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Advances in the study of intraclonal variation in *Gracilaria chilensis* (Rhodophyta)

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Abstract: The search for causes of intra-clonal variation in *Gracilaria chilensis* has lead to studying the effects of physiological and development factors, pathogenic infections, genetic instability and sporeling coalescence on this response. Results suggest intra-clonal variation is a frequent process that could explain morphological variation among thalli, physiological variation among ramets and local population variation. Intra-clonal variation, in addition, has significant importance with respect to strain selection of economically important seaweeds.

Résumé : La recherche des causes des variations somaclonales chez *Gracilaria chilensis* a conduit à étudier les effets des facteurs physiologiques, du développement, des infections par des pathogènes, de l'instabilité génétique et de la coalescence des spores sur cette réponse. Les résultats de ces études suggèrent que la variation somaclonale est un processus fréquent qui peut rendre compte des variations morphologiques des thalles, des variations physiologiques entre ramètes et des variations dans les populations locales. De plus, la variation somaclonale revêt une importance particulière dans le cas de la sélection de souches d'algues marines cultivées.

Keywords: Intra-clonal variation, *Gracilaria*, sporeling coalescence, genetic variability, chimeric tissues.

Introduction

Intra-clonal variation is defined as the occurrence of significant phenotypic differences among ramets derived from the same genet. This process is frequent among clonal invertebrates and land plants (Buss, 1985; Harper, 1985; Silander, 1985; Watkinson & White, 1986) and known to result from the single or interacting effects of four principle factors. These include differences in the microenvironment surrounding each ramet during growth, physiological or developmental differences among genetically similar ramets, localized infections by pathogens and genetic changes differentially affecting one or a few ramets. The last three factors may induce significant differences among ramets derived from the same genet even when incubated under similar culture conditions.

Similar studies of inter-clonal variation with seaweeds were initiated only in the last decade. Up until then, the assumption that most seaweeds are unitary individuals has restricted their population and genetic studies to models derived from such kinds of organisms only (Santelices, 1999). However, several types of seaweeds, including the species of *Gracilaria*, grow and propagate by self-replication of genetically identical units (Cook, 1985; Jackson et al., 1985). When natural or artificial thallus fragmentation occurs in these seaweeds, each of the

resulting units has the capacity to function on its own and to regenerate the whole plant. In fact, commercial farming of species of *Gracilaria, Eucheuma* and *Chondrus* is based on their clonal propagation capabilities. Therefore, it should not be surprising, that since the early 70's the pertinent literature on large-scale cultivation of the above genera contains evidence of intra-clonal variation in seaweeds.

This study first reviews the kinds of intra-clonal variation so far described for various seaweed species and then concentrates on the recent advances achieved in the study of factors inducing intra-clonal variation in seaweeds.

Intra-clonal variation in seaweeds

Intra-clonal variants should be searched among mitotically replicated stages occurring in the life history of any given seaweed. In the red algae there are two such stages, branches (ramets) produced by cell divisions of lateral initials and mitotically replicated carpospores.

Pigment and morphological variants are the most frequently described types of intra-clonal changes in growing branches and branchlets. Colour or morphological changes generally appear first at the tip of branches and branchlets. As growth progresses and the branchlet elongates, part of the clone becomes a different colour or develops a different morphology (Fig. 1). Eventually, such a part of the clone may become separated from the rest of the clone and propagated within the population, producing a new colour or a new morphological variant in the population (Santelices & Varela, 1993). These new colour or morphological variants have been reported to arise spontaneously in experimental or pilot cultivation of several commercial red algae, including species of *Gracilaria, Chondrus* and *Kappaphycus* (see Patwary & van der Meer, 1992 and Santelices, 1992 for reviews).

