Persistence of intraclonal variation in *Gracilaria chilensis* Bird, McLachlan & Oliveira (Gracilariales, Rhodophyta) through successive generations of cuttings

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Key words: generations of cuttings, genetic instability, *Gracilaria*, intraclonal variation, somaclonal variation

Abstract

In seaweeds, interclonal variation originates as variation within the clone, and the stability of the new variant may facilitate or complicate strain selection practices. High frequency changes would require fairly continuous selection processes, while high persistence characters would require less effort to use the variability added by intraclonal variation. This study evaluates the persistence of intraclonal variation in *Gracilaria chilensis* Bird, McLachlan & Oliveira through successive generations of cuttings. Stock clones maintained for three years in laboratory cultures were incubated under controlled conditions. Significant intraclonal variations in growth rate were detected within six months and successive cutting experiments indicated that this variation may or may not be associated with morphological changes. Physiological (growth) variations not associated with morphological changes tended to disappear or be modified in the next generation of cuttings. On the other hand, intraclonal variation in growth associated with morphological changes persisted through successive generations of cuttings. These morphological changes affect the entire frond and can be propagated from fragments as small as 1 cm long, a capacity that facilitates vegetative propagation of the new variety.

Introduction

Intraclonal variation is any significant phenotypic difference exhibited by ramets derived from a single genet (i.e. thallus). Among red seaweeds, pigment and morphological variants are the most visible and, therefore, the most frequently described type of variants. However, physiological variants also occur (see Santelices 2001, for a review).

The most important factors inducing intraclonal variation are differences in the microenvironment surrounding each ramet, physiological and developmental differences among ramets, highly localized pathogen infections or genetic changes restricted to one or a few ramets (Santelices & Varela, 1993; Santelices *et al.*, 1995). In the case of *Gracilaria chilensis*, laboratory cultures have shown a lack of physiological differences among vegetative ramets (Santelices, 2001). The main pests of this species seem to be externally visible epiphytic algae, as the species has been found to be free of endophytic algae, fungi and bacteria. Nevertheless, localized genetic changes, as detected by DNA-fragment polymorphism, seem to be frequent. Mitotic recombinations and other kinds of DNA changes seem to be occurring in various magnitudes during branch production (Meneses *et al.*, 1999; Meneses & Santelices, 1999). Thus, as growth occurs and biomass accumulates, genetic variability increases.

G. chilensis and other types of clonal seaweeds may propagate by fragmentation of ramets. In fact, commercial crops are harvested and propagated by thallus fragmentation. If a significant intraclonal difference arises in a given ramet, and that ramet becomes separated from the rest of the plant and then re-established, these differences previously recognized as intraclonal variation become interclonal variation. Genetically-based interclonal changes are most important for strain selection practices. Such changes can be regarded as an additional source of variation for clonal organisms besides the well-known inter-individual (intergenet) variation. Thus, interclonal variation could either increase or decrease the magnitude of improvement of a given character selected by traditional means (e.g. inter-genet selection). However, its use would strongly depend on the capacity of the interclonal change to propagate and persist through successive generations of cuttings. In this study, we experimentally evaluated the stability and persistence of two spontaneous variants of *Gracilaria chilensis* through four successive generations of cuttings maintained under controlled cultivation conditions for a total of 800 days.

Materials and Methods

Vegetative branches from a cystocarpic thallus (B_2) of *Gracilaria chilensis* collected on May 31, 1993 in Maullín, southern Chile (41°36' S; 73°36' W) and maintained for three years in laboratory stock cultures were used in all experiments. A four cm-long branch of this plant was initially grown under controlled conditions of temperature (14–16°C), photoperiod (12 h light: 12 h dark), and photon-flux density (50 ± 10 μmol $m^{-2}s^{-1}$), inside a 500 ml beaker filled with 300 ml of SWM-3 culture-medium (McLachlan, 1973) and gentle air bubbling. The culture medium was changed every six days.

After two months of culture, nine, 1 cm-long apical tips of branchlets were sectioned from the original branch and incubated for 120 days in individual, previously labeled containers, under the above controlled conditions. These were called the first generation cuttings. As the thalli grew, incubation volumes were increased gradually from 200 ml to 1,000 ml and also exchanged weekly. At the end of the experiment, wet-weights (blotted weight) of the nine ramets were measured and four of them (ramets 2, 3, 5 and 7) were used to obtain the second generation of cuttings. These four ramets were selected because they clearly exhibited different growth rates.

