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COALESCING RED ALGAE EXHIBIT NONINVASIVE, REVERSIBLE CHIMERISM[1]

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Chimerism is produced by the somatic fusion of two or more genetically distinct conspecific individuals. In animals, the main cost of fusion is competition between genetically different cell lineages and the probability of original cell line replacement by more competitive invasive lines, which limits its natural frequency (3%–5%). In red and brown seaweeds, chimerism is widespread (27%–53%), seemingly without the negative outcomes described for animals. The rigidity of cell walls in macroalgae prevents cell motility and invasions. In addition, in moving waters, most somatic fusions involve the holdfast. Histological observations in laboratory-built bicolor macroalgal chimeras indicated that upright axes emerge from the base of plants by proliferation and vertical growth of discrete cell groups that include one or just a few of the cell lineages occurring in the holdfasts. Laboratory experiments showed growth competition between cell lineages, thus explaining lineage segregation during growth along originally chimeric erect axes. Genotyping of the axes showed more heterogeneous tissues basally, but apically more homogeneous ones, generating a vertical gradient of allele abundance and diversity. The few chimeric primary branches produced, eventually became homogenous after repeated branching. Therefore, coalescing macroagae exhibit a unique pattern of post-fusion growth, with the capacity to reverse chimerism. This pattern is significantly different from those in animals and land plants, suggesting chimerism is a biologically heterogeneous concept.

Key index words: **cell competition; cell lineage segregation; chimerism; coalescence; upright axes formation**

Abbreviations: **DNA, deoxyribonucleic acid; PCR, polymerase chain reaction**

Natural chimerism is produced by the somatic fusion of two or more conspecific genetically different individuals. The process is phylogenetically widespread, occurring in fungi, slime molds, colonial invertebrates, humans, other mammals, land plants and macroalgae (Rinkevich 2011). Natural chimerism offers both benefits and costs. The main benefit is increased body size, and the consequent reduction in susceptibility to mortality factors. The costs have been studied mainly in invertebrates; cell lineage competition (somatic selection sensu Buss 1987, or diplontic selection sensu Klekowski and Kazzarinova-Fukshansky 1984) may result in competitive replacement of the original cell line (wildtype). In organisms with motile cells, such as animals, any competitively superior cell lineage may invade different tissues, becoming the new cell line systemic (Hughes 2002). Thus, in animals, the main cost is related to cell motility and may have consequences such as cancer and developmental instability (Pineda-Krch and Lehtilä 2004, Puill-Stephan et al. 2012).

The rigidity of cell walls in land plants and macroalgae prevents cell mobility and the spread of potentially invasive cell lines (Buss 1987, Hughes 2002). Therefore, both groups have been considered (Hughes 2002, Clarke 2013) as representing a similar type of chimerism, different from that of animals. However, the relative importance of the two sources of intra-organismal genetic heterogeneity (mosaicism ad chimerism) seems to be different in the two groups of produced. In land plants, the principal cell lineages remain dependent on apical differentiation and growth. Therefore, the expansion probabilities of new genetic variants occur only when they arise in growth meristems. Consequently, studies on intra-organismic genetic heterogeneity in

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land plants have focused mainly on apical mosaicism (mutations in meristems), with limited attention paid to somatic fusion or chimerism (Reusch and Boström 2011). In macroalgae, both processes are known to be common. Somatic mutations and mosaicism have been described in many red algae (Van der Meer 1990, Monro and Poore 2009), while the capacity to fuse or coalesce with conspecific has been known since the work of Rosenvinge (1931). More recent studies (Santelices et al. 1999, González et al. 2014, 2015, Santelices and González 2014) have confirmed the widespread occurrence of interindividual fusions in red and brown algal species and their importance in the formation of chimeric individuals.

The frequency of chimera formation in macroalgae seems to be much higher than in land plants. In fact, ongoing studies (González and Santelices 2016) have found a high representation of chimeric individuals in natural kelp populations (27%–53%). In red macroalgae, chimera may be formed by somatic fusions of spores, sporelings or grown thalli (examples in Santelices et al. 1999, 2003). In these organisms, somatic fusions also increase individual size, survival, and tolerance to stress conditions (Santelices et al. 2010, Santelices and Alvarado 2011, Medina et al. 2015). However, the negative effects described for chimera in invertebrates, such as development instability, disruptions in the formation of reproductive structures or reductions in reproductive potential, seem to be absent.

Fragmentary evidence suggests that the events following somatic fusions in macroalgae might be different from equivalent processes in land plants. Due to the hydrodynamic conditions of seaweed habitats, macroalgae coalescence often involves supporting structures only (e.g., holdfast or base, Santelices 2004). In many macroalgae, including various red algae and kelps, upright axes emerge by proliferation and the vertical growth of discrete groups of cells that arise from the holdfast or from some basal tissues (Gabrielson and Garbary 1986, Santelices and Alvarado 2011). These new uprights may contain cell lineages from one partner or another in the chimeric base; rarely would it include cell lineages from all partners (Santelices 2004). In addition, if a chimeric upright could be formed with the occurrence of more than one cell lineage, then interlineage differences in growth rates, if they exist, could segregate the cell lineages by forming different axes or different parts of the same axis. Thus, upright axes and branches could revert or partially reduce the magnitude of chimerism produced after the fusion of two or more partners. In this study, we tested this hypothesis using coalescing Rhodophyta.

The idea that the upright formation of each axis involves only a discrete group of basal cells is an important condition of the above hypotheses. Using laboratory-built bicolor chimera of *Mazzaella laminarioides* and *Gracilaria chilensis*, we studied cell lineage segregation during the initiation of axial filaments and the resulting axes formation. In addition, in experimental germlings of the above two species, we measured the patterns of holdfast use in the formation of a progressively larger number of axes.

Differences in growth rates between cell lineages is also a key component of the hypothesis, as growth differences should help to segregate genetically different cell lineages, decreasing chimerism and increasing genetic homogeneity in the uppermost portions of the erect axes. Differences in growth rates and branching patterns between clones (wildtype and green) were measured in laboratory-built bicolor chimeras and in control (single color) plants of *G. chilensis*.

