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EVOLUTIONARY GENETICS OF ALLORECOGNITION IN THE COLONIAL HYDROID *HYDRACTINIA SYMBIOLONGICARPUS*

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Abstract.—Many sedentary, clonal marine invertebrates compete intensively with conspecifics for habitable space. Allorecognition systems mediate the nature and outcome of these intraspecific competitive interactions, such that the initiation of agonistic behavior and the potential for intergenotypic fusion depend strongly on the relatedness of the contestants. The dependence of these behaviors on relatedness, along with the extraordinary precision with which self can be discriminated from nonself, suggest that allorecognition systems are highly polymorphic genetically. However, allotypic specificity of this sort could be produced by any number of genetic scenarios, ranging from relatively few loci with abundant allelic variation to numerous loci with relatively few alleles per locus. At this point, virtually nothing is known of the formal genetics of allorecognition in marine invertebrates; consequently, the evolutionary dynamics of such systems remain poorly understood. In this paper, we characterize the formal genetics of allorecognition in the marine hydrozoan *Hydractinia symbiolongicarpus*. *Hydractinia symbiolongicarpus* colonizes gastropod shells occupied by hermit crabs. When two or more individuals grow into contact, one of three outcomes ensues: fusion (compatibility), transitory fusion (a temporary state of compatibility), and rejection (incompatibility, often accompanied by the production of agonistic structures termed hyperplastic stolons). Observed patterns of compatibility between unrelated, half-sib pairs, and full-sib pairs show that unrelated and half-sib pairs under laboratory culture have a very low probability of being fusible, whereas full sibs have a roughly 30% rate of fusion in experimental pairings. The genetic simulations indicate that roughly five loci, with 5–7 alleles per locus, confer specificity in this species. In ecological terms, the reproductive ecology of *H. symbiolongicarpus* should promote the cosettlement of kin, some of which should be full sibs, and some half sibs. Thus, there is potential for kin selection to play a major role in the evolution of the *H. symbiolongicarpus* allorecognition system. In genetic terms, this system conforms to theoretical predictions for a recognition system selected to distinguish among classes of kin, in addition to self from nonself.

Key words.—Allorecognition, *Hydractinia symbiolongicarpus*, transmission genetics.

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The life cycles of many sedentary clonal animals typically exhibit a combination of extensive asexual proliferation, indeterminate growth, exceptional longevity (reviewed in Jackson 1985; Harvell and Grosberg 1989), and limited dispersal of asexual or sexual propagules (reviewed in Jackson 1986). These life-history attributes, in turn, promote tissue contacts between isogeneic (self) and allogeneic (nonself) conspecifics (Grosberg 1988) and often lead to intense intraspecific competition for space (Buss 1990). In numerous sponges, cnidarians, bryozoans, and colonial ascidians, the outcomes of allogeneic interactions range from no apparent response, through intergenotypic fusion, to active cytotoxic rejection and even aggression (reviewed in Karlson 1980; Buss et al. 1984; Grosberg 1988; Buss 1990).

As in many social insects and vertebrates that modify their behavior according to the individual identities or relatedness of conspecifics (Michener and Smith 1987; Breed and Bennett, 1987; Blaustein et al. 1987; papers in Hepper 1991), a growing number of field and laboratory studies on aquatic, clonal invertebrates show that neither rejection and aggression, nor fusion, randomly occur with respect to the genotypes of interacting conspecifics (Buss 1982, 1987; reviewed in Grosberg 1988; Buss 1990). The initiation of cytotoxic or agonistic behavior generally depends on the relatedness of contestants: interactions between clonemates and close relatives usually do not elicit cytotoxicity or aggression, whereas interactions between more distant relatives often do. Likewise, somatic fusion usually occurs between clonemates and close relatives. The dependence of cytotoxicity, aggression, and fusion on relatedness, along with the precision of discrimination (reliabilities [sensu Crozier and Dix 1979] often exceed 95%), together suggest that the allorecognition systems governing these behaviors enhance individual fitness by mediating responses with respect to kinship (Hamilton 1964), and are underlain by highly polymorphic recognition loci (reviewed in Grosberg 1988; Grafen 1990).

In the absence of breeding studies, patterns of allorecognition specificity alone reveal little about the formal genetics of allorecognition (Curtis et al. 1982; Grosberg et al. 1985; Stoddart et al. 1985; Grosberg 1988). Yet, it is just such information that sets the foundation for understanding the function of allorecognition systems, how allotypic diversity evolves, and the utility of allorecognition assays for deciphering the genetic structure of natural populations (Crozier 1987; Grosberg 1988; Grafen 1990). This is because the precision with which genetically based cues (i.e., allotypes) can be used to distinguish among different individuals, and classes of relatives, depends on the distribution of allelic variation within and among loci, along with the rules of genetic matching that elicit a particular class of behavior, such as rejection, aggression, or fusion (Crozier and Dix 1979; Getz 1981, 1982; Beecher 1982; Curtis et al. 1982; Lacy and Sherman 1983; Crozier 1987; Grosberg 1988; Bull and Pease 1989; Reeve 1989).

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Our understanding of the genetic basis of allorecognition specificity in animals (including kin recognition) is limited to a few mammals (notably *Mus musculus*; reviewed in Potts and Wakeland 1990; Brown and Eklund 1994), and colonial ascidians, notably members of the genus *Botryllus* (reviewed in Weissman et al. 1988; also see Raftos and Briscoe 1990 on solitary ascidians). In *Botryllus*, the primary fusion/rejection response is controlled by a single, highly polymorphic locus, in which the sharing of one, or both, codominant alleles leads to fusion, and the absence of a shared allele leads to nonfusion or cytotoxic rejection (Oka and Watanabe 1957; Sabbadin 1962; Scofield et al. 1982). Studies on other botryllid ascidians are consistent with this model (Yund and Feldgarden 1992); however, in other ascidian families, the genetics appear to be slightly more complex, perhaps involving several loci (Raftos 1991). Beyond this, little is known of the formal genetics of allorecognition in clonal organisms, especially in taxa exhibiting allorecognition-mediated behaviors more complex than fusion/nonfusion reactions. Thus, in contrast to our growing understanding of the molecular, formal, and population genetics of many gametic incompatibility systems in angiosperms (reviewed in Clark 1992; Charlesworth 1995) and mammalian historecognition systems (Potts and Wakeland 1990; Brown and Eklund 1994), the assumptions underlying genetic models of the evolution of invertebrate allorecognition and kin recognition systems (e.g., Grosberg and Quinn 1986, 1988; Neigel 1988; De Boer 1995) remain suspect. Furthermore, to the extent that the formal genetics of allorecognition permit allotypically distinct conspecifics to fuse, the reliability of allorecognition-based analyses of clonal and kin structure in natural populations is compromised (reviewed in Grosberg 1988).

From ecological, morphological, cytological, and developmental perspectives, the mechanisms governing the nature and outcome of intraspecific competitive interactions between colonies of hydroids in the genus *Hydractinia* are now better understood than in perhaps any other clonal animal (reviewed in Ivker 1972; Buss et al. 1984; Buss and Grosberg 1990; Shenk and Buss 1991; Müller et al. 1987). *Hydractinia symbiolongicarpus* Buss and Yund, like many other members of the Family Hydractiniidae, colonizes gastropod shells inhabited by hermit crabs (Yund et al. 1987; Buss and Yund 1989). Befitting its name, *H. symbiolongicarpus* usually settles on shells occupied by the hermit crab *Pagurus longicarpus* (Buss and Yund 1989). In many cases, several of the demersal, sexually produced larvae of *H. symbiolongicarpus* colonize a single shell and metamorphose into small, sessile feeding polyps (Yund et al. 1987; Yund and Parker 1989; pers. obs.). As the resulting colonies grow asexually, they compete for space, frequently leading to the competitive exclusion of all but one of the contestants (Yund et al. 1987; Buss and Yund 1989; Yund and Parker 1989; Buss and Grosberg 1990). Early laboratory studies of allorecognition showed that when two allogeneic colonies (i.e., nonclonemates) grew into contact, rather than simply not fusing (as in many colonial ascidians, bryozoans, and sponges), one of the colonies (and usually both) produced a set of specialized, nematocyst-laden, tubular extensions of the gastrovascular system, termed hyperplastic stolons (Schijfsma 1939; Ivker 1972). These hyperplastic stolons proliferate, and as they do, they swell by recruiting large numbers of nematocysts (Buss et al. 1984). The nematocysts discharge into an allogeneic opponent and cause substantial necrosis (Buss et al. 1984). In contrast, when isogeneic colonies (i.e., clonemates) grew into contact, the gastrovascular systems of the two colonies fused.

Despite our detailed understanding of the principal morphological, ontogenetic, and cytological features of allorecognition in *Hydractinia*, little is known conclusively of the genetic rules that govern whether an allogeneic interaction will lead to fusion, transitory fusion, or rejection. Preliminary data from a number of studies (e.g., Teissier 1929; Crowell 1950; von Hauenschild 1954, 1956; Müller 1964; Ivker 1972) revealed that close relatives fused considerably more often than distantly related individuals, suggesting that allorecognition specificity had a genetic basis. Later studies (Yund et al. 1987; Shenk and Buss 1991) confirmed these observations, and extended them in several fundamental ways. First, it is now clear that in natural populations, virtually all (i.e., >95%) allogeneic interactions produce an incompatibility response, involving the unilateral or bilateral deployment of hyperplastic stolons (Yund et al. 1987; our unpubl. obs.). Second, Shenk and Buss (1991) confirmed von Hauenschild's (1954) observation of a third class of intergenotypic interaction in *H. symbiolongicarpus*, transitory fusion, in which two colonies, either parents and their offspring or a pair of full sibs, initially fuse, and subsequently unilaterally or bilaterally reject each another. Most recently, Mokady and Buss (1996), based on a series of incrosses and backcrosses derived from a single mated pair (one member of which was highly inbred) argued that, as in the ascidian genus *Botryllus*, a single, highly polymorphic locus with multiple codominant alleles controls allorecognition in *H. symbiolongicarpus*

In this paper, we characterize key features of the transmission genetics of allorecognition in *H. symbiolongicarpus*. Because of offspring inviability in our attempts to generate large numbers of F_2 progeny and conduct F_1 backcrosses (also see von Hauenschild 1954, 1956; Mokady and Buss 1996), our approach to unraveling the formal genetics of allorecognition in this species combines two principal components. We first characterize patterns of compatibility and incompatibility between full- and half-sibling offspring from laboratory matings. Second, we develop a simulation model that varies the number of loci, the number of alleles per locus, and the rules of genetic matching involved in allorecognition. We then use this model to determine the parameter values that best correspond to empirically observed frequencies of fusion, transitory fusion, and rejection between closely and distantly related individuals.

Materials and Methods

Collection, Husbandry, and Mating Procedures

We collected *H. symbiolongicarpus*–encrusted gastropod shells (all *Littorina littorea*), inhabited by the hermit crab *Pagurus longicarpus*, from the shallow sandflats of Barnstable Harbor, MA. We transported the shells carrying colonies of *H. symbiolongicarpus* back to our laboratory in Davis, CA, where we sorted reproductively mature male from female colonies. We discarded any shells carrying more than a single

colony. To ensure that these colonies remained sexually isolated, we maintained them (on their hermit crab hosts) at 16–17°C in two separate aerated seawater aquaria, one for males and one for females. Male and female colonies remained separated for at least one week before we initiated any experimental matings, enough time to ensure both that no foreign sperm survived among the females and that female colonies spawned all eggs fertilized prior to collection (Levitan and Grosberg 1993).

To characterize patterns of allorecognition responses among full sibs, we first initiated matings between five haphazardly selected male-female pairs of colonies. We used the offspring from these matings to establish five corresponding full-sib compatibility matrices (see below). We also mated two additional male and two female colonies in all four combinations. We used the offspring from these matings to establish four additional full-sib compatibility matrices. This mating design also produced two sets of maternal half-sibs, which we used to establish two half sib compatibility matrices.

