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Growth-related intraclonal genetic changes in *Gracilaria chilensis* (Gracilariales: Rhodophyta)

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Abstract *Gracilaria chilensis* exhibits noticeable intraclonal variation, some of which is presumed to result from mitotic recombinations or other types of DNA turnover associated with replication activities during cellular division. To test this, genetic variability (determined by multilocus fingerprinting markers using the randomly amplified polymorphic DNA technique, RAPD) and total growth were simultaneously measured over time in clonal replicates of *G. chilensis* incubated under controlled laboratory conditions. The results suggest that genetic variability increases as growth occurs and biomass accumulates, supporting the hypothesis of growth-related increases in genetic heterogeneity. For species massively propagated by thallus fragmentation in either naturally or farmed populations, growth-dependent genetic changes may constitute a powerful means of generating intra-population variation without thalli becoming reproductively mature, and, as a consequence, bypassing meiosis and/or sexual recombination.

Introduction

Clonal propagation is common in many groups of organisms, including several phyla of the Metazoa (reviewed by Hughes and Cancino 1985) land plants (Silander 1985), and seaweeds (Santelices and Varela 1993), and can occur through several mechanisms including budding, fission, parthenogenesis and fragmentation (Jackson 1985).

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Although asexually-originated fragments have been classically considered as identical copies of the genet originating them (Harper 1977), several sources of variation among ramets are now known to occur (Buss 1985). Point mutation, inversions, deletions, and other sources of somatic variation arising from mitotic errors result in genetic differences between clones. The importance of this variation in natural populations of species with vegetative propagation is unknown, but Bradshaw (1965) and Silander (1985) have reported high genetic diversity in natural populations of the clonal land plants *Trifolium repens* and *Spartina patens*, respectively.

Several types of seaweeds, including *Gracilaria* spp., grow and propagate by self-replication of genetically identical units, and should be regarded as clones (Santelices and Varela 1993). However, the assumption that most seaweeds consist of independent individuals has delayed the study of their intra-clonal variability, although mitotic recombinations have been documented in *G. tikvahiae* (Van der Meer and Todd 1977), and the involvement of transposable genetic elements is suspected in the occurrence of unstable mutations in the ramets of three species of *Gracilaria* (Van der Meer and Zhang 1988).

Artificial and natural populations of *Gracilaria chilensis* can be formed by thallus fragmentation. However, population levels are mostly maintained by means of vegetative growth, bypassing sexual recombination. In this species, fragments from the same thallus as well as spores from the same cystocarp display notable differences in morphology and growth rates when cultured under the same conditions. This phenomenon indicates an underlying genetic divergence in a system that otherwise was assumed to be homogeneous (Santelices and Varela 1993).

If the intra-clonal variation detected in *Gracilaria chilensis* is the result of mitotic recombinations or other types of DNA-turnover mechanisms (Dover 1982) resulting from replication activities during mitosis, then, in vegetatively-growing clones of this species, genetic variability should increase with growth rate. This means

that the probabilities of DNA-turnover will increase at the same rate as cellular divisions occur. To test this, we grew clonal replicates of *G. chilensis* under controlled laboratory conditions.

Genetic variability, as measured by multilocus fingerprinting markers (RAPD technique) and total growth were simultaneously measured over time.

Materials and methods

To determine if genetic variability in *Gracilaria chilensis* increases with increasing biomass, three experiments were performed.

Unisporic axes (Experiment 1)

We used a female gametophytic plant with three upright axes grown from a single spore. The fertile sporophyte had been collected from Maullin, Puerto Montt, southern Chile (41°37′S; 73°35′W) four months earlier. The female plant was cultivated in the laboratory under controlled conditions of temperature (14 °C ± 2 C°), irradiance (50 μmol m^{-2} s^{-1}), and photoperiod (12 h light:12 h dark) for 2 mo, by which time the axes were 5 to 6 cm long with a few branchlets. Since the three upright axes originated from a single spore, they were considered clonal replicates.