The second generation cuttings, 10 per ramet, were incubated in a similar way and under the same controlled conditions as first generation cuttings. However, after 90 days of incubation, two of the ramets exhibited notable differences in growth rates. One of them (ramet 5.1) had a fast growth rate and a morphology similar to its corresponding clonal replicates. This clone was named the 'physiological variant'. The other ramet (ramet 2.9.A) also exhibited a fast growth rate but a different branching pattern (see results). This clone was named the 'morphological variant'.

To allow full expression of the growth differences of these clonal replicates, the incubation of second generation cuttings was extended for 170 days and the volume of the growth media increased to 2,000 ml. At the end of the experiment, wet weights of the 40 clones used as second generation cuttings were measured and the inter-ramet differences in growth rates tested by ANOVA (Snedecor & Cochrane, 1967).

Third generation cuttings were used to estimate persistence of the 'morphological' and 'physiological' variants arising from the second generation cuttings. Thus, seven apical tips (1 cm long) were excised from the physiological variant (ramet 5.1) and four apical tips from the replicate ramets 5.2 and 5.8. Similarly, seven apical tips of branches arising from the morphological variant (ramet 2.9A) were excised and incubated. Two apical tips of branches from ramet 2.9, which had not exhibited morphological variation, and four apical tips from a clonal replicate from ramet 2 (ramet 2.1) were used as controls to compare morphology and growth rates after 210 days of incubation.

Fourth generation cuttings were used to compare growth rates and persistence of morphological changes in the morphological variant. Ten apical tips were excised from each of two clones exhibiting morphological variations (ramets 2.9.A.4 and

2.9.A.6) and compared with a replicate clone (ramet 2.1.3) without morphological variation. Fourth generation cuttings were incubated for 90 days as indicated above. Thallus length, blotted weight and number of branchlets of each ramet were recorded at the end of the experiment. Inter-ramet differences in growth rates were tested by ANOVA, followed by an *a posteriori* test (Tukey test).

Results

First generation cuttings:

Cuttings 7 and 9 from *G. chilensis* genet B_2 showed the lowest daily growth rates (1.6–1.75%), while cuttings 2 and 6 had the highest growth rates (2.5–2.8%) (Fig. 1).

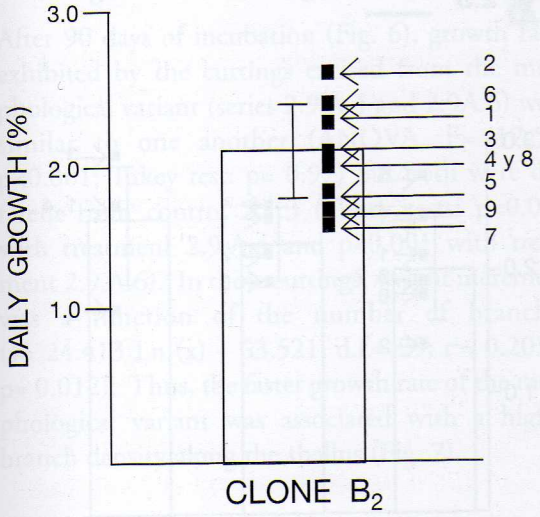

Fig. 1. Daily growth rates (%) of first generation cuttings (1 to 9) after 120 days in culture. Column represents average growth rate and bar, standard error. Clones 2, 3, 5 and 7 were selected for further experiments.

Second generation cuttings:

The ramets excised from first generation cuttings 2, 3, 5 and 7 after 170 days of incubation (Fig. 2) reproduced the growth variation already described for the first generation cuttings, but the pattern of variation was not related to the growth differences found among first generation cuttings. Average growth rates of ramets derived from the two faster growing first generation cuttings (2 and 3) were similar ($F = 0.47956$; $p<0.69852035$) to the values exhibited by the slower growing first generation cuttings (5 and 7).

