Cah. Biol. Mar. (2001) 42 : 45-51

Insights on the biology of *Gracilaria chilensis* using molecular techniques.

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Abstract: Studies in populations of *Gracilaria chilensis* are incipient along the coasts of Chile. Although this is the species that has driven more attention in research studies, due to its commercial value, these have been most frequently oriented to the development of management and cultures. Rather than population studies *per se*, the purpose of population surveys has been an attempt to include the phenetic variability occurring in *Gracilaria chilensis* in taxonomic research. This has been accomplished, within and between populations using morphological features as well as molecular techniques. In fact, the application of molecular techniques in this area is fairly recent in *Gracilaria chilensis*. Nevertheless, the use of these techniques, concomitant to studies oriented to disclose the basic biology of this taxon, have been able to show characteristics particular to it. The first is the frequent vegetatively spreading by fragmentation in *Gracilaria chilensis*, allowing the existence of large stocks of clonal thalli. The second is the occurrence of spore coalescence during the germination process, which ultimately results in a thallus morphologically resembling a single individual but genetically heterogeneous. The third is the occurrence of mitotic recombination at the tip of the branches during the process of active growth. These three characteristics imply a population genetic dynamics that differs from that of organisms with purely meiotic and sexual means of recombination.

Résumé : Des études de populations de *Gracilaria chilensis* ont récemment débuté tout le long de la côte chilienne. Bien que cette espèce ait retenu le plus l'attention dans les programmes de recherche, en raison de sa valeur commerciale, ces derniers ont été orientés le plus souvent sur la gestion de la ressource ou le développement des cultures. Plutôt que des études de populations en soi, les objectifs des observations se sont confondus avec celui d'une caractérisation de la variabilité phénotypique dans le cadre d'études taxonomiques de *G. chilensis*. Cela a été accompli, au sein et entre populations, à l'aide de caractéristiques et de techniques morphologiques et moléculaires. En fait, l'emploi des techniques de biologie moléculaire dans ce domaine est relativement récent chez *G. chilensis*. Cependant, ces techniques utilisées dans le cadre d'études des caractéristiques de base de la biologie de cette espèce se sont montrées capables de révéler des caractéristiques particulières à ce taxon. La première concerne la reproduction végétative par fragmentation du thalle, courante chez *G. chilensis*, produisant de larges stocks de thalles apparemment clonaux. La seconde concerne la coalescence des spores pendant le processus de germination, ce qui donne un thalle ressemblant morphologiquement à un individu, mais génétiquement hétérogène. La troisième est l'existence de recombinaison mitotique dans les apex lors de phases de croissance active. Ces trois caractéristiques impliquent une dynamique dans la génétique des populations qui diffère de celles d'autres organismes à recombinaison d'origine purement méiotique lors de la reproduction sexuée.

*Keywords***:** *Gracilaria chilensis*, mitotic recombination, vegetative propagation, clonal thalli, RAPD, RFLP.

Introduction

Gracilaria spp. have been harvested in Chile for several decades due to its commercial importance in the elaboration of agar. A heavy exploitation without any legal regulation eventually led to the exhaustion of natural populations. This fact forced the development of management programs (Santelices et al., 1984; Poblete, 1986; Westermeier, 1986) and of massive cultures (Santelices & Doty, 1989). These farming efforts have been largely localized in areas where natural populations originally existed or where *Gracilaria* natural stands still remain (Santelices & Fonck, 1979; Romo et al., 1979). These areas showed certain environmental characteristics that facilitate *Gracilaria* growth. Furthermore, some of these areas showed plants with higher average growth rates than those from other localities and this was attributed to the occurrence of different species or strains being cultured. Under this assumption, the plants that had these desirable characteristics were used as source of material to start or replace thalli growing in other localities. Thus, eventually the cultivated stands of *Gracilaria* became a combination of native and transplanted thalli that entangled the recognition of the possible different species or strains present at each locality.

The need for identification of those commercially important species and the fact that the taxonomy of the genus was currently revised around the world, generated several studies on *Gracilaria* along the Chilean coasts. Major attempts in species differentiation and recognition were made using all possible morphological characters that could be evaluated such as: branching level and characteristics, axes girth, apex shape, number and size of cortical and medullary cells among others.

