

usually four in *I. tenuirostris* and up to five in *E. huenei*. The forefin is about half the length of the mandible, as in *I. tenuirostris*, whereas in *E. huenei* it is relatively longer, often exceeding the length of the mandible. There are 12 elements in the longest digit (commencing with the epipodials¹⁶), which is within the observed range for *I. tenuirostris*, but there are usually more in *E. huenei* (up to 23). Fin structure, however, is often variable within species, especially in *E. huenei*⁵.

Aside from its longer snout, the skull of *E. costini* is similar to that of *I. tenuirostris* (compare Fig. 2b, a); this results in their having similar orbital ratios, but their other mandibular ratios, and their cranial ratios, are disparate (Table 1). However, if the snout of *I. tenuirostris* is increased in length until the ratio of skull length/mandibular length corresponds to that of *E. costini*, their cranial and mandibular ratios converge (Table 1). *E. costini* could therefore be derived from *I. tenuirostris* by an elongation of the snout.

E. costini is cranially similar to *E. huenei*, except in having a relatively longer mandible (Fig. 2b, c); this is reflected in the similarity of their cranial ratios, and the dissimilarity of their mandibular ratios (Table 1). However, if the mandible of *E. costini* is reduced to 400 mm, giving the same value for the ratio mandible length/skull length as in *E. huenei* (0.51), its mandibular ratios converge with those of *E. huenei* (Table 1). *E. huenei* could therefore be derived from *E. costini* by mandibular reduction.

If *E. huenei* evolved from a long-snouted ancestor like *I. tenuirostris*, through an intermediary like *E. costini*, what mechanism might have been involved? A change in growth rates of the rostrum and mandible during ontogeny seems plausible, and some insight is gained by studying ontogeny in *Xiphias*. Larval swordfish have mandibles extending to the tip of the skull¹⁷⁻¹⁹ whereas in adults the mandible is only about one-third of the skull length. These profound changes are effected by relatively small differences in growth rates between the mandible and rostrum (C. McG., in preparation). A small increase in the growth rate of the rostrum in an ichthyosaur like *I. tenuirostris* could therefore give rise to a descendant like *E. costini* with an elongated rostrum. This, in turn, could give rise to *E. huenei* by a reduction in the growth rate of the mandible. Such minor modifications would presumably require relatively small genetic changes, but the morphological, and consequent biological changes would be profound.

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The genetic control and consequences of kin recognition by the larvae of a colonial marine invertebrate

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The evolution of altruism, cooperation and sociality should be favoured by mechanisms promoting interactions among relatives^{1,2}. In turn, the opportunity for such interactions should be enhanced where related individuals are spatially associated. The simplest explanation for association of kin invokes philopatry, or limited, dispersal³. Alternatively, kin recognition—which is known from a broad array of taxa^{4,5}—can produce similar associations. Neither the prevalence of kin recognition, nor aggregations of kin, are by themselves sufficient to demonstrate that kin recognition plays an important role in the production of nonrandom associations of relatives. To have such a role, kin recognition must promote or inhibit associations of kin beyond the effects of other processes, notably dispersal, that modify spatial patterns. Here we report the results of field experiments showing that sibling planktonic larvae of the sessile colonial ascidian *Botryllus schlosseri* settle in aggregations that are much stronger than expected from dispersal distance effects alone. Laboratory experiments indicate that larvae distinguish kin on the basis of shared alleles at a highly polymorphic histocompatibility locus known to regulate fusion between adult colonies. This kin recognition mechanism, along with limited dispersal of larvae, promotes co-settlement of histocompatible individuals. Consequently, the probability of fusion between adult colonies is far greater than that expected if larvae settled randomly.

Botryllus schlosseri colonies are founded after a sexually produced planktonic tadpole larva attaches to a firm substratum and metamorphoses. Repeated cycles of asexual multiplication produce a colony of morphologically and genetically identical zooids, connected by a blood vascular system. As in many colonial marine invertebrates, the larvae of several oviparous ascidians can metamorphose soon after release into the plankton⁶⁻¹⁰. In the case of *B. schlosseri*, our laboratory and field observations, as well as those of others¹¹, demonstrate that larvae can metamorphose—and many do—soon after escaping from their natal colony.

