Not like *Botryllus*: indiscriminate post-metamorphic fusion in a compound ascidian

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Fusion trials between metamorphs of the aplousobranch compound ascidian *Diplosoma listerianum* indicated that chimera formation was not dependent on relatedness. Similar, high rates of union were observed between full siblings, half siblings, unrelated individuals from the same population, and individuals from two geographically distant localities. This is in contrast to the well-studied ascidian genus *Botryllus*, in which a highly polymorphic allorecognition system governing the fusion–non-fusion reaction (colony specificity) largely limits fusion to close relatives. Fusion in *Botryllus* establishes a vascular chimera throughout which stem cells may circulate, promoting cell lineage competition between the fusion partners. The restriction of fusion to close kin in *Botryllus* is thought to reduce the inclusive fitness costs of competitive interactions between cell lineages within the chimera. In contrast to *Botryllus*, modules (zooids) of a *D. listerianum* colony are not interlinked by blood vessels, seemingly precluding the exchange of stem cells. The apparent absence of strict colony specificity in *D. listerianum* is thus in keeping with the predictions of the *Botryllus* model for the maintenance of allorecognition polymorphism.

However, colony specificity has been reported in other species of aplousobranch ascidian that also lack a common vascular system. In these, the threat of migrating blood-borne stem cells cannot be responsible for the presence of colony specificity. One possibility, requiring experimental investigation, is that stem cells could perhaps migrate between zooids by another route, such as through the matrix of the colonial tunic. Even in the absence of stem cell exchange, cheating on the costs of colony maintenance and defence could also produce selective forces favouring colony specificity. In compound ascidians, this could involve unequal contribution to extrazoidal structures, principally the tunic and related tissues. This consideration seems potentially relevant to the lack of discrimination during fusion in *D. listerianum*, since extrazoidal somatic investment in this species appears minimal, severely limiting the scope for this other form of cheating.

The various possible modes of exploitative interaction between fused colonies are not mutually exclusive, and offer fundamentally similar explanations for colony specificity. If none of them can be shown to occur in non-botryllid species possessing colony specificity, the generality of the *Botryllus* model may require re-evaluation.

Keywords: *Diplosoma listerianum*; *Botryllus*; fusion; chimera; allorecognition; kin selection

1. INTRODUCTION

The Fu/HC (fusibility/histocompatibility) locus that governs fusion of neighbouring colonies in the compound ascidian genus *Botryllus* has been the subject of considerable interest as the putative precursor of the vertebrate major histocompatibility complex (e.g. Scofield *et al.* 1982a,b; Weissman *et al.* 1990; Saito *et al.* 1994) and remains the foremost model for protochordate allorecognition. Fusion to produce a chimera initially involves the tunic (an organic matrix within which the colony modules, or zooids, are embedded), plus the vascular system which interconnects the zooids. Following fusion, blood circulates between the zooids of the new partners, which may thereby exchange germ-line cells and totipotent somatic stem cells. Within the zooids of the recipient, these cells may respectively develop as gametes, and found somatic lineages (Sabbadin & Zaniolo 1979; Pancer *et al.* 1995; Stoner & Weissman 1996). Since the Fu/HC locus is hypervariable (Grosberg & Quinn 1986;

Rinkevich *et al.* 1995), the requirement for partial matching at this locus generally restricts fusions to close relatives. This constraint is expected to reduce the inclusive fitness costs of somatic and germ-cell parasitism within the partnership, while retaining the advantages of chimera formation, and it has been postulated that somatic compatibility systems evolved to alleviate the costs of cell lineage competition in this way (Buss 1982). A second, similarly well-studied, sessile marine invertebrate also fits these ideas. The cnidarian *Hydractinia* shares with *Botryllus* the association between a highly discriminatory allorecognition system governing the nature of interactions between colonies (e.g. Grosberg *et al.* 1996) and the potential for cell lineage parasitism within a chimera (Müller 1964; Buss & Shenk 1990).

From these considerations, reduced or absent colony specificity would be expected in species in which the costs of chimerism were greatly diminished. Here, we test this prediction by investigating the pattern of fusion of metamorphs in a compound ascidian in which the zooids are

not interconnected by a common vascular network, so that exchange of stem cells between fusion partners appears impossible. We thereby re-evaluate the *Botryllus*–*Hydractinia* model for the generation and maintenance of allorecognition polymorphism in sessile invertebrates, and examine the degree to which *Botryllus* spp. can be taken as representative of the compound ascidians in general.

