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GRAFT REJECTION IN SPONGES

GENETIC STRUCTURE OF ACCEPTING AND REJECTING POPULATIONS

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Graft rejection frequencies in a population of the sponge *Ectyoplasia ferox* are reported in relation to the complexity of the histocompatibility system in this species. The frequency of graft acceptance is high but we show that, despite assumptions in the literature to the contrary, this does not imply that sponges accepting each other's grafts are genetically identical. This paper reports that graft-accepting pairs of sponges can have dissimilar plasmalemmal proteins. In addition, a theoretical analysis is presented of the types of histocompatibility systems that would account for the present results and those of others. We concluded that there is no evidence that sponges have highly polymorphic histocompatibility systems. The paper also reports on the histology of graft rejection and discusses some of the ecological relevance of the findings.

Hildemann et al. (*1, 2*) have suggested that graft acceptance or rejection between members of natural populations of invertebrates can be used to determine whether or not separate colonies are the genetically identical products of asexual reproduction. In studies of the sponge *Callyspongia diffusa* and the coral *Montipora verrucosa*, they found no cases of graft acceptance in 480 and 890 pairings, respectively. From these data they argued that there must be such extensive polymorphism for histocompatibility molecules that essentially all colonies arising from separate sexual processes will be histoincompatible ("the uniqueness of the individual principle").

Genetic control of quite modest complexity could give compatibility results of the type described by Hildemann et al. (*1, 2*), however. Imagine a histocompatibility system of L loci with A alleles at each locus. Furthermore, assume that (1) all alleles have the same frequency; (2) the loci are in linkage equilibrium; and (3) each tested individual is a separate, diploid sexual product. The probability of two compatible types encountering one another (or being chosen for a grafting experiment) depends on the criteria governing fusion. If fusion occurs whenever two individuals share one or both alleles at each histocompatibility locus, then the overall probability of fusion is $((4A^2 - 6A + 3)/A^3)^L$. If fusion requires that both alleles at each locus be shared, then the probability drops to $((2A - 1)/A^3)^L$.

Table 1 presents these probabilities under a variety of simple genetic control systems for each of the two fusion criteria. If one assumes the more extreme, second criterion, then one could expect the results reported by Hildemann et al. (*1, 2*) with a genetic system of four loci with four alleles at each locus. One noteworthy feature of the calculations based on the second criterion is that very small decreases in the number of loci or the number of alleles per locus within this range can generate substantially higher probabilities of finding histocompatible colonies originating from separate sexual events.

The data available on the percentage of allograft acceptances in natural populations of invertebrates are in fact extremely variable. The values reported include 0.7% for 1479 colonies of the gorgonian *Eunicella stricta* (*3*), 32% for 50 non-neighbors in the sponge *Hymeniacidon sp.* (*4*), and 82% for 11 colonies of the hydrozoan *Hydractinia echinata* (*5*). The variability revealed in these studies is difficult to interpret, however, because of the tremendous differences in geographical range in the source material. The study by Curtis (*4*) was in an area of about 1 x 10^4 m^2. Hildemann et al. (*1, 2*), in contrast, have consistently paired individuals from different areas, several kilometers apart. Thus, one might argue that the high graft acceptance probabilities reported by Curtis (*4*) reflect only the fact that there is likely to have been considerable asexual propagation within a limited geographical region (*6*). On the other hand, the low probability of graft acceptance between individuals from widely separated areas indicates that the populations do not share any histocompatibility alleles, but cannot be used to infer that the number of alleles within any one population is necessarily high. It is thus impossible to establish the level of genetic polymorphism for histocompatibility molecules from grafting data alone; one must have an independent measure of the genetic structure of the populations being studied.

Here we present data on grafting experiments using the marine sponge *E. ferox*, along with electrophoretic data on their plasmalemmal proteins. The logic behind this study was that any indication of protein differences between pairs of individuals showing allograft acceptance would disprove Hildemann's hypothesis that histocompatibility implies clonal identity. *E. ferox* was chosen both because of its abundance on the reefs of Discovery Bay, Jamaica, and because we have never detected any eukaryotic contaminants (commensals, parasites, adventitious organisms) in this species. Plasmalemmal proteins were analyzed because contaminating prokaryotic proteins could be removed prior to electrophoresis. (Prokaryotic plasmalemmae are normally surrounded by a cell wall resistant to the methods of cell disruption used, and they also differ in density from eukaryote plasmalemmae.)