If each upright axis rises from a chimeric holdfast, extending up and culminating in a genetically more homogeneous branch, then a vertical pattern of allelic abundance and genetic relatedness should exist along each branch. Allelic characterization and genetic relatedness in vertical axes were measured in the green and wildtype clones of *G. chilensis* and in the laboratory-built bicolor plants.

While the above hypothesis could explain the reversal of chimerism, it cannot explain the persistence of chimeric branches in fully branched individuals. This type of branch (red/green) was found in low frequency in previous studies with bicolor plants of *G. chilensis* (Santelices et al. 1996). In this study, we quantitated the frequency of this type of branch in bicolor plants and followed the future of the green and red tissues in successive branching events.

In summary, these five topics: cell lineage segregation, pattern of holdfast use, comparison of clonal growth rates, allelic abundance in vertical axes, and the fate of chimeric branches were the focus of this study.

MATERIALS AND METHODS

All the studies were carried out with field-collected or laboratory-grown individuals of *G. chilensis* Bird, McLachlan et Oliveira and *M. laminarioides* (Bory) Fredericq. Populations of both species may contain green mutants and wildtype (red) individuals. Plants of *G. chilensis* were collected in southern Chile (ca: 41°37′ S, 73°36′ W) in the localities of Pudeto and Maullín. The individuals of *M. laminarioides* were gathered by hand from mid-intertidal zones in Maitencillo, central Chile (32°30′ S, 71°29′ W). In all the localities and in both species, the green individuals exhibited very low frequency in space and time (1%–5% of field frequency).

After collecting, the plants were transported in refrigerated containers (4°C) to the laboratory in Santiago. There, they were briefly rinsed with running freshwater to remove epiphytes and debris; they were then maintained for 24 h in filtered seawater under controlled conditions of temperature (14 ± 2°C), irradiance (20 ± 5 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$) and photoperiod (12:12 LD). When necessary, sporulation was induced by immersing previously dehydrated mature cystocarps in microfiltered seawater (0.45 μm). Dehydration was performed for 1–2 h at room temperature. When necessary, different densities of spores were incubated following

published protocols for the cultivation of the above two species (Santelices et al. 1999, 2010). Spores and sporelings were incubated under the above controlled conditions.

Cell lineage segregation. To characterize cell lineage segregation and to confirm the involvement of a discrete number of cortical cells during the initiation of the axial filaments and upright formation, we used the natural interclonal differences in pigmentation between the wildtype and green clones.

Sixty-day-old bicolor specimens of each species formed by the coalescence of 20 green and 20 red spores were used. A total of 15 individuals from each species was incubated for 60 d and then fixed using a 4% solution of formaldehyde in seawater. Twenty-four hours after fixation, holdfasts were sectioned using a Leitz freezing microtome. Sections, without staining, were mounted on slides using 50% Karo® corn syrup. Histological sections were studied over the next 3 d to clearly distinguish the natural pigmentation of the cells forming the erect axes. Observations were made using a Nikon Optiphot-2 microscope equipped with a cool SNAP-Proof Color camera (Media Cybernetics, Silver Spring, MD, USA). Cells and tissues were photographed and measured using Image Proplus v4.5 software (Media Cybernetics).

Patterns of holdfast use. To confirm the involvement in upright formation of discrete groups of basal cells, we compared the total holdfast surface with the diameter of each axis' insertion in the holdfast. Comparisons included sporelings of various ages exhibiting different holdfast diameters and different densities of erect axes.

The experiments were performed with wildtype red individuals of both species. Sporulation was induced as described above and sporelings were cultivated under the above controlled conditions for up to 150 d. In order to obtain germlings with different holdfast sizes and number of erect axes, we varied the number of spores forming the coalescing germlings. In the case of *M. laminarioides*, eight replicate sporelings were formed with 2, 10, 20, 40, or 80 coalescing spores cultivated for 75 d. In the case of *G. chilensis*, 6–7 replicate sporelings formed by 2, 10, 20, 30, or 40 spores were used and incubated for 70 d.

Originally, all sporelings were incubated in small petri dishes (50 × 10 mm) filled with SFC culture medium (Correa and McLachan 1991) changed weekly. However, in the case of *G. chilensis*, at week 6 of incubation, germlings were moved into aerated 250 mL flasks, each containing 200 mL of SFC culture medium, changed every 4 d.

At the end of the incubation period, all germlings were photographed under a Nikon Stereomicroscope (SMZ-10A) equipped with a cool SNAP-Proof Color camera (Media Cybernetics) or with a Nikon DS-Fi 1 camera using the program NIS Elements F 3.2. The holdfast diameter of each individual germling and the basal diameter of each erect axis arising from the holdfast were measured using the program Image Proplus v4.5 software (Media Cybernetics). The data were used to calculate the percentage of holdfast surface used in the formation of each new axis.

Comparison of clonal growth rates. Growth rate comparisons between the green and the red (wildtype) lineages were made with individuals of *G. chilensis* only. The number of primary branches produced by each lineage in the presence or absence of the other one was measured as an indicator of the effects of each lineage on the other. Experimental plants were bicolor, formed by a total of 10 or 20 spores, containing a combination of equal numbers of red and green spores (e.g., 5 + 5 or 10 + 10). The control individuals were made by a similar total number of spores (10 or 20) of only a single lineage (red or green).

Five replicate sporelings per treatment were cultivated under the above controlled culture conditions. During the first 60 d of culture, dishes were placed on top of rotatory shakers set at 100 rpm. Thereafter, the plants were transferred to aerated 250 mL flasks, each containing 200 mL of SFC medium, exchanged every 4 d. After 130 d of incubation, all green and red axes arising from a common holdfast were distinguished by color and counted. The significance of the differences among treatments was calculated using one-way ANOVA and *t*-test (Sokal and Rohlf 1995), aided by Minitab 16 statistical software (Minitab Inc., State College, Pennsylvania, USA).

