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Genetic structure of *Botryllus schlosseri* (Tunicata) populations from the Mediterranean coast of Israel

Guy Paz[1, 2,*], Jacob Douek[1], Caiqing Mo[1], Menachem Goren[2], Baruch Rinkevich[1]

[1]National Institute of Oceanography, Israel Oceanographic and Limnological Research, PO Box 8030, Haifa 31080, Israel
[2]Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

ABSTRACT: *Botryllus schlosseri*, a subtidal cosmopolitan tunicate species, is abundant in many rocky shores along the Mediterranean and other European seas. A highly polymorphic microsatellite locus (BS-811) elucidated *Botryllus* population genetics in 3 localities along the Israeli coast (at Shikmona, Caesarea and Michmoret, 12 to 36 km apart from each other, north to south, respectively) during 8 seasonal samplings (2 yr). Four other loci were studied only in the Michmoret population. The analysis on 1156 scorable colonies revealed a high number of alleles per locus (up to 15 to 38 alleles), unique alleles for each population (8 to 13 alleles per locus), high heterozygous deficiency, rapid seasonal changes in allele frequencies at all sites, and the existence of natural chimeric colonies. Locus BS-811, which was studied in all 3 localities, revealed 34 to 40 alleles per locality, and a total of 64 alleles, of which 48% were unique to one or other of the localities. Only 24 colonies from the 1156 studied were heterozygous on this locus. These results are discussed together with recent outcomes from *B. schlosseri* populations sampled in New Zealand, the east and west coasts of the USA and the Adriatic Sea, Croatia.

KEY WORDS: *Botryllus schlosseri* · Chimerism · Heterozygous deficiency · Israel · Microsatellites · Population genetics

INTRODUCTION

The colonial tunicate *Botryllus schlosseri* (subfamily Botryllinae) is a cosmopolitan subtidal urochordate species recorded in the North Sea; all European waters; the eastern and western continental shelves of North America; south and east Asia (India, Japan, Hong Kong, Korea); Vancouver Island, Canada; North to South Africa; the Great Barrier Reef Australia; southern New Zealand and more (Berrill 1950, Tokioka 1953, Dybern 1969, Luckens 1976, Plough 1978, Kott 1985, Rinkevich et al. 1995, 1998, Lambert & Lambert 1998, Ben-Shlomo et al. 2001, Stoner et al. 2002). Colonies are found both in very shallow intertidal water and at almost 200 m depth; on rocky and other hard bottom habitats; above and under stones; on algae and sea weeds; on piling, floats and other artificial substrata; within marinas and in the wild (Sabbadin 1969, Rinkevich et al. 1998). It is believed that this species originated from the Mediterranean Sea, and has been introduced into new habitats worldwide through man-mediated dispersal mechanisms, mainly by being attached to boat hulls or to floating objects (Berrill 1950, Skerman 1960, Lambert & Lambert 1998, Ben-Shlomo et al. 2001).

Botryllus schlosseri populations worldwide revealed high polymorphism on either the morphological or the molecular characteristics studied. These include colonial color morphs (Sabbadin & Grazini 1967, Sabbadin 1979), microsatellite alleles (Pancer et al. 1994, Stoner et al. 1997, 2002, Paz 1999, Ben-Shlomo et al. 2001, Rinkevich et al. 2001) and allorecognition elements (Rinkevich et al. 1995, 2001). *Botryllus* populations across the world may significantly differ on specific genetic elements, or conversely may share the same alleles on other gene loci. For example, while hundreds of allorecognition assays did not reveal any shared allele on the tunicate fusibility locus in east

*Email: pazguy@ocean.org.il

versus west USA coast population analyses (Boyd et al. 1990, Rinkevich & Weissman 1991), about 4.4% of the assays performed on California versus Mediterranean (Israel) populations revealed shared alleles on this locus (Rinkevich et al. 1992). By employing a population genetic analysis on 5 microsatellite loci, Ben-Shlomo et al. (2001) have revealed high polymorphism and heterozygote deficiencies within 6 New Zealand *Botryllus* populations. These populations are thriving in natural coastal and man-made habitats that have been subjected to significant *Botryllus* invasions during the last century. Similar outcomes from the same microsatellite loci were recorded in natural populations from Istra Peninsula, Croatia (Rinkevich et al. 2001) and in 8 populations from the east and west coasts of the USA (Stoner et al. 2002). These results may suggest partial inbreeding in wild *Botryllus* populations (Ben-Shlomo et al. 2001, Stoner et al. 2002).

Using the protocol of colony allorecognition assay, Rinkevich et al. (1995) studied polymorphism at the fusibility haplotype in 3 *Botryllus* populations along the Mediterranean coast of Israel, 12 to 36 km apart from each other. Several 100s of fusibility alleles were confined to each one of these adjacent populations, revealing an unprecedented extensive polymorphism at this locus. It was proposed that this characteristic is maintained through an overdominant selection, which is enhanced by the threat of germ/somatic cell parasitism, resulting from the gregarious settlement of kin colonies (Grosberg & Quinn 1986) and the inevitable consequences of allogeneic fusions (Pancer et al. 1995, Stoner et al. 1999). Interestingly, interpopulational allorecognition assays performed between colonies of these 3 closely situated *Botryllus* populations failed to reveal shared fusibility alleles. It is therefore of further interest to evaluate polymorphism and shared/non-shared alleles on additional genetic elements to characterize the genetic population structures of the Israeli *Botryllus* populations.

