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EVOLUTION OF ALLORECOGNITION IN BOTRYLLID ASCIDIANS INFERRED FROM A MOLECULAR PHYLOGENY

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Abstract.—Despite the functional and phyletic ubiquity of highly polymorphic genetic recognition systems, the evolution and maintenance of these remarkable loci remain an empirical and theoretical puzzle. Many clonal invertebrates use polymorphic genetic recognition systems to discriminate kin from unrelated individuals during behavioral interactions that mediate competition for space. Space competition may have been a selective force promoting the evolution of highly polymorphic recognition systems, or preexisting polymorphic loci may have been coopted for the purpose of mediating space competition. Ascidian species in the family Botryllidae have an allorecognition system in which fusion or rejection between neighboring colonies is controlled by allele-sharing at a single, highly polymorphic locus. The behavioral sequence involved in allorecognition varies in a species-specific fashion with some species requiring extensive intercolony tissue integration prior to the allorecognition response, while other species contact opposing colonies at only a few points on the outer surface before resolving space conflicts. Due to an apparent species-specific continuum of behavioral variation in the degree of intercolony tissue integration required for allorecognition, this system lends itself to a phylogenetic analysis of the evolution of an allorecognition system. We constructed a molecular phylogeny of the botryllids based on 18S rDNA sequence and mapped allorecognition behavioral variation onto the phylogeny. Our phylogeny shows the basal allorecognition condition for the group is the most internal form of the recognition reaction. More derived species show progressively more external allorecognition responses, and in some cases loss of some features of internal function. We suggest that external allorecognition appears to be a secondary function of a polymorphic discriminatory system that was already in place due to other selective pressures such as gamete, pathogen, or developmental cell lineage recognition.

Key words.—Allorecognition, ascidian, behavioral evolution, histocompatibility, space competition.

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Highly polymorphic loci form the genetic basis of recognition systems in diverse taxa from fungal and angiosperm mating compatibility loci to invertebrate and vertebrate histocompatibility loci (e.g., reviews in: de Nettancourt 1977; Buss and Green 1985; Grosberg 1988a; papers in Grosberg et al. 1988; Potts and Wakeland 1990; Cook and Crozier 1995; Kahmann and Bolker 1996; Nauta and Hoekstra 1996). The evolution and maintainance of highly polymorphic loci in natural populations has been attributed to a variety of evolutionary forces, but their existence remains an empirical and theoretical mystery. Potential evolutionary scenarios have invoked a variety of selective and nonselective forces operating either within the organism or externally to mediate intra- or interspecific challenges (e.g., Buss and Green 1985; Klein 1987; Grosberg 1988a; papers in Grosberg et al. 1988; Harvell 1990; Potts and Wakeland 1990; Lee and Vacquier 1992; Brown and Eklund 1994; De Boer 1995; Grosberg et al. 1996; Parham and Ohta 1996; Apanius et al. 1997). Existing evolutionary forces may have promoted the de novo appearance of highly polymorphic recognition loci to function in their current context, or those evolutionary pressures may be responsible for the cooption of preexisting molecular systems (e.g., discussion in: Burnet 1971; Buss 1982; Buss et al. 1984; Harp et al. 1988; Weissman 1988; Haig 1996).

Many clonal invertebrate taxa (e.g., sponges, cnidarians, bryozoans, and ascidians) possess tissue recognition systems that mediate naturally occuring competition between genotypes for limited space (as reviewed in Grosberg 1988a; Buss 1990; Lang and Chornesky 1990). The broad phyletic distribution of tissue recognition systems in invertebrates has led to speculation that these early metazoan historecognition systems may contain components that are the evolutionary antecedents of vertebrate tissue recognition systems (e.g., Burnet 1971; Scofield et al. 1982b; Buss and Green 1985; Weissman 1988; Du Pasquier 1989; Humphreys and Reinherz 1994). This hypothesis is intriguing given the absence of natural selective scenarios for vertebrate historecognition systems. Since vertebrates do not, in general, exchange tissue between individuals (although maternal/fetal tissue interactions are a notable exception), the selective forces that promoted the evolution of the vertebrate historecognition system are enigmatic. Since many invertebrates do possess historecognition loci that are of obvious selective importance during space competition, an understanding of the evolution of invertebrate allorecognition systems should shed light on mechanisms promoting the development of highly polymorphic loci in a broad range of taxa (e.g., Burnet 1971; Buss and Green 1985; Grosberg 1988a; Weissman 1988; Du Pasquier 1989; Brown and Eklund 1994; Davidson 1994; Humphreys and Reinherz 1994).

Single-species studies have been used frequently to address the question of evolutionary forces responsible for allorecognition systems in clonal invertebrates. Empirical studies have attempted to measure the costs and benefits of fusion, rejection, and aggression within a single species (e.g., Buss 1982; Rinkevich and Weissman 1992; Ayre and Grosberg 1995, 1996; Chadwick-Furman and Weissman, in press). Theoretical approaches have modeled costs and benefits to look for evolutionary stable strategies that would lead to the evolution or maintenance of highly polymorphic loci (e.g., Cro-

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zier 1988; Neigel 1988; Grosberg and Quinn 1989). In contrast, we take a multispecies phylogenetic approach to the evolutionary puzzle of highly polymorphic loci and recognition systems by looking specifically at the evolution of allorecognition behavior in a clade of ascidians. Construction of phylogenies, followed by independent character-mapping, has proved an extraordinarily powerful means of gaining insight into evolutionary processes in many areas including behavior (e.g., Brooks and McClennan 1991; Maddison and Maddison 1992; Martins 1996).

