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Environmental split between germ cell parasitism and somatic cell synergism in chimeras of a colonial urochordate

Baruch Rinkevich* and Irena Yankelevich

Israel Oceanographic and Limnological Research, National Institute of Oceanography, Tel Shikmona, POB 8030, Haifa 31080, Israel

*Author for correspondence (e-mail: buki@ocean.org.il)

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Summary

Colonies of the urochordate *Botryllus schlosseri* may fuse upon contact if they share common alleles on the highly polymorphic fusibility/histocompatibility locus. While, in these chimeras, one of the partners is usually morphologically eliminated (resorbed), circulating totipotent cells of the inferior genotype on the resorption phenomenon may parasitize either the soma or the germ line of the winner. Here, we show an environmental split of the two stem cell lineages that may develop germ cell parasitism *vs* somatic cell cooperation. Each naturally formed *Botryllus* chimera can be a composite of component genotypes created through two unlinked parasitic germ and somatic cell lineage interactions. The germ line parasitism is inherited through a pedigree. Conversely, by using amplified fragment length polymorphism (AFLP) and microsatellite alleles as polymorphic genetic markers, and seawater temperature as the variable environmental factor, we documented that the somatic constituent of chimeric zooids was shifted from one genotype to another, in accordance with the changes in seawater temperatures. This variable somatic state of chimerism in the field may, thus, carry benefits to the chimeral entity, which presents synergistically, at any time, the best-fitted combination of its genetic components.

Key words: *Botryllus*, chimerism, fusion-rejection, green-beard allelism, stem cell.

Introduction

A typical colony of the urochordate *Botryllus schlosseri* is composed of a few to hundreds of similar-size modules (zooids) arranged in star-shaped structures (systems), embedded in a semi-transparent gelatinous-like matrix, the tunic. All zooids within a system and all systems within a colony are interconnected *via* a ramified vascular system, ending in sausage-like termini of blood vessels in the peripheral colony zone. The colony can be split experimentally or naturally to more than a single subclone. Each such subclone usually continues to thrive independently, forming a large independent colony.

Colonies and subclones grow by lateral expansion on the substrate. When different colonies meet their peripheral ampullae under natural or laboratory conditions, they may anastomose to form a vascular parabiont (cytomictical chimera; Rinkevich and Weissman, 1987a). This occurs if they share one or both alleles on a highly polymorphic haplotype (Oka and Watanabe, 1957; Sabbadin, 1982; Scofield et al., 1982), the fusibility/histocompatibility (Fu/HC; Weissman et al., 1990) locus. Thereafter, during a period of a few days to several months, massive apoptosis and phagocytosis processes, called ‘colony resorption’, morphologically eliminate all zooids originating from one of the genotypes within a chimera (Rinkevich and Weissman, 1987a; Weissman et al., 1990; Rinkevich et al., 1993). This phenomenon appears to be genetically controlled by multi-level hierarchical organization of the Fu/HC and additional histocompatibility alleles (Rinkevich et al., 1993).

It has been suggested that the development of such a complex, genetically based, self–nonself recognition system emerged as an adaptation for weakening the threat of cell lineage competition and parasitism (Buss, 1982; Grosberg and Quinn, 1986), a possible scenario resulting from natural fusions in the wild (Ben-Shlomo et al., 2001). However, by using polymorphic microsatellite markers, recent studies (Pancer et al., 1995; Stoner et al., 1999) have demonstrated that, even after complete morphological resorption of one partner in an anastomosed entity, the blood, the soma and the germ cells of the ‘winner’ partner are, in many cases, chimeric, pointing to cell lineage parasitism (Pancer et al., 1995; Stoner and Weissman, 1996; Magor et al., 1999; Stoner et al., 1999). Moreover, it is also common to find outcomes where the whole mass of gonads, as well as the soma, is derived from the resorbed genotype. The germ cell parasitism events are reproducible, show hierarchical patterns and are sexually inherited, as shown by breeding experiments (Stoner et al.,

1999). Somatic cell parasitism, on the other hand, while reproducible under controlled mariculture conditions and characterized by hierarchical organization, does not reveal the trait of sexual inheritance through a pedigree (Stoner et al., 1999). Therefore, gametic and somatic competitive routes, although reproducible, appear to be unlinked (Stoner et al., 1999; Magor et al., 1999).