Pigment variants also exist among mitotically replicated carpospores, as van der Meer & Zhang (1988) reported in species of *Gracilaria*. They found masses of reddish

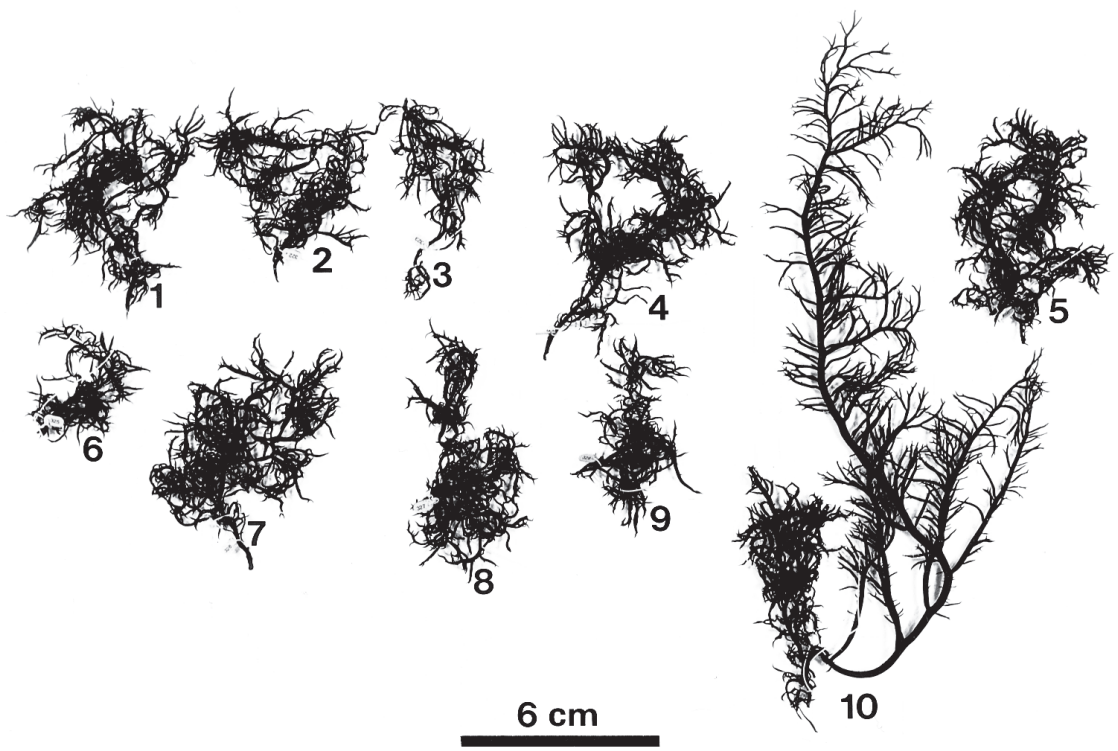

Figure 1. Morphological variant arising from a clonal replicate of *Gracilaria chilensis*. The 20 fragments in this and the next figure were excised as 1 cm-long branchlets from a single, unisporic, laboratory-grown plant. The 20 clonal replicates have been cultivated for 230 days. The morphological variant (replicate Nº 10) grows at four times the average rate of the other nine fragments.

Figure 1. Variant morphologique provenant d'un clone de *Gracilaria chilensis*. Les 20 fragments de cette figure et de la suivante ont été excisés de branches de 1 cm de long d'un thalle unique, monosporique et cultivé en conditions contrôlées au laboratoire. Les 20 clones obtenus ont été cultivés pendant 230 jours. Le variant morphologique (replicat nº 10) croît quatre fois plus vite que les neuf autres fragments.

carpospores, in addition to massive production of green carpospores, in green (mutant) cystocarps formed from a green fertile portion of a green female mutant fertilized with spermatia produced from a green male thalli. Many of the tetrasporophytes derived from the reddish carpospores were of the red wild-type.

Physiological variants are more difficult to detect but also they have been found in mitotically replicated growth stages of *Gracilaria chilensis* (Santelices & Varela, 1993). Experimental incubation of populations of sporelings, each grown under similar culture conditions and derived from carpospores shed by the same cystocarp exhibited significant differences in growth and performance both at 30 days and 100 days of growth. On the other hand, ramets derived from the same genet and grown under similar conditions also exhibited significant variation in growth rates (Fig. 2).