Allelic abundance and relatedness in vertical axes. This study was conducted for *G. chilensis* only, since specific microsatellites (Guillemin et al. 2005) existed prior to our studies. For genetic characterization, we used tissues from the axes grown in the previous experiment. After counting, all axes (red and green) were carefully sectioned from their respective holdfast and tagged. The most basal portions (2 cm) and apical (2 cm) portions of each axis were then sectioned and placed in a previously tagged sterile plastic tube with silica. Each holdfast was also sectioned into two pieces and kept in tagged tubes with silica. Genomic DNA from the tissues was extracted using the phenol-chloroform method described by Sambrook et al. (1989) and modified by González et al. (2012). For DNA extraction and microsatellite genotyping, we used five microsatellite loci and the protocols developed by Guillemin et al. (2005). Amplifications were carried out using forward primers with a fluorescently labelled M13 tail (Schuelke 2000). PCRs were made as described by Guillemin et al. (2005) and González et al. (2014), including 0.2 μm of fluorescently labelled (FAM, VIC, PET or NED) M13 universal primer with specific cycling conditions for each species. For genotyping, 1 μL of the PCR product was added to 22 μL formamide and 0.5 μL LIZ-400 size standards. The mixture was run on ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystems, Hitachi, Japan) and analyzed using Peak Scanner software v1.0 (Applied Biosystems, Foster City, California, USA). All results were recorded manually. Fragment sizes were entered into Microsoft Excel for further analysis.

We estimated the locus richness at the holdfast (H) and axes portions (Basal: A_B and Apical: A_A). Comparisons among these different levels were made by a one-way ANOVA (Sokal and Rohlf 1995). In order to distinguish between mosaicism and chimerism, we estimated their relatedness, defined as the probability that two individuals (2n) share an allele due to recent common ancestry. We used the Queller and Goodnight estimator (Queller and Goodnight 1989) in GenA1EX 6.1. Relatedness analysis was performed on genotypes based on the five loci. When QG was less than 0.24, samples were considered unrelated individuals, but if QG was equal to 1 (maximal similitude among samples), they were considered clone samples. The definition of chimerism was restricted to values (−0.25 to 0.25) exhibited by unrelated samples from the same plant. Since coalescence among half-sibling spores can occur in the wild, we considered the remaining QG ranges (0.9–0.25) as mosaicism, restricting coalescence and chimeric formation to nonrelated individuals only. This classification ranking has been used in the detection of chimerism in the natural populations of the coral *Acropora millepora* (Puill-Stephan et al. 2009).

The fate of chimeric branches. Observations on the long-term outcomes of chimeric branches were made for *G. chilensis* only. A total of 100 branches from 20 bicolor plants were used. Plants were cultivated for 300 d under the controlled conditions described previously. The extended cultivation time allowed us to observe, and genetically characterize, the nature of primary and secondary branchlets, especially in the chimeric branches. Relatedness analysis (Queller and Goodnight 1989) was performed on the genotypes of branches and branchlets, using the same five loci described above.

RESULTS

Cell lineage segregation. Histological observations indicated that 30–45 d after spore germination and crust formation, groups of transversely dividing axial cells had arisen from the surface of the crustose base, leading to a number of axial filaments as described by Dixon (1973), Gabrielson and Garbary (1986), and Santelices and Alvarado (2008). Upright shoots were observed to have originated from cells in almost any location in the crustose disk . Our experiments with bicolor plants exhibited chimeric holdfasts with mixes of red and green cells from both of the lineages considered.

Axis primordia in *M. laminarioides* (Fig. 1A) were uni- or bicolor, rounded or ovoid, formed by 10–30 apical cells (mean = 19.7 ± 1.4), and reached 45–210 μm diam (mean = 168 ± 18.2). Primordia in *G. chilensis* (Fig. 1B) were more cylindrical and narrower, formed by 4–10 (mean = 7.0 ± 0.38) apical cells (45–152 μm diam., mean = 85.8 ± 5.6), frequently unicolor although bicolor (chimeric) could also be found, albeit in low frequency (1%).

As the upright shoot elongated, chimeric axes exhibited segregation between cell lineages, seemingly due to interlineage differences in elongation rates. Faster growing lineages held the top position in the growing axes. Groups of cells from the second lineage remained at a lower level or formed a separate axis. Therefore, the primordia of upright axes appeared to be chimeric and genetically heterogeneous at their most basal portion, but most of them became genetically less diverse and homogeneous at their apical portions (Fig. 1, A and B).

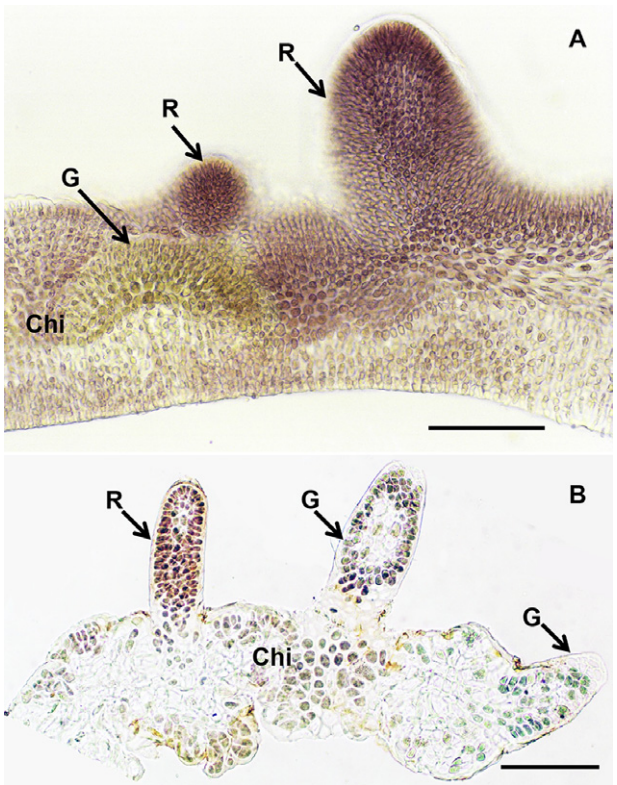

FIG. 1. Semi-thin transections through the holdfast of a bicolor plants constructed in the laboratory with two strains (red and green) of the species, scale bar = 100 μm. (A) Chimeric plantlet of *Mazzaella laminarioides* (Rhodophyta). (B) Chimeric plantlet of *Gracilaria chilensis* (Rhodophyta). R: Red lineage, G: Green lineage, Chi: Chimeric tissues, scale bar = 100 μm.

Quantitation of the segregation process carried out with 50 growing primordia of *M. laminarioides* indicated that chimeric tissues (mixes of green and red cells) were found in 70%–75% of the young primordia shorter than 5 mm long. The remaining primordia rose directly from a holdfast portion containing cells of a lineage similar to the branch (e.g., red (R) in Fig. 1).