MATERIALS AND METHODS

Grafts were carried out using SCUBA equipment on in situ *E. ferox* at 20- to 23-m depth on the west edge of the sand channel at Mooring 1, Discovery Bay, Jamaica. Sponges of this species occurred in seven main groups over a distance of about 25 m at this site, see Figures 1 and 2. Each group occupied an area of about 2 m^2. Noncoalescence (nonconfluence) defined by van de Vyver (*7*) was observed to occur naturally between four pairs of individuals. In addition, sponge material for grafting into these sponges was obtained from the sites known as Rio

TABLE 1. Probabilities of two identical histocompatibility types encountering one another for genetic control systems of one to four loci (L) with varying number of alleles (A) at each locus[a]

L = 1			L = 2			L = 3		L = 4	
A	C1	C2	A	C1	C2	C1	C2	C1	C2
2	8.8×10^{-1}	3.8×10^{-1}	2	7.7×10^{-1}	1.4×10^{-1}	6.7×10^{-1}	5.3×10^{-2}	5.9×10^{-1}	2.0×10^{-2}
5	5.8×10^{-1}	7.2×10^{-2}	3	6.9×10^{-1}	3.4×10^{-2}	4.7×10^{-1}	3.4×10^{-3}	3.7×10^{-1}	1.2×10^{-3}
15	2.4×10^{-1}	8.6×10^{-3}	4	4.5×10^{-1}	1.2×10^{-2}	3.0×10^{-1}	1.3×10^{-3}	2.0×10^{-1}	1.4×10^{-4}
45	8.6×10^{-2}	9.8×10^{-4}	5	3.4×10^{-1}	5.2×10^{-3}	2.0×10^{-1}	3.7×10^{-4}	1.2×10^{-1}	2.7×10^{-5}

[a] Results for two fusion criteria (C1, C2) are presented (see text).

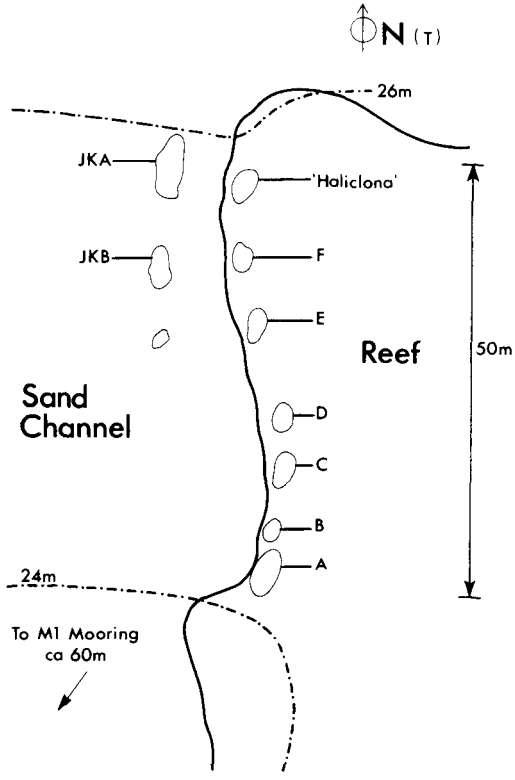


FIGURE 1. Map of area in the West Fore Reef area, Discovery Bay, Jamaica in which the main local groups of sponges were located. The letters refer to the groups with the same notation in the text of this paper. Survey approximate.

