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The ancient Levantine *Botryllus schlosseri* (Tunicata): population genetics landscape under frequent natural disturbances

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Abstract

The colonial ascidian *Botryllus schlosseri* is a cosmopolitan species commonly found in sheltered temperate marine environments, primarily marinas and harbors. Upon release, *B. schlosseri* larvae settle immediately on hard substrates in the vicinity (< 50 cm) of maternal colonies, suggesting genetically structured populations on fine spatial scales. We used seven microsatellite loci to characterize long-term (2004-2008) genetic structures of a *B. schlosseri* population residing underneath stones at Michmoret Harbor (along the Israeli Mediterranean coast), revealing a persistent genetic structure through time. Further, Michmoret Harbor was exposed to episodes of natural disturbances (October 2006 and November 2007), during which the entire population was eradicated from shallow waters. These disturbances provided a unique opportunity to study the population genetic dynamics of newly established population of a marine invasive species within its geographic origin. The results revealed a 4-year-long structured single population in Michmoret Harbor that was homogeneous at the time but not at the micro-geographic scale. The *B. schlosseri* population from Michmoret Harbor showed no genetic changes, in contrast to changes in the distribution of allele frequencies that were reported in other *B. schlosseri* populations in other parts of the world after natural catastrophic events.

Keywords: Population genetics; Established population; *Botryllus schlosseri*; Microsatellites; Heterozygote deficiency.

Introduction

The native range of the colonial ascidian *Botryllus schlosseri* is thought to include the Mediterranean Sea and European Atlantic waters (Reem *et al.,* 2017), but this species has been introduced into a wide range of temperate habitats worldwide, primarily through man-mediated dispersal mechanisms. The success of this species is mainly attributed to the dispersal of colonies via attachment to ship hulls, floating objects, and hard-shelled organisms rather than to spreading of its short-lived pelagic larvae (Van Name, 1945; Berrill, 1950; Lambert & Lambert, 1998; Rinkevich *et al.*, 1998b; Ben-Shlomo *et al.*, 2001; Paz *et al.,* 2003). Currently, colonies are found along the coasts of the Mediterranean Sea and in all European waters, on the eastern and western continental shelves of North America, and in Asia (India, Japan, Korea, Hong Kong), Australia, Tasmania, New Zealand, South Africa, and more (Berrill, 1950; Tokioka, 1953; Dybern, 1969; Luckens, 1976; Plough, 1978; Kott, 1985; Lambert & Lambert, 1998; Rinkevich *et al.*, 1998a; Ben-Shlomo *et al.*, 2001; Stoner *et al.*, 2002; Ben-Shlomo *et al.*, 2010; Reem *et al.*, 2013b; Karahan *et al.*, 2016; Reem *et al.*, 2017; Reem *et al*., 2018). *B. schlosseri* colonies encrust hard bottom substrates in shallow waters from intertidal areas down to > 690 m depth (Arroyo *et al.*, 2021), above and under stones, on algae and sea weeds, on floats and other artificial substrata, and within marinas and in the wild (Sabbadin, 1969; Rinkevich *et al.*, 1998a). Employing molecular tools, studies unveiled five divergent clades that suggested a complex of five cryptic species (Bock *et al.*, 2012), yet Reem *et al.* (2022) recently reported that while *B. schlosseri* represents a widely variable species, there is not enough evidence for its designation as a species complex.

Using microsatellite loci, researchers conducted population genetic studies of *B. schlosseri* populations from various locales around the world and found that high polymorphism and heterozygote deficiency are two general attributes of all studied populations, spanning from New Zealand to the South and North American coasts to northern Europe and to the Mediterranean Sea basins (Rinkevich *et al.*, 2001; Stoner *et al.*, 2002; Paz *et al.*, 2003; Ben-Shlomo *et al.*, 2010; Reem *et al.*, 2013b; Karahan *et al.*, 2016; Reem *et al.*, 2017). Ben-Shlomo *et al.* (2001) reported high polymorphism and heterozygote de-

ficiencies within six New Zealand *Botryllus* populations based on data from five microsatellite loci. Similar results were recorded in populations from Istra Peninsula, Croatia (Rinkevich *et al.*, 2001) and in populations from the USA (Stoner *et al.*, 2002). Together these data suggest partial inbreeding in wild populations (Ben-Shlomo *et al.*, 2001; Rinkevich *et al.*, 2001; Stoner *et al.*, 2002). Reem *et al.* (2013a) and Karahan *et al.* (2016) characterized the population genetics fluctuations of *B. schlosseri* from marinas located in Santa Cruz and Moss Landing, CA, USA from seven sampling sessions throughout a period of 12 years. They reported high numbers of alleles per microsatellite locus, rapid fluctuations in allelic frequencies between different years, high numbers of unique alleles, and frequency fluctuations of the most common alleles. These findings supported previous findings by Paz *et al.* (2003). Long-term studies (Paz *et al.*, 2003; Reem *et al.*, 2013b) have further revealed high mutation rates in the microsatellite alleles.

