Allorecognition and Microsatellite Allele Polymorphism of *Botryllus schlosseri* from the Adriatic Sea

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Summary. *Botryllus schlosseri*, a compound ascidian is found worldwide in temperate and subtropical shallow waters. This species, which originated in the Mediterranean Sea, has become cosmopolitan via the transportation of colonies attached to ship hulls. Two types of genetic markers (allorecognition alleles on the tunicate fusibility-histocompatibility locus and molecular markers, microsatellites) have been used here to characterize native populations of *B. schlosseri* in the Istra Peninsula, Croatia (the Northern Adriatic Sea). In this area, ship transportation is locally restricted, reducing the possibility of gene flow from remote populations. Allorecognition assays revealed that fusion and rejection responses are similar to those recorded previously from other populations. Pairwise allorecognition assays performed on all combinations (n=120) from 16 colonies resulted in 14.9% rejections, due to the appearance of 10-19 calculated fusibility alleles, not equally frequent. Genetic diversity of two populations was tested by four polymorphic microsatellite loci (4-11 alleles). Hardy-Weinberg exact test for loci revealed a significant heterozygote deficiency, suggesting partial inbreeding. Comparisons with other populations worldwide further indicate the high polymorphism characteristic to this species.

Key words. *Botryllus schlosseri*, Histocompatibility, Genetic structure, Molecular markers

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Introduction

Botryllid ascidians (subfamily Botryllinae) are a small but widely distributed group of subtidal organisms, common inhabitants of fouling assemblages. It is now almost 20 years that researchers have used different taxa of this group as important model organisms in a variety of scientific disciplines such as evolutionary ecology, invertebrate allorecognition, developmental biology and cell biology, concentrating primarily in the worldwide species *Botryllus schlosseri*. *B. schlosseri* is found in all European waters, the eastern and western coasts of North America, Australia, New Zealand, Japan, Korea, India and in many other localities (cited in Berrill 1950; Tokioka 1953; Dybern 1969; Luckens 1976; Plough 1978; Kott 1985; Rinkevich et al. 1995, 1998). This species, most likely of Mediterranean Sea origin, has become cosmopolitan via the transportation of colonies attached to ship hulls (Berrill 1950; Skerman 1960).

It is therefore of great interest to reveal the genetic structures of *B. schlosseri* populations worldwide, introduced and native populations alike, to formulate a comprehensive genetic portrait of this species. Two different types of genetic markers have been used for these populational comparisons, the morphological consequences of histocompatibility alleles operations (Boyd et al. 1990; Rinkevich and Weissman 1991; Rinkevich et al. 1995) and the molecular markers of microsatellites (Pancer et al. 1994; Stoner et al. 1997; Paz 1999, Ben-Shlomo et al. in press). Previous studies on a variety of *B. schlosseri* ecological aspects revealed that different populations can be divided into distinct local subpopulations exhibiting microgeographic differences in life history patterns (Sabbadin and Graziani 1967; Sabbadin 1972, 1978; Grosberg 1991; Rinkevich et al. 1998). On the other hand there are a variety of genetically controlled life history patterns of this species that are shared even between remote populations (Rinkevich et al. 1998). Detailed studies on additional *B. schlosseri* populations will be therefore of significant value to elucidate its whole spectrum of life history patterns and genetic characteristics.

This manuscript aims in studying polymorphism levels of allorecognition alleles and microsatellite loci in the relatively isolated *B. schlosseri* population from the Istra Peninsula, northern Adriatic Sea. As in other Mediterranean and European sites (Dybern 1969; Rinkevich et al. 1998; unpublished results), *B. schlosseri* colonies in this area encrust the shallow water stones undersurface. At 30m depth, they overgrow upper surface areas of stones lying on soft bottoms (Müller et al. 1994). Ship transportation in the area is locally restricted, reducing the possibility of gene flow from remote populations.

Materials and Methods

Sampling

B. schlosseri colonies were collected from shallow waters (mainly <1 m depth) in 3 localities along the Istra Peninsula, north (near Institute Ruder Boskovi) and south (marina) of the town of Rovinj and within Limski Canal situated 8km north to Rovinj. Samples for microsatellite analyses were collected only from the first 2 localities, 17 colonies from the Institute region and 28 from the Marina. In each place, colonies were haphazardly collected from underneath stones. *Botryllus* colonies in the Limski canal were very sparse and few were collected after a long search. For allorecognition assays, colonies were peeled off stones by single-edge razor blades and tied by cotton threads to 5 x 7.5cm glass slides, one colony per slide. The collected colonies were usually >1m from each other, reducing the chances of sampling kin colonies. For microsatellite analyses, colony samples (at least one system in size) were placed separately into 1.5ml vials containing 240µl lysing buffer (Graham 1978), homogenized, extracted with 240µl phenol/ chloroform/isoamyl alcohol solution and shipped to the laboratory in Haifa, Israel.