The present study followed these 3 *Botryllus* populations over a 2 yr period. One highly polymorphic microsatellite locus was studied during 8 seasonal samplings at all 3 sites, while the other 4 microsatellite loci were studied in greater detail on the collected DNA samples from colonies of a single population (Michmoret).

MATERIALS AND METHODS

Animal sampling. During the 2 yr course of the study, 1193 colonies of *Botryllus schlosseri* (1156 scorable DNA samples) were sampled from rocky intertidal (0.5 to 1 m) waters at 3 localities along the Mediterranean coast of Israel (from Shikmona 32° 50′ N, 34° 58′ E in the north, to Caesarea 32° 30′ N, 34° 54′ E and Michmoret 32° 24′ N, 34° 52′ E in the south). Approximately 50 colonies/sites were sampled each time, 4 times yr^{-1}, from spring 1995 to autumn 1997 in each season. The colonies were randomly collected from distances of at least 1 m apart, to reduce the chances of sampling kin colonies (Grosberg & Quinn 1986), or ramets of the same genotype.

In this type of area, *Botryllus schlosseri* colonies are easily collected from underneath stones, lying on the sandy bottom or in piles (Rinkevich et al. 1998). The localities, however, differed in the numbers of stones per m^2, the highest number being in Michmoret and the lowest in Shikmona (Rinkevich et al. 1995). Colonies were removed from the substrate using single-edge razor blades. A tissue sample of at least 1 system containing up to 20 zooids was taken from each colony for DNA extraction. The stones were then placed back at their original position, as carefully as possible.

DNA extraction. Each tissue sample was placed in the field, separately, in a 1.5 ml vial containing 100 µl lysis buffer (0.25 M Tris Borate pH 8.2, 0.1 M EDTA, 2% SDS, 0.1 M NaCl). Samples were homogenized and 20 µl $NaClO_4$ (5 M) followed by 120 µl of phenol/chloroform/isoamyl alcohol solution (25:24:1 v:v:v) were added. The vials were transferred to the laboratory at the National Institute of Oceanography, Haifa, for further work. In the laboratory the samples were thoroughly mixed (1 min) and centrifuged (14 000 rpm [10 000 × *g*] for 5 min). The aqueous phase was collected, added to the same volume of chloroform-isoamyl alcohol (24:1 v:v), thoroughly mixed (1 min) and centrifuged (14 000 rpm [10 000 × *g*] for 5 min). Genomic DNA visible at this stage in the center of the vial was precipitated by centrifugation with 100% absolute alcohol in the cold (4°C, 14 000 rpm [10 000 × *g*] for 15 min). The alcohol was removed and the DNA was washed with 70% ethyl alcohol (volume × 2), centrifuged (14 000 rpm [10 000 × *g*]) dried up in a ventilated hood, dissolved in 50 µl sterile double distilled water, and diluted 1:20 in double distilled water. DNA samples were kept in a cold room (4°C) for 1 mo or at –20°C for longer periods.

Microsatellite typing. Five *Botryllus schlosseri* microsatellites, BS-811 (Pancer et al. 1994), PB-29, PB-41, PB-49 and PBC-1 (Stoner et al. 1997), were amplified by polymerase chain reaction (PCR) with specific primers and according to the following recommended conditions (96°C, 60 s; 20× [72°C, 60 s; 55°C, 60 s; 96°C, 60 s], 72°C, 60 s; 6°C; Ben-Shlomo et al. 2001). DNA was labelled with [^{35}S]αd′ATP in a 20 µl reaction mixture, containing PCR buffer 0.1 mM d′NTP's, polymerase 1 unit, 0.1 mM primers and 0.5 µg $µl^{-1}$ DNA. After the PCR, 4 µl of stop solution (Bromophenol

blue/Xylene Cyanol) was added to the reaction mixture that had been kept in cool conditions (4°C). Four µl of the PCR product mixture was loaded on standard 50 cm long plates (Biorad); 6% polyacrylamide denaturating gel electrophoresis (2.1 kV; 40 W; 35 mA), with a known sequence of Blue Script SK plasmid (Stratagene) as the size marker. Several samples from different gels were rerun on the same gel to ensure a consistent reading among the gels.

The gels were attached to Whatman paper, dried (80°C, 140 min), and exposed to film (X-OMAT AR, Eastman Kodak). Time exposure to the film was 24 h or more, depending on the strength of the microsatellite bands.