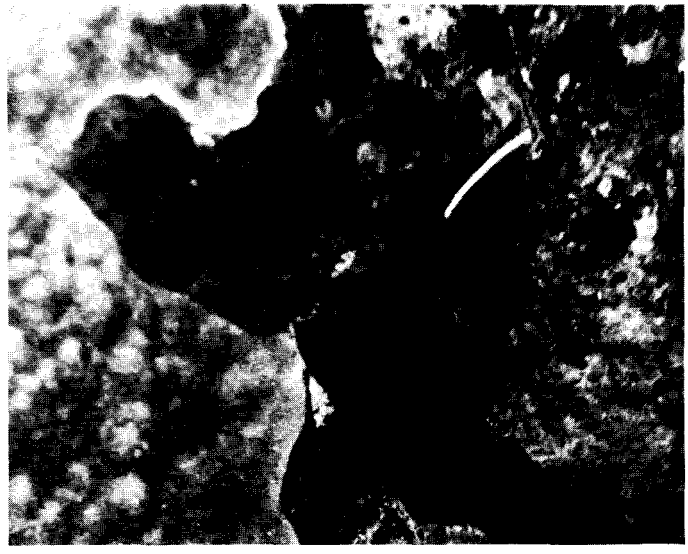


FIGURE 2. An underwater photograph of part of local group A showing "accepted" and "rejected" grafts 5 days after the operations. One sponge, A2, appears as a dark patch with prominent osculae and a white plastic tag in it. An accepted graft can be seen very faintly just to the lower left of the tag and is clearly fused into the host very well, a rejected graft can be seen in the lowest lobe of this sponge showing clear demarcation from the host.

Bueno (4 km west distant), LTS (0.25 km west distant), East Fore Reef 80 (0.5 km east distant), and Pear Tree Bottom (5 km east distant). The grafting technique was identical to that described by Curtis (*4*). Acceptance or rejection was scored at 5 days and again at 7 to 9 days. Acceptances showed fusion of pinacoderms within 4 days, while rejected grafts failed to form any fusion with the host. This criterion does allow the possibility that inept grafts which fell out for mechanical reasons might be scored as rejections but since no autografts fell out and all scored rejected grafts were seen in place for at least 4 days, this possibility seems unlikely.

Plasma membrane preparations for polyacrylamide gel electrophoresis (PAGE) were prepared from freshly collected material obtained from individuals with known graft acceptance and rejection histories. The sponges were broken into fragments of approximately 1 cm side and dissociated by the EDTA method introduced by Curtis (*8*). The single cells were centrifuged in calcium and magnesium-free seawater at 1000 *g* for 5 min and the supernatant was discarded. The cell pellet was mixed with 3 volumes of homogenization medium (sucrose, 0.32 M; KCl, 0.001, $MgCl_2$, 0.0001 M; Tris, 0.01 M, pH 8.0) and homogenized with a Dounce homogenizer at 4 C. Aprotinin was added at 50 K units/ml^{-1} and mercaptoethanol to 0.01 M to reduce proteolysis. The homogenate was then centrifuged at 10,000 *g* for 1½ hr at 4 C and the supernatant then frozen in Dry Ice and shipped back in this storage to the United Kingdom. Upon receipt, the supernatants were thawed and diluted 4-fold with 0.01 M Tris-HCl (pH 8.0) and centrifuged at 100,000 *g* for 2 hr. The pellets were collected as crude membrane fractions. Electron microscopy of specimens from other species of sponge processed by this technique shows that typical plasmalemmas, uncontaminated by other organelles, are prepared by this technique.

The crude membrane factions were run on PAGE systems using the technique of Laemmli (*9*) at gel concentrations in the gradient gel between 6 and 12.5%. Care was taken to make loadings as equal as possible, about 40 μg of protein being loaded per track. Fibronectin, bovine serum albumin, and cytochrome *c* were used as molecular weight markers. The gels were fixed with acetic acid-methanol mixtures when Coomassie blue staining was used and with 10% glutaraldehyde when the silver staining method attributable to Oakley et al. (*10*) was applied.

