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A Colonial Invertebrate Species that Displays a Hierarchy of Allorecognition Responses

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Abstract. When two colonies of the compound ascidian *Botryllus schlosseri* come into contact with each other, they either fuse or reject. This allorecognition is governed genetically by multiple, codominantly expressed alleles at a single, highly polymorphic haplotype called the fusibility/histocompatibility (Fu/HC) locus. Two colonies sharing one or both alleles at this locus can fuse via their extracorporeal tunic blood vessels. Thereafter, in laboratory studies, one partner in the chimera is usually resorbed. The direction of resorption appears to be inherited, as multiple subclones of asexually-derived individuals from colony A always resorb paired subclones from colony B, independent of laboratory conditions or colony age.

We established 121 pairs of chimeric partners by fusions of relatives from four generations within a pedigree, all homozygotes (AA line) at their Fu/HC haplotype. This was carried out by self- and defined-crosses done in the laboratory on two outbred founder colonies (each AB at the fusibility locus) which were taken from the field. We found that the resorption phenomenon is characterized by a linear hierarchy within each generation of colonies, which is expressed by the existence of at least 5 intermediate groups. However, the time for resorption did not correlate with the position in the hierarchy. Analysis of resorption hierarchies between different generations revealed that mother colonies always resorbed their self crossed offspring. More interesting, colonies low in the hierarchy within a specific generation reproducibly resorbed the self crossed offspring of a superior kin. Chimeras between defined-crossed offspring of different generations revealed nontransitive types of hierarchies which were correlated with the relative position of each colony in the linear hierarchy established for the colonies within each generation. We propose that colony resorption in colonial botryllid ascidians is controlled by several allorecognition elements that determine a resorption hierarchy.

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Introduction

The compound ascidian *Botryllus schlosseri* is a cosmopolitan metazoan of the subfamily Botryllinae, inhabiting shallow waters abundantly throughout the world, especially in harbors. Adults are made of several to hundreds of genetically identical units (each one is called a zooid), which are grouped in typical star-shape structures (systems), and are embedded within a translucent, gelatinous matrix, the tunic. All systems, as well as zooids within a single system, are interconnected to each other by a network of blood vessels, which bear spherical to elongate termini (called ampullae) near the surface of the tunic, between the systems and around the borders of the colony.

Colonies originate from a sexually produced tadpole larva. After a short free-swimming phase, the larva attaches to the substrate, resorbs its tail components, and undergoes metamorphosis to a founder individual, the oozooid. Oozooids grow by a typical asexual budding (blastogenesis), a cyclic phenomenon: *i.e.,* every six to seven days all parental zooids in a colony are synchronously resorbed and a new generation of buds matures to the zooid stage. Each adult zooid can give rise to one to four buds per generation (Boyd *et al.,* 1986). At the end of each blastogenic cycle, all of the zooids of one generation are resorbed. This event, called "takeover", is characterized by a massive phagocytosis, and is completed within 24 h (Harp *et al.,* 1988). During takeover, some

buds can be resorbed together with their parent, and thus the total number of zooids in a colony can either increase, remain constant, or decrease.

Allorecognition by complex metazoans can result in a variety of manifestations of histoincompatibility. The most detailed information concerning the genes that encode histocompatibility determinants, and the cells and receptors that recognize these determinants, comes from studies of mouse and man. In these species, as in all vertebrates tested, a single, highly polymorphic haplotype of linked histocompatibility genes—called the major histocompatibility complex (MHC)—is the primary determinant of rapid graft rejection mediated by allospecific T lymphocytes (Klein, 1986). However, when grafts are exchanged between individuals that share both MHC alleles, but are otherwise genetically distinct, a T cell-mediated graft rejection occurs, albeit at a slower pace than when MHC mismatched grafts are applied (Bevan, 1975; Loveland and Simpson, 1986). The genes encoding these determinants—almost certainly proteolytically derived peptides that are embedded in an MHC protein cleft (Bjorkman *et al.*, 1987) and thereby presented to MHC-restricted, allospecific T cells—are called minor histocompatibility (H) antigens. Genetic studies indicate that, with any two distinct mouse strains, the combinatorial association of minor H antigenic peptides with highly polymorphic MHC genes results in the elaboration of tens of alloantigens, encoded by genes residing on all (or nearly all) chromosomes (Bailey, 1978; Johnson, 1981; Zaleski *et al.*, 1983; Klein, 1986).

