Pattern of settlement and natural chimerism in the colonial urochordate *Botryllus schlosseri*

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Abstract Colonies of the cosmopolitan urochordate *Botryllus schlosseri* that share one or both alleles at a single allorecognition locus (Fu/HC) and come into tissue contacts, may fuse and form a mixed entity, a chimera. *Botryllus* populations worldwide exhibit unprecedented extensive polymorphism at this locus, a result that restricts fusions to kin encounters. This study aims to compare spatiotemporal configurations in source and introduced *B. schlosseri* populations, residing on natural and man-made substrata, respectively. By using four microsatellite loci, we tested genetic consanguinity of colonies settled naturally along spatial vectors on both, natural (native populations) and man-made (introduced) substrates. Four populations were studied. Results revealed that *B. schlosseri* colonies, on both substrate types, assemble in groups of relatives that share similar microsatellite profiles. We suggest that this pattern of settlement promotes the formation of chimeras, which evoke conflicting interactions: cooperation between different somatic cell lines that constitute the colonial soma and competition between germ cells that inhabit the chimera gonads. Under natural conditions, the chimera may allow genetic flexibility that

depends on joint genomic fitness of its partners. This is probably one of the life history characteristics that led to the worldwide distribution success of this species.

Introduction

Sessile colonial organisms compete intensely with conspecifics (Hart and Grosberg 1999) and with members of other taxa (Grosberg 1981) for growing space. Conspecific interactions are unique as they may result in either aggressiveness or in natural tissue transplantations (fusions and chimera formation), a phenomenon reported from over nine marine phyla (Buss 1982; Grosberg 1988; Chadwick-Furman and Rinkevich 1994). Some taxa have further developed highly polymorphic histocompatibility systems (Rinkevich et al. 1995; Hart and Grosberg 1999) that are used to mediate allogeneic interactions. The development and preservation of the polymorphism in histocompatibility systems have been attributed to a variety of evolutionary forces (Grosberg and Quinn 1989; Grosberg and Hart 2000; Rinkevich 2002), but their nature remains an empirical and theoretical challenge (Rinkevich 2002). Recently, De Tomaso and Weissman (2004) showed that in the laboratory heterozygotes of the histocompatibility locus had a significantly advantage over homozygotes sibling.

One of the best examples for histocompatibility complexity in the invertebrates is the allorecognition responses in the cosmopolitan colonial urochordate, *Botryllus schlosseri*. When two *Botryllus* colonies grow into tissue contacts, they either parabiose through peripheral blood vessels, forming a single entity (a chimera) or develop

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inflammatory reactions (a phenomenon called colony rejection—Oka and Watanabe 1960; Sabbadin 1962; Scofield et al. 1982). This allorecognition is controlled by a single, highly polymorphic fusibility locus, termed Fu/HC (Fusibility/Histocompatibility; Weissman et al. 1990). Chimerism is established between allospecifics that share either one or both Fu/HC alleles, whereas rejecting pairs share no Fu/HC allele. Natural populations of *Botryllus* exhibit unprecedented extensive polymorphism at this locus with several hundred alleles per population (Rinkevich et al. 1995). Thus, arbitrary tissue contacts between conspecifics will favor fusions almost only between relatives.

Resident adults of *B. schlosseri*, as many other sessile organisms, usurp the space, restricting settlement of larvae to unoccupied areas (Osman and Whitlatch 1995) or to adjacent parental colonies (Rinkevich and Weissman 1987). Grosberg and Quinn (1986) studied settlement patterns of *B. schlosseri* larvae in a single population (within a marina, Atlantic coast USA) and showed that in this anthropogenic influenced habitat, (a) sibling larvae settle in aggregations, at higher rates than would be expected from random dispersal distance effects alone, and (b), that Fu/HC compatible oozooids settle in close proximity to each other; therefore, chimerism is clearly related to spatial distribution of offspring. Yund and O'Neill (2000) further found low level of genetic differentiation over small spatial scales for another USA east coast population.

Aggregations of histocompatible genotypes promote the formation of chimeras in the field, resulting from fusions of kin colonies and these chimeras may thrive for considerable time periods (Chadwick-Furman and Weissman 1995). Natural chimerism in *Botryllus* populations is probably a worldwide common phenomenon (Stoner and Weissman 1996; Ben-Shlomo et al. 2001; Paz et al. 2003). From available genetic surveys it seems that the frequency of chimeric colonies is much higher in invading populations, thus, in a young population of New Zealand (less than 150 years old) and Japan more than 8% of the tested colonies were in a chimeric state (Ben-Shlomo et al. 2001; Ben-Shlomo and Rinkevich unpublished data) while in the Mediterranean populations of Israel and Croatia only less than 3% of the colonies were found to be chimeric (Paz et al. 2003; Ben-Shlomo and Rinkevich unpublished data).