The lengths of the microsatellite alleles were read directly from the autoradiograms, according to the respective band of the marker. Only the strongest band was determined as the real microsatellite allele. When several weak bands (stutter bands) appeared below a strong band, the colony was considered as homozygous on that specific microsatellite locus. When 2 strong bands followed by stutter bands were recorded, the colony was considered as heterozygous on the microsatellite locus. The colony was considered as a chimera when 3 to 4 strong bands were revealed by stutter bands (Fig. 1).

DNA analysis. Expected heterozygosity was calculated according to Nei's gene diversity (Nei 1987). Unbiased estimates of Hardy-Weinberg (HW) exact p-value were computed by the Markov-chain method using Genepop, Version 3.1D (Raymond & Rousset 1995). The significance level was determined after 20 batches and 1000 interactions each. F_{ST} between different sampling dates was calculated according to Schneider et al. (1997) following Ben-Shlomo et al. (2001).

RESULTS

Microsatellite loci in the Michmoret population

A total of 78 different alleles were scored in the Michmoret population by the 5 microsatellites studied (15 to 38 alleles per locus; Tables 1–5). Allele distributions at each locus revealed a few major and many rare alleles (details below). Observed heterozygosity was lower than expected in all loci. A HW exact test for all loci on all sampling dates (Genepop Version 3.1d) revealed heterozygote deficiency at a p-value (HW equilibrium) of 0.0000 and highly significant F_{ST} p-values. In some cases (locus PB-49, summer and autumn 1995, winter 1997 samples; locus BP-29, summer 1995 sample; locus BS-811, winter and spring 1997 samples: a total of 239 colonies), colony samples exhibited 100% homozygotes. Another common characteristic is the appearance of natural chimeras. *In situ* allogeneic contacts between *Botryllus schlosseri* colonies can result in colony fusions and chimerical formation. Chimeric individuals, which were regarded here as colonies showing 3 to 4 (Fig. 1) different bands on a specific microsatellite locus, were found in all 5 loci studied. Altogether, 19 chimeras (Tables 1–5; up to 25% in a specific population: locus PBC-1, winter 1997 sample) were identified, some of them by only a single microsatellite locus. This number of chimeras probably does not reflect the real level of chimerism in *B. schlosseri* populations from Michmoret, as only samples that showed at least 3 bands were considered as real chimeras; it is possible that some heterozygous colonies (2 bands) or even homozygotes may actually be chimeras as well. Chimeras were found in the first year of the study in all 4 seasons (54% of all chimeras) and in the following year in 2 seasons, but in high frequencies (up to 8 chimeras; 46% of all chimeras).

Locus BS-811

A total of 36 alleles (9 to 17 alleles per sampling date) were scored among 394 colonies at Michmoret

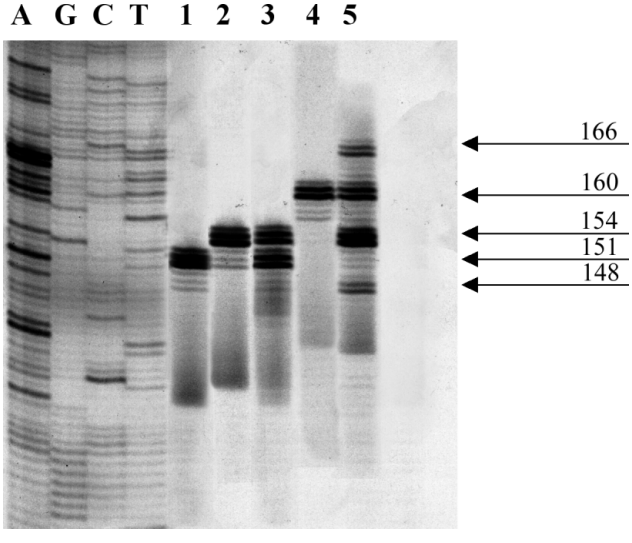

Fig. 1. *Botryllus schlosseri.* Autoradiograph of PB-29 microsatellite, showing 5 different alleles. Allele size was determined by strongest and highest band. Lanes A, G, C and T are a known sequence of Blue Script SK plasmid used here as a marker size. Lanes 1, 2 and 4 show homozygous colonies possessing 1 allele each; lane 3 shows heterozygous colony with 2 alleles; and lane 5 shows chimeric colony possessing 4 alleles. Genotypes of samples are: Sample 1, allele size = 151 bp; Sample 2, allele size = 154 bp; Sample 3, allele sizes = 151 bp/154 bp; Sample 4, allele size = 160 bp; Sample 5, allele sizes = 148 bp/151 bp/160 bp/164 bp

Table 1. *Botryllus schlosseri.* Locus BS-811. Microsatellite allele-frequency distribution in Michmoret population throughout the 2 yr period. H_0, H_e = observed and expected heterozygosity, respectively