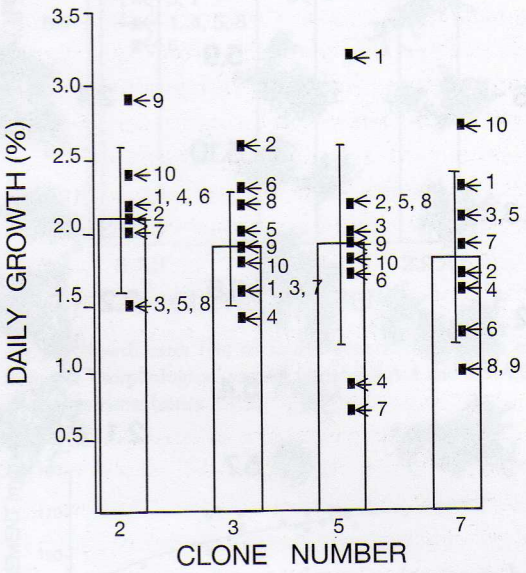

Fig. 2. Daily growth rates (%) of second generation cuttings after 170 days in culture. Columns represent average growth rates and bars, standard errors. Note the growth rates of ramet 2.9 (the 'morphological' variant) and ramet 5.1 (the 'physiological' variant).

Two branchlets derived respectively from first generation cuttings 2 (branchlet 2.9) and 5 (branchlet 5.1) exhibited size and weight increments visibly larger than the other nine replicate branchlets in each treatment (Fig. 2). Branchlet 2.9 also exhibited a different branching pattern (Fig. 3) that was recognized as a 'morphological' variant. Branchlet 5.1 maintained the original morphology and it was characterized as a 'physiological' variant. Branches in the clonal replicates and in the 'physiological variant' (ramet 5.1 in Fig. 3) were short (up to 5 mm long), incurved and irregularly disposed along the axes. In the 'morphological variant' (ramet 2.9.A in Fig. 3), branches were up to 2 cm long, straight, terete or gradually compressed to the tip and disposed in opposite, subopposite or distichous form along the axes.

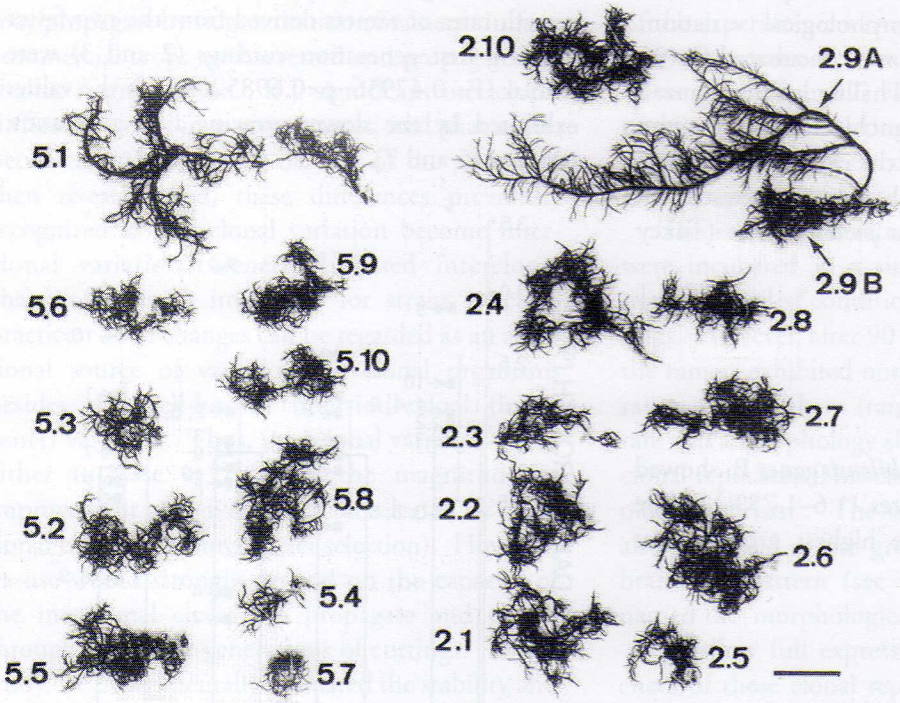

Fig. 3. The 'morphological' (clone 2.9.A) and the 'physiological' (clone 5.1) variants together with their respective clonal replicates. Incubation time 170 days. Scale = 3 cm.