For each mating, we chose a pair of shells, one carrying a single ripe male and the other a single ripe female *H. symbiolongicarpus*, and placed this male-female pair in an aerated 4-liter seawater aquarium, also held at 16–17°C. We induced the colonies to spawn by holding the mated pairs in complete darkness for 48–72 h, and then directing a bright light at each aquarium for 2–3 h (Bunting 1894; Ballard 1942; Levitan and Grosberg 1993). Forty-eight hours later, we used a pipette to harvest all planula larvae that developed from the fertilized eggs. In several matings, we harvested larvae on 2–3 successive days, to give at least 200 larvae per mating.

We then transferred these larvae into 35-mm plastic petri dishes containing ≈5ml of 53mM CsCl, mixed 1:1 with full-strength 0.22 μ filtered seawater. The larvae remained in this solution for 2–4 h, when they began to contract and initiate metamorphosis (Müller 1973). At this point, we individually transferred the larvae to separate glass slides (4.5 cm × 6.0 cm), submerged horizontally in dishes of filtered seawater at room temperature. Twenty-four to 48 hours later, when the larvae had completed metamorphosis into primary feeding polyps, we used a pipette to feed each polyp with a dense suspension of two day-old brine shrimp (*Artemia salina*) nauplii. The next day, we arranged all of the slides carrying metamorphosed offspring into plastic racks, that held the slides vertically. We subsequently maintained all resulting colonies in 650 liter recirculating seawater aquaria at 16°C, and fed them daily (for 3–6 h) to repletion with 2–3 day-old brine shrimp nauplii.

At the same time, we also established clonal explants of the parental colonies by excising with a scalpel a small section of ectodermal mat containing several feeding polyps (gastrozooids) from the stock colonies on the hermit crab shells (Ivker 1972). We secured each of these explants to a glass slide under a loop of 1.8 kg test nylon monofilament; 5–7 days later, by which time the colonies had attached to the slides, we removed the monofilament loops. We fed these colonies in the same manner and at the same time as their offpring.

Compatibility Assays of Full and Half Sibships

Three to four months after metamorphosis, and before they became sexually mature, the F_1 colonies from all of the nine matings reached sizes ranging from several hundred (≈ 1–2 cm^2) to several thousand polyps (≈ 10–20 cm^2). We used clonal explants from these stock colonies to build 11 complete compatibility matrices. Matrices A–I tested all possible pairwise interactions among full sibs (n = 8-19 offspring per matrix); matrices J and K tested responses between maternal half sibs (n = 10 offspring per matrix).

We established each pairing by excising a small piece of tissue carrying three to four gastrozooids from the sexually immature stock colonies of each contestant in a pairing, and then positioning these explants under monofilament threads approximately 5 mm apart on glass slides. In initial trials, we established three or more replicates of each pairing; however, like others before us (e.g., von Hauenschild 1954, 1956; Yund et al. 1987; Buss and Grosberg 1990), we never detected variation among replicates in the outcome of a compatibility test. Consequently, we generally used only a single pairing to characterize the outcome of a compatibility test between each unique combination of sibling genotypes. As described below, in several instances, outcomes of compatibility tests were ambiguous; in such cases, we repeated tests, although the outcomes usually remained ambiguous.

At weekly intervals, we observed each pairing in a matrix under a dissecting microscope, recorded whether contact had occurred between the clonal explants, and if so, the nature of the encounter. We classified the outcomes of the compatibility tests into four categories.

Fusion.—When gastrovascular connections formed between the two sibling explants, usually accompanied by visible exchange of fluid and particles between the explants, and persisted for at least seven months after initial contact, we scored the interaction as compatibility. During this period, at least one, and often both, explants attained reproductive maturity. In some cases, one or both members of a fused pair died before the end of the seven-month period of observation. In such cases, provided that the stock cultures for the both members of a pairing survived, we repeated the pairing. When we could not repeat a test, and if we found no signs of incompatibility prior to death, we scored such interactions as fusions.

Rejection.—When we found no evidence of gastrovascular connection after the tissues of the two explants grew into contact, we scored the interaction as an incompatible response (i.e., rejection). If such a response elicited the production of hyperplastic stolons by one, or both, explants (i.e., aggressive incompatibility), or when extensive tissue necrosis occurred along the zone of contact between the explants, we terminated observations. As described by Buss and Grosberg (1990), in some cases, especially when an interaction did not involve stoloniferous tissue, there was neither a hyperplastic, nor a necrotic, response. In these cases of passive incompatibility, which we scored as rejections, we followed interactions for at least seven months to ensure that explants remained unfused.

Transitory Fusion.—Some interactions between sibling explants showed initial fusion (including exchange of gastro-

vascular fluid) between two colonies, followed by rejection at all points of contact between the explants. Rejection could be either aggressive (involving unilateral or bilateral production of hyperplastic stolons), necrotic, or passive. von Hauenschild (1954) reported comparable behaviors between siblings in *H. echinata* and initially classified such transitory events as incompatibility. In a subsequent paper (von Hauenschild 1956), he scored transitory fusions as compatible interactions. Shenk and Buss (1991) described the morphological features of transitory compatibility in *H. symbiolongicarpus* and showed that in at least one set of parent-offspring interactions, the onset of the rejection phase of the interaction was associated with the attainment of sexual maturity by the offspring. We did not consistently find such an association (unpubl. obs.).

Unresolved Interactions.—In a small fraction ($\leq$ 8.2% in any given matrix; 3.7% over all 11 matrices), of interactions, we could not assign the outcome of an interaction to any of the three categories listed above, even after seven months of observation (also see von Hauenschild 1954). In the majority of these ambiguous interactions, different areas of interclonal contact simultaneously and persistently involved fusion and rejection. In calculating pairwise similarity indices (see below), we denoted such unresolved interactions by empty cells in a compatibility matrix.

Allorecognition Assays of Field Populations

To estimate the frequencies of fusion, transitory fusion, and rejection in the population of *H. symbiolongicarpus* in Barnstable Harbor, in 1993 we established 98 pairs of compatibility tests (as described above) using explants from 196 field-collected colonies, each from separate hermit crab shells.

Genetic Simulations

We first attempted to analyze our empirically derived patterns of inheritance of fusion, transitory fusion, and rejection, with simple one- or two-locus models of particulate inheritance. Like our predecessors studying the genetics of allorecognition specificity in *H. echinata* (von Hauenschild 1954, 1956; du Pasquier 1974), we could not explain transmission of these traits using one or two loci with codominant or dominant alleles. We therefore developed a simple genetic model that simulated our breeding protocol and generated replicated compatibility matrices analogous to those we analyzed in our empirical study. Our basic approach substantially modifies the kin recognition models (with self-matching and codominance) of Getz (1981), as well as Lacy and Sherman (1983).

In principle, four genetic attributes of allorecognition systems govern whether the outcome of an intergenotypic interaction leads to fusion, transitory fusion, or rejection: (1) the numbers of loci that confer allotypic specificity; (2) the number of alleles per locus; (3) the allelic dosage rules that specify how many alleles two individuals share at each locus; and (4) the threshold levels of overall (i.e., multilocus) allotypic similarity necessary to produce each of the three observed outcomes. The analysis therefore consisted of two sets of simulations, both of the same general form. The first set explored the influence of different values of the following three parameters on the total number of allorecognition alleles across loci shared by pairs of full sibs, half sibs, and randomly selected individuals: (1) the number of independent loci, L, additively controlling allorecognition specificity; (2) the number of equally frequent alleles, n, at each locus; and (3) the allelic dosage rules (see next paragraph). The second set examined which combinations of L, n, and dosage rules best matched our empirically measured frequencies of fusion, transitory fusion, rejection, and compatibility types among full sibs, half sibs, and randomly paired individuals.

TABLE 1. Alternative dosage rules for scoring the number of alleles shared by two genotypes at a hypothetical allorecognition locus (A). Subscripts denote allelic identities. The values corresponding to each genotypic combination refer to the number of alleles shared by the two genotypes under the 0, 1, 2, and 0, 1, 2, 4 dosage rules.

	Genotype I			
	A_1A_1	A_1A_2	A_2A_2	A_2A_3
Genotype II				
1. 0,1,2 Dosage Rules				
A_1A_1	2	1	0	0
A_1A_2		2	1	1
A_2A_2			2	1
A_2A_3				2
2. 0,1,2,4 Dosage Rules				
A_1A_1	4	2	0	0
A_1A_2		2	2	1
A_2A_2			4	2
A_2A_3				2

The number of alleles shared at each locus (i.e., dosage rules) can be scored in several ways (Table 1). The first approach, like that of Cotterman (1940, cited in Crow and Kimura 1970) and Curtis et al. (1982), assumes that a tested pair of siblings can share 0, 1, or 2 alleles at any given locus (i.e., 0, 1, 2 rules). Alternatively, allelic dosage effects at a locus can be scored such that only when all four alleles are identical (e.g., A_1A_1 versus A_1A_1) is a score of 4 assigned (i.e., 0, 1, 2, 4 rules).

The first step in the simulations generates parental allotypes by randomly drawing pairs of codominant alleles (with replacement) from a pool of n alleles, for L loci. For each iteration, the simulation randomly selects three parents (one female and two males), then separately "mates" the female to both males to generate ten offspring per mating (or 20 offspring per trio). Thus, each iteration produces two full sibships, which are each other's half sibs.

The simulation then specifies the multilocus allotypes of each offspring by randomly choosing a single allele from each parent at each locus. Each iteration of this process therefore generates multilocus allotypes for two full sibships of ten progeny, and then calculates the allotypic similarity of each pair of full sibs (45 unique pairings per full sibship) as the ratio of the actual number of alleles shared to the number of possible matches. This procedure also calculates the number of alleles shared and allotypic similarity between pairs of offspring from the two matings, which are maternal half sibs (100 unique pairings per half sibship). The simulation repeats this process for 100 iterations for each combination

of 1–5 loci, 2–10 alleles per locus, and both sets of dosage rules.

To generate allorecognition outcomes from the simulated values of allotypic similarity, threshold proportions of overall allotypic similarity necessary to produce fusion, transitory fusion, and rejection must be superimposed on the simulated values. We chose these threshold proportions by inspecting the numerical output from the first set of simulations, and searching for the threshold for each combination of L and n that minimized the difference between the simulated and observed mean frequencies of fusion, transitory fusion, and rejection for both full and half sibs (see Results). The groupings giving the best fit differed for each combination of L and dosage rule; however, we held the groupings constant for all values of n for each combination of L and dosage rule.

We then used these groupings in a second set of simulations to generate (1) expected frequencies of fusion, transitory fusion, and rejection among randomly selected individuals and (2) the expected proportion of unique compatibility groups for full and half sibships. Each iteration of the simulation for each combination of parametric values produces a set of two full sibships (10 offspring per sibship), and, as above, allows for a half-sib comparison. To generate frequencies of fusion, transitory fusion, and rejection, the model once again calculates the overall number of alleles shared by all unique pairs of full and half sibs for each "mating," then assigns an outcome to each pairing based on whether the proportion of shared alleles across loci is above or below the critical threshold value for fusion, transitory fusion, or rejection. After 500 iterations of each combination of parametric values, the simulations estimate the mean proportions (and standard deviations) of fusions, transitory fusions, and rejections expected in full and half sibships, as well as the population as a whole. These simulations also yielded estimates of the frequency of unique compatibility groups in a 10 × 10 matrix.

Results

Compatibility Assays

Compatibility Frequencies.—The number of siblings in each of the 11 sets of matrices varied from 8 to 19 offspring (Table 2); thus, the number of unique allogeneic pairings in each matrix varied from 28 to 171. The frequencies of each type of outcome are derived solely from the unique allogeneic pairings in each matrix (Table 2); unresolved interactions are not included. We calculated all descriptive statistics based on frequencies, proportions or percentages from arcsine-transformed data, with reported values back-transformed to the original units.