Allele size (bp)	1995		1996				1997	
	Summer (n = 46)	Autumn (n = 54)	Winter (n = 49)	Spring (n = 47)	Summer (n = 52)	Autumn (n = 49)	Winter (n = 43)	Spring (n = 54)
146					0.02			
186			0.02				0.07	
188			0.02				0.12	
200			0.08		0.06	0.02		
202	0.02	0.03	0.06	0.13	0.02	0.02	0.05	
204	0.07		0.02			0.08	0.05	
212		0.06						
214		0.04						
218			0.08	0.01		0.08		
220	0.09		0.06		0.06	0.04	0.02	
222		0.02	0.10		0.06		0.07	0.07
224	0.02	0.02						0.09
226		0.02	0.06	0.04	0.02	0.04		
228	0.23	0.06	0.11	0.02	0.02	0.02	0.14	
230	0.15	0.07	0.10	0.02		0.03		
232	0.11	0.13	0.04		0.08	0.10	0.07	
236		0.01		0.08				
238	0.17	0.02	0.02	0.10	0.08	0.12		
240	0.02	0.19	0.10	0.08	0.09	0.12	0.14	
244								0.20
246	0.03	0.15	0.11	0.14	0.08	0.04	0.14	0.22
248				0.04	0.06			0.04
250	0.04	0.19		0.04	0.06	0.16		
252				0.04	0.06	0.02		
262					0.16		0.02	
264			0.01		0.04		0.02	
270							0.02	
272					0.06	0.09	0.07	
280				0.02				0.07
282				0.02				0.11
284				0.06				
286				0.10				0.13
288								0.06
290				0.04				
316	0.02							
320	0.02							
No. of alleles	13	14	16	17	17	15	14	9
H_0	0.02	0.04	0.02	0.02	0.02	0.02	0.00	0.00
H_e	0.87	0.87	0.93	0.92	0.92	0.91	0.90	0.86
No. of chimeras		1	1			1		

(Table 1). Three chimerical colonies (exhibiting 3 bands each) were recorded in the samples of autumn 1995 and 1996, and winter 1996. Only 1 allele (246 bp) was found in all sampling dates throughout the 2 yr study, revealing frequencies of 3 to 22%, while 17 alleles (47.2% of all alleles) were recorded in only 1 to 2 sampling dates each, exhibiting frequencies of 1 to 20%. Allele frequencies of the Michmoret population are highly dynamic. For example, allele 228 (appeared in 7 out of 8 samples) was the most common allele in summer 1995 (23%) but rare 1 yr later (2%, summer 1996; Table 1). The most common allele in summer 1996 (262 bp, 16% frequency) was not found on any of the 4 preceding sampling dates and appeared later only in winter 1997 as a rare allele (Table 1). The highest frequent allele (244 bp) in the spring 1997 sample (20%) was not recorded in any of the 340 colonies scored on the 7 following sampling dates (Table 1).

The level of observed heterozygosity for all sampling dates (0.00 to 0.04, Table 1) deviated significantly from the expected (0.86 to 0.93; $p < 0.001$), showing high heterozygote deficiency. To confirm that this heterozygote deficiency was a genuine phenomenon and not a technical failure, we re-ran part of the DNA samples by re-amplifying them in low

Table 2. *Botryllus schlosseri.* Locus PB-29. Microsatellite allele-frequency distribution in Michmoret population throughout the 2 yr period. H_0, H_e = observed and expected heterozygosity, respectively

Allele size (bp)	1995		1996				1997	
	Summer (n = 33)	Autumn (n = 47)	Winter (n = 37)	Spring (n = 42)	Summer (n = 41)	Autumn (n = 44)	Winter (n = 37)	Spring (n = 42)
146				0.01		0.05	0.03	0.05
148				0.02	0.05	0.03	0.04	
150					0.26			
151	0.06			0.05			0.09	
152	0.44		0.23	0.18	0.17		0.09	
153	0.21		0.03	0.13		0.13	0.04	
154			0.38	0.15	0.01	0.07	0.05	0.32
155	0.24		0.03	0.06	0.06	0.09		0.19
156	0.02	0.19	0.22	0.01	0.12		0.05	0.29
158		0.21	0.08	0.01	0.02		0.24	
160		0.59	0.03	0.37				
161	0.03					0.25	0.01	
164		0.01	0.01			0.39	0.15	0.18
166					0.30		0.19	
No. of alleles	6	4	8	10	8	7	11	5
H_0	0.00	0.38	0.13	0.11	0.15	0.11	0.19	0.33
H_e	0.70	0.57	0.75	0.78	0.79	0.75	0.86	0.74
No. of chimeras			1			2	1	

Table 3. *Botryllus schlosseri.* Locus PB-41. Microsatellite allele-frequency distributions in Michmoret population throughout the 2 yr period. H_0, H_e = observed and expected heterozygosity, respectively