Both, Grosberg and Quinn (1986) and Yund and O'Neill (2000), studied spatial settlement of *B. schlosseri* colonies from the USA Atlantic coasts, less than 200 years old introduced populations. Demographic traits of invading populations do not necessarily represent the same life history traits as the original populations, presumably coming from the Mediterranean Sea basin (Van Name 1945; Berrill 1950; Stoner et al. 2002). Newly founded populations are expected to show lower gene diversity relative to their source populations and thus the probability

of co-settlement of sibling colonies may be significantly higher. However, demographic analyses, like the genetic consanguinity of *B. schlosseri* populations along a spatial vector, were not performed in either one of the Mediterranean Sea or the invading populations.

Comparing the settling patterns in ancient established populations, with contemporary and younger populations that reside primarily in anthropogenic impacted habitats, is furthermore important for understanding evolutionary and ecological processes that take place in *Botryllus* spatiotemporal aggregates. This study aims, therefore, to compare spatiotemporal configurations in source and introduced *B. schlosseri* populations, residing on natural and man-made substrata, respectively. By using highly polymorphic microsatellite loci, we study here patterns of adult distribution in two native *B. schlosseri* populations from the Mediterranean Sea and in two introduced invasive populations residing in the east and the west coasts of the USA. We hypothesize, that resulting from founder effect, the level of genetic similarity between colonies would be higher in invading populations relative to their original populations. This descent similarity combine with within marinas dense inhabitants of invading populations may further promote the formation of high frequency of chimeric colonies.

Material and methods

Colony sampling

Tissue samples from *B. schlosseri* colonies were collected along transects from four different shallow water localities, representing natural and man-made habitats worldwide. Two native populations, inhabiting undersurface stones, were chosen along the Israeli Mediterranean coast (0.5–1.5 m depth, Caesarea and Mikhmoret sites, 10 km apart). In both natural habitats the stones were dispersed, lying above a sandy substratum that lacked *Botryllus* colonies. A single transect of several tens of meters was run in each locality parallel to the shoreline. All stones underneath the transect line and 25 cm on either side, were inspected. In both Israeli sites, colonies of *B. schlosseri* were absent from small stones or from stones that were partially buried in the sand, whereas underneath larger stones, several colonies per stone were often found in close proximity to one another. The distances between colonies residing underneath each stone were calculated to the nearest centimeter. Distances between two sampled stones were usually several decimeters to a few meters but never exceeded 10 m. Two recently introduced populations, residing in shallow water artificial substrates, were sampled from underneath the floating dock at the marina in Woods Hole, MA, and from a submerged rope in Santa Barbara harbor,

CA (east and west coasts of the USA, respectively). In contrast to the native populations from the Mediterranean Sea, both introduced American populations were highly crowded. For tissue collections, a plastic ruler was tied to the dock, at a depth of 20 cm, parallel to sea level (Woods Hole) or to a vertically submerged rope (Santa Barbara). All *Botryllus* colonies that came into contact with the ruler were observed and sampled. The sampling scales were therefore inconsistent from natural sites to the artificial sites but represented, in duplicate, the two most common distribution types characteristic of *Botryllus schlosseri* populations, worldwide.

Microsatellite typing

DNA extraction and microsatellite typing of colonies followed Ben-Shlomo et al. (2001). Each individual colony was typed by three to four highly polymorphic microsatellite loci. Several samples from different gels and different populations were re-run on the same gel to ensure unity of reading between the gels and the sampled populations. Allele identification and genotyping were determined directly from the autoradiographs.