All isogeneic (i.e., involving explants from the same colony) and all but two parent-offspring pairings fused permanently (unreported data). Among the full-sib allogeneic pairings, rejection occurred considerably more frequently than fusion and transitory fusion (Table 2: $\bar{x}$ = 47.2%). Overall, fusion was the next most common outcome in the full-sib matrices ($\bar{x}$ = 28.4%), followed by transitory fusion ($\bar{x}$ = 21.5%). However, in four (D, E, F, and I) of the nine full-sib matrices, transitory fusion was more common than fusion. The frequency of unresolved contests ranged from 0–8.2% ($\bar{x}$ = 3.4%). Relative to frequencies of fusion and transitory fusion, rejection frequencies remained fairly constant across matrices, perhaps because this response was easiest to score unambiguously. Frequencies of fusion also appeared to decline as the size of the matrix increased, whereas transitory compatibility frequencies increased with the size of the matrix (Table 2).

Table 2. Percentages of fusion, transitory fusion, and rejection, and frequencies of unique compatibility types (see text) in full and half sibships of *Hydractinia symbiolongicarpus*. Matrices A through I consist of full-sib pairings; matrices J and K are maternal half-sib pairings.

Matrix	Matrix size	Fusion (%)	Transitory fusion (%)	Rejection (%)	Frequency of unique compatibility types
1. Full-sib matrices					
A	8 × 8	39.3	10.7	46.4	0.875
B	13 × 13	39.7	14.1	46.2	0.923
C	17 × 17	26.5	23.5	48.5	1.0
D	18 × 18	20.3	22.2	52.3	1.0
E	19 × 19	15.8	29.2	46.8	1.0
F	10 × 10	28.9	33.3	37.8	1.0
G	10 × 10	37.8	13.3	48.9	1.0
H	10 × 10	33.3	22.2	44.4	1.0
I	10 × 10	17.8	28.9	53.3	1.0
Mean		28.4	21.5	47.2	
Upper 95% CI		35.8	27.9	50.7	
Lower 95% CI		21.3	15.7	43.7	
2. Half-sib matrices					
J	10 × 10	2.0	24.0	74.0	0.875
K	10 × 10	2.0	41.0	57.0	0.600
Mean		2.0	35.2	65.8	

In the two half-sib matrices, rejection frequencies ($\bar{x}$ = 65.8%; n = 2) were generally more than double those of transitory fusion ($\bar{x}$ = 35.2). In contrast to the full-sib matrices, fusion rarely occurred among half sibs ($\bar{x}$ = 2.0%).

In all 11 matrices, a minimum of 10–20% of trios reflected intransitive compatibility relationships (i.e., sib 1 fuses with 2, 2 with 3, but 1 does not fuse with 3; or 1 rejects 2, 2 rejects 3, but 1 fuses with 3). Such intransitivities suggest that siblings need not share all allotypic determinants in order to be fusible (reviewed in Grosberg 1988).

Number of Unique Compatibility Types.—In seven of the nine full-sib matrices, each sib had a distinct pattern of response. Thus, the number of unique compatibility types (defined by individual patterns of allorecognition response to other members of the compatibility matrix) in each matrix matched the number of individuals in the matrix. However, in each of matrices A and B, two sibs (which in both cases fused) shared an identical pattern of response to all of their other sibs (Table 2).

In the two half-sib matrices, both the absolute number and frequency of unique compatibility types were considerably lower than in the full-sib matrices. At first sight, this result appears to be counterintuitive, because on average half sibs should be more allotypically distinct from one another than full sibs; thus, there should be more, rather than fewer, unique compatibility types. The higher frequency of rejections among half sibs is consistent with this expectation. However, to the extent that fusion requires a higher degree of allotypic

matching than does rejection, the proportion of unique compatibility groups in a compatibility matrix need not be positively related to the level of allotypic disparity among the genotypes used to construct the matrix. For example, consider the extreme case in which all individual genotypes rejected all other genotypes: all genotypes would have the same pattern of allorecognition response, yet none would have the same allotype. Because rejection occurred more frequently between half-sib compared with full-sib pairs, individuals are more likely to match in their responses to other individuals in the matrix, and there should be relatively fewer unique compatibility types. The outcomes of the simulations are consistent with this result (see Fig. 5 C, D).

Patterns of Similarity among Full Sibs.—Even when every individual in a matrix possesses a unique set of responses to its siblings, some siblings may be more similar to each other than to other siblings in their overall patterns of response. An analysis of patterns of allotypic similarity could, in principle, provide some insight into the formal genetics of allorecognition. For example, in the case of the single-locus allorecognition system in *Botryllus*, a mating between parents heterozygous for different allorecognition alleles (e.g., $A_1A_2 \times A_3A_4$), yields four equally frequent groups of allotypes among the full-sib offspring. Offspring in each of these groups will share an allele with siblings from two of the other groups, yielding an overall within-sibship probability of fusion of 0.75 (Oka 1970; Scofield et al. 1982). As the number of independent loci controlling the expression of allotypic specificity increases, so, too, should the number of possible allotypic classes among F_1's (Crozier 1987).

We analyzed patterns of similarity in allorecognition response within each of the full sibships using the following procedure. We first constructed a similarity index for all pairs of individuals in a sibship by taking the ratio of the number of cases in which both members of a pair matched in their responses to each other and their sibs to the total number of possible matches. (If, in a given comparison between two individuals, one or the other member of the pair lacked an observation for a specific interaction, then we reduced the number of possible matches by one.) For example, in a 10 × 10 matrix, if two sibs had identical responses to six sibs (including themselves) but differed in their responses to the remaining four sibs, we assigned a similarity index of 0.6. In calculating these similarity indices, we implicitly and unavoidably assumed that fusions, transitory fusions, and rejections each provide comparable information about genetic similarity. We further recognize that the power to distinguish patterns of similarity is highest when frequencies of each class of outcome are similar. Thus, in the half-sib matrices, in which rejections are extremely common (and fusions are rare), the power to identify patterns of similarity is necessarily weaker than in the full-sib matrices. For this reason, we did not calculate similarity indices, or analyze patterns of similarity, in the half-sib compatibility matrices.

In the *Botryllus* mating described above, the frequency distributions of similarity indices will reflect the four distinct groupings of F_1 allotypes produced by the simple genetics of allorecognition. Thus, the mean similarity index (as calculated above) based on shared patterns of allotypic response between all pairs of full sibs is 0.75, the same as the fusion frequency. However, no pair of full sibs actually has this value: a plot of the frequency distribution of similarity indices would show that half of the similarity indices equal 1, and the other half take a value of 0.5. If, on the other hand, comparable overall compatibility frequencies were controlled by numerous independent loci, the mean similarity index would remain the same; however, the frequency distribution of similarity indices should cluster closer to the mean value, yielding a smaller variance in the distribution of similarities than if relatively few loci controlled the expression of compatibility. We therefore compared for the nine full-sib matrices the observed variances of similarity indices to simulated variances generated by shuffling observed frequencies in each matrix.

TABLE 3. Mean similarity indices and variances based on observed and simulated patterns of tissue compatibility for the nine (A–I) full-sib matrices. Mean observed similarities quantify the resemblance among siblings within a matrix, based on their overall patterns of allorecognition response to each other. The simulated values characterize resemblance based on randomly assigned outcomes drawn from the observed frequency distribution of fusions, transitory fusions, and rejections in each matrix, over 100 iterations.

Matrix	No. of pairings	Observed mean	σ^2 of observed mean	Simulated mean	σ^2 of simulated mean	(±99% CI)
A	28	0.388	0.114	0.392	0.033	($\pm 1.16 \times 10^{-4}$)
B	78	0.387	0.072	0.392	0.020	($\pm 3.26 \times 10^{-3}$)
C	136	0.392	0.048	0.360	0.015	($\pm 5.63 \times 10^{-4}$)
D	153	0.383	0.037	0.384	0.014	($\pm 5.04 \times 10^{-4}$)
E	171	0.343	0.042	0.366	0.015	($\pm 3.94 \times 10^{-4}$)
F	45	0.307	0.046	0.320	0.026	($\pm 1.86 \times 10^{-4}$)
G	45	0.356	0.091	0.389	0.026	($\pm 1.69 \times 10^{-4}$)
H	45	0.369	0.151	0.348	0.027	($\pm 1.89 \times 10^{-4}$)
I	45	0.358	0.046	0.351	0.023	($\pm 1.48 \times 10^{-4}$)

The shuffling procedure randomly assigned an outcome of (1) fusion, (2) transitory fusion, (3) rejection, or (4) unresolved to each cell (representing a unique pairwise test) in each of nine matrices, the sizes of which corresponded to the nine full-sib matrices described earlier. We drew the outcomes without replacement from a distribution corresponding to the frequencies observed in the full-sib matrices. For each of 100 iterations of each matrix, the simulations calculated all pairwise similarities between the full sibs, the mean similarity for each iterated matrix, and the variance of that mean. In so doing, we generated 100 estimates of the within-matrix variance, based on the shuffled data from each of the original nine matrices. We then asked whether the single observed variance fell within the 99% CI of the mean variance for the corresponding simulated matrices.

The mean empirically derived similarities ranged from 0.307–0.392 for each of the nine full-sib matrices (Table 3). The mean similarities for the corresponding shuffled outcomes ranged from 0.320–0.392 (Table 3). The distributions of both the observed and simulated similarity indices fit the assumptions of normality (Kolmogorov-Smirnov test; all $P > 0.3$); consequently we did not transform the data. In every pairwise comparison between observed and simulated similarities, the means did not differ significantly (t-test; all $P > 0.5$). However, in all nine comparisons, the variances of