Specifically for the problem of allorecognition loci, a phylogenetic approach has been considered on a broad, whole metazoan scale in general verbal terms (e.g., Buss and Green 1985; Harvell 1990; Sima and Vetvicka 1990). Some authors have pointed out the need for a more careful phylogenetic analysis of the distribution of allorecognition systems in metazoans (e.g., Harvell 1990). However, since the genetic basis (and ecological details) of most of these invertebrate allorecognition systems are known only in the most cursory fashion, inferences of selective scenarios based on phylogenetic inference at this taxonomic scale are currently limited. Even within the phylum Cnidaria, a multitude of effector structures have been described, and there is no reason to suppose these structures are homologous across the phylum (Buss et al. 1984; Buss 1990).

Here, we use a narrower-scale phylogenetic approach (intrafamily-level phylogeny), to infer the ancestral state and directional evolution of allorecognition behavior in the ascidian family Botryllidae. This group of colonial protochordates are an ideal clade for a comparative approach to the evolution of a tissue allorecognition system. Species of colonial ascidians in the botryllid family use a simple genetically controlled allorecognition system to mediate competition for space between neighboring colonies (Oka and Watanabe 1957, 1960; Sabbadin 1982; Scofield et al. 1982a,b). In botryllids, the single locus controlling the fusion/rejection determination is highly polymorphic in natural populations (Milkman 1967; Mukai and Watanabe 1975a,b; Scofield et al. 1982a,b; Grosberg and Quinn 1986; Rinkevich and Saito 1992; Yund and Feldgarden 1992; Rinkevich et al. 1994, 1995). Depending on whether at least one allele at this locus is shared between these competing conspecific colonies, individuals may fuse tissue and become a potentially chimeric individual, or they may reject each other's tissue and establish a new boundary between them. The phylogenetic character-mapping approach taken here is based on the fact that botryllids show species-specific differences in allorecognition behavior. In the botryllid family, the apparent similarity of responses, effectors, and genetics of the locus, combined with the close phylogenetic relationship of the species involved, make this a good group to use for inferences of the selective forces involved in the evolution and maintenance of the allorecognition locus.

Botryllid colonies, like all colonial ascidians, consist of individual feeding and reproductive units called zooids that are encased in a common outer covering, the tunic. Zooids in a single colony are connected to each other and to sausage-shaped terminal blood-filled bulbs at the tunic periphery by a common blood vascular system. Botryllid colonies frequently grow in crowded conditions in a sheetlike form on solid substrata where they encounter other conspecifics (e.g., Grave 1933; Yamaguchi 1975; Grosberg 1988b; Berman et al. 1992). Interactions between contacting colonies occur as a set of species-specific behaviors involving the outer body covering (tunic) and terminal blood-filled bulbs (ampullae) at the periphery of each colony. The allorecognition interaction involves both cellular (blood and tunic) and humoral (plasma) components associated with the tunic and ampullae of each colony. During a rejection reaction between two colonies, classic cytotoxic responses are visible under the light microscope (e.g., Scofield and Nagashima 1983).

Naturally contacting conspecific colonies show species-specific behavioral variation in the degree of intercolony tissue integration required for a recognition response to occur (Taneda et al. 1985; Hirose et al. 1988; Saito et al. 1994; Fig. 1; Table 1). This behavioral variation produces an effective difference in the location of the allorecognition response between species, with variation in this clade from limited external intercolony contact to extensive internal tissue integration. At one end of the spectrum, recognition follows minimal outer tissue contact between the two colonies as their growing edges contact and push up against each other. There may be limited blood cell leakage from the terminal ampullar vessels and perhaps partial fusion of outer tunics at selected points of contact. At the other extreme in allorecognition behavioral sequences are other species that completely fuse tunics along the contacting border and then subsequently fuse blood vessels and actually exchange blood cells within the fused vessels before the recognition reaction occurs. Between these two extremes are a number of species showing intermediate levels of intercolony fusion and blood exchange before recognition occurs. All botryllid species tested to date show the allorecognition response in encounters with conspecifics (reviewed in Rinkevich 1992; Hirose et al. 1994; Saito et al. 1994).

A phylogeny of the botryllids based on morphological systematics is not possible at this time due to uncertainty about the reliability of characters (Monniot and Monniot 1987; Monniot 1988; Boyd et al. 1990). Here, we present a phylogeny of the botryllid ascidians using a nuclear ribosomal DNA locus independent of the allorecognition locus itself. This allows us to independently evaluate the evolution of the group, and subsequently map the allorecognition behavior trait onto the phylogeny. We use this approach to infer, based on the basal character state and the pattern of character transformation in subsequently more derived species, which selective forces were primary in the evolution of allorecognition behavior in the botryllids and which selective forces were likely secondary. If internal evolutionary pressures such as defense against internal pathogens, somatic or germ cell parasitism, gametic incompatibility systems, or developmental lineage sorting led to the evolution of this locus, then species with the most internal allorecognition behaviors should be basal. Conversely, if external pressures such as energetic efficiency in space competition were primary selective forces for this locus, then a species with the most limited type of tissue interaction is expected to be basal in the botryllid phylogeny.

