# Morphological, ultrastructural, and genetic characterization of coalescence in the intertidal and shallow subtidal kelps *Lessonia spicata* and *L. berteroana* (Laminariales, Heterokontophyta)

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**Abstract** Coalescing macroalgae may fuse with conspecifics, forming genetically heterogeneous entities known as chimera. This process has been shown in taxa from roughly half the red algal orders and in the *Codium* species, a green alga. Field observations indicate that common and dominant kelps along central Chile exhibit a fused holdfast. We evaluated whether such fusions are true coalescence processes in *Lessonia spicata* and *Lessonia berteroana*. To this end, we characterized the ultrastructural event involved in holdfast fusion in the laboratory. Additionally, coalescence in natural populations was quantified by measuring the frequency of individuals with genetically heterogenic stipes within the same holdfast. Results indicate that coalescence appears as a frequent process in laboratory, mostly restricted to intraspecific fusions. During fusion, the meristodermatic cells located in the contact area modify their morphology and reduce the number of plastids, mitochondria, and cell inclusions. The cell wall becomes much thinner and develops plasmodesmata, enhancing communication with equivalent cells of the other coalescencing individual. Stipe genotyping indicates that there is a widespread occurrence of chimerism in both species and genetic heterogeneity is increasing directly with the increasing number of stipes. The combination of results suggests that kelp frequently coalesce in the field, and the histological response observed approaches that of red algae. Since kelps are part of the dominant vegetation in low intertidal and shallow subtidal beds, the adaptive values of coalescence in these species should be evaluated. It is concluded that coalescence and chimerism are evolutionary convergent processes, occurring in all three major groups of seaweeds.

**Keywords** Coalescence · Chimerism · Kelps · *Lessonia* · Microsatellites · Heterokontophyta

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## Introduction

Interorganismal fusions and chimera formation occur in several major groups of organisms including slime molds, protists, macroalgae, fungi, land plants, invertebrates, and vertebrate animals (see Pineda-Krch and Lehtila 2004, for reviews). In seaweeds, such fusions are well known among conspecifics of similar or different karyological phases, sizes, or ages in 7 of the 12–15 orders of Rhodophyta (see Santelices 2004, for review) and in some genera of Chlorophyta (González and Santelices 2008).

Several biological benefits have been described for coalescing individuals including increased size, which is especially important during recruitment since larger sizes increase survival rates (Santelices et al. 1999, 2010; Santelices and Alvarado 2008). Larger initial sizes later result in a larger number of erect axes and branches, higher productivity, and higher fertility during later ontogenic stages (Santelices et al. 1999, 2011; Morley et al. 2003; Shaughnessy 2004). The histological and ultrastructural changes following coalescence in red algae suggest that the process is more complex than a

single fusion of the basal part of the plant. During red algal recruitment and sporeling development, there occurs the establishment of secondary pit connections between neighboring cells from different clusters of spore derivatives. The clusters may later grow together surrounded by a common cell wall (Santelices et al. 1996, 1999). In the case of the green macroalgae *Codium* sp., the process seems to be simpler, principally restricted to intertwining and mixing of the growth filaments from two or more genetically different partners (González and Santelices 2008).

No formal description of coalescence has been documented in brown algae, but pertinent reports are contradictory. After reviewing several studies describing kelp sporophytes with adjacent, seemingly fused holdfasts, Wernberg (2005) concluded that kelp aggregates were much more superficial than the coalescence processes described for red algae. More recently, Vásquez et al. (2008) and Segovia (2009) have reported recurrent interindividual fusions in populations of *Lessonia berteroana* Montagne, resulting in chimeric, plurigenotypic organisms, as described in red algae. However, the nature of the fusion process and the histological and ultrastructural changes involved in brown algal coalescence are yet to be explored. Using populations of the intertidal and shallow subtidal kelp *Lessonia spicata* (Suhr) Santelices and *L. berteroana*, in this study, we first confirmed the frequent occurrence of plurigenotypic individuals of these species in two study localities in central Chile. Then, we cultured sporophytes of both species under controlled laboratory conditions, thereby inducing intraspecific fusions in order to describe the histological and ultrastructural changes involved in the process.