Allorecognition Assays

Colonies that were attached to glass substrates and grew, were shipped in insulated containers to the laboratory at Haifa. We employed the CAA protocol (Colony Allorecognition Assay; Rinkevich et al. 1995) to analyse fusibility at the morphological level. In these assays, subclones were carefully isolated from large colonies by razor blades, and allogenetically paired on glass slides, so they contacted one another through extending ampullae. Outcomes are usually developed in less than 72h. Several assays were sacrificed for histological observations. Sample were fixed in 2.5% glutaraldehyde in sea water for 3h at room temperature, dehydrated and embedded in JB-4 according to the manufacture's protocol (Polysciences, Inc., Warnington, PA). Sections (2µm) were stained by Delafield's hematoxilin eosin.

Estimating the number of allorecognition alleles on *B. schlosseri* fusibility locus is based on a single locus, partial-matching genetic model of multi-codominantly expressed alleles (Oka and Watanabe 1960; Sabbadin 1962; Scofield et al. 1982). During the experiments, the colonies were kept in 17 l standing seawater tanks, aerated by air stones in a temperature-controlled room (20°C). CAAs were observed daily and cleaned during the observations by small brush. Food was supplied daily (Rinkevich and Shapira 1998).

Microsatellite Typing

Four *B. schlosseri* microsatellites, BS-811 (Pancer et al. 1994), PB-29, PB-41 and PBC-1 (Stoner et al. 1997), were determined by PCR amplification following Ben-Shlomo et al. (in press). Unbiased estimated of Hardy-Weinberg extract *P*-values were computed by the Markov chain method using GENEPOP, version 3.1d (Raymond and Rousset 1995). The significance level was determined after 20 batches and 1000 interactions each. F_{st} was calculated using the program ARLEQUIN, version 1.1 (Schneider et al. 1997), and the significance level for the overall values was determined after 1023 permutations, and for the population pairwise F_{st} values after 99856 permutations. The population pairwise differentiation test (AMOVA) included 900,000 Markov chain steps.

Results

Allorecognition on the Morphological Level

B. schlosseri colonies from Istra, Croatia possessed morphologically the color morphs and general characteristics as of the Israeli populations (Rinkevich et al. 1998). The same holds for allorecognition responses. The first morphological response was revealed when the tips of marginal ampullae were reciprocally positioned in tip-tip orientation. In compatible assays, the tunic matrices of both partners fused (usually in limited areas) and ampullae of both or of a single colony penetrated into the tunic matrix of the other genotype. Fusions were established in

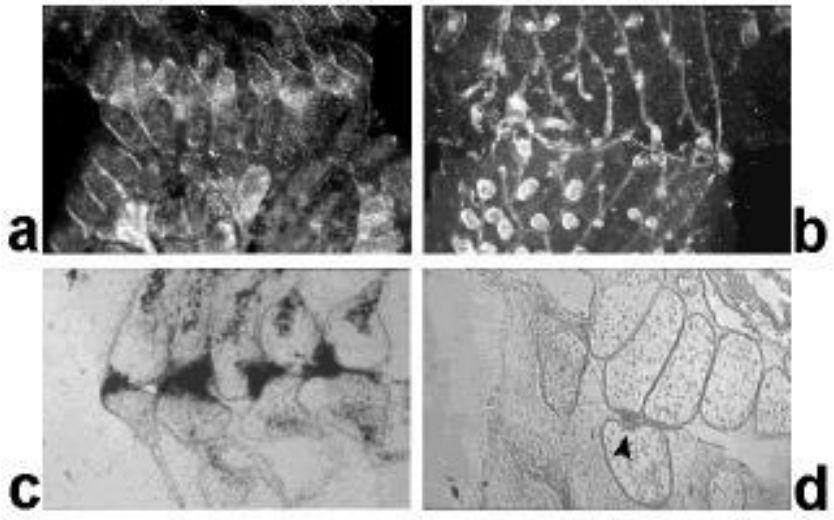


Fig 1. Allogeneic interactions between non-compatible colonies. **a**. Tips of ampullae possess aggregations of bright cells in contact area, x13. **b**. Necrotic lesions left in interacting area after retreating of most contacting ampullae, x13. **c**. Points of rejection, POR's as seen in (phase) inverted microscopy, x50. **d**. Histology section of interacting ampullae. Initial formation of POR's (*arrowhead*), x50.