Allele size (bp)	1995		1996				1997	
	Summer (n = 39)	Autumn (n = 47)	Winter (n = 47)	Spring (n = 47)	Summer (n = 49)	Autumn (n = 47)	Winter (n = 38)	Spring (n = 39)
160				0.03		0.06	0.03	0.03
162					0.02		0.01	0.01
164					0.03	0.02	0.01	0.01
170		0.29	0.03	0.06	0.07	0.07	0.05	0.04
172	0.03		0.37	0.04			0.05	
174	0.21		0.06	0.14		0.06	0.03	
175			0.09				0.01	
176	0.22	0.41	0.06	0.11	0.08	0.04	0.03	0.06
177				0.05				
178	0.13		0.06			0.06	0.03	0.01
180	0.18	0.23	0.11	0.1	0.06		0.01	0.08
182	0.19			0.11	0.19	0.12	0.22	0.05
184			0.01	0.13	0.27	0.05	0.11	0.03
186	0.04		0.06	0.11		0.06		0.03
188	0.01	0.06			0.01	0.06	0.03	0.09
190			0.02	0.13		0.06	0.01	
192					0.04			
194						0.1	0.04	0.08
196					0.22	0.04		
198								0.12
200						0.15	0.16	0.22
202							0.09	0.09
204						0.02	0.08	0.06
No. of alleles	8	4	10	11	10	15	18	16
H_0	0.13	0.30	0.06	0.13	0.10	0.30	0.23	0.08
H_e	0.82	0.69	0.83	0.89	0.82	0.92	0.89	0.89
No. of chimeras	2			1				

stringent conditions (annealing at 45°C, 40 cycles; data not shown). This procedure increased the number of scorable alleles as a few rare alleles appeared on only some of the sampling dates. The significant heterozygote deficiency, however, was exhibited even after taking these alleles into consideration. Some of these new bands, on the other hand, may be regarded as spurious, resulting from the permissive conditions, or they may reveal additional cases of chimerism.

Locus PB-29

Fourteen alleles were elucidated among 323 scorable individual colonies (4 to 11 alleles per sampling date; Table 2). Although the core sequence of locus PB-29 is based on 3-mer tandemly repeats (Stoner et al. 1997) most alleles differ from their nearest neighbours by only 1 to 2 bp, revealing a high mutation rate at this locus. None of the alleles was found in all 8 seasons sampled and 3 (20%) appeared in only 1 to 2 samples each. As in locus BS-811, allelic repertoire and distribution differed from 1 sampling date to the other. For example, the most frequent allele in the first sampling season (152 bp; 44%) was missing on the next sampling date (autumn 1995). The most frequent allele in autumn 1995 (160 bp, 59%) was not found in the preceding sample and was reduced to 3% on the following sampling date. Allele 166 bp, the most common allele in summer 1996 (30%) and 1 of the 2 most frequent alleles in winter 1997 (19%) was not recorded on the other 6 sampling dates. Allele 150 bp appeared only once (summer 1996) but at high frequencies (26%). Also, the distribution of the 3 most common alleles (154, 155, 156 bp) varied among seasons, revealing values of 1 to 38% (6 sampling dates), 3 to 24% (6 sampling dates) and 1 to 29% (7 sampling dates) frequencies, respectively (Table 2). Observed heterozygosity was lower than expected (H_0 = 0.00 to 0.38; $p < 0.01$). Four chimerical individuals, showing 3 to 4 different bands each, were found in winter and autumn 1996 and winter 1997 samples.

Table 4. *Botryllus schlosseri*. Locus PB-49. Microsatellite allele-frequency distribution in Michmoret population throughout the 2 yr period. H_0, H_e = observed and expected heterozygosity, respectively

Allele size (bp)	1995		1996				1997	
	Summer (n = 31)	Autumn (n = 46)	Winter (n = 40)	Spring (n = 43)	Summer (n = 47)	Autumn (n = 32)	Winter (n = 32)	Spring (n = 42)
138			0.01					
168	0.03			0.01		0.02	0.03	
176					0.05		0.13	0.02
185				0.10	0.07	0.01	0.06	
190				0.02	0.03	0.14		
198			0.08	0.03				0.04
200			0.05		0.07			
202	0.03			0.06	0.10	0.02	0.09	0.11
204		0.04	0.11			0.20	0.03	
206		0.04		0.15	0.03		0.09	0.02
208		0.12	0.33		0.15	0.01	0.09	0.14
210		0.04	0.03	0.15		0.20		0.14
212	0.10	0.04		0.03				0.05
213				0.05	0.08			
214		0.09						
220				0.13	0.16	0.22	0.13	
226		0.02		0.05		0.04		0.07
228	0.23	0.38		0.07	0.05		0.13	0.20
230	0.10	0.20		0.07				
232	0.35				0.08		0.19	0.15
236			0.20	0.07	0.05	0.06		
238	0.16	0.02	0.14				0.03	
242			0.05					0.02
244			0.01		0.07	0.07		
262								0.02
No. of alleles	7	10	10	14	13	11	11	12
H_0	0.00	0.00	0.10	0.16	0.32	0.22	0.00	0.19
H_e	0.78	0.79	0.81	0.90	0.91	0.84	0.88	0.88
No. of chimeras	1							

Table 5. *Botryllus schlosseri*. Locus PBC-1. Microsatellite allele-frequency distribution in Michmoret population throughout the 2 yr period. H_0, H_e = observed and expected heterozygosity, respectively