In the chimeric primordia, the cell mix (bicolor with green and red) appeared to extend from the base up to 25% of the basalmost portion of the shorter axes. In longer chimeric young axes (e.g., 3.0 mm long), the mixed tissue reached 10%–15% the axes length, while in the grown axes (6–15 cm tall), chimeric tissues were not present, probably outgrown by the lineage that formed them.

The general pattern of lineage segregation in *G. chilensis* was similar, but with variations due to the differences in holdfast structure and thickness. About 48% of the 50 primordia examined lacked the chimeric cell mixing at their base, rising directly from holdfast cells of a similar lineage (see axis green (G) in Fig. 1B). The basal portion of the remaining 26 primordia exhibited chimeric cell mixing, but, infrequently, they extended beyond 10% of the most basal portion of the upright shoot. Axes longer than 1.0 mm did not show any chimeric tissue at their base and most of the axes were of a single lineage only.

These results support the idea that the upright formation of each erect axis involves discrete groups of cells only and that lineage segregation occurs during elongation of originally chimeric axes. In our experiments, lineage segregation with the formation of genetically homogeneous unicolor axes occurred in 100% of the studied samples of *M. laminarioides*. The corresponding value for *G. chilensis* was 99%. Red and green axes genotyping, as well as what occurred with the few chimeric axes produced, are further addressed below.

Patterns of holdfast use. Under the experimental conditions used, the germlings of *M. laminarioides* developed a total holdfast area of 0.3–12.1 mm^2. Those of *G. chilensis* ranged from 0.5 to 3.6 mm. The number of erect axes produced increased asymptotically as the holdfast surface increased ($y = 1.7353 \ln(x) + 2.8128$, $r^2 = 0.520$, $n = 40$, $P < 0.001$ for *M. laminarioides* and $y = 2.8665 \ln(x) + 5.0004$, $r^2 = 0.2033$, $n = 34$, $P = 0.007$ for *G. chilensis*). However, the proportion of the holdfast area utilized for axis production never exceeded 64% in

M. laminarioides and 30% in *G. chilensis*. In both species, the proportion of the holdfast surface used for axis initiation decreased significantly (Fig. 2, A and B) as the number of axes increased in increasingly larger holdfasts. Thus, the data of holdfast use from both species provide additional support to the idea that upright axes in red algae begin with the growth of discrete, small groups of basal cells, each being just a portion of the diversity of genotypes occurring in the holdfast. The limited number of cells in these discrete cell groups would facilitate the segregation process of cell lines as described above.

Comparison of clonal growth rates. Numerical results (Table 1) indicated that the total number of branches produced by the 10-spore bicolor plants did not differ from the number of primary branches produced by the green or the red controls (ANOVA test: $F_{2,14} = 0.75$, $P = 0.493$). A similar result was found in equivalent comparisons using the 20-spore plants (ANOVA test: $F_{2,14} = 1.33$, $P = 0.301$). However, numerical differences became significant when the type of branch in the bicolor plants was considered. The number of green branches in the 10-spore bicolor plant (Table 1) was significantly higher (t-test: 6.00, df = 6, $P = 0.001$) than the number of red branches. Equivalent differences were found with the 20-spore plants. In both treatments, the green lineage produced over twice as many branches as the red lineage (t-test = 5.06, df = 5, $P = 0.007$).

Differences were also significant when comparing the number of branches produced by the red controls with the number of red branches produced by the bicolor plants (t-test 10-spores = 4.15, df = 5, $P = 0.009$; 20-spores = 6.35, df = 7, $P = 0.0001$). In both cases, there was a notable reduction in the number of red branches produced by the bicolor plants. Since the number of red spores used in the bicolor plants was only half the number of those used in their corresponding red controls, we also compared the number of red branches produced by the 10-spore and the 20-spore bicolor plants (5 and 10 red spores used in their respective incubation). Results (t-test = -1.81, df = 6, $P = 0.120$) indicated no significant differences. Thus, the significant reduction in the number of red branches formed by the bicolor plants as compared to their controls apparently occurred as a result of the effects of the green cell lineage growing in the same individuals as the red ones.

Results with the green lineage were somewhat different. There were no significant differences in the number of green branches produced by the 10-spore control plants and the bicolor plants (t-