tip-base orientation. In non-compatible assays (Fig. 1a-d), tunic matrices did not fuse and ampullae engaged reciprocally through both cortical layers. Following this stage, peripheral contacting ampullae of one or both partners were distinguished by aggregations of blood cells in their tips that developed (within 24-72h) in some places to dark brown points of rejection (PORs). Other cases revealed also blood cell infiltration into the tunic matrix.

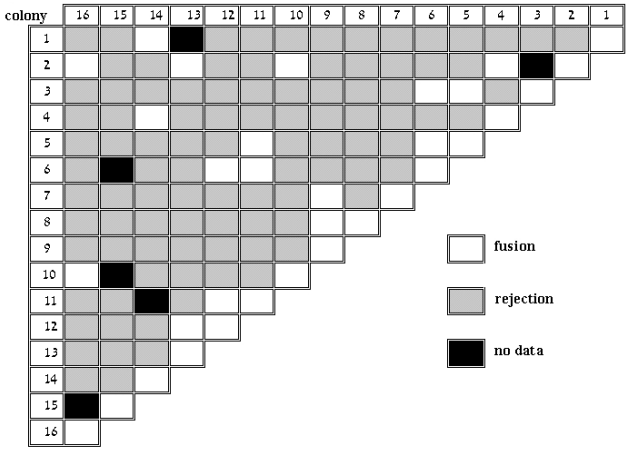
Pairwise Allorecognition Assays

Sixteen *B. schlosseri* colonies were sampled from 3 localities (4 from the Limski Canal, 4 from the marina and 8 near the Institute Ruder Bošković). A panel of 120 pairwise allorecognition combinations and 16 autogeneic assays was performed. All autogeneic assays resulted in fusions (Fig. 2a). Six allogeneic combinations gave no conclusive results. Out of the remaining assays, 97 (85.1%) resulted in rejections and 17 (14.9%) in fusions. A fusibility allele chart for allorecognition alleles revealed that the 16 participating colonies possess 10-19 different Fu/HC alleles, assigned into 4 distinct groups in which intergroup assays resulted in only rejection outcomes (Fig. 2b). One of the groups had all the 8 colonies collected near the Institute Ruder Bošković and one colony from the marina. Two groups contained one and three colonies each, only from the Limski Canal. The fourth group contained 3 marina collected colonies. Out of the maximum possible 19 alleles, 10 (52.6%) were assigned, each, to a single colony alone and only 4 alleles (21.1%; i, m, p, q; Fig. 2b) appeared in 3 different colonies each.

Microsatellite Analysis

A total of 26 different alleles were found in the four tested microsatellites, 4-11 alleles per locus (Fig. 3). Observed heterozygosity was lower than expected in most loci. A Hardy-Weinberg exact test for all loci and the two populations revealed a *P*-value of 0.0000 (HW equilibrium). Allele frequencies varied between population. An interesting difference between the 2 populations appeared in PBC-1 where the Institute population was monomorphic on the 193bp allele (n=10), and in the marina population (n=5) its frequency was only 0.1 (Fig. 3). However, AMOVA differentiation tests between the two populations studied revealed no significant differences ($P>0.25$).

a



b

Colony \ Allele	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	Estimated # of alleles
L 15	+	+																		
M 8			+	+																
M 9			+		+															
M 7					+	+														
L 1							+		+											
L 14								+	+											
L 4									+	+										
M 10											+		+							
I 16												+	+							
I 2													+	+						
I 13														+	+					
I 12															+	+				
I 11																+	+			
I 6																+		+		
I 5																	+	+		
I 3															+				+	
		+	+		+				+				+	+	+	+	+	+		Minimum: n=10
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Maximum: n=19

Fig. 2. Colony allo- and auto-recognition assays. **a.** Pairwise recognition outcomes. **b.** Estimated number of Fu/Hc alleles.