Allorecognition is also genetically defined in botryllid ascidians, as manifested by several distinct phenomena. Colonies that meet naturally in the wild or under laboratory conditions, very soon after initial contact, either fuse their adjacent extracorporeal tunic blood vessels to form a natural vascular parabiont (cytomictical chimera), or undergo a rapid series of inflammatory phenomena culminating in rejection, and the formation of a fibrous barrier between them (Scofield *et al.*, 1982; Taneda *et al.*, 1985; and literature therein). This colony specificity phenomenon is determined by a single, highly polymorphic fusibility/histocompatibility (Fu/HC) locus (or haplotype). In laboratory experiments, if two individuals sharing a single allele (or both alleles) at this locus fuse at some later time, the genetic colonial descendants (zooids) from one partner in the chimera are all resorbed by massive phagocytosis, leaving the genetic descendants of the other colony intact (Rinkevich and Weissman, 1987a, b, 1989, 1992; Weissman *et al.*, 1990). This phenomenon, called colony resorption, typically occurs at the end of a blastogenic cycle, when the new generation of zooids fails to develop to the mature phase, or does not develop at all (Rinkevich and Weissman, 1987a). Moreover, colony resorption also appears to be controlled genetically, insofar as all subclones from colony A will resorb all subclones from colony B, whatever the laboratory microenvironment in which the subclones are reared. The microenvironmental variables tested include different temperature regimens, types of food, running *versus* standing seawater systems, and the number of asexual generations separating the founder of the colony and the particular subclone tested (Taneda *et al.*, 1985; Rinkevich and Weissman, 1987a, b; 1989, 1992).

Here we provide evidence that the resorption of nonidentical partners in a chimera of the colonial tunicate *Botryllus schlosseri*, from Monterey, California, is at least partly controlled by additional recognition elements unlinked to the Fu/HC haplotype of this species. These colony resorption responses have a hierarchial property in that dominant, intermediate, and inferior responses are maintained through many asexual cycles, independent of environment.

Materials and Methods

Animals

Botryllus schlosseri colonies were kept in 17 l glass tanks supplied with 50–70 ml/min of filtered seawater that had been preconditioned in a large plastic holding tank containing 235 l. The water in each glass tank was aerated with an airstone and maintained at 18°C (with a 50 W aquarium heater). The animals were fed daily with 0.55 gr/tank of powdered Similac (a milk substitute) and subjected to a 14:10 hour light:dark regimen. Colonies were grown on 5 × 7.5 cm glass slides, one colony per slide, and kept vertically in slots of glass staining racks, within the glass tanks.

Colony allorecognition assays (CAAs)

Only large, healthy colonies were used. Small pieces of growing edges (subclones, ramets; containing one to three systems each) were isolated by careful dissection from each colony without injuring their surrounding ampullae. Subclones from two genetically distinct colonies were paired on glass slides, so that they contacted one another with their extended ampullae. They were fastened to the slides by placing them in a moisture chamber for 30–45 min before transferring them into the 17 l running seawater tanks. All of the paired subclones were in the range of size differences which does not affect directionality in the resorption phenomenon (Rinkevich and Weissman, 1987a) and were observed under the binocular stereomicroscope every day until they formed a well-organized chimera (Rinkevich and Weissman, 1987a, 1988, 1989). Thereafter they were observed 2 to 3 times a week. During the observations, colonies were cleaned with soft, small brushes to remove debris, fouling organisms, and trapped food particles. The substrate around the colonies was carefully cleaned with small pieces of razor blade.

Experimental procedures

To analyze further the possible role of heritable elements in colony resorption other than the Fu/HC locus, we carried out self and defined crosses from one of the Fu/HC homozygotic strains that are raised in our laboratory (Boyd *et al.*, 1986), the Monterey AA haplotype. The genealogical tree relevant to the present study is illustrated in Figure 1, which shows the pedigree of four successive generations.