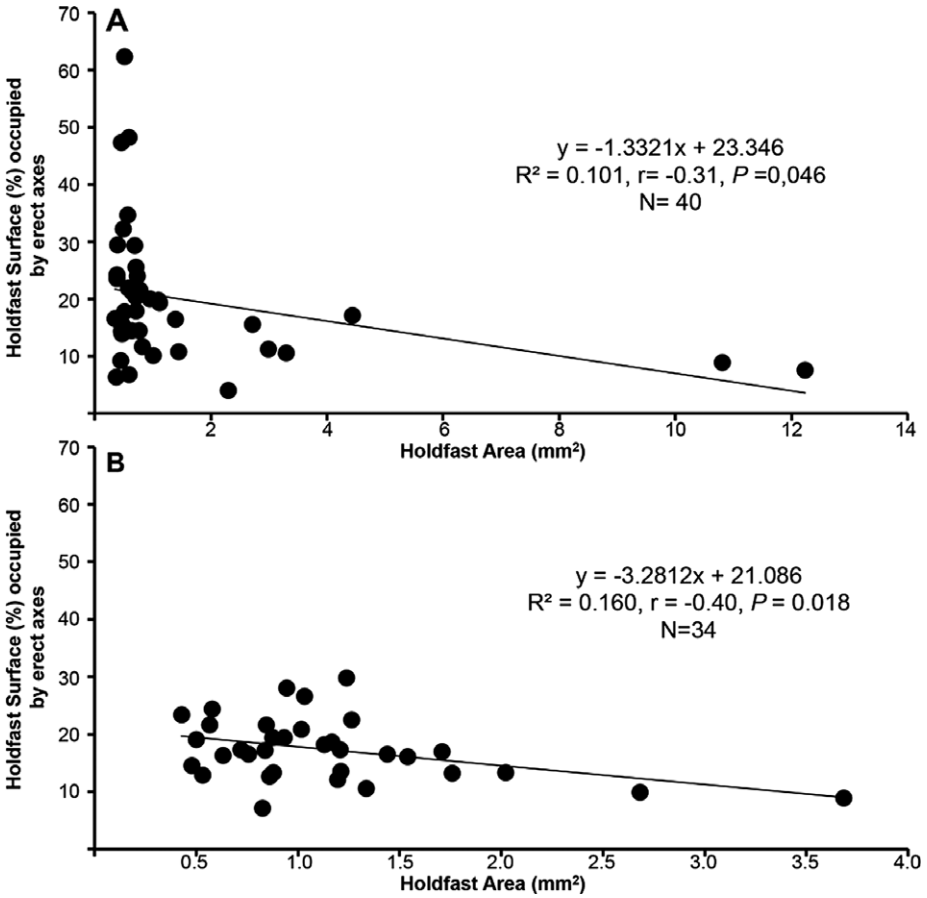

FIG. 2. Patterns of holdfast use. Relationship among holdfast surface (%) occupied by erect axes versus the holdfast area. (A) *Mazzaella laminarioides*, (B) *Gracilaria chilensis*.

TABLE 1. Number of primary branches produced by bicolor plants and their respective controls (green and red) formed from 10 and 20 spores.

	10 Spores					20 Spores				
	Unicolor		Bicolor			Unicolor		Bicolor		
Replica Number	Green control	Red control	Green	Red	Total	Green control	Red control	Green	Red	Total
1	6	3	5	0	5	10	9	5	1	6
2	5	5	6	2	8	6	8	5	2	7
3	9	8	3	1	4	8	10	5	4	9
4	9	4	5	1	6	12	11	6	3	9
5	3	4	4	1	5	13	6	6	1	7
Mean	6.4	4.8	4.6	1.0	5.6	9.8	8.8	5.4	2.2	7.6
SE	1.17	0.86	0.51	0.32	0.68	1.28	0.86	0.24	0.58	0.60

test = 1.41, df = 5, $P = 0.216$). However, differences were significant in equivalent comparisons using the 20-spore plants (Table 1; t-test = 3.37, df = 4, $P = 0.028$). Similar to the case of the red lineage, variations in the number of spores between the bicolor plants and controls did not seem to explain these differences. In fact, the number of green branches produced by the 10 or the 20-spore bicolor plants was statistically similar (t-test = 1.96, df = 7, $P = 0.090$); a lack of significance was also found when comparing the number of green branches generated by 10-spore or 20-spore bicolor plants (t-test = 1.41, df = 5, $P = 0.216$). Therefore, it seems likely that red algal spores also have an effect on the branching abundance of the green lineage, especially at higher spore densities.

Allelic abundance and relatedness in vertical axes. The allelic studies conducted with the green and red controls, as well as with the bicolor plants, indicated that some alleles are exclusive to the red cell line (i.e., alleles 317, 193, 203, 294, 190; Table 2) while others are exclusive to the green cell line (i.e., alleles 315 and 201; Table 2). In the control plants (red or green), allelic richness did not vary from the base to the tip of the primary branches (Fig. 3, A and B). In bicolor plants, the holdfast showed both types of alleles to be present in the cells of the two lineages (green and red). As indicated previously, 99% of the primary branches are either red or green and their allelic richness remains similar from the base to the tip. Consequently, allelic richness in bicolor plants was significantly higher in the chimeric, bicolor holdfasts (including alleles from green and red lineages) than in the genetically more homogeneous red ($F_{2,233} = 26.9$, $P < 0.001$) or green axes ($F_{2,237} = 72.1$, $P < 0.01$, Fig. 4, C and D).

Results from the relatedness test (Queller and Godnight test) comparing the holdfast (H), base of branches (Basal:A_B) and the apical portion of branches (Apical:A_A) support the above findings. For example, relatedness values between holdfasts and axes in unisporic red plants (H × H, H × A_B, H × A_A, Fig. 4A) were high (e.g., QG values between 0.75 and 1.00). QG values were also high (e.g., 0.9–1.00) in other comparisons between different portions of the axes and there is a similar occurrence with the green plants (Fig. 4B). By contrast, comparisons of different parts of the bicolor plants resulted in low QG values. For example (Fig. 4C), comparisons between holdfast parts (H × H) or between holdfast and red or green branches resulted in QG values of about −0.25 to +0.25. Relatedness comparisons of red versus green branches (e.g., Fig. 4C, Red vs. Green: $A_B \times A_B$ or $A_A \times A_A$) also resulted in low QG values, which were consistent with the allelic differences just described for these two types of lineages.

The fate of chimeric branches. Only 1% of the total number of axes examined (100) were chimeric. This occurred in a plant rising from a chimeric holdfast, which generated four primary axes: one red (see number 1 in Fig. 5A), two green (see numbers 2 and 3 in the Fig. 5A), and one bicolor or chimeric (Chi_1 in the Fig. 5A). A close up of the bicolor axis (Chi_1) showed both red (R) and green (G) tissues without mixing (Fig. 5). In addition, three secondary red axes (R_1–R_3), three secondary green axes (G_1–G_3) and two secondary bicolor (chimeric) axes (Chi_2 and Chi_3) emerged from this chimeric axis (Fig. 5C). The genetic characterization of the primary axis Chi_1 in the different sections sampled from the basal to the apical portion of the chimeric axis (letters a-f in Fig. 5C), indicated that most of the tissues were genetically similar to that of red axes (e.g., Chi_1a-Chi_1d with QG values >0.59 compared to red axes, Table 3). However, a combination of red and green alleles (chimeric tissues) was found at the apical portions of the chimeric axis (see samples Chi_1e and Chi_1f in Table 3). On the other hand, genetic characterization of the secondary axes indicated that red (R_1–R_3) and green (G_1–G_3) axes showed exclusively red and green alleles, with high genetic relatedness between them (Table 3). Thus, the data suggested that most of new axes formed by the chimeric axis segregated into the original red and green lineages, reducing the amount of chimeric tissue.

TABLE 2. Allele frequency of each locus studied in unisporic and bicolor plants of *G. chilensis* cultured under laboratory conditions.