The didemnid ascidian *Diplosoma listerianum* (Milne Edwards) shares the same basic colony form as *Botryllus*, forming patches or sheets of small zooids enclosed by tunic, which consists of a fibrous tunicin (cellulose) matrix within which several types of cell are dispersed. In this didemnid the tunic forms a thin-walled sac enclosing an extensive water-filled cloacal space in which the zooids are suspended (Berrill 1950; Mackie & Singla 1987). Each zooid has its own inhalant siphon, but numerous zooids use a common exhalant opening in the tunic. Zooids are relatively independent and are only connected by the common tunic. Vascular ampullae passing into the tunic from individual zooids are blind-ended and do not anastomose. Any single zooid within a colony may bud independently, giving rise to two descendants without the direct involvement of other zooids (e.g. Berrill 1935). Colonies are dynamic structures, constantly changing shape, capable of moving slowly and sometimes splitting (Della Valle 1908). Individual zooids appear to change their relative positions within the tunic over hours or days, so that those originating in different parts of a colony may subsequently become intermingled (Bishop & Ryland 1991). Sexually produced embryos are brooded within the substance of the tunic. Larvae of *D. listerianum* metamorphose to produce two-zooid (oozooid plus first blastozooid) juvenile colonies which, like the adults, can move slowly across the substratum.

Initial indications that promiscuous fusion might occur in *D. listerianum* involved the formation, following settlement, of fusion masses incorporating several metamorphs (J. D. D. Bishop, unpublished observations). Larvae released by the various *D. listerianum* colonies on single kelp fronds collected from Port Erin Bay, Isle of Man, British Isles, were allowed to co-settle and metamorphose in the laboratory on glass microscope slides. There was no evidence of gregarious settlement (in agreement with the findings of Schmitt (1982a), but overall density was very high. Within 24 hours of metamorphosis, a large proportion of metamorphs that had settled in close proximity, or had subsequently moved together, fused to produce chimeric colonies. These chimeras could be extensive: the largest consisted of 90 zooids and resembled a partially grown colony, but in fact represented 45 metamorphs. These observations stimulated the experiments described below which investigated the possibility of kinship-independent fusion in *D. listerianum*. The temporal stability of the resulting chimeras was also investigated.

2. METHODS

(a) *Fusion of metamorphs*

(i) *Basic protocol*

Post-metamorphic fusion was studied in *D. listerianum* using a protocol similar to the oozooid allorecognition assay employed in botryllids (Scofield *et al.* 1982b; Hirose *et al.* 1988; Rinkevich

& Weissman 1992). Larvae were allowed to metamorphose, and metamorphs were then gently detached from the substratum within 24 h of settlement, without damaging the tunic. Metamorphs were placed together in pairs in the wells of polystyrene 60 µl × 10 µl microwell plates (Nunc, Life Technologies, Paisley, Scotland) submerged horizontally in still seawater. After 24 h the metamorphs had reattached and were observed under the dissecting microscope. Any pairs with missing or damaged zooids were noted, and excluded from subsequent analysis. The plates were then placed vertically in small tanks in standard culture conditions (stirred water and microalgal food: Ryland & Bishop (1990)) for a further 24 h. Each pair of metamorphs was then scored under a dissecting microscope as fused (tunics fused, cloacal spaces generally, but not necessarily, confluent) or unfused (tunics touching but unfused, or not touching).

(ii) *Origin of biological material*

Trials were performed using progeny from two distinct parental sources: pairwise matings of laboratory stocks and open fertilization of wild colonies. The laboratory matings used cultured clones originating from Queen's Dock, Swansea, Wales (QDS). The parental clones had been founded and maintained in culture as previously described (Ryland & Bishop 1990; Bishop *et al.* 1996), and included a subset of those used by Bishop *et al.* (1996), which are referred to below by their original letters; their genetic relatedness was investigated by Bishop *et al.* (1996) by estimation of RAPD band-sharing using multiple primers. An additional laboratory clone, *L*, was founded in July 1995 by a larva from QDS. Metamorphs from QDS-derived cultured material are referred to in table 1 by a combination of their parents' reference letters; thus progeny of clones *E* and *F* are referred to as *EF*.