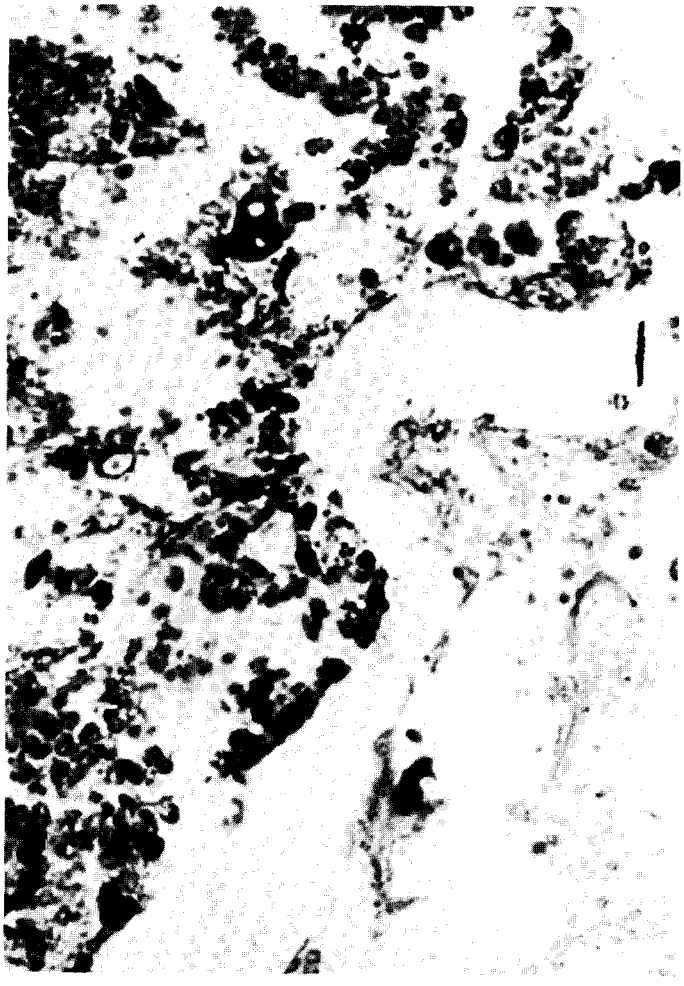


FIGURE 3. Photomicrograph of a junction between host and graft which has been rejected four days after grafting. Mallory trichrome after formol-seawater fixation. The graft has been effectively depleted of cells and only collagenous elements are left. The host contains choanocyte chambers and many other cells which stain darkly. Bar is 20-μm long.

RESULTS

Grafts were made between 121 individuals of *E. ferox*. Autografts were carried out on 45 individuals. After 4 days all autographs and a proportion of "allografts" were accepted. The tissues of accepted grafts joined those of the host with no gap, collagen barrier, or sign of discoloration between graft and host. Grafts were observed at time intervals up to 5 weeks after grafting and no sign of early fusion followed by late rejection was seen. When rejection took place, it was clearly visible within 4 days of grafting. Rejection was assessed as a failure of the graft to fuse with the host with a clear gap between the two. There was no discoloration of the host, although on occasion the graft became slightly yellower. The majority of rejected grafts survived in place for the whole period of observation (up to 5 weeks) but some fell out after a few days.

Histological examination of the grafts and surrounding host tissues proved difficult because of the heavy spiculation of this species. However, examination shows that unlike the situation in Hymeniacidon (Kerr, unpublished data), no collagen barrier is deposited between graft and host. Rejected grafts show (by 3 days) a decellularized zone near the contact with the host, while accepted grafts do not show this (see Figs. 3 to 5). Possibly the cells have migrated away from the contact zone in this decellularization. Choanocyte chambers seem to be the first cell structures to disappear in rejected grafts, i.e., by 2 days (Fig. 5). At this time the contact zone with the graft has pinacoderm, archaeocytes, and probably collenocytes. Later the area becomes totally decellularized. There is no clear evidence of cytolysis in the decellularized zones.

The number of graft acceptances and rejections is set out in Table 2. Overall, slightly less than one-half of the allografts were accepted. All grafts from noncoalescent neighbors were rejected, a situation observed in other sponge species, e.g., Hymeniacidon (*4*). Grafts derived from sponges geographically remote to their hosts were rejected more frequently than those derived from sponges within the same "local group" (Fisher exact probability test, $P = 0.04$). There was also a parallel but nonsignificant trend ($P = 0.13$) of decreasing acceptance frequency with increasing distance between donor and host for grafts done between local groups. Seven of the 62 pairs of reciprocal grafts made between members of the same local group gave conflicting results (i.e., one acceptance and one rejection).

FIGURE 4. Choanocyte chambers in a healthy graft (2 days) that has been accepted; 20-μm bar.

FIGURE 5. Choanocyte chambers in a rejected graft after 2 days. Note decellularization; 20-μm bar.