We chose the nuclear small ribosomal subunit (18S rDNA) locus for sequence information because it had previously

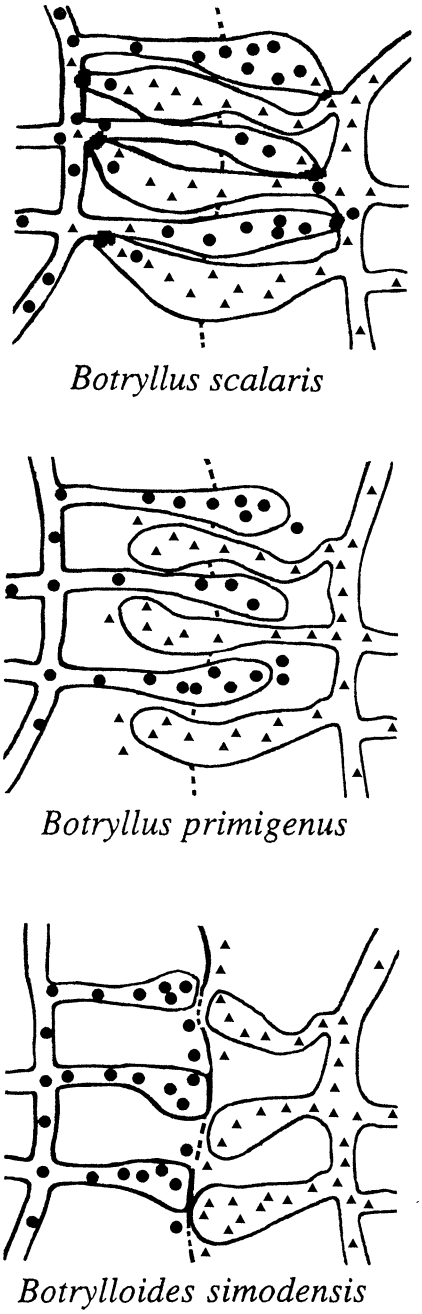

FIG. 1. Location of the allorecognition response in three species of botryllids. Each of the three illustrations show two colonies, on the right and left sides of the drawing, and the interaction between them. The tunic border is delineated by a thin black line vertically drawn down the center of the illustration. A dashed line indicates areas of potential tunic fusion between the two colonies. The blood vascular system of each colony, including the protruding ampullar ends, are shown containing round and triangular black blood cells within the vessels. Blood cells outside the vessels indicate leakage into the tunic.

shown to be appropriate for construction of molecular phylogenies at the family level in ascidians, including the family Styelidae under consideration here (Hadfield et al. 1995). To root the botryllid phylogeny, we use solitary styelids, the solitary group basal to the colonial botryllids (Berrill 1950; Monniot et al. 1991) as the closest outgroup to the botryllids. We also include more distant outgroup species: from the Mogulidae (another family in the same order, Stolidobranchia) and from a different order (Phlebobranchia).

TABLE 1. Botryllid species and locations used in this study and location of the species-specific allorecognition response (Taneda et al. 1985; Hirose et al. 1988; Saito et al. 1994; Y. Saito, unpub. obs.). Notes on ingroup (botryllid) samples included in this phylogeny: species designations attached to some samples in this study are not well resolved in the current literature. In particularly difficult cases, the samples are labeled in parentheses here by collecting location. Further molecular work is in progress to resolve the taxonomic identity of these populations.

Species	Stage of allorecognition response	Geographic location
B. scalaris	ampullar fusion	Japan
B. primigenus	ampullar penetration	Japan
B. sexiens	ampullar fusion?	Japan
B. fuscus	partial fusion of tunic (fusion only in cut assays)	Japan
B. simodensis	partial fusion of tunic	Japan
B. leachi	unknown	Italy
B. diegense? (WHD)	unknown	U.S., NE: Woods Hole, MA
B. diegense? (SD)	unknown	U.S., SW: Mission Bay, CA
B. violaceous	partial fusion of tunic (fusion only in cut assays)	U.S., W: Monterey, CA

MATERIALS AND METHODS

Specimens

The botryllid species sampled for this phylogeny (Table 1) include all species known to exhibit the most extreme differences in allorecognition behavior. Also included are most species described as having intermediate sorts of allorecognition behavior. In addition, in the full analysis, some specimens are included from botryllid populations where life-history and reproductive traits have been described, or where accidental introductions have raised questions about the identity of newly invading species.

At the most extreme end of the behavioral spectrum of botryllid allorecognition, ampullar fusion (the complete membrane fusion of the vascular systems of opposing colonies and subsequent exchange of blood cells between the two colonies), has been described in *Botryllus scalaris* (Saito and Watanabe 1982) and is also thought to be the allorecognition behavior of *B. sexiens* (Y. Saito, unpubl. obs.). Following extensive tunic and vascular fusion and blood cell exchange, elements in the blood (cellular and plasma) interact between the two colonies. In a rejection reaction, these interacting cells turn dark and lyse. Parts of the shared vascular system and tunic area become necrotic and the two colonies both shrink away from the zone of interaction. Fused vascular elements may be pinched off from the parent colony and left to degenerate in a necrotic zone. Both colonies erect a thick fibrous barrier in this zone that is easily visible with a light microscope.

A less extensive interaction between opposing colonies involving extensive outer tunic fusion and penetration of one colony's ampullae into the opposing colony's tunic (but, without fusion between opposing colonies' ampullae) is found in *B. primigenus* (Oka 1970; Taneda et al. 1985). Here blood and plasma interactions occur within the tunic, but outside the vascular systems, as blood cells and plasma are leaked into the shared tunic area. Again, as in the vessel-fusing species described above, if the colonies reject, a dark necrotic reaction is observed around the interacting vessels and cells. Vessels are then pinched off and abandoned in a necrotic area that is demarcated by a thick fibrous barrier between the two colonies.

In *Botrylloides simodensis, B. violaceous*, and *B. fuscus*, the outer surface of the animal's body is the site of allogenic tissue fusion and rejection reactions (Hirose et al. 1988, 1990, 1994). Fusion of the outer colony covering (tunic) occurs only at limited points of contact in these species. In this last group of species, blood cells and plasma leak at the points of contact between the two colonies and cytotoxic reactions occur within the tunic between blood cells and plasma of the opposing colonies. In the final phase of the rejection reaction in these species, a new border composed of a thin, fibrous material is formed between the opposing colonies (Hirose et al. 1990).

