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**Abstract:** The colonial ascidian *Botryllus schlosseri* is a cosmopolitan, marine filter feeder, introduced as a laboratory research organism in the 1950s. Currently, it is widely used in many laboratories to investigate a variety of biological questions. Recently, it has become a species of concern, as it is an invasive species in many coastal environments. Here, we review studies on the geographical distribution of the species, sexual and asexual reproduction in the field, tolerance to temperature, salinity and anthropogenic activity, polychromatism, enzymatic polymorphism, and the genetic basis of pigmentation. Studying the relationship between genetic polymorphism and the adaptation of *B. schlosseri* to environmental stress is a challenge of future research and will improve our understanding of its evolutionary success and invasive potential.

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## Abstract

The colonial ascidian *Botryllus schlosseri* is a cosmopolitan, marine filter feeder, introduced as a laboratory research organism in the 1950s. Currently, it is widely used in many laboratories to investigate a variety of biological questions. Recently, it has become a species of concern, as it is an invasive species in many coastal environments. Here, we review studies on the geographical distribution of the species, sexual and asexual reproduction in the field, tolerance to temperature, salinity and anthropogenic activity, polychromatism, enzymatic polymorphism, and the genetic basis of pigmentation. Studying the relationship between genetic polymorphism and the adaptation of *B. schlosseri* to environmental stress is a challenge of future research and will improve our understanding of its evolutionary success and invasive potential.

## 1. Introduction

*Botryllus schlosseri* is a colonial ascidian (Tunicata), widely distributed in shallow subarctic and temperate waters of both the hemispheres (Ben-Shlomo et al., 2001, 2010; Reem et al., 2013a,b). Colonies live in shallow waters of the littoral zone, including the intertidal zone or adjacent subtidal zone, up to a depth of 200 m. The species prefers sheltered areas of harbours and marinas (Hiscock, 2005) on either natural or artificial substrates, including rocks, algae, eelgrass, bivalves, solitary tunicates, floating docks, wharf pilings, and aquaculture plants. In macrofouling biocoenoses of hard substrata, *B. schlosseri* strongly competes with other benthic filter feeders, such as barnacles and mussels, for space and food (*i.e.*, suspended phytoplankton, zooplankton and organic matter).

Due to its fast-growing colonies, adaptive abilities and high genetic diversity (Reem et al., 2013b), *B. schlosseri* is an invasive species able to exploit new environments, potentially displacing native species and disrupting community dynamics (Harms and Anger, 1983; Schmidt and Warner, 1986). Once it becomes established, it is very difficult to eradicate, thus presenting a concern for the aquaculture industry (Carver et al., 2006; Arens et al., 2011). The species is now considered cosmopolitan, living in eurythermal and mildly euryhaline conditions of all epicontinental waters except the Antarctic Circle, and it is classified as “globally secure” or without conservation status. **The success of such species as invader is mainly related to its post-larval mode of dispersal by rafting of colony fragments on macrophytes rather than by spreading through larval swimming and its ability to adapt to new habitats. (Worcester, 1994).**

**Although the species was firstly described by Schlosser (1756) and Pallas (1766) in the Cornwall coasts, its native range is thought to include the Mediterranean and Black seas (Berrill, 1950). This assumption is supported by genetic studies based on the analyses of microsatellite loci, revealing allelic diversity between Mediterranean and Atlantic coast populations of Europe related to recent post-glacial invasions (Ben-Shlomo et al., 2006). A high level of polymorphism (more than 60 alleles for a single locus) was found in Mediterranean populations (Paz, 1999; Paz et al., 2003); this level decreases to 17 alleles at the same locus in Monterey Bay, California (Stoner, 1997) and to between 4 and 20 alleles for 5 loci in New Zealand (Ben-Shlomo et al., 2001). These studies support that idea that *B. schlosseri* is of Mediterranean origin and suggest that a limited number of different individual colonies founded each of the introduced populations worldwide, as reported in Fig. 1A. According to this view, the species spread to the whole European coasts, probably because of post-glacial period dispersal, extending to Spain, Portugal, France, Great Britain, the Netherlands, Denmark, Germany, Sweden, Norway and the Faroe Islands (Ben-Shlomo et al., 2001, 2006; Cohen, 2005). From Europe, it was presumably introduced into several regions worldwide, likely as fouling organism on ship hulls or floating debris; these regions include the Mediterranean part of the Suez Canal (reported since 1869, down to Lake Timsah), the east continental shelf of North America (reported since 1841), the Gulf of Mexico (reported since 1887), India (reported since 2006), Far East (Japan, Korea, Hong Kong with first sporadic reports in 1929 and 1952), the Great Barrier Reef and southern Australia (reported since 1905), New Zealand (reported since 1922), Tasmania (reported since 1928), the west coast of North America (reported since the mid-1940s), the west coast of South America (reported since 1948), South Africa (reported since 1955), and the east coast of South America (reported since 1964) (Rinkevich et al., 1998a, NIMPIS, 2002, Paz et al., 2003; Carver et al., 2006; Reem et al., 2013a). The current trend of invasiveness appears to be both the Northern latitudes (Iceland from North Europe and Alaska from the U.S. north-west coast) and the tropic-equatorial ones (west coast of Central and Southern America, the latter from populations of New Zealand). Scanty information can be obtained about the recent spotted colonisation of the west coast of India, probably from various populations of the south and east coasts of Africa.**

High polymorphism and heterozygote deficiency appear as common attributes of all the investigated populations of *B. schlosseri* (Ben-Shlomo et al., 2001, 2008; Rinkevich et al., 2001; Reem et al., 2013a, b). Recently, further insights

1 into the dispersal potential and the degree of genetic differentiation among populations and subpopulations have been  
2 gained using molecular techniques. Yund and O'Neil (2000) noted that genetic differentiation may occur over very  
3 short distances (8 to 21 m) and that the patterns were consistent with inbreeding and genetic drift models.

4 The eastern and western coasts of North America had different founders over different invasion periods (Stoner et  
5 al., 2002). The east coast population is similar to the European one. The California population is younger than the New  
6 Zealand population and is the result of different founders, with continuous introductions from either European or Asian  
7 populations (Stoner et al., 2002) within a period of time shorter than the introductions to New Zealand. The same events  
8 occurred in western coastal populations in South America, which were subjected to introductions from either European  
9 or South Pacific populations (Ben-Shlomo et al., 2010). In New Zealand, the allele distribution pattern significantly  
10 differs among subpopulations **probably** due to both limited gene flow and founder effects, resulting in heterozygote  
11 deficiency at most loci. The latter may also reflect high levels of natural chimerism as a result of aggregated settlement  
12 of sibling larvae (Ben-Shlomo et al., 2001).

13 Recently, Bock and collaborators (2012) characterised the phylogenetic and population genetic structure of  
14 European and North American populations using cytochrome c oxidase subunit I, nuclear 18S rRNA and 10  
15 polymorphic microsatellite loci. They **reported** that the species comprises at least five putative, previously unrecognised  
16 cryptic species that are morphologically indistinguishable, three of which are likely reproductively isolated from one  
17 another. One of these putatively cryptic species is widespread globally, whereas its sibling species are highly restricted  
18 geographically, limited to the Mediterranean coast of Spain and the English Channel. The genetic divergence among  
19 these lineages represents approximately 4.3-11.0 Myr of evolutionary history, likely starting during the Messinian  
20 Salinity Crisis of the Mediterranean Sea, which caused habitat fragmentation. Some intra-specific variability in *B.*  
21 *schlosseri* has also been reported using detailed whole-mitogenome comparisons, suggesting on-going speciation events  
22 (Griggio et al., 2014).

## 33 **2. Anatomy of a colony**

34 A colony is a clone and is derived from the metamorphosis of a single, tadpole-like larva into a sessile zooid that  
35 founds the new colony; this zooid is known as an oozoid as it is derived from a fertilised egg. Interestingly, the  
36 founder oozoid contains the bud that will give rise to a blastozooid (a zooid derived from blastogenesis; i.e., budding).  
37 As a blastozooid can form more than a single bud, the colony grows through repeated cycles of budding. Zooids are  
38 arranged in star-shaped systems (8-12 blastozooids), with their individual oral siphons opening anteriorly and their  
39 atrial siphons converging into a common, cloacal cavity in the centre of each system (Fig. 1B). The cloacal cavity is  
40 connected to the environment through the cloacal siphon. Adult zooids bear buds (primary buds) that, in turn, bear  
41 budlets or secondary buds. In a colony, individuals are interconnected by a general vascular apparatus ( the colonial  
42 circulatory system) that crosses the colonial tunic, i.e., the extrazoidal collagenous matrix rich in cellulose, typical of  
43 tunicates (Brunetti and Burighel, 1969; Burighel and Brunetti, 1971; Gasparini et al., 2007).

44 Cyclical generation changes or take-overs occur during the colonial lifespan, wherein the adult generation is  
45 replaced by the subsequent one (represented by primary buds) and new budlet primordia appear (Fig. 1C). A  
46 blastogenetic cycle is defined as the period of time from one take-over to the next and includes seven main  
47 developmental phases (Manni et al., 2007, Gasparini et al., 2014).

48 The replication and succession of genetically identical individuals provides the colony with strong regulatory  
49 ability demonstrating the adaptive value of both the colony lifestyle and the population dynamics of the species  
50 (Sabbadin, 1979). This homeostasis represents the main feature of colonial life, based on a somatic plasticity that allows  
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1 the continual repetition of blastogenetic cycles and the continuous adjustment a of colonial growth rate according to  
2 environmental conditions, regulating the number of individuals per generation and the number of co-existing  
3 generations (Sabbadin, 1966; Gasparini et al., 2014). For example, under high stress conditions (such as when all  
4 blastozooids are removed from the colony and only the tunic with its vessels and cells remains), the colony can rebuild  
5 **itself** by vascular budding, *i.e.*, by the aggregation and organisation of a few multipotent blood cells into a new bud  
6 (Sabbadin et al., 1975).  
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### 10 **3. Life history**