Allele size (bp)	1995		1996				1997	
	Summer (n = 37)	Autumn (n = 48)	Winter (n = 41)	Spring (n = 46)	Summer (n = 44)	Autumn (n = 38)	Winter (n = 28)	Spring (n = 33)
144					0.05		0.04	
150				0.02	0.02		0.04	
154				0.02	0.01		0.05	
156							0.02	
176			0.07		0.05	0.17	0.14	
178	0.04			0.02				
180				0.04	0.01		0.07	0.02
182	0.01	0.09	0.04					0.03
184			0.20	0.07	0.03			
186	0.08		0.05	0.03	0.01		0.04	
188	0.07	0.02	0.06	0.01	0.01		0.07	0.09
190		0.10	0.04	0.15	0.01	0.13		0.09
192	0.11		0.07	0.02				0.06
196		0.47	0.11	0.02				0.14
198	0.03	0.16		0.04	0.02			0.48
200	0.03	0.10	0.06	0.03	0.01			
202				0.05	0.11			
204	0.12	0.05	0.17	0.04	0.06	0.11		
206	0.11		0.05	0.08	0.16	0.22	0.20	0.03
208	0.12				0.05		0.04	
210	0.08		0.09	0.17	0.23	0.18	0.11	0.03
212	0.01			0.16	0.07			
218						0.08		0.03
219	0.01					0.03		
220					0.05	0.03		
221	0.05				0.02			
222	0.03							
225	0.04				0.02			
226	0.03							
230	0.01					0.05	0.20	
236	0.01							
No. of alleles	19	7	12	17	20	9	12	10
H_0	0.42	0.42	0.24	0.22	0.24	0.26	0.21	0.03
H_e	0.92	0.72	0.89	0.90	0.89	0.85	0.87	0.73
No. of chimeras			3				7	

Locus PB-41

Twenty-three alleles were revealed among 353 scorable colonies (4 to 18 alleles per sampling date; Table 3). Only 1 allele (176 bp; 4.3% of all alleles) was found on all 8 sampling dates and 6 (26.1%) on only 1 to 2 sampling dates each. As before, high differences in allele frequencies were recorded between seasons. The most common allele, 176 bp, was dominant (41%) in a single season (autumn 1995) but less frequent (3 to 6%) on the other 5 dates. Allele 182 bp, the most common (22%) in winter 1997, reduced to 5% frequency a few months later and allele 174 bp, the most common in summer 1995 (21%), was absent 1 yr later. Allele 172 bp, the most common allele in the winter 1996 sample (37%) appeared in only 3 additional samples but at low frequencies (3 to 5%; Table 3). Allele repertoire also differed among seasons and years. For example, only 4 alleles were recorded in autumn 1995, compared with 15 alleles in autumn 1996, in the same number of 47 scorable colonies. Even when a similar number of alleles was found for the 2 yr samples in a specific season, many of them were not the same. Only 4 of the 8 alleles found in summer 1995 overlapped with the 10 alleles of the summer 1996 sample. The most common allele in summer 1996 (184 bp; 27%) was missing in summer 1995, and vice versa with allele 174 bp, 1 of the 2 most frequent alleles in summer 1995 (Table 3). Observed heterozygosity (0.06 to 0.30) was significantly lower than expected ($p < 0.01$). Three chimerical individuals were found on 2 sampling dates (summer 1995, spring 1996), showing 3 to 4 different bands each.

Table 6. *Botryllus schlosseri.* Locus BS-811: General population characteristics in the 3 Israeli populations

Characteristic	Population studied			Total
	Michmoret	Caesarea	Shikmona	
No. of scorable colonies	394	387	375	1156
No. of alleles	36	40	34	64
No. of homozygous colonies	387	377	364	1128
No. of heterozygous colonies	6	8	10	24
No. of chimeras	3	2	1	6
No. of unique alleles at specific site	10	13	8	31
Frequency of unique alleles per site	0.28	0.33	0.24	0.48

Locus PB-49

Twenty-five alleles were detected among 313 scorable *Botryllus* colonies (7 to 14 alleles per sampling date; Table 4). None of the alleles was recorded in all 8 seasons sampled and 6 (24%) appeared only 1 to 2 times. Allele 228 bp, the second most frequent (23%) in the first sampling season and the most frequent (38%) in the second sampling season, disappeared in the following sample (winter 1996), but returned and was highly frequent on the last 2 sampling dates. Allele 190 bp, one of the most common alleles in autumn 1996 (14%) appeared at low frequencies (2 to 3%), in only 2 other samples. Four alleles, 138, 168, 242, and 262 bp, that were rare (1 to 5%) appeared on 1 to 4 sampling dates each. Other alleles that appeared on up to 4 sampling dates each (176, 185, 190, 198, 200, 204, 212, 213, 214, 220, 226, 230, 232, 236, 238 bp) exhibited changes in frequencies on consecutive sampling dates from rare to dominant (1 to 35% frequencies). As before, the level of observed heterozygosity (0.00 to 0.32) significantly deviated from that expected (0.78 to 0.91; $p < 0.001$), showing heterozygote deficiency. A single chimerical colony (3 bands) was recorded in summer 1995.