Population genetic parameters of the *B. schlosseri* populations in the eastern basin sites of the Mediterranean Sea displayed stronger genetic structures, higher differentiation, and larger genetic distances compared to those in the western sites (Reem *et al.*, 2013b, 2017). These results may be due to abiotic ecological differences between the basins and/or restricted connectivity or a higher genetic drift among the eastern sites (Lee, 2002; Reem *et al.*, 2017). The high genetic structure characteristic of the worldwide-distributed *B. schlosseri* populations attest to long-lasting and established populations, and the high genetic diversity values obtained for the Mediterranean Sea suggest the possibility that the Mediterranean Sea is likely the site of origin for this species (Reem *et al.*, 2017). However, microsatellite loci and COI haplotypes indicated that even populations from two closely situated sites located in the eastern basin (Glyfada, Greece and Michmoret, Israel) are distinct and presumably genetically isolated from the others (Reem *et al.*, 2017).

In this study, we used a panel of microsatellite loci to elucidate the population genetic structure of the *B. schlosseri* population from Michmoret Harbor (in the Israeli Mediterranean Sea) at two micro-scales: (a) long-term (4 years) analyses at the harbor to evaluate yearly fluctuations, gene flow, and genetic consanguinity of colonies settled naturally along a spatial vector; and (b) long-term (4 years) genetic parameters at the microgeographic, single stone scale. The Michmoret Harbor population is unique compared to all previously studied *B. schlosseri* populations for two reasons: (a) the local *B. schlosseri* population likely is a natal resident that has been in this area for thousands of years; and (b) the population has loose biological connectivity with other populations in the Levant (Rinkevich *et al.*, 1995; Paz *et al.*, 2003) and in the Mediterranean Sea, as the area has been a main active harbor for about 2000 years (after Herod's construction of Caesarea; https://www.biblewalks.com/michmoret). Therefore, Michmoret Harbor appears to be a stable and relatively isolated site.

Materials and Methods

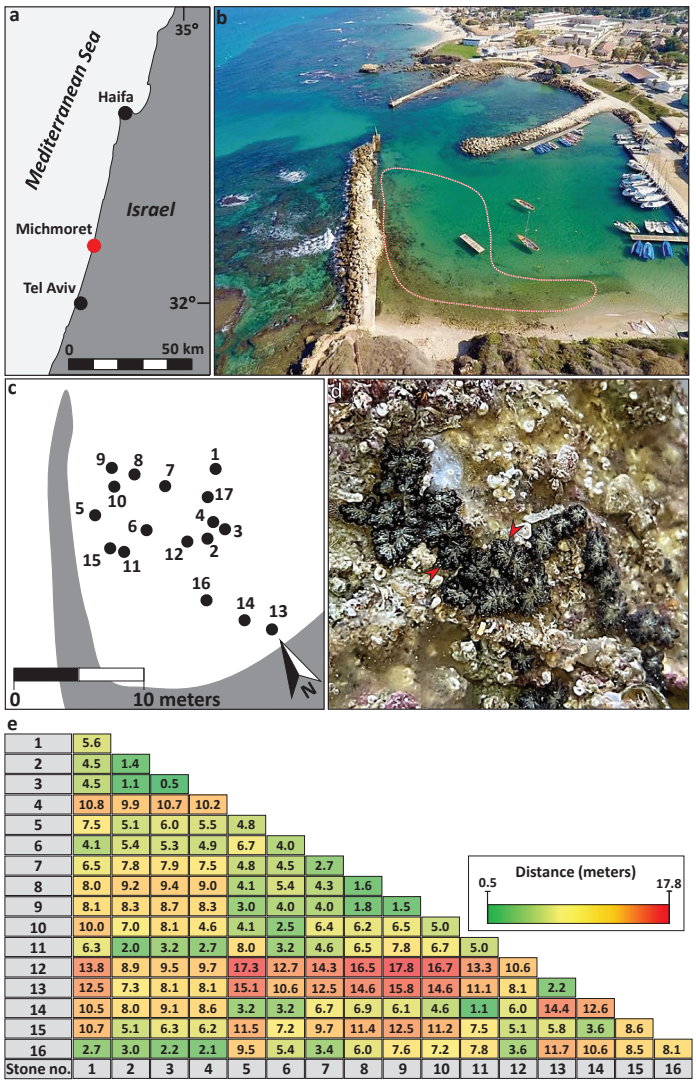
Collection and preparation of genetic material

Colonies of *B. schlosseri* were sampled from underneath tagged stones (n = 17) that were laying on the soft sediment in shallow waters (0.3-1 m depth) in Michmoret Harbor (32.401944° N, 34.865833° E; Fig. 1a-c) over a period of 4 years (2004-2008). Stone sizes ranged from 16 to 42 cm long and 14 to 35 cm wide (mean size 27X21 cm). The numbers of colonies found on the stones fluctuated between sampling sessions, and no correlation between genetic diversity and stone size was detected. Samplings were performed lengthwise along a 20 × 15 m rectangle from underneath tagged stones (Fig. 1d,e; 0.5-17.8 m away from each other) that were repeatedly sampled twice a year for the entire time period. Whole colonies or colonial fragments were removed from their substrates using single-edge razor blades. Sampling repeatedly from the underside of the same stones over 4 years may have impacted the genetic composition at this micro-scale. However, this sampling protocol ensured a comparable potential effect on all stones sampled throughout the study period.