	Unisporic										
	Red control (N = 16)			Green control (N = 16)			Polysporic bicolor (25 red and 25 green spores, N = 20)				
	H	A_B	A_A	H	A_B	A_A	H	A_BRed	A_ARed	A_BGreen	A_AGreen
Locus 2											
293	0.313	0.250	0.094	0.000	0.000	0.000	0.000	0.005	0.005	0.000	0.000
301	0.000	0.000	0.000	0.625	1.000	1.000	0.513	0.273	0.268	0.894	0.894
315	0.000	0.000	0.000	0.375	0.000	0.000	0.025	0.000	0.000	0.096	0.096
317	0.688	0.750	0.906	0.000	0.000	0.000	0.463	0.722	0.727	0.010*	0.010*
Locus 3											
189	0.000	0.000	0.000	0.500	0.500	0.313	0.088	0.010*	0.010*	0.490	0.490
193	0.281	0.094	0.188	0.000	0.000	0.000	0.013	0.227	0.227	0.000	0.000
199	0.219	0.406	0.219	0.500	0.500	0.688	0.425	0.258	0.258	0.500	0.495
203	0.500	0.500	0.594	0.000	0.000	0.000	0.475	0.505	0.505	0.010*	0.015*
Locus 4											
259	0.406	0.438	0.406	0.500	0.500	0.500	0.513	0.343	0.369	0.500	0.515
294	0.594	0.563	0.594	0.000	0.000	0.000	0.425	0.586	0.581	0.005*	0.005*
296	0.000	0.000	0.000	0.500	0.500	0.500	0.063	0.071	0.051	0.495	0.480
Locus 5											
190	1.000	1.000	1.000	0.000	0.000	0.000	0.600	0.914	0.914	0.000	0.000
199	0.000	0.000	0.000	0.500	0.500	0.500	0.150	0.081	0.081	0.500	0.500
201	0.000	0.000	0.000	0.500	0.500	0.500	0.250	0.005*	0.005*	0.500	0.500
Locus 6											
354	0.663	0.520	0.525	0.995	1.000	0.500	0.500	0.688	1.000	1.000	1.000
357	0.338	0.480	0.475	0.005	0.000	0.500	0.500	0.313	0.000	0.000	0.000

H: Holdfast tissues. A_B: tissues from basal portion of axes. A_A: tissues from apical zones of axes. Gray color indicates the allele exclusively for each red and green color strain. Asterisk asterisk indicates chimeric tissue with both red and green alleles.

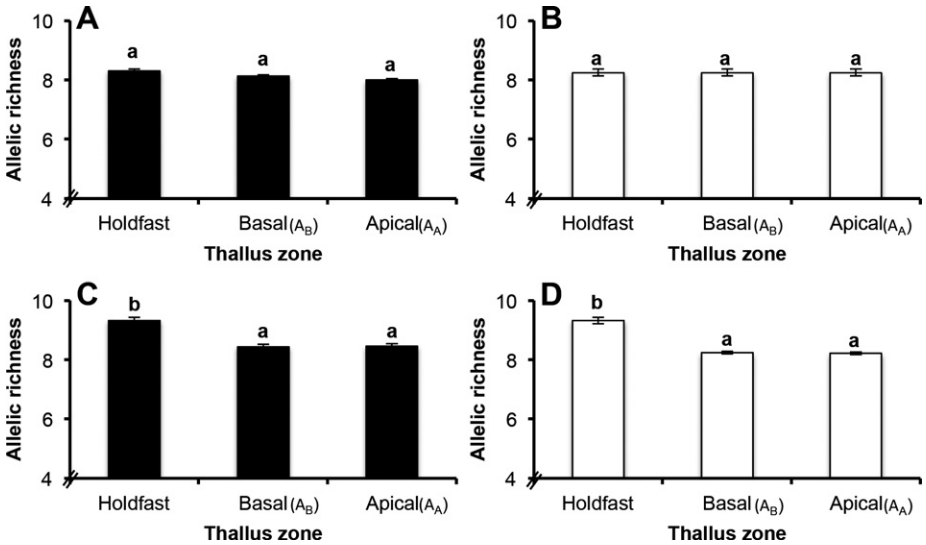

FIG. 3. Allelic richness (mean ± SE) using 5 microsatellites loci at Holdfast (H), Basal (A_B), and Apical zones of thallus (A_A) of the *Gracilaria chilensis* plants constructed in the laboratory. (A) Unisporic red plants. (B) Unisporic green plants. (C) Red axes and holdfasts of bicolor plants. (D) Green axes and holdfasts of bicolor plants. Different letters above the bars indicate significant differences.

DISCUSSION

The combination of results obtained in this study suggests that chimeric individuals of coalescing Rhodophyta may reverse chimerism. Three factors, including patterns of upright axes formation, the fate of the few chimeric branches arising from the chimeric holdfasts and cell lineage segregation appear to be key factors in this process.

In the coalescing red algae studied, once the crustose holdfast had been formed, erect axes were differentiated from one another by the activity of discrete groups of cells arising from the base of the plants. These axes sometimes arose from genetically homogeneous portions of the disk , forming homogeneous erect axes. Others originated from genetically heterogeneous tissues, containing a cell mix at their most basal portions. As these chimeric axes elongated, cell segregation occurred and one cell lineage appeared to dominate over the other, forming apically genetically homogeneous axes. The histological observations and quantitations, the discrete axis area in relation to the total holdfast area, as well as the vertical differences in allelic abundance and diversity between the chimeric holdfast area and the genetically more homogeneous upper part of erect axes,