To determine the contribution of larval settlement behaviour to the spatial arrangement of sibling adult colonies, we examined the recruitment pattern of sibling colonies (<4 weeks old) that were founded by larvae, all derived from the same cross, and carrying a rare genetic marker. We identified the marker from an electrophoretic survey of 512 colonies living on the side of the Marine Biological Laboratory Supply Dock in the Eel Pond at Woods Hole, Massachusetts, USA. At the phosphoglucose isomerase locus (*PGI*), five electromorphs occurred, one of which (*PGI-fast*) was present in only two of the colonies surveyed. We cross-fertilized these two colonies, then assayed the F₁ colonies for *PGI-fast* homozygotes. One F₁ homozygote was selected as the source colony for marked larvae, and was positioned at the centre of a 1-m diameter circular asbestos-cement panel horizontally suspended 0.5 m below the dock. All zooids in a sexually mature *B. schlosseri* colony ovulate synchronously approximately weekly. Just before each ovulation, we returned the source colony to the laboratory, where we mated it with a sibling colony homozygous for the same marker allele, then repositioned the source colony on the panel. We left the panel in position for 4 weeks, then mapped and removed all *B. schlosseri* colonies that had recruited. Colonies were frozen at -80 °C, then analysed electrophoretically. We mapped the panel before any recruited colonies had reached sexual maturity; thus,

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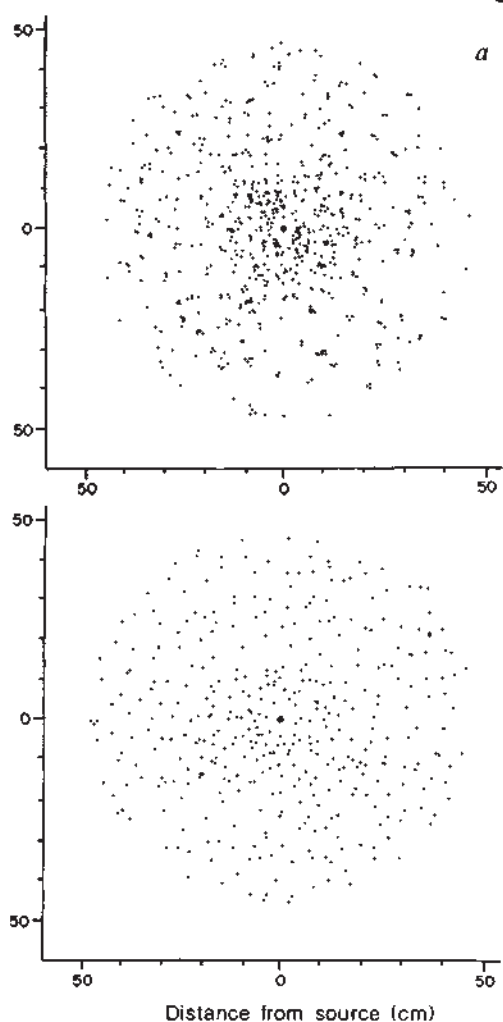


Fig. 1 Maps of the dispersion of *Botryllus schlosseri* (which settled on the undersurface of a 1-m circular asbestos-cement panel suspended below the Marine Biological Laboratory Supply Dock at Woods Hole, Massachusetts). Small dots depict the locations of colonies. The large dot marks the location of the source colony. *a*, Map of F₁ colonies (identified by the presence of the *PGI-fast* allele, see text) derived from source colony. *b*, Map of colonies not carrying the *PGI-fast* allele, thus, not derived from the source colony.

colonies homozygous for the *PGI* marker allele were assumed to be siblings originating from the source colony at the centre of the panel. Recruits not carrying the marker allele must have immigrated.