11 The first studies on the life history of *B. schlosseri* populations were conducted by Grave (1933) at Woods Hole,  
12 MA, in the North Atlantic, and by Chadwick-Furman and Weissman (1995a,b) at Monterey Bay, CA, in the North  
13 Pacific.  
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16 Woods Hole colonies grow rapidly during the summer, with the number of zooids doubling every two or three  
17 days. Colonies reach the sexual maturity after 35-50 days from the metamorphosis but most of them do not survive the  
18 winter season. Large colonies can be found at the end of winter (Grave, 1933).  
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21 Colonies in Monterey Bay have rapid growth, intense sexual reproduction and short lifespans ranging from 3 to 8  
22 months and this time interval is seasonally dependent. When transferred in laboratory aquaria, growth slows down,  
23 reproduction ceases and colony can live for more than two years (Chadwick-Furman and Weissman, 1995b).  
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26 According to Millar (1952), in *Botryllus* population from the Ardrossan harbour (Ayrshire, Scotland), along the  
27 Scottish Atlantic coast, the colonial lifespan does not overrun one year. Most of the winter colonies extend over a  
28 surface less than 300 mm<sup>2</sup>, corresponding to 1-12 systems of zooids. Larger colonies, 500-800 mm<sup>2</sup> wide (15-20 zooid  
29 systems), started to appear from late spring (April-May) up to the end of July. They disappear in late summer and are  
30 replaced by newly settled, small colonies representing the overwintering generation.  
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33 In the Mediterranean area the first description of *B. schlosseri* biological cycle is owed to Sabbadin (1955b) for  
34 populations of the Lagoon of Venice (Fig. 2A). He collected data from 1952 to 1955 on population trends throughout  
35 the year, growth rates, the duration of the life cycle, the replacement of generations, and the sexual reproductive cycle,  
36 and compared these observations with the contemporaneous observations of Millar **for Scotland** (1952). Differences in  
37 lifespan related to latitude were emphasised because the biological cycle is affected by the particular thermal regime of  
38 the Lagoon of Venice environment. In the lagoon, the composition of the population was arbitrarily divided into three  
39 frequency classes based on the number of zooid systems per colony. In the spring (April), the sudden and extensive  
40 appearance of a new generation with mature gonads causes a significant decline in the number of large colonies (more  
41 than 50 systems; Fig. 2B). Small colonies (up to 10 systems) overlap the existing population and are overtaken, in turn,  
42 by new oozoids, abundant throughout the summer and much of the autumn. They become increasingly rare toward the  
43 end of the year and are absent from January to March, when the monthly average seawater temperature reaches 5-6°C.  
44 Only at the end of the autumn and during the winter, when no sexual reproduction occurs but blastogenesis is very  
45 active, do large colonies appear again. However, the population undergoes a significant decline in the late autumn and  
46 early winter due to the low initial abundance of large colonies and the loss of the previous year generation. Large  
47 colonies are only abundant at the end of winter. As a result, in the Lagoon of Venice, the colony lifespan usually lasts  
48 12-20 months, *i.e.*, the period from the first appearance of the colonies to the summer or autumn of the following year;  
49 therefore, there is continuous overlap of generations from two different years (Fig. 2B).  
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1 Along the Mediterranean coast of Israel, where seawater temperatures never drop below 10°C, *B. schlosseri* live on  
2 the undersurface of bottom stones: colonies are more abundant in summer-autumn with larger colonies appearing in  
3 spring (Rinkevich et al., 1998a).

4 In the early 1970s, a comprehensive study was conducted in the Lagoon of Venice to answer some remaining  
5 questions on the life cycle of *B. schlosseri*. Artificial panels were submerged at five stations in the southern basin that  
6 varied in the intensity of tidal flow and the amount of organic material introduced from urban wastewater. The  
7 population frequency throughout the year, reproductive period, number of annual sexual generations, trends in sexual  
8 maturation in relation to the thermal regime, duration of the life cycle, growth pattern and morphology of the mature  
9 colonies were investigated (Brunetti, 1974). This research provided the first detailed results on the auto-ecology of this  
10 species in the Lagoon of Venice, some of which are summarised below:

11 a) Larval settlement on panels is strongly conditioned and inhibited by exposure to tidal current.

12 b) Maximum population and colony growth occurs along canals rich in organic material from urban wastewater.

13 c) In the absence of competition for space, colonies initially grow isometrically on a circular fouling surface;  
14 however, when they reach a diameter of 20-25 cm, isometric growth ends, and vertical growth from the centre of each  
15 colony begins. This process leads to the formation of folds, and the colony assumes the characteristic form of a pendant,  
16 typical of colonies on piles.

17 Subsequent studies revealed that, in the Lagoon of Venice, the life cycle of *B. schlosseri* does not include the  
18 hibernation phase **observed** in the related species *Botrylloides leachii* **when** temperature decreases to below 10°C. In the  
19 latter species, during the winter months, all filter-feeding zooids and buds disappear, and colonies are reduced to a  
20 matrix consisting of the tunic and its vasculature full of pigmented haemocytes. In spring, colonies reactivate and a  
21 period of intensive growth begins, culminating with sexual reproduction and the successive death of the colony  
22 (Brunetti, 1976; Burighel et al., 1976).

23 **Collectively, these studies highlighted geographic differences in average colony size, colony growth rates, onset of  
24 reproduction, numbers of larvae and oozoids, seasonal appearance, intra- and inter-specific competition. They revealed  
25 one of the most interesting aspects of the life history of this species; namely, the variation in reproductive seasonality  
26 across the broad distribution area of the species, with the reproductive period being restricted to a brief summer season  
27 at higher latitudes.**

### 3.1 Asexual reproduction

31 The colonial growth rate is determined by the duration of the blastogenetic cycles and depends on various biotic  
32 and abiotic factors. In theory, during the transition from a blastogenetic generation to the subsequent one, the colony  
33 growth rate is exponential due to bilateral budding. However, the budding rate is not equal among all zooids and is  
34 strongly affected by both environmental conditions and food availability. Unfavourable conditions can cause some of  
35 the colonial buds to degenerate, sometimes resulting in negative growth and a reduction in the number of adult zooids in  
36 the colony in the subsequent generation.

37 In the Lagoon of Venice, in spring (between April and May), as temperatures increase from an average **value** of  
38 18°C to 21°C, the duration of the zooid lifespan (from zooid appearance as a bud to its resorption at take-over)  
39 decreases from 21.5 to 15.7 days. In the summer (June-July), the zooid lifespan reaches a minimum duration of 13 days,  
40 when the seawater temperature increases to an average **value** of 26°C. In the autumn (November), when the temperature  
41 falls below 10°C, the zooid lifespan **and the duration of the blastogenetic cycle** are greatly extended, up to 22 days and  
42 65 days, **respectively** (Sabbadin, 1955b). As far as colonial growth is concerned, it **remains** largely constant after the

1 first five blastogenetic generations. The maximum colonial growth by blastogenesis, with the formation of large  
2 pendants, occurs during the winter, when sexual reproduction is arrested. This growth may also be enhanced by the  
3 abundant phytoplankton provided by the winter tidal current (Brunetti, 1974).

4 Interestingly, populations from the Mediterranean Israeli coast reared in the lab under different temperatures, show  
5 a lag phase of colonial growth after the 1<sup>st</sup>-3<sup>rd</sup> blastogenetic generation, followed by an exponential growth phase (5<sup>th</sup>-  
6 8<sup>th</sup>- blastogenetic generations), a plateau phase (around the 10<sup>th</sup> blastogenetic generation), with approximately 1 bud per  
7 zooid, and a final degeneration phase, with less than 1 bud per zooid, which, frequently, leads to colony death  
8 (Rinkevich et al., 1998b).

9 In the wild, the colony lifespan is also influenced by sexual reproduction. Blastogenesis slows during the season of  
10 sexual reproduction, which continues as the temperature remains high. Accordingly, zooids at the periphery of colonies  
11 from the Mediterranean coast of Israel form more buds and less eggs than zooids in the centre (Rinkevich et al., 1998b).  
12 A reduction in budding effort has been observed in the iteroparous colonies (see below) of Woods Hole (Grosberg,  
13 1988). The onset of sexual reproduction also marks the onset of a senescence phenomenon that rapidly leads to colony  
14 death without initial effects on the growth rate, as it is related to a decrease in the renewal capacity of the tunic. Initially,  
15 large and opaque necrotic areas covered with bacteria appear in the colonial matrix, which subsequently gradually  
16 extend throughout the entire colony (Brunetti, 1974; Brunetti and Copello, 1978; Grosberg, 1988).

### 24 3.2 Sexual reproduction

25 Mature egg production begins when the average seawater temperature reaches 10-11°C (Sabbadin, 1955b);  
26 therefore, the period of sexual reproduction varies with latitude. In Scotland, it is mainly concentrated in June-August  
27 (Millar, 1952); in the Lagoon of Venice, it begins at the end of March and peaks between April and late October  
28 (Brunetti, 1974); in Woods Hole (Grave, 1933; Grosberg, 1988) and Monterey Bay (Chadwick-Furman and Weissman,  
29 1995b), it spans from late spring to early autumn. In the Gulf of Naples (Lo Bianco, 1909) and the Gulf of Tunis (Pérès,  
30 1954), it occurs throughout the year and similar behaviour was observed for the Israeli Mediterranean coast, with a peak  
31 of reproduction in the spring (Rinkevich et al., 1998a).

32 In Woods Hole, Grosberg (1988) reported the presence of two types of colonies: a semelparous type, frequent in  
33 spring and early summer and producing only one clutch of eggs before dying, and an iteroparous type appearing in mid  
34 and late summer, producing at least 4 clutches (one clutch per blastogenetic cycle). The number of eggs per zooid  
35 ranged from 8, in semelparous colonies of early and mid-summer, to 4 or fewer in iteroparous colonies of late summer.  
36 A maximum of five eggs per zooid has been reported for the Israeli population (Rinkevich et al., 1998b), whereas two-  
37 ten eggs per zooid were observed in British waters (Hiscock, 2005).

38 In Tomales Bay and Monterey Bay, colonies undergo intense reproductive activity from late spring to early autumn  
39 (Worcester, 1994; Chadwick-Furman and Weissman, 1995b). In Monterey, they produce up to 10 clutches of eggs, with  
40 each zooid containing up to 5 eggs, with a maximum fecundity of 8,000 eggs per colony. Colonies born in late autumn  
41 overwinter as small juveniles and postponed reproduction until the following spring (Chadwick-Furman and Weissman,  
42 1995b).

43 In the Lagoon of Venice, four sexual generations are usually produced during the year (Brunetti, 1974): one in the  
44 spring, two in the summer and one in the autumn. A five-year study on the reproductive cycle and distribution of  
45 several ascidian species at 25 stations (artificial panels on canal boundary piles) in the southern basin of the Lagoon of  
46 Venice (Fig. 2A) demonstrated that, based on both frequency and distribution, *B. schlosseri* was one of the best adapted  
47 species and exhibited one of the longest reproductive period (Fig. 2C); this species also exhibited the highest mortality

1 during the first part of the period of sexual reproduction in wintering populations (Brunetti and Menin, 1977). In the  
2 same environment, the formation of mature gonads is typical of the largest colonies. In young colonies, gonad  
3 primordia appear in the buds only upon reaching a certain size (4<sup>th</sup>-5<sup>th</sup> blastogenetic generation), independent of  
4 temperature, but maturation does not occur below 10°C. Similar observations were reported by Grosberg (1988) for the  
5 Woods Hole semelparous colonies.  
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7 In the lagoon of Venice, colonies of both the spring and summer generations reproduce at 50-60 days of age (at 18-  
8 20°C) and consist of more than ten zooid systems (Brunetti, 1974), although the lower limit is represented by colonies  
9 with three zooid systems (Sabbadin, 1955b). Chadwick-Furman and Weissman (1995a) observed that, in Monterey Bay,  
10 giant chimeric colonies resulting from the fusion of genetically compatible colonies reached sexual maturity more  
11 rapidly than smaller, unfused colonies.  
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13 In the Lagoon of Venice, as well as in Woods Hole (Grosberg, 1988), the autumn generation is the wintering one,  
14 with zooids showing developing testes and immature ovaries. Gonads are present even in the coldest months of the  
15 winter, with large colonies producing eggs that do not complete their development. As a consequence, due to the  
16 thermal regime, part of the new generation cannot reproduce sexually within the year of its formation and therefore has  
17 a breeding season during the following year (Brunetti, 1974). Consistent with the observations in the Lagoon of Venice,  
18 overwintering and wintertime reproductive sterility was also documented in Woods Hole and Cape Cod populations by  
19 Grosberg (1988), who suggested that egg production and fecundity rates might be affected by food availability, and in  
20 Monterey populations by Chadwick-Furman and Weissman (1995b). More recently, Newlon and collaborators (2003)  
21 observed that changes in environmental conditions alter the reproductive strategy, such that more productive and  
22 warmer environments tend to favour egg production and colder, food-limited sites favour testis maturation.  
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### 31 **3.3 Life quality: influence of salinity and temperature on survival, growth and reproduction**