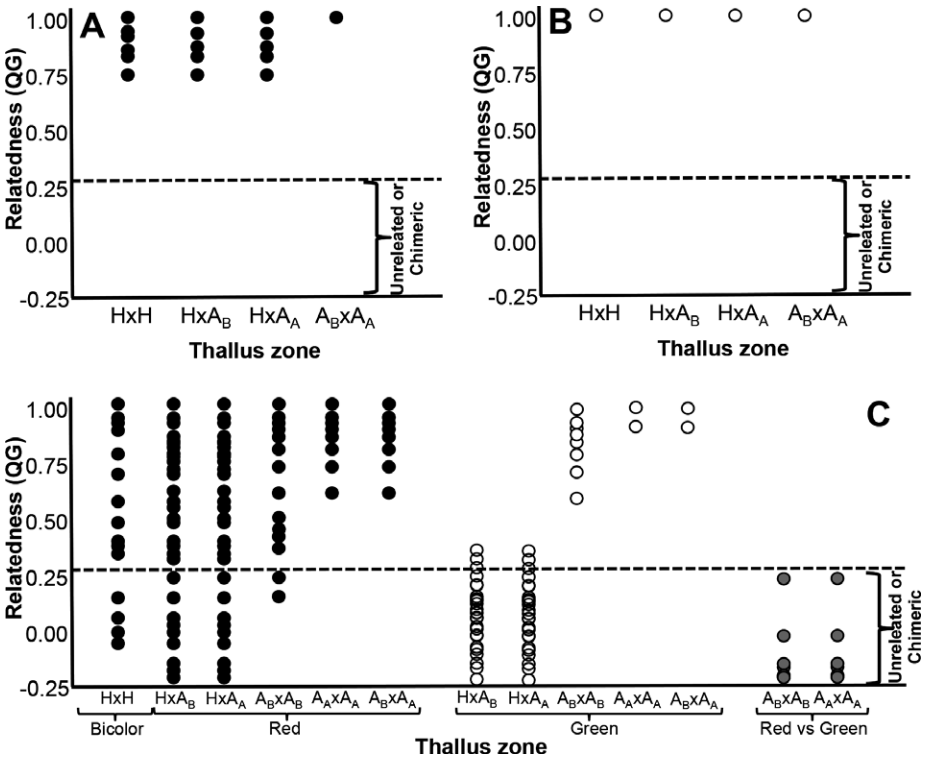

FIG. 4. Genetic relatedness (QG) at Holdfast (H), Basal (A_B), and Apical zones of thallus (A_A) of the *Gracilaria chilensis* plants constructed in the laboratory. (A) Unisporic red plants. (B) Unisporic green plants. (C) Bicolor plants with red and green axes.

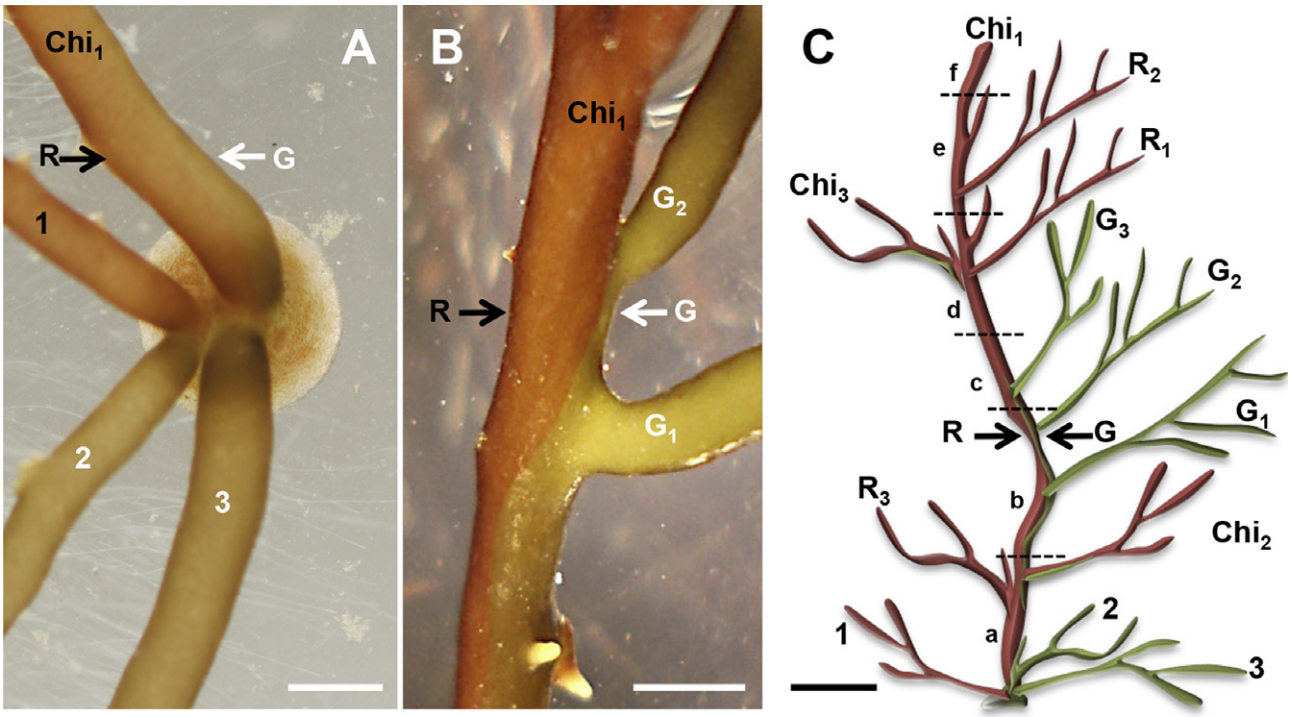

FIG. 5. Bicolor plants of *Gracilaria chilensis* constructed in the laboratory. (A) Red (1), green (2–3) and chimeric (Chi1) axes emerged from the same bicolor holdfast, after 70 d of culture. R: red cell lineage. G: green cell lineage, scale bar = 600 μm scale bar = 600 μm. (B) Bicolor or chimeric primary axis (Chi_1) composed of red (R) and green (G) cell lineages, after 300 d of culture G1 and G2 are secondary axis with green color, scale bar = 2.5 2.5 mm. scale bar = 2.5 mm. (C) Graphic representation of the bicolor plants which showed one red axis (1), two green axes (2–3) and one chimeric or bicolor axes (Chi_1). Then, numerous secondary axis emerged with green color (G_1–G_3), red color (R_1–R_3) and bicolor (Chi_2–Chi_3) a–f: different portions of the same chimeric axe (Chi1) from basal to apical portions, scale bar = 1 cm.

TABLE 3. Relatedness value (QG) between different axes from the chimeric plant.