In the field, each tissue sample was placed separately into a 1.5 ml vial containing 200 µl of lysis buffer (0.25 M Trisborate pH 8.2, 0.1 M EDTA, 2% SDS, 0.1 M NaCl(and homogenized. Next, 40 µl of5 M $NaClO_4$ were added, followed by 240 µl of phenol/chloroform/isoamyl alcohol solution (25:24:1 v:v:v). The vials were transferred to the laboratory at the National Institute of Oceanography, Haifa, Israel and kept at 4°C for further analysis. In the laboratory, the samples were thoroughly mixed (1 min) and centrifuged (14000 rpm [10000 × g] for 15 min). The aqueous phase was collected and added to the same volume of chloroform/isoamyl ethyl-alcohol (24:1; v:v), and the solution was thoroughly mixed (1 min) and centrifuged (14000 rpm [10000 × g] for 15 min). Genomic DNA was precipitated by adding 100% cold alcohol, stored overnight at –20°C, and centrifuged for 15 min (4°C, 14000 rpm [10000 × g]). The alcohol was removed, and the DNA was washed with 70% ethyl-alcohol (volume × 2), centrifuged (14000 rpm [10000 × g]), dried up in a hood, dissolved in 50 µl of sterile double distilled water (DDW), and diluted at 1:100 in DDW. DNA samples were kept in a cold room (4°C).

Microsatellite genotyping

Seven microsatellite loci (PBC-1, PB-29, PB-41 (Stoner *et al.*, 1997), BS2, BS8, BS9 (Austin *et al.*, 2011), and BS-811 (Pancer *et al.*, 1994)) were 5' labeled with one of the following fluorescent dyes: VIC, 6-FAM, NED, or PET (Thermo Fisher Scientific, Waltham, MA, USA). They were amplified by polymerase chain reaction (PCR) under the following conditions: 94°C for 2 min followed by 32 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 45 min followed by storage at 10°C. Microsat-



Stone no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	5.6															
2	4.5	1.4														
3	4.5	1.1	0.5													
4	10.8	9.9	10.7	10.2												
5	7.5	5.1	6.0	5.5	4.8											
6	4.1	5.4	5.3	4.9	6.7	4.0										
7	6.5	7.8	7.9	7.5	4.8	4.5	2.7									
8	8.0	9.2	9.4	9.0	4.1	5.4	4.3	1.6								
9	8.1	8.3	8.7	8.3	3.0	4.0	4.0	1.8	1.5							
10	10.0	7.0	8.1	4.6	4.1	2.5	6.4	6.2	6.5	5.0						
11	6.3	2.0	3.2	2.7	8.0	3.2	4.6	6.5	7.8	6.7	5.0					
12	13.8	8.9	9.5	9.7	17.3	12.7	14.3	16.5	17.8	16.7	13.3	10.6				
13	12.5	7.3	8.1	8.1	15.1	10.6	12.5	14.6	15.8	14.6	11.1	8.1	2.2			
14	10.5	8.0	9.1	8.6	3.2	3.2	6.7	6.9	6.1	4.6	1.1	6.0	14.4	12.6		
15	10.7	5.1	6.3	6.2	11.5	7.2	9.7	11.4	12.5	11.2	7.5	5.1	5.8	3.6	8.6	
16	2.7	3.0	2.2	2.1	9.5	5.4	3.4	6.0	7.6	7.2	7.8	3.6	11.7	10.6	8.5	8.1

Fig. 1: (a,b). *B. schlosseri* sampling locations at Michmoret Harbor (in the Israeli Mediterranean Sea), with the sampling area (20×15 m) marked by a dotted red line. (c) A detailed mapping of the sampling area showing the 17 marked and repeatedly sampled stones. (d) A colony of *B. schlosseri* from underneath a stone at Michmoret Harbor. (e) Color scale chart showing the distances between the 17 sampled stones.

ellite genotyping was initially performed by the senior author. Unsuccessful amplifications were re-genotyped and double-checked by the senior author as well as another author, until a consensus was reached. Samples with more than two peaks were deleted from the analyses.

The 16 µl reaction mixture contained 8 µl of PCR reaction mix (2×Taq PCR MasterMix (Tiangen, Beijing, China)), 5 pmol of fluorescent primer, 6 µl of DDW, and 1 µl of DNA solution (1:100). PCR products were visualized on 2% agarose gels, and PCR products of four different primer pair sets were mixed in equal proportions. One µl of the mixture was added to 8.6 µl of Hi-Di Formamide (Applied Biosystems, Thermo Fisher Scientific) and 0.4 µl of 50–500 base pairs (bp) of LIZ size marker (MAPMARKER DY632). Microsatellite genotyping was performed at Rapport Medical School using 3500xl genetic analyzer (Life Technology, Carlsbad, CA, USA). Fluorescence peaks were converted to base pairs using GeneMaper software (Life Technology) according to the LIZ size marker.