## Materials and methods

We studied two populations of kelp species from central Chile. Individuals of *L. spicata* were collected in Maitencillo (32°37′ S, 71°25′W), 50 km to the north of Port Valparaíso. Specimens of *L. berteroana* were collected at Punta Choros (24°14′S, 71°27′W), 60 km north of Coquimbo. Sampling in both populations was at random, and the only requirement was that the individuals to be removed would be found at least 1 m away from the prior removal.

Morphological and ultrastructural studies of coalescence

*Sporophyte cultivation and induction to coalescence* Fertile sporophytic fronds from 14 individuals collected in Maitencillo (*L. spicata*) and Punta Choros (*L. berteroana*) were brought to the laboratory inside wet plastic bags in refrigerated containers. To stimulate spore release in the laboratory, fragments of mature sori were cleaned with running tap water, rinsed several times with sterile seawater, dehydrated at 14 °C for 2 h, placed into petri dishes (140×20 mm) with a

glass slide at the bottom, and rehydrated with cold (5–8 °C) seawater. Petri dishes were then incubated for spore release for 2–4 h at 14 °C, under cool white fluorescent tubes (20 W/54 RSS day, Philips, Brazil) yielding a photon flux density of 35–45 μmol photons m$^{-2}$ s$^{-1}$. After spore release and attachment (2–3 days), slides with gametophytes were transferred to new petri dishes with 100 mL of culture medium (SFC) (Correa and McLachlan 1991) and incubated in controlled conditions (constant temperature 12°±2 °C, irradiance 20±10 μmol photons m$^{-2}$ s$^{-1}$, photoperiod 12:12 h LD), exchanging the enriched seawater (SFC) two times a week.

Sporophytes appeared after 10–13 days of gametophyte incubation, and 30 days later, a 1-mm-diameter disk was evident in 90 % of the 800 sporophytes being incubated. They were placed in multiplates (TTP), each with six 3.5-cm-diameter and 1.5-cm-deep wells. Coalescence was induced by placing two sporophytes in close proximity within each well in a multiplate. Multiplates were placed in culture chambers under standard culture conditions (12°±2 °C, 20± 10 μmol photons m$^{-2}$ s$^{-1}$, 12:12 h LD). These culture conditions are well known (Avila et al. 1985; Oppliger et al. 2011) to be optimal to cultivate gametophytes and young sporophytes of *Lessonia* species. Proximity between coalescencing sporophytes was maintained by entangling their rhizoids. Culture medium was changed every 4 days. The rate of coalescence was not the same in all wells, but the first coalescent individuals were found around 10 days after inducing coalescence. The rest were maintained either until the sporophytes were dead or coalescence had occurred (no longer than 20 days after induction). The process was similar in the two species studied.

*Ultrastructural studies* Twenty-five coalescing individuals from each species were fixed with 3 % glutaraldehyde, 1 % paraformaldehyde, and 0.1 % caffeine in filtered seawater for 2 weeks at 5 °C. Postfixation was as described in Santelices et al. (1996), but using 2 % O$_5$O$_4$ tetroxide and 1 % potassium ferricyanide followed by dehydration in an ethanol series. Samples were then embedded in Spurr's resin. Staining of semi-thin and thin sections of the contact area between both sporophytes was done according to Reynolds (1963) and Santelices et al. (1996). Observations were made in a Nikon Optiphot-2 microscope, and a Philips Tecnai 12 transmission electron microscope.

Frequency of plurigenotypic chimeric individuals

Natural chimerism was estimated as the frequency of genetically heterogeneous organisms in each population. A genetically heterogeneous individual was defined as one exhibiting genetically different stipes among the total number of stipes growing from a single holdfast. In each population, a total of 14 individuals with holdfast diameters of 10–30 cm were

collected. After removal, holdfast dimension (length, width, and diameter) and number and length of stipes from each plant were measured. A paper towel-dried portion of each stipe was kept in a sealed, previously numbered bag with 15 g of silica gel. After sampling, bags with stipe fragments were stored in the laboratory, at room temperature, until DNA extraction was performed (30–45 days after).