Figure 1*a* and *b* show respectively maps of marked and unmarked colonies. To test for spatial association among siblings, we computed the Clark and Evans¹² index of aggregation (*R*) based on nearest-neighbour distances between sibling colonies. *R* is the mean nearest-neighbour distance observed, divided by that expected if dispersion were random. Thus, randomly arranged individuals are expected to yield a value for *R* of 1.0. Values of *R* significantly <1 indicate aggregation, whereas values >1 indicate overdispersion. The sibling colonies were aggregated as tested by Clark and Evans' significance criteria (*R* = 0.769; *P* < 0.001). The unmarked immigrant colonies were significantly overdispersed (*R* = 1.227; *P* < 0.001). This overdispersion could result from larval behaviour, or could simply be an artefact of spacing due to colony growth¹³. In any case, the degree of aggregation of siblings differs even more from that of immigrants than it does from random (*t*-test; *P* < 0.001).

Figure 1*a* shows that sibling colonies are clustered around the source colony, perhaps because *B. schlosseri* larvae can settle

upon release into the plankton. To separate any aggregation caused by sibling recognition from that due to limited dispersal, we constructed a null model based on a bootstrap analysis¹⁴ of the nearest-neighbour data. The locations of colonies were expressed in polar coordinates with the origin at the centre of the source colony: pseudosamples with the same distributions of distances and directions were chosen independently and randomly, with replacement, from the actual coordinates of sibling colonies. The randomized distance and direction coordinates are thus independent in the pseudosamples. This randomization procedure should preserve in the pseudosamples the overall aggregating effect of dispersal distance alone. However, smaller-scale aggregations due to interactions between immediate neighbours will be broken up by the randomization of the angular coordinates. For 500 randomizations, we calculated the value of *R* produced by the random model. In all cases, the values of *R* under the random model were substantially greater (mean = 0.897; s.d. = 0.023; range 0.824–0.964) than the observed *R* = 0.769. This analysis demonstrates that limited dispersal explains only about half the deviation of mean nearest-neighbour distance from that expected if settlement were random.

The histocompatibility system known to control fusion and rejection between adult *Botryllus* colonies may provide a mechanism by which larvae recognize their siblings. Histocompatibility is controlled by a single mendelian locus such that two colonies sharing one or both alleles can fuse by their blood vascular systems. When colonies do not share an allele, rejection, accompanied by necrosis at the site of allogeneic contact, occurs^{15–17}. Fusion frequencies among pairs of *Botryllus* colonies collected in the field have been reported to range from 4 to 8% (refs 18, 19). In our studies on the Marine Biological Laboratory Supply Dock, of 500 pairs of colonies collected haphazardly along a 20-m transect, 22 pairs (4.2%) fused. According to the method of Curtis *et al.*²⁰, at equilibrium, approximately 100 equally frequent histocompatibility alleles would produce the fusion frequency observed in the Eel Pond population. Given such a high level of polymorphism at the histocompatibility locus, the likelihood that any two colonies will share a histocompatibility allele should depend primarily on their pedigree relatedness.

Because the settled colonies in the field experiments were all full siblings, shared alleles at loci other than the histocompatibility locus could have been used for sibling recognition. Accordingly, we designed laboratory experiments to test whether larval settlement location is better predicted by the histocompatibility type or the relatedness of resident individuals. With a breeding programme using colonies of known histocompatibility genotypes (determined by fusion assays), in a factorial design, we varied independently the effects of pedigree relatedness and histocompatibility genotype of larvae and residents on distance between settled larvae and previously attached residents. Thus, there were four experimental treatments: (1) the residents and larvae were inbred, and shared one histocompatibility allele; (2) the residents and larvae were inbred, but did not share a histocompatibility allele; (3) the residents were fourth-generation outbred descendants of the parents of the larvae, with one shared histocompatibility allele; and (4) the same as (3), but residents and larvae did not share a histocompatibility allele. In outline, the breeding programme was as follows: for treatments (1) and (2), homozygous resident colonies of known histocompatibility genotype (*AA*) were derived from crosses between heterozygous sibling colonies (*AB*). Unfusable homozygous colonies (*BB*) were isolated from the same cross. In treatment (1), introduced larvae (*AB*) were derived from a cross between the *AA* and *BB* full-sibs. In treatment (2), introduced larvae (*BB*) were derived from a *BB* × *BB* cross from the same sibship used to generate the larvae in treatment (1). Therefore, the introduced larvae in treatments (1) and (2) bear the same pedigree relatedness to the residents, but differ in the presence