Since environmentally controlled variability was detected in most of these morphological characters, it was mandatory the use of alternative means to study the identity of species, ecotypes and strains possibly involved in this complex. In addition, the frequent difficulty in finding male reproductive structures in natural and artificial stands of *Gracilaria*, considered one of the most definitive features for identification, also forced the use of other techniques for taxa recognition rather than morphological features. At the same time, molecular techniques, originally developed in other organisms, were being applied to a number of seaweeds (Goff & Coleman, 1988; Bird et al., 1990; Patwary et al., 1993; Dutcher & Kapraun, 1994; Ho et al., 1995; Van Oppen et al., 1995).

The following is a synthesized account of the application of some of these techniques to studies in *Gracilaria* spp. in Chile and the repercussions that some of the results obtained have had on different areas of its research.

Taxonomic Studies

The commercial importance of *Gracilaria* is probably the main reason this alga has received plenty of attention in seaweed descriptions and records of the Chilean coasts (Levring, 1960; Santelices, 1988; Ramírez & Santelices, 1991). Although several species have been described, all share a cylindrical thallus with an irregular branching pattern that makes species distinction remarkably difficult. The establishment of the characteristics of the male spermatangia (Yamamoto, 1975; 1978; 1984) as a clear specific character allowed the determination with certainty of two species of the order Gracilariales, *Gracilaria chilensis* (Bird et al., 1986) and *Gracilariopsis lemaneiformis* (Dawson et al., 1964) in Chile. Nevertheless, as a result of the transplants performed among natural and artificially cultured beds, it was not clear whether more than these species occurred.

The pursuing of the taxonomic determination of the possible species, subspecies or strains of the genus *Gracilaria* distributed along the coasts of Chile, induced the use of combined characters, morphological and molecular, in the study of populations (Meneses, 1996b). Previous to the application of molecular techniques, some attempts were made utilizing multivariate methods to analyse only morphological features (León, 1990, Meneses, 1996a). Some tendencies towards morphological segregation were reported, however these were often related to the prevailing conditions under which the plants were growing rather than being ground for taxa distinction.

Comparisons among six populations in five different localities based on 15 vegetative characters using cluster analysis (UPGMA) resulted in the clustering of two basic groups each encompassed neighboring and distant populations along the coast of Chile (Meneses, 1996a). The first group encompassed populations from Maullín (41º40’ S, 73º45’ W), Niebla (39º51’S, 73º081W) and Coquimbo (29º58’S, 71º22’W), and the second group included Lenga (36º45’S, 73º11’W), Coquimbo and Chañar (27º33’ S, 70º01’ W). Both groups clearly separated the populations according to the respective morphologies of their plants. The first group included short, bushy, densely branched thalli, while the second included long slender plants scarcely branched. However, Chañar was an artificial culture, started with plants that came originally from a natural population from Maullín and both localities did not share the same cluster. Likewise, the two populations sampled in Coquimbo belonged to different morphological clusters although one was artificial and the other a relict of a natural population. The reason that these populations were grouped differently was simply that the morphology of their plants differed due to the distinct habitats in which they grew. While one was located in sandy bottom in the open

bay (3-6m depth), the other was growing on rocks in the shallow subtidal. Thus, morphologies had drifted apart according to the environmental conditions although the thalli had the same origin (Meneses, 1996a).

Later the same populations were analysed applying a molecular technique, RAPD (Random Amplified Polymorphic DNA) to the DNA from samples of each of these populations. This technique (Welsh & McClelland, 1990; Williams et al., 1990) consists on the generation of arbitrary DNA fragments by amplification using randomly generated primers. These primers (usually 10-mer) would annealed with those complementary sequences present in the DNA sample and thus the enzyme polymerase (also added to the reaction solution) would build fragments of different size. Different DNA sequences will result in a battery of polymorphic DNA fragments that may be used as a "finger print" to recognize each sample. The DNA of a species similar in morphology to those taxa from Chile, but from an allopatric population (China), *Gracilaria tenuistipitata*, was included in this study for comparative purposes (outlier). As a result, the variability obtained in the fragments of DNA from the different populations indicated that the degree of differentiation among populations was not enough to warrant separation at the species level. Therefore its was concluded that, at least representatives of these populations, did all belong to *Gracilaria chilensis* (Meneses, 1996b).