Statistical analyses

Weighted observed heterozygosity (Ho), gene diversity (expected heterozygosity, He), and unbiased estimates of Hardy-Weinberg (HW) exact P-values were computed by the Markov chain method using the GENEPOP web application (Raymond & Rousset, 1995). Deviation from HW equilibrium was determined after 1000 dememorization steps and 10 batches of 2000 permutations per batch. The inbreeding coefficient (F_{IS}; Weir & Cockerham, 1984) and allelic richness (AR) were estimated with FSTAT 2.9.3 (Goudet, 1995) using the rarefaction approach (El Mousadik & Petit, 1996) to avoid bias due to different sampling sizes. Multivariate tests (Pillai's trace, Wilks' lambda, Hotelling's trace, Roy's largest root) based on allele frequencies were performed between the sampling sessions using SPSS software. The number of different alleles (Nas), private alleles (PAs), and the allele frequencies were calculated using GenAIEx 6.5 software (Peakall & Smouse, 2012). Chi-square tests for significance of allele frequency fluctuations between two sampling peri-

ods were performed using Excel software. GenAIEx 6.5 software was used to calculate Dest (an estimator of actual differentiation; Jost, 2008) values by performing 9999 permutations and to conduct the Mantel test to examine the correlation between geographic and genetic distances (based on unbiased Nei genetic distance; Hedrick, 2000). For the Mantel test, Cohen's d effect size was calculated in order to estimate the magnitude of the effect and to determine if it supports the results of the statistical calculation (a meaningful effect could exist despite the lack of statistical power to detect a significant level due to a too small sample size).The genetic variation F_{ST} and the overall F_{IS} were calculated using analysis of molecular variance (AMOVA) following Michalakis & Excoffier (1996), with 9999 permutations using GenAIEx 6.5 software. Population assignment (Paetkau *et al.*, 1995, 2004) was calculated using GenAIEx, and for each sampling, a log likelihood value was calculated using the allele frequencies of the respective population (sampled stone). The sample was assigned to the population (stone) with the smallest value (converted negative log-likelihood values to positive numbers while multiplying by -1). We used Micro-Checker software to avoid scoring errors due to null alleles (Van Oosterhout *et al.*, 2004), and suspected samples were re-genotyped.

For the long-term analysis, we performed additional cluster analyses, including three different distance measures (the squared Euclidean distance, a modified squared chord distance, and the Manhattan [or city block] distance), and one similarity measure (a modified Morisita's similarity coefficient). For each population (sampling session), the frequency of each allele was calculated separately for each marker. Three different amalgamation procedures were conducted using MVSP software (Kovach Computation Services, 2013): unweighted pair group method with arithmetic mean, minimum variance (Ward's method), and furthest neighbor (complete-linkage clustering).

Results

General

Eight sampling sessions were conducted during the 4-year study. In six of the sessions, 545 colonies were sampled: October 2004 (n = 122), April 2005 (n = 101), November 2005 (n = 50), April 2006 (n = 88), May 2007 (n = 111), and June 2008 (n = 73). Animals were not found during October 2006 and November 2007, as the lower parts of the tagged stones were sunk in sand, reflecting natural disturbance events that occurred sometime before the sampling sessions. Microsatellite analyses were performed on two levels: between the six sessions that yielded animal samples (n = 545) and between stones that each had >15 colonies during the whole period (n = 360). Population genetics analyses were then performed on (a) stones divided into two geographically separated groups (eastern [stones 13, 14, and 16] and western [stones 1, 7, 8, and 9]; Fig. 1c) and (b) between repeatedly sampled stones containing ≥ 10 colonies for each sampling session that were sampled during at least three sessions (stones 2, 3, 7, and 15).

Time-series sampling analyses

B. schlosseri population genetics parameters from Michmoret Harbor for the four sampling years (2004–2008; Table 1) revealed high polymorphism in all seven microsatellite loci used. Locus BS811 was the most polymorphic, with 48 alleles; PB29 was the least polymor-

Table 1. Genetic diversity parameters for six sampling sessions where colonies were found, including mean (based on seven microsatellite loci) number of different alleles (Na), observed and unbiased expected heterozygosity (Ho and He), average inbreeding coefficient F_{IS}, number of private alleles (PA), and allelic richness (AR).

Pop		Parameter							
		N	Na	Na/N	Ho	He	F_{IS}	PA	AR
Oct 2004	Mean	107.571	8.571	0.080	0.404	0.540	0.215	0.429	6.687
	SE	5.468	3.199		0.075	0.104	0.078	0.297	
Apr 2005	Mean	91.000	9.286	0.102	0.363	0.550	0.265	1.000	7.276
	SE	3.423	3.790		0.050	0.099	0.096	0.690	
Nov 2005	Mean	45.143	4.857	0.108	0.252	0.515	0.445	0.000	4.718
	SE	2.521	1.550		0.066	0.106	0.103	0.000	
Apr 2006	Mean	79.857	8.429	0.108	0.450	0.627	0.233	0.429	7.132
	SE	3.763	3.169		0.041	0.070	0.100	0.297	
Oct 2006	–	–	–	–	–	–	–	–	–
May 2007	Mean	94.714	9.000	0.102	0.371	0.573	0.310	0.143	6.838
	SE	7.849	3.443		0.072	0.097	0.095	0.143	
Nov 2007	–	–	–	–	–	–	–	–	–
Jun 2008	Mean	62.000	7.429	0.120	0.275	0.517	0.413	0.143	6.329
	SE	5.214	2.869		0.059	0.094	0.105	0.143	