Two outbred colonies, each AB at the fusibility locus, were taken from the Monterey marina and served as the founders of this strain. The fusibility of each offspring was determined through CAAs by removing subclones from the main colony and placing them with subclones from other colonies (Rinkevich and Weissman, 1988). The present study focuses on the AA strain. Five healthy colonies of the AA strain served as parent colonies for the next generation offspring by self-crosses and defined-crosses. Self-crosses result from the fertilization of the eggs of one subclone (ramet) from a specific colony with the sperm from another ramet from the same colony, in the absence of competing sperm. The fastest growing and healthiest offspring colonies were either used in the experiments, with subclones being taken for fusibility assays (Rinkevich and Weissman, 1988), or they were used to produce the next generation of the AA line. Subclones from the indicated colonies were isolated, placed side-by-side on colony fusion plates, as described previously, and observed closely to record colony resorption (Rinkevich and Weissman, 1987a).

Results

Colony resorption hierarchies within different generations

Thirteen chimeras were derived by fusion between the four surviving AA colonies of generation II (Fig. 2a). In five cases (38.5%), the partners within the chimeras disconnected before resorption was complete (average time for disconnection 64 ± 21 days). Colony P111R was involved in four of these cases.

The resorption between this group of colonies (average time for resorption 48 ± 23 days) is characterized by a linear hierarchy. In this hierarchy, colony P21R is the "superior" partner, in that in the absence of dissociation, it resorbs all other colonies of this generation. In contrast, colony P94R is the "inferior" partner, as it is resorbed by the other three members of this generation (Fig. 2a). The time for resorption does not correlate with position in the hierarchy; subclones from the inferior P94R colony were resorbed by subclones from the superior P21R colony at the same, or even a slower pace than between the subclones of the two intermediate members (P32R and P111R; Fig. 2a). During the phase of chimerism, from the day of fusion up to the day of complete resorption, the superior partners increased in zooid numbers by asexual budding by 35–300%.

Eleven chimeras were derived by fusion between the self-crossed offspring of generation III themselves, the offspring of P111R and P21R (Fig. 1 and the two diagrams on the left of Fig. 2b). Out of 11 cases, one chimera died and one disconnected. Two hierarchies emerged when the resorption patterns were observed. Another hierarchy was

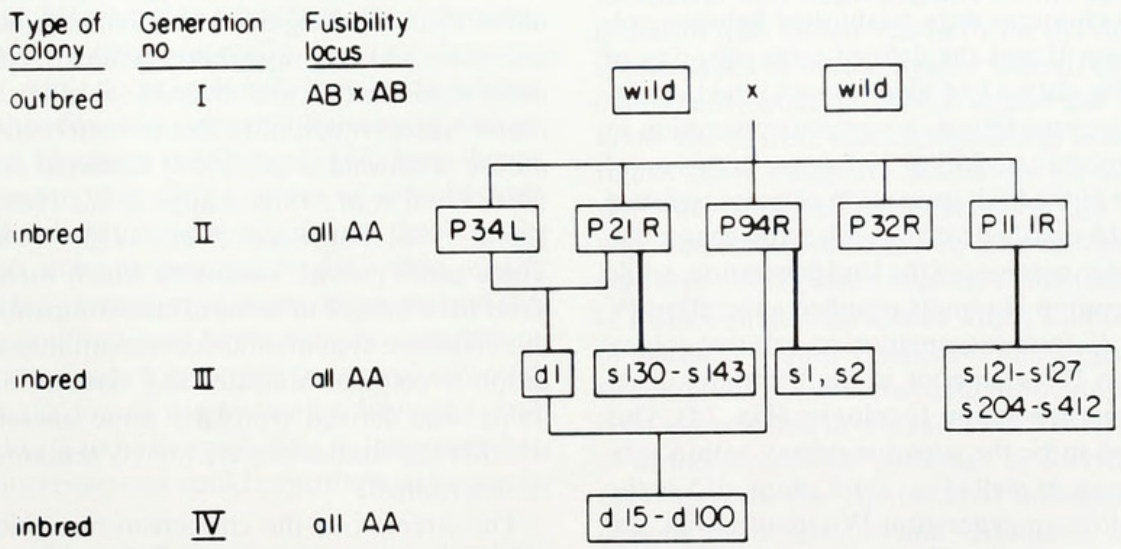

Figure 1. The pedigree of four successive generations of Monterey *Botryllus schlosseri* used in this study. Two independent outbred colonies, typed as Fu/HC AB, were designated generation I and were mated to give rise to generation II of Fu/HC AA colonies. Colonies of generations III and IV (all AA on the Fu/HC haplotype) are designated in running numbers with a preface letter which denotes the type of crossing: d = defined crossed colony; s = self-crossed colony. Heavy lines represent the pedigree of self-crossed colonies.