Sample	R_1	R_2	R_3	Chi_1a	Chi_1b	Chi_1c	Chi_1d	Chi_1e	Chi_1f	Chi_2	Chi_3	G_1	G_2	G_3
R_1														
R_2	1.00													
R_3	0.66	0.62												
Chi_1a	0.59	0.59	0.52											
Chi_1b	1.00	1.00	0.62	0.59										
Chi_1c	1.00	1.00	0.62	0.59	1.00									
Chi_1d	1.00	1.00	0.62	0.59	1.00	1.00								
Chi_1e	0.09	0.09	−0.22	−0.28	0.09	0.09	0.09							
Chi_1f	0.09	0.09	−0.22	−0.28	0.09	0.09	0.09	1.00						
Chi_2	1.00	1.00	0.66	0.59	1.00	1.00	1.00	0.09	0.09					
Chi_3	1.00	1.00	0.62	0.59	1.00	1.00	1.00	0.09	0.09	0.62				
G_1	0.24	0.24	0.24	0.61	0.36	0.36	0.36	−0.31	−0.31	0.32	0.35			
G_2	0.24	0.24	0.24	0.61	0.36	0.36	0.36	−0.31	−0.31	0.32	0.35	0.64		
G_3	0.24	0.24	0.24	0.61	0.36	0.36	0.36	−0.31	−0.31	0.32	0.35	1.00	0.64	

Chi_1: primary chimeric axe which emerge from a bicolor holdfast. R_1–R_3: secondary red branches. G_1–G_3: secondary green branches. Chi_2-Chi_3: secondary chimeric branches. a–f: different portions of the same axe from basal to apical. Gray color indicates chimeric or unrelated tissues.

are all consistent with our interpretation. We have no experimental evidence as to the factors responsible for the cell segregation of the two lineages, but physiological differences or local processes of inhibition might play such a role. Our experiments with bicolor plants strongly suggest the inhibitory effects of one cell lineage over the other.

The very low abundance of truly chimeric branches arising from the chimeric holdfast was somewhat unexpected and suggested a rather strict process of cell lineage segregation during axis formation. In just a few axes (1% of the total number of branches observed in our experiment) segregation was incomplete, and the apical portions of branches contained more than one cell lineage. Observations of these plants and branches indicated that, in the next branching generation, most of the new branches were unicolor, attesting to complete segregation. Only in the apices of a few branchlets was there still evidence of the remains of chimeric tissue, likely to be segregated in the next branching generation and thus reversing the chimerism process completely.

The pattern of upright formation exhibited by *M. laminarioides* and *G. chilensis* demonstrates a clear difference with land plants, in which meristems are generally concentrated at the apices of roots and shoots. Expansions of new genetic lines occur when they arise in meristematic areas, which explains why studies on genetic heterogeneity in land plants have focused mainly on apical mosaicism, rather than on somatic fusion or chimerism (Reusch and Boström 2011).

Due to the presence of a wall limiting cell motility, various authors (e.g., Buss 1987, Hughes 2002, Clarke 2013) have included land plants and macroalgae as being representative of a similar type of chimerism. Data gathered in this study suggest the two above groups of primary producers follow very different processes, partially determined by their distinct patterns of body formation and growth.

The negative interaction between different cell lineages is an additional factor that may help to reverse chimerism. As discussed by several authors (e.g., Gill et al. 1995, Fagerström et al. 1998, Poore and Fagerström 2000), mutant cells in a given organism may result in extinction, lead to fixation in a meristem, producing a genetically homogeneous new entity; alternatively, they may persist at a level somewhere between extinction and fixation. In the latter two cases, mitotic cell lineages may develop with phenotypic consequences expressed as a new character given in a ramet or individual. Although seaweeds are a rich source of somatic mutation (Russell 1986, Van der Meer 1990, Santelices 2001, Monro and Poore 2009), their evolutionary significance has only recently been explored, and with special reference to clonal species. In these taxa, cell lineages rather than sexual offspring may act as units of phenotypic selection (Monro and Poore 2004). Thus, intraclonal genetic variation may potentially help clonal organisms to evolve adaptively in the absence of sex (Monro and Poore 2009).

Our experimental studies with bicolor plants showed the branching effects of intra-organismal cell lineage interactions. Comparisons between branching in the respective control (green and red) and the bicolor plants indicated that the active propagation (branching) of one cell lineage (e.g., the green mutant) could result in a reduction in the number of branches produced by the other lineage (e.g., the red one). Both lineages could fully express themselves when forming the control, but the negative effect of one over the other was evident whenever the two lineages were placed to grow together, forming a single entity. Total exclusion of one type of lineage did not occur under our experimental conditions, but it may occur in sporelings formed by a larger proportion of the competitively dominant clone (in this case the green mutant). Total exclusion would also result in chimerism reversal in the next generation of branches.

It could be argued in our experiments that the green or red plants were not adequate controls for

the bicolor plants because, although they were formed by a similar total number of spores, the specific number of green or red spores was different. However, comparisons based on the number of spores (e.g., 10 green spores in the 20-spore bicolor plants vs. the 10-spore control) led to equivalent results. They also suggested the probability of potentially negative interactions between different cell lineages. Thus, these results support predictions by Monro and Poore (2004, 2009) on the evolutionary importance of cell lineages in macroalgae, as well as provide an experimental model that might help to understand better the effects of different selection factors on intra-organismal selection.

A few overall conclusions can be formulated from our results. The various processes, now described as chimerism, are heterogeneous. We were able to distinguish between invasive (as in animals) and noninvasive (as in land plants and macroalgae) chimerism. These two types of processes differ by the presence or absence of mobile and invasive cell lines. In turn, among organisms exhibiting noninvasive chimerism, we were able to distinguish between reversible (red algae) and nonreversible (land plants) processes. The distinction of several types of chimerism suggests that perhaps very different biological processes are mistakenly grouped under a common name.

Contrary to literature predictions (e.g., Buss 1987, Hughes 2002), competitive interactions do occur between nonmotile cells. In this case, motility is replaced by growth. Competitive interactions do also occur between cell lineages in red algal chimeras, facilitating cell segregation during upright axis formation, thus reducing not only genetic heterogeneity in the upright tissue but also determining the replacement of one type of cell by another.

Chimeric red algae exhibit differences in allelic richness between different plant parts. Holdfasts, the most common site of somatic fusion, generally exhibit the highest allelic abundance and diversity. Cell segregation and potential competitive interactions among cell lines may decrease the number of alleles at the upper parts of axes, increasing their genetic homogeneity. This study seems to be the first of its kind to show a consistent pattern of morphological differences in allelic abundance within an organism.

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