Ten, 2 mm-long fragments were transversally cut off each of the three upright axes and incubated for 200 d in three individual, labeled, containers under controlled temperature and photoperiod conditions with no air-bubbling. Irradiance was increased to 70 ± 5 μmol m^{-2} s^{-1}, the culture medium was enriched with SFC medium (Correa and McLachlan 1991). After 90 d the container volume was increased 1 to 2 liters, and gentle air-bubbling was then introduced to all containers. The culture medium was changed every 6 d.

Apice whorls began growing from the periphery of each axis fragment during the first few days of culture under controlled conditions. After 60 d culture, each of the 30 fragments was blot-dried and weighed. The apices of three of the newly formed branches (top 2 mm) were excised for DNA extraction. Weighing and extraction were repeated after 125 and after 200 d culture.

For DNA extraction, excised apices were added to 1.5 ml Eppendorf tubes containing 200 μl homogenization buffer (250 m*M* Tris HCI, pH 8.2, 250 m*M* NaCI, 25 n*M* EDTA, 0.1% sodium dodecyl sulphate) and silicon carbide [<400 mesh (Fluka)] and then ground with a hand pestle. DNA extraction was carried out by adding an equal volume of chloroform:isoamyl alcohol (26:1 by vol) and frequently vortexing over a period of 10 min. The aqueous fraction was then separated by centrifugation at 12 000 rpm for 10 min. DNA was precipitated by adding 100% cold ethanol to this fraction in new tubes and subsequently freezing at −18 °C for 30 min to 1 h. Samples were then centrifuged for 20 min at 13 000 rpm and subsequently washed with 70% (v/v) cold ethanol for an additional 5 min. Pellets were air-dried and re-suspended in 50 μl TE buffer (10 m*M* Tris, 1 m*M* EDTA) pH 8.0, at 40 °C with occasional gentle agitation. Crude extracts were kept frozen for as long as 2 wk. DNA crude extracts were purified using a Wizard DNA clean-up system (Promega) just before polymerase chain-reaction (PCR) processing of the samples.

We used 15 to 20 ng DNA crude extract (1.5 μl) for each 20 μl PCR solution. A mixture of 100 μ*M* each of deoxynucleotide (dATP, dTTP, dCTP, dGTP) (Amersham, England), 0.5 U Taq DNA polymerase, 2 μl Taq DNA polymerase buffer, 3 m*M* $MgCl_2$ (Promega), and 1.0 μ*M* 10-mer primers (primers from the OPX series of Operon Technologies) was used per sample. PCR reactions were carried out in a programmable thermal controller PTC-100 (MJ Research, Inc., USA), starting with 3 initial cycles (1 min at 95 °C, 1 min at 37 °C and 1 min at 71 °C) followed by 40 cycles of 30 s per cycle at 94 °C for denaturation, 30 s at 40 °C for annealing, and 1 min extension time at 72 °C, followed by 5 min at 72 °C to allow complete extension of the amplified fragments. PCR reactions were repeated at least twice to ensure replicability. Negative controls of the reaction solution with ultrapure water instead of DNA were included in each run. RAPD–PCR products were separated in 1.5% agarose (Amresco) gels in TBE buffer (45 m*M* Tris-borate, 1 m*M* EDTA) run at 120 V, and stained with ethidium bromide. Patterns of RAPD products obtained in the gels were photographed under 312 nm UV light with a Nikon camera fitted with an orange filter (Cokin A002) and loaded with 100 ASA T-Max Kodak film. When negative controls revealed bands at the lower end of the gel, these bands were omitted from the analysis of the experimental solutions.

Of 20 primers, 14 generated polymorphic bands. Band patterns were recorded as 0 or 1 based on the absence/presence, respectively, of bands. Similarity matrices [Jaccard index calculated with NTSYS (Exeter Software)] were obtained for each pair of samples within clones and between clonal replicates for these 14 primers. The range of similarity indices thus obtained was used to plot the distribution of frequencies obtained each time the ramets were examined and weighed. Their distributions over time were compared with a Wilcoxon signed-ranks test (Siegel and Castellan 1988) for paired replicates.