Physiological variants, as compared to morphological or pigment variants, are more difficult to detect as their identification normally requires controlled experimental conditions and statistical analysis. Care has to be taken to handle all ramets in a given experiment in the same way and to provide identical growth conditions to all replicates in order to reduce differences in the micro-environment around the ramets. In addition, physiological or developmental differences among ramets have to be reduced as much as possible by the use of ramets of similar orders of branching, equivalent position along the axes and similar number of branchlets. To identify the individual ramet as a variant departing significantly from the confidence limits of the population mean value, a correlation between performance and another character has to be established. In the case of *Gracilaria chilensis*, experimental incubation under controlled conditions indicated that biomass increments were a function of initial weight as long as the algal biomass was maintained within the carrying capacity of the culture medium (Santelices & Varela, 1993; Santelices et al., 1995). The magnitude of the departure of extreme data points could then be tested against the confidence limits of the population determined by the regression equation.

Causes of intra-clonal variation in *Gracilaria chilensis*

The same kinds of factors causing intra-clonal variation in other organisms affect seaweeds. As indicated previously, these factors include differences in the microenvironment surrounding each ramet, physiological and developmental differences among ramets, localized genetic changes and pathogens affecting only a part of the genet. In recent years, sporeling coalescence has been added, a factor that seems to be exclusive of seaweeds (Santelices et al., 1996; 1999).

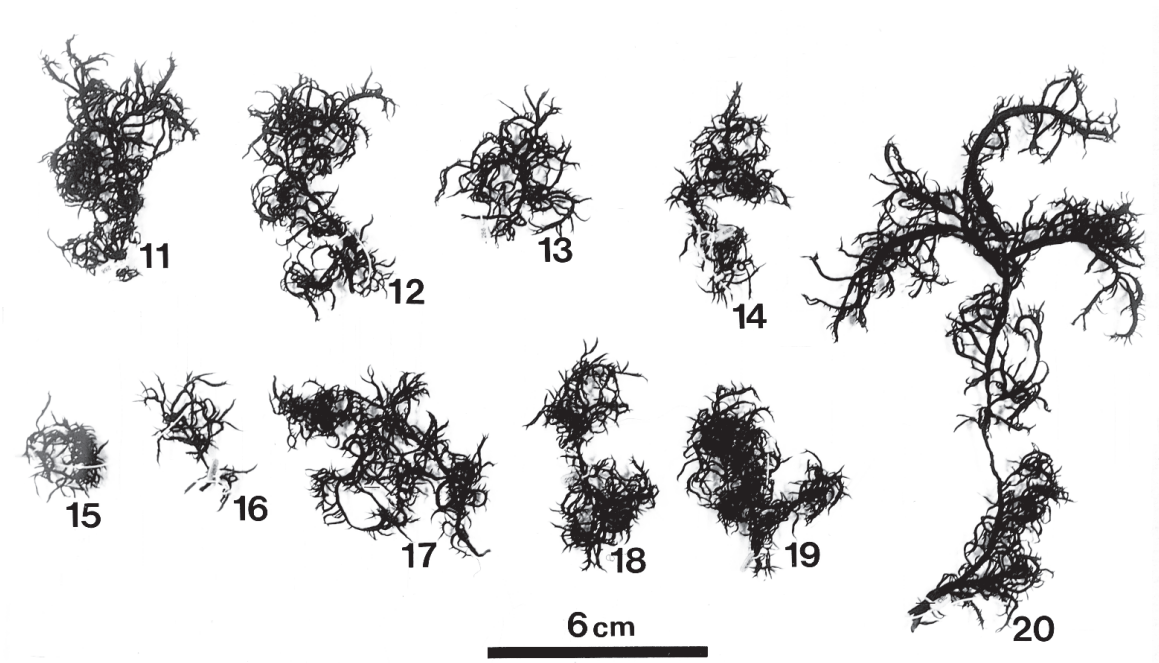

Figure 2. Physiological variant of *Gracilaria chilensis*. In this case the clonal variant (thallus Nº 20) maintains the original branching pattern but its growth rate is three times the average rate of the other nine fragments.