established by analyzing the outcome of 20 CAAs carried out between the defined-cross offspring of generation IV (right diagram, Fig. 2b). In this set, three chimeras disconnected and one died. The average time for resorption between self-crossed offspring (20 ± 18 days) was significantly shorter than the average time for resorption between defined-cross offspring (69 ± 43 days; $P < 0.001$, t test). As before (Fig. 2a), a linear hierarchy emerged from the analyses of the interactions within generations III and IV (Fig. 2b), and there was no correlation between the time to resorption and level in the hierarchy. The results illustrated in Fig. 2b also indicate the existence of at least five intermediate levels in the resorption hierarchy.

Colony resorption hierarchies between different generations

Fifty chimeras were generated between colonies of generation II and their self-crossed offspring of generation III, by assaying pairs of similar-sized ramets between parents vs. its own offspring, and pairs of generation II colonies vs. offspring of a kin colony (Fig. 2c). Death of the chimera or a disconnection was recorded in eight (16%) of the cases. In the other 42 cases, no matter how large the colonies were at the time of fusion, generation II ramets resorb generation III ramets (average time 42.4 ± 25.9 days). This result was obtained either when parent-offspring chimeras or chimeras of a generation II colony vs. self-crossed offspring from a kin were done. Most interestingly, generation II inferior colonies in the resorption hierarchy reproducibly resorbed the self-crossed offspring of a superior kin, such as the cases of P94R, P111R and P32R vs. offspring of P21R (Fig. 2c). In addition, similar to the cases shown in Fig. 2b, a resorption hierarchy is also found between the self-crossed offspring of colony P94R (Fig. 2c).

Twenty-seven chimeras were established between colonies of generation II and the defined-cross offspring of generation IV (Fig. 2d); 12 of them (44.4%) died or disconnected. The average time to a complete resorption in the other 15 chimeras was 56.1 ± 26.8 days. In this set of experiments, five of the IVth generation colonies resorbed and two (marked by dashed arrows with arrowheads; Fig. 2d) started to resorb colonies of the IInd generation, while in 10 cases, generation II ramets resorbed generation IV ramets (Fig. 2d). A closer examination reveals that colony d20 of generation IV is superior in the hierarchy of resorption to all four generation II colonies (Fig. 2d). This colony was found to be the superior colony within generation IV offspring as well (Fig. 2b). Colony d15 is the most inferior colony in generation IV colonies (Fig. 2b) and is resorbed by generation II colonies as well (Fig. 2d). Colony P94R (the inferior colony of generation II, Fig. 2a) was resorbed in all cases where a successful chimera was followed with generation IV colonies (Fig. 2d), whereas colony P21R (the superior colony of generation II, Fig. 2a) was inferior in the resorption hierarchy only to colony d20 of generation IV colonies (Fig. 2d).

Discussion

The colony resorption phenomenon is limited to individuals that are not genetically identical, since two genetically identical isolates from a single parent colony will meet, fuse, and give rise by asexual budding to growing colonies (Rinkevich and Weissman, 1987a). The studies of tunicate colony resorption reported here and previously (Rinkevich and Weissman, 1987a, b, 1989, 1990, 1992; Weissman *et al.*, 1990) reveal a unique hierarchical organization in *Botryllus schlosseri* chimeras. Fusion in the laboratory between two colonies that are Fu/HC homozygotes (*i.e.*, AA *vs.* AA), but that are not genetically identical or Fu/HC heterozygotes (*i.e.*, any combination of AX *vs.* AY), leads to colony resorption. All ramets from a superior colony will resorb fused ramets of an inferior colony, implying that other resorption elements, most likely encoded at other genetic loci, are responsible. Because the mother colony ramets usually resorb ramets from their more inbred progeny ramets, a simple hierarchy is difficult to explain. Perhaps heterozygotes at these loci are more likely to resorb homozygotes; or perhaps the general "fitness" of progeny of a self-cross allows a weaker resorption locus to emerge superior in colony resorption. The second suggestion is much less plausible, since the "performance" of the studied self-crossed homozygotes (either in survivorship, reproductive outputs, or growth rates) in our laboratory conditions (Boyd *et al.*, 1986) was as good if not better than that of the control, more heterozygotic colonies (Ishizuka and Rinkevich, in prep.).