Table 1 Two-way analysis of variance on the distance between settled larvae and the nearest resident colony as a function of resident/larval relatedness and histocompatibility types

Source	Sum of squares	Degrees of freedom	F value (P)
Relatedness	57.2	1	1.35 (>0.25)
Histocompatibility type	3,708.5	1	87.46 (<0.001)
Replicate	39.8	2	0.47 (>0.63)
Relatedness/ histocompatibility	174.1	1	4.10 (0.04)
Error	9,964.6	235	

Table 2 Summary of the mean distances between settled larvae and the nearest resident colony according to experimental treatment

Treatment	Mean (mm)	n
Siblings, no shared alleles	14.70	60
Siblings, one shared allele	7.80	60
Outbred, one shared allele	5.15	60
Outbred, no shared alleles	13.92	60

n, Number of observations for each treatment.

of an A histocompatibility allele. For treatments (3) and (4), either AA or BB larvae were introduced into Petri dishes with outbred residents carrying an A allele. These residents were derived after sequential outcrossing with different colonies taken from the field, none of which carried either an A or B allele. After each generation, a colony carrying the A allele was identified (with a fusion assay) and used for subsequent matings. After four generations of outcrossing, single zooids from a heterozygous colony carrying the A histocompatibility allele, and an unknown second allele (differing from B), were used as residents.

For each treatment, we established three replicates, each with 20 resident colonies. The resident colonies were founded by attaching single zooids from a colony of known genotype to random positions on the submerged undersurfaces of the tops of 150-mm plastic Petri dishes. Twenty-four hours later, the dishes were filled with seawater, sealed and kept in the dark. We then introduced 20 larvae through a port in the side of each dish according to the above treatments. One day later, we measured the distance from each settled larva to the nearest resident.

The results of an analysis of variance show that the relatedness of residents and larvae has no discernible effect on nearest-neighbour distances (Table 1). In contrast, where larvae and residents shared a histocompatibility allele, the larvae settled significantly closer to the residents (Table 1). Indeed, the mean nearest-neighbour distance between larvae and residents sharing a histocompatibility allele is roughly half that observed when no allele is shared (Table 2). Table 1 shows a marginally significant interaction between the two main effects that is difficult to interpret. However, the minor contribution that this interaction makes to the overall sum of squares suggests that the interaction is biologically unimportant in these experiments.

Our experiments do not eliminate the possibility that larval kin recognition and histocompatibility are closely linked, rather than identical, genetic traits. However, because larval kin recognition increases the likelihood of adjacent colonies being fusible—an otherwise improbable event given the polymorphism of the histocompatibility locus—there are functional grounds for expecting the two processes to be controlled by a single locus.

The promotion of co-settlement of histocompatible colonies, coupled with the restriction of fusion to closely related

genotypes, indicate that colony fusion may be beneficial, but only among kin. In colonial organisms such as *Botryllus*, fusion may benefit one, or both, members of a chimaera in several ways. First, colony fusion immediately increases colony size. Survivorship is known to be size-dependent in several colonial ascidians (refs 21, 22 and our unpublished observations), sponges^{23,24}, cnidarians²⁵⁻²⁸ and ectopods^{29,30}; hence, fusion may reduce the likelihood of total mortality^{29,30}. Second, if the onset of reproduction is size-dependent—as it appears to be in several cnidarians^{28,31} and ascidians^{6,21}—then fusion may lower the age at first reproduction of both members of the chimaera³². In colonial organisms where growth rate is size-dependent^{33,34}, fusion may also increase the probability of subsequent survival and reproduction. Indeed, fusion among juvenile colonial organisms is well known^{32,35}. Third, a chimaera may have different physiological attributes from either component colony, potentially increasing the range of environmental tolerance³². Finally, fused botryllid ascidians (like many other clonal taxa^{36,37}) freely exchange cells that can differentiate into gametes³⁸, and the rate of differentiation may vary genetically³⁸. Under these conditions, somatic cell parasitism, in which one member of a chimaera increases its reproductive output at the expense of the other, can occur³². However, because the polymorphism and genetics of the *Botryllus* histocompatibility system limit fusion almost entirely to closely related individuals, the parasitic losses, in evolutionary terms, would be considerably less than if the fused colonies were unrelated^{1,39}.