32 Studies of colony growth using both qualitative and quantitative approaches were conducted on colonies reared in  
33 the laboratory and wild colonies from the Lagoon of Venice (Beghi and Brunetti, 1978; Brunetti and Copello, 1978). A  
34 "quality index" was established based on nine characters (developmental stages of the blastogenetic generations in a  
35 colony, feeding, circulatory system, tunic, morphology of ampullae, pigmentation, arrangement of zooids in the  
36 systems, cardiac contractions, general aspect of the colony), along with an associated conventional score of life quality.  
37 To quantitatively assess colony growth, a mathematical approach was developed using i) a linear regression of the  
38 logarithm of zooid number at generation  $n$  (exponential growth hypothesis) and ii) a linear regression of the growth rate  
39 (divergence from exponential growth) calculated for 4-5 subsequent generations under various temperature-salinity  
40 combinations (values ranging from 3 to 28°C and from 16 to 44‰ for temperature and salinity, respectively). The mean  
41 values of the trend of the two regression lines were used as indices of growth. These qualitative and quantitative indices,  
42 developed from observations of colonies reared in the laboratory, were used to investigate the combined effects of  
43 temperature and salinity on survival, growth and reproduction (Brunetti et al., 1980). *B. schlosseri* appears to be  
44 sufficiently eurythermal and euryhaline to thrive in coastal, but not estuarine, environments. Unlike *B. leachii*, adult  
45 colonies of *B. schlosseri* (i.e., beyond the 5<sup>th</sup> blastogenetic generation) exhibit a greater tolerance range to experimental  
46 conditions than do young ones (i.e., from the oozoid stage to the 5<sup>th</sup> blastogenetic generation). Both survival and  
47 colonial growth appear to be primarily influenced by temperature, rather than salinity. The optimum temperature range  
48 ranges from 11 to 26°C. These values correspond to the lowest and highest thermal levels for sexual reproduction, the  
49 latter value occurring in the surface waters of the Lagoon of Venice in July and August. The optimum salinity range  
50 ranges from 25 to 40‰, with a mean value of 33‰ that corresponds to the annual mean salinity in the Lagoon. At 16‰,  
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1 a structural simplification of zooids occurs before colonial death, with reductions in the number of oral tentacles and  
2 rows of stigmata and an irreversible alteration change in bud growth.

3 In other Mediterranean areas, such as the Israeli coast, Rinkevich and collaborators (1998) also reported that  
4 temperatures significantly influence colony density. The greatest colony **abundance** occurs during the spring (April,  
5 temperature 18.8°C) and the lowest occurs in the winter (December-February, temperature <17°C). **In agreement with  
6 these observations, in Monterey Bay, colonies grow better at 20°C rather than 24°C (Boyd et al., 1986).**

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9 Recently, a study on the effects of temperature and salinity on the survival and growth of colonies collected from  
10 the coasts of British Columbia confirmed the broad tolerance of this species. The colonies survived environmental  
11 conditions of 10-25°C and 14-38‰, exhibited positive growth at 10-25°C and 20-38‰, and **reached** the largest sizes at  
12 15-20°C and 20-38‰. At 9°C and 17‰, the colony size remained constant, as only one bud per zooid was produced  
13 during each blastogenetic cycle. Between 0°C and 5°C or at 5‰, all colonies underwent an irreversible degeneration:  
14 the blood vessels narrowed, blood flow slowed, and the zooids decreased in size, becoming densely pigmented and  
15 disorganised (Epelbaum et al., 2009).  
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19 A shift in the seasonal abundance of *B. schlosseri* from late autumn and winter to summer and early autumn,  
20 related to changes (decreases) in seasonal seawater temperatures, has been reported for the Gulf of Maine: similar shifts  
21 may have significant impacts on benthic communities allowing the spreading of colonial ascidians in non-indigenous  
22 environments (Dijkstra et al., 2007).  
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25 In the Lagoon of Venice, a subsequent investigation was conducted to evaluate the interactive effects of  
26 temperature and salinity on sexual and asexual reproduction (Brunetti et al., 1984). These parameters do not influence  
27 the formation of the gonad primordia in the buds up to the 5<sup>th</sup> blastogenetic generation, supporting the hypothesis that  
28 their presence is solely dependent on the age of the colony. In contrast, beyond the 5<sup>th</sup> blastogenetic generation, both  
29 colonial growth and gonad maturation are strongly influenced (ovaries more than testes) by environmental parameters  
30 and show **opposite thermo-salinity** optima. Temperature is the major factor influencing asexual reproduction; but at any  
31 given salinity, increasing temperature has a negative influence on growth and a positive influence on sexual maturity.  
32 Brunetti et al. (1984) **provided** support for the hypothesis that different ecological requirements for sexual and asexual  
33 reproduction are advantageous for the species: in the spring, high temperatures assure zooid survival in young colonies,  
34 whereas in sexually mature colonies of the late spring to autumn, the temperature-dependent slowing of growth is not  
35 detrimental because the colonies are at the end of their life cycle; in winter, low temperatures warrant the survival and  
36 growth of sexually immature colonies, favouring them in spatial competition.  
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39 A seasonal pattern in sexual reproduction was reported in Mediterranean areas, where reproductive activity is  
40 recorded **all the** year-round. The peak of reproduction occurs in the spring (April) and is correlated with colony size but  
41 not seawater temperature. (Rinkevich et al., 1998).  
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44 To complement this research on sexual reproduction, a preliminary investigation of larval metamorphosis was  
45 conducted (Brunetti and Beghi, 1982) **that** demonstrated a direct influence of environmental salinity values beyond the  
46 salinity range tolerated by the species on the metamorphosis rate.  
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### 49 **3.4 Life quality: influence of anthropogenic activities**

50 The ability to adjust growth and reproductive strategy in response to environmental conditions allows *B. schlosseri*  
51 to be highly resilient to environmental change (Carver et al., 2006). Anthropogenic impacts on *B. schlosseri* populations  
52 affect their establishment worldwide and alter the original structure of the benthic community. In the Lagoon of Venice,  
53 where human activity has persisted for centuries, the effects of pollution (from industrial, urban, agricultural, and port  
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activities), variation in lagoon depth, changes of substrata, and maritime traffic can be monitored. In this complex ecosystem, chemical contamination is a primary influence of its ecological status (e.g., macrobenthic biodiversity). A recent screening for the ecological risk assessment of different classes of pollutants for the benthic community of the Lagoon of Venice revealed that the greatest risks in the areas nearest the industrial district of Porto Marghera were related to the presence of mercury, arsenic and nickel, which exceeded the Threshold Effect Level in the upper 15-cm layer of sediment (Critto et al., 2005). In this environment, *B. schlosseri* transitioned from a pollution-sensitive phenotype to one **tolerating** long-term heavy-metal contamination. Other studies confirmed that it is one of few species tolerant to extremely polluted conditions (Naranjo et al., 1996; Lambert and Lambert, 2003). In contrast, *B. schlosseri* appears to be very sensitive to biocides that were recently introduced into the formulations of antifouling paints. These paints are widely used on boats and submerged artificial structures and contain organotin compounds and their alternatives. The use of these latter compounds increased after the definitive ban of tin-based paints by the International Maritime Organisation in 2003. Therefore, as a filter-feeding benthic invertebrate, cosmopolitan along the coastal environment of temperate latitudes, *B. schlosseri* can serve as an effective bioindicator for ecotoxicological studies of antifouling biocides in both the wild (Cima and Ballarin, 2008) and the laboratory (Cima and Ballarin, 2004; Cima et al., 2008; Menin et al., 2008; Cima and Ballarin, 2012). It can also be used to investigate the mechanism of action of xenobiotics at the cellular level. In particular, it can be used to study the toxic effects of xenobiotics on the immune system, on which organism survival and, consequently, species fitness at the biocoenosis level depend.

*B. schlosseri* is an important component of the hard-substratum biocoenoses in the Lagoon of Venice. Recently, sixty years after Sabbadin's first study (1955b) of population distribution and thirty years after Brunetti's first ecological analysis (1974) of the hard-substratum biocoenoses on artificial panels, a two-year progression of the macrofouling community was analysed at stations in the southern basin of the Lagoon of Venice. This included the development of an integrated bioindex for evaluating ecological status according to the Water Framework 2000/60/EC Directive (Cima and Ballarin, 2013). The researchers found that, as in previous studies, the dominant taxa during the summer (June-August) are represented by serpulids, bryozoans, tunicates and, to a lesser extent, cirripeds. Conversely, during the autumn (September-December), a relative climax is reached which remains stable until December: more than 90% of the community comprises solitary and colonial tunicates, predominantly botryllids (Cima and Ballarin, 2013). This finding strongly contrasts the results of previous studies, in which *Mytilus galloprovincialis* was the dominant species during the final stage of the ecological succession in the autumn. Major changes in the ecological succession of the lagoon have occurred during the past thirty years in association with the progressive abandonment of mussel farming. This trend has favoured the expansion of ascidians over bivalves as the dominant filter-feeding organisms. Thus, the climax community of hard substrata in the Lagoon of Venice can presently be described as a "*Botryllus* community", consisting primarily of colonial **botryllid** ascidians, such as *B. schlosseri* and *B. leachii* and to a lesser extent, solitary ascidians, bryozoans, barnacles, serpulids, chlorophytes and rhodophytes. This climax community also confirms the successful invasion of *B. schlosseri* inside its source area (Cima and Ballarin, 2013).

#### 4. Spatial-temporal dynamics of populations in the Lagoon of Venice: a case study

The first ecological observations of the ascidian populations of the Lagoon of Venice, conducted by Salfi (1946), revealed that *B. schlosseri* and the solitary ascidian *Ciona intestinalis* represented the dominant species of ascidian fauna in two biotopes: wooden piles bordering the navigable canals and the eelgrass (*Zostera*) beds lining the bottom of the lagoon to the edges of the canals (Fig. 2A,D-E). Together with sponges and polychaetes, colonies of *B. schlosseri* settle on piles and grow over other fouling organisms such as hydroids, bryozoans, mussels, barnacles and solitary

ascidians. In the eelgrass beds, the colonies are protected by the vegetation and have fewer competitors, settling on *Zostera* leaves or even bryozoans, algae and solitary ascidians. In 1965, Sabbadin and Graziani observed that the species was very tolerant to strong environmental changes, but that large numerical fluctuations occurred depending on substratum availability. Rapid expansions in coverage occurred during the season of sexual reproduction, when the areas of fouling substratum increased; coverage decreased in winter, when these areas were reduced. In contrast, along the Israeli Mediterranean coast, no correlation between substratum size and colony number was found, as the only substratum was the undersurface area of stones (Rinkevich et al., 1998).

In the Lagoon of Venice, populations fluctuate both seasonally and among different years, and the genetic structure is affected by the following factors (Sabbadin 1978):

- 1) a discontinuity in the distribution of colonies between the two biotopes (piles and eelgrass leaves);
- 2) individual dispersal is limited by the short (hours) duration of the free-swimming larval stage, shaped by the tidal flow entering the harbour-canals, and concentrated along the internal canals, with some minor overflow into the adjacent shallower waters;
- 3) larvae preferentially settle near the parental colonies, and larval crowding produces competition for space, which is greatly enhanced by colonial growth.

Following Giard (1872), who noticed that along the Breton coasts, the pigmentation of *B. schlosseri* colonies varied among areas of the littoral zone, Sabbadin (1969) used colour morphs to characterise the populations living in the two different biotopes of the Lagoon of Venice and to evaluate the structure and dynamics of the populations. Moreover, due to challenges in the direct genotyping of heterozygous genotypes of colour morphs, a pioneering study on enzyme polymorphism was developed (Sabbadin 1978).