Botryllus chimeras are common in nature (Ben-Shlomo et al., 2001). Several studies (Buss, 1982; Grosberg and Quinn, 1986; Rinkevich and Weissman, 1992; Rinkevich and Shapira, 1999; Rinkevich, 2002) were set up to define the evolutionary significance of natural chimeras by evaluating the fitness costs and benefits of chimerism as compared with the state of genetically homogeneous entities or by analysing bi-*vs*-multi-partner chimeras. No definite benefit was recorded in the kingdom Animalia for any case of chimerism. Conversely, scientific interest focused on the possible threat of germ cell parasitism in chimeras, which is particularly relevant for organisms where germ cell sequestration remained undetermined until late in ontogeny or when their full attainment along the life span of the organism was not achieved (Buss, 1982, 1983). No such analysis has been devised for a case where germ cell parasitism and somatic cell parasitism split. In the present study, we test the hypothesis that directionality of somatic cell parasitism in *Botryllus* chimeras is a plastic trait. We found that, under adverse environmental conditions, the chimera entity continuously responds to natural selection forces by exhibiting different phenotypic combinations of its genetic components. This leads to two conflicting types of interactions that are simultaneously exhibited by the two genotypes: germ cell parasitism *vs* somatic cell synergism.

Materials and methods

Animal maintenance

Botryllus schlosseri Pallas 1766 colonies selected for the experiments came from our laboratory stocks and originated at Monterey Marina, CA, USA. They were raised and maintained at 20°C as described previously (Rinkevich and Shapira, 1998). Colonies were paired for compatible combinations that share at least one common allele at the Fu/HC locus (Scofield et al., 1982; Weissman et al., 1990; Magor et al., 1999) and thus would fuse upon contact *via* their peripheral ampullae. Colonies were divided into several subclones at least one month before their use. Subclones from each colony were acclimated to temperature changes (15°C, 25°C) for one month and then paired with similar-sized subclones of the compatible partner and placed on glass slides fitted within glass racks (Rinkevich et al., 1993; Pancer et al., 1995; Rinkevich and Shapira, 1999). Fusions were established within 48–72 h after first ampulla-to-ampulla contacts. Chimeras were observed twice per week for the first two weeks and thereafter once per week. Basic maintenance conditions were as follows: 17 litre tanks standing seawater system, in which water was exchanged twice per week, at temperatures of 15°C, 20°C or 25°C. Tanks were held in a 15°C incubator room. Small heaters maintained the water temperature at 20°C and 25°C in the appropriate tanks. Water was changed using temperature-adjusted seawater-holding tanks. Data were gathered morphologically with respect to colony size (number of zooids at a specific time), disconnection between partners, path and directionality of morphological resorptions and sexual reproduction.

Tissue processing

Tissue samples were taken from zooids deprived of gonads. A longitudinal incision was made between the atrial and branchial siphons with a fine needle. Both edges of the incision were retracted with fine forceps, and zooids were lifted and removed with a fine needle and forceps. In sexually matured colonies, the male gonads were carefully removed with this needle, washed several times with sterile (0.22 μm) seawater and each was placed in a small Petri dish submerged in a small drop of seawater. The tip of a sharp needle was used to release the sperm, which were collected using a Pasteur pipette. Blood was collected by cutting (with industrial metal blades) the peripheral ampullae of colonies growing on glass slides that were wiped beforehand with soft towels and placed under a dissecting microscope. Released blood cells and haemolymph were collected using pulled glass micropipettes.