Data analysis

Weighted observed heterozygosity (Ho), gene diversity (expected heterozygosity, He) and the inbreeding coefficient (Fis) were computed by the Markov chain method using GENEPOP (Raymond and Rousset 1995), version 3.4 (2003). To determine the spatial distribution of adult colonies in nature we calculated, for each location, the correlation coefficient between the genetic distance and the physical distance for the different pairs of individuals. Genetic distances between individuals were calculated using the index suggested by Lynch (1990). Thus, for any marker, if both individuals have the same genotype (having two alleles in common), the individual genetic distance between them for that marker is 0; if they share only one allele, where one individual is homozygote and the other is heterozygote, the distance is 1/3; if they share only one allele, and both are heterozygotes, the distance is 1/2; if the two individuals have no common alleles, the distance is 1. The overall genetic distance between the two individuals is the Euclidean measure (that is, the square root of the sum of squares) of the genetic distances over all the markers. Note that this measure of genetic distance (or similarity) incorporates similarity not only due to identity-by-descent, but also due to chance (that is, identity-by-state).

Physical distances are measured in meters for all four populations. Since the settlement of the Israeli populations is discrete, following stone positions, we measured physical distances for these populations also in ''stone'' units,

that is, the distance between two colonies is expressed as the number of stones separating these colonies. Clustering was performed using Ward's method (squared distances and minimum variance amalgamation). Different colonies which occupy the same stone have the same stone number.

If there are N individuals on a transect, there are $\frac{1}{2}N(N-1)$ different pairs of distances, which are, nevertheless, statistically dependent. Thus the significance of the computed correlation coefficient was estimated using a self-made programmed Mantel's test. The test considers all possible permutations between the pairs of distances, and calculates the correlation coefficient for each permutation. The proportion of permutations with a correlation coefficient larger than or equal to the observed correlation coefficient is taken as the P value for rejecting the null hypothesis of no correlation between the physical and the genetic distances. Since the number of possible permutations can be quite huge, the common practice is to take a random sample of the possible permutations. Thus, in our analysis, we sampled 1000 permutations in each case. Individuals that were not typed for all the microsatellite markers were eliminated from the transect analysis. Since for a large proportion of the Woods Hole sample the PB41 genotyping was missing, this marker was not used for the Woods Hole transect analysis. A few individuals revealed, each, microsatellite loci with more than two different alleles, indicating that they are indeed chimeras. In these cases, two of the alleles were chosen at random for the genetic distance calculations.

Results

Although sampling along a transect may result in underestimated genetic variability, both the native and the introduced populations exhibited high levels of gene diversity and natural chimerism (Table 1). A total of 184 *B. schlosseri* colonies were sampled. Three microsatellite loci (BS811, PB29 and PB41) were highly polymorphic in all tested populations (Table 1), revealing 56 different alleles for the four studied populations. The forth microsatellite locus (PB49) was informative for the USA populations only (additional nine alleles). Observed heterozygosity was lower than expected in all tested loci. This is valid also when samples that show no result were considered as homozygote for a null allele. The average number of alleles (A) was 7.07 ± 5.25 (mean±SD), and the average gene diversity (He) was 0.67 ± 0.17. A Hardy-Weinberg exact test for all loci and all populations revealed a highly significant heterozygote deficiency ($P < 0.0001$; H_0: HW equilibrium).

Botryllus schlosseri colonies in native and introduced populations settle in aggregations as groups of colonies exhibiting similar microsatellite profiles, as can be inferred from the positive correlation coefficients between genetic and physical distances in all four populations (Table 2).

Table 1 Total number of alleles (A), observed (H_o), expected heterozygosity (H_e) and number of chimeras. N = number of sampled colonies

Locus		Israel		USA	
		Caesarea	Michmoret	Woods Hole	Santa Barbara
BS-811	A	23	13	7	7
	H_e	0.93	0.89	0.70	0.89
	H_o	0.44	0.21	0.32	0.10
	Chimera	5	—	3	—
	N	54	42	31	23
PB-29	A	7	3	5	4
	H_e	0.73	0.35	0.40	0.71
	H_o	0.2	0	0.26	0.14
	Chimera	1	—	1	—
	N	41	39	47	26
PB-41	A	7	4	3	4
	H_e	0.67	0.57	0.48	0.66
	H_o	0.31	0.27	0.11	0.61
	Chimera	—	—	—	—
	N	54	49	17	20
PB-49	A			7	5
	H_e			0.76	0.68
	H_o			0.14	0.12
	Chimera			—	—
	N			36	26
Fis (3 loci)		0.58	0.72	0.50	0.65

While the correlation coefficients are not very large, they are all significantly positive (For each population, $P < 0.001$, Table 2). The aggregation of colonies having similar microsatellite profiles is also illustrated graphically, by cladograms which amalgamate colonies according to their genetic similarity (Fig. 1). In both populations, different colonies which occupy the same stone (that is, having the same stone number) clumped together (according to their genetic similarity) in the same cluster or in adjacent clusters.