#### 4.1 Polychromatism

The polychromatism of *B. schlosseri* has attracted the attention of researchers since the end of the nineteenth century. Colonies can exhibit a variety of attractive colours, including blue, silver, orange and reddish (Fig. 3A-F). The pigments can be uniformly distributed or form bands on the dorsal side of the zooids. The variability of colour morphs is so great that Giard (1872) considered them as different species. However, after Bancroft (1903) evidenced that the descendants of a single colony can vary in pigmentation, colour was considered a highly polymorphic character, and colonial ascidian pigmentation is considered an inadequate character for ascidian systematics (Sabbadin and Graziani, 1967a).

Polychromatism is a function of the distribution and different proportions of pigment cells, nephrocytes and orange cells (Fig. 3G-M). These cells are large haemocytes (30-40  $\mu\text{m}$  in diameter) with their cytoplasm occupied entirely by a few large vacuoles containing pigment granules. Pigment cells contain blue and/or reddish pigment, whereas nephrocytes, also called purinic cells, contain whitish, refracting pigment. In both cell types, pigment granules are in Brownian motion inside the cell vacuoles.

Pigment cells can be distinguished based on the ultrastructure of their granules (Burighel et al., 1983). The blue pigment is in the form of bluish granules, 150 nm in size, of prismatic morphology with a roundish, square or rectangular profile, with strongly electron-dense concentric layers and a slightly electron-dense central area (Fig. 3K). It confers colours ranging from blue to violet and emerald to blastozooids. The reddish pigment appears later than the blue one during colonial development and involves most of the blue cells, whose pigment gradually turns reddish-brown. Reddish granules are 300-500 nm in size, roundish or rectangular in shape, with an optically empty central area flanked by few parallel, electron-dense layers (Fig. 3L). The number of pigment cells increases over the colony lifespan: they

1 accumulate in sites where blood circulation is slow, such as in the blind, sausage-like termini (ampullae) that line the  
2 periphery of the colonial vasculature. The whitish pigment is located in the nephrocytes. It is stocked in large vacuoles  
3 in the form of granules of geometric form, primarily cylindrical or parallelepipedal (Fig. 3M). Nephrocytes contain a  
4 small amount of cytoplasm with few organelles, with a peripheral nucleus; like pigment cells, vacuoles occupy most of  
5 the cell volume (Milanesi and Burighel, 1978). Sabbadin and Tontodonati (1967) demonstrated that the refracting  
6 pigment consists of uric acid concretions, consistent with the role of nephrocytes in accumulating metabolic nitrogenous  
7 wastes. Nephrocyte crowding confers a whitish colour to the dorsal side of the zooids in the form of either two narrow,  
8 parallel stripes running from one siphon to the other, or as two wide stripes subdivided in smaller areas, with antero-  
9 posterior variable extensions. In addition, they can form peristomatic rings around the oral siphon.

10 The orange pigment is stored within the orange cells. Orange cells are 10-15  $\mu\text{m}$  in size and contain vacuoles 2  $\mu\text{m}$   
11 in diameter; they are morphologically similar to cytotoxic morula cells (Fig. 3J; Ballarin and Cima, 2005). Orange cell  
12 crowding produces an orange colour of varying intensity, particularly around the oral siphon. Orange pigment cells are  
13 clearly recognisable in oozoids, whereas the blue and whitish pigment cells make their appearance during larval  
14 metamorphosis but require several blastogenetic generations before their full expression.

15 Pigmentation inheritance was used to study the efficiency of cross and self-fertilisation in *B. schlosseri* (Sabbadin,  
16 1971) and germ cell recycling in the colony (Sabbadin and Zaniolo, 1979). Recently, pigment cells were analysed in the  
17 colonial ascidian *Ecteinascidia turbinata*, and a migratory cell population resembling neural crest cells was described  
18 (Jeffery et al., 2004). These cells emerge from the neural tube, migrate into the body wall and siphon primordia, and  
19 then differentiate as pigment cells. These are distinguished by their orange colour and star-shaped morphology. Similar  
20 migratory cells were also found in *B. schlosseri* in a study using natural pigmentation morphs to investigate  
21 relationships between tyrosinase expression and body pigmentation (Jeffery, 2006). These results prompted the  
22 hypothesis that migratory cells with some of the features of vertebrate neural crest cells are present in ascidians and are  
23 responsible for body pigmentation in the common ancestor of the tunicates and vertebrates.

#### 24 4.1.1 Genetics of pigmentation in *B. schlosseri*

25 Experiments involving fusion and separation of colonies of different colour morphs showed that, following the  
26 fusion of an orange colony with a colony lacking orange pigment (thus creating a new parabiotic colony), the part of  
27 the chimeric colony that derived from the latter developed, within a few days, rings of orange pigment cells around the  
28 siphons after (Sabbadin, 1959, 1962). The orange pigment was conserved in successive generations, even if the two  
29 colonies were separated anew (Fig. 4A). This phenomenon occurred even if the two original colonies were separated  
30 after only a brief period of fusion, before the formation of the orange ring. These experiments suggest a transfer of  
31 orange pigment cell precursors via blood, as mature pigment cells share many features of terminally differentiated cells  
32 and seem unable to multiply (Burighel et al., 1983). In contrast, neither the characters “intersiphonal band of purine  
33 pigment” nor “peristomatic rings of purine pigment” were transmitted during the parabiosis experiments (Sabbadin,  
34 1959, 1962) (Fig. 4 B,C). This is probably due to the fact that both the intersiphonal bands and the peristomatic rings  
35 are formed by fixed nephrocytes adhering to the basal lamina of sinus epithelia of particular body regions representing  
36 homing sites for these cells.

37 To avoid using chimeric colonies, which are abundant in the wild, the inheritance of pigmentation was tested using  
38 colonies obtained from larvae reared in laboratory (Sabbadin, 1959a,b, 1962, 1964; Sabbadin and Graziani, 1967a,b).  
39 Several cross- and self-fertilisation experiments were performed, and the resulting colony phenotypes were assessed  
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based on five characters: orange pigment, peristomatic rings, intersiphonal band, blue pigment, and reddish pigment.

The main results were as follows:

1) *Character “orange pigment”*

- i) Colonies lacking orange pigment, either crossed or self-fertilised, always produced colonies without orange pigment (Fig. 4D);
- ii) A colony with orange pigment crossed with a colony lacking orange pigment produced descendants with orange pigment (Fig. 4E);
- iii) These descendants (F1), crossed or self-fertilised, produced descendants (F2) with and without orange pigment in a 3:1 ratio (Fig. 4F);
- iv) F1 descendants with orange pigment crossed with a colony without pigment (as a counter check) produced descendants (F2) with and without orange pigment in a 1:1 ratio (Fig. 4G).

These experiments demonstrate that the character “orange pigment” is a simple Mendelian character with an allele “presence of pigment” (A) dominant over the allele “absence of pigment” (a).

2) *Character “peristomatic rings”*. Similar cross- and self-fertilisation experiments performed with colonies with and without peristomatic rings indicated that this character is a simple Mendelian character, with the allele “presence of peristomatic rings” (F) dominant over the allele “absence of peristomatic rings” (f).

3) *Character “intersiphonal band”*. This character was analysed using crosses between colonies lacking bands (indicated with b), colonies with bands formed by two parallel stripes (labelled as B<sub>2</sub>) and colonies with dotted bands (labelled as B<sub>1</sub>) (Fig. 5). Experiments showed that:

- i) crosses between colonies without bands (b) always produced colonies without bands (Fig. 5A);
- ii) a colony with two parallel strips (B<sub>2</sub>) crossed with a colony without bands (b) produced descendants (F1) with phenotypes B<sub>2</sub> and b in a 1:1 ratio (suggesting that the parental (P) colony B<sub>2</sub> was heterozygous, with B<sub>2</sub> dominant over b) (Fig. 5B);
- iii) the F1 colony B<sub>2</sub>, putatively heterozygous, crossed with a B<sub>1</sub> colony produced colonies B<sub>2</sub> and B<sub>1</sub> in a 1:1 ratio; no b colonies were produced (Fig. 5C). These results suggest that the colony B<sub>1</sub> was homozygous B<sub>1</sub>B<sub>1</sub>. Therefore, the descendants (F2) with phenotype B<sub>1</sub> were B<sub>1</sub>b, and descendants with phenotype B<sub>2</sub> were B<sub>2</sub>B<sub>1</sub>, with B<sub>2</sub> dominant over B<sub>1</sub>;
- iv) as a countercheck, B<sub>1</sub> descendants (F2) crossed with a colony without bands (bb) produced new descendants (F3) B<sub>1</sub> and b in a 1:1 ratio (Fig. 5D), and B<sub>2</sub> descendants (F2) crossed with a colony without bands (bb) produced new descendants (F3) B<sub>2</sub> and B<sub>1</sub> in a 1:1 ratio (without b) (Fig. 5E).

These experiments indicated that the character “intersiphonal band” is controlled by three alleles at the same locus: B<sub>2</sub>, B<sub>1</sub>, b. B<sub>2</sub> (parallel strips) and B<sub>1</sub> (subdivided bands) are dominant over b (absence of band). Moreover, B<sub>2</sub> is dominant over B<sub>1</sub> (Fig. 5F).

4) *Character “blue pigment”*. The inheritance of this character involves a single pair of alleles: a dominant allele for the absence of the pigment (Bl) and a recessive one for pigment presence (bl) (Fig. 6). This conclusion derives from the following findings:

- i) blue colonies crossed with other blue colonies always produced blue colonies (Fig. 6A);
- ii) a colony without blue pigment crossed with a blue colony yielded only colonies (F1) without blue pigment (Fig. 6B);
- iii) these descendants (F1) back-crossed to blue colonies produced descendants (F2) with and without blue pigments in a 1:1 ratio (Fig. 6C);

1 iv) crossing unpigmented F2 colonies produced offspring (F3) with unpigmented and blue colonies in a 3:1 ratio  
2 (Fig. 6D).

3 5) Character “reddish pigment”. The inheritance of this character involves a pair of alleles: one for the presence  
4 of reddish pigment (*R*) and one for the absence (*r*), with the former dominant over the latter (Fig. 7). Sabbadin’s  
5 experiments revealed that:

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7 i) crossing colonies lacking reddish pigment (*rr*) only produced colonies lacking reddish pigment (Fig. 7A);  
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9 ii) reddish colonies (*RR*) crossed with colonies without pigment (*rr*) produced reddish descendants (F1, *Rr*) (Fig.  
10 7B);  
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12 iii) crossing these descendants (F1, *Rr*) yielded the two phenotypes (F2) of reddish colonies and colonies lacking  
13 reddish pigment in a 3:1 ratio, respectively (Fig. 7C).  
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15 These experiments produced 48 different phenotypes (including some never observed in the Lagoon of Venice)  
16 (Sabbadin and Graziani, 1967a,b) (Fig. 8). Phenotypes derived from the segregation of four pairs of alleles (*A/a*, *F/f*,  
17 *Bl/bl*, *R/r*) at four different loci and of three alleles (*B<sub>2</sub>/B<sub>1</sub>/b*) at a fifth locus.  
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19 Lastly, to study the relationships between loci controlling pigmentation, the allele pairs *A/a*, *B<sub>2</sub>/b*, *Bl/bl*, and *R/r*  
20 were examined according to these combinations of loci pairs: *A-B*, *A-Bl*, *A-R*, *B-Bl*, and *Bl-R* (Sabbadin and Graziani,  
21 1967b; Sabbadin, 1977). These alleles are responsible for the presence/absence of orange pigments, intersiphonal bands,  
22 and blue and reddish pigments. The analysis of many controlled crosses evidenced that locus *A* is independent of the  
23 loci *B*, *Bl* and *R*; *Bl* is independent of *B* and *R*; and *A* and *B* are independent. In conclusion, at least three of the 16 pairs  
24 of chromosomes in the diploid complement of *B. schlosseri* (Colombera, 1969) carry loci for pigmentation.  
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#### 30 4.1.2 Colour morphs in the wild