Other botryllid samples included in the full phylogenetic analysis include *Botrylloides leachi* from Venice Lagoon, Italy, and specimens collected from Woods Hole, Massachusetts, and San Diego, California, that may both represent the species *B. diegensis* or *B. leachi*. Although detailed descriptions of the natural allorecognition behavior of these species or populations are not generally agreed upon or have not been published, these samples were included in the phylogeny because they represent historically and ecologically important populations, and because their taxonomic status relative to other botryllids is in question. Current work is in progress to resolve their systematic identities and allorecognition behavior (C. S. Cohen, University of New Hampshire).

In some cases, where taxonomic details separating sympatric species are particularly difficult to resolve, field-collected specimens were transferred to glass slides and raised in a controlled environment to confirm diagnostic reproductive and life-history traits. This culturing work was carried out for all of the samples obtained from Japan.

DNA Sequences

Specimens were used either fresh or after freezing or ethanol preservation. Matching voucher specimens were kept in ethanol and formalin where possible for morphological comparisons.

Molecular studies with ascidians, and botryllids in particular, have been difficult due to the presence of secondary compounds associated with the DNA (Kumar et al. 1988; Pancer et al. 1995). Experimentation with a wide variety of extraction techniques permitted this comparative study of botryllid species with varying body chemistry. For this study, we developed an extraction protocol for ethanol-preserved species to facilitate shipping of samples from distant locations.

DNA was extracted and PCR-amplified generally following the methods and using the primers of Hadfield et al. (1995) with the following modifications. Most material was preserved in 70% or 95% ethanol. Ethanol-preserved samples were blotted on Kimwipes to remove as much ethanol as possible and then finely minced with a razor blade before extraction. Some extractions either included CTAB in the initial extraction buffer or in a second round of purification after the initial extraction. Some other extractions used a Chelex 100 resin (BioRad) protocol (adapted from Walsh et al. 1991).

Agar bands were purified using a handpacked glasswool spin column or a QIAquick gel extraction column (Qiagen Inc, Chatsworth, CA) and precipitated one or two times with 100% ethanol and 3M NaAc, pH 5.2. PCR products were directly sequenced or cloned with the TA Cloning Kit (Invitrogen, San Diego, CA). Most sequencing was carried out with an Applied Biosystems, Inc., automated sequencer. Several sequences were also sequenced manually using the Promega Cycle Sequencing kit and DNA labelled with 33P. Forward and reverse sequencing or sequencing of a second sample was carried out for each species.

Phylogenetic Analyses

Outgroup Choice.—Trees were rooted with outgroup species of different genetic distances (based on commonly accepted phylogenies of ascidians; Berrill 1936; Kott 1969; Monniot and Monniot 1973; Wada et al. 1992). The full complement of outgroups, used in the parsimony analysis, includes other species from the family Styelidae outside the botryllid clade (sometimes recognized as a subfamily, Botryllinae; Berrill 1950), another species from the order Stolidobranchiata in the family Molgulidae, and a species from another order, Phlebobranchiata. Outgroup sequences were obtained from GenBank (accession numbers are listed in Hadfield et al. 1995).

Different methods of phylogenetic inference were used to compare the results obtained with full and partial datasets using methods that make different assumptions about the data (Felsenstein 1988; Hillis et al. 1993; Swofford 1996). Phylogenetic analyses were carried out with programs from the PHYLIP software package (vers. 3.56c; Felsenstein 1993) for distance and maximum-likelihood analyses and PAUP vers. 3.1.1; Swofford 1990) for the parsimony analysis. Genetic distances between sequences were estimated using Kimura's two-parameter model in the DNADIST program. Phylogenetic inference according to the neighbor-joining method was carried out using the NEIGHBOR program. Statistical confidence estimates expressed as bootstrap values were obtained using SEQBOOT, and consensus trees were generated with the program CONSENSE. Trees were drawn with PAUP (parsimony) or with the hypercard program Tree Draw Deck (Gilbert 1990), which makes use of the programs DrawTree and DrawGram from PHYLIP.

Using parsimony and distance, we experimented with different combinations of ingroup and outgroup taxa to see which nodes might be subject to change (Lecointre et al. 1993; Swofford 1996). For the distance tests, we restricted the outgroups to two to three species following the suggestion

TABLE 2. Pairwise distance matrix of all 15 species used in phylogenetic analyses. Numbers below the diagonal represent absolute distances and numbers above the diagonal are mean distances (corrected for missing data). Species abbreviations as in Figure 2.

Ascid		0.127	0.120	0.113	0.117	0.104	0.107	0.104	0.117	0.117	0.121	0.134	0.150	0.101	0.142
fus	39		0.026	0.062	0.042	0.062	0.062	0.062	0.023	0.029	0.026	0.040	0.139	0.059	0.056
leach	37	8		0.039	0.032	0.042	0.042	0.045	0.003	0.010	0.007	0.020	0.125	0.036	0.036
scal	35	19	12		0.052	0.045	0.052	0.042	0.039	0.039	0.042	0.056	0.141	0.039	0.058
prim	36	13	10	16		0.058	0.045	0.055	0.029	0.036	0.033	0.046	0.131	0.049	0.045
Splic	32	19	13	14	18		0.023	0.013	0.042	0.042	0.046	0.059	0.134	0.029	0.061
Cfin	33	19	13	16	14	7		0.016	0.042	0.049	0.046	0.059	0.128	0.026	0.055
Pcorr	32	19	14	13	17	4	5		0.046	0.045	0.049	0.062	0.134	0.023	0.058
WHD	36	7	1	12	9	13	13	14		0.007	0.003	0.016	0.129	0.039	0.036
simo	36	9	3	12	11	13	15	14	2		0.003	0.023	0.132	0.039	0.042
SD	37	8	2	13	10	14	14	15	1	1		0.02	0.129	0.043	0.039
viol	41	12	6	17	14	18	18	19	5	7	6		0.143	0.056	0.049
Mblei	46	42	38	43	40	41	39	41	39	40	39	43		0.125	0.131
Dgro	31	18	11	12	15	9	8	7	12	12	13	17	38		0.059
sex	44	17	11	18	14	19	17	18	11	13	12	15	40	18	