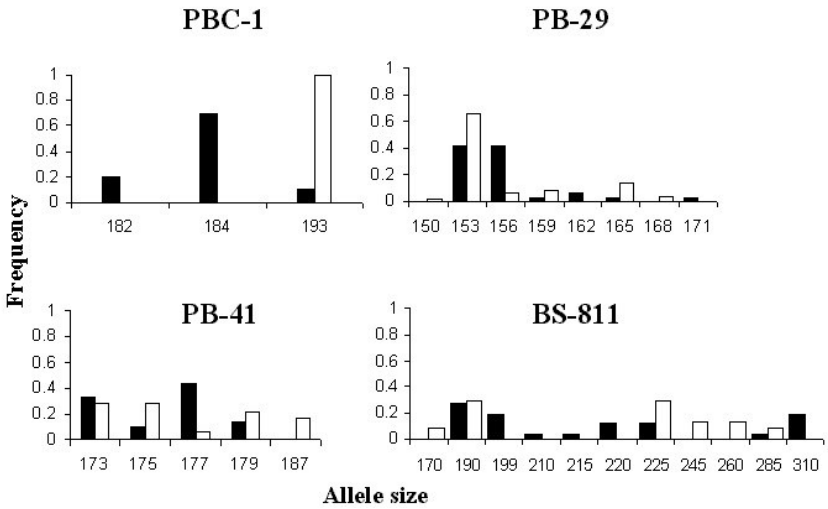


Fig. 3. Allele frequency histograms. Black bars are for the institute population, empty bars for the marina population.

Discussion

Pairwise Allorecognition Assays

As in other studies *B. schlosseri* populations (reviewed in Rinkevich et al. 1995) the Fu/HC alleles reveal unprecalented extensive polymorphism. More than half of the deduced maximal possible no. of alleles (10/19) were assigned to only a single colony. Although the number of colonies assayed for their fusibility alleles repertoire is low to reveal the total number of Fu/HC alleles in the Istra population, it is evident that also this native populations is highly polymorphic. Such high polymorphism calculated as hundreds of alleles in worldwide distributed populations should be further taken into consideration when studying the biology of this species. Botryllid ascidians possess highly complex and polymorphic systems of effector and allorecognition mechanisms (including

fusion and rejection, resorption and germ/ somatic cell parasitism) all controlled by variety of histocompatibility genes (Scofield et al. 1982; Rinkevich 1993).

Previous predictions that allorecognition alleles reveal high polymorphism to reduce rates of natural occurring fusions (Grosberg and Quinn 1986) may further be questioned by recent results showing high rates of natural chimerism (Ben-Shlomo et al. in press).

Heterozygote Deficiency

The results of the microsatellite analysis revealed a significant heterozygote deficiency. Similar outcomes of heterozygote deficiency on microsatellite loci were found in all already sampled *B. schlosseri* populations, including New Zealand North and South Islands (Ben-Shlomo et al. in press), Monterey, California (Stoner et al. 1997), and the Mediterranean coast of Israel (Pancer et al. 1994; Paz 1999). Such deviations from expected levels that had been recorded repeatedly on several loci on animals collected from different localities by different laboratories and at different times, should reflect a genuine characteristic of *B. schlosseri.* Non random mating is most likely the reason for this. Although *B. schlosseri* colonies are hermaphrodites, sperm release from gravid colonies does not start until about 1-2 days after ovulation, effectively preventing self-fertilization (Yund et al. 1997). However, Grosberg and Quinn (1986) showed in field experiments, an aggregated settlement of sibling *Botryllus schlosseri* planktonic larvae. Such aggregations can form small-scale subpopulations, a situation that promotes mating between sibling colonies (Sabbadin 1978; Grosberg and Quinn 1986, Rinkevich et al. 1998).

Interpopulation Comparison

The two populations sampled are geographically closed, separated by the city of Rovinj. However, they exhibit different allele distributions, as best recorded by the PBC-1 locus. The marina population was monomorphic on this locus (193bp allele), whereas in the institute, the population possessed 3 alleles, of which 193bp frequency was minimal, only 0.1. Diffrences in allele frequencies between the populations are also reflected in locus PB-29 (allele 156bp was the most common [0.40] in the institute but rare [0.06] in the marina), and in locus PB-41 (allele 177bp was the most frequent [0.43] in the institute and rare [0.06] in the marina). The results suggest that gene flow between these populations is minimal. Differentiation tests between populations, however, were not significant. This may result from the small sample size, especially as in PBC1 locus, in which 10 colonies gave scorrable result in the Institute population, and only 5 in the Marina. Larger sample size, and/or repeated sampling in different years can pinpoint whether this phenomenon is genuine or only a sampling error.

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