For microsatellite analyses, samples were placed, separately, into 1.5 ml vials containing 240 μl lysis buffer (Graham, 1978), homogenized and extracted with 240 μl phenol:chloroform:isoamylalcohol (25:24:1). DNA samples were precipitated in ethanol, resuspended in water and used for microsatellite analyses, as described previously (Pancer et al., 1995; Stoner et al., 1999). In the present study, all tissue samples were typed along with microsatellite PB-41 (Stoner and Weissman, 1996; Stoner et al., 1997). In the amplified fragment length polymorphism (AFLP) analyses, for each genotype, 5–12 specific loci (resolved bands on the sequencing gel) were first assigned as described previously (Rinkevich et al., 1998). Since AFLP loci can differ by as much as 10-fold in their ability to be amplified from low concentrations (Rinkevich et al., 1998), a semi-quantitative evaluation was established as follows: appearance of <20% of genotypic specific bands revealed only traces of the corresponding genotype's DNA.

Results and discussion

We studied consequences of somatic *vs* germ cell parasitism in specific chimeric combinations that grow simultaneously under the three different assigned water temperature regimen (15°C, 20°C, 25°C). Eight *Botryllus* genotypes were arranged in four combinations of Fu/HC compatible pairs, and 3–6 chimeras were established from each pair. Somatic and gametic tissues from the chimeras were harvested and genetically assayed by a PCR-based technique that used amplified unique microsatellite alleles (Pancer et al., 1995; Stoner and Weissman, 1996; Stoner et al., 1999; Ben-Shlomo et al., 2001) or by colony-specific AFLP fingerprints