Out of 55 colonies samples in Caesarea (a native population), 5 colonies (9.1%) were identified as chimeras (that is, having at least three distinct alleles in any of the three examined loci). This is, of course, an underestimate of the true frequency of chimeras, as real chimeras can occur which are undetectable in our study (that is, chimeras which for each locus, the total number of different alleles for both participants is no more than two). Nevertheless,

the probability that a mixture of two colonies will yield no more than two alleles at each of the three loci examined is only 1.3%. Hence only a negligible fraction of the chimeras are hidden, while 98.7% of the chimeras are detectable.

The picture is slightly different for the introduced population of Woods Hole. Here, 4 of the 48 colonies (8.3%) were identified as chimeras. However, due to the smaller number of alleles in that population, the probability that a true chimera will be undetected is larger—12.4% for the four loci system examined in Woods Hole, raising the estimated frequency of chimeras to around 9.5%.

Discussion

As any unoccupied subtidal space available for settlement to sessile marine assemblages is limited, the intense com-

Table 2 Correlation coefficients between the genetic distance and the physical distance for the different pairs of individuals

Location	Number of markers	Number of individuals	Number of pairs	Correlation coefficient (r)	Estimated P Value
Caesarea Metric distance	3	40	780	0.111	<0.001
Caesarea Stone distance	3	40	780	0.131	<0.001
Mikhmoret Metric distance	3	33	528	0.288	<0.001
Mikhmoret Stone Distance	3	33	528	0.268	<0.001
Woods Hole (East Coast USA)	3	22	231	0.260	<0.001
Santa Barbara (West Coast USA)	4	19	171	0.415	<0.001

Fig. 1 Distribution profiles of
B. schlosseri populations
inhabiting shallow water
Mediterranean habitats.
Numbers reveal stones with at
least four individuals (**A**.
Caesarea) or two individuals (**B**.
Mikhmoret). The genetic
distances between colonies were
calculated using Lynch's
measure (see Methods).
Clustering was performed using
Ward's method (squared
distances and minimum
variance amalgamation).
Different colonies which
occupy the same stone have the
same stone number. (**C**) Plots
presenting the relationship
between spatial distance and
genetic similarity
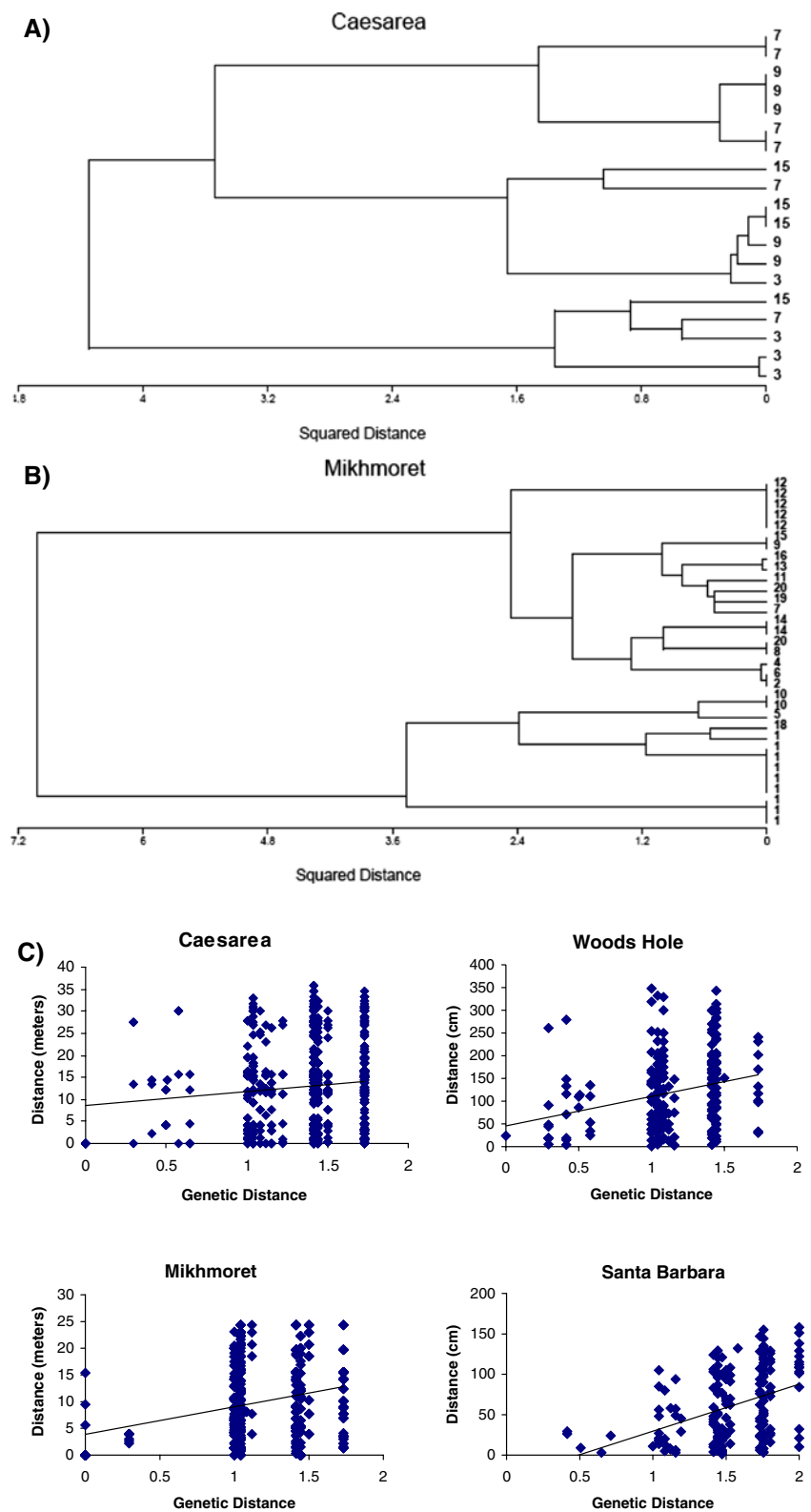
petition for space results in arrays of inter- and intraspecific competitions (Grosberg 1981; Connell 1985; Pineda and Caswell 1997; Hart and Grosberg 1999). Theoretical models suggest that these interactions should reveal selection modes acting on species-community properties and on spatial structures (Johnson and Seinen 2002). Selection should favor self-restraint and cooperation when relatedness between neighbors is high, such as in some