31 Populations from different Mediterranean areas show significant differences in the frequency of pigmented  
32 phenotypes. The most important conclusion resulting from statistical analyses of data from colonies collected from the  
33 two biotopes described above (wooden piles and eelgrass) in the Lagoon of Venice was that the population is  
34 subdivided into microgeographical subpopulations that differ significantly in the phenotypic frequencies of the  
35 presence/absence of the orange pigment and the double intersiphonal band, which involve two independent loci  
36 (Sabbadin, 1969; Sabbadin and Graziani, 1965, 1967b). This is probably related to the diversity and the ecological  
37 isolation of the biotopes which prevent larval movement from the eelgrass to the piles bordering the canals (Sabbadin,  
38 1972; 1973).  
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44 In the seaward portion of the Lagoon of Venice, highly significant differences in phenotype frequencies were  
45 observed among different habitats, even within the same area, suggesting a different adaptive value of the morphotypes  
46 for different biotopes. Frequencies were homogeneous within the same biotopes even in very remote areas, indicating  
47 high levels of gene flow. In contrast, observations in the inland areas, such as the landward portion of the Lagoon,  
48 where colonies live in very shallow waters that are intersected by canals with a polarised tidal current, revealed that  
49 populations were fragmented into highly differentiated subpopulations even within the same biotope, likely due to the  
50 inability of larvae to traverse the canals, limiting gene flow (Sabbadin, 1972; 1973).  
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54 In 1972, Sabbadin found significant differences in the frequency of one of the phenotypes among populations from  
55 three regions: the Lagoon of Venice, the Tyrrhenian coast near Civitavecchia and Golfo Aranci in Sardinia. Within the  
56 same population, no significant fluctuations in phenotypic frequencies were observed among samples of the same year  
57 at the same station; however, changes in frequency over time could potentially occur due to high mortality caused by  
58 cold winter, especially at the highest latitude.  
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1 Similar observations on restricted gene flow resulting from limited larval dispersal were made by Grosberg (1987)  
2 in North America, and by Rinkevich and collaborators (1998) along the Israeli Mediterranean coast. The latter authors  
3 confirmed Sabbadin's observations that populations of this species are divided into local subpopulations exhibiting  
4 microgeographic differences in life-history patterns and morphological characteristics over only hundreds of metres.  
5 Israeli populations are characterised by a low variety of colour morphs. Three main morphs were recognised in natural  
6 Israeli populations: 1) the brown morph (including brownish, blackish and whitish colours), which was dominant at all  
7 localities (>80% of the colonies); 2) the intersiphonal double-band morph and 3) the orange morph, which was either  
8 rare or absent at all localities.  
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#### 11 **4.2 Enzyme polymorphism**

12 The spatial-temporal population dynamics of *B. schlosseri* in the Lagoon of Venice were studied using native  
13 electrophoresis of colony homogenates on polyacrylamide gels and the specific reactions of four enzymes: non-specific  
14 esterase, malate dehydrogenase (MDH), superoxide dismutase (SOD), and phosphoglucose isomerase (PGI) (Sabbadin  
15 and Tontodonati 1976). Electropherograms of single colonies showed different bands with enzyme activity, including a  
16 zone of lower electrophoretic mobility and high individual variability. Five phenotypes were recognised on the basis of  
17 differences in the four slowest bands of the non-specific esterase: in the order of increasing mobility, they were 1) all  
18 present, 2) all present except for the first, 3) all present except the first two, 4) the first three absent, and 5) all absent.  
19 Genetic control by four co-dominant alleles at one locus was suggested for both MDH and SOD. Their loci (*mdh-1* and  
20 *sod-1*) are independent and present with one band in the homozygous condition and three bands, including a hybrid one,  
21 in the heterozygous condition. Ten phenotypes were revealed for both MDH and SOD (Sabbadin 1978), and more than  
22 ten were revealed for PGI (Sabbadin 1982). At both the *mdh-1* and *sod-1* loci, the frequency of extreme alleles was very  
23 low (< 1%), which explains the rarity of some phenotypes in the wild. This hypothesis was tested and confirmed by  
24 crosses (Table 1), which also produced genotypes that have never been found in the wild due to their low frequency  
25 (Sabbadin, 1978).  
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27 Population fragmentation into subpopulations in the Lagoon of Venice was confirmed by analysis of enzyme  
28 polymorphisms in colonies collected at different times of year and at different stations (Table 2). According to Berrill  
29 (1950), who argued that the larvae of *B. schlosseri* are adapted for site selection and settlement rather than dispersal,  
30 microgeographical fragmentation was the result of a very limited dispersal range, upon which gene flow among  
31 populations and subpopulations depends. Although high genetic similarity arising from a high degree of inbreeding  
32 within subpopulations was expected (due to ovoviviparity and larval settlement near the parental colony, with strong  
33 competition for space), the subpopulations show significantly different allelic frequencies. This result reflects the nature  
34 of the substratum and the distance and exposure to tidal currents that influence larval dispersal (Sabbadin and  
35 Tontodonati, 1976; Sabbadin, 1978). Recently, Hiscock (2005) observed that although most larvae remain within a few  
36 meters of the parental colony and settle in less than one day, the dispersal potential depends on the local hydrodynamic  
37 conditions; it is also dependent on the recruitment of other invertebrates (Sams and Keough, 2013). Studies of the  
38 genetic control of polychromatism through an extensive series of crosses led to the discovery of the five loci reported  
39 above, two of which are closely associated with each other; along with discovery of the linkages between the SOD and  
40 PGI loci and between the MDH locus and the orange pigment locus (Table 1; Sabbadin, 1977, 1978, 1982).  
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#### 59 **5. Conclusions**

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1 *B. schlosseri* is an invasive species able to overpopulate its native zones, colonise non-native natural areas and  
2 spread causing changes in the structure and composition of benthic communities (Colautti and MacIsaac, 2004).  
3 Anthropogenic activities, by disturbing the environments, enhance the dissemination of exogenous species and offer  
4 many sources of transport opportunity contributing to their diffusion (Tyrrell and Byers, 2007). *B. schlosseri* reproduces  
5 rapidly and larvae can settle on a variety of solid natural and artificial surfaces including ship hulls, docks, piers,  
6 cultured shellfish, gravel, rocks, metals, tires, plastic, styrofoam, rope, fibreglass, wood, causing damages to the  
7 environment and human economy. **In addition, adult rafting can recruit colonies to new environments and should be**  
8 **considered in studies of population genetics and structure of biocoenosis (Worcester, 1994).** Where dominant, it may  
9 out-compete other organisms for food and space, altering natural community dynamics and threaten aquaculture, fishing  
10 and other coastal and offshore activities. On the other hand, the presence of non-indigenous and pollution-tolerant  
11 ascidian species can be useful to evaluate water quality and its effect on biodiversity and in the development of benthic  
12 communities in polluted marine environments (Carman et al., 2004). Therefore, the elucidation of the factors which  
13 shape species distribution, in particular genetic subdivision of the species and the evolution of differential invasion  
14 abilities, is an important goal in population genetics. As regards *Botryllus*, many authors have emphasised the need for a  
15 taxonomic re-evaluation using molecular data because an accurate identification is fundamental for understanding the  
16 genetic variance, the extent of tolerance and the evolution of both the species and its invasiveness capability (Bock et  
17 al., 2012; Griggio et al, 2014). Geographical and biological barriers to gene flow in this species remain largely  
18 unexplored, and few data are available on the differentiation of genetic groups as a function of their geographical  
19 distribution and invasion success. Knowledge of the genetic relationships among subpopulations/**colour morphs** and  
20 their adaptation to pollution is a challenge for future research on this species and may lead to a better comprehension of  
21 its evolutionary success and invasive potential.  
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### 32 **Acknowledgements**

33 This paper is dedicated to Prof. A. Sabbadin, who introduced us to the study of *Botryllus*. From 1952 to retirement  
34 in 1991 and beyond (he was active in the laboratory until 2005, at the age of 85), he set up the culture conditions in the  
35 lab, studied the colonial life cycle with respect to zooid development, refined the descriptions of the stages of zooid  
36 development, investigated the relationships between zooid and bud development, pioneered the study of allorecognition  
37 and the underlying genetics, investigated the genetics of chromatic polymorphism, and conducted pioneering  
38 experiments on bud transplantation, bud ablation and germ cell exchange between fusing colonies. In his last years of  
39 research activity, he focussed on immunobiology and the role of haemocytes in the recognition of non-self.  
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## Figure legends

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3 **Figure 1.** **A.** Worldwide distribution and species richness of *B. schlosseri*. The assumed native areale of the species is  
4 indicated by the dotted ellipse. Numbers refer to the date of first reports in various coastal localities (modified  
5 from Computer Generated Native Distribution Map, IPCC A2 scenario, for *B. schlosseri*. www.aquamaps.org,  
6 version of Aug. 2013); arrows suggest the presumed dispersal pathways as deduced by data available in the  
7 literature. **B-C.** Single-system colonies of *B. schlosseri*. **A**, ventral view of a colony at mid-cycle; **B**, dorsal view  
8 of a colony at take-over. **a**: ampullae; **b**: bud; **bb**: branchial basket; **bl**: budlet; **e**: embryo; **en**: endostyle; **in**:  
9 intestine; **mv**: marginal vessel; **rv**: radial vessel; **st**: stomach; **t**: tunic. Scale bar: 0.5 mm.

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15 **Figure 2.** **A.** Map of the southern basin of the Lagoon of Venice showing localities from which samples of *B. schlosseri*  
16 were collected by Sabbadin beginning in 1955 (1-3: biotope of piles marking the canals; 4: biotope of eelgrass  
17 beds). Orange circles and yellow squares indicate settlement during spring and summer, respectively, observed by  
18 Brunetti and Menin (1977) on artificial panels along canals of varying hydrodynamic characteristics. **B.** Trends in  
19 the frequency of colony numbers per size class; data were collected monthly in the Lagoon of Venice during 1954.  
20 Black squares: large colonies; empty squares: small colonies (modified from Sabbadin, 1955b). **C.** Settlement  
21 periods of various ascidian species of the Lagoon of Venice (upper bars): dark green bars show period of  
22 maximum settlement. Sexual reproduction periods (lower, blue bars): ranges of seawater temperatures at which  
23 sexual reproduction occurs are indicated on the right (modified from Brunetti and Menin, 1977). **D-E.** Colonies of  
24 *B. schlosseri* in natural biotopes of the Lagoon of Venice, growing as pendants on piles marking canals (**D**) and as  
25 small epiphyte chains on *Zostera* leaves (**E**).

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33 **Figure 3.** Polychromatism in *B. schlosseri*. **A-D.** Colonies in dorsal view. **A**, orange morph; **B**, red morph; **C**,  
34 intersiphonal band in form of two narrow, parallel strips; **D**, blue morph with intersiphonal band in form of  
35 fragmented strips. **E-F.** Examples of the five pigmentation patterns: **E**, pigment; **F**, colour markings. **G-M.**  
36 Pigment cells of *B. schlosseri*. **G-J**, living cells viewed under light microscope showing blue (**G**) and reddish (**H**)  
37 pigment cells, nephrocyte (**I**) and orange cell (**J**); the latter is flanked by morula cells (arrowheads). **K-M**, details  
38 of pigment granules from vacuoles of blue (**K**), reddish (**L**) pigment cells, and purinic granules of nephrocyte  
39 (**M**), viewed under transmission electron microscope. Scale bar: 1 mm in **A**, **B**; 2.5 mm in **C**, **D**; 10  $\mu\text{m}$  in **G-J**;  
40 0.35  $\mu\text{m}$  in **K**, 0.5  $\mu\text{m}$  in **L**, 0.2  $\mu\text{m}$  in **M**.