Metamorphs from fertilizations in the wild were the progeny of colonies growing naturally on glass plates (150 mm × 150 mm) in three submerged racks (160 mm × 160 mm × 240 mm) in Queen Anne's Battery Marina, Plymouth, England (QAB). This site has extensive connection to the open sea and supports a large natural population of *D. listerianum*. Racks were separated by respective distances of 2.3, 11.0 and 13.3 m. Plates from the racks, each bearing several colonies, were transferred from the marina into aerated seawater in small tanks in the laboratory. Metamorphs for experimentation were collected daily from the walls of each tank for up to 5 days after removal of their brood parents from the wild.

(iii) *Fusion trials*

Five fusion trials were conducted. Successive batches of progeny for each trial were collected daily over a period of time, and various pairings with different degrees of relatedness were set up in haphazard order in a new microwell plate each day. Trials 1–3 used metamorphs of known relatedness (full sibling, half sibling and unrelated) from laboratory crosses of QDS clones to investigate the possible effect of pedigree relatedness on fusion. The three trials were carried out in 1995, 1996 and 1997 respectively.

In trial 4, fusion rates were assayed between QAB metamorphs as follows: pairs of metamorphs derived from brood-parents on different racks; pairs from different settlement plates on the same rack; and pairs originating on the same plate. Differences in average relatedness between these categories were not investigated, but, if differences exist, pairs from different racks would be expected to have the lowest mean value of kinship, while at the other extreme an unknown

Table 1. *Fusion between pairs of metamorphs of Diplosoma listerianum*

(QDS: progeny from laboratory crosses of cultured specimens derived from Queen's Dock, Swansea. QAB: progeny of wild colonies on racks of settlement plates in Queen Anne's Battery, Plymouth. Scores shown as number of pairs fused:unfused (percentage fused). Results given of $R \times C$ tests of independence using G -test with Williams' correction (Sokal & Rohlf 1995).)

trial	QDS metamorphs with different degrees of relatedness (letters denote parentage of metamorphs (see text))			G
	sib	half-sib	unrelated	
1	<i>EF + EF</i> 32:21 (60.4)	<i>EF + BE</i> 24:26 (48.0)	<i>EF + BC</i> 27:25 (51.9)	1.654, $0.5 > p > 0.1$
2	<i>EF + EF, AL + AL</i> 19:14 (57.6)	—	<i>EF + AL</i> 21:15 (58.3)	0.004, $1 > p > 0.9$
3	<i>EF + EF</i> 49:32 (60.5)	<i>EF + AE</i> 38:44 (46.3)	<i>EF + BC</i> 45:40 (52.9)	3.269, $0.5 > p > 0.1$
1 + 2 + 3	100:67 (59.9)	62:70 (47.0)	93:80 (53.8)	4.943, $0.1 > p > 0.05$
QAB metamorphs				
	same plate	same rack, different plates	different racks	
4	14:13 (51.9)	15:10 (60.0)	94:76 (55.3)	0.350, $0.9 > p > 0.5$
QDS metamorphs paired with QAB metamorphs				
5	99:70 (58.6)			

proportion of pairs from the same plate might share a common brood-parent.

In trial 5, fusion was assayed between QAB and QDS metamorphs. QDS metamorphs in this trial originated from four laboratory crosses involving seven laboratory clones. The two field locations from which these metamorphs were derived are separated by *ca.* 340 km of sea, estimated from charts as the shortest series of straight lines around headlands.

(b) *Stability of chimeras*

To study the temporal stability of established chimeric associations, four chimeras, each founded by the fusion of two unrelated QDS metamorphs (*EF* and *AL*) in fusion trial 2, were grown in culture for 36 or 44 days, during which time colony growth by asexual budding took place. The individual zooids within each colony were then fingerprinted using randomly amplified polymorphic DNA (RAPD) markers. Molecular methods followed Bishop *et al.* (1996), except that DynaZyme II (Flowgen Instruments, Sittingbourne, UK) with 1.65 mM MgCl₂ was used rather than *Taq* polymerase for colonies II and IV.

3. RESULTS

(a) *Fusion of metamorphs*

During fusion, the tunics of touching metamorphs became continuous, and tunic material originally separating the respective water-filled cloacal spaces of the participants parted, establishing an initial connection that enlarged to produce a single, communal cloacal space containing the zooids of both partners. No recognizable boundary persisted between fusion partners and the single resulting colony showed no sign of its dual origin. The vascular ampullae emanating from individual zooids did not appear to be involved in the process of tunic

fusion or to interact with each other subsequently, and no vascular connections were observed.