of Swofford (1996) and alternately picking combinations of ingroup and outgroup species from the full 15-species dataset. For the maximum-likelihood analysis, using DNAML in PHYLIP, we used as many species as computationally possible including important members of the ingroup exhibiting the range of allorecognition behavior. Outgroup members are limited to two solitary Styelidae and one solitary *Ascidia* in the order Phlebobranchiata. Bootstrap values for the distance and maximum-likelihood analyses were generated with the PHYLIP programs SEQBOOT, DNAML with input order jumbled three times, and CONSENSE.

RESULTS

Sequences from the most variable central portion of the nuclear small subunit ribosomal locus were trimmed to 314 bases. This portion of 18S rDNA was chosen for analysis based on the surprisingly high levels of variability previously found in molgulid ascidians (Hadfield et al. 1995). Hadfield et al. (1995) suggest that relative to other taxa, the small subunit ribosomal DNA in ascidians either has a faster rate of mutation (as also found in *Drosophila*; Friedrich and Tautz 1995), or the divergence of these lineages is more ancient than previously suspected.

Sequences were initially aligned using the program GeneWorks (Intelligenetics). Alignments were adjusted by eye. Sequences obtained for this study are deposited in GenBank under accession numbers AF008422–AF008429. Accession numbers of previously sequenced species are found in Hadfield et al. (1995). Estimation of the transition/transversion ratios for the dataset showed an approximately 2:1 ratio and approximately equal frequencies of all bases (ranging from 0.20 to 0.33).

Species Identifications

The identity of the location-specified samples in this study remains in question at present. Neither of the specimens of uncertain taxonomic identity (WHD and SD) had 18S sequences that showed complete matching with any other sample thus far. *Botrylloides diegense*? (SD) and *B. diegense*? (WHD), which could potentially both have been *B. leachi* or *B. diegense*, instead appear to be different from each other and from Italian *B. leachi* as well.

Phylogenetic Analyses

Because of the close relationships between these species (as shown in the distance matrix, Table 2), the assumption of similarity of evolutionary rates between taxa, an assumption that may be crucial to robust phylogenetic analysis, is strong in this study (e.g., Swofford 1996). This assumption is apparently less critical to maximum-likelihood methods (Hillis et al. 1993), and our results with maximum likelihood are in good agreement with analyses using parsimony and distance methods. In the parsimony analysis, of 314 total sites 64 were variable (20.0%) and 31 were informative (9.9%).

The botryllid ingroup is supported in all analyses. Botryllid monophyly is not questioned in any ascidian systematic literature, and there is no reason to suppose lack of monophyly here. All analyses placed colonial botryllids as derived with respect to the solitary styelids. These analyses do not provide information on the particular relationship of specific solitary styelid species to the botryllids, and there are many more species of solitary and colonial styelids (including colonial species outside the botryllid clade) that would likely need to be added to the phylogeny to gain this information. Placement of *B. scalaris* as the basal botryllid is particularly well supported by the molecular results. Bootstrap support measures for the placement of *B. scalaris* were 74% using the full parsimony analysis, 88% in a neighbor-joining distance analysis, and 92% in a maximum-likelihood analysis.

Concerning the branching order of the botryllids, we consistently found the same topology with regard to the placement of *B. scalaris* as basal, followed by other species in the genus *Botryllus* (*B. primigenus* and *B. sexiens*), and finally, *Botrylloides* species as most derived. *Botrylloides* appears as a monophyletic cluster in 84% of the maximum-parsimony bootstraps. In the full parsimony analysis, *Botryllus primigenus* and *B. sexiens* appear as a sister group to the *Botrylloides* species, and both groups are derived with respect to *B. scalaris*.

DISCUSSION

Tree Topology and Allorecognition Character Mapping

Phylogenetic analyses using parsimony, distance, and maximum-likelihood algorithms all agreed on the placement of