TABLE 2. Graft acceptance and rejection in *E. ferox*

	Accept	Reject
All grafts		
Autografts	45	0
Noncoalescent pairs	0	7
Local group "allos"	47	49
Distant groups		
Separation < 100	6	11
Separation > 100	0	8
Paired grafts (included in above)		
Both accept	30	0
Both reject	0	25
1 accept 1 reject	7	7

Statistical analysis of "local" verus "distant" acceptance and rejection

Local distant comparison

	Acc.[a]	Rej.
Local	47	49
Distant	6	19

Fisher's exact probability test, P = 0.0403

"Near" and "far distant" comparison

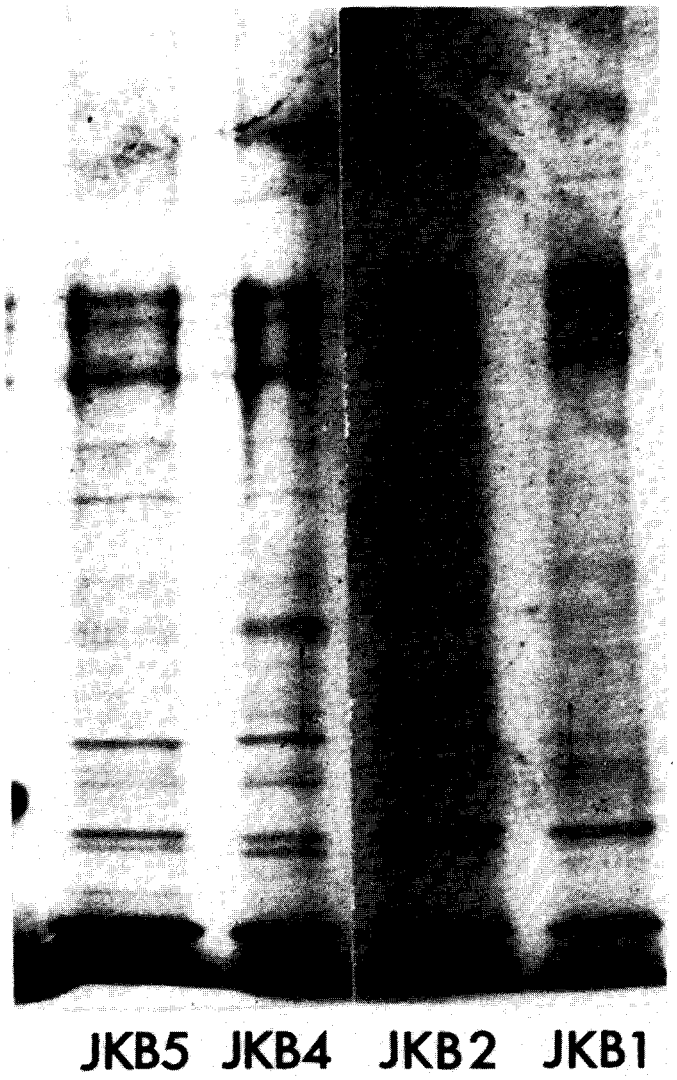
	Acc.	Rej.
<100 m	6	11
>100 m	0	8

Fisher's exact probability test, P = 0.1290

[a] Acc., accept; rej, reject.

PAGE of plasmalemmal proteins from six groups of accepting sponges, in the Mooring I area, was carried out. The respective acceptance groups were: A1 + A2, Bβ + BB + B1 + B4 + E2 + E4, B6 + B7, D1 + D2, and JKB1 + JKB2 + JKB4 + JKB5. Each letter group identifies a local group (see Fig. 1). The acceptance groups show that acceptance can occur between relatively remote groups.

Examination of the electrophoretic results (see Figs. 6 to 10 for example) shows that, as might be expected, the individual sponges give a range of molecular weight bands that are very



FIGURES 6 to 10. Photographs of PAGE tracks of plasmalemmal extracts from sponge individuals. Coomassie blue staining. Gel concentration ranges 6 to 12.5%. The lower molecular weight ranges of the tracks are shown, 60K being near the top of each photograph and 15K very close to the lower ends. In two cases the photographs have been processed by the elimination of tracks that were underloaded, but in every case each photograph is of part of a single gel. These photographs show the appreciable nonidentity in plasmalemmal proteins between sponges which accept grafts from each other. The arrows marked 1 or 2 on the sides of the tracks refer to some of the major differences in banding. The ls refer to bands that appear to be present in one and absent in another pair or triplet of accepting sponges, 2s refer to bands that may have shifted. Not all differences are indicated by arrows.