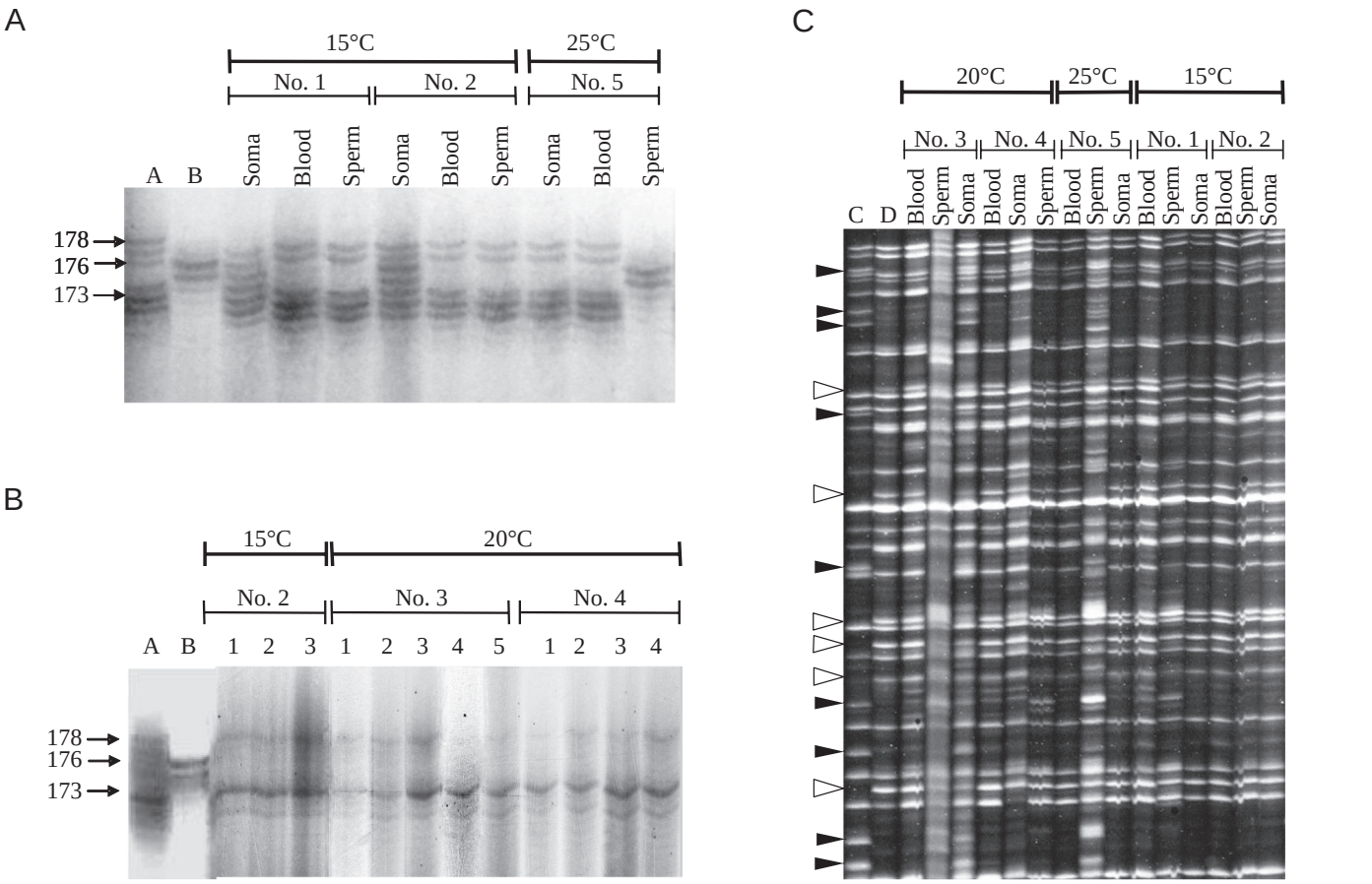

Fig. 1. The outcome of chimerism under variable water temperature regimen. (A) Representative cases for two water temperature regimen (15°C, 25°C) are depicted. Microsatellite profiles (locus PB-41) of three *Botryllus* chimeras (nos. 1, 2, 5; see Table 1), 8 months following allogeneic fusions of genotypic combination A *vs* B. Allele sizes of genotype A (alleles 173 bp and 178 bp) and genotype B (homozygotic on allele 176 bp) are marked on the left. The soma of both 15°C chimeras is typed as AB whereas at 25°C it is typed as A. (B) Typing of three offspring from chimera no. 2 (15°C; see Table 1) and nine offspring from chimeras 3 and 4 (20°C). All offspring reveal the A genotype only. (C) Part of the amplified fragment length polymorphism (AFLP) profiles of chimerical combinations (a negative, to better depict the AFLP loci, is marked as white bands). Left two lanes: genotypes C and D; specific bands are marked with arrowheads (filled for genotype C, open for genotype D). The panel depicts an example of chimeras under 15°C, 20°C and 25°C. The two soma samples at 20°C reveal cases of mixed chimeras. The three soma samples at 15°C and 25°C reveal cases in which frequencies of the other partner's alleles are <20%.

(Rinkevich et al., 1998). The competitive relationships between the interacting *Botryllus* genotypes were determined by the observed ranking of cell lineage parasitism.