Polysporic axes at 14 °C (Experiment 2)

To evaluate whether the genetic origin of the experimental thalli had affected the results, Experiment 1 was replicated, but with the difference that the three tetrasporophytic axes used as the source for the 10 fragments of the experiment originated from different carpospores. Each of these carpospores were taken from a different cystocarp. This experiment tested the replicability of the results of the first experiment with the added potential of revealing a larger genetic variability, since carpospores from different cystocarps are the products of different sexual fusions.

Polysporic axes at 18 °C (Experiment 3)

A third experiment replicated the second, but was conducted at 18 °C in an attempt to increase the growth rates of the new branchlets originating from the fragments. Frequency distributions obtained with similarity indexes were compared between experiments with the Kolmorogov–Smirnov two-sample test (Siegel and Castellan 1988).

Results

Unisporic axes (Experiment 1)

By Day 60, new branches were emerging from the thallus fragments of *Gracilaria chilensis*, and measured an average of 1.2 cm in length. The wet weight of all fragments was <1 mg (Fig. 1A); a full range of weights could not be obtained because the scale used for

►

Fig. 1 *Gracilaria chilensis*. Incubation of unisporic thalli at 14 °C (Experiment 1). **A** Cumulative biomass for each of 30 fragments used in experiment [*Thicker line with black circles* average values; *arrowheads below x-axis* points in time (days) when apices were excised from branchlets growing from fragments and DNA was extracted]; range of wet weight exhibited by fragments is shown. **B** Variation in wet weight of fragments derived from each of three upright axes on Day 200 of culture. **C** Histograms of similarity indices between thallus fragments; values derived from banding pattern obtained from multilocus fingerprinting markers (RAPD technique) at times indicated on abscissa of **A**