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47 **Figure 4.** **A-C.** Design of Sabbadin's experiments of fusion and separation. Experiments show the transmission of the  
48 character "orange pigment" through the common vasculature (**A**), whereas the characters "intersiphonal band of  
49 purine pigment" (**B**) and "peristomatic rings of purine pigment" (**C**) are not transmitted. **D-G.** Scheme of the  
50 crossing experiments for the study of the inheritance of the character "orange pigment". The allele A ("presence  
51 of pigment") is dominant over the allele a ("absence of pigment"). See text for details.

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56 **Figure 5** **A-E.** Summary of inheritance of the character "intersiphonal band". The character is controlled by three  
57 alleles at a single locus: B2 ("parallel strips"), B1 ("subdivided bands"), and b ("absence of band"). The allele B2  
58 is dominant over B1; both are dominant over b. See text for details. **F.** Dominance relationships among alleles for  
59 the character "intersiphonal band".

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**Figure 6 A-D.** Summary of inheritance of the character “blue pigment”. The character is controlled by two alleles at a single locus: bl (“presence of blue pigment”) and Bl (“absence of blue pigment”); the latter is dominant over bl. See text for details.

**Figure 7 A-C.** Summary of inheritance of the character “reddish pigment”. The character is controlled by two alleles at a single locus: R (“presence of reddish pigment”) and r (“absence of reddish pigment”); R is dominant over r. See text for details.

**Figure 8.** Pigmentation characters and colour morphs of *B. schlosseri* found in the Lagoon of Venice. For each morph, a blastozoid is shown; the seven blastozoids at the bottom represent the legend for each single character (grey: absence of blue and/or reddish pigments).

**Table 1**

Genetic analysis of the two alleles identified by the electrophoretic mobility of their products (S = slow; F = fast) at each locus of three enzymes in *B. schlosseri* and linkage relationships between enzyme and pigmentation loci (Sabbadin, 1978; 1982). The observed phenotype ratios in italics mark the presence of linkage relationships.

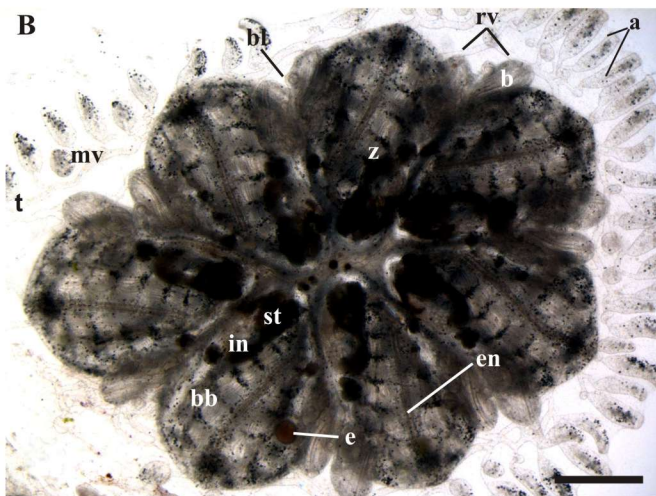
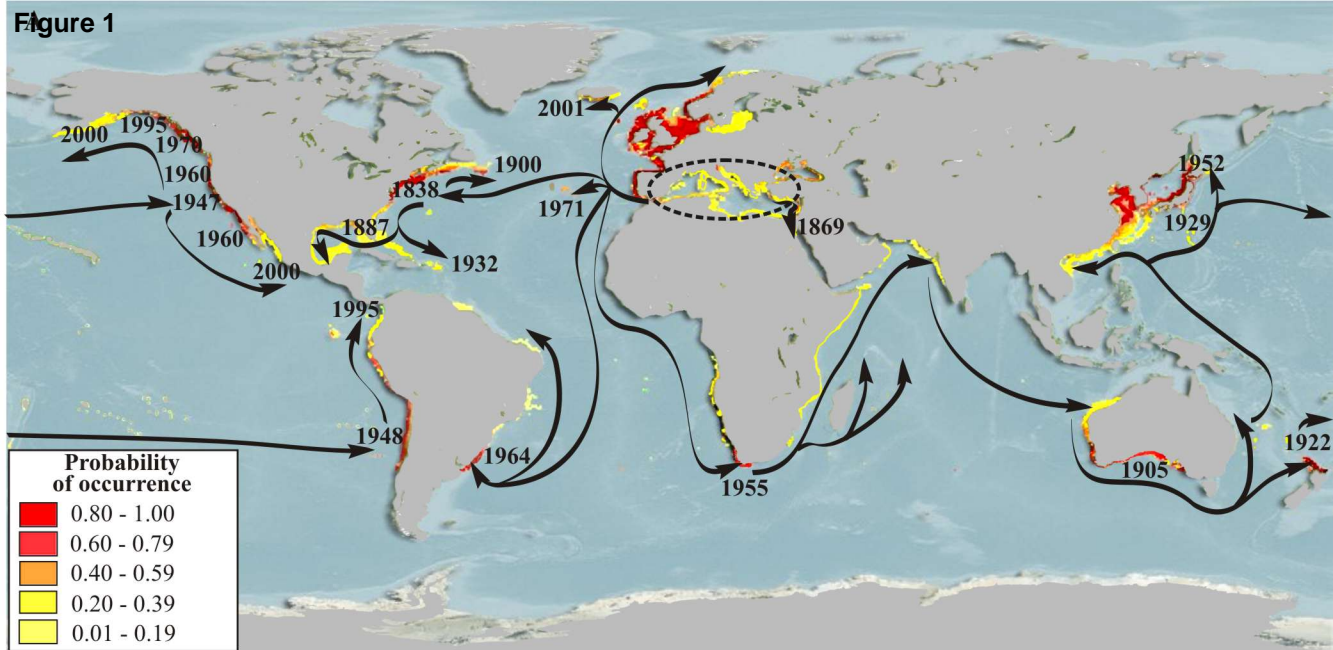
Loci	Parental genotypes	Offspring phenotypes	Expected phenotype ratio	Observed phenotype ratio
MDH	SS x SS	S (11)	1	1
	SF x SF	S (28), SF (59), F (28)	1:2:1	0.9:2:0.9
	FF x FF	F (281)	1	1
	SS x FF	SF (105)	1	1
	SF x FF	SF (133), F (159)	1:1	0.9:1.1
SOD	SS x SS	S (227)	1	1
	SF x SF	S (16), SF (37), F (19)	1.2:1	0.9:2:1
	FF x FF	F (37)	1	1
	SS x FF	SF (9)	1	1
	SF x SS	S (185), SF (194)	1:1	0.9:1
PGI	SS x SS	S (165)	1	1
	FF x FF	F (60)	1	1
	SS x FF	SF (89)	1	1
	SF x SS	S (147), SF (149)	1:1	0.9:1
MDH/SOD	SF,SF x FF,SS	SF,S (5), F,SF (10), SF,SF (3), F,S (6)	1:1:1:1	0.8:1.6:0.5:1
SOD/PGI	SF,SF x SS,SS	S,S (2), S,SF (106), SF,S (101), SF,SF (2)	1:1:1:1	0.03:2:1.9:0.03
MDH/A	SF,Aa x FF,aa	SF,A (6), F,A (57), SF,a (49), F,a (4)	1:1:1:1	0.2:2:1.7:0.1
SOD/A	SF,Aa x SS,Aa	SF,A (13), S,A (9), SF,a (1), S,a (4)	3:3:1:1	3.5:2.5:0.9:1.1
MDH/B	SF,Bb x FF,bb	SF,B (14), F,B (7), SF,b (11), F,b (7)	1:1:1:1	1.4:0.7:1.1:0.7
SOD/B	SF,Bb x SS,Bb	SF,B (11), S,B (21), SF,b (4), S,b (5)	3:3:1:1	2.1:4.1:0.8:0.9
MDH/R	SF,Rr x FF,rr	SF,R (9), F,R (8), SF,r (9), F,r (15)	1:1:1:1	0.9:0.8:0.9:1.5
SOD/R	SF,Rr x SS,rr	SF,R (12), S,R (9), SF,r (7), S,r (4)	1:1:1:1	0.7:1.1:0.9:0.5

Colours mark the alleles of different loci: MDH = malate dehydrogenase; SOD = superoxide dismutase; PGI = phosphoglucose isomerase; A = orange pigment; B = intersiphonal band; R = reddish pigment

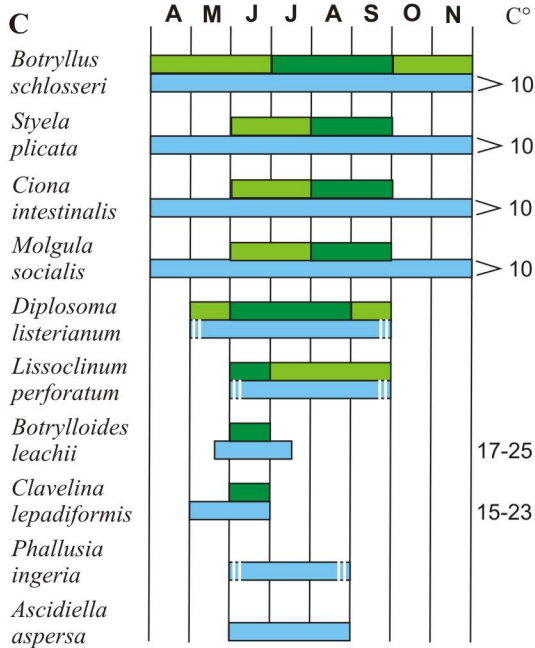
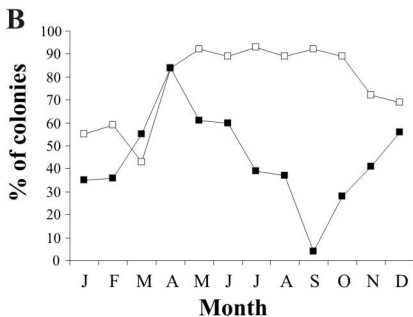
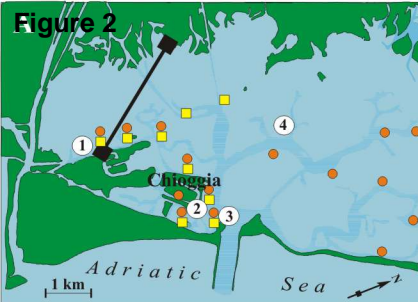
**Table 2**

Mean allele frequency (S = slow; F = fast) and heterozygote frequency (SF) at two enzyme loci, MDH (malate dehydrogenase) and SOD (superoxide dismutase), from pooled colonies of *B. schlosseri* at four stations in the Lagoon of Venice representative of two biotopes (Sabbadin, 1978; 1994).