After initial limited fusion of tunics, the metamorphs occasionally moved apart but remained clearly joined for some time by one or more strands of tunic under tension. Complete separation was then sometimes accomplished by tearing of the tunic of one or both partners, leaving zooids partly or largely naked. However, viewed under the dissecting microscope this process was not accompanied by discernible inflammation or necrosis in the region of contact, and similar stretching and tearing of the tunic may occur during fission of non-chimeric colonies, suggesting that no true rejection reaction was observed during the trials with metamorphs.

Frequencies of fusion of QDS metamorphs ranged between 46 and 60% in trials 1–3 (table 1 and figure 1), and did not differ significantly between degrees of relatedness, either within individual trials or when all the data were combined. Although the combined data approached significance, the lowest frequency of fusion was recorded at the intermediate level of relatedness (half-sib).

Frequencies of fusion of pairs of QAB metamorphs in trial 4 ranged between 52 and 60%, and did not differ significantly according to the respective origins of the two metamorphs (table 1). In trial 5, pairs of metamorphs from the two different localities (QDS–QAB) fused with each other at a similar frequency to the trials within localities (table 1 and figure 1). The respective fusion rates did not differ significantly between unrelated QDS pairs (trials 1–3), different-rack QAB pairs (trial 4) and QDS–QAB pairs (trial 5) ($n=173$, 170 and 169, respectively; $R \times C$ test of independence using G -test with Williams' correction (Sokal & Rohlf 1995): $G=0.838$, $0.9 > p > 0.5$).

Figure 1. Frequency of fusion (± 1 s.e.) of pairs of *Diplosoma listerianum* metamorphs. QDS: progeny from laboratory crosses of cultured specimens derived from Queen's Dock, Swansea. QAB: progeny of wild colonies on settlement racks in Queen Anne's Battery, Plymouth. QDS fusions at three levels of pedigree relatedness are combined data from fusion trials 1–3 (see table 1).

(b) Stability of chimeras

DNA fingerprinting revealed that two of the four originally chimeric colonies (colonies I and II) were still chimeric, the zooids belonging to two distinct genotypes (figure 2), while in the other two colonies one partner had apparently been lost, leading to the detection of a single genotype after the typing of all zooids (table 2). Three of the four original colonies (I, III and IV) had split naturally into separate pieces (ramets) by the time of scoring. In the persistent chimera that had undergone fission (colony I), zooids of both genotypes were present in all three of the resulting ramets. It should be noted that, although DNA extracted from the tunics of chimeras showed representation of both genotypes, no evidence was seen for the admixture of clonal genotypes within individual zooids, i.e. zooidal lineages remained distinct (cf. Pancer *et al.* 1995; Stoner & Weissman (1996) for contrasting data from *Botryllus schlosseri*). The same was true in other *D. listerianum* chimeras, similarly analysed by RAPD, produced by the fusion of ramets of unrelated adult laboratory clones; in these, banding profiles of individual zooids were identical to those of the original unfused clones after six months (Sommerfeldt & Bishop 1999).

4. DISCUSSION

Fusion in *Botryllus* occurs only between colonies sharing at least one allele at the Fu/HC locus (Oka & Watanabe 1960; Sabbadin 1962; Scofield *et al.* 1982b). Since this locus is hypervariable, with up to 300 alleles in natural populations (Grosberg & Quinn 1986; Rinkevich *et al.* 1995), fusion between unrelated colonies is rare. Encounters between related colonies may be promoted by limited dispersal of larvae (Grosberg 1987) and by their preferential co-settlement on the basis of shared alleles at the Fu/HC locus (Grosberg & Quinn 1986). Colony

Figure 2. Gel of RAPD amplification products from 20 individual zooids of colony grown from chimera of two *Diplosoma listerianum* metamorphs. Samples are from ramet 2 of colony I (see table 2). Outer lanes (1 and 23) are molecular size markers. Zooids of two distinct genotypes are present (lanes 3, 5–7, 9, 11 and 13–22 versus lanes 4, 8, 10 and 12); sample of tunic (lane 2) shows influence of both genotypes.