FIGURE 6. Acceptance group JKB, sponges 1, 2, 4, and 5. Note differences.

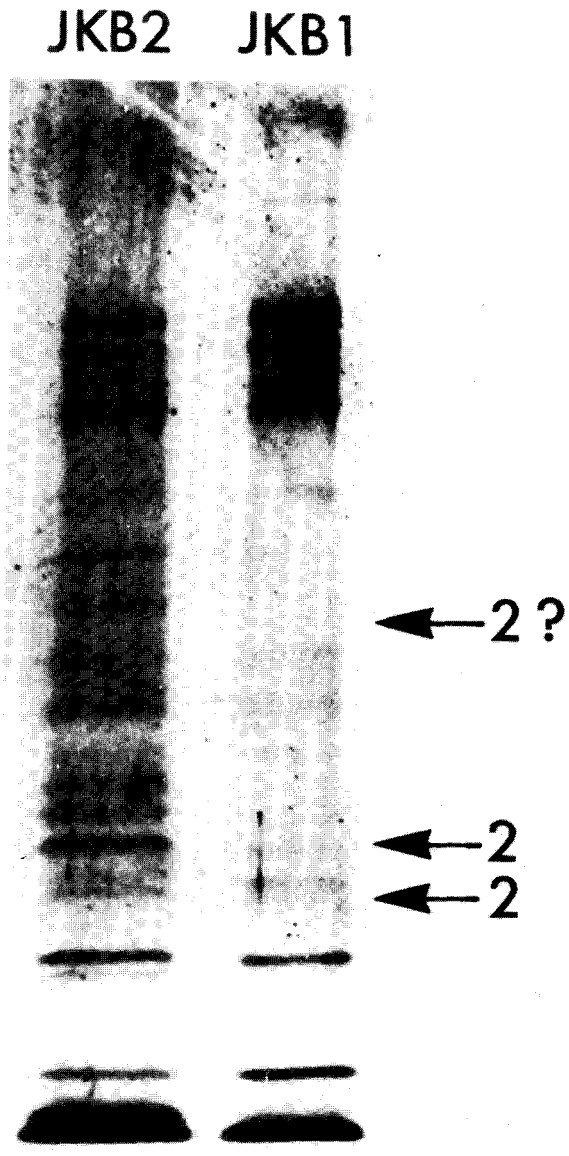


FIGURE 7. Acceptance group JKB, details of differences between sponges 1 and 2.

similar from one sponge to another. In Figure 6 we show the results for all but one member of an acceptance group, one member being omitted because insufficient sample material was recovered. In Figure 7 results for two members of this group are shown in more detail and differences indicated by arrows in the margin of the figure. Note that the majority of bands appear at approximately equal intensity, indicating little difference in loading: thus, missing bands cannot be attributable to inadequate loading. If two or more sponges show differences in plasmalemmal proteins, these might be of two different types: (1) one or more proteins present in one sponge at a particular molecular weight might be absent in plasmalemmas of another sponge. It is in fact unlikely that two proteins from one sponge would appear at exactly the same position in a track and still less likely that they would be both present in one and absent in the other sponge. (2) Small differences in molecular weight of a component might be found from one sponge individual to another because of changes in amino acid sequence. The resolution of the electrophoretic method is probably insufficient to show up changes corresponding to less than 10 amino acid deletions in the lower molecular weight protein. Obviously, if displacements become considerable it might be impossible to identify them as such and confusion with simple deletions in one member of a pair would be possible. Two-dimensional electrophoresis might give still more indication of variation but sample sizes precluded this method of investigation. Considerable differences in staining intensity of one particular band compared with a band at the same position in another track from a different individual (and with the general level of staining) might represent genetic differences between two individuals, but we have chosen to ignore such differences though they may possibly be seen in Figure 7.