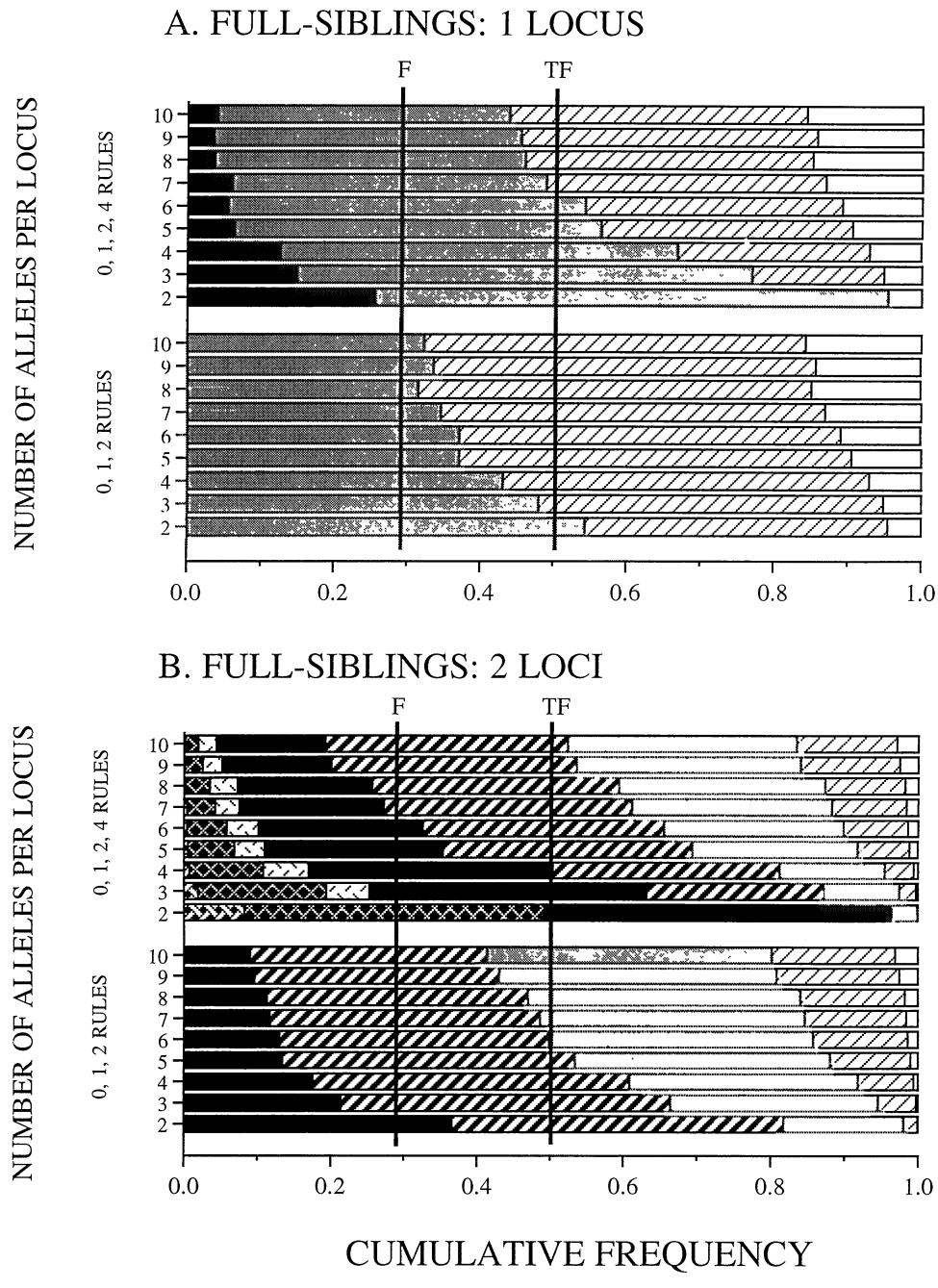

FIG. 1. Composite bar graphs based on genetic simulations showing the expected number of shared alleles, summed across all loci, for full-sib pairings. Each shading pattern corresponds to a different allelic sharing category (see inset key). Each panel (A–E) shows simulated results for 1–5 loci, respectively. The upper set of bars in each panel show results for the 0, 1, 2, 4 dosage rules (see text); the lower set shows results for the 0, 1, 2 dosage rules. The lines labeled "F" and "TF" divide the histograms into regions whose length corresponds to the observed frequencies of fusion, transitory fusion, and rejection in full sibships.

the observed mean similarities were lower than the mean simulated variances, and fell below the 99% CI of the simulated variances (Table 3).

Allorecognition Assays in Field Populations

All 96 pairings between haphazardly chosen members of the population of *H. symbiolongicarpus* from Barnstable Harbor elicited a rejection response. This estimate of rejection frequencies has a lower 99% CL of 95.32% (Sokal and Rohlf 1981, Table 23, p. 160). As described in the next section, we used this lower value to constrain our search for realistic combinations of parameters in the simulation models.

Genetic Simulations

The simulations assessed how variation in three key genetic parameters affects patterns of allotypic similarity and

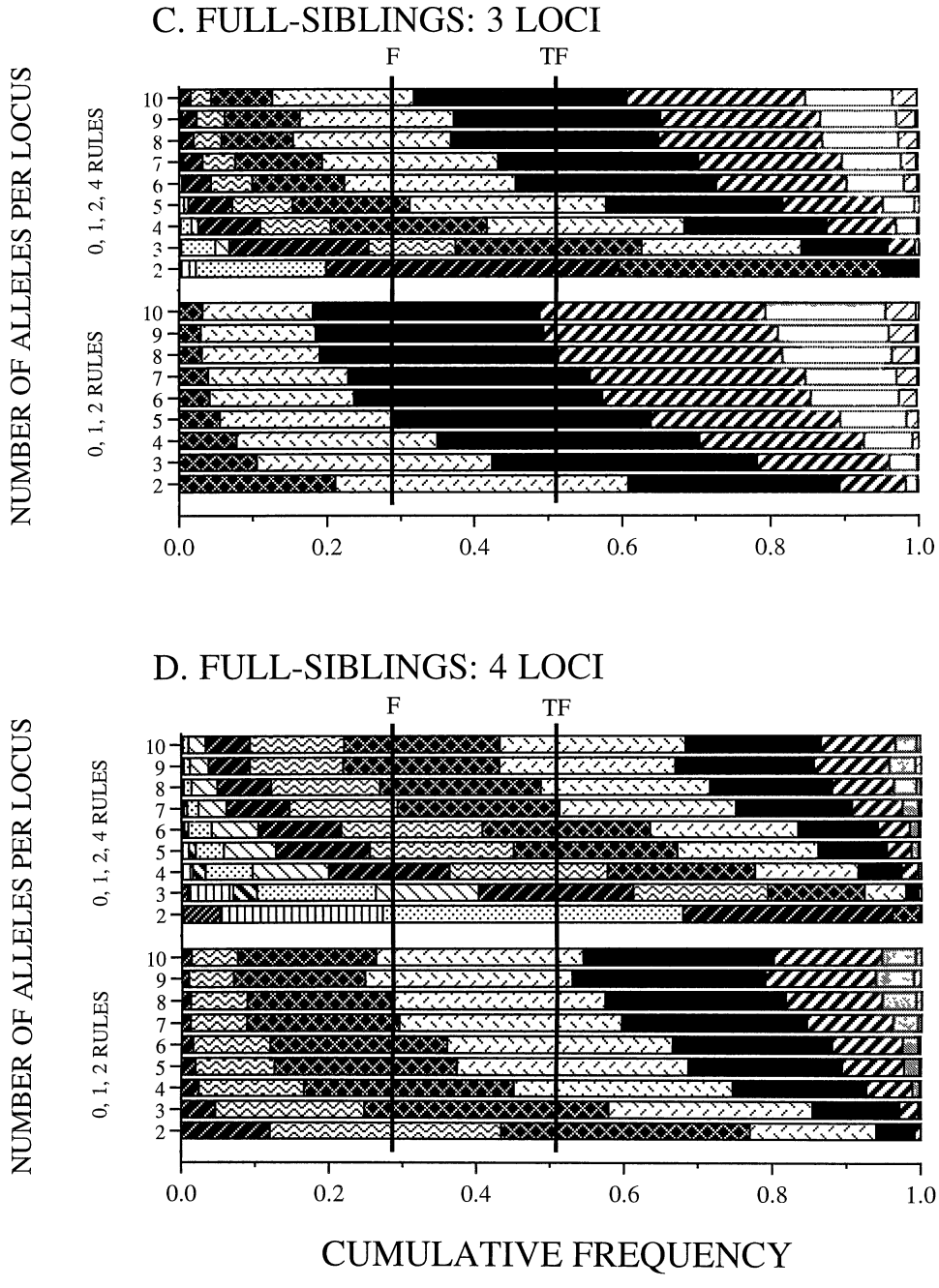

FIG. 1. Continued.

compatibility frequencies among (1) full sibships; (2) half sibships; and (3) randomly drawn pairs using a simple additive model. Because we also had empirically estimated frequencies of unique compatibility types in full and half sibships, we simulated these data, too. In outline, the overall simulation procedure involved a three-step process. First, we explored the effects of varying (1) number of allorecognition loci (L: 1–5); (2) number of alleles per locus (n: 2–10); and (3) dosage rules (0, 1, 2 and 0, 1, 2, 4) on the **total** number of alleles shared by full and half sibs across all L loci. This value can range from 0 through 20 (with five loci and 0, 1, 2, 4 dosage rules). For instance, with two independent loci, the maximum number of alleles at which two individuals can match equals four for the 0, 1, 2 dosage rules, and eight for the 0, 1, 2, 4 dosage rules.

Next, we used this information to identify threshold proportions of allotypic similarity (i.e., number of allorecognition alleles actually shared/number of alleles potentially shared) for different combinations of L and n that yielded frequencies of fusion, transitory fusion, and rejection that most closely matched our empirical estimates.

Finally, we used these threshold proportions in a second set of simulations to generate expected rejection (because rejection was the only outcome we observed in the pairings

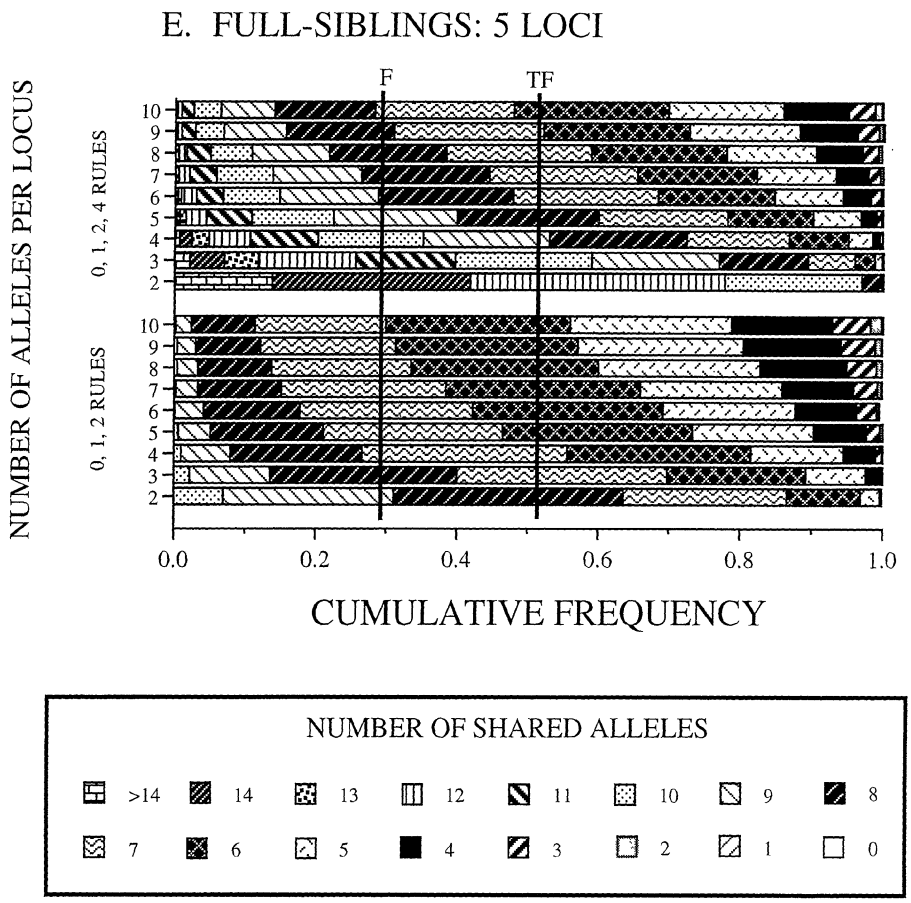

FIG. 1. Continued.

between field-collected colonies) frequencies among randomly selected pairs of genotypes, as well as frequencies of unique compatibility types expected in full and half sibships.

Trial Simulations.—We first compared output from the simulation model to numerical solutions of a simple analytical model developed to predict compatibility frequencies (Curtis et al. 1982). This analytical model, like our simulation, assumes that allorecognition specificity is controlled by L independent loci (in linkage equilibrium), with n equally frequent alleles at each locus. It then gives the probability that two genotypes drawn randomly from an essentially infinite population will be compatible or incompatible (i.e., fuse or reject). The model uses only 0, 1, 2 dosage rules and generates expectations depending on whether (1) only one allele per locus need be shared for two individuals to be compatible (i.e., $\geq$ 50% allelic sharing at each locus) or (2) both alleles at each locus must be shared (although both individuals could be homozygous for a particular allele, or both could be heterozygous for the same two alleles).

Our simulated estimates of populationwide fusion frequencies (based on 1000 iterations) and analytical calculations based on Curtis et al. (1982) are virtually identical. For example, with $L = 1$, and $n = 50$ under case 1 above, the analytical model gives an expected fusion frequency of 7.76%, whereas the simulation gives 7.9%. For the same values of L and n, we also ran 1000 iterations of the simulation using our 0, 1, 2, 4 dosage rules (which for case 1 corresponds to 0.25 sharing; i.e., a pair will fuse when they share one or more alleles out of four possible matches). This simulation also produces a mean fusion frequency of 8.4%, demonstrating that in a single-locus allorecognition system, fusion/rejection frequencies alone cannot be used to distinguish between 0, 1, 2 and 0, 1, 2, 4 dosage rules.