Another potential consequence of close association of kin is increased inbreeding. Laboratory studies suggest that self-fertilization is deleterious in *B. schlosseri*⁴⁰ and, in some congeners, syngamy may not occur if sperm and egg carry the same histocompatibility allele^{41,42}. However, there is no evidence of inbreeding depression in the Eel Pond population of *Botryllus schlosseri*⁴³. Indeed, studies of other marine invertebrates in which closely related individuals are likely to cross-fertilize provide meagre evidence for inbreeding depression^{9,44}.

Botryllus schlosseri appears to be one of the first species where a functional—and perhaps evolutionary—link between kin recognition, historecognition and the enhancement of colony fusion has been experimentally demonstrated. The prevalence of limited dispersal⁹ and historecognition systems among sessile clonal marine invertebrates³² suggests that such a link may be found in other clonal organisms. Recent studies of congeneric mice show that haplotypic differences in the *H-2* region affect mating behaviour⁴⁵; thus, histocompatibility markers could mediate kin recognition in other taxa. Whether the study of marine invertebrates will provide useful insights into the more complex dynamics of kin recognition in mobile, social vertebrates is unclear, but the analogies remain intriguing.

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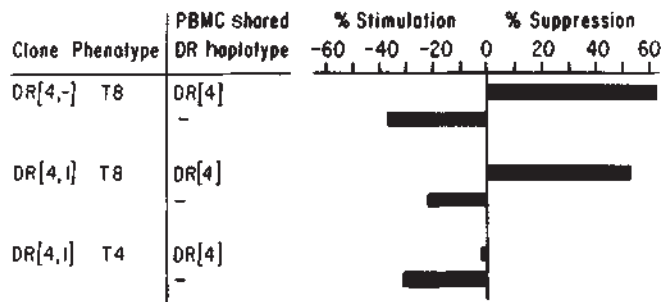


Fig. 1 HLA-D-restricted lepromin-induced suppression of Con A responses of normal peripheral blood mononuclear cells (PBMC) by T8 clones from lepromatous leprosy skin lesions.

Methods. Ellipsoid skin biopsy specimens obtained under local anaesthesia (1% lidocaine with adrenaline) from lepromatous leprosy patients, classified according to the criteria of Ridley and Jopling¹⁰, were stripped of epidermis and subcutaneous fat and placed in a tissue sieve fitted with a 64- μ m-mesh filter (Bellco Glass Inc.) on a Petri dish containing RPMI 1640 medium with 10% fetal calf serum¹². The tissue was cut into small pieces with a surgical scalpel and extruded through the mesh with a glass rod. The lymphocytes were isolated by Ficoll-Paque (Pharmacia) centrifugation, labelled with fluorescein isothiocyanate-conjugated monoclonal antibodies T4 and T8 (Coulter Immunology), and sorted using a FACS IV (Becton-Dickinson). Long-term culture lines were successfully established from T lymphocytes derived from skin lesions of lepromatous patients. T4 and T8 lymphocytes sorted by FACS to greater than 90% purity were seeded in 96-well flat-bottom Linbro microtitre plates (Flow Laboratories) at 1,000 cells per well. To each well were added 5×10^5 lethally irradiated (5,000 rad) allogeneic feeder peripheral blood mononuclear cells, RPMI 1640 with penicillin/streptomycin, 10% human AB serum and 10% IL-2 (Electronucleonics). Cultures were incubated at 37°C in 5% CO₂. Every 3 or 4 days thereafter, the cultures were monitored with an inverted phase microscope, transferred to additional wells when necessary and fed with 50% fresh IL-2-containing medium. To avoid suppression from adherent suppressor cells found in blood of lepromatous patients, the lines were cultured with allogeneic or no feeder cells. Some lines were maintained for longer than 11 months using these techniques. Lines from two lepromatous patients (J.G. and B.P.) were selected for subcloning. Clones were obtained by limiting dilution by seeding 0.3 cells per 20 μ l Terasaki well with 10% AB serum, 5-10% IL-2 and 10^4 γ -irradiated allogeneic feeder PBMC for 1 week¹³. Positive wells were transferred to a 96-well plate and expanded as above, using 5×10^4 feeder cells, and then to 24-well plates using 5×10^3 feeder cells. When ready for study, T-lymphocyte clones were separated from non-viable cells over Ficoll-Paque and the phenotype was determined by FACS. Antibodies used included T4, T8, anti-HLA-DR (Becton-Dickinson) and anti-IL-2 receptor (Tac, kindly provided by Dr T. Waldmann, or from Becton-Dickinson). Measurement of antigen-induced suppressor activity of lesion derived T-lymphocyte clones was performed using the method of Mehra *et al.*³. 5×10^4 Cells of a particular T8 clone were admixed with 2×10^5 normal PBMC per microtitre well with and without Dharmendra lepromin (1:10). Con A (2.5μ g ml⁻¹) was added and ³H-thymidine incorporation of triplicate cultures measured at 3 days. Per cent suppression is calculated as $100 - (\text{c.p.m. Con A} + \text{lepromin} / \text{c.p.m. Con A}) \times 100$. For each clone, three HLA-DR-matched (A.K., M.R., H.K.) and three HLA-DR-mismatched (B.L., G.P., M.S.) donors were used and the results were averaged. A non-reactive T4 clone derived from a lepromatous lesion was substituted for the T8 clones to control for the addition of cells. The c.p.m. above background obtained in the Con A controls averaged 27,471. The results obtained with the T8 clones admixed with the HLA-DR-matched cells differed significantly ($P < 0.001$) when compared with either the T8 clones admixed with HLA-DR-mismatched cells or the T4 'filler' clone assayed with HLA-DR-matched cells, using the z-test approximation to the test for relative difference in Poisson data on actual c.p.m.