phic, with just 3 alleles; and loci BS2, BS8, BS9, PB41, and PBC1 had 8, 6, 7, 6, and 12 alleles, respectively (Table S1). The range of the mean Na/locus was 4.9-9.3, and AR values ranged from 4.7 to 7.3 (Table 1). Mean number of PAs, AR, and the number of different alleles after normalization to sampling size (Na/N) remained stable between sessions within a single year and between years, even in sampling sessions following the disturbances (Table 1). The unbiased He showed stability over time, with slight variation between April 2006 (0.627) and November 2005 (0.515) (Table 1). Multivariate tests showed no significant differences between the six samplings sessions ($p = 0.44$; Table S2). All F_{IS} values (Table 1) were positive, and overall F_{IS} calculated via AMOVA was 0.46 ($p < 0.001$) for the analysis between the six sampling sessions. This result revealed deviations from HW equilibrium (heterozygote deficiency) in the *B. schlosseri* population at Michmoret. Heterozygote deficiency was further revealed for all loci in all populations using the HW exact test (GENEPOP; $p < 0.001$).

The most common two (three on locus BS811) microsatellite alleles (Fig. 2, Table S3) were prominent throughout the entire six sampling sessions but fluctuated between the different sampling dates. A significant difference between the sampling sessions was detected for locus PB41 (alleles 174 bp and 176 bp) and locus PBC1 (alleles 211 bp and 214 bp), which were the most common alleles at all sampling sessions (Table S3). For the most polymorphic BS811 locus, two of the three dominant common alleles differed significantly between sampling sessions (alleles 211 bp and 304 bp, Table S3). The frequency of the most common allele at locus BS2 (174 bp), locus PB29 (152 bp), and locus BS8 (192 bp(did not differ significantly between sampling sessions and were not impacted by the disturbance events (shown as two slash symbols with empty spaces between; Fig. 2).

The overall population differentiation showed low differentiation between the sampling sessions (AMOVA; $F_{ST} = 0.05$; $p < 0.001$; Fig. 3a). All pairwise F_{ST} and Dest (Table S4) values were statistically significant ($p < 0.01$), which further confirmed low differentiation (F_{ST}: 0.05–0.1 and Dest: 0.03–0.09) between the two late sampling sessions (May 2007 and June 2008) and the earliest sampling sessions (October 2004, April 2005, November