If the above view is correct, then there may be positive selection for tunicates heterozygous for several allorecognition loci. Allorecognition in colonial tunicates therefore represents a histocompatibility system of considerable genetic sophistication and diversity, rivalling the MHC and minor histocompatibility loci in vertebrates, such as the mouse (Eichwald *et al.*, 1958; Eichwald and Weissman, 1966; Graff *et al.*, 1966; Lappe *et al.*, 1969; Graff, 1978; Klein, 1986; Townsend *et al.*, 1986; Weissman, 1988). These genes provide means by which each individual is likely to be unique in terms of histocompatibility. Whether this elaborate system of histocompatibility and allorecognition in colonial tunicates and vertebrate histocompatibility was derived from the same ancestral genes, or whether the similarities are merely semantic, remains to be determined.

The strength of the chimerism-resorption system, as defined by the period needed for a complete resorption, is extremely variable, from one week to five months (Fig. 2). At least part of the variability in the time for resorption may have been caused by experimental manipulations (such as the length and the structure of the fusion areas,

the numbers of anastomizing blood vessels, etc.; Rinkevich and Weissman, 1989), rather than by genetic factors. A similar characteristic of variability is also found in the murine minor histocompatibility loci (Klein, 1986), where there appear to be at least 50–100 distinct histocompatibility loci, with histocompatibility genes scattered throughout virtually every chromosome.

That the diversity of genetic types in the MHC of the vertebrates is the result of past selection for resistance to different diseases is a persistent speculation (Black and Salzano, 1981; Robertson, 1982; Hedrick and Thomson, 1983). This suggests that an animal heterozygous for the MHC antigens may respond much more efficiently to a wider range of pathogens than a homozygote, and that polymorphism may be maintained by heterozygous advantage or heterosis. In contrast, Flaherty (1988) has proposed that the high degree of mammalian MHC polymorphism has been established and maintained because of a constant, but promiscuous, heterozygote advantage. That is, no particular MHC allele has selective advantage; rather, all heterozygotes are favored over all homozygotes. This promiscuous heterozygote advantage would lead to a large allelic pool, because rare alleles would be favored and lead to more heterozygotes in the population. Other authors have proposed previously that heterozygote advantage might be involved in mammalian MHC evolution (Galton, 1967; Robertson, 1982; Hughes and Nei, 1988).

In general, heterosis refers to allelic combinations in which a heterozygote (*i.e.*, AB) has greater fitness than either of its homozygotes (AA or BB) (reviewed in Grosberg, 1988). We therefore postulate that the phenomena of fusion and chimeric resorption of Fu/HC compatible botryllid ascidians may provide substantial fitness benefits. If the dominant resorption of offspring settling near, and fusing with, maternal colonies (Rinkevich and Weissman, 1987b) is due to heterozygote advantage, then chimeric resorption linked to chromosomally dispersed "resorption" loci may serve to promote chromosomal heterogeneity, and therefore may provide substantial fitness benefits. Indeed, Grosberg and Quinn (1986) have shown in field experiments that sibling larvae of *B. schlosseri* settle non-randomly in aggregations; siblings that cosettle in these clusters share at least one Fu/HC allele, which should lead to the formation of more chimeras, and the resorption of a significant part of the *Botryllus* population. If colony resorption occurs in nature, the survivors would not only be at the top of the resorption hierarchy, but also are more likely to be heterozygotic at the resorption loci; thus colony resorption could contribute to heterosis benefits.

Four classes of benefits: genetic variability, developmental synergism, mate location, and size-specific ecological processes, have been attributed to the chimeric state (Buss, 1982). Despite these proposed benefits and those that heterosis might engender, however, there are potential costs to fusion, as well as mechanisms that could prevent this advantage from being passed on. The result of mixing genetically distant cell lines could result in germ-cell or somatic-cell parasitism when one member of the chimera could parasitize the other (Buss, 1982; Rinkevich and Weissman, 1987c). It has been reported (Sabbadin and Zaniolo, 1979; Rinkevich and Weissman, 1987c) that short-term chimeras of *Botryllus* colonies result in free exchange of germ cells, and that one individual in the chimera may gain a disproportionate share of gametic output even after the separation between both members in the chimera (Sabbadin and Zaniolo, 1979).