*DNA extraction and stipe genotyping* Genomic DNA from the stipes was extracted using the phenol–chloroform method described by Sambrook et al. (1989) and modified by González et al. (2012). We explored nine microsatellite loci previously developed for those species (under the name of *Lessonia nigrescens*) by Faugeron et al. (2009). Amplifications were carried out using forward primers with a fluorescently labeled M13 tail (Schuelke 2000). Following Faugeron et al. (2009), PCRs were made 12.5 μL and contained 25 ng of template DNA, 0.2 μm of each primer, 0.2 μm of fluorescently labeled (FAM, VIC, or NED) M13 universal primer, 100 μm of each dNTP, 2 mm of $MgCl_2$, 1.25 μL 10× PCR buffer, and 0.5 U Taq DNA polymerase (Invitrogen). Cycling conditions consisted of an initial denaturing step of 5 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at the specific temperature (see Faugeron et al. 2009 for details), 2 min at 72 °C, and a final elongation step at 72 °C for 5 min. 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 310 (Applied Biosystems, USA) and analyzed using Peak Scanner Software version 1.0 (Applied Biosystems). Polymorphisms were evaluated in a total of 91 *L. spicata* stipes, with an average (±SE) of 6.03 (±0.53) of stipes per plant. In the same way, 86 stipes were genotyped in *L. berteroana* with an average of 6.14 (±0.53) stipes per plant. Together with the frequency of chimeric plants in the population, we measured the percentage of genetically different stipes per plant and the number of polymorphic loci for each chimeric plant. Moreover, the relationship between the number of genetically different stipes and the total number of stipes for each species was estimated using Pearson's correlation coefficient. All the analysis was performed using the software STATISTICA (http://www.statsoft.com), and comparisons between populations and species were done using *G* test (Sokal and Rohlf 1995).

## Results

### Morphological and ultrastructural studies of coalescence

Morphological and histological changes during holdfast coalescence in both species of *Lessonia* are very similar.

Therefore, the following description is representative of changes in *L. spicata* and *L. berteroana*. A transection (semi-thin section) through the individual disk before fusion (Fig. 1a–c) shows a thick external cell wall in the ectodermatic (meristodermatic) cells (see arrow in Fig. 1b), which also exhibits an abundant number of organelles (chloroplast (c) and polyphenol (p), Fig. 1c). Ten days after placing the sporophytes in close proximity, the disks fused with each other while still evidencing the narrow contacting zone between the two disks. During the subsequent days, the fused disks increase in size and the contact zone becomes continuous from one to the other holdfast (Fig. 1d–f). Similar transections across fusing disks indicate that the cell wall of ectodermatic cells, near the fusion areas (arrow in Fig. 1e, f), is becoming much thinner. Later, the rounded epidermic cell changes shape, adopting a polygonal shape, increasing size from 20 to 50 μm, and reducing very significantly the number of subcellular organelles (see dotted arrows in Fig. 1f). Thirty days after the initial fusion, only a rounded, elongated disk could be distinguished. In completely fused disks, the prior meristodermatic cells cannot be separated from typical cortical cells, being larger in size and with thin cell walls. Occasionally, a slightly thickened cell wall remains, indicating the previous existence of the fusion zones.

Observations of holdfast shape of growing sporophytes suggest that the disks often are able to fold. Contacting parts may fuse as previously described, experiencing similar histological changes as those described for interindividual fusions. Plasmodesmata (see arrows in Fig. 1g–i for *L. spicata* and Fig. 1j for *L. berteroana*) can be found among fused epidermatic cells that have become cortical cells after fusion, suggesting effective communication among coalesced, previously individual disks. Numerous bacteria are often found bordering the thick external cell wall of individuals in close proximity (see arrows in Fig. 1k for *L. spicata* and Fig. 1l for *L. berteroana*). Equivalent bacteria are not found bordering thick non-coalescing cell walls.

