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Transmission Genetics of Allorecognition in *Hydractinia symbiolongicarpus* (Cnidaria: Hydrozoa)

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ABSTRACT

Allorecognition is ubiquitous, or nearly so, amongst colonial invertebrates. Despite the prominent role that such phenomena have played both in evolutionary theory and in speculations on the origin of the vertebrate immune system, unambiguous data on the transmission genetics of fusibility (*i.e.*, the ability of two individuals to fuse upon tissue contact) is lacking for any metazoan outside of the phylum Chordata. We have developed lines of the hydroid *Hydractinia symbiolongicarpus* (Phylum Cnidaria) inbred for fusibility and here report results of breeding experiments establishing that fusibility segregates as expected for a single locus with codominantly expressed alleles, with one shared allele producing a fusible phenotype. Surveys of fusibility in field populations and additional breeding experiments indicate the presence of an extensive allele series.

INVERTEBRATE allorecognition, first demonstrated by Bancroft (1903) in a colonial ascidian (Phylum Chordata), was recognized shortly thereafter by observations of similar phenomena in the near basal eumetazoan phyla Porifera and Cnidaria (*e.g.*, Buss 1982, 1985; Grosberg 1988). Such phenomena are now known to characterize most substrate-bound colonial invertebrates and have excited unusual interest in a number of disciplines. Specifically, allorecognition phenomena represent a challenge to conventional population genetic explanations for the maintenance of genetic variation (Grosberg 1988) and have also attracted the attention of evolutionary theorists as an exemplar of conflicts between units-of-selection (Buss 1982, 1987). Additionally they have prompted debate among comparative immunologists as to whether such phenomena are homologous to aspects of the vertebrate immune response (Burnet 1971; Hildemann *et al.* 1977; Scofield *et al.* 1982; Weissman *et al.* 1990).

Despite the ubiquity of invertebrate allorecognition, the cell surface molecules responsible and the genes encoding them remain unknown. Indeed, even classical genetic analysis is largely lacking. While limited data (F_1 progenies of crosses between wild-type strains) has been reported for sponges, cnidarians, bryozoans and ascidians, in only two taxa are any intercross, incross and backcross data available (terminology after Green 1981). These include the colonial ascidian *Botryllus schlosseri* (Oka and Watanabe 1957; Scofield *et al.* 1982) and the cnidarian *Hydractinia echinata* (Hauenschild 1956). Allorecognition in the former is controlled by a single locus, such that the fusible phenotype appears if at least one codominant allele is shared. Recent studies have demonstrated that this apparent simplicity masks a complex set of modifying loci that influence the fate of fused individuals (Rinkevich *et al.* 1993).

Hydractinia displays a recognition response after contact of allogeneic tissue involving either fusion or rejection (Hauenschild 1956). The latter is accompanied by a pronounced effector response involving site-specific differentiation, transport, and triggering of the "stinging organelles" (*i.e.*, nematocysts) distinctive to this phylum (Buss *et al.* 1984). In the mid-1950s Hauenschild (1956) reported breeding experiments, the results of which he interpreted as supporting a model of transmission identical to that subsequently established for the ascidian *Botryllus*. At the time, Hauenschild's data were the most exhaustive available for any invertebrate. However, as Hauenschild himself noted, his data were neither completely consistent with a simple one-locus model (especially unexpected phenotypes of F_1, F_2 and in the progeny of a $F_2 \times F_1$ backcross) nor did they preclude alternative interpretations. Similarly inconclusive (unpublished) findings have been obtained in our laboratory. Subsequent discovery of the occurrence of ontogenetic regulation of the fusibility phenotype (Shenk and Buss 1991), not unlike that reported for the ascidian, and of tissue-specificity in the expression of the rejection response (Buss and Grosberg 1990), introduced further complications. These findings led us to inbreed lines for fusibility and to utilize the inbred animals to establish unambiguously the mode of transmission.

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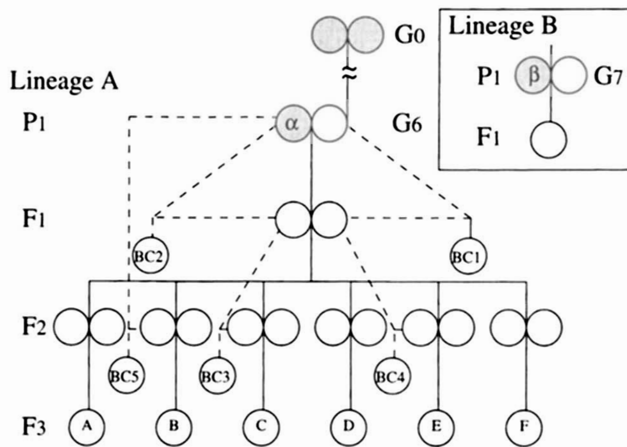

FIGURE 1.—Schematic of the mating program in lineages A and B (inset). Wild-type colonies are shaded. Dashed lines represent backcrosses. Fusibility of offspring of different classes of lineage A to the reporter strain is reported in Table 1. Numbers of backcrosses and letters indicating different F_3 classes correspond to those used in Tables 1 and 2.