Thus, while the "heterosis" concept favors fusion as a pathway for selection against the less vigorous partner (including its soma and the germ line), the "somatic cell parasitism" concept, if reproducible and functional in nature, could lead to the survival of blood cells (especially the totipotent stem cells) from the resorbed partner, in effect cancelling the advantages gained by heterozygote-dominated resorption. In chimeras, therefore, several contradicting processes might play a role in colony survival until complete resorption occurs. However, successful domination of a feeding surface by chimeras should effectively prevent colonization of that surface by other competitor species. Whether the resorption "winner" or "loser" gives rise to the germ line that will successfully give rise to offspring is a critical evolutionary issue. Clearly, much more effort will be needed to elucidate the processes occurring within *Botryllus* chimeras in the field.

The present and previous studies on tunicate resorption (Rinkevich and Weissman, 1987a, b, 1989, 1990, 1992) used non-inbred *Botryllus* colonies. In order to study the individual histocompatibility gene and proteins of the mouse MHC, it was necessary to deal with the problems of multiple histocompatibility loci, extensive H-2 polymorphism and heterozygosity of the H-2 genes. These obstacles were circumvented by the development of three special types of mouse strains: inbred, congenic, and recombinant congenic, which is also the reason why we know the murine histocompatibility system better than in any other vertebrate. Studies on the colonial tunicate *Botryllus schlosseri* have revealed the Fu/HC system which resembles in some ways the vertebrate MHC (Scofield *et al.*, 1982), and a multilevel hierarchial organization of histocompatibility alleles which lead to the resorption of partners within chimeras (this study). The genetic structure of the protochordate histocompatibility system is only now being slowly revealed. Therefore, the analysis of histocompatibility pathways in *Botryllus* inbred lines may elucidate the sophisticated immunological systems of both protochordates and vertebrates, and their evolution.