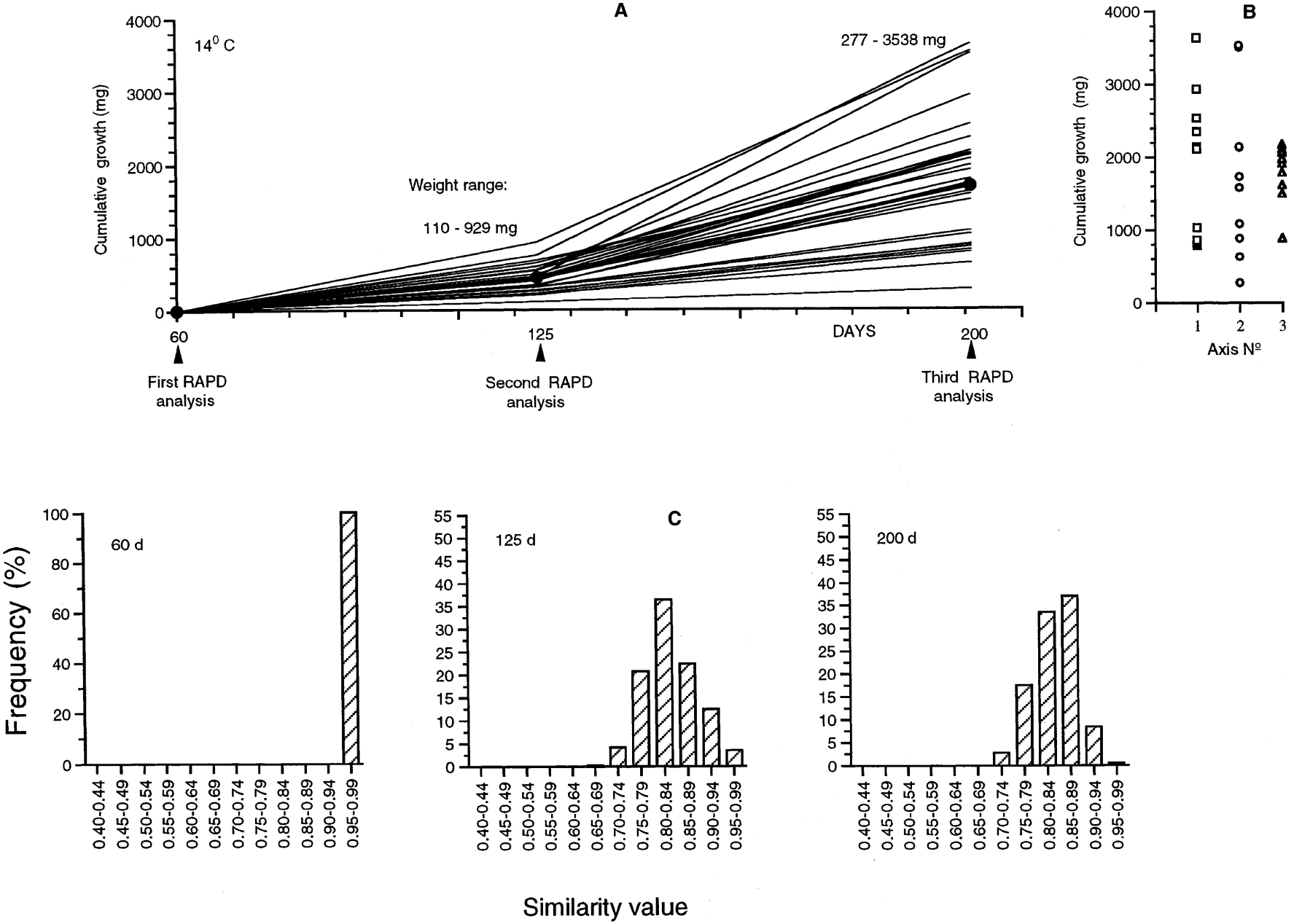
A
14⁰ C
277 - 3538 mg
Weight range:
110 - 929 mg
Cumulative growth (mg)
4000
3000
2000
1000
0
60
125
200
DAYS
First RAPD analysis
Second RAPD analysis
Third RAPD analysis
B
Cumulative growth (mg)
Axis №
1
2
3
C
60 d
125 d
200 d
Frequency (%)
100
80
60
40
20
55
50
45
40
35
30
25
20
15
10
5
0.40-0.44
0.45-0.49
0.50-0.54
0.55-0.59
0.60-0.64
0.65-0.69
0.70-0.74
0.75-0.79
0.80-0.84
0.85-0.89
0.90-0.94
0.95-0.99
Similarity value