intraspecific interactions (Frank 2003). Kin aggregation on the limited available substrate, as is evident in *B. schlosseri* populations (Grosberg and Quinn 1986), should have endorsed intra-familial competition. Chimera formation is a suitable and appropriate arena to solve or intensify these intra-specific conflicts (Rinkevich 2002, 2004). By repressing aggressive outcomes between intra-familial partners, the chimera may serve as a joint vehicle where the whole communal entity better compete other organisms for vacant space. Moreover, chimerism produces an entity which has a greater store of genetic variability and hence a wider range of physiological qualities (Rinkevich and Yankelevich 2004).

Does chimerism contribute to the global distribution of *Botryllus* populations? The current study show that both native and introduced populations show similar mode of kin co-settlement in aggregates. Hence, introduced populations found to be highly crowded within marinas, while native populations are dispersed. In general, newly established *Botryllus* populations are characterized by lower genetic diversity resulting from founder effect (Ben-Shlomo et al. 2001; Stoner et al. 2002; Ben-Shlomo et al. 2006). However, reduction in genetic diversity is not necessarily disadvantageous. One controversial example is the invasive Argentine ant *Linepithema humile* that became a major pest species. Tsutsui et al. (2000) and Tsutsui et al. (2003) reported that in the USA reduced genetic diversity and reduced intraspecific aggregation lead to widespread ecological success. Nonetheless, Giraud et al. (2002) and Pedersen et al. (2006) reported that introduced Argentine ants populations in Europe lost only limited level of genetic diversity and the relatedness between nests mates were close to null.

Aggregations of sibling *B. schlosseri* colonies (Grosberg and Quinn 1986; Yund and O'Neill 2000) can increase the probability for the formation of chimeras, as revealed for both, native and introduced populations (Table 2). About 9% of the colonies in Caesarea (native) and Woods Hole (introduced; Table 1) were chimeras. This is an underestimate value. We defined chimera as colony that exhibit more than two different alleles on any microsatellite locus (Figure 4 in Ben-Shlomo et al. 2001). Based on number alleles found at each population we estimated the probability that a mixture of two colonies will yield no more than two alleles at each of the loci examined. We found that only a negligible fraction of the chimeras are hidden and both types of populations harbor similar frequencies of chimeric colonies. However, additional chimeras could not be depicted stemming from the characteristic heterozygote deficiency recorded in *B. schlosseri* populations (found at every locus and in all populations studied to date; Table 1; and Ben-Shlomo et al. 2001; Stoner et al. 2002; Paz et al. 2003;

Ben-Shlomo et al. 2006). Thus, because introduced populations are characterized by colonies that are highly crowded and because they exhibit lower gene diversity, it is possible that the actual level of chimerism in introduced population is much higher.