Figure 2. Variant physiologique de *Gracilaria chilensis*. Dans ce cas le clone variant (thalle nº 20) maintient le patron de ramification initial mais son taux de croissance est trois fois supérieur à celui des neuf autres fragments.

Studies with *Gracilaria chilensis* developed over the last five years have advanced our understanding of the effects of most of these factors on intra-clonal variation.

Physiological and developmental factors

Physiological or developmental differences among ramets may induce intra-clonal variation in morphology, growth or performance. In the case of *Gracilaria chilensis*, a few such differences seem to be important. Removal of the apical tip of branches in some strains may induce increments in higher orders branching (Santelices et al., 1995). Thus, differences in branching patterns and consequently in growth and performance among genetically identical ramets, may be induced by the removal (e.g. through grazing) of the apical tip of a ramet.

Experimental studies (Santelices & Varela, 1995) also have shown that elongation rates and rate of weight increase in young ramets (up to 20 cm long) are a function of the initial length of the ramet, suggesting that intercalary growth makes a significant contribution to growth and performance at these young stages. Thus, slight differences in initial length among genetically similar ramets may cause significant variability in growth rates and performance at least during these early stages of development.

Reproductive state of the ramet also is a factor to consider. In all experiments, weight and length increments shown by vegetative ramets were significantly greater (Santelices & Varela, 1995) than the values shown by fertile cystocarpic branches. These results are consistent with resource allocation theory, which predicts that reproduction imposes a cost on an organism (De Wreede & Klinger, 1988; Santelices, 1990). Therefore, differentiation of reproductive structures in a given ramet may induce intra-clonal differences in growth and performance. While cystocarps are macroscopic and visible to the naked eye, spermatia and tetraspores in this species are microscopic and dispersed over the whole thalli. Therefore, it is not always easy to detect the presence of this factor which otherwise could induce significant variation among branches of the same clone.

Pathogen infections

Localized infections within a clone also may induce intraclonal variation in phenotypic responses. The effects of pathogens may be highly localized or more widespread, depending on the age, cell wall composition (Craigie et al., 1992), the structural, metabolic (Evans et al., 1978; Correa & McLachlan, 1991; Pickering et al., 1993) and reproductive (Correa & Sánchez, 1996) characteristics of the host.

In the case of *Gracilaria chilensis*, the main pests appear to be epiphytic algae. The species composition, frequency, prevalence and average loads of these epiphytes vary among habitats and cultivation systems (see Buschmann et al., 1995 and 1997 for reviews). On the other hand, the species seems to be free of endophytic algae, fungi and bacteria. The only important endophyte so far isolated from *Gracilaria chilensis* is an amoeba able to induce whitening of the alga, tissue softening and thallus fragmentation and decay (Correa & Flores, 1996).

Genetic changes

As stated earlier, intraclonal variation also can arise from localized genetic changes. These may include somatic (mitotic) recombinations, gene duplications as a result of errors during mitosis, or changes derived from the presence of mobile genetic elements (transposons). Some of these sources of variation have been identified in several species of *Gracilaria*. Although the frequency of mitotic recombinations in *G. chilensis* is unknown, in *G. tikvahiae* it is high (van der Meer & Todd, 1977) and they may occur more than once in a cell line.

If mitotic recombinations, or other sorts of DNA turnover, occur due to replication activities during mitosis, then, in vegetatively growing clones such as those of *Gracilaria chilensis*, genetic variability should increase with growth, as the probabilities of DNA turnover increases with the rate at which cellular divisions occur. To test this idea, clonal replicates of *Gracilaria chilensis* were grown under controlled laboratory conditions, simultaneously measuring genetic variability as detected by DNA-fragment polymorphism using RAPDs-PCR and total growth. The results suggest (Meneses et al., 1999) that as growth occurs and biomass accumulates, the genetic variability increases. Therefore, clones being massively propagated by thallus fragmentation, either in naturally grown or in farmed populations are suffering much more genetic changes than previously thought. They might constitute a powerful mean to generate intra-population variation even under environmental conditions not allowing successful completion of the sexual cycle.