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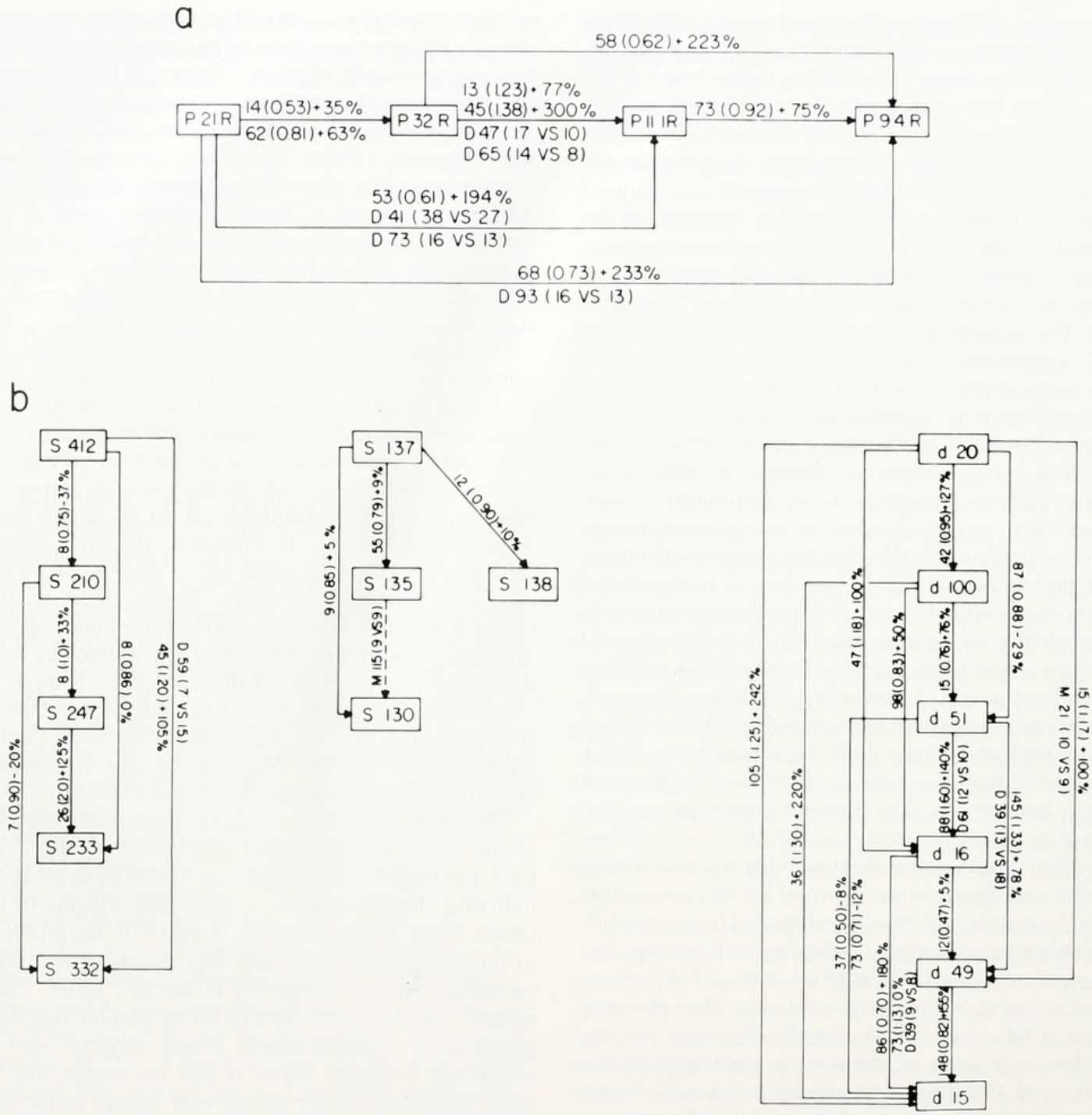
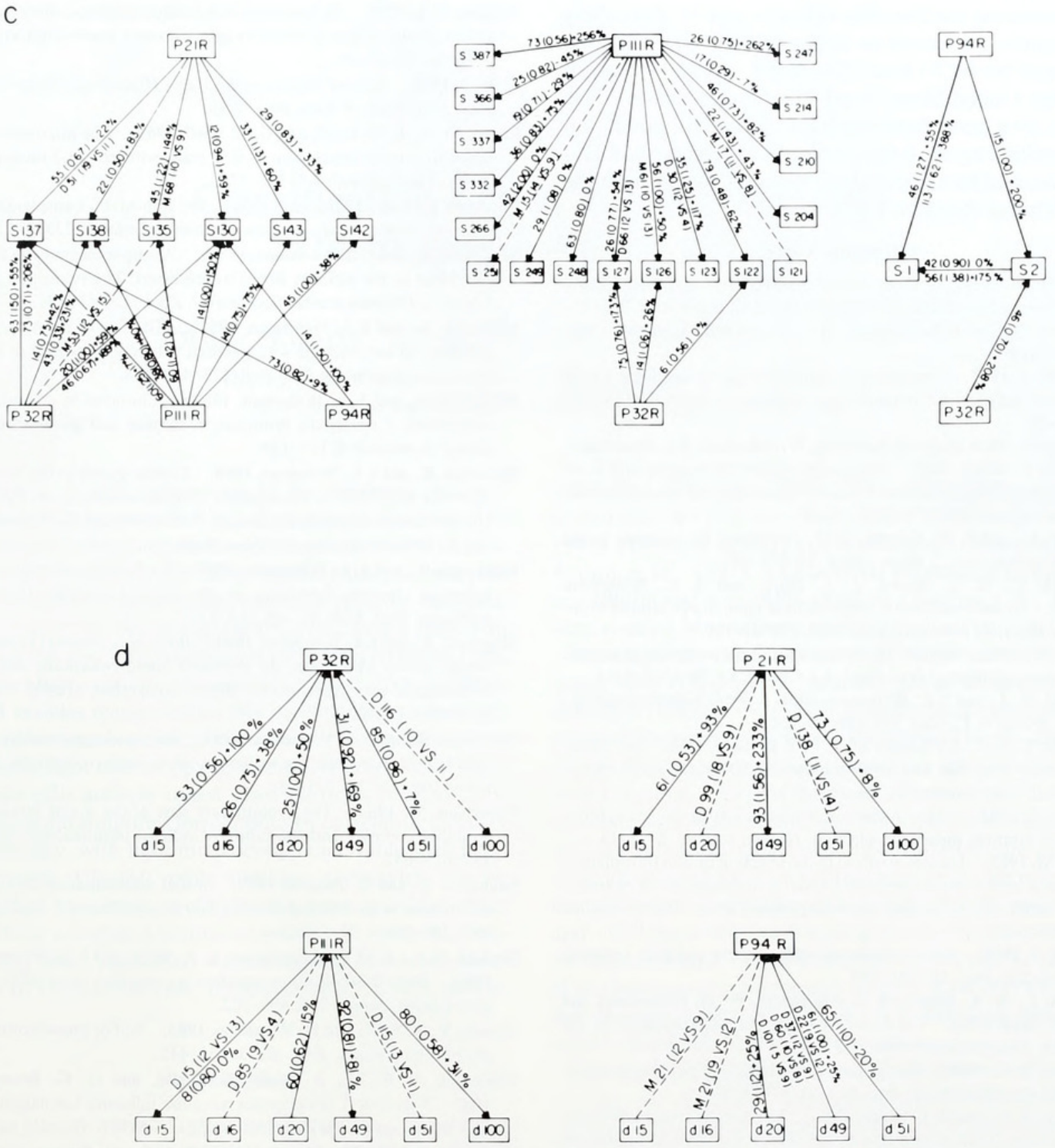