Chimeras are known from studies on algae, sponges, hydroids, corals, bryozoans and ascidians. These groups dominate sessile assemblages on hard marine substrates (Grosberg 1981, 1988; Sommerfeldt et al. 2003). The fitness consequence of the chimeric state, however, is still obscure. Following chimera formation one of the partners is morphologically resorbed, leading to cell lineage competitions (Rinkevich 2002). Laboratory and field experiments showed that both somatic cell and germ-line parasitism are common in chimeras (Pancer et al. 1995; Stoner et al. 1999). The somatic constituent that is replaced weekly by a blastogenic cycle (Berrill 1950), may be shifted in chimeras from one genotype to another, in accordance with changes in environmental conditions (Rinkevich and Yankelevich 2004). This attribute may carry advantages to the chimera entity, which synergistically presents to natural selection forces, at any time, the most fitted combination of its genetic components. Moreover, larger botryllid chimeras may successfully control feeding substrates and effectively prevent colonization of that surface area by other competitive species. This is especially true in chimeras made of several partners (Rinkevich and Shapira 1999; Rinkevich 2002, 2004). Chimerism may also involve a trade-off mechanism scrutinizing between selection pressures at the individual level and the kin level.

We found a spatial clustering of genetically similar individuals in *B. schlosseri*. While the absolute values of our genetic similarity measure incorporate not only similarity due to identity-by-descent, but also due to identity-by-state, they clearly suggest the spatial clustering of genetically related individuals. This is the result of short-range dispersal and rapid settlement of propagules (Grosberg 1987) on the one hand and tolerance to kin on the other. This tolerance, resulting in chimerism between allogeneic compatible individuals, can be viewed as an alliance between selfish partners. Alliance, since following competition for a limited substrate there is an advantage to larger body-sized entities which is acquired through chimerism; and selfishness, since only one of the partners in the association of two genotypes succeeds to contribute to the germ line (Stoner et al. 1999; Rinkevich and Yankelevich 2004). The apriori uncertainty as to which individual germ line will be expressed in the reproductive niches enables the formation of this partnership, at least at its early stage. Reduced intra-partnership antagonism and the formation of a better fit entity may develop only if the partners are relatives.

This *Botryllus* life-history attribute resembles the pleometric nest formation, associated with the density of founding young queens in social insects, such as ants (Tschinkel 1993 and references therein) and termites (Thorne 1982). The presumed advantage of these queen associations over the single-queen nest is in the ability to produce a larger number of workers in a shorter time, as this ability can be a crucial factor in territorial disputes between neighboring nests. In most of the pleometric species, only one of the queens from each founding group survives in the mature colony, following intra-colony struggles. Strassmann et al. (1989) pointed out that in pleometric wasps (of the genera *Polistes* and *Mischocyttarus*) the founding queens are indeed genetically related. In the ant species *Gnamptogenys striatula*, where there are multiple queens per nest, Giraud et al. (2001) reported that the relatedness among nestmate queens is very high.

The settlement strategy in *B. schlosseri* is not based solely on kin aggregation. Although numerous genetically related colonies are established in a nearby location, probably next to their emergence place, some larvae disperse away and are found on distant substrates (Fig. 1). This is evidenced also by the relative low correlation coefficient values recorded between the genetic distance and the physical distance for different pairs of individuals. This complex settlement mode may also promote crossing between adjacent, non-genetically related colonies. The apparent strategy for splitting the risk further guarantees gene flow and genetic heterogeneity. The results showing larval settlement on distance substrates further demonstrate that spatial clumping is not the outcome of low dispersal ability and that dispersal effects are insufficient to explain the recorded population genetic structures. The ability to repress intra-familial competition of aggregated kin by chimerism on one hand, and the capability to disperse and establish colonies on distant substrates on the other (in both native and introduced populations), may add to our understanding of how this taxon becomes a successful cosmopolitan invader species (Berrill 1950; Lambert and Lambert 1998).

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