(Leu 2a) phenotype were present in excess in lepromatous granulomata as compared with tuberculoid⁷⁻¹⁰. Because the surface markers do not necessarily reflect lymphocyte function within lesions¹¹, simple procedures were developed to extract 10^5 - 10^6 lymphocytes from biopsies of leprosy skin lesions¹². The phenotypic distribution of these cells as determined by fluorescence-activated cell sorter (FACS) analysis was similar to that obtained using immunoperoxidase staining of the tissue sections (T4:T8 = 0.5:1 in lepromatous and T4:T8 = 2.0:1 from tuberculoid lesions). In lepromatous patients, the T4/T8 ratio of extracted cells was significantly different from that in blood, indicating that contamination from peripheral blood was negligible. Because we assumed that the lymphocytes in the lesions may be involved in specific immune reactivity and were likely

Genetically restricted suppressor T-cell clones derived from lepromatous leprosy lesions

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Leprosy is a spectral disease in which immune responses to *Mycobacterium leprae* correlate with the clinical, bacteriological and histopathological manifestations of disease^{1,2}, so study of its pathology provides insights into immunoregulatory mechanisms in man. At the tuberculoid pole, patients have few lesions in the skin which contain rare organisms and are able to mount strong cell-mediated immune responses to *M. leprae* antigens. In contrast, at the lepromatous pole, patients have disseminated skin lesions containing large numbers of acid-fast bacilli and are selectively unresponsive to antigens of *M. leprae*. *M. leprae*-induced suppressor cells derived from peripheral blood have been reported to be active *in vitro*³⁻⁶, yet their *in vivo* significance has remained unclear. Because the focal point of the immune response to *M. leprae* is the skin lesion consisting of lymphocytes and macrophages, we have recently developed methods for isolating lymphocytes from skin biopsies of leprosy patients. We report here that two T8 clones derived from lepromatous leprosy skin biopsies, in the presence of lepromin, suppress concanavalin A (Con-A) responses both of peripheral blood mononuclear cells and of T4 clones in an HLA-D (HLA, histocompatibility locus antigen)-restricted manner. Moreover, these T8 clones suppressed responses of HLA-D-matched, but not HLA-D-mismatched antigen-responsive T4 clones to *M. leprae* antigens, indicating that T-cell suppression is major histocompatibility complex (MHC)-restricted at some level in man.

Initial immunohistological studies of skin lesions from patients across the spectrum revealed that cells bearing the T8