Unfortunately, there are no simple analytical expressions that allow the calculation of compatibility frequencies for more complex genetic systems in which there are more than two outcomes to an interaction. Moreover, the formal genetics of allorecognition govern the expected number of compatibility types expected within a sibship. As mentioned previously, the only clonal invertebrate for which the formal genetics of allorecognition are known is the colonial ascidian *Botryllus* (Oka and Watanabe 1957; Oka 1970; Sabbadin 1962): here, a mating between two individuals heterozygous for allorecognition alleles should produce four classes of allotypes.

Our simulation model, using both 0, 1, 2 and 0, 1, 2, 4 dosage rules (under case 1), with $L = 1$ and $n = 100$ alleles, produces a mean of 3.8 unique compatibility types for both sets of dosage rules. Because of the finite number of alleles and crosses used in the simulation, this value is slightly lower

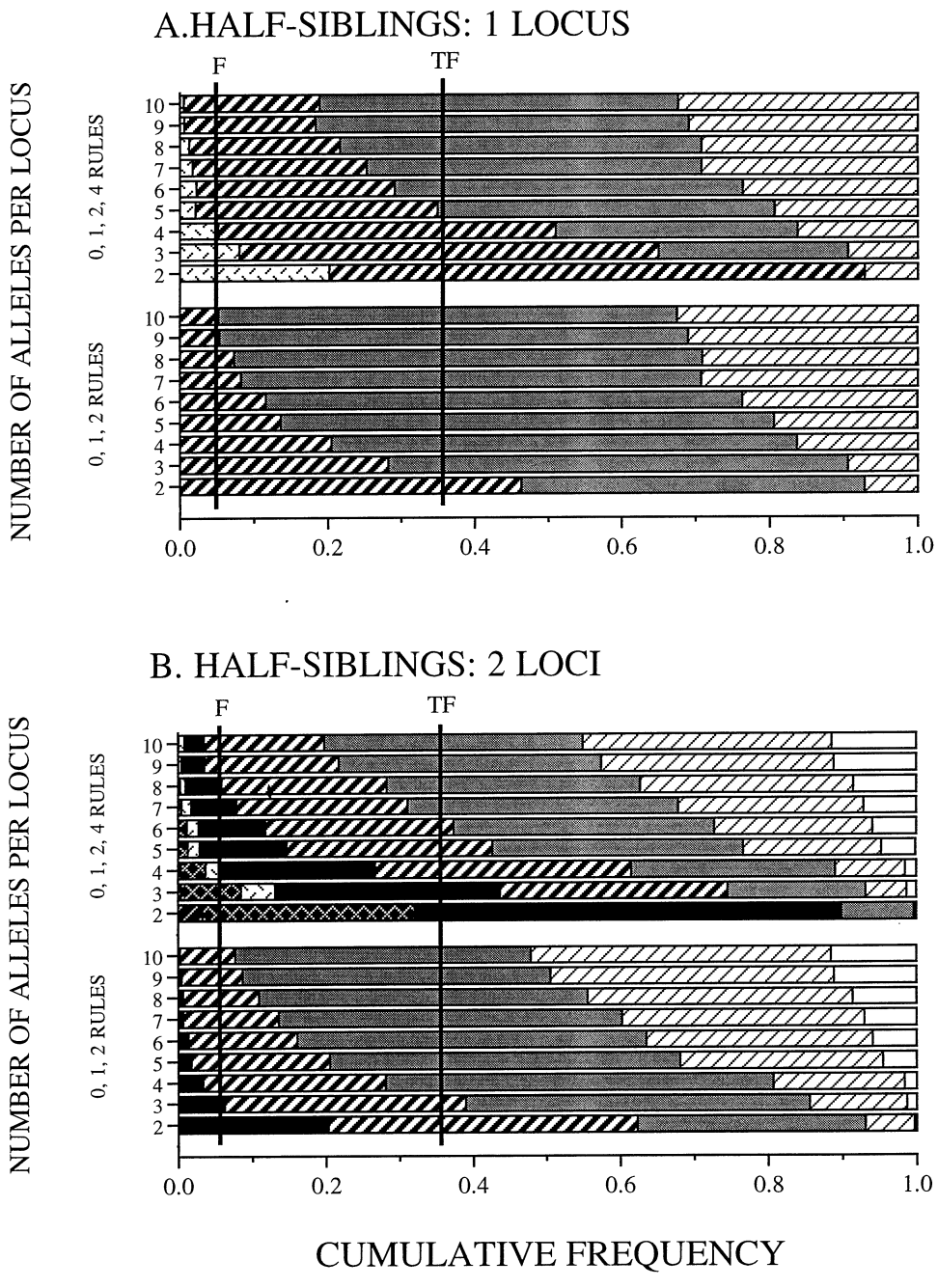

FIG. 2. Composite bar graphs based on genetic simulations showing the expected number of shared alleles, summed across all loci, for half-sib pairings. Each shading pattern corresponds to a different allelic sharing category (see inset key). Each panel (A–E) shows simulated results for 1–5 loci, respectively. The upper set of bars in each panel show results for the 0, 1, 2, 4 dosage rules (see text); the lower set shows results for the 0, 1, 2 dosage rules. The lines labeled "F" and "TF" divide the histograms into regions whose length corresponds to the observed frequencies of fusion, transitory fusion, and rejection in half sibships.

than the predicted value of four. Consequently, not all matings involved parents carrying four different allorecognition alleles. Thus, we can confirm for a limited set of parameter values that our simulation model produces realistic output in terms of overall fusion frequencies, and frequencies of unique compatibility types.

Simulated Patterns of Allelic Sharing in Full and Half Sibships.—Figures 1 and 2 show the output of these simulations as a series of composite bar graphs for full and half sibs, respectively. These are analogous to kingrams (Getz 1981). Each bar in the graphs consists of sections coded with a shading pattern corresponding to a specific number of alleles, summed across all L loci, shared by a pair of siblings. The length of each shaded section of each bar reflects the proportion of full- or half-sib pairs, summed across all of the 200 groups (45 pairings per group) of full siblings or 100 groups (100 pairings per group) of half siblings, that fell into a specific allelic sharing category (i.e., share a given number

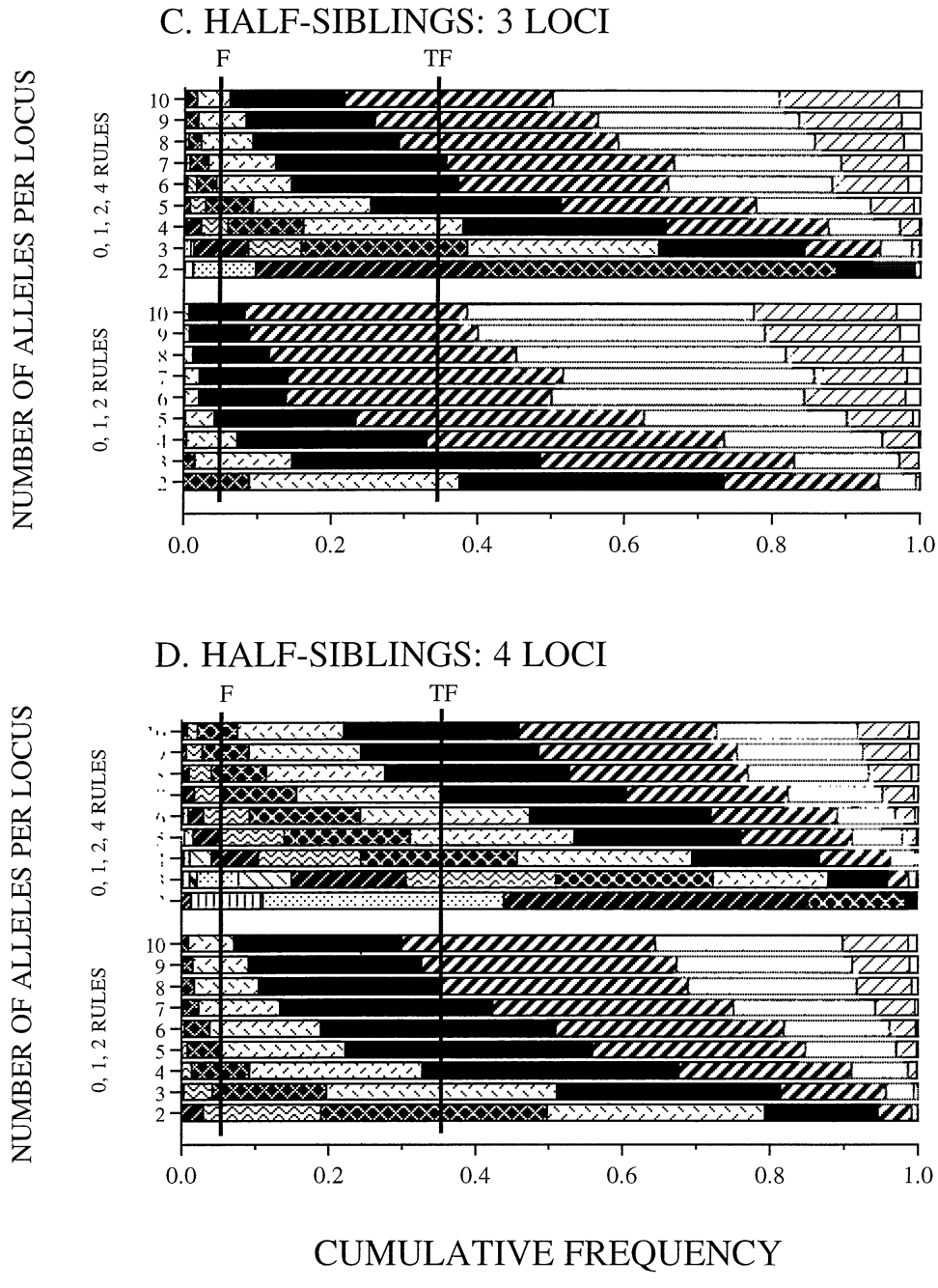

FIG. 2. Continued.

of allorecognition alleles). The different panels in the figure compare results for full- and half-sibling pairs and different values of L. Within a panel, one group of bars shows the results for the 0, 1, 2 dosage rules, with the other group showing the results for the 0, 1, 2, 4 dosage rules. In other words, each bar amounts to a frequency distribution showing the proportion of sibling pairs that share a given number of allorecognition alleles for a specific combination of L, n, and dosage rules (also see Getz 1981).

For all combinations of L and n, the proportion of full- and half-sib pairs in each allelic sharing category shows a roughly symmetrical unimodal distribution, with relatively few pairs sharing few or many alleles. For a given combination of L and n, the modal category of allelic sharing for half sibs is always less than that for full sibs, consistent with differences in relatedness. For instance, under the 0, 1, 2, 4 dosage rules, with $L=5$ and $n=5$, the modal allelic sharing category for full sibs is eight alleles (Fig. 1E), whereas for half sibs, the modal value is six alleles (Fig. 2E). In general, as n increases the modal category shifts toward allelic sharing categories reflecting fewer overall matches. Thus, as n increases (within the limits we explored), so, too, does the per-locus probability that the parents of a given sibship will be heterozygous for different alleles. Consequently, the overall

TABLE 4. Groupings of allelic sharing categories and number of alleles per locus (n) that minimized the total deviation between observed and simulated frequencies of fusion, transitory fusion, and rejection, according to the number of loci (L) and dosage rules. Values represent the proportion of shared alleles (P) necessary to produce a particular allorecognition outcome.