In a different study, *Gracilaria* from the locality of Lenga (36º45'S, 73º11'W) showed 3-4 different phenotypes that included variations in morphology and color (Gonzalez et al., 1995). A molecular technique previously used in algae (Goff & Coleman, 1988; Bird et al. 1990), RFLP or Restriction Fragment Length Polymorphism was applied to organellar DNA from these phenotypes to detect whether they were the result of different taxa. RFLP is a technique consisting in the addition of endonucleases or restriction enzymes to isolated organellar DNA. Since each enzyme digests the DNA in a particular sequence of bases the amplification of the products of this digestion is a number of fragments of different molecular size that can be separated by electrophoresis in an agarose gel. RFLP showed no polymorphism in the fragments of plastidial DNA among the previously distinguished phenotypes (Gonzalez et al., 1995).

Variations in morphology in those taxa of cylindrical *Gracilaria* where the thallus has an irregular branching pattern (Abbott & Norris, 1985) are frequently determined by the growing conditions thus, interpreted as a result of phenotypic variability. Color however, may be a different phenomenon. Gradients of colour in *Gracilaria*, as well as in other genera of Rhodophyta, are usually the result of growing exposed to different light conditions. In fact, in plants of *G. chilensis*, the same thallus shows an intense red color in those portions of the plant that are buried in the sand while it is light brownish in the exposed axes. However, colour variants not resulting from environmental conditions but deriving from genetic recombination (van der Meer, 1979; van der Meer & Zhang, 1988) are well known in the genus and are useful genetic markers (van der Meer & Todd, 1977). The lack of polymorphism detected among the colour phenotypes in Lenga could be a result of the use of RFLP, which is known to detect less polymorphism than other molecular techniques (Rafalski, 1994), rather than the occurrence of purely phenotypic colour variants.

Mosaicism in *Gracilaria chilensis*

Spore coalescence, a different phenomenon involving colour variants in some cases, has been studied in *Gracilaria chilensis*, (extensively analysed in B. Santelices' contribution to this workshop). Genetic variation in *G. chilensis* as a result of spore coalescence has not been evident until it was observed with genetically coloured variants (green and red- Santelices et al., 1996). Juvenile plants were obtained in laboratory by growing spores from different colour variants (polysporic) that eventually coalesced and formed a single disc from which upright axes originated. A retarded display of the green colour was observed in these polysporic plants after 70 weeks of growth in laboratory. This could be either a result of some sort of developmental dominance of one cell variant over the other or, a result of the differential interaction of certain environmental conditions with each cell type. However, the most interesting fact was that not only did green axes arise from the adhesion disc but that green branchlets were sharply originated from red branches (Santelices et al., 1996). Under these assumptions, it is difficult to explain the appearance of colour variants in branchlets, unless some genetic recombination has occurred. Coalescence has been obtained in laboratory conditions, not only among carpospores of the same cystocarp, but also among spores of different cystocarps of the same thallus, among spores of different thalli of the same or different life-history phases, and among spores from different species. This indicates high tissue compatibility within the genus not yet tested in nature, something that could be done with molecular techniques probably other than RFLP (Parker et al., 1998).

