tracker orange), the *'left' half* (the slug's anterior is towards the *lower left*). **c** The stalk seems to be composed mainly of 46a3 cells except at the very posterior. **d**–**f** show that the dyes played no role in this. 46a3 amoebae were stained with both dyes and reconstituted in a 1:1 ratio. **d** Reconstituted slug of 46a3 (anterior towards the *upper left*), **e** fluorescence of cell tracker blue in the same slug and **f** fluorescence of cell tracker green in same slug. Both dyes seem to be uniformly distributed. Slug lengths are approximately 0.380 mm in **a**–**c** and 0.35 mm in **d**–**e**

Table 2 Allocation of amoebae to spore formation (intra-hectare chimeras)

Mix	Genetic similarity	Degree of fairness	Spores in chimeric fruiting bodies (%)		
			46a3	46c6	46d2
46a3+46c6	0.64±0.08	0.31	23.8±5.4	76.2±5.4	–
46c6+46d2	0.74±0.09	0.75	–	42.8±2.7	57.2±2.7
46a3+46d2	0.70±0.05	0.40	29.1±4.6	–	71.9±4.6
46a3+46c6+46d2	0.69±0.05	0.88 0.88 (46a3:46c6) 1.06 (46c6:46d2) 0.93 (46a3:46d2)	31.1±3.5	35.5±5.5	33.4±2.2

The degree of fairness in spore contributions is defined as the ratio of the mean number of spores formed by the first genotype in a chimeric fruiting body divided by the mean number of spores formed by the second. The ratio would be 1 if the strains contributed to forming spores in proportion to their mixing ratio. In the mix of all three strains, the genetic similarity is that expected between any two amoebae picked at random. About 10–35 fruiting bodies and 200 spores from each fruiting body were scored in each experiment. Each result is the mean±SD of at least three independent experiments

the part of any strain for either left or right. To rule out an effect of dye colour in causing this unexpected pattern, amoebae of the same strain were stained with two different fluorescent dyes and mixed in a 1:1 ratio; they always showed complete intermixing (Fig. 5d–f).

Contributions to spore formation

Do the genotypes in a chimera contribute equally to spore formation? A strain's relative contribution to the spore population depended on which strain it is mixed with (Table 2). 46a3 contributed significantly fewer spores than expected when mixed with either 46c6 (t=11.89, 2 df, p<0.001) or 46d2 (t=11.40, n=3, p<0.001); 46c6 contributed significantly fewer spores when mixed with 46d2 (t=6.53, 2 df, p=0.001). In terms of their effectiveness in forming spores in pairwise mixtures, the hierarchy may be expressed as 46d2>46c6>46a3. However, when all three strains from the 46th hectare were mixed evenly, the relative number of spores contributed by them was also approximately 1:1:1 (pairwise comparisons: t≤1.17, 2 df, p≥0.23; Table 2). The relative contribution to spore formation in pairs of these three strains approached 1 as their genetic similarity approached 1 (Table 2). 50c1, which was comparatively poor at forming a chimera with any 46th-hectare strain (Table 1), contributed more to spore formation than 46a3 and about the same as 46c6 or 46d2 (Table 3). FACS confirmed that when two strains were mixed, the fraction of spores contributed by a strain was roughly comparable irrespective of whether all fruiting bodies or only chimeric fruiting bodies were monitored (Table 3).

The number of spores formed divided by the number of amoebae plated, which we call SFE, forms an important component of fitness. As defined, the SFE is a group-level trait; if interpreted as the probability that an amoeba differentiates into a spore, it can be thought of as an individual trait. The mean SFE was much the same for the 46th-hectare strains—it ranged from 51.3 to 55.3%. However, as Table 4 shows, when developed together with another strain, the SFE could stay essentially unchanged (46a3 with 46c6 and 46d2 with 46a3), decrease significantly (46a3 with 46d2) or increase marginally (46c6 with 46d2). Interestingly, the combined SFE from a mixture of two 46th-hectare strains was about the same as, or smaller than, the SFE of either strain when it developed by itself: a strain does not improve its productivity by joining another strain.