In the first Fu/HC compatible combination, two partners (A and B) were categorized with the Fu/HC unlinked microsatellite locus PB-41 as: partner A = heterozygous alleles 173 bp and 178 bp; partner B = homozygous allele 176 bp (Fig. 1A). Six chimeras made of subclones from partners A and B were randomly split into three temperature regimen (15°C, 20°C, 25°C) groups. Chimeras were monitored for up to 10 months and were sampled (blood, gonads and total zooid tissue) four times during this period (Table 1; Fig. 1). In two cases (chimeras 1, 6; Table 1), one of the partners was morphologically resorbed within the first two months after chimeral establishment, and in four cases (chimeras 2–5), stable chimeras (Rinkevich and Weissman, 1987b) were established 8–10 months after allogeneic fusions. In a single case (chimera 6; Table 1), a chimeral death (Rinkevich and Weissman, 1987b) was recorded. In chimeras that had been maintained for 8 and 10 months after fusion at 15°C and 20°C temperature regimen, the zooidal soma consisted, in most cases, of both partners. On the other hand, in the 25°C treatment, genotype B disappeared from the soma of 2-month-old chimeras but appeared in the male gonads. Some of the outcomes for blood cells were consistent with the sperm analyses (Table 1), pointing to the existence of circulating germ cells in the blood (Sabbadin and Zaniolo, 1979). Male gonads in chimeras that had been grown at 15°C and 20°C revealed only the A genotype. This outcome was further confirmed by self-crosses made with both of the 20°C chimeras and one of the 15°C chimeras (chimera 2; Table 1) at age 8–10 months. These crosses were made to test the extent to which the microsatellite analysis of sperm was a good predictor of progeny production by gametes from both testes and

ovaries. Fifteen progenies were collected and tested; all revealed the A genotype only (Fig. 1B depicts 12 offspring).

The other three sets of *Botryllus* chimerical combinations were sampled 1.5–2 months after chimeral establishment (Table 2; Fig. 1C). Using AFLP fingerprinting profiles (Rinkevich et al., 1998), it was possible to elucidate, independently, the sensitivity of each specific AFLP locus in a mixture of genotypes and to define cases where only traces of the genotypic DNA of interest are found (Table 2). In two of the three genotypic combinations (C–D and G–H), the fingerprints of the chimeric soma differed between those grown in ambient temperature (20°C) and most of those grown in the two extreme maintenance conditions (15°C, 25°C; Table 2; Fig. 1C). In chimeric combination C–D, both genotypes appeared in the two 20°C chimeras while only genotype D was recorded in the soma of two of the chimeras grown under extreme temperatures (chimeras 1 and 5) and only traces were recorded in the third chimera (chimera 2). In zooidal soma analyses of combination G–H, genotype H (but not G) was expressed only in 20°C chimeras, as opposed to the chimeras grown at extreme temperatures, where both genotypes appeared simultaneously. Sperm identity was consistent and

Table 1. *Germ and somatic cell parasitism in the first set of six chimeras developed from fusion between subclones of the compatible genotypes A and B*

Sampled tissue	Chimeral age (months)	15°C				20°C				25°C			
		Chimera 1		Chimera 2		Chimera 3		Chimera 4		Chimera 5		Chimera 6	
		A-side	B-side	A-side	B-side	A-side	B-side	A-side	B-side	A-side	B-side	A-side	B-side
Soma	2	Resorbed	A, B	A	A, B	A, B	A	A	A, B	A	A	A	Resorbed
	5	Resorbed	A, B	A	A, B	A	A, B	A	A, B	A	A		cd
	8	Resorbed	A, B	sc (A, B)		A	A, B	sc (A, B)		sc (A)			cd
	10	Resorbed	A, B	na		sc (A)		sc (A, B)		sc (A)			cd
Sperm	2	Resorbed	A	A	A	A	A	A	A	A, B	B	AB	Resorbed
	5	Resorbed	A	A	A	A	A	A	A	B	B		cd
	8	Resorbed	A	sc (A)		A	A	sc (A)		sc (B)			cd
	10	Resorbed	–	sc (–)		sc (A)		sc (A)		na			cd
Blood	2	Resorbed	A	A	A, B	A, B	A	A	A	A	A	A	Resorbed
	5	Resorbed	A	A	A, B	A	A	A	A	A	A		cd
	8	Resorbed	A	sc (A)		A	A	sc (A)		sc (A)			cd
	10	Resorbed	A, B	sc (A)		sc (A)		sc (A)		sc (A, B)			cd

A- and B-side refer to the original genotypes within the chimera where partners are morphologically distinguishable. sc refers to cases of stable chimerism (Rinkevich and Weissman, 1987b), the establishment of a long-lived chimera where partners are morphologically indistinguishable from each other. Morphological resorption (Rinkevich and Weissman, 1987b; Weissman et al., 1990; Rinkevich et al., 1993) of a specific partner is marked as 'resorbed'. cd refers to chimeral death (Rinkevich and Weissman, 1987b). (–), male gonads were not found; na – no available data.