Fig. 1

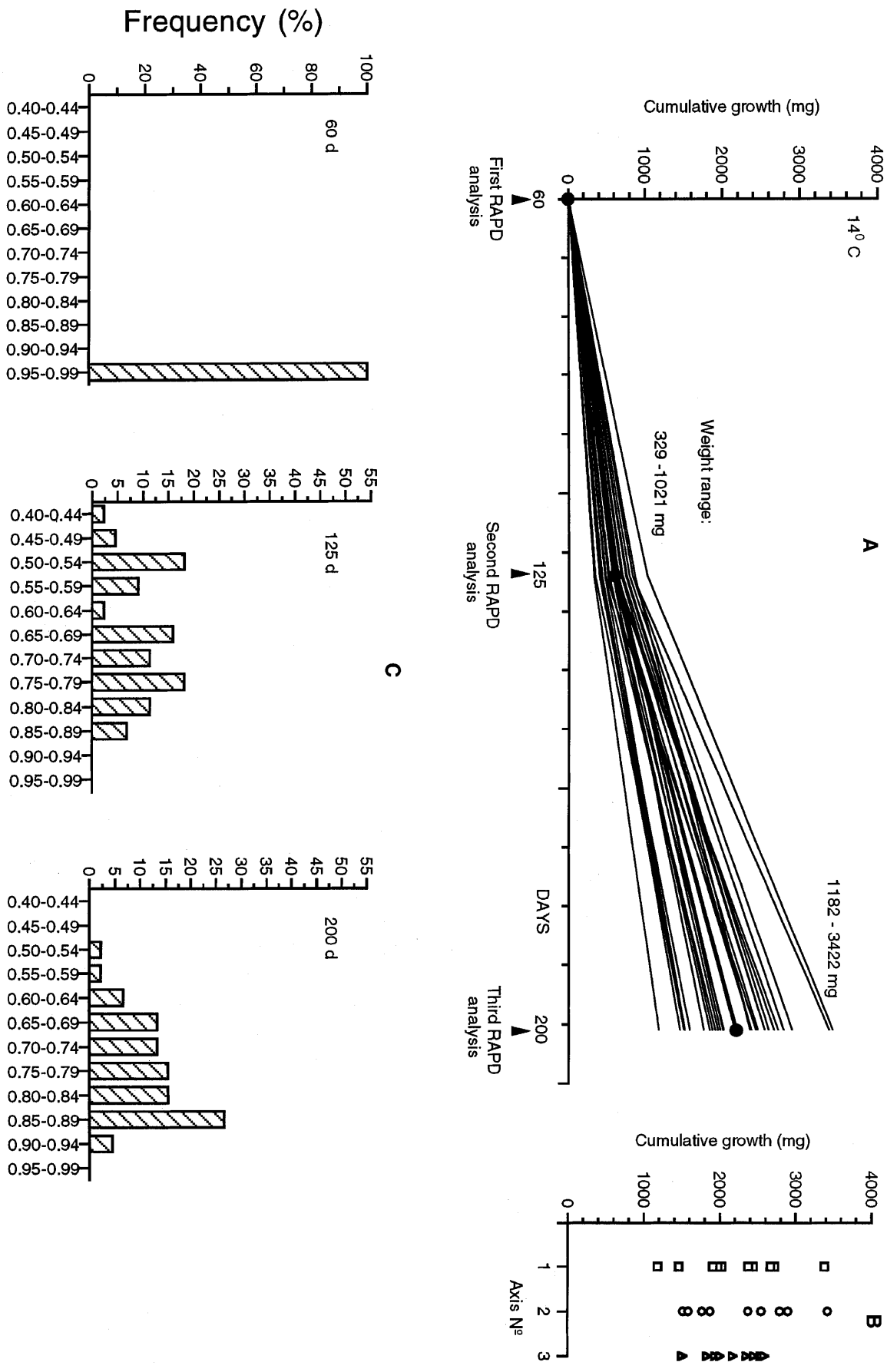
A
Cumulative growth (mg)
4000
3000
2000
1000
0
14° C
Weight range:
329 -1021 mg
1182 - 3422 mg
First RAPD analysis
Second RAPD analysis
Third RAPD analysis
60
125
200
DAYS
B
Cumulative growth (mg)
4000
3000
2000
1000
0
1
2
3
Axis Nº
C
Frequency (%)
100
80
60
40
20
0
60 d
125 d
200 d
0.40-0.44
0.45-0.49
0.50-0.54
0.55-0.59
0.60-0.64
0.65-0.69
0.70-0.74
0.75-0.79
0.80-0.84
0.85-0.89
0.90-0.94
0.95-0.99
55
50
45
40
35
30
25
20
15
10
5
0
Similarity value

Fig. 2

◀

Fig. 2 *Gracilaria chilensis*. Incubation of polysporic thalli at 14 °C (Experiment 2). Details as in legend to Fig. 1

weighing the samples did not record values of <1 mg. By Day 125, the average wet weight of the fragments (plus branches) was 441 mg (±33 SE); by Day 200, the average weight was 1815 mg (±158 SE). The data for each individual fragment (Fig. 1B) highlight the range of intraclonal variation at Day 200, revealing extensive overlapping in the range of variability of the different fragments derived from the three upright axes.