	0, 1, 2 dosage rules				0, 1, 2, 4 dosage rules			
Number of loci (L)	Alleles per locus (n)	Fusion	Transitory fusion	Rejection	Alleles per locus (n)	Fusion	Transitory fusion	Rejection
2	3	$P \geq 0.90$	$0.70 \leq P < 0.90$	$P < 0.70$	7	$P \geq 0.49$	$0.37 \leq P < 0.49$	$P < 0.37$
3	4	$P \geq 0.80$	$0.60 \leq P < 0.80$	$P < 0.60$	5	$P \geq 0.49$	$0.40 \leq P < 0.49$	$P < 0.40$
4	7	$P \geq 0.70$	$0.60 \leq P < 0.70$	$P < 0.60$	7	$P \geq 0.43$	$0.38 \leq P < 0.43$	$P < 0.38$
5	9	$P \geq 0.65$	$0.55 \leq P < 0.65$	$P < 0.55$	6	$P \geq 0.45$	$0.36 \leq P < 0.45$	$P < 0.36$

allotypic similarity among siblings across loci will also decrease, as reflected in the downward "migration" of a given coded section as n increases. Of course, as n gets very large, the magnitude of this effect will decrease. Finally, for a given combination of n and dosage rules, the number of loci, L, has no obvious effect on the position of the modal category relative to the total number of possible matches.

Simulated Frequencies of Compatibility in Full and Half Sibships.—We next identified groupings of allelic sharing categories to yield frequencies of fusion, transitory fusion, and rejection in full and half sibships that best approximated our empirical findings. In so doing, we assumed that sibling pairs sharing relatively large numbers of alleles would be most likely to fuse, those sharing intermediate numbers would undergo transitory fusion, and that pairs sharing relatively few alleles would reject. The vertical lines superimposed on each panel of Figures 1 and 2 divide the frequency distributions into three regions, the lengths of which correspond to the observed mean frequencies of fusion (to the left of the line labeled "F"), transitory fusion (between the lines labeled "F" and "TF"), and rejection (to the right of the line labeled "TF").

For each combination of L, n, and dosage rule, we divided the frequency distribution of allelic sharing categories into three adjacent groups, such that each grouping yielded a total frequency that most closely corresponded to the empirically estimated mean frequencies (from Table 2) of fusion, transitory fusion, and rejection. In practice, we accomplished this for each combination of L and dosage rule by searching for the grouping of allelic sharing categories for each value of n that produced the smallest value when we summed the absolute values of the differences between the simulated and observed frequencies of fusion, transitory fusion, and rejection for both full and half sibs. We selected the grouping and value of n that minimized the deviation, then used that grouping to calculate threshold proportions of overall sharing that could be applied to all values of n for each value of L. For example, under the 0, 1, 2, 4 dosage rules, with $L = 3$ (hence the maximum number of alleles that can be shared equals 12), the closest correspondence between simulated and observed compatibility frequencies occurs with $n = 5$ and the following groupings of allelic sharing categories: $\geq$ 6/12 alleles shared yields fusion; 5/12 alleles shared yields transitory fusion; and $\leq$ 5/12 alleles shared yields rejection. These groupings give simulated frequencies of fusion, transitory fusion, and rejection for full sibs equaling 30.9% (cf. 28.4% from Table 2), 26.6% (cf. 21.4% from Table 2), and 42.5% (cf. 47.2% from Table 2), respectively. For the same set of groupings, the fit of simulated to observed frequencies for half sibs is considerably poorer than for full sibs, with the simulated frequencies of fusion, transitory fusion, and rejections equaling 9.4% (cf. 2.0%), 16.4% (cf. 32.5%), and 74.2% (cf. 65.5%), respectively. The total deviation between all six simulated and observed frequencies with these groupings equals 44.2%.

Table 4 reports the groupings of allelic sharing categories (or proportion of shared alleles, P) that minimize the total deviation between simulated and observed frequencies of fusion, transitory fusion, and rejection. Figure 3 shows the deviations for all combinations of L and n, and both sets of dosage rules, based on this procedure. For the 0, 1, 2 dosage rules, the combination of $L = 3$, $n = 4$, and $P \geq 80\%$ for fusion, produces the smallest difference ($= 0.48$) between the summed observed and simulated frequencies of fusion, transitory fusion, and rejection (Fig. 3A). In general, for all values of L under the 0, 1, 2 dosage rules, $n = 4$ gives the smallest deviation between simulated and observed frequencies. Under the 0, 1, 2, 4 dosage rules (Fig. 3B), the combinations of $L = 2$ and $n = 7$, and $L = 3$ and $n = 5$ (with fusion requiring $P \geq 0.49$), both produce the smallest total deviations ($= 0.42$). With these dosage rules, and for all values of $L > 2$, values of $n = 5$, 6, or 7 yield the smallest deviations between simulated and observed frequencies.

Simulated Compatibility Frequencies between Randomly Drawn Pairs of Genotypes.—We finally used these groupings of allelic sharing categories in a second set of simulations designed to estimate values of fusion, transitory fusion, and rejection between 500 pairs of randomly drawn individuals for all combinations of L, n, and dosage rules. This allowed us to limit further our search to only those combinations of dosage rules, L, and n that gave rejection frequencies approximating our observed frequency of 100% (lower 99% CL = 95.3%) among randomly paired colonies from natural populations.

Under the 0, 1, 2 dosage rules, simulated rejection frequencies do not appear to be very sensitive to number of loci and match or exceed the lower 99% CI of the observed frequency when there are at least six or seven alleles per locus (Fig. 4A). The 0, 1, 2, 4 dosage rules yield a similar pattern (Fig. 4B); however, for $L = 2$, a rejection frequency or more than 95% requires at least 10 alleles per locus.

Simulated Frequencies of Unique Compatibility Types.—The second set of simulations also generated estimates of the frequencies of unique compatibility types within full sibships

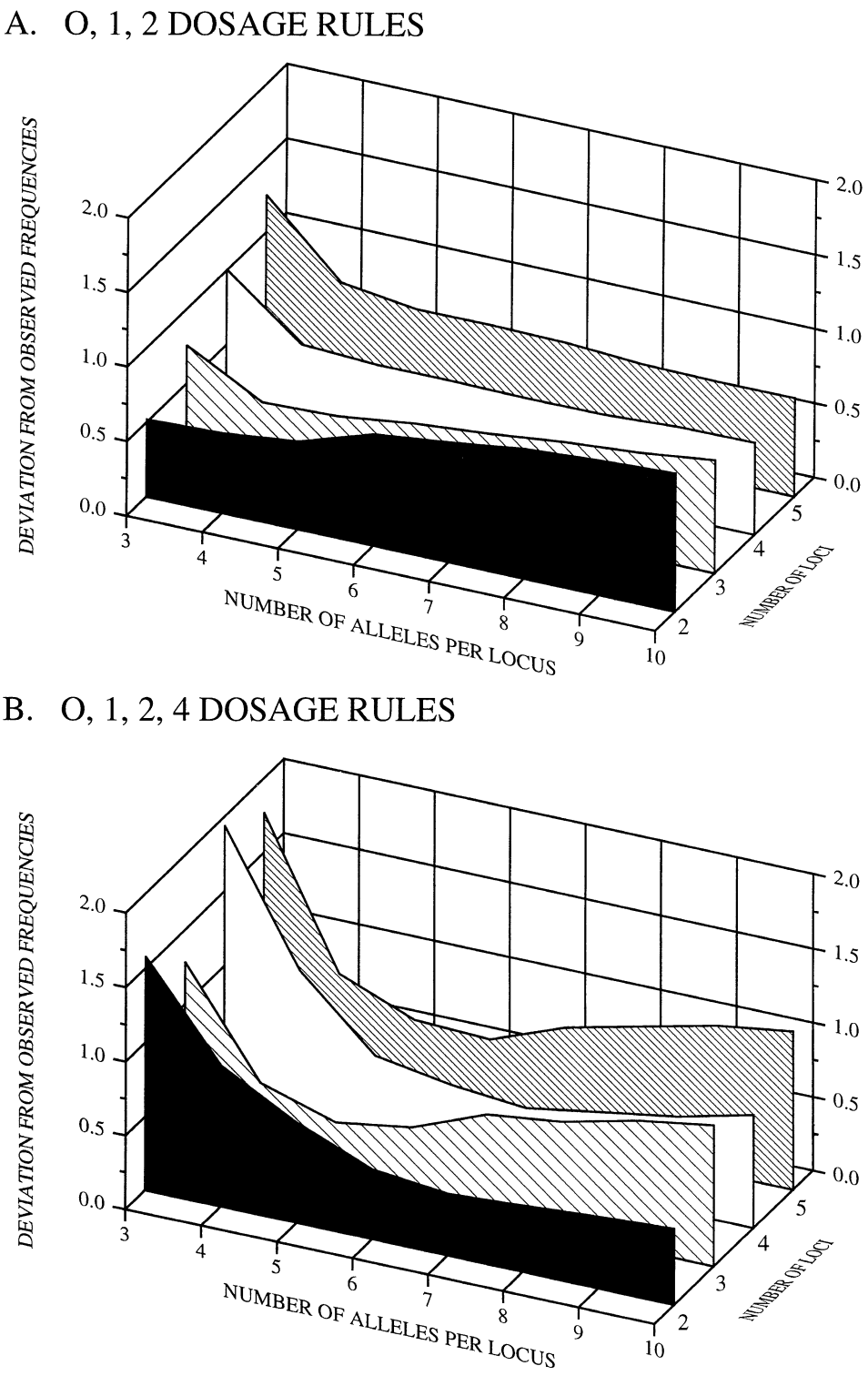

FIG. 3. Summed deviations between simulated and observed frequencies of fusion, transitory fusion, and rejection according to the number of loci and number of alleles per locus conferring allotypic specificity. (A) Deviations for the 0, 1, 2 dosage rules. (B) Deviations for the 0, 1, 2, 4 dosage rules.

(of 10 each) expected when paired with full and half sibs (Fig. 5). The observed values of the frequency of unique compatibility types for the full-sib pairings ranges from 0.875 through 1.0 ($\bar{x} = 0.99$), with most types distinguished by two or more differences in their patterns of compatibility. The simulated values for full-sib pairings, for both sets of dosage rules, increase with both L and n, and approximate the empirical values only when there are at least four loci and a

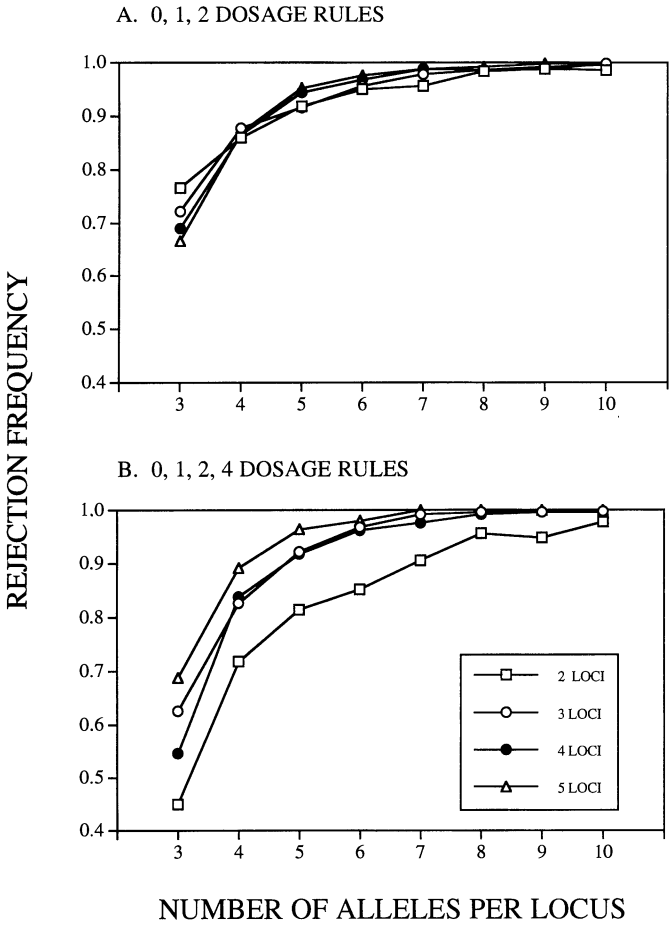

FIG. 4. Simulated rejection frequencies between randomly chosen pairs of individuals, as a function of number of loci and number of alleles per locus. (A) Frequencies for the 0, 1, 2 dosage rules. (B) Frequencies for the 0, 1, 2, 4 dosage rules.

minimum of six alleles per locus, regardless of the dosage rules used (Fig. 5A, B).