Table 2. *Germ and somatic cell parasitism in 1.5–2-month-old chimeras comprising three different combinations of compatible* Botryllus *genotypes (termed C–D, E–F, G–H)*

Chimeral combination (X *vs* Z)	Sampled tissue	15°C				20°C				25°C	
		Chimera 1		Chimera 2		Chimera 3		Chimera 4		Chimera 5	
		X-side	Z-side	X-side	Z-side	X-side	Z-side	X-side	Z-side	X-side	Z-side
C–D	Soma	sc (D)		sc (C*, D)		Resorbed	C, D	Resorbed	C, D	Resorbed	D
	Sperm	sc (C*, D)		sc (C*, D)		Resorbed	C, D	Resorbed	C*, D	Resorbed	C, D
	Blood	sc (C, D)		sc (C*, D)		Resorbed	C, D	Resorbed	C, D	Resorbed	D
E–F	Soma	sc (E, F)		nd		E	E, F	nd		sc (E, F)	
	Sperm	sc (E)				E	E			sc (E)	
	Blood	sc (E)				E	E, F			sc (E, F)	
G–H	Soma	sc (G, H)		nd		Resorbed	H	G, H	H	Resorbed	G, H
	Sperm	sc (G, H)				Resorbed	G, H	G, H	G, H	Resorbed	G, H
	Blood	sc (G, H)				Resorbed	G, H	G, H	G, H	Resorbed	G, H

X- and Z-side refer to the original genotypes within the chimeras. For abbreviations and definition of terms, refer to Table 1. nd, not done; *, less than 20% of specific alleles of the corresponding genotypes appeared in the AFLP analysis; traces of the corresponding DNA signature.

unchanged at all temperature regimens and in all three chimeral combinations (12 chimeras). Only in combination E–F did soma fingerprints appear to be the same at all temperatures.

Thus, this study shows that the somatic constituent of chimeric *Botryllus* entities is a plastic event. The competitive (parasitic) relationships of interacting genotypes, under variable environmental conditions (seawater temperature was used here as an example of an environmental parameter), are frequently altered. This outcome contradicts the state of germ cell parasitism, where almost no alterations were recorded (Tables 1, 2; Stoner et al., 1999) and where directionality was probably dictated genetically by primitive germ cell lineage hierarchy (Stoner et al., 1999).

Natural chimerism is a common phenomenon, not only in the *Botryllus* system. Chimeras from a variety of protists, plants and animals are documented in nature (Buss, 1982). Several studies (Buss, 1982; Rinkevich and Weissman, 1987b, 1992; Rinkevich and Shapira, 1999) have dealt with the evolutionary significance of chimerism by evaluating fitness costs and benefits of the chimeral entity, relative to the state of genetically homogeneous individuals. Of all the different suggested classes of benefits for chimerism (none has been recorded in organisms more developed than primitive slime molds and algae; Rinkevich and Shapira, 1999), one has focused on the possible synergistic impact of both partners' genetic constituents on the chimeric fitness (Rinkevich and Weissman, 1987a; Rinkevich and Shapira, 1999). It was suggested (Buss, 1982) that since a chimera has a greater store of genetic variability, and hence a wider range of effective physiological qualities and characteristics, this organismic state may tolerate a greater range of environmental variation than the organismic state of genetically homogeneous entity.