RAPD variability expressed as percentage of similarity indices (Fig. 1C) indicated no detectable genetic polymorphism (Jaccard index = 1) up to Day 60. By Day 125, new banding patterns were visible in the agarose gels, differing between samples. These new patterns resulted in lower similarity values between sample pairs. As a result, the frequency distributions of the similarity indices changed and dispersed along the x-axis (Fig. 1C). By Day 200, the sequence of the Jaccard indices between samples had changed, resulting in a significant difference in the distribution of the indices between this and the 125 d distribution (Wilcoxon signed-ranks test, $T = 21$, $P = 0.0156$).

Polysporic axes at 14 °C (Experiment 2)

Results at 14 °C for upright axes derived from different spores (Fig. 2) revealed the same general pattern as for unisporic axes, with some important differences. As for unisporic axes, the wet weight of fragments and branches was negligible up to Day 60. By Day 125 the average wet weight had increased to 599 mg (±30 SE) and by Day 200 had attained 2178 mg (±104 SE) (Fig. 2A). The average fragment weight at Day 200 was significantly higher (ANOVA, $F = 25.362$, $df = 58$, $P < 0.0001$) than for unisporic axes, despite similar experimental conditions.

Divergence in wet weight among fragments increased as biomass increased (Fig. 2A). However, the total variability by Day 200 (Fig. 2B) was less than that seen in unisporic axes. Even though in Experiment 2 each upright axis derived from a different carpospore, there was a similar amount of overlap in the range of values for the different fragments.

The RAPD technique revealed no difference in the banding pattern of samples after 60 d culture (Jaccard index = 1). Genetic variability appeared after 125 and 200 d culture (Fig. 2C), but did not differ significantly among these two periods (Wilcoxon signed-ranks test, $T = 32$, $P = 0.5171$). The similarity values were lower than those obtained for unisporic axes, and were also more heterogeneous, with a significantly different distribution pattern on both Day 125 and Day 200 (Kolmorogov–Smirnov test, $D = 0.889$, $P < 0.001$; and $D = 0.176$, $P < 0.001$, respectively).

Polysporic axes at 18 °C (Experiment 3)

Biomass increments at 18 °C followed a pattern similar to that in Experiments 1 and 2 (Fig. 3A). However, while the range of wet weight variability (Fig. 3B) was similar to that of polysporic thalli at 14 °C [$\bar{x} = 703$ mg (±27 SE)], the average wet weight per fragment by Day 200 ($\bar{x} = 2.280$ mg) was significantly lower.

As in Experiment 2, the banding pattern of RAPD variability at 18 °C did not differ between samples up to Day 60. Genetic variability then appeared at Day 125 (Fig. 3C), but showed no further significant change up to Day 200 (Wilcoxon signed ranks-test, $T = 19.5$, $P = 0.4727$).

Although there was an initial (up to Day 60) lack of DNA variability in polysporic ramets from both Experiments 2 and 3, measurements in variability at 125 and 200 d culture differed significantly between both experiments, with the lowest variability observed in Experiment 3 (Kolmorogov–Smirnov test, $D = 0.154$, $P < 0.01$; and $D = 0.156$, $P < 0.01$; for Days 125 and 200, respectively).

Comparison of the 18 °C results (Experiment 3) with those obtained for unisporic ramets at 14 °C (Experiment 1) revealed higher genetic variability at Days 125 and 200 (Kolmorov–Smirnov test, $D = 0.299$, $P < 0.001$; and $D = 0.238$, $P < 0.001$) at 18 °C, apart from the higher variability in growth ranges at 14 °C for the unisporic ramets.