This alternative molecular technique considered was Random Amplified Polymorphic DNA (RAPD). During the process of RAPD standardization in order to analyse the samples of *Gracilaria* species, polymorphism was detected among DNA samples extracted from different apices of the same thallus. This was first interpreted as an artefact of the technique resulting from the small amounts of DNA obtained from a single apex. Nevertheless, testing larger

amounts of DNA obtained by using more material, the same results (although less frequent) were observed among different axes of the same plant. These results were not surprising since *Gracilaria chilensis* is known to grow thalli from an adhesion disc that results from the germination of coalescent spores (Muñoz & Santelices, 1994). The variability detected by RAPD could mean that the thallus did not originate from the genetic material of a single spore, but was the result of the germination of combined spores. The artificial construction of monosporic and polysporic thalli (Santelices et al., 1996) allowed the comparison of DNA variability occurring in different axes of the same thallus and among thalli of different origin. DNA polymorphism was present in those axes of polysporic thalli but absent from monosporic thalli. Results indicated that spore coalescence derived in the production of plants that were a genetic mosaic and partially explained the frequent occurrence in nature (Prieto et al., 1991) of cystocarpic and tetrasporic branches in the same thallus.

Clonal variability

Unexpected variability of a different type has been found in laboratory cultures of *Gracilaria chilensis*. Germlings from spores gathered from the same cystocarp and grown in the same Petri dish displayed an increasing variability in growth rates through time. Careful observations suggest that carpospores from the same cystocarp are the result of a single event of fertilization thus, all carpospores should be genetically identical. This fact implies that no variability should be detected and, if that is the case, this should be a result of environmental effect. It is not uncommon to show differential growth rates, in thalli growing under different micro environmental conditions within the same dish (Santelices & Varela, 1993). However, this variable should not differ in more than one order of magnitude. Similar results were obtained from branches obtained fragmenting a single thallus and culturing them under identical conditions (Santelices & Varela, 1993). Posterior analysis with RAPD of the latter fragments indicated the occurrence of polymorphism after regular growth periods.

To test whether genetic variability was correlated to particular environmental factors in *G.chilensis,* the following experiments were performed. Individual fragments from ten axes of each of three gametophytic plants (a total of 30 fragments) were cleaned, sonicated and isolated in culture. Once the fragments had developed six to seven apices each, total DNA was extracted from three apices of each fragment, suspended in TE buffer and kept frozen under –20ºC. This procedure was repeated every two months for four to six months, depending on the experiment. Experiments were designed in order to evaluate possible effects of several environmental conditions, which included temperature, salinity, light intensity, nutrients (phosphate and nitrate) and copper concentration upon the genetic variability detected. RAPD was performed using thirteen 10-mer primers (Operon Technologies, Inc.) to detect polymorphism among the samples with time and under each set of conditions.

Experiments evaluating the effect of temperature in genetic variability resulted in the lack of polymorphism among samples of DNA obtained when the apices were initially formed in thalli under different temperature conditions. After a 125-day – growing – period, the initially identical bonding patterns obtained through RAPD changed significantly among samples generating detectable variability. A third growth – period of 200 days again changed the banding patterns of DNA, affecting the previous variability but with no identifiable increasing or decreasing tendencies. Therefore, no defined trends were found correlated to differences in temperature (Meneses et al., 1999), except that variability was generated over the experimental period. Mitotic recombinations at the apical level of branches (zones of active growth in *Gracilaria*) are suggested as responsible for these genetic changes that strongly depend on the strain (clone) used for the experiment.

With the exception of the experiments testing the effect in temperature, which was done twice, using only two different temperatures and 30 samples at each (Meneses et al. 1999), the other factors included more conditions and lower number of replicates. Therefore, results obtained until now are not conclusive.

Nevertheless, certain tendencies may be suggested. In these last cases, the experimental set up consisted on three ramets (fragments of branches) of each of two genets (genetically different plants) of *Gracilaria chilensis* cultured under each set of conditions. Genets were grown and analysed independently. Since the temperature experiment suggested that mitotic rate (growth rate) was important in the generation of genetic variability, growth rate under the different conditions of each factor tested had to be controlled and uniform for all ramets. When ramets showed differences in growth rate within experiments these were discarded.

Five nitrate concentrations (3, 6, 2000, 4000 µM l^{-1} and seawater), five phosphate concentrations (0.5, 1, 100, 200 µM l^{-1} and seawater), five copper concentrations (0,10,20, 40 and 80 µg l^{-1}) and three different conditions of salinity were tested (20,25 and 30‰). In all experiments, different degrees of DNA polymorphism were observed after two months of culture. No particular tendencies were found in terms of a direct or inverse correlation between genetic variability and a particular environmental factor. However, all measurements, made after apices were grown

for a period of time under the experimental conditions, displayed the occurrence of genetic variability.