The case of 50d8

On its own, the development of 50d8 is unexceptional. When stained and unstained amoebae of 50d8 were mixed and allowed to develop, stained and unstained spores were present in equal proportions in the resulting fruiting bodies (not shown). 50d8 co-aggregated with 50c1 but did not do so with any of the 46th-hectare strains. In 50c1 + 50d8 mixtures, 18% of the fruiting bodies were chimeric. This was much smaller than in the intra-46th-hectare mixtures but about the same as in the 50c1 + 46th-hectare mixtures (Table 1). In the chimeric fruiting bodies, 50c1 contributed significantly more to the spore population than 50d8 (59.7

Table 3 Allocation of amoebae to spore formation (inter-hectare chimeras)

Strains	Genetic similarity	50c1 spores (all fruiting bodies) (%)	50c1 spores (chimeric fruiting bodies only, n=3 experiments) (%)
50c1:46a3	0.71±0.05	53.0±4.6	58.0±6.4
50c1:46c6	0.65±0.07	60.0±3.1	51.9±3.8
50c1:46d2	0.70±0.1	53.0±2.8	53.6±5.2

Exponentially growing amoebae of strains isolated from about 400 m apart were starved, mixed in a 1:1 ratio and allowed to develop on plates. About 10–35 fruiting bodies and 200 spores from each fruiting body were scored in each experiment. The results are given as the mean±SD of three independent experiments. In chimaeric fruiting bodies, 50c1 contributes significantly more to spore formation than 46a3 (t=3.01, 2 df, p=0.02)

Table 4 Spore-forming efficiencies (SFEs) measured in isolation and in chimeras

Strains	Combined SFE	SFE of the first component		SFE of the second component	
		On its own	When mixed	On its own	When mixed
46a3 and 46c6	52.8±10.6	51.3±0.4	51.5±4.9	53.5±2.1	61.3±5.2*
46a3 and 46d2	45.9±3.0	51.3±0.4	39.6±7.8*	55.3±6.7	51.8±13.1

Spore forming efficiency (SFE) is defined as the number of spores belonging to a strain divided by the number of amoebae of that strain that were plated initially. The combined SFE is the total number of spores formed as a percentage of the total number of amoebae plated. The results given are means±SD of two independent experiments. (A few macrocysts were seen on all plates.) *The SFE of 46c6 goes up marginally when mixed with 46a3 (t=1.97, 1 df, p=0.07) and that of 46a3 goes down significantly when mixed with 46d2 (t=2.12, 1 df, p=0.02)

vs 40.3%, SD=5.3%, 1 df, t=3.66, p=0.04). This was also true when spores from all fruiting bodies, chimeric and clonal, were counted using FACS (63.1±1.69% for 50c1 when all fruiting bodies are combined vs the previous 59.7±5.3%, t=0.86, 1 df, p=0.2). In chimeric slugs, 50d8 segregated in a 'left–right' manner from 50c1 (not shown), just as the 46th-hectare strains did (Fig. 5).

No chimeric fruiting bodies were seen in mixtures of 50d8 with any strain belonging to the 46th hectare. The reason was that the development of 50d8 was largely inhibited. When plates that contained a 46th-hectare strain combined with 50d8 were harvested after 1 week and scored for spores, essentially all the spores belonged to the 46th-hectare strain. FACS was carried out on samples of 10,000 spores formed on plates where 50d8 amoebae had been mixed in a 1:1 ratio with those of 46a3, 46d2 and 46c6; the fraction of 50d8 spores was 3.2, 3.1 and 3.1%, respectively. Overall, the 50d8 amoebae remained unaggregated. This was confirmed after 1 week when stained amoebae were washed off the plates and observed under the microscope; all belonged to 50d8.

We tried to see whether extracellular factors could account for the developmental arrest of 50d8 in these mixtures. Freshly starved amoebae of 50d8 were deposited on a PBA plate in the form of a drop of KK2 buffer containing 2×10^6 amoebae. A cellulose acetate filter paper (pore diameter 0.2 μm) was placed gently on the drop, and 2×10^6 amoebae of another strain were layered on top of the filter paper. A reciprocal experiment was carried out on a separate plate, with the strain that was initially below the filter paper now being placed above. In control experiments, the same strain was put both below and above the filter paper. The experiments were carried out in duplicate and repeated on 3 days. 50d8 amoebae aggregated and fruited normally when separated by a filter paper from any of the 46th-hectare strains. Not surprisingly, many more fruiting bodies were formed when 50d8 was placed above the filter paper than when placed below. (When below the filter paper, strains were inconsistent in development and fruiting. Occasionally, delicate fruiting bodies could be seen emerging from underneath the paper, sticking out of the rim.)