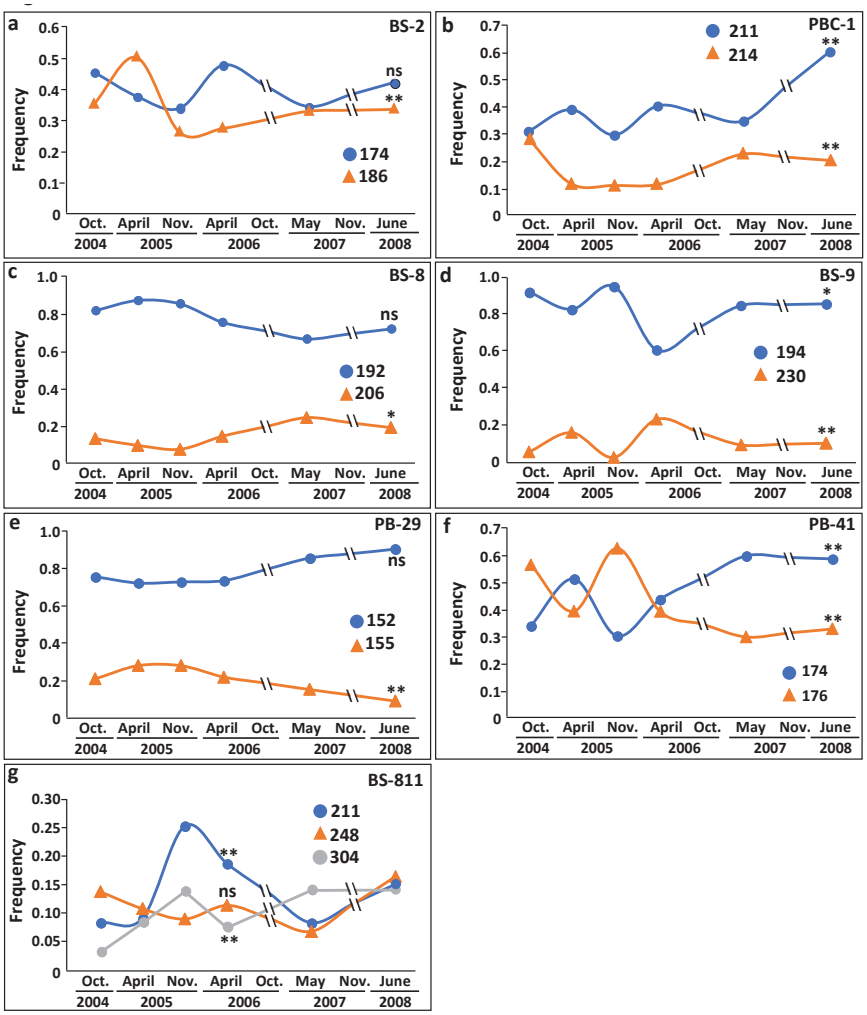


Fig. 2: Allelic frequencies for the two most abundant alleles at each of the seven microsatellite loci (three in locus BS811) within each of the sampling dates (2004–2008) at Michmoret Harbor. The most common allele/locus is marked in blue, followed by the second most common (orange) and the third most common for locus BS811 (grey). October 2006 and November 2007 reflect sessions without sampling due to disturbance events (marked as two slash symbols with empty spaces between). *$p < 0.05$; **$p < 0.01$; ns = not significant ($p > 0.05$), Chi-square test.

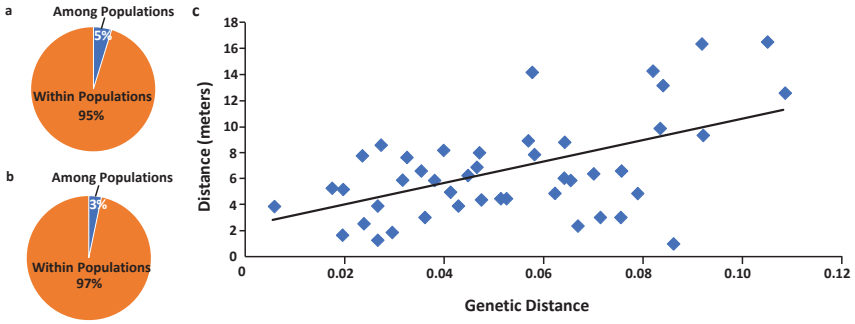


Fig. 3: F_{ST} using AMOVA (9999 permutations) between the six sampling sessions (a) and between the stones (b). (c) Mantel test (9999 permutations) for genetic distance between stones (a pairwise test based on unbiased Nei genetic distance; p = 0.035 and r = 0.536). All sampling sessions were used (n = 360).

2005, and April 2006). The cluster analysis dendrogram for the six *B. schlosseri* sampling sessions (Fig. 4, Fig. S1a–c) revealed continuity between the years, with no visible effect of the two disturbance events (before and after October 2006 and November 2007). It is important to note that although the four genetic distance/similarity matrices employed are based on different approaches, they yielded similar results.

Analyses of the genetic relations among colonies from different stones

The overall F_{IS} value between the different stones was 0.49 (AMOVA; $p < 0.001$), revealing deviation from HW equilibrium and heterozygote deficiency. Pairwise F_{ST} between the stones showed very low, yet statistically significant, differentiation results for 43 of the 45 pairwise combinations of stones, with $F_{ST} < 0.05$ (Tables S5, S6). Further, the overall differentiation between the sampled stones was low, albeit statistically significant (AMOVA, $F_{ST} = 0.03$; $p < 0.001$, Fig. 3b). The population assignment of *B. schlosseri* specimens sampled from stones containing ≥ 15 colonies/stone categorized them into self populations (the sample was assigned to the stone of origin) or other population (the sample was assigned to another stone). The analysis revealed that 68% (n = 246 (of the sampled animals were defined as other, while only 32% (n = 114) were assigned as self (Fig. 5, Table S7).

The allele frequency analyses for the two geographically disparate groups of stones (eastern [stones 13, 14, and 16] and western [stones 1, 7, 8, and 9]) (Fig. 1) revealed that they shared most common alleles (Table S8), with low overall differentiation between the groups (AMOVA, $F_{ST} = 0.027$; $p < 0.001$). The Mantel test for correlation between geographic and genetic distances for all colonies in the highly populated stones (≥ 15 colonies/stone; total of 360 colonies) revealed modest (r = 0.53) but statistically significant correlation (Fig. 3c). In addition, the high Cohen's d (1.269) result of the Mantel test indicated a strong effect size, thus corroborating the finding of a correlation between geographic and genetic distances among the stones.

Discussion

The *B. schlosseri* population from Michmoret Harbor most likely represents an ancient population that has existed alongside human civilization using its natural anchorage, located on the north side of the mound. When active, it represented one of the deepest natural anchorages along the Israeli coast (3–7 m water depth). It was made of natural kurkar ridge, which was partly submerged, forming small islands offshore (*Israel Antiquities Authority; http://www.antiquities.org.il/article_eng.aspx?sec_id=16&subj_id=143*). This type of anchorage was used as early as the Middle Bronze period (2000–1750 BC), during the Iron Age, and until Herod's construction of Caesarea, at which point this marine city lost its importance as the central port of the area. During the Mameluke period (1250–1516 AD), this anchorage was used seasonally or in low capacity (https://www.biblewalks.com/michmoret). With its reduced importance