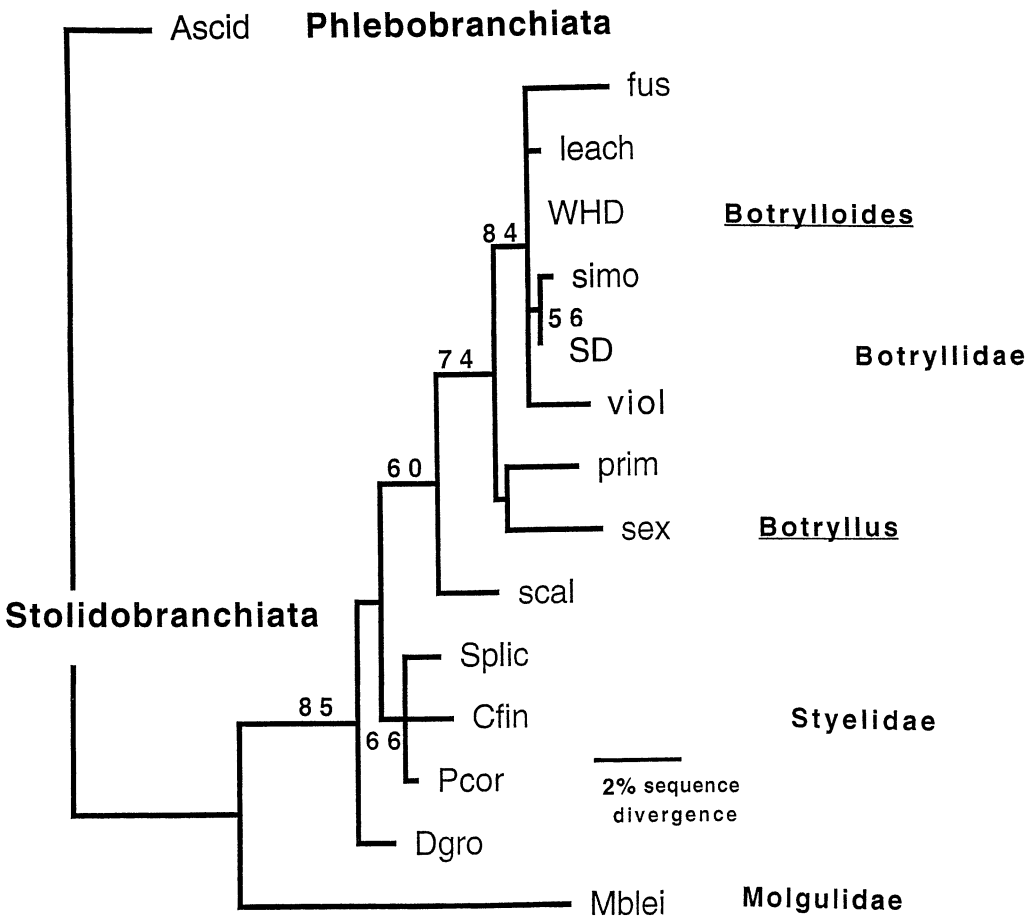

FIG. 2. Full 15-species phylogenetic tree produced with parsimony analysis. Phylogenetic analyses using parsimony, distance, and maximum-likelihood algorithms all agreed on the placement of all botryllid species where bootstrap support is indicated at the nodes in Figures 2 and 3. Outgroup species to the botryllids include the following solitary ascidians: (1) order Phlebobranchiata, family Ascidiidae: Ascid, *Ascidia ceratodes*; (2) order Stolidobranchiata, family Molgulidae: Mblei, *Molgula bleizi*; (3) order Stolidobranchiata, family Styelidae: Dgro, *Dendrodoa grossularia*; Pcor, *Pelonaia corrugata*; Cfin, *Cnemidocarpa finmarkiensis*; Splic, *Styela plicata*. Ingroup botryllid species are: scal, *Botryllus scalaris*; sex, *Botryllus sexiens*; prim, *Botryllus primigenus*; fus, *Botrylloides fuscus*; leach, *Botrylloides leachi*; simo, *Botrylloides simodensis*; viol, *Botrylloides violaceous*. The tree shown is a strict consensus of the six shortest trees found using the branch and bound method with the furthest addition sequence option in PAUP, vers. 3.1.1. These six trees all had an equal length of 122 steps and a consistency index (CI) of 0.820 (HI = 0.180). Numbers at nodes represent percent of 100 bootstraps that support that node.

all botryllid species where bootstrap support is indicated at the nodes in Figures 2 and 3. Analyses using three types of phylogenetic algorithms (parsimony [Fig. 2], distance, and maximum likelihood [Fig. 3]), which make different kinds of assumptions about the data and the process of molecular evolutionary change, are in agreement on the basal state and pattern of evolution within the botryllid clade. Further, the topology of the tree regarding the basal botryllid species and the subsequent botryllid branching arrangement were robust to changes in the composition and number of ingroup and outgroup species. In particular, there is a high level of bootstrapped confidence using all analyses for the placement of *B. scalaris* as the basal botryllid in this phylogeny. Phylogenetic reconstructions using DNA sequence data from the 18S nuclear rDNA locus strongly support the hypothesis of internal allorecognition as the basal state in the botryllids.

There is also a high level of support for the subsequent branching order from species with internal or intermediate allorecognition behaviors (*B. sexiens* and *B. primigenus*) to the *Botrylloides* species with the most external forms of allorecognition behaviors (*B. simodensis, B. fuscus*, and *B. violaceous*). The allorecognition mechanisms of some species are unknown either from lack of study or ambiguity concerning species identifications (e.g., *B. diegensis* and *B. leachi*). *Botryllus sexiens* is thought to behave similarly to *B. scalaris* (Y. Saito, unpubl. obs.).

Interestingly, two of the most derived species, *B. fuscus* and *B. violaceous* have outer tunic recognition abilities, but appear to have lost that ability internally (Hirose et al. 1994). This ability is assessed using cut colony assays where the outer ampullar regions of two colonies are cut away and the internal areas are juxtaposed on a slide. Most botryllid species tested thus far show similar rejection responses in fusion assays using whole colonies (outer ampullar contacts) and cut colonies. However, *B. fuscus* and *B. violaceous*, species that do not naturally undergo intercolony internal tissue con-