Locus PBC-1

Thirty-one alleles were revealed among 315 scorable colonies (7 to 20 alleles per sampling date; Table 5). None of the alleles was found in all 8 seasons sampled and 12 alleles (38.7%) were recorded in only 1 to 2 sampling seasons. As before, allele frequencies and repertoire differed between repeated sampling of the same season. For example, both autumn samples (7 and 9 alleles respectively) shared only 2 alleles, and both summer samples (19 and 20 alleles) had only 11 alleles in common. The most frequent allele in autumn 1995 (196 bp; 47% frequency) was not found in autumn 1996, while 2 of the most common alleles in autumn 1996 (206 and 210 bp, 22 and 18% frequency, respectively) were not found in the autumn 1995 sample. The 2 most common alleles in winter 1997 (206, 230 bp; 20% each) were rare or missing (5 and 0%, respectively) in the winter 1996 sample. The level of observed heterozygosity (0.03 to 0.42) deviated significantly from that expected (0.72 to 0.92; $p < 0.01$), exhibiting heterozygote deficiency. Ten chimeras were found showing 3 to 4 bands each. Three of these chimeras were found in winter 1996 (7.3% chimerism), and 7 chimeras were found in winter 1997 (25.0% chimerism).

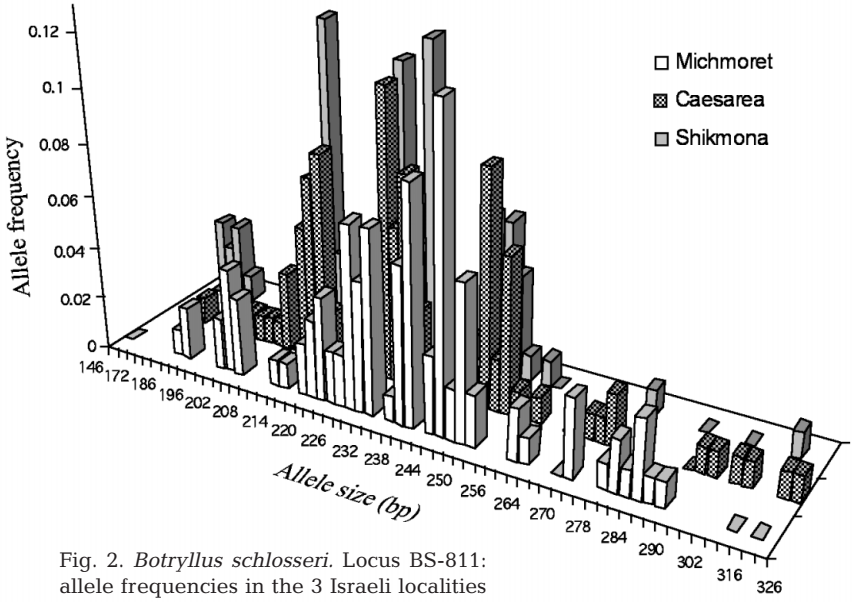

Fig. 2. *Botryllus schlosseri.* Locus BS-811: allele frequencies in the 3 Israeli localities

Locus BS-811 in Shikmona, Caesarea and Michmoret

Analysis of locus BS-811 throughout the 2 yr study revealed a total of 34 alleles in Shikmona, 40 alleles in Caesarea and 36 in Michmoret (a total of 64 different alleles; Table 6) among 1156 scorable colonies (n = 375, 387, 394 colonies, respectively; Table 6). Only 5 alleles (202, 228, 230, 232 and 246 bp) were found in all sampled populations, while a total of 31 alleles were unique to a single location (Table 6), exhibiting frequencies of 23.5% unique alleles in Shikmona, 32.5% in Caesarea and 27.8% in Michmoret. Six chimerical colonies were recorded, 3 in Michmoret, 2 in Caesarea and 1 in Shik-

mona. Of the 5 common alleles, 3 (228, 230, 232 bp) reached the highest frequencies (7.37, 7.33 and 7.29% within the Israeli populations, respectively), and together with the 5.78% frequency at allele 246 bp and the 1.77% value for allele 202 bp (Fig. 2) amounted to 29.54% Israeli allele frequencies. Therefore, the other 59 alleles in total contributed to only about 70% of allele frequencies. Different alleles marked different frequencies at each sampling site (Fig. 2).

The level of observed heterozygosity per season, as well as the level of observed heterozygosity per site (Shikmona H_0 = 0.03, H_e = 0.97, Caesarea H_0 = 0.03, H_e = 0.94 and Michmoret H_0 = 0.02, H_e = 0.97) deviated significantly from that expected, showing that the whole Israeli populations are characterized by the state of heterozygote deficiency.

DISCUSSION

The 3 *Botryllus schlosseri* populations studied along the Israeli coast revealed: (1) a high number of alleles per locus (up to 64 alleles in locus BS-811); (2) within each site, rapid changes in allele frequencies among different seasons and years; (3) a high number of unique alleles per locality in geographically adjacent populations; (4) heterozygote deficiencies in all sampling seasons and localities; and (5) the existence of natural chimerism.