For half-sib pairings, the observed frequency of unique compatibility type ranges from 0.60 through 0.875. In contrast to the outcomes of full-sib pairings, the simulated values for half-sib pairings decline with increasing values of n for both sets of dosage rules, although increasing values of L have a less pronounced effect on the frequency of unique compatibility types for the 0, 1, 2, 4 dosage rules (Fig. 5C, D). Overall, values of L = 3–5, with three to five alleles per locus, gave the best fit to observed frequencies. However, the correspondence for half-sib pairings between observed and simulated values under both sets of dosage rules is difficult to evaluate for at least two reasons: (1) we only empirically analyzed two half-sib matrices and (2) because fusion was rare and rejection common (Table 2), half sibs appeared to be far more similar to each other in their interactions with half sibs compared with full sibs, often distinguishable by only a single difference (usually a transitory fusion).

DISCUSSION

This study, along with Mokady and Buss (1996), shows that *H. symbiolongicarpus*, like the vast majority of encrusting, clonal animals, can distinguish self from unrelated conspecific nonself with nearly unerring precision (reviewed in Buss 1987; Grosberg 1988). To the extent that this specificity is genetically controlled, the loci governing invertebrate allorecognition specificity may exhibit polymorphism that rivals, or exceeds, levels recorded at loci in the vertebrate Major Histocompatibility Complex (Potts and Wakeland 1990; Hughes and Nei 1992; Brown and Ecklund 1994; Hedrick 1994), as well as loci associated with gametophytic incompatibility systems in angiosperms (Ebert et al. 1989). High levels of allotypic specificity could, however, result from any number of genetic alternatives, ranging from one or a few loci with tens to hundreds of alleles per locus (characteristic of populations of botryllid ascidians: Milkman 1967; Mukai and Watanabe 1975a,b; Scofield et al. 1982; Grosberg and Quinn 1986; Rinkevich and Saito 1992; Yund and Feldgarden 1992; Rinkevich et al. 1994) to numerous independent loci with relatively low levels of allelic variation (Curtis et al. 1982).

The ability to distinguish among these genetic alternatives, and to circumscribe the rules governing compatibility, determines how the expression of allorecognition-dependent behaviors influences the evolution of allotypic variation. Barring substantial functional and phylogenetic constraints, the formal genetics of allorecognition may also reflect whether selection operates (or has operated) to facilitate distinguishing among different categories of relatedness (kin recognition) or to minimize errors in distinguishing self from nonself. For example, the models of Getz (1981, 1982) and Lacy and Sherman (1983) together show that genetically based kin discrimination controlled by "moderate numbers of alleles (about five) per locus at a moderate number of loci are more efficient [in terms of reliability] than systems with large numbers of alleles at a small number of loci or a small number of alleles at a large number of loci" (Crozier 1987, p. 65). In contrast, for a fixed number of allorecognition alleles or "traits" (sensu Lacy and Sherman 1983), the power to discriminate self from unrelated nonself is highest when allelic variation is distributed among relatively few loci (Curtis et al. 1982; Lacy and Sherman 1983).

Our data show that, as in botryllid ascidians, allotypic identity is not a prerequisite for compatibility in *H. symbiolongicarpus* (also see von Hauenschild 1956): if it were, allorecognition responses in any sampled trio should be strictly transitive (e.g., Neigel and Avise 1983a,b; Grosberg et al. 1984; Grosberg 1992). To the contrary, we found many instances among full and half sibs of intransitive allorecognition responses involving not only fusion, but transitory fusion and aggressive rejection, as well. This observation, along with the high frequency of fusion between parents and offspring, suggests that fusion does not require complete allotypic identity (reviewed in Grosberg 1988), and that allorecognition assays cannot fully portray clonal structure in *H. symbiolongicarpus*, or any other clonal organism unless fusion depends on genotypic identity.

This study also generally confirms prior studies of the inheritance of allotypic specificity in *Hydractinia*, in showing that the likelihood of tissue fusion declines according to the relatedness of interacting pairs (e.g., Teissier 1929; Crowell 1950; Hauenschild 1954, 1956; Müller 1964; Ivker 1972;

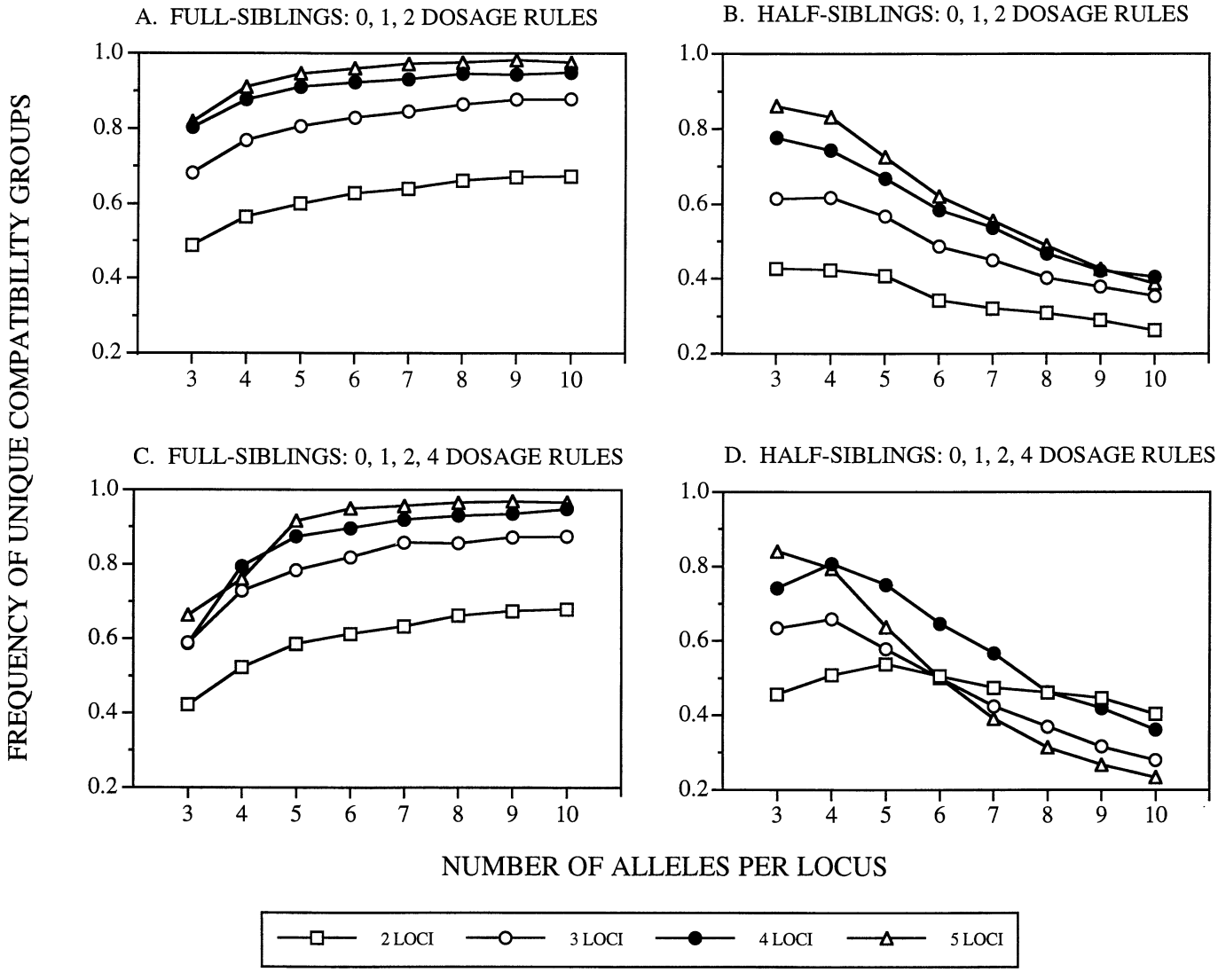

FIG. 5. Frequency of unique compatibility types expected in full sibships (A and B) and half sibships (C and D) according to number of loci, number of alleles per locus, and dosage rules.

Mokady and Buss 1996). Previous attempts to go beyond this state of affairs in *Hydractinia* (1) employed relatively few compatibility tests, making it difficult to distinguish statistically the predictions of alternative genetic models (e.g., von Hauenschild 1954, 1956; Müller 1964; Ivker 1972; Mokady and Buss 1996); (2) used offspring ultimately derived from a few or a single matings in compatibility tests, minimizing the likelihood of identifying all loci that are segregating in a population for allorecognition (e.g., Mokady and Buss 1996), or (3) failed to score the full spectrum of outcomes between conspecifics (e.g., Teissier 1929; Crowell 1950; von Hauenschild 1954, 1956; Müller 1964; Ivker 1972; Mokady and Buss 1996). As such, these investigations could not thoroughly reveal the distribution of allotypic diversity within and among loci, or identify patterns of allotypic similarity among relatives. Given the widespread technical obstacles to formal genetic analysis of allorecognition in virtually all clonal invertebrates (including *Hydractinia*), we sought to distinguish among alternative genetic mechanisms and rules of matching that could produce such specificity by comparing observed patterns of inheritance of allorecognition responses among full and half siblings with expectations from simple genetic simulations.

The single-locus, multiple codominant allele system (with a single shared allele producing fusion) that appears to control the acute fusion/rejection response in botryllid ascidians is currently the simplest, empirically supported genetic model for invertebrate allorecognition. Mokady and Buss (1996) recently argued that a comparable system operates in *H. symbiolongicarpus*. One would therefore expect that in a highly polymorphic population, in a mating between two unrelated individuals from the field (which are presumably heterozygous for different alleles), approximately 25% of full sibs would not share an allele at the locus, and therefore reject each other. Similarly, 50% of half sibs would lack a shared allele and thus reject one another. With fewer alleles, or in a mating between relatives, rejection frequencies would be lower still. Mokady and Buss (1996) did not explicitly report

compatibility data for full sibships and half sibships. However, we **consistently** found rejection frequencies in full and half sibships that substantially exceeded the predictions of the model proposed by Mokady and Buss (1996). We therefore considered more complex genetic systems.

To the extent that the structure and assumptions of the simulations reflect the genetics of allorecognition in *H. symbiolongicarpus*, several lines of evidence argue that along the continuum of genetic models that could confer allotypic specificity, relatively few loci with a moderate number of alleles govern allotypic specificity in this species. First, if many (i.e., > 10) independent loci with relatively few alleles per locus governed specificity, then all siblings should have comparable patterns of allorecognition response (Crozier 1987; Barnard 1990) and should be equally similar (or dissimilar) in their patterns of allorecognition response. At the other extreme, if one locus (as in botryllid ascidians) or a few loci with many alleles per locus confer high levels of specificity (at the level of the population), then there should be groups (representing distinct allotypes) within sibships, each of which exhibits a fairly distinctive pattern of allorecognition response to other groups. Comparison of the observed to randomly generated distributions of similarities in patterns of allorecognition response among full sibs shows that the observed variances were significantly and consistently higher than the randomly generated variances. This, in turn, indicates that there are distinguishable groups of similar allotypes within sibships of *H. symbiolongicarpus*, a pattern expected if relatively few loci conferred specificity (Crozier 1987).