### Frequency of plurigenotypic chimeric individuals

Not all primers gave reliable amplifications for the populations of the two *Lessonia* species studied. Moreover, the locus showed differences in dominance (homo versus heterozygosis), size range of locus (bp), and polymorphism of locus by holdfast (chimeric individual) (Table 1). In general terms, the populations of the two species studied exhibited a high level of natural chimerism (70–90 %) despite a significant difference in abundance of plurigenotypic individuals in both populations ($G=16.5$, $df=1$, $p<0.0000$). In *L. spicata*, 10 out of the 14 individuals sampled (71.4 %) were chimeric, while in *L. berteroana*, 13 out of the 14 individuals sampled (92.8 %) were chimeric.
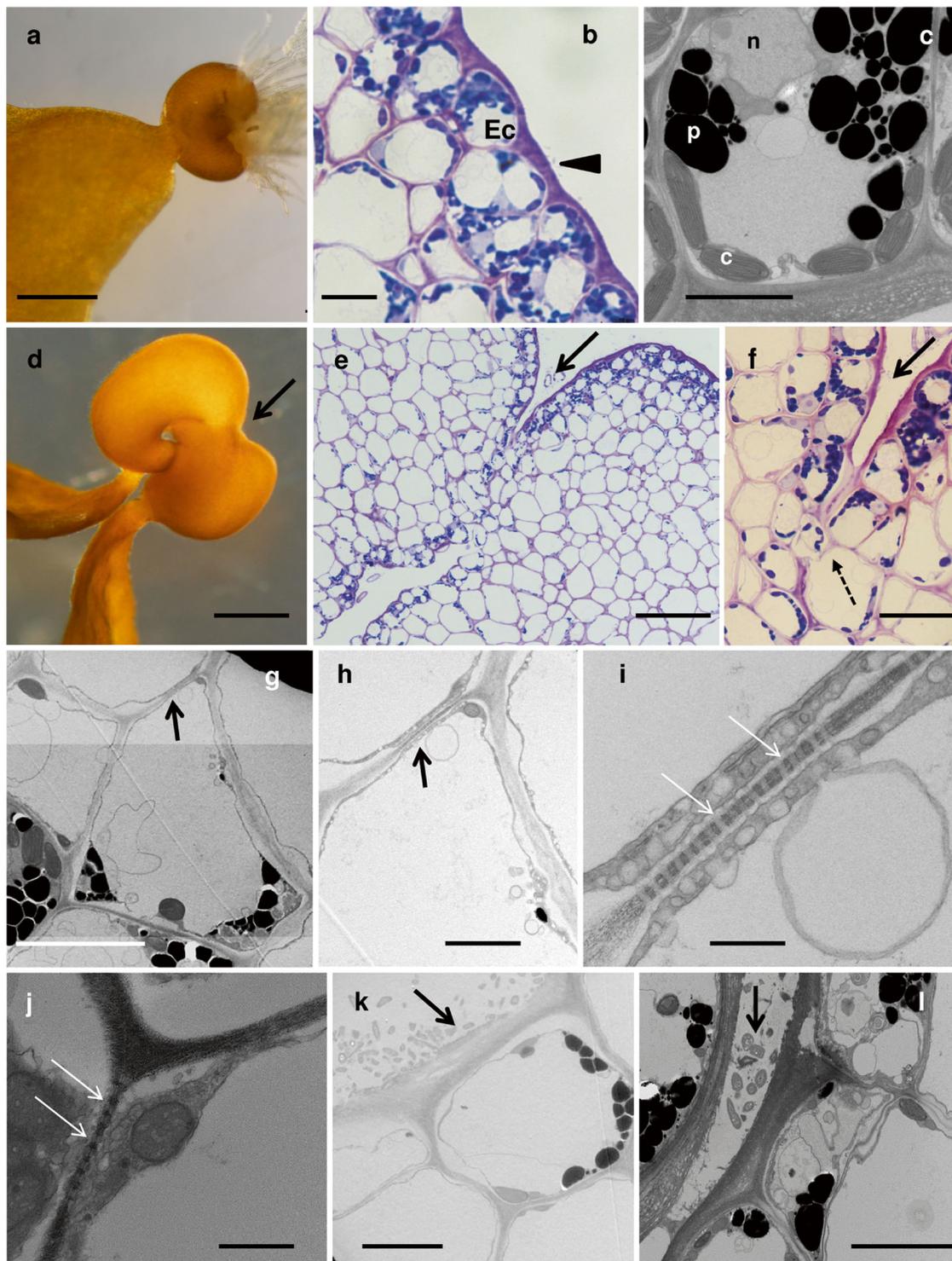
**Fig. 1** Coalescence process in *Lessonia* in laboratory conditions. **a** Individual sporophyte of *L. spicata*. *Scale bar* =500 μm. **b** Median transection to the individual holdfast shows a thick external cell wall (*arrow*) in the ectodermatic (meristodermatic) cells. *Scale bar* = 10 μm. **c** Normal epidermic cell with nucleus (*n*), numerous chloroplasts (*c*), and polyphenol (*p*). *Scale bar* = 5 μm. **d** Two individuals of *L. spicata* after 15 days of fusion. *Arrow* shows contact zone between holdfast. *Scale bar* =1 mm. **e**. Semi-thin section of the contact zone of *L. spicata*. *Scale bar* =100 μm. **f** Close-up of **e**, showing that the cells located at the initial of contact zone (see *arrow*) are starting their morphological change. *Dotted arrow* shows increase in size and lack of organelles in cells. *Scale bar* =10 μm. **g** Fine section showing modified cell shape (polygonal) and plasmodesmata between cells (*arrow*). *Scale bar* =10 μm. **h** Close-up in plasmodesmata, in contact zone of two neighboring cells. *Scale bar* =2 μm. **i** Close-up of plasmodesmata showing numerous cellular contacts between neighboring cells (see *arrows*) in *L. spicata*. *Scale bar* =500 nm. **j** Close-up in zone with complete process of coalescence between cells, contacted by plasmodesmata in *L. berteroana* (see *arrows*). *Scale bar* = 500 nm. **k, l** Close-up between cell in close proximity with numerous bacteria (*arrow*) bordering the thick external cell wall of individuals in *L. spicata* and *L. berteroana*. *Scale bar* = 5 μm