These detailed results are of significant importance, as although this globally distributed sedentary marine species has been studied in a variety of sites worldwide, population genetics analyses using molecular tools such as microsatellites have been performed in only a few localities, i.e. New Zealand (Ben-Shlomo et al. 2001), the Croatian Istra peninsula, the Adriatic Sea (Rinkevich et al. 2001), and on the Pacific and Atlantic coasts of the USA (Stoner et al. 1997, 2002).

As described, the Israeli populations revealed high numbers of alleles per locus. Locus BS-811 is the most diverse and revealed, in 1156 scorable colonies, a total of 64 alleles. In the New Zealand populations (Ben-Shlomo et al. 2001), 131 scorable colonies exhibited 20 alleles on this locus, all within the size range of the Israeli alleles repertoire. In Croatia, 11 BS-811 alleles were found in 16 *Botryllus schlosseri* colonies (Rinkevich et al. 2001). When further comparing the Israeli and the Croatian populations, only 2 of the 5 alleles at microsatellite PB-41 (175 and 177 bp) appeared in the Israeli population of Michmoret, and a size difference of 1 nucleotide was recorded for the other 3 alleles. At locus PB-29 (8 alleles in Croatia), the shortest 4 alleles in the Croatian populations (150, 153, 156 and 168 bp) overlapped with the alleles repertoire of the Israeli populations. As for the PBC-1 locus, 2 out of the 3 Croatian alleles (182 and 184 bp) appeared in the Israeli populations. When comparing the Israeli populations with the Californian populations, major differences in allele repertoire are confined to the 4 microsatellite loci analyzed (PB-29, PB-41, PB-49 and PBC-1). Of the total 23 alleles (Stoner et al. 1997) only 6 (26.1%) overlap with the Israeli populations, while the rest are a similar size to the Israeli alleles, with size differences of 1 to 2 nucleotides.

In spite of the rapid changes in allele sizes and frequencies, the Israeli populations (Michmoret, Caesarea and Shikmona), which are located only 12 to 36 km from each other, are characterized by a high number of locality-specific alleles. As a result, only 6 alleles on microsatellite BS-811 (9.4% of the alleles) were found at the 3 localities throughout the 2 yr study. Similar outcomes were found in microsatellite PBC-1 in 2 Croatian populations located about 1 km from each other (Rinkevich et al. 2001). One of the populations was monomorphic having a single allele only, while in the other, the frequency of this specific allele was 0.1. These results resemble the outcomes of a study performed on the fusibility locus at the same 3 Israeli populations (Rinkevich et al. 1995), which revealed no shared fusibility alleles as compared to the high numbers of these alleles (479 to 560 alleles per locality).

In the Israeli populations all 5 microsatellite loci revealed a significant heterozygote deficiency, a phenomenon documented in other populations of *Botryllus schlosseri* in Croatia (Rinkevich et al. 2001), New Zealand (Ben-Shlomo et al. 2001) and the USA (Stoner et al. 1997, 2002), as well as other localities (France, Portugal, UK, east coast of the USA; B. Rinkevich unpubl. data). Such deviations from expected levels in all studied *B. schlosseri* populations reflect a genuine biological phenomenon characteristic to this species (Ben-Shlomo et al. 2001). Nonrandom mating, inbreeding and limited gene flow are probable explanations of such a deficiency (Chakraborty & Jim 1992).

Self-fertilization may not account for this worldwide observed heterozygote deficiency. *Botryllus schlosseri* is a hermaphroditic species (Berril 1950); however, sperm release from gravid colonies starts only 1 to 2 d after ovulation, effectively preventing selfing (Yund et al. 1997). On the other hand, cosettlement of genetically related colonies is promoted in *B. schlosseri* by the use of the kin recognition mechanism along with limited dispersal of larvae (Grosberg & Quinn 1986). This phenomenon may promote partial inbreeding resulting from mating between siblings (Ben-Shlomo et al. 2001), increasing heterozygote deficiency to levels higher than that expected from random settlement.

The aggregate settlement of tadpole larvae (Grosberg & Quinn 1986), the frequent natural catastrophes that eradicate populations (Rinkevich et al. 1995, 1998; a phenomenon which also resulted in strong differentiation between sampling dates in allele repertoire and allele frequencies) and the limited dispersal of swimming larvae (Grosberg & Quinn 1986), all help in forming small-scale subpopulations in which gene flow is somewhat restricted, further promoting heterozygote deficiency.

This study, along with previous work (Paz 1999, Ben-Shlomo et al. 2001, Rinkevich et al. 2001, Stoner et al. 2002) are directed at learning about the genetic structure of *Botryllus schlosseri* populations worldwide, residing in short distance localities and in different continents. Additional studies on other populations of this cosmopolitan species are required to evaluate their genetic characteristics for the formulation of a comprehensive genetic structure, and for the elucidation of its global invasion routes.

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