Discussion

The results of this study suggest a parallel pattern of genetic change in *Gracilaria chilensis* under all experimental conditions tested. As growth increased and biomass accumulated, genetic variability, as detected by RAPD analysis, increased. After an initial growth period, the genetic changes varied, with no recognizable trend. Equivalent studies have not been performed on other seaweeds, therefore no comparisons are possible. However, the recorded changes are consistent with the hypothesis that genetic variability increases with growth, probably as a result of mitotic recombinations and other types of DNA-turnover mechanisms. Mitotic recombinations have been documented in other species of *Gracilaria* (Van der Meer and Todd 1977), and have been suggested for *Lophocladia trichoclados* (Van Oppen 1995: pp 119–140).

As growth continued, genetic changes also continued in unisporic ramets, contributing to increase rather than decrease similarity between samples. Close examination of the banding patterns shows that by Day 125 changes in the banding pattern (disappearance or appearance of one or more bands) were, in many cases, only partially featured among the branchlets sampled, resulting in high dissimilarity values. By Day 200, all branchlets examined were genetically homogeneous; their banding pat-

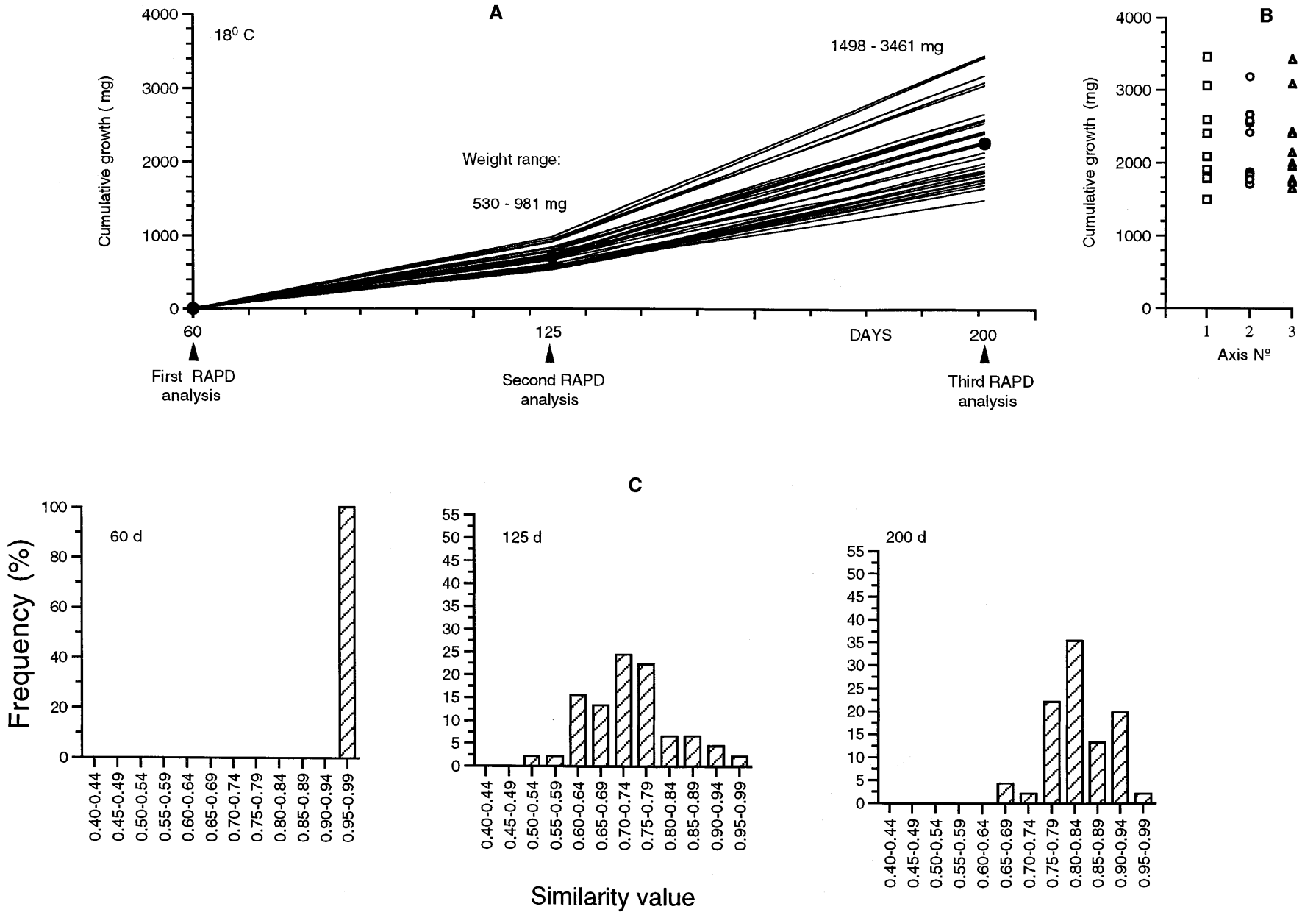
A
18⁰ C
1498 - 3461 mg
Weight range:
530 - 981 mg
Cumulative growth (mg)
4000
3000
2000
1000
0
60
125
DAYS
200
First RAPD analysis
Second RAPD analysis
Third RAPD analysis
B
Cumulative growth (mg)
Axis Nº
C
60 d
125 d
200 d
Frequency (%)
0.40-0.44
0.45-0.49
0.50-0.54
0.55-0.59
0.60-0.64
0.65-0.69
0.70-0.74
0.75-0.79
0.80-0.84
0.85-0.89
0.90-0.94
0.95-0.99
Similarity value