MATERIALS AND METHODS

Two wild-type colonies of *H. symbiolongicarpus* (G_0, Figure 1) were collected at Lighthouse Pt., CT. These colonies were mated and larvae metamorphosed onto small (<*ca.* 4 mm^2) plastic chips using conventional methods (BLACKSTONE and BUSS 1991). Offspring were maintained individually in 7-ml tubes (Falcon 2027) at room temperature (22–24°), continuously rolled on a serology mixer. Animals were typically fed every other day with 3–5-day-old nauplii of *Artemia salina*, with daily water changes of 0.45 μm-filtered, pasteurized (to 90°), artificial seawater (Reef Crystals).

Offspring of the original wild-type colonies were tested for fusibility to one another using the rapid polyp assay developed by LANGE *et al.* (1992). Polyps excised from fusible colonies and held with their cut ends appressed, develop continuous ectodermal and endodermal cell layers and share a common gastric cavity within 12–24 hr, whereas incompatible polyps separate. To verify the equivalence of fusibility determined using the polyp assay with the naturally occurring condition of stolonal interactions, 15 pairwise tests of both the polyp assay and the conventional stolonal assay (SHENK and BUSS 1991) were established and found to yield identical results.

Successive generations of brother-sister inbreeding between fusible offspring from the original two wild-types produced a line in which all variability in fusibility was eliminated by the fourth generation (G_4). On the basis of these data, the lineage was deemed homozygous for fusibility and a G_6 animal from this lineage mated to an incompatible wild-type colony (designated as α in Figure 1). Subsequently, the conventional intercross/backcross/incross analysis schematized in Figure 1 was established (lineage A) and the fusibility of offspring assessed relative to the inbred (G_6 and G_7 animals) "reporter strain".

Two approaches were used to assess the diversity of fusibility types in field populations. First, 20 wild-type colonies were collected from a nearby locality (Old Quarry Harbor, Guilford, CT) and fusibility assayed in all 190 pairwise allogeneic combinations. Second, a new wild-type colony (β in Figure 1), known by assay to be incompatible with both the first wild type (α) and the reporter strain, was mated to the reporter strain (lineage B, Figure 1) and the offspring of this cross tested for fusibility with F_3 animals of known genotype from lineage A.

RESULTS AND DISCUSSION

Table 1 summarizes the segregation of fusibility in lineage A. Segregation is Mendelian, with either no deviation of expected to observed values or with deviations well within statistical expectations for all 13 crosses. The results are in complete agreement with a model of *Hydractinia* allorecognition as a one-locus trait, with codominant expression of alleles, such that one shared allele yields a fusible phenotype. The results of fusibility assays between F_3 offspring of defined crosses/incrosses (Table 2) enhance confidence in this model.

The set of genetic states of the parents and those proposed to generate a fusible phenotype are the determinants of predicted frequencies of any model. A diversity of fusibility criteria may be imagined, involving one to *n* loci. In polyfactorial cases, alleles at different loci may be further hypothesized to control the observed response in a diversity of ways (*e.g.*, controlling expression additively or not, being variously linked, etc.). The suggested single locus model is the simplest and hence most parsimonious of a universe of possible models. It is, nonetheless, prudent to consider alternative polyfactorial models that might generate results identical to the monofactorial case. We have been unable to generate a plausible set of fusibility rules compatible with our data for models involving a small number of loci (see Table 3 for a nonexhaustive treatment of alternative models for $n \leq 3$), with one exception.

The sole exception is the following polyfactorial case: P_1 individuals (reporter strain and wild type α) share one or both alleles at all but one loci (the total number of shared alleles is between $n-1$ and $2n-2$), the reporter strain is completely homozygous, and a single shared allele at each locus is required to permit fusibility. Under these conditions there will always be a proportion of F_1 offspring in which all loci but one will be homozygous for the same allele as the reporter strain and one locus will be heterozygous (thus "imitating" a monofactorial system with respect to the reporter strain, by "silencing" all loci but one). Such F_1s will generate exactly the same expected segregation of fusibility to the reporter strain as obtained in this study (*i.e.*, F_2, F_3 and all backcrosses).