Differences in banding for the three individuals Bβ, B4, and E4 which all accepted each other's grafts are shown in Figure 8. Similar differences between the accepting pair A1 and A2 are shown in Figure 9. Sponges D1 and D2, which accepted each other's grafts, have no detectable differences (Fig. 10). This lack of differences was seen in the other members of the acceptance groups not illustrated in Figure 8, although the absence of a visible difference cannot be taken as proof of identity. Clearly, there are differences in the plasmalemmal proteins of many of the sponges which accept grafts from each other.

It is noticeable that the gel band patterns from sponges in different acceptance groups, which reject members of another group, are more unalike than those within acceptance groups, which suggests that components of the tracks do not originate from unsuspected protein contaminants from other organisms.

DISCUSSION

The differences seen in the presence or position of bands of protein in the PAGE gels shows that in sponges which accept each other's grafts, there are genetic differences at other loci. This provides experimental disproof of the hypothesis of Hildemann et al. (*1*, *2*). In other words, graft acceptance is in no way a demonstration of clonal identity.

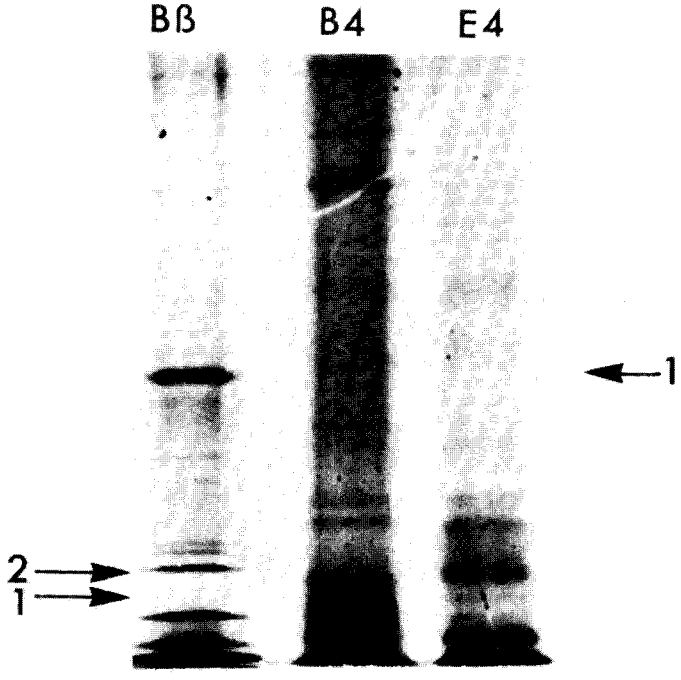


FIGURE 8. Acceptance group with Bβ, B4, and E4. Note marked differences.

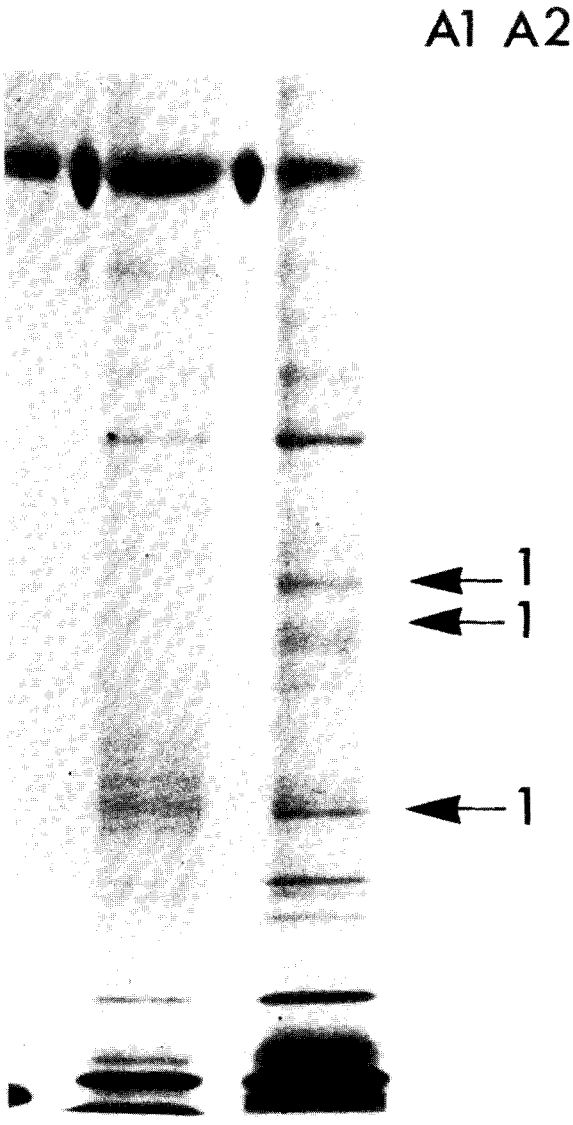


FIGURE 9. Acceptance groups A1 and A2.