We ignore whether this increasing variability is a result of base changes in nuclear or organellar DNA that could be consequence of errors during mitotic divisions. Actively growing apices, where no sexual or meiotic recombination takes place depend exclusively on mitosis for increasing thallus bulk and length in *Gracilaria*. In *G. chilensis* potentially meristematic cells do not seem to be restricted to apical meristems, in fact observation of adventitious branches and the newly-formed apices from thallus fragments indicate the presence of cortical cells with meristematic potential (Santelices & Varela, 1995). Occurrence of these cells could explain the outgrowth of branches with different morphology than the rest of the thallus occasionally observed in *Gracilaria*.

The assumption of lower variation in clonal populations seems to be unwarranted at least among the species studied in this aspect (Jackson, 1985). Apomictic species in terrestrial plants have demonstrated that the evolutionary potential of many of these plants is enhanced since apomixis is facultative. In these species, of which *Gracilaria chilensis* is an example, sexual and asexual individuals coexist; therefore sexual recombination would give sufficient genetic variation that can be propagated vegetatively. This is especially significant since the proliferation of appropriate genotypes often implies rapid colonization and exploitation of new habitats. Vegetative propagation in *G. chilensis* is performed in the form of fragmentation, a frequent phenomenon in macroalgae (Lobban & Harrison, 1994) although rarely considered as a means to maintain populations.

However, the suggested occurrence of somatic variation in *G. chilensis* thalli, would indicate that the dispersion of fragments of a thallus is not the actual spreading of a suitable genet into multiple units, but the dispersion of genetically different units.

Somatic variation is well documented in terrestrial plants (Hartmannn & Kester, 1975) where genetic and chromosomic changes occurring in different parts of the plant and caused by different mechanisms (deletions, inversions, mutations, etc.) may be perpetuated when these parts become independent of the parental plant (Grant, 1975). This can be a significant source of phenotypic variation in plant organs or only part of an organ (i.e. chimeras or mosaics). Information of somatic variation in terrestrial plants is mainly based in tissue-culture studies and it has been called somatoclonal variation. It basically consists on a broad spectrum of genetic differences observed in several traits within the same individual. Genetic mosaics of this type tend to be common when plants suffer physical damage (Whitham & Slobodchnikoff, 1981). In *Gracilaria chilensis*, as well as other commercial seaweeds, meristems are present in each of the branches of the thallus (Santelices & Varela, 1995) and genetic variation within the thalli will be spread by natural or artificial cloning of them, increasing the complexity of strain selection.

In conclusion, results so far suggest that we do have in *Gracilaria chilensis* an organism that can combine two simultaneous processes 1) the alternatives between genetic homogeneity and variability and 2) vegetative propagation and a *Polysiphonia* – type life history. Tetrasporangia formation in asexual individuals undergoes meiotic recombination thus generating genetically distinct thalli. Due to fragmentation any of these genetic variants is potentially able to propagate vegetatively, therefore increasing the number of identical genetic units throughout the population. A similar situation would occur with sexually reproductive individuals where recombination resulting from outcrossing may also be perpetuated by eventual fragmentation of the newly formed individuals. Under conditions that may be limiting, or totally restrict the completion of the life cycle, or allow the occurrence of only one of the two processes, meiosis and sexual recombination, these clones will be able to persist. New recombinants occurring in some of the branches slightly different form the original clonal plant could be dispersed by fragmentation. This strategy would ensure genetic variability in an additive form to those asexually reproducing populations whereas genetic variability in the form of dominance variability and epistasis would occur in those populations reproducing by sexual means.

Acknowledgements

This review is a synthesis of results obtained directly from research supported by FONDECYT Grants 1930014 and 1960646. I would like to extend my gratitude to research assistants, professors and colleagues from the Department of Ecology of the Pontificia Universidad Católica de Chile who provided me with laboratory assistance and fruitful discussions on this subject.

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