**Table 1** Characterization of dominance, size range (bp), and chimeric holdfast (%) by microsatellites studied in *L. spicata* and *L. berteroana*

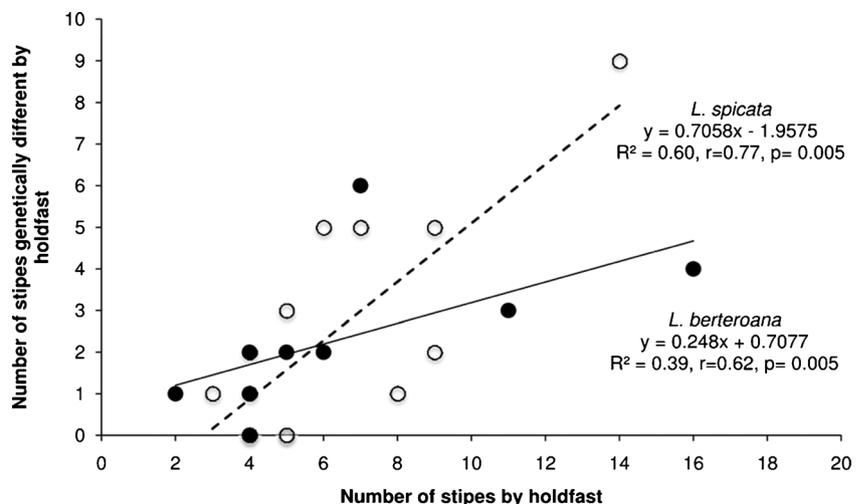| Locus | *L. spicata* | | | *L. berteroana* | | |
|---|---|---|---|---|---|---|
| | Dominance | Size range (bp) | Chimeric holdfast (%) | Dominance | Size range (bp) | Chimeric holdfast (%) |
| LESS2D1 | Homozygote | 314 | 0.0 | Homozygote | 295-300 | 57.1 |
| LESS1T3 | Homo/heterozygote | 135-152-171 | 21.4 | Homo/heterozygote | 135-156-165-171 | 28.6 |
| LESS1D4 | Homo/heterozygote | 165-171 | 28.6 | Homo/heterozygote | 168-171-188 | 28.6 |
| LESS1T9 | Homo/heterozygote | 254-284-289-292 | 21.4 | No amplification | – | – |
| LESS1T11 | Homo/heterozygote | 154-254 | 28.6 | Homozygote | 154 | 0.0 |
| LESS1D17 | Homo/heterozygote | 299-360-370-380 | 57.1 | Homo/heterozygote | 284-299-316 | 21.4 |
| LESS2D22 | Homo/heterozygote | 117-121 | 0.0 | Homo/heterozygote | 127-137 | 0.0 |
| LESS2D25 | Homozygote | 228 | 0.0 | Homo/heterozygote | 255-262-264-295 | 42.8 |
| LESS2D26 | Homozygote | 153 | 0.0 | Homozygote | 150-155-157 | 14.2 |

In terms of polymorphic stipes, *L. spicata* showed an average of 47.7±6.2 % of genetically different stipes in the same individual, a value that is not statistically different ($G = 0.427$, $df = 1$, $p = 0.513$) from that of the polymorphic stipes found in *L. berteroana* with an average of 43.1±4.5 % genetically heterogeneous stipes. In the same way, the chimeric individuals of both species did not show significant differences in the number of loci that detect chimeric holdfasts (Table 1). However, different loci can capture chimeric individuals between species. For example, LESS1D17 is the loci with the largest genetic variability among the stipes in *L. spicata*. By contrast, LESS2D1 is the loci with higher chimeric individual in *L. berteroana* (Table 1). In both species, the relationship between total number of stipes and number of genetically different stipes was positive and statistically significant ($r = 0.77$, $p = 0.005$ in *L. spicata* and $r = 0.62$, $p = 0.005$ in *L. berteroana*; Fig. 2).