It should be emphasized that PAGE gels only reveal the presence or absence of a gene product or an appreciable change in molecular weight of a gene product; sequence changes are unlikely to affect position on the gel. Jones (*11*) has also noted that structural proteins tend to be less variable than soluble enzymes (although a moderate amount of variation was detected in our study). Consequently, the method we have used will, if anything, tend to underestimate the frequency with which histocompatible individuals differ genetically at other loci. Use of grafting data alone could yield a gross overestimate of the median proportion of the population which members of a single clonal group represent.

It is more difficult to assess the likelihood of kin relationships among genetically distinct individuals. In *E. ferox*, circumstantial evidence suggests that sexually produced offspring may settle relatively close to their parents. During the course of these experiments, we saw sponges releasing larvae on mucus threads, perhaps indicating a limited degree of larval dispersal. The lower level of acceptance for grafts made between geographically more remote sponges is consistent with this interpretation, although infrequent long-distance asexual propagation could also generate this pattern (and we have no data on plasmalemmal proteins for pairs of remote, histcompatible individuals). The occasional nonreciprocity of results in the two-way grafting experiments also parallels those obtained in grafts between inbred parents of two strains of mice and their F_1 offspring. If the parents are homozygous, grafts from a parent to F_1s are accepted but grafts from F_1 to a parent are rejected.

We can still say very little about the genetics of histocompatibility for *E. ferox*, largely because we have inadequate information on graft acceptance between geographically remote individuals. Within the confined area of extensive study, a simple system of perhaps one locus and a small number of alleles seems likely, but we could conceivably obtain results comparable to those of Hildemann et al. (*1, 2*) for graft experiments done over greater distances. There is no reason to expect a uniform genetic pattern for all invertebrate species, however. If the primary adaptive significance of the mechanisms underlying graft rejection were to differ (e.g., competition for space, prevention of inbreeding (*12*), defense against pathogens), the selective forces acting on the genetic control systems might well produce divergent evolutionary results, even within taxa of colonial invertebrates.

If graft acceptance can occur between genetically unalike individuals in sponges, we might expect that on occasion natural

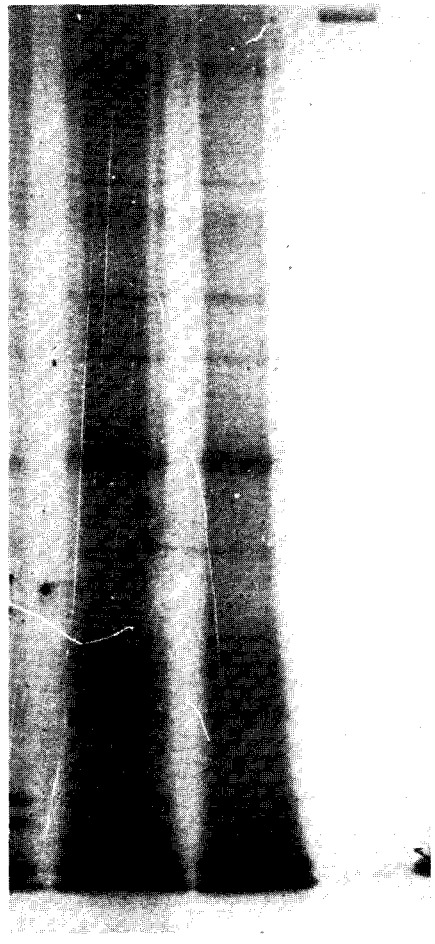


FIGURE 10. Acceptance groups D1 and D2 which appear to be identical.

chimeras would form. The frequency of these however would be lower than the frequency of graft acceptance between separate individuals since some of these animals will be genetically identical.

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