Fig. 3

◀

Fig. 3 *Gracilaria chilensis*. Incubation of polysporic thalli at 18 °C (Experiment 3). Details as in legend to Fig. 1

tern was again identical, although it differed from the pattern at the beginning of the growth period (Day 60).

Although our results support the hypothesis of growth-related increases in genetic heterogeneity in *Gracilaria chilensis* over a certain period of time, they do not suggest a strict relationship between growth variability and genetic variability. In our experiments, the quantification of genetic variability was based on total genomic DNA, including organellar and nuclear DNA. Therefore, some of the genetic changes observed might not be related to growth, and therefore not be expressed as changes in growth rates.

On the other hand, growth rates are known to be effectively influenced by a diversity of other factors. Under the controlled conditions of the present experiments, the original make-up of the thallus cultivated was of paramount importance. A growth-rate comparison of the two experiments at 14 °C shows a much wider range for fragments excised from unisporic than from polysporic thalli. This is consistent with the fact that the number of fragments excised from the unisporic thalli was three times greater than the number excised from each of the polysporic axes, leading to a wider sample of intraclonal variants. The inclusion in this wider sample of a rather large number of clonal fragments with low growth rates contributed to the significant differences found among treatments. Nevertheless, the fact that the range of genetic (DNA) variability exhibited by the 30 fragments excised from a single thallus was lower than the variability shown by an equal number of fragments excised from three genetically different thalli further suggests that variation in growth in this species in not related to genetic variability estimated as DNA polymorphism. However, the data on growth-rate variation as well as those on DNA variability indicate that, depending on the organic level at which intraclonal variation is estimated (i.e. physiological or genetic), it is as important as interclonal variation in terms of population variability.

Polysporic thalli incubated at 14 and 18 °C exhibited a similar range in growth rate. However, the occurrence at 14 °C of fragments with faster growth rates resulted in significant differences between these and the fragments incubated at 18 °C. These results thus suggest that under the culture conditions used, intraclonal variability is more important than a 4 C° temperature difference in determining growth rates.

In *Gracilaria chilensis*, completion of the life-cycle is rarely observed. Many of the naturally-growing populations lack fertile gametophytic phases (see Santelices and Doty 1989 for review), at the same time, both wild and farmed stands are massively propagated by thallus fragmentation. Growth-related genetic changes such as those detected in this study, and which constitute the basis of intraclonal variability, may constitute a powerful means of generating genetic variation within populations where thalli do not become reproductive and do not undergo meiosis or sexual recombination.

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