The analysis of lineage A alone, therefore, cannot exclude the possibility of multiple loci as an alternative to multiple alleles in this exceptional case. However, additional lines of evidence serve to diminish and eventually dismiss this possibility. The field survey of fusibility designed to assess whether this locus supports multiple alleles yielded one pairwise fusion and 189 pairwise rejections. This result calls for at least five unlinked loci with two possible alleles in the population for each locus to produce the variability observed in the sample alone (*i.e.*, four unlinked loci will produce only $2^4 = 16$ different fusibility types, and more than one fusion will be observed among 20 sampled colonies). With this (and

TABLE 1

Fusibility data

Cross[a]	Monofactorial model[b]: Genotype: Parents[d]	Offspring[e]	Expected fusion/rejection (%)	Results: n	Observed fusion/rejection	χ^2 test[c]: G value	Significance
F_1	*ff* × *rq*	*fr*, *fq*	100/0	18	18/0		
F_2	*fr*/*fq* × *fr*/*fq*	1*ff*, 2(*fr*, *fq*), 1(*rr*, *rq*, *qq*)	75/25	34	26/8	0.040	NS
F_3[f]							
A	*fr*/*fq* × *ff*	1(*fr*, *fq*), 1*ff*	100/0	19	19/0		
B	*fr*/*fq* × *fr*/*fq*	1*ff*, 2(*fr*, *fq*), 1(*rr*, *rq*, *qq*)	75/25	23	17/6	0.014	NS
C	*fr*/*fq* × *rr*/2*rq*/*qq*	1(*fr*, *fq*), 1(*rr*, *rq*, *qq*)	50/50	18	12/6	2.039	NS
D	*ff* × *ff*	*ff*	100/0	12	12/0		
E	*ff* × *rr*/2*rq*/*qq*	*fr*, *fq*	100/0	12	12/0		
F	*rr*/2*rq*/*qq* × *rr*/2*rq*/*qq*	*rr*, *rq*, *qq*	0/100	14	0/14		
Backcrosses							
F_1							
BC1	*fr*/*fq* × *ff*	1(*fr*, *fq*), 1*ff*	100/0	14	14/0		
BC2	*fr*/*fq* × *rq*	1(*fr*, *fq*), 1(*rr*, *rq*, *qq*)	50/50	13	8/5	0.699	NS
F_2							
BC3	*fr*/*fq* × *fr*/*fq*	1*ff*, 2(*fr*, *fq*), 1(*rr*, *rq*, *qq*)	75/25	13	10/3	0.026	NS
BC4	*ff* × *fr*/*fq*	1(*fr*, *fq*), 1*ff*	100/0	9	9/0		
BC5	*fr*/*fq* × *rq*	1(*fr*, *fq*), 1(*rr*, *rq*, *qq*)	50/50	8	3/5	0.505	NS

Offspring of the different crosses in lineage A (see Figure 1) were assayed for fusibility to the reporter strain.

[a] See Figure 1.

[b] Under the following assumptions: one-locus, condominantly expressed alleles, with one shared allele generating a fusible phenotype; the inbred line (G_6) is homozygous and the wild type is heterozygous, with no allele shared between the two.

[c] Log likelihood ratio test (SOKAL and ROHLF 1981). G values were not corrected to facilitate rejection of the proposed model.

[d] In most crosses, parents were involved for which it was not necessary to assign a specific genotype. In these cases, the genotype presents the mixture of genotypes in the population from which these individuals were taken (*e.g.*, *fr*/*fq* is a mixture of *fr* and *fq* heterozygotes).

[e] Genotypes in parentheses (producing equal phenotypes) represent alternative possibilities depending upon the actual genotypes of the parents crossed.

[f] F_2 parents were assigned specific genotypes following fusibility tests to both the reporter-strain (G_6, G_7) and the wild type (α). Numbers of backcrosses and letters indicating different F_3 classes correspond to those used in Figure 1 and Table 2.

larger) number of loci, the probability of finding a second wild type (β) that will reject both the first wild type (α) and the reporter strain is sufficiently low to reject the multiloci option ($P < 0.05$ for five loci; P is the probability product of the possible genotypic combinations for α and β). Moreover, this simple analysis is conservative—since one shared allele is sufficient for fusion, heterozygosity must be extremely rare, or the number of loci involved much higher than five, to produce the variability demonstrated in the field sample.

Further support for the proposed model was obtained by testing F_1 offspring of lineage B for fusibility with F_3s of lineage A produced by defined crosses/incrosses. These F_1s are expected to carry one reporter strain allele and one allele that is foreign to both the reporter strain and the first wild-type colony (α). Three

TABLE 2

Fusibility between F_3 offspring of lineage A

Monofactorial model[a]: Genotype[b]: Colony 1	Colony 2	Expected fusion/rejection (%)	Results: n	Observed fusion/rejection
ff (D)	*ff* (D)	100/0	10	10/0
ff (D)	*fr*, *fq* (E)	100/0	10	10/0
ff (D)	*rr*, *rq*, *qq* (F)	0/100	10	0/10

[a] Under the following assumptions: one-locus, codominantly expressed alleles, with one shared allele generating a fusible phenotype; the inbred line (G_6) is homozygous and the wild type is heterozygous, with no allele shared between the two.