Third generation cuttings:

The growth patterns exhibited by third generation cuttings after 210 days of incubation differ between the physiological variant, the morphological variant and the respective controls. Growth rates of branchlets excised from second generation cuttings 5.1, 5.2 and 5.8 (Fig. 4) exhibited the typical growth variation already described for the two previous incubations. Some ramets (e.g. 5.1.5, 5.8.3) grew faster than others taken from the same cutting, but the average value among all replicates did not differ significantly ($F=0.479562$; $p<0.6985203$). A similar homogeneity in average growth was exhibited also by the series of ramets excised from the physiological variant (cuttings 5.1.1 to 5.1.7).

In contrast, growth rates of the ramets excised from the 'morphological' variant (treatment 2.9.A, Fig. 5) were significantly faster (ANOVA, $F= 45.06264$; $p<0.001$) than the specific growth rates of the other two controls (2.9.A vs 2.9.B, Tukey test = $p<0.001$; 2.9.A vs 2.1, Tukey test = $p<0.001$). The two controls (2.9.B and 2.1) had similar growth rates (Tukey test, $p= 0.70$).

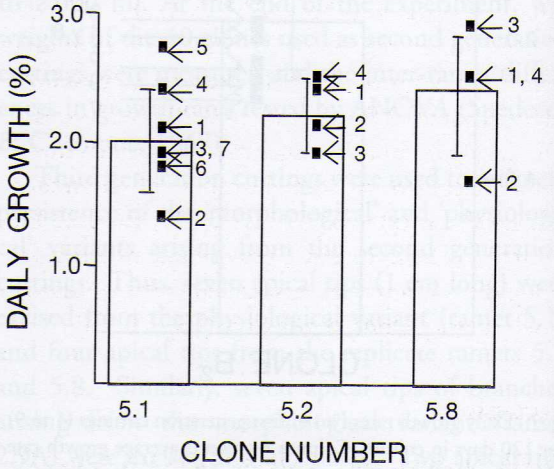

Fig. 4. Growth rates (%) of third generation cuttings excised from the 'physiological' variant (series 5.1) and two clonal replicates, after 210 days in culture. Column represents average growth rates and bars, standard errors.

Third generation cuttings exhibited, under the controlled culture conditions used, an external morphology and a branching pattern similar to the second generation cuttings from which they originated.

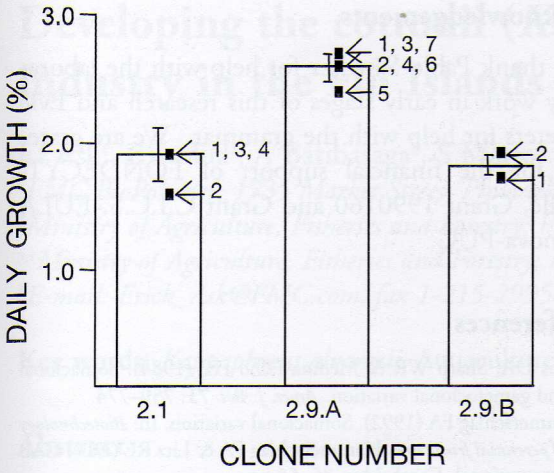

Fig. 5. Growth rates (%) of third generation cuttings excised from the 'morphological' variant (series 2.9.A) and two clonal replicates, after 210 days in culture. Column represents average growth rates and bars, standard errors.

Fourth generation cuttings:

After 90 days of incubation (Fig. 6), growth rates exhibited by the cuttings excised from the morphological variant (series 2.9.A.4 and 2.9A.6) were similar to one another (ANOVA, F= 17.51; $p<0.001$; Tukey test: p= 0.97) but both were different from control 2.1.3 (Tukey tests: $p<0.001$ with treatment 2.9.A.4 and $p<0.001$ with treatment 2.9.A.6). In these cuttings, weight increment was a function of the number of branches ($y= 24.413 \text{ Ln } (x) + 63.521$; d.f.= 29; $r^2= 0.2033$, p= 0.012). Thus, the faster growth rate of the morphological variant was associated with a higher branch density along the thallus (Fig. 7).