Second, the genetic simulations revealed for both dosage rules that two or three loci, with 3–7 alleles per locus consistently minimized the differences between observed and simulated values of compatibility frequencies in full and half sibships. More specifically, the smallest deviations occurred under the 0, 1, 2, 4 dosage rules, when $L = 2$ and $n = 7$, or $L = 3$ and $n = 5$. Comparison of observed to simulated frequencies of (1) unique compatibility types in full-sib matrices and (2) rejection frequencies in random pairings indicates that at least three loci, with seven (or more) alleles per locus produces the best match. With more loci, or more alleles per locus, the fit between observed and simulated values generally worsened (unpubl. simulations), an outcome consistent with the existence of distinguishable allotypic groupings within sibships.

There is an alternative approach to estimating the number of loci controlling allorecognition specificity, based on observed fusion frequencies between full sibs and half sibs. Assume, as in botryllid ascidians, that paired individuals must share at least one allele at each of L loci in order to fuse ("partial matching" sensu Curtis et al. 1982) and that there is essentially an infinite number of alleles in a population (also see the "individualistic model" of Crozier and Dix 1979). The probability that two full sibs from a mating between unrelated parents will share at least one allele at a locus that is identical by descent (ibd) will then be 0.75, whereas the probability that a pair of half sibs will share at least one (and only one) allele ibd will be 0.50. The probability that full sibs will be fusible is therefore $(0.75)^L$, and for half sibs $(0.5)^L$. Given that the mean observed fusion frequencies among full-sib and half-sib *H. symbiolongicarpus* equaled 0.284 and 0.02, respectively, the corresponding values of $L = 4.37$ and 5.64. Substitution of an integer value of $L = 5$ into the original expressions gives an expected fusion frequency of 0.237 (cf. $L = 4$, $L = 6$, and $L = 8$, which yield frequencies of 0.316, 0.178, and 0.100, respectively) for full sibs and 0.031 (cf. $L = 4$, $L = 6$, and $L = 8$, which yield frequencies of 0.063, 0.016, and 0.004, respectively) for half sibs. Thus, with values of L ranging from 4–6, the estimated fusion frequencies for full sibs falls within the 95% confidence intervals of the observed mean, and that for half sibs is remarkably close to the observed value of 2%. Under an infinite alleles assumption, with eight or more loci, the correspondence between observed and theoretical values worsens considerably. If five loci confer allotypic specificity in *H. symbiolongicarpus*, then under the partial matching rules decribed above (which are quite similar to those under the 0, 1, 2, 4 dosage rules: Table 4), five equally frequent alleles per locus would produce a rejection frequency between randomly paired individuals of 93.2%, and eight alleles per locus would yield 98.8% rejections (Curtis et al. 1982). Taken together, the two modeling approaches, using somewhat different allelic sharing algorithms, agree that a moderate number of allorecognition loci (3–7), with a minimum of five to seven equally frequent alleles per locus, confer allotypic specificity in *H. symbiolongicarpus*.

If five loci carrying six equal frequent alleles per locus governed allotypic specificity in *H. symbiolongicarpus,* then in a large population there could be as many as 4.08×10^6

$$\left(\text{i.e., } \left(\frac{n(n+1)}{2}\right)^L\right)$$

unique allotypes. Such high levels of allotypic diversity could, at least in part, represent a balance between the generation of new allotypic variants by mutation and their loss via drift (Neigel 1988; Brown and Eklund 1994). Alternatively, some form of negative frequency-dependent or overdominant selection, acting either pleiotropically (e.g., through host-pathogen coevolution: Bremermann 1980; Potts and Wakeland 1990; Hamilton et al. 1990; Parham and Ohta 1996) or directly, could foster the persistence of allotypic variation. The crucial role of allorecognition systems in mediating the nature and outcomes of intraspecific spatial competition in *Hydractinia,* and many other sessile, clonal invertebrates (Buss 1990), suggests that natural selection acting directly on the ability to distinguish conspecific self from nonself (Getz 1982; Grosberg and Quinn 1988; Yund and Feldgarden 1992), or among classes of kin (Lacy and Sherman 1983; Grosberg 1988) plays a critical role in the maintenance of allotypic polymorphism in these organisms.

In the case of cnidarians such as *Hydractinia*, in which allorecognition systems determine whether individuals will fuse or fight, frequency-dependent selection acting at the level of the individual can favor the accumulation of allotypic variation when the fitness costs of intergenotypic fusion exceed the benefits of aggression (Grosberg and Quinn 1988). Several studies suggest that intergenotypic fusion may enhance competitive ability and lower age at first reproduction for one, or both members of a chimera. Fusion may also allow partners to monopolize a shell, and prevent the subsequent colonization by competitors, more rapidly than a single col-

ony could. However, there may also be substantial costs to intergenotypic fusion in terms of intraspecific parasitism, pathogen transmission, and developmental instability (Mukai 1976; Sabbadin and Zaniolo 1979; Buss 1982, 1987; Sabbadin and Astorri 1988; Shenk and Buss 1991; Grosberg 1992; Rinkevich and Weissman 1992a,b). Similarly, agonistic behavior is one of the principal ways that clonal cnidarians can acquire space occupied by other organisms; however, the production and deployment of structures that mediate aggressive interactions can exact substantial costs in terms of future growth and reproductive potential (Buss 1990; Buss and Grosberg 1990; Ayre and Grosberg 1995, 1996). No studies comprehensively quantify these costs and benefits. Moreover, because the genetic polymorphism that characterizes most invertebrate allorecognition systems should restrict fusion to close relatives and aggression to more distantly related individuals, the costs and benefits of both types of behavior must be adjusted according to kinship of the interactants (Buss and Green 1985; Grosberg and Quinn 1986; Reeve 1989; Grafen 1990), an unknown quantity in most instances.

From a genetic perspective, the allorecognition system of *H. symbiolongicarpus* has attributes that imply that both individual and kin selection play important roles in the evolution of allotypic specificity. If fusion with any other individual, regardless of kinship, were costly (or aggression beneficial), then, given a fixed number of allotypes, selection should favor the evolution of an allorecognition system that requires full, rather than partial, allotypic matching for compatibility (Curtis et al. 1982; Lacy and Sherman 1983). On the other hand, if the costs and benefits of fusion or aggression depended on the relatedness of interactors, then selection should favor the evolution of an allorecognition system that minimizes errors in classifying individuals according to relatedness, but does not necessarily require allotypic identity for fusion. Getz (1981, 1982), along with Lacy and Sherman (1983), showed that with a discrete character, "self-referent" model and a fixed number of "traits" (alleles in the case of our model), the genetic system that minimizes recognition errors between full sibs, half sibs, and unrelated individuals entails the distribution of allotypic variation across roughly five loci.

The combination of high allotypic diversity and the requirement that to be compatible individuals share a substantial proportion of allorecognition alleles virtually guarantees that unrelated individuals will be incompatible, and suggests that self/nonself discrimination is an important component of the evolution of allotypic specificity in *H. symbiolongicarpus* (Curtis et al. 1982; Grosberg 1988). However, the partial matching system of *H. symbiolongicarpus*, like that of *Botryllus*, permits fusion between full sibs at a relatively high frequency, while limiting half-sib fusion probabilities to approximately 2%. Moreoever, we estimated that allotypic diversity could be ascribed to between three and six loci in *H. symbiolongicarpus*, roughly the same number of loci that minimizes error in discriminating between full and half sibs (Lacy and Sherman 1983). Thus, the genetics of allorecognition in *H. symbiolongicarpus* suggest that the ability to distinguish full-sibs (and parents) from half-sibs and more distantly related individuals (and not just self from nonself) is selectively critical.

From an ecological perspective, the incidence of multiply colonized hermit crab shells and the probabilities of interacting with kin versus unrelated individuals (along with the costs and benefits of fusion and aggression) will dictate the strength of selection acting to promote the evolution of allotypic diversity, and whether selection favors discrimination among different classes of kin or self from nonself (Hamilton 1964). At the peak of the recruitment season in the Barnstable Harbor population of *H. symbiolongicarpus*, 113 of 306 (37%) shells carried more than one *H. symbiolongicarpus* recruit (unpubl. obs.), a figure comparable to estimates from other sites (Yund et al. 1987; Yund and Parker 1989).

The association of *H. symbiolongicarpus* colonies with a mobile host (hermit crab–occupied gastropod shells) implies that there should be little persistent kin structure, at least at the level of the population. Consequently, allotypes should be distributed fairly randomly **among** shells. At the level of **individual** shells, however, three aspects of the reproductive ecology of *H. symbiolongicarpus* could promote the cosettlement of members of the same clutch. First, female *H. symbiolongicarpus* synchronously release hundreds to thousands of ripe eggs (Bunting 1894; Yund et al. 1987). Second, eggs, developing embryos, and larvae quickly sink, and remain benthic even in strongly aerated containers (unpubl. obs.). Finally, the demersal planulae larvae can attach to (and metamorphose on) a passing hermit crab shell within 24 h of fertilization (Müller 1973), leaving little opportunity for extensive dispersal prior to metamorphosis.

Because fertilization occurs externally in *H. symbiolongicarpus*, there is ample scope for multiple paternity within a clutch. Depending on the frequency of multiple paternity, some members of a clutch will be full sibs, whereas others will be half sibs. Thus, when two, or more, siblings settle on the same shell, some interactions will involve full sibs, whereas others will involve half sibs. To the extent that fusion represents altruistic behavior, or the costs and benefits of fusion and aggression vary according to the relatedness of interacting genotypes, cosettlement of full sibs, half sibs, and unrelated individuals on a single shell (which they compete to monopolize) sets the stage for the operation of kin selection and the evolution of an allorecognition system that distinguishes not only self from nonself, but also full sibs from half sibs. At this point, we have no direct evidence that cosettling *H. symbiolongicarpus* larvae are more closely related (and have a higher probability of fusing) than would be expected in a well-mixed population. In general, species with limited dispersal and a high probability of encountering kin, should have allorecognition systems whose genetics promote discrimination among different classes of kin. Conversely, species with more extensively dispersing propagules, and hence with a relatively low probability of encountering kin, should have allorecognition systems whose genetics favor discimination of self from conspecific nonself.

An alternative view holds that fusion with self alone is beneficial (as might be the case when a colonial organism grows around obstructions or is damaged), with selection favoring the ability to distinguish self from nonself, rather than among different classes of kin (Waldman 1987; Grafen

1990; Feldgarden and Yund 1992). Thus, the partial matching allorecognition system of *H. symbiolongicarpus*, which permits fusion between about 30% of full sibs, could simply reflect functional and phylogenetic constraints on the nature of the molecules that confer allotypic identity, and the recognition mechanisms that distinguish among allotypes, rather than the operation of kin selection on the recognition system. However, in some sponges (e.g., Neigel and Avise 1983a; Wulff 1986), cnidarians (e.g., Neigel and Avise 1983b), and ascidians (Raftos and Briscoe 1990), full allotypic identity appears to be required for fusion, demonstrating that both full-matching and partial-matching allorecognition systems can evolve in the same phyla.

In contrast to the progress in documenting the phylogenetic distribution of invertebrate allorecognition systems (reviewed in Grosberg 1988), our understanding of the moleculuar, cellular, genetic, and ecological processes that govern the evolution of allorecognition systems continues to lag far behind (Buss 1982, 1987; Grosberg 1988; Potts and Wakeland 1990; Brown and Eklund 1994). As our knowledge of (1) the formal and molecular genetics of allorecognition (Getz 1981; Lacy and Sherman 1983; Crozier 1987; Grosberg 1988; Ratnieks 1991; Mokady and Buss 1996); (2) the genetic structure of natural populations (Jackson 1985, 1986; Grosberg 1991; Hughes et al.1992; Knowlton and Jackson 1993; Nauta and Hoekstra 1994); (3) the costs and benefits of fusion and aggression (Grosberg 1988; Nauta and Hoekstra 1994; De Boer 1995); (4) mutation rates to novel allotypes (Yund and Feldgarden 1992); and (5) effective population size (Hedgecock 1994) grows, it will become possible to evaluate the relative contributions that selective and nonselective processes, as well as individual and kin selection, make to the evolution of allorecognition systems and allotypic specificity in sessile, clonal organisms.

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