Discussion

On account of their unusual life cycle and the ease with which they can be manipulated, the CSMs are ideal for examining whether a particular cellular or multicellular trait is an adaptation for social behaviour. Should fruiting body formation be viewed as a group adaptation or as an adaptation at the level of the individual cell? If the former, does it depend on genetic relatedness within the group—that is, could kin selection be a possible contributing factor (Bonner 1982)? Or is social behaviour in the CSMs better viewed as the outcome of phenotypic selection between the individual amoebae that constitute a group (Atzmony et al. 1997)? The individual-level selection point of view implies that the outcome is inherently stable, meaning that it cannot successfully be exploited by a social parasite. However, individual selection does not ipso facto account for constant cell-type proportions. On the other hand, a fixed ratio of stalk cells to spores can be shown to follow from a group-selection model with clonal groups (Nanjundiah 1985) or with a kin-selection model that aims for an evolutionarily stable strategy in which each clone in the group attempts to maximize its reproductive fitness (Matsuda and Harada 1990). Unfortunately, in both cases, the outcome is vulnerable to social parasites (Kaushik and Nanjundiah 2003). The present study involves behaviour in homogeneous social groups vis-à-vis heterogeneous groups; and, with the help of RAPD, we have tried to relate the findings to a measure of overall genetic similarity. RAPD analysis has been shown to possess strong discriminating power in studies of genetic relatedness in bacteria (Clerc et al. 1998), plants (Huang et al. 2000) and mammals (Ratnayeke et al. 2002). In the present case, reproducibility was high, with the average coefficient of variation in the similarity index S being 9%. However, our experimental design does not address the issue of kinship, nor do we have explicit measurements of genetic relatedness. Given this, our findings can offer evidence that makes one or the other model appear plausible, but cannot refute either. At the same time, we can rule out some simple-minded possibilities; for instance, that genetic heterogeneity automatically leads to more selfish behaviour (i.e., a higher allocation to the spore pathway relative to the stalk pathway). The most striking feature of our observations is this: in many ways, *D. giganteum* amoebae show that they prefer to be associated with others belonging to their own genotype. However, when that preference cannot be ensured and they go through development as part of a chimera, their behaviour is not thereby any more selfish than usual.

With regard to social behaviour in genetically heterogeneous groups, three aspects of a chimeric fruiting body are

of interest. One is the relative allocation of strains within a fruiting body to the stalk and spore pathways ("Does one strain exploit the other?"). The second is the productivity, or SFE, of a strain that participates in a chimeric fruiting body when compared with its SFE when developing alone ("Which is better, developing with clonal relatives or as part of a chimera?"). Thirdly, there is the overall SFE of a chimeric fruiting body in comparison with the SFE of either component ("Is the likelihood of selfish behaviour more in a genetically heterogeneous group than in a clonal group?").

In relation to these aspects, the salient features of our observations may be listed as follows:

(a) For a given degree of genetic similarity, the probability that a fruiting body is chimeric is higher when both strains originate from locations in close proximity than when they originate from locations that are far apart. When the strains are derived from locations that are close by, the higher the genetic similarity, the larger the fraction of fruiting bodies that is chimeric (Table 1). A hypothesis that can explain these observations is that chimera formation depends in part on adaptive features of the phenotype such as sensitivity to a chemoattractant, the strength of intercellular adhesion and so on. To some extent, such features depend on the genotype. In addition, within what is broadly the same genotype (for instance, between different strains of the same species), local variations in the phenotype may exist. Analogous to the neutral phenotypic variations posited by Bonner and Lamont (2005), they may represent alternative adaptations to the same environment, with the difference that here we are referring to members of the same species. Phenotypic differences of this sort may make chimerism more likely, or more successful, between geographically close strains than those that are isolated from far apart.

(b) When a chimera is formed, cells tend to sort out and cluster with others of the same genotype (Figs. 2, 3 and 5).

Bonner and Adams (1958) showed that when mixed, different species of CSMs could aggregate together and then form separate fruiting bodies; different strains of the same species (*Dictyostelium mucoroides*) could sort out within the same aggregate and the sorting persisted (as in the present study) in spore masses. Here we find that it is possible for two strains of the same species to form chimeric fruiting bodies and, at other times, to co-aggregate and later sort out, giving rise to pure fruiting bodies (Fig. 2). In light of the observation of Bonner and Adams, we interpret this to mean that what are species-level differences in one context may be strain-level differences in another context. Sorting out along the anterior–posterior axis may reflect pre- and post-aggregation variations in motility, chemotactic ability or adhesiveness. Lateral (left–right) sorting, a new finding, could also be a consequence of differences in the strength of intercellular adhesiveness within and between strains. We note that slugs are well known to display a longitudinal 'fault line': sometimes they break up spontaneously down the middle into two bilaterally symmetric parts (Raper 1940).