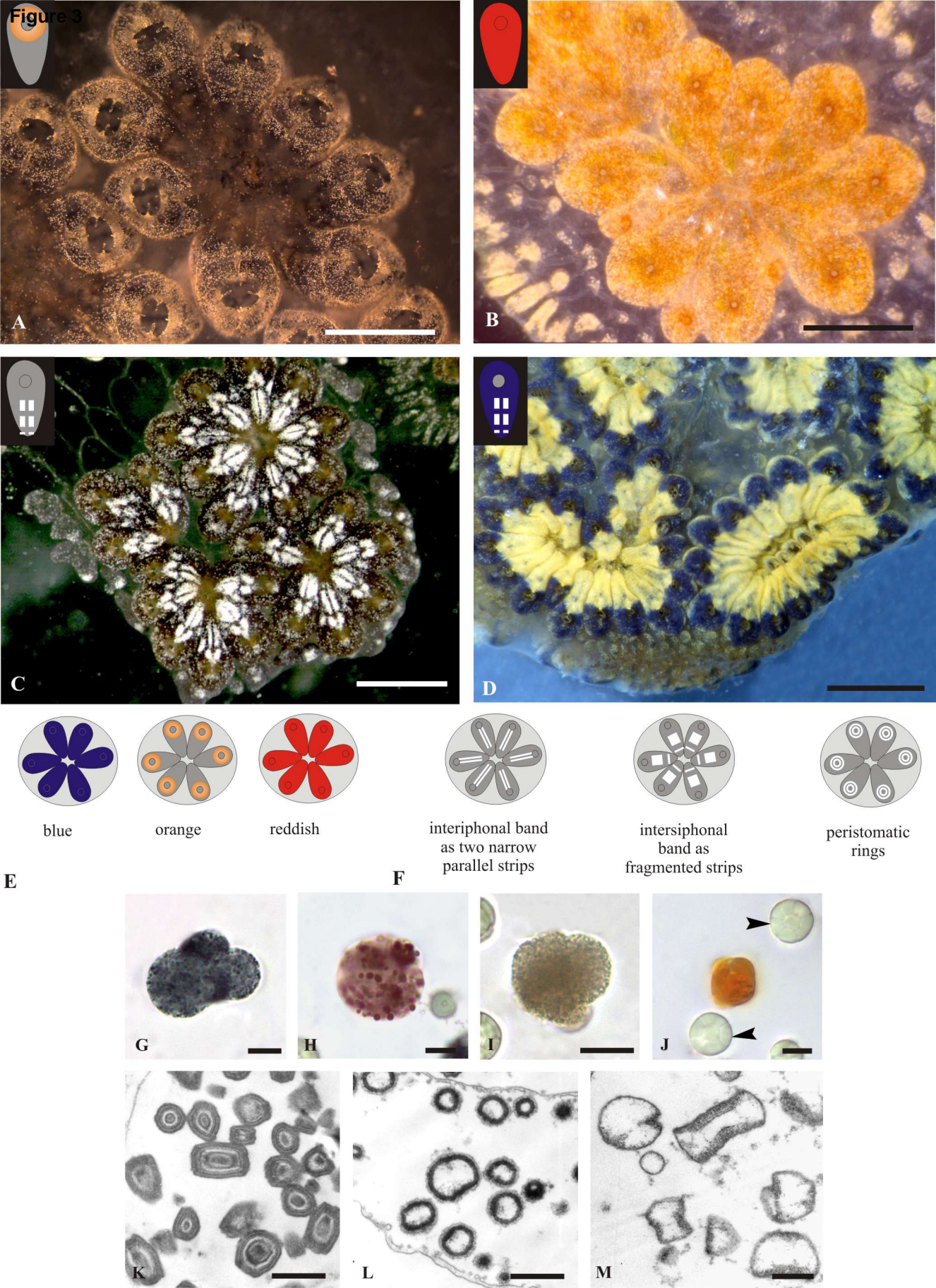
Station	Mean allele frequency		Heterozygote (SF) frequency	
	S	F	Expected	Observed
	MDH		MDH	
A (piles)	14.4	85.6	25.8	24.7
B (piles)	14.6	85.4	26.3	24.7
C (piles)	16.2	83.8	29.1	28.3
D (eelgrass)	5.7	94.3	11.2	11.9
	SOD		SOD	
A (piles)	77.2	22.8	36.3	38.6
B (piles)	72.0	28.0	39.4	38.6
C (piles)	79.6	20.4	33.4	33.2
D (eelgrass)	91.0	9.0	17.4	16.5