Table 2. *RAPD* fingerprinting of zooids removed from colonies each founded as a chimera of two *Diplosoma listerianum* metamorphs

colony	days in culture	ramet	no. of zooids present	no. of zooids finger- printed	primer(s)	ratio of zooidal geno- types
I	44	I.1	52	26	Y15	11:15
		I.2	32	20	Y15	4:16
		I.3	8	8	Y15, R02	2:6
II	36	—	27	17	Y15	7:10
III	36	III.1	12	12	Y15, R02	12:0
		III.2	7	7	Y15, R02	7:0
IV	36	IV.1	20	20	Y15	20:0
		IV.2	7	7	Y15, Y01	7:0

specificity is fully developed at the metamorph stage (Scofield *et al.* 1982b; Hirose *et al.* 1988; Rinkevich & Weissman 1992), which in *Botryllus* consists of the single oozoid. Chimeras of Fu/HC-compatible non-clonemates of *Botryllus* in laboratory culture are often unstable. One of the partners is commonly resorbed within days to months, losing its zooidal representation in the partnership, or the partners may physically disconnect (Rinkevich & Weissman 1987, 1992; Sabbadin & Astorri 1988). However, these phenomena may not be prevalent in more natural conditions (Chadwick-Furman & Weissman 1995).

The high level of fusion reported here between unrelated *D. listerianum* metamorphs, including those derived from geographically distant populations, and the potential for coexistence of unrelated partners through several cycles of zooidal budding during subsequent colony growth, indicate that colony specificity in this didemnid is much less developed. Furthermore, natural division of one chimera did not involve re-separation of the partners, but produced a series of chimeric ramets. If fusibility in *D. listerianum* were under the control of a locus like Fu/HC, with the same matching rules as in

Table 3. *The occurrence of colony specificity and colony-wide vascular networks in compound ascidians*

(F, fusion; R, rejection; I, indifference (non-fusion without particular reaction); —, not tested. Control of fusion by single locus with multiple codominant alleles: **, confirmed in controlled laboratory crosses; *, consistent with results of multiple fusion trials with wild material.)

classification	vascular network	species	contact of		colony specificity	references ^a
			growing edges	cut surfaces		
ENTEROGONA						
Aplousobranchiata						
Polyclinidae	no	<i>Aplidium yamazii</i>	F or R	F or R	yes	1
		<i>A. constellatum</i>	F or R	—	yes	2
Polycitoridae	no	<i>Polycitor proliferus</i>	I	F	no	3
Didemnidae	no	<i>Didemnum moseleyi</i>	F or R	F or R	yes	4
		<i>Diplosoma listerianum</i>	F or I	—	no	this study
Phlebobranchiata						
Perophoridae	yes	<i>Perophora viridis</i>	F or R	—	yes	2
		<i>P. bermudensis</i>	F or R	—	yes	2
		<i>P. japonica</i>	F or R	—	yes	5
		<i>P. orientalis</i>	I	F	no	4
		<i>P. sagamiensis</i>	F or R	—	yes	6
		<i>Ecteinascidia tortugensis</i>	F or R	—	yes	2
PLEUROGONA						
Stolidobranchiata						
Botryllidae	yes	<i>Botryllus schlosseri</i> **	F or R	F or R	yes	7, 8, 9, 10
		<i>B. primigenus</i> **	F or R	F or R	yes	4, 11, 12
		<i>B. scalaris</i>	F or R	F or R	yes	13
		<i>Botrylloides simodensis</i> *	F or R	F or R	yes	4, 14, 15
		<i>B. violaceus</i>	F or R	F	yes	16
		<i>B. fuscus</i> *	F or R	F	yes	17
		<i>B. diegense</i> *	F or R	—	yes	18
		<i>B. leachii</i> (**?)	F or R	—	yes	19
		<i>Symplegma reptans</i>	F or R	F or R	yes	4

^a References: 1, Watanabe & Taneda (1982); 2, Freeman (1970); 3, Oka & Usui (1944); 4, Mukai & Watanabe (1974); 5, Koyama & Watanabe (1981); 6, Koyama & Watanabe (1982); 7, Bancroft (1903); 8, Sabbadin (1962); 9, Scofield *et al.* (1982a); 10, Scofield *et al.* (1982b); 11, Oka & Watanabe (1957); 12, Oka & Watanabe (1960); 13, Saito & Watanabe (1982); 14, Mukai & Watanabe (1975); 15, Saito & Watanabe (1976); 16, Hirose *et al.* (1988); 17, Hirose *et al.* (1994); 18, Yund & Feldgarden (1992); 19, Svoboda & Scofield (1989).