Within chimeras of *Botryllus schlosseri*, parasitic germ lines hitchhike and pass throughout successive generations without being visible to natural selection forces (Pancer et al., 1995; Stoner et al., 1999; Rinkevich, 2002, in press). Hitchhiking onto the soma of positively selected genotypes provides the parasitic forms with the inevitable advantage of establishing new progenies. However, this may eventually turn into a Pyrrhic victory (Rinkevich, 2002, in press) by causing possible development of super-parasitic entities, specialized in allogeneic invasion and germ cell parasitism. Three evolutionary selected mechanisms (diversification of fusibility allele repertoire, the establishment of multichimeric entities and the induction of programmed life spans) reduce opportunities for parasitic forms to hitchhike to a high frequency with selected genotypes and may shape more benign germ cell parasitic forms that share overlapping future expectations with their hosts (Rinkevich, 2002). These benign forms are expected to contribute cells for somatic functions, forming entities with fitnesses that depend on the combined genomic fitness of the partners, as seen in the present study. It should also be taken into consideration that, while intraspecific interactions within chimeras go on, larger botryllid chimeras successfully control feeding substrates. This should effectively prevent colonization of that surface area by other competitive species and/or may increase interspecific competitive abilities (Rinkevich and Shapira, 1999).

The apparent synergism for fine-tuning a plastic combination of the genetic components in *Botryllus* chimerical soma (to better fit adverse environmental conditions) may represent a typical form of a green-beard (Dawkins, 1976) allelism on historecognition elements. All components of a green-beard effect (Dawkins, 1976; Haig, 1996; Keller and Ross, 1998; Riley and Gordon, 1999; Queller et al., 2003) – a detectable phenotypic feature (the Fu/HC allele), the ability to recognize this feature and the ability to respond [in the *Botryllus* system: three sets of different responses are expressed towards genotypes possessing or not possessing this feature: fusion and rejection (Oka and Watanabe, 1957; Sabbadin, 1982; Scofield et al., 1982), allogeneic resorption (Rinkevich and Weissman, 1992; Rinkevich et al., 1993), somatic and germ cell parasitism (Rinkevich and Weissman, 1987a; Pancer et al., 1995; Stoner and Weissman, 1996; Magor et al., 1999; Stoner et al., 1999; Rinkevich, 2002)] – are present in *Botryllus* chimeras and are mediated by a single green-beard allorecognition haplotype.

In the wild, any single *Botryllus* population at any specific time represents unprecedented extensive polymorphism of Fu/HC alleles (up to several hundred; Rinkevich et al., 1995), a phenomenon that may reduce the chances of forming natural chimeras. However, preferential gregarious settlement of Fu/HC compatible oozooids in nature (Grosberg and Quinn, 1986) results in compatible allogeneic contacts and high percentages of *Botryllus* chimeras (Ben-Shlomo et al., 2001). In the case of a natural chimera (i.e. carrying A–B *vs* A–C allorecognition alleles), the shared selfish allorecognition 'A' allele will always attain its 50% share in the germ line, regardless of any germ line hierarchical combination (i.e. A–B or A–C colony is the winner). At the same time, chimeric fitness is synergistically fine-tuned by all its genetic constituents to fit changes in environmental conditions (such as seawater temperature). This cooperation on the somatic level clearly benefits the chimera as compared with genetically homogenous *Botryllus* colonies. In the *Botryllus* system, therefore, a single allorecognition green-beard allelism directly assesses allelic kinship and simultaneously modifies two 'conflicting' responses within a whole chimeric entity (germ cell parasitism *vs* somatic cell cooperation). It not only 'ensures' its proportional transmission to subsequent generations but also enhances fitness of the entity (the vehicle; sensu Dawkins, 1976) that houses the germ line. The genotype that wins the soma is, thus, neither sporadic nor random, and the synergistic expression of somatic constituents is dictated by selfish genetic elements working through green-beard effects. It is also interesting to note that the green-beard gene in the red fire ant system (Keller and Ross, 1998) is also hallmarked by widespread polymorphism.

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