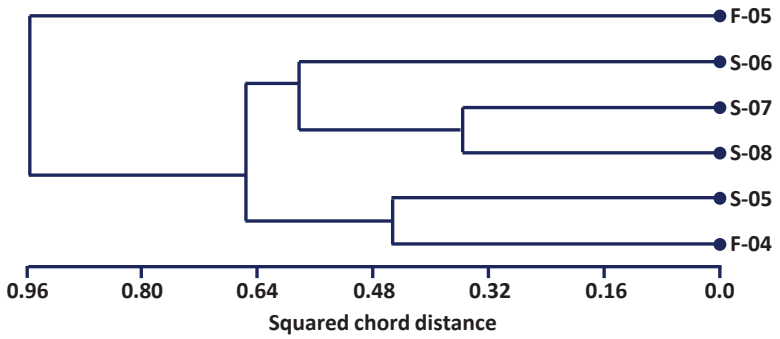


Fig. 4: A cluster analysis dendrogram for the relationships between the six *B. schlosseri* seasonal samplings conducted from 2004 to 2008; the squared chord distance and minimum variance clustering were used in the analysis. S, Spring; F, Fall.

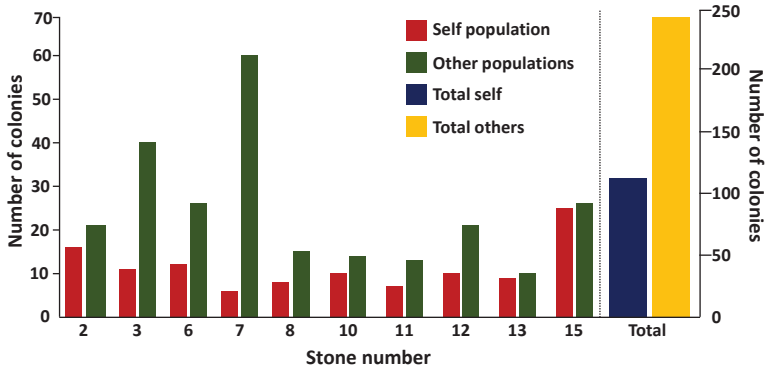


Fig. 5: Population assignment results for highly populated stones (≥ 15 colonies/stone; 10 stones). Columns show either a self population (stone of origin; red) or other populations (from other stones; green). The total sum is shown on the right.

as the regional port, Michmoret Harbor likely lost its biological connectivity with other populations in the Levant and in the Mediterranean Sea, allowing the establishment of a stable and relatively isolated (Rinkevich *et al.*, 1995; Paz *et al.*, 2003) *B. schlosseri* population that is one of the most ancient *B. schlosseri* populations known today (Reem *et al.*, 2017).

In this study, we sampled the *B. schlosseri* population from Michmoret Harbor from the same natural stones over time. This approach differed from that of most former *B. schlosseri* population genetics studies that relied on samples taken from manmade artificial substrates and/or recently established populations (Stoner *et al.*, 2002; Ben-Shlomo *et al.*, 2010; Reem *et al.*, 2013a, b; Karahan *et al.*, 2016; Reem *et al.*, 2017). Working on this highly established natural population (likely with thousands consecutive local generations), the sampling protocol allowed us to analyze the Michmoret Harbor population on three disparate levels: multiple seasonal/yearly, within stone, and before vs. after a natural disturbance (stones with colonies buried below a pile of sand).

The *B. schlosseri* population from Michmoret Harbor deviated from HW equilibrium, as has been reported for other *B. schlosseri* populations worldwide (Ben-Shlomo *et al.*, 2001; Stoner *et al.*, 2002; Paz *et al.*, 2003; Ben-Shlomo *et al.*, 2006; Yund *et al.*, 2007; Ben-Shlomo *et al.*, 2010; Reem *et al.*, 2013a; Karahan *et al.*, 2016). Results of the present and other studies indicate that heterozygote deficiency, most probably caused by inbreeding, is a common attribute of *B. schlosseri* populations. Grosberg & Quinn (1986) described an aggregated settlement of sibling *B. schlosseri* larvae that may result in small-scale subpopulations, which is a situation that would promote mating between sibling colonies (Sabbadin 1979; Grosberg & Quinn 1986; Rinkevich *et al.*, 1998a). The overall differentiation periods and pairwise F_{ST} and Dest among all pair-populations revealed low differentiation between the sampling sessions. These results also showed modest changes in the microsatellite allele frequencies over a period of 4 years.

A previous (1995–1997) population genetics study conducted in Michmoret Harbor (Paz *et al.*, 2003) used a panel of microsatellite loci, of which four (BS811, PB29, PB41, PBC1) were also used in the present study. Both studies reported heterozygote deficiency. In contrast to our results, Paz *et al.* (2003) detected rapid changes in the dominant alleles among the eight sampled seasons during two consecutive years. Moreover, most of these dominant alleles did not correspond with the dominant alleles in the present study (except from alleles 174 and 176 in locus PB41 (year 1995) and allele 152 in locus PB29 (summer 1995)). However, many of the alleles/microsatellite loci in the present study were detected in the previous study conducted a decade earlier (11/46, 2/3, 7/7, 3/12, for loci BS811, PB29, PB41, PBC1, respectively). Further, the previous analyses (years 1995-1997) revealed more alleles per microsatellites compared to this study (years 2004-2008) in three out of four shared microsatellite loci (excluding BS811, which was the most polymorphic locus in both studies). Furthermore, they were characterized by high mutation rates, probably to compensate for the documented heterozygote deficiency (Reem *et al.*, 2013a).