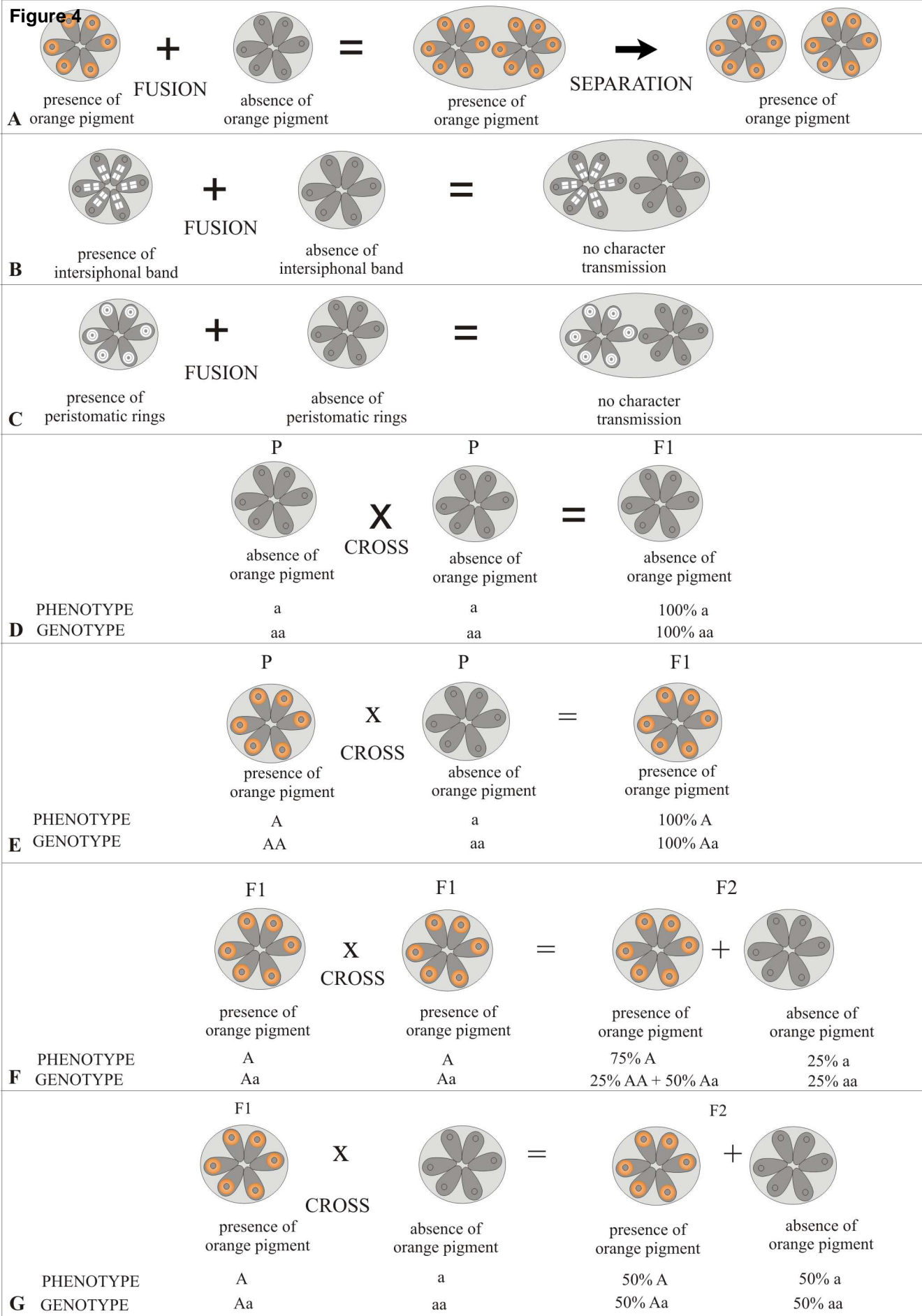
**FIG. 1**



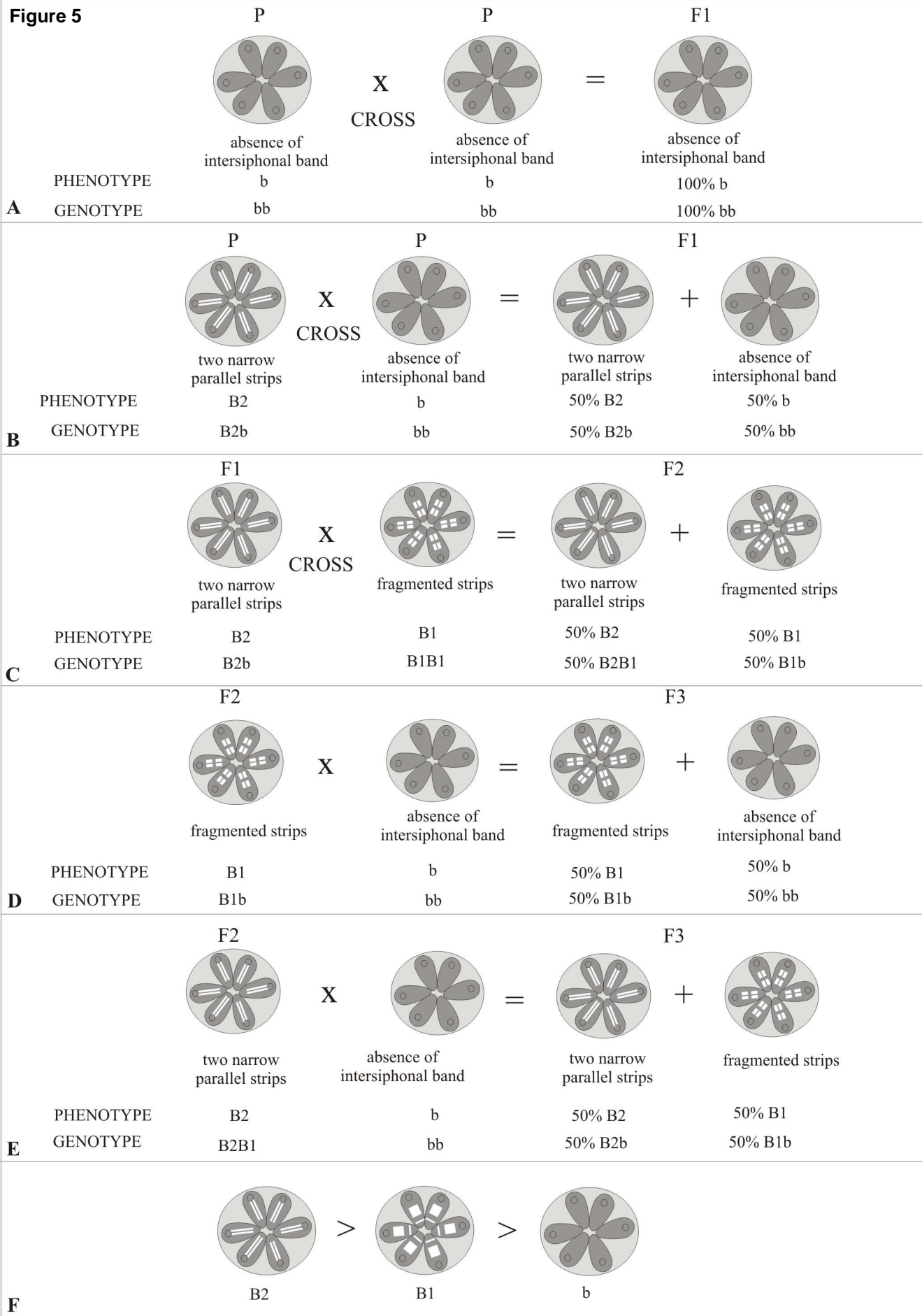
**FIG. 2**

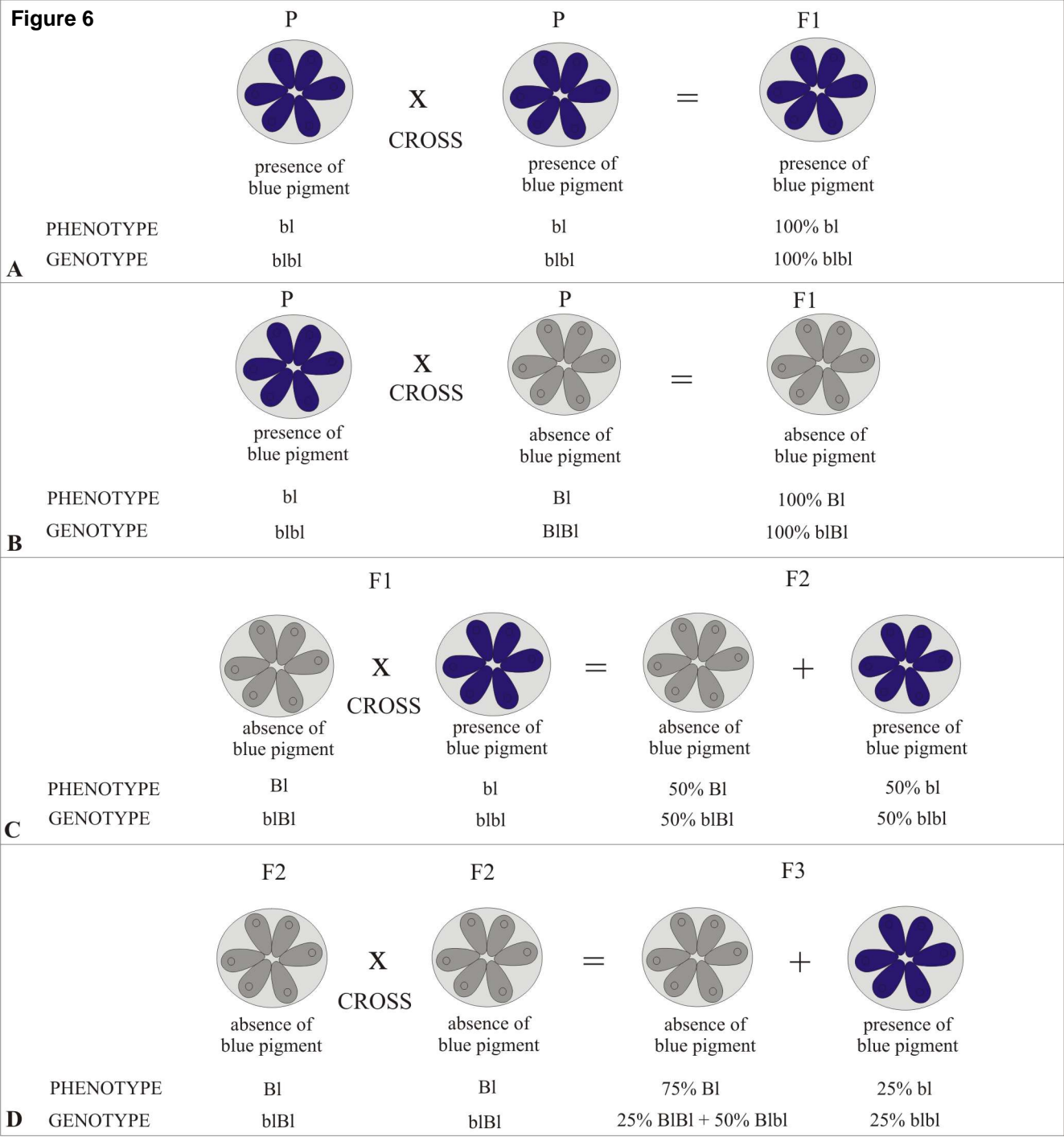


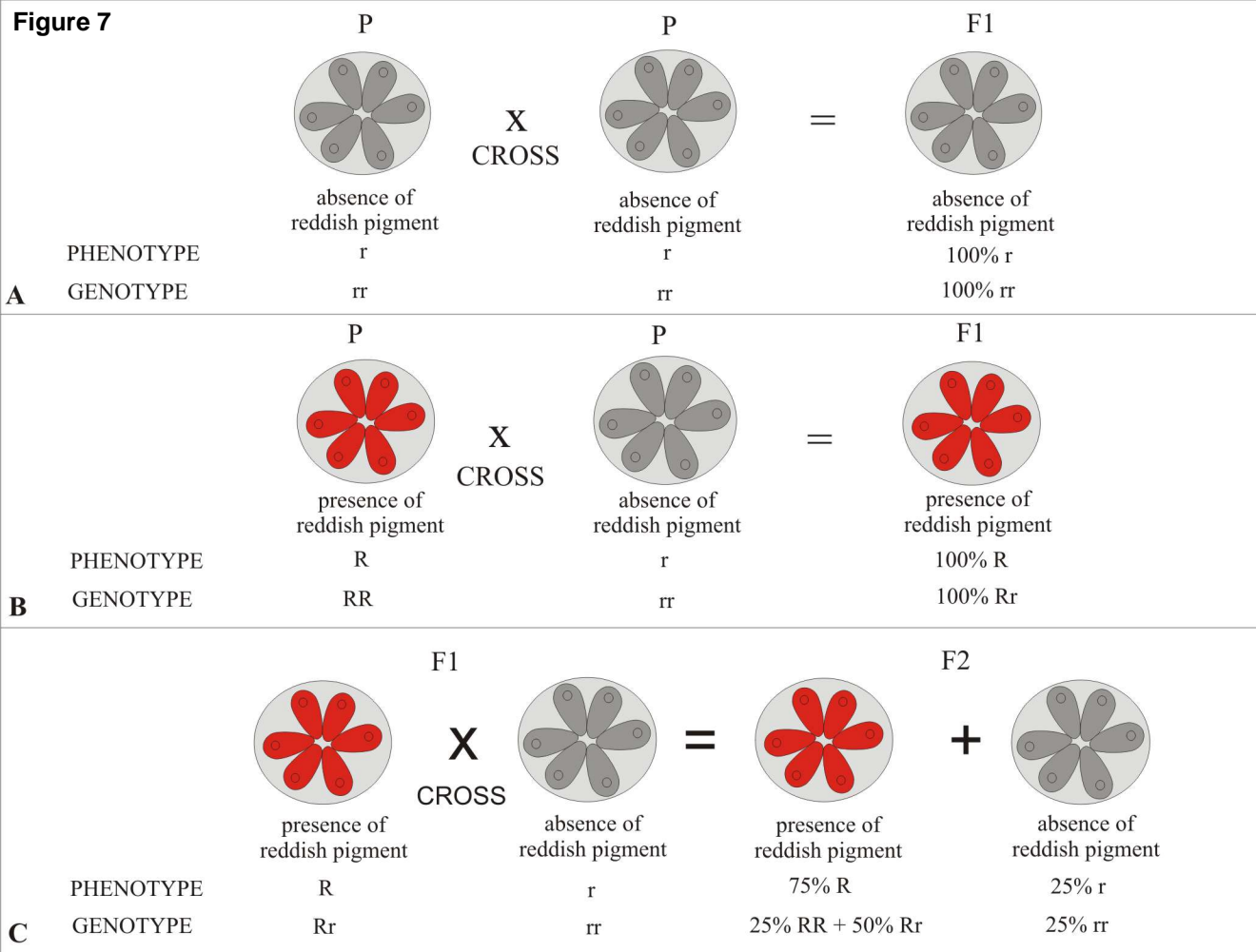
**FIG. 3**



**FIG. 4**

**Figure 5****FIG. 5**

**Figure 6****FIG. 6**



**FIG. 7**

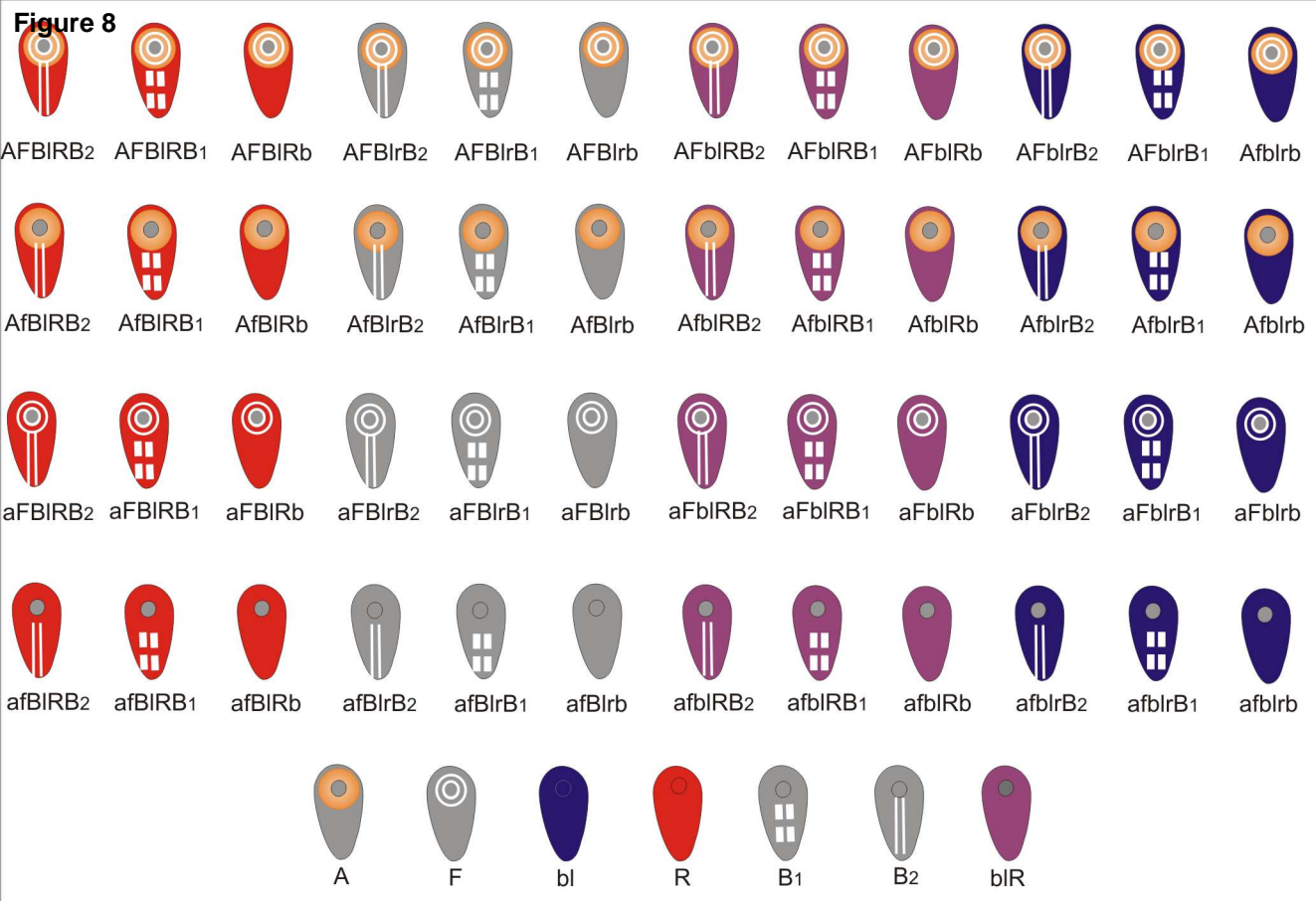


Fig. 8