Botryllus and in a highly polymorphic state, very improbable allelic combinations in the pairs of unrelated parents would be necessary to obtain results similar to those in the fusion trials detailed here. Nor does it appear that the fusion reported here between unrelated *D. listerianum* metamorphs is purely a function of their early ontogenetic stage: fusion is permitted between unrelated adult clones of *D. listerianum* at relatively high frequency (A. D. Sommerfeldt and J. D. D. Bishop, unpublished observations).

The reported fusion percentages of *D. listerianum* metamorphs cannot be interpreted as definitive rates of compatibility, but may be regarded as minimum estimates of potential fusibility, reflecting our particular experimental protocol. Thus, although contact without fusion was observed during some of the individual pairings, it is additionally highly probable that, in a proportion of cases of non-fusion, the two metamorphs happened to move apart immediately after being placed together in the well and did not subsequently make sustained contact during the trial. The fusion rate observed would therefore be expected to depend on the exact size and physical configuration of the trial arena. These factors were kept constant during the reported trials by the use throughout of a single batch of microwell plates.

Fusion of different genetic individuals to form a chimera is potentially cooperative behaviour under kin selection (Buss 1982). If so, the degree of discrimination of the fusion–non-fusion reaction should reflect how the costs and benefits of fusion depend on the relatedness of the protagonists (Hamilton 1964; Buss 1982; Grafen 1990). The main potential cost of fusion may be competitive interactions between members of a chimera. Asymmetrical transfer and maturation of germ-line cells may enable one partner in a *Botryllus* chimera to dominate the gametic output of the association; this situation may continue even after the reseparation of partners or resorption of the zooids of the reproductively dominant genotype (Sabbadin & Zaniolo 1979; Pancer *et al.* 1995; Stoner & Weissman 1996). In addition, circulating stem cells may also establish somatic lineages within the zooids of the other partner (Pancer *et al.* 1995; Stoner & Weissman 1996). Restricting fusion to relatives will partially offset the costs incurred by the loser of these interactions, via compensatory indirect contributions to its inclusive fitness through the reproductive output of the winner (Buss 1982).

According to this argument, the ramified vascular network interconnecting the zooids is of crucial significance to colony specificity in *Botryllus*, allowing the

transfer of stem cells between zooids and zooidal generations and thus opening the way for competition between the partners' respective cell lineages. Critically, in didemnids such as *D. listerianum* the vascular ampullae arise separately from individual zooids and there is no common vascular system. Zooids are thus relatively independent and there is no apparent route for the exchange of cells between them. The potential for cell lineage competition therefore appears to be much reduced in didemnids. Through their differing vascular organization, *Botryllus* spp. and *D. listerianum* constitute contrasting examples in which divergent levels of somatic incompatibility appear to match predictions relating the degree of kin discrimination to the potential costs of cooperation (Hamilton 1964; Buss 1982; Grafen 1990). However, before accepting this conclusion, it is necessary to consider available information on fusion in colonial ascidians as a whole.

A compilation of published information on the occurrence of colony specificity in ascidians is given in table 3, based on that presented by Saito *et al.* (1994). Under the established hypothesis for *Botryllus*, well-developed allorecognition would be predicted in other species in which zooids are interconnected by a vascular network, independent stem cells are free to circulate, and fusion with conspecific tissue potentially occurs with appreciable frequency. The existence of colony specificity in all tested species of botryllids is in keeping with this idea. The majority of species in the Perophoridae also directly comply, if it is assumed that stem cells might be exchanged via the vascular stolons that link zooids in this family. The absence of specificity in one species of perophorid (*Perophora orientalis*) in which uncut stolons do not undergo either autogenic or allogenic natural fusion is also in agreement with predictions.

However, aplousobranch ascidians do not possess a colony-wide vascular system. While the absence of colony specificity in a non-fusing species of polycitorid, *Polycitor proliferus*, is of no particular significance to assessing the possible role of vascular exchange, colony specificity has been reported in two other families within the order, the Polyclinidae and Didemnidae, and the existence of allorecognition in these cannot be explained by the potential migration of blood-borne stem cells between zooids. One possibility is that this exchange may nevertheless occur, but via another route. The tunic contains numerous motile cells, and the possible transfer of stem cells between zooids of a chimera via the tunic matrix at least deserves consideration. This possibility could be assessed by genetic analyses similar to those used to investigate the establishment of alien cell lineages in *Botryllus* (Sabbadin & Zaniolo 1979; Sabbadin & Astorri 1988; Pancer *et al.* 1995; Stoner & Weissman 1996). Investigation of chimeras of aplousobranchs from this viewpoint would be of considerable interest. (The possible mixing of cell lines within perophorid chimeras could profitably be subjected to a similar approach.)