Similar to studies that revealed genetic differentiation of *B. schlosseri* populations over very short distances (Grosberg, 1991; Yund & O'Neil, 2000), the Michmoret population showed significant microgeographic differentiation in the form of overall differentiation between the sampled stones ($F_{ST} = 0.03$) or between pairwise stones ($F_{ST} < 0.05$). The population assignment results showed that most of the samples (68%) were assigned to other stones and only 32% were assigned to their natal stones (Fig. 5, Table S7). The same trend of allele frequencies was obtained between the two geographically separated groups (eastern (stones 13, 14, and 16) and western (stones 1, 7, 8, and 9(), indicating a homogeneous population. The Mantel test between geographic and genetic distances (Fig. 3c) revealed a modest yet statistically significant positive correlation with a meaningful effect size (high Cohen's d), probably due to the limited natural dispersal of the larvae (Grosberg, 1987; Rinkevich *et al.*, 1998a). The contrasting results between the ancient Michmoret population (this study, Paz *et al.*, 2003) and the rapid fluctuations in the more recently established populations (Reem *et al.*, 2013a; Karahan *et al.*, 2016) are probably related to the lack of current connectivity between the Michmoret population and other Levantine populations.

During this study, the *B. schlosseri* population in Michmoret Harbor was exposed to frequent natural dis-

turbances during which colonies were buried below a pile of sand; afterwards, not a single animal was found. This provided a unique opportunity to study the genetic structure dynamics of a newly constructed population over a prolonged period. Previously, Karahan *et al*. (2016) studied a newly constructed *B. schlosseri* population following a natural catastrophe (flood) and found significant fluctuations in PAs during the post-catastrophe sampling period. However, we found no changes in the PA repertoire, and it remained stable throughout the sampling period despite the two disturbance events in Michmoret Harbor. Further, the overall differentiation and pairwise F_{ST} and Dest revealed low differentiation in the comparison of data from before and after the disturbances (that occurred before the sampling sessions of October 2006 and November 2007), indicating long-term changes that were unrelated to the natural disturbances (also revealed by the alleles frequencies).

Restricted gene flow plays an important role in the evolution of microgeographic genetic differentiation in sessile and sedentary organisms (Grosberg, 1991). Sabbadin & Graziani (1967) reported differentiation in the *B. schlosseri* population from the Venetian Lagoon at a larger spatial scale (about 1 km), while Grosberg (1991) reported homogeneity of allelic frequencies and consistently small values of F_{ST} in the Eel Pond (Woods Hole, MA, USA) population. This latter result implies that levels of gene flow within a scale of few meters were too high to permit microgeographic differentiation. This premise was further supported by results from a study of the *B. schlosseri* population from the Damariscotta River Estuary in Maine, USA (Yund & O'Neil, 2000). The discrepancy between populations may also arise from different environmental conditions that reflect various levels of turbulent mixing and advection. For example, the Venetian Lagoon population resides in tidal channels with high current speeds, whereas the Eel Pond population lies in very protected waters (Grosberg, 1991). Further, while Hiscock (2008) observed that the most short-lived larvae remain within a few meters of the parental colony, Sams & Keough (2013) reported that the dispersal potential of larvae depends on local hydrodynamic conditions. If differences in levels of mixing (hence gene flow) underlie differences in the breeding system, then one would expect newly established populations in well-mixed waters to remain panmictic over many generations. In poorly mixed waters, however, after initial colonization by immigrants from elsewhere, levels of inbreeding should increase through time as siblings co-settle and the frequency of consanguineous mating increases (Grosberg, 1991).

Our results reveal the presence of a long-term homogeneous population of *B. schlosseri* in Michmoret Harbor, even following disturbances, as the new populations did not differ from the pre-disturbance status or other sampling dates during the 4-year study. The high F_{IS} observed in the newly established *Botryllus* population further suggests that the sampled colonies within the harbor are seemingly the same as the outside-harbor populations, possibly because the water flush by currents and waves provided a supply of larvae to the within harbor area immediately following the re-exposure of the sand-buried stones. Moreover, the *B. schlosseri* colonies residing in Michmoret (outside and inside the harbor) belong to a single ancient population separated by a perforated breakwater (constructed just seven decades ago when the marine youth school Mevo'ot Yam was established; *https://en.wikipedia.org/wiki/Mevo%27ot_Yam*) that does not serve as an effective barrier to out-harbor propagules. Future research that includes the influence of currents on the dispersal of *B. schlosseri* larvae would lead to a better understanding of the re-settlement patterns of this species in Michmoret.

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Supplementary Data

The following supplementary information is available online for the article:

Fig. S1: Cluster analysis dendrograms for the relationships between the 6 Botryllus schlosseri seasonal sampling (populations), using (a) the Manhattan distance and furthest neighbor clustering, (b) the squared Euclidean distance and minimum variance clustering and (c) the Morisita's similarity coefficient with UPGMA clustering, S- Spring and F- Fall. 04-08, refers to years 2004-2008.