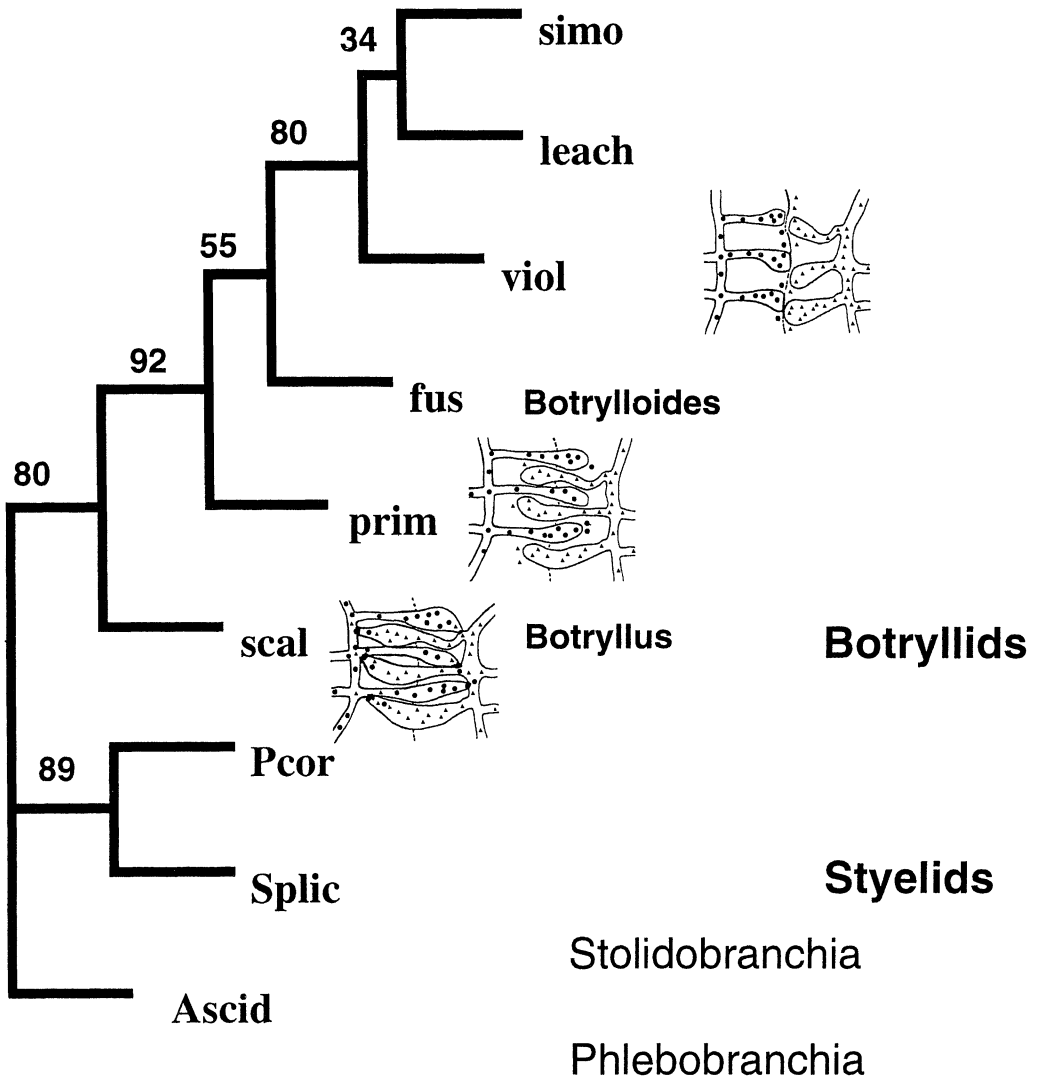

FIG. 3. Maximum-likelihood phylogeny of a subset of the original 15 species concentrating on the botryllids. Adjacent cartoons of interacting tunic and ampullar areas for three species show the location of the allorecognition response.

tact, appear to accept all fusions using cut assays, including fusion with individuals that they reject in whole colony assays. Interestingly, *B. simodensis*, another of the species showing the most external form of allorecognition behavior, does show rejection in cut assays (Hirose et al. 1990).

While little is known about physiological mechanisms regulating allorecognition and allowing this behavioral variation in intercolony integration to occur between species, it seems likely that all species share components of a common effector system (Taneda et al. 1985; Hirose et al. 1990). Scofield and Nagashima (1983; Scofield 1987) suggest that the continuum in allorecognition behavior represents different species-specific thresholds for activation of effector responses for rejection. Hirose et al. (1994) propose that differential distribution of allorecognition effector components in various species, and specifically, a concentration of the effectors at the tunic where allorecognition normally occurs in *B. fuscus* and *B. violaceous*, and an absence of those effectors at interior locations in the colony, may explain the lack of a rejection response found in cut colony assays of these species (as described above). Experimentation with a variety of different species in an effort to isolate blood components of the recognition system has revealed some common components, which are discussed below.

Solitary and Colonial Ascidian Allorecognition Systems

Identification of internal allorecognition as the ancestral state is compatible with observations using allogeneic and syngeneic blood mixtures in solitary ascidians. As previously mentioned, solitary ascidians are ancestral to colonial ascidians according to modern phylogenetic reconstructions of ascidian evolution using either morphological (Berrill 1936, 1955; Kott 1974; Monniot et al. 1991) or molecular characters (Wada et al. 1992). Several species of solitary ascidians show cytotoxic reactions in allogeneic blood mixtures (e.g., *Halocynthia roretzi*: Fuke 1980; Sawada and Ohtake 1994; *Styela plicata*: Raftos and Hutchinson 1995). Cytotoxicity has been

observed in allogeneic botryllid blood mixtures as well (Taneda and Watanabe 1982; Saito and Watanabe 1984; Ballarin et al. 1995). Both solitary and colonial ascidians show a variety of cellular and humoral allorecognition response characteristics including lectin-binding; response to vertebrate antigens; antimicrobial activity, and phagocytic, cytotoxic, and encapsulation behaviors (Ballarin et al. 1993, 1994; Millar and Ratcliffe 1994; Parrinello et al. 1996). For botryllids specifically, allorecognition responses at the blood level are observed as a darkening either within the cells involved or associated with blood cells that have leaked out of their ampullar vessels and contacted cells or plasma from the opposing colony. The darkening has been shown to be associated with a phenoloxidase reaction in at least one species (Ballarin et al. 1995).