## Discussion

The set of results described in this paper suggests that intraspecific coalescence and the subsequent natural chimerism is a common response in populations of two species of intertidal *Lessonia* occurring along the Chilean coast. Chimerism values between stipes of 71 and of 93 % among individuals of *L. spicata* and *L. berteroana* populations indicate a high frequency of the process, while histological and ultrastructural studies of coalesced disks confirm that the nature of the process is more complex than the simple disk fusion occurring during plant ontogeny.

The histological and ultrastructural changes that occur during coalescence primarily involve meristodermatic cells (ectodermic) of the disks in contact. The cell wall of these thick ectodermal cells thins considerably. Simultaneously, a reduction in subcellular organelles, mainly plastids, a significant increase in cell size that doubles or triples in size, and a

**Fig. 2** Pearson's correlation between the number of genetically different stipes and the total number of stipes for *L. spicata* (*open circles*) and *L. berteroana* (*black circles*)



*L. spicata*
y = 0.7058x - 1.9575
R² = 0.60, r=0.77, p= 0.005

*L. berteroana*
y = 0.248x + 0.7077
R² = 0.39, r=0.62, p= 0.005

change in shape, to a polyhedral or rounded form, occur. In later development stages, plasmodesmata can be found between both partner cells that have come into contact. The set of ultrastructural changes described above that involves the transformation of ectodermal into cortical cells has only been observed in *Lessonia* and seems to characterize the coalescence processes among developing kelps. Ongoing studies are finding similar responses among individuals of other species of *Lessonia* and *Macrocystis pyrifera*. The establishment of plasmodesmata between cells of different disks calls to mind the establishment of secondary pit connections between coalescing red alga cells (Santelices et al. 1999). However, the ultrastructural changes observed in ectodermatic disk cells in *Lessonia* sporophytes were not previously observed in any other types of alga.

The collection of cellular modifications and ultrastructural changes that occur during coalescence processes is interpretable as the removal of barriers that could interfere with the displacement of photosynthates inside the disk. From the study by Lobban (1978), it is known that photosynthate translocation exists along the length of the kelp plant. Translocation is especially important for the survival of the basal parts of these plants that are often shadowed by the upper parts of its own canopy. Given that the disk is the portion of the plant that provides support against the pulling effect exerted by the water on the plant (Denny 1985), the energy requirements are high, and therefore, the uninhibited movement of photosynthates inside the disk, without barriers to its displacement, may be paramount. Future experimental studies of substance translocation in the disk with different types of barriers should provide additional information regarding this interpretation.

Studies of red algae (reviewed in Santelices et al. 2011) have shown that coalescence increases survival during early recruitment due to the larger size of the coalescent plants. In later development stages, the coalescing plants form a canopy with more branches and greater productivity, and during reproductive periods, the increased number of branches and blades results in a greater number of fertile branches and more fertility, compared to individual non-coalescing plants. It is not known whether similar or equivalent adaptive advantages occur in coalescing *Lessonia* plants, but future experimental work should test these ideas. The adaptive value of coalescence could be particularly important, both from the ecological and economical perspective, given the biological and commercial importance of the kelp species in the intertidal and subtidal environments of temperate-cold waters in both hemispheres.

The combination of results obtained in this study suggests that coalescence is a common process in kelp species from the central Chilean coast. The process exhibited three distinctive characteristics in *Lessonia* species: First, it is a convergent response, due to similitude to coalescence described in Rhodophyta species, and it probably occurs in many other types of kelp. Second, it involves morphological responses at histological level, including modification of cellular layers and formation of plasmodesmata. Third, in natural conditions, it involved the formation of genetically heterogeneous entities; thus, genetic heterogeneity might correspond to an adaptive trait, associated to coalescence, providing increased variability and consequently different tolerance in the chimeric individual.

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