Figure 2. a. A hierarchy in the resorption between four colonies of generation II (refer to Fig. 1). The arrowheads point to the inferior partner. Numbers printed along the arrows refer to, respectively: days for complete resorption, zooid ratio (in parentheses, calculated as: the number of zooids in the inferior/superior partners on the day of fusion), percent increase or decrease of zooids in the superior partner from the day of fusion until a complete resorption of the inferior partner. The letter D refers to a case where a disconnection between the partners in a specific chimera occurs. In that case, the numbers along the arrow indicate: days from fusion to disconnection, number of zooids of the left colony on the day of fusion vs. the number of zooids of the right colony (in parentheses). Disconnection between the partners within a *Botryllus* chimera is one of the variations in the outcome to chimera formation, resulting from unsuccessful fusion (Rinkevich and Weissman, 1989), reciprocal resorption (Rinkevich and Weissman, 1987a, 1989), or from a retreat growth phenomenon (Rinkevich and Weissman, 1988). These physiological-genetic-morphological parameters may lead to early separation between the partners before a complete resorption of the inferior partners in a chimera is obtained (Rinkevich and Weissman, 1988, 1989). The hierarchial tendency in the resorption phenomenon is, in most of the cases, already observed before separation between the candidates cancels this reaction. However, we did not count disconnection even when figuring hierarchy. In each such case, at least one additional chimera, where full resorption was accomplished, is assayed. It should be noted, however, that the incompleted results of disconnections are always in agreement with the results where resorption is completed. Subclone sizes may alter the direction of chimera resorption. However, this occurs only when



the subordinate partner is much larger than the winner. All subclones used in the present study were matched to pairs with zooid ratios, below that may reverse the direction of resorption. b. Hierarchy in the resorption within the self-crossed offspring of generation III (the two left schemes) and within the defined-cross offspring of generation IV (refer to Fig. 1). The letter M refers to a case where the chimera dies. In that case, the numbers along the arrow indicate: days from fusion until the death of the chimera, number of zooids of the left and the right partners, respectively, on the day of fusion (in parentheses). A dashed arrow with an arrowhead points to a case where the direction of resorption is evident; however, the chimera either died or the partners disconnected before the resorption was completed. Additional subclones for doing new chimeras were absent; therefore, the hierarchy in resorption was not fully determined. c. Hierarchy in the resorption between generation II colonies and the self-crossed offspring of generation III. A dashed arrow without an arrowhead indicates a case where hierarchy is not evident before interactions of the partners in a specific chimera were interrupted by chimera mortality or disconnection. In those cases, no more chimeras were done because of the lack of additional subclones. d. Hierarchy in the resorption between generation II colonies and the defined-cross offspring of generation IV. Dashed arrow with arrowhead indicates two cases where hierarchy became evident before the interactions in the CAAs were interrupted by disconnection.

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