An alternative explanation for the maintenance of colony specificity is potential cheating through unequal sharing of the energetic costs of colony protection and maintenance. Disproportionate contribution to supportive functions was envisaged as a component of cell lineage competition by Buss (1982). The tunic and related tissues

(including, if present, the common vascular system) of colonial ascidians represent extrazoidal components of the colony, produced from zooidal resources, to which partners in a chimera might not contribute fairly. This possible exploitative interaction does not involve exchange of cells between partners. Polyclinid ascidians generally form substantial colonies, with the zooids completely embedded in thick tunic, and the expense of tunic production and potential for cheating may therefore be considerable. In contrast, investment in tunic is low in many didemnids, which generally have a low ratio of tunic to zooid biomass compared to other colonial ascidians (Turon & Becerro 1992). Unequal investment in tunic production therefore appears a less convincing explanation for the reported colony specificity in the case of *Didemnum moseleyi* than in polyclinids. Nevertheless, a distinction may be drawn within the didemnids between genera such as *Didemnum*, in which the tunic includes numerous calcareous spicules produced by the zooids, and *Diplosoma listerianum*, in which spicules are absent and the tunic is particularly insubstantial, offering even less scope for inequitable investment by fusion partners.

The orthodox taxonomic division of ascidians into the orders Enterogona and Pleurogona is supported by molecular phylogenetic analysis, but cuts across the distinction between solitary species and budding, colonial forms (Wada *et al.* 1992; Satoh 1994). This implies that one, or conceivably both, of the lifestyles has arisen more than once. A considerable diversity of modes of bud formation exists between different families (e.g. Nakauchi 1982; Satoh 1994) and it has, for instance, been argued that ascidian coloniality had separate origins in stolidobranchs and aplousobranchs (Berrill 1935, 1950). Phylogenetic relationships within the Ascidiacea, when elucidated, may therefore prove highly relevant to understanding the origin and significance of colony specificity (cf. the recent phylogenetic analysis of allorecognition mechanisms within the botryllids by Cohen *et al.* (1998)). A truly convincing theory for the existence of relatively sophisticated somatic compatibility in ascidians would have to encompass not only the full range of colonial forms, but also unitary species. Although outside the scope of this paper, the occurrence of allorecognition in solitary ascidians of both orders, in the form of rejection of tunic allografts and allogenic reactions between blood cells (e.g. Reddy *et al.* 1975; Fuke & Nakamura 1985; Raftos 1994), also requires explanation. The tunics of adjacent conspecific solitary ascidians in natural aggregations may fuse (Schmidt 1982b; Raftos 1994), although whether this involves establishment of common vascular circulation has not been specifically reported.

The various forms of competitive interaction within chimeras discussed above are not mutually exclusive, and could of course co-occur in the same species. Through its combination of insubstantial tunic and disjunct vascular system, *D. listerianum* seems very close to the theoretical trustworthy compound ascidian hypothesized by Grafen (1990, p. 52), in which inability to cheat within a chimera was predicted to lead to loss of variability at the allorecognition locus, and thus to indiscriminate fusion. The contrast between *Botryllus* spp. and *D. listerianum* thus seems to support notions of the importance of cell lineage competition for the accumulation of allotypic

polymorphism. Although it is not known whether strict colony specificity has been lost or was never developed in the ancestors of *D. listerianum*, the reported existence of colony specificity in other aplousobranchs suggests the former. If so, fusion of *D. listerianum* metamorphs might exemplify the derivation of by-product mutualism ('no-cost cooperation'; Dugatkin, 1997) from kin-selected cooperative behaviour. The results suggest that natural populations of *D. listerianum* may consist in significant part of chimeras of unrelated partners, and prompt the question of what direct individual fitness advantage accrues from fusion in this protochordate. Unlike the costs, the compensatory benefits of chimerism have proved elusive in *Botryllus* and other colonial marine invertebrates (Rinkevich 1996; Maldonado 1998).

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