Additional experiments with *Gracilaria chilensis* have shown rapid and significant changes in genetic variability after being transferred to new environments (Meneses & Santelices, 1999). Transferring wild individuals to controlled laboratory conditions has resulted in reductions of genetic variability as laboratory-produced branchlets exhibited less variability (evaluated as total DNA polymorphism) than branchlets in plants recently collected in the field. Transfers from the controlled laboratory conditions to large-scale cultivation systems additionally reduced the total range of variation, increasing the similarity values among branchlets.

An additional point emerging from the above studies (Meneses & Santelices, 1999) relates to the importance of the external environment in determining these changes. Due to growth-related changes in genetic variation, the emergence of new branchlets often results in new gene combinations. Since the external environment always influences the gene combination to be fixed by selection, the performance of a ramets becomes thus a continuous reaction between genotype and environment, expressed at any particular time and place. The performance is likely to change either due to genetic changes or to environmental modifications.

Sporeling coalescence

Sporelings of *Gracilaria chilensis* have the ability to form basal crusts that may grow together, forming a completely coalesced mass that subsequently develop into a single plant (Maggs & Cheney, 1990; Muñoz & Santelices, 1994). Therefore, spore coalescence might allow the occurrence of unitary thalli that in fact correspond to genetically different, coalesced individuals. If that is the case, plant portions derived from these chimeric individuals may exhibit dissimilar growth responses (= intra-clonal variation) even when incubated under similar abiotic conditions.

Testing the above hypothesis included various approaches (Santelices et al., 1996). TEM observations of early stages of sporeling coalescence indicated that polysporic plantlets were formed by coalescence of groups of spores and its derivatives. Even though adjacent cells in two different groups may fuse, these groups maintained an independent capacity to grow and form uprights. Construction and growth of bicolour individuals further confirmed the chimeric nature of the coalesced individual. Coalesced, bicolour holdfasts had green and red cells, which subsequently produced green and red uprights, respectively. Individuals fronds were also chimeric, as indicated by the production of green and red branchlets from single, red uprights. The existence of mixed tissues was further substantiated by random amplified polymorphic DNA (RAPD's) analysis. The banding pattern produced by branchlets of a unisporic thallus was consistently monomorphic, whereas the pattern produced by the polysporic thallus were polymorphic. Growth rate of polysporic thalli had larger data dispersal and variation coefficients than oligosporic or monosporic thalli. Therefore, all results supported the hypothesis that spore coalescence may cause intra-clonal variation. In turn, spore coalescence result in higher variability in physiological responses as consequence of the existence of chimeric thalli.

More recent studies (Santelices et al., 1999) have shown that coalescence is widespread among members of roughly half of the number of Orders presently distinguished in the Florideophycidae, including species in the Ahnfeltiales, Corallinales, Gigartinales, Gracilariales, Halymeniales, Palmariales and Rhodymeniales. Therefore, intra-clonal variation should be widespread among species of these algal groups.

Conclusion

The combination of results gathered with *Gracilaria chilensis* suggests that intra-clonal variation should be a type of response frequently found in wild and cultivated populations of red algae. As expected, several physiological and developmental factors, as well as localized pathogen infections may induce inter-ramets differences in performance. The pattern emerging from measurements of genetic variability, as detected by DNA-fragment polymorphism, suggests a fast and dynamic change which is coupled to growth and occurs in a continuous reaction with the environment. Thus, genetic changes, due to somatic recombinations and other kinds of DNA turnover are always occurring in various magnitudes during branch production. Studies on sporeling coalescence suggest, on the other hand, the probability that many organisms considered unitary would exhibit intra-clonal variation due to their chimeric nature derived from sporeling coalescence. Coalescence seems to be widespread among red algae, confirming the expectation that intra-clonal variability due to tissue mosaicism should be frequent among red seaweeds.

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