[b] Letters in parentheses correspond to notation of F_3 offspring in Table 1 and Figure 1.

TABLE 3

Alternative fusibility models and the deviation of their predictions from fusibility data for lineage A

No. of unlinked loci	Alleles shared at P1[a]	Fusibility criterion[b]	Node in lineage A tree deviating from model[c]	Statistical tests: χ^2 test[d]	Statistical tests: Binomial distribution[e]
1	0	2	$\underline{F_1}$		
	1	1	$\underline{P_1}$		
		2	F_1		3.8×10^{-6}
2	0	1	F_2	$P < 0.025$	0.008
		2	$\underline{F_1}$		
	1 or 2 at one locus	1	no deviation		
		2	$\underline{F_1}$		
	1 at each locus	1	$\underline{P_1}$		
		2	F_1		1.5×10^{-11}
	3	1	$\underline{P_1}$		
		2	F_1		3.8×10^{-6}
3	0	1	F_2	$P < 0.001$	1.89×10^{-5}
	1	1	F_2	$P < 0.025$	0.008
	1 or 2 at each of two loci	1	no deviation		
		2	$\underline{F_1}$		
	3–5, distributed over all loci	1	$\underline{P_1}$		
		2	F_1		3.8×10^{-6}

[a] The reporter strain is assumed to be homozygous at all loci.
[b] The number of alleles at each locus that have to be shared between two colonies to make them fusible.
[c] Some models predict either no fusibility in F_1 (100% fusion observed) or fusibility of P_1 colonies (rejection observed). These cases are underlined, and no statistical tests are required.
[d] Log likelihood ratio test; observed frequencies were adjusted by continuity correction (SOKAL and ROHLF 1981).
[e] For F_1, where $n < 25$, only exact binomial probabilities were calculated (see SOKAL and ROHLF 1981).

such offspring were tested for fusibility against an F_3 strain known to be heterozygous (*i.e.*, *fr* or *fq*) and another F_3 strain known not to bear the *f* allele (*i.e.*, *rr*, *rq* or *qq*). Fusion was observed to heterozygous F_3s and rejection observed for F_3s not carrying the *f* allele (a total of six tests).

While our findings collectively establish a monofactorial control of fusibility in *Hydractinia*, we would still expect subsequent work with these or other lineages to reveal modifiers of the expression of this locus. This prediction is based on the following: allorecognition is known, from *in situ* field experimentation, to directly influence survivorship of *Hydractinia* (YUND *et al.* 1987; YUND 1991) and natural selection rarely leaves traits so directly impacting fitness unmodified; the fusibility response is known to display ontogenetic regulation (SHENK and BUSS 1991) and the transmission of this trait remains uninvestigated; and, finally, the ambiguity in HAUENSCHILD's original results (1956), which motivated our inbreeding program at the outset implies an influence of genetic background. While the F_1 of HAUENSCHILD's lineage B deviates from expected only with respect to the proportions of expected phenotypes ($P < 0.001$, log likelihood ratio test with continuity correction), the following progenies produced in his study include phenotypes that are not predicted by any simple model considered in either his work or ours: F_1 of lineage A (3% showed an unexpected phenotype); two F_2 progenies of reciprocal crosses of F_1 from both lineages (11 and 14%); offspring of a backcross $F_2 \times F_1$ (97%). A possible explanation originally suggested by HAUENSCHILD was: "... these alleles can be influenced by other genes of lower expressivity or penetrance, which only operate in specific combinations" (translated by C. MCFADDEN). The process of inbreeding, employed in the current study to produce a "reporter-strain", apparently has minimized genetic background heterogeneity to a degree that a simple underlying mode of inheritance can be detected.

The monofactorial mode of transmission in the eumetazoan *Hydractinia* is strikingly similar to the widely known findings in the protochordate *Botryllus* (OKA and WATANABE 1957; SCOFIELD *et al.* 1982) and raises the possibility that clonal invertebrate allorecognition is similarly controlled across such taxa. The question remains, however, whether any similarity is attributable to equivalence in selection regimes molding genes of different ancestry to common features or whether the similarly is attributable, in some measure, to common descent. This issue, and the related issue of the oft-hypothesized ancestor-descendant relationship of these genes to the vertebrate MHC, must await isolation of this locus and characterization of its product(s). Clearly, the isogenic and congenic lines that we are currently developing will prove of use in this regard.

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