When spores of the same genotype occupy contiguous clusters—reflecting, presumably, the contiguous clusters of cells seen in the slug (Figs. 2, 3, 4, 5)—one must conclude that following dispersal there is a high probability that the neighbours of a spore belong to its own genotype. The implication is that in the next generation, there is a high probability that aggregations will involve clones. The propensity for clonal groups to be favoured over non-clonal groups would seem to be the opposite of what is seen in *Dictyostelium discoideum*, but that is not so. A chimeric slug of *D. discoideum* migrates farther, and presumably ensures more efficient dispersal, than a slug belonging to a single genotype; but that happens only if the latter contains fewer cells than the chimera (Foster et al. 2002). Basically, this is because large slugs move faster than small slugs (Bonner et al. 1953). However, between two equally sized slugs, the one whose amoebae belong to a single clone does better than the one that is genetically heterogeneous (Foster et al. 2002). So in the case of *D. discoideum* too, it would appear that given the opportunity of joining an ongoing aggregation, cells would prefer to join others of the same clone over an equal number belonging to a different clone.

Why might *D. giganteum* be predisposed to form clonal social groups? It may be that homogeneous slugs and fruiting bodies are more stable than heterogeneous ones and so have been favoured by selection. Another possibility is that measures that favour genetic purity would act as an automatic defence against social parasites. Such defence may be important, given that under natural conditions fruiting bodies can contain spores belonging to at least nine genotypes (Kaushik and Nanjundiah 2003).

(c) One strain can inhibit the development of another. With the 50d8–46th-hectare mixtures, the inhibition depends on cell–cell contact. It disappears when the strains are separated by a 0.2-μm filter, a pore size that should permit signalling by diffusible extracellular factors. In a separate study not reported here, we found that amoebae of one strain can inhibit the growth of another. Either form of inhibition would imply that one strain is excluded from a growing or developing colony of another strain. The consequence would be that the genetic purity of a strain is maintained.

(d) The proportions of spores in chimeric fruiting bodies need not be the same as the proportions in which the amoebae are mixed (Table 2). On the face of it, one strain appears to exploit the other, as also observed in *D. discoideum* (Strassmann et al. 2000). When a strain forms more spores than a second, and in another chimera the second strain does better than a third, it turns out that the first does better than the third (Table 2; symbolically, 46c6>46a3, 46d2>46c6 and 46d2>46a3). The transitive nature of this relationship could be taken to imply that SFE is an invariant, an intrinsic property of a strain. That might justify referring to a strain as a 'cheater' or as an exploiter of another strain. However, the situation is more complicated than that. 46a3, which forms fewer spores than either partner in two separate binary mixtures, does significantly better when both are present (Table 2). The observation that 50d8 can form a chimera with 50c1, and 50c1 can do so with any 46th-hectare strain, but 50d8 develops very poorly when mixed with any 46th-hectare strain, also points to a similar complexity. In short,

the efficiency with which cells belonging to one strain sporulate depends strongly on the presence of other strains. The absence of an invariant hierarchy is evidence of non-linear interactions between cells, interactions that are sensitive to the social environment.

(e) The productivity, meaning overall SFE, of a chimeric group is about the same as, or smaller than, the SFE of either strain by itself (Table 4). If we equate spore formation to selfish behaviour, this means that amoebae do not behave more selfishly just because they find themselves in the company of amoebae belonging to a different genotype. When considered along with (d) above, the implication seems to be that once an amoeba is committed to become part of a chimeric fruiting body, whether it forms a stalk cell or a spore has more to do with epigenetic traits than with genetic relatedness per se. This speculation is reinforced by what we have learnt from *D. discoideum*. In its case, two classes of inputs influence the pathway of differentiation: subtle cell-to-cell heterogeneities within clonal populations of amoebae (supposedly raised in the same 'uniform' environment), and self-organisation via intercellular signalling (for reviews, see Gross 1994; Kawli and Kaushik 2001; Kaushik and Nanjundiah 2003).

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