It remains to be established that the blood components in solitary ascidians that are involved in internal recognition functions are homologous to blood components involved in the allorecognition reaction of colonial ascidians (Satoh 1994). Laboratory-graft rejection experiments in solitary ascidians indicate that for some species the histocompatibility matching system apparently requires complete matching of all alleles at more than one locus, while for others a single shared allele, as in the botryllid recognition system, appears to be sufficient for graft acceptance (Raftos and Briscoe 1990; Rinkevich 1992; Davidson 1994). Since solitary ascidians rarely, if ever (but see Schmidt 1982), undergo natural fusion of intact individuals, the existence of homologous cellular and blood-based recognition systems in solitary and colonial ascidians argues for selective forces other than space competition as the evolutionary impetus for an allorecognition system subsequently coopted for botryllid allorecognition. As research on blood-based ascidian effector systems and the genetics of historecognition responses advances, it will be interesting to look for homologies between solitary styelids and colonial botryllids. The existence of clear species identifications (based on molecular data) and a phylogenetic context for interspecific variation should contribute substantially to mechanistic studies on the physiology and genetic basis for the allorecognition response.

Concordance of the Molecular Phylogeny with Other Characters

A detailed morphological character analysis is not available at this time for the botryllids due to ambiguities in systematic characters in this group (Monniot and Monniot 1987; Monniot 1988). However, this phylogeny does agree with some previous suggestions on the ancestral type of allorecognition behavior based on morphological and behavioral observations (Taneda et al. 1985; Hirose et al. 1988, 1994; Saito et al. 1994). Historically, morphological analysis of this group has separated the botryllids into two genera, *Botryllus* and *Botrylloides*. The placement of *Botrylloides* species as derived with respect to *Botryllus* species is supported by developmental and reproductive characters (Berrill 1947, 1950; Mukai 1977; Saito and Watanabe 1985; Mukai et al. 1987; Table 3). For example, all described *Botrylloides* species to date have separate specialized pouches where embryos are brooded, however few *Botryllus* species have specialized brood structures and most hold their embryos free in the peribranchial chamber (Berrill 1950).

TABLE 3. Botryllid life history characters (Berrill 1936, 1947, 1950, 1955; Kott 1974; Saito et al. 1981a,b; Saito and Watanabe 1982, 1985; Mukai et al. 1987; Monniot et al. 1991). Comparison of the two genera shows an increase in larval size at hatching, a decrease in the number of eggs per zooid, and an increase in development time between *Botryllus* and *Botrylloides* species.

Species	Larval size (mm)	# of eggs/ zooid	Embry. dvt. time (d)	Type of brooding
Botryllus				
scalaris	1.5	2–4	4–5	no pouch
primigenus	1.5–1.7	2–4	4–5	pouch
sexiens	1.5–1.8	4	4–5	no pouch
Botrylloides				
fuscus	2.6–2.7	usu. 1	12–14	pouch
simodensis	1.7–2.0	1 or 2	4–5	pouch
leachi	2	2	"a few days"	pouch
violaceous	3	1 or 2	>30	pouch

Saito and Watanabe (1985) propose a transformation series of correlated reproductive characters with increasing specialization and parental care for embryos directionally from *Botryllus* species to various *Botrylloides* species (and other authors have noted these trends, e.g., Berrill 1947, 1950; Sabbadin et al. 1992). Characters (listed in Table 3) associated with the transformation series include general trends from *Botryllus* species to *Botrylloides* species of fewer eggs per zooid, increasing development time per embryo, increasing larval size at hatching, and development of more enclosed brood structures. Similar correlated trends in some of these developmental traits have been proposed for a number of ascidian families (Berrill 1935; Kott 1969).

While two generic names for botryllids are still in general use, a reanalysis of reproductive and other morphological characters that are the basis for the separation has led to a proposed synonomization of the genera (Monniot and Monniot 1987; Monniot 1988). This molecular phylogeny does not contradict the generic distinction since the *Botrylloides* and *Botryllus* species each cluster together, however this clustering in the molecular phylogeny does not necessarily justify separate generic status either.

Phylogenetic Hypotheses on Allorecognition

Application of a comparative phylogenetic approach to the evolution of invertebrate allorecognition systems has been considered at much broader scales (e.g., across phyla, Du Pasquier 1989; Harvell 1990; Davidson 1994; and more specifically within the phylum Cnidaria; Buss et al. 1984; Buss 1990). However, because analysis at these higher levels most likely deals with nonhomologous allorecognition effector structures (Buss 1990) and the genetics of allorecognition for nearly all of these systems is currently unknown, the question of selective forces promoting the evolution of the polymorphic recognition loci functionally related to these structures becomes more complicated. The advantage of using the botryllid clade for a phylogenetic analysis of allorecognition lies in the fact that there is substantial behavioral variation based on a single effector system.

Phylogenetic reconstruction of the evolution of allorecognition behavior using molecular systematics in this study shows that allorecognition at the whole colony level for purposes of space competition is unlikely to have been the original evolutionary force selecting for a highly polymorphic recognition system in botryllid ascidians, and perhaps, other colonial protochordates as well. In styelid ascidians, it appears more likely that internal discriminatory functions selected for blood-mediated recognition mechanisms in solitary ascidians, and these systems were subsequently coopted for external space competitive purposes in colonial botryllids. Whether this pattern of internal to external allorecognition function in botryllids is reflected in the evolution of allorecognition systems in other colonial invertebrates remains to be seen.

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