Discussion

Growth variation among clonal replicates appeared repeatedly in the various experiments performed in this study. However, as in previous studies (Santelices & Varela, 1993; Santelices *et al.*, 1995), these frequent growth differences could not be explained clearly although all ramets in a given experiment were handled in the same way and care was taken not to affect the growing tips. Potential microenvironmental differences around the ramets were reduced as much as possible and only similar

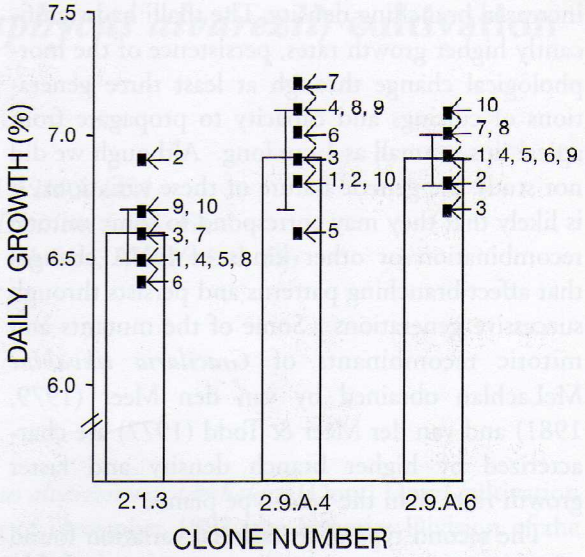

Fig. 6. Growth rates (%) of fourth generation cuttings excised from the 'morphological' variant (series 2.9.A.4 and 2.9.A.6) and a replicate clone (series 2.1.3).

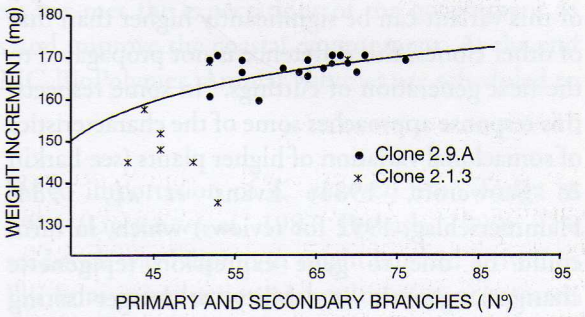

Fig. 7. Weight increments as a function of number of branches in fourth generation cuttings. Regression values include the morphological variant (series 2.9.A) and the control clone (series 2.1.3).

orders of branching or ramets with approximately similar number of branchlets were used for comparative purposes.

In addition to the frequent growth variations, our results also exhibit two types of significant differences in growth rates that can be identified as intraclonal variants. One of them is persistent through successive generations while the other appears and disappears along successive generations of cuttings.

In this study with *Gracilaria chilensis*, the most persistent type of intraclonal variation was associated with significant morphological changes due to

increased branching density. The thalli had significantly higher growth rates, persistence of the morphological change through at least three generations of cuttings and capacity to propagate from apical tips as small as 1 cm long. Although we did not study the genetic nature of these variations, it is likely that they may correspond to some mitotic recombination or other kinds of DNA changes that affect branching patterns and persists through successive generations. Some of the mutants and mitotic recombinants of *Gracilaria tikvahiae* McLachlan obtained by van den Meer (1979, 1981) and van der Meer & Todd (1977) are characterized by higher branch density and faster growth rates than the wild-type plants.

The second type of intraclonal variation found in our studies is more puzzling, as it may appear spontaneously under controlled conditions. Nevertheless, it does not persist along different generations of cuttings. Although the growth rate of this variant can be significantly higher than that of other clones, this difference is not propagated to the next generation of cuttings. In some respects, this response approaches some of the characteristics of somaclonal variation of higher plants (see Larkin & Scowcroft, 1981; Evans *et al.*, 1984; Hammerschlag, 1992 for reviews) which, in turn, could be due to gene expression (epigenetic changes), genetic instability or longer-lasting genetic changes. At present, there are not enough data to genetically characterize this response, other than to document its occurrence and the possibility that it could complicate strain selection. Genetic studies are needed to clarify the observed physiological and morphological variations in this economically important species.

Overall, our results suggest that the most permanent type of intra-clonal variation could be used in strain selection practices to increase the magnitude of the difference in the selection character beyond that obtained through genet selection. It remains to be seen if these more permanent, positive variations could be induced under laboratory conditions to add predictability to the selection process.

Acknowledgements

We thank Pablo Sánchez for help with the laboratory work in early stages of this research and Evie Wieters for help with the grammar. We are grateful for the financial support of FONDECYT-Chile, Grant 1990160 and Grant C.I.C.S.-EULA Genova-PUC.

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