Organellar DNA restriction analysis of four morphotypes of *Gracilaria* from Lenga, VIIIth Region, Chile

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Gracilaria *is an important seaweed resource of Chile, with a wide distributional range along the coast, yet there is uncertainty about the exact number of taxa present. The data of this study introduce the use of a molecular approach in an attempt to solve this problem. Four morphotypes collected from a natural bed located in Lenga (36°45'S; 73°10'W) were assayed for DNA sequence polymorphism utilizing the RFLP technique. Total DNA extraction and organelle DNA purification were performed according to the methodology of Rice and Bird (1990). Organellar DNA was digested with thirteen restriction endonucleases. The electrophoretic separation in an agarose gel of digested organelle DNA showed identical patterns for all the morphotypes. Concurrent preliminary data from hybridization trials among the morphotypes revealed that the four were interfertile. DNA data reported herein, plus that from hybridization and anatomical studies of reproductive structures, has led us to conclude that all four of the morphotypes correspond to* ***Gracilaria chilensis****.*

Key terms: *taxonomy, RFLP, morphological variants, color variants.*

INTRODUCTION

Gracilaria is considered a taxonomically difficult genus. Until 1985, only two main taxa were recognized in Chile: *G. lemaneiformis* (Bory) Weber Van Bosse, distributed from Coquimbo (29° 56'S, IVth Region) to Chiloé (42° 30'S, Xth Region), and *G. verrucosa* (Hud.) Papenfuss, from Dichato (36° 33'S, VIIIth Region) to Quetalmahue (41° 52'S, Xth Region) (Etcheverry, 1958; Kim, 1970; Candia and Kim, 1977; Romo *et al*, 1979; Santelices and Fonck, 1979).

Prior to 1990, taxonomic studies of *Gracilaria* in Chile had been based solely on such traditional criteria as morphology, reproductive anatomy and hybridization. Based on those data, Bird *et al* (1986) described, from plants collected in Bahía La Herradura (IVth Region), Cerro Verde (VIIIth Region) and Río Maullín (Xth Region), a new species: *Gracilaria chilensis*. According to them, this taxon had been previously cited either as *G. verrucosa* or *G. lemaneiformis*. Also, they doubted the occurrence of *G. verrucosa* in Chile, and agreed without reservation that *G. lemaneiformis* is an important component of the Chilean flora. The three species referred to are morphologically similar, while differing only in the structure of the spermatangia, namely: "*textori*-type" in *G. chilensis*, "*verrucosa*-type" in *G. verrucosa* and "*chorda*-type" in *G. lemaneiformis*.

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Recognizing that *G. lemaneiformis* now has been included in the genus *Gracilariopsis* (Fredericq and Hommersand, 1989), and *G. verrucosa*'s presence is doubtful, it would appear that *G. chilensis* is the only species present in the Chilean beds.

More recently, Candia (1988) and Candia and Reyes (1993) analyzed length of thalli, branching pattern, color, presence or absence of reproductive structures and provenance of the samples, and recognized the existence of a number of morphotypes of *Gracilaria* in beds from the VIIIth and Xth Regions. Also, Santelices and Ugarte (1990) studying the physiological responses of four distantly distributed populations of *Gracilaria*, concluded that only three of their samples (from Antofagasta, Coquimbo and Talcahuano) corresponded with *G. chilensis*, while the other (from Maullín) was a different species of *Gracilaria*, as yet to be identified.

While the morphological data provided by Candia (1988) and Candia and Reyes (1993) suggest the possible existence of more than one taxa, preliminary interfertility trials (Candia, unpublished observations) indicate that all of them would correspond to only one taxon. Therefore, it would appear justified the application of new techniques to resolve this particular question.

The incorporation, at the end of the past decade, of DNA analyses to solve taxonomic problems, has been a great help in obtaining a better understanding of the interspecific phylogenetic relationships in this genus (Goff and Coleman, 1988; Bird *et al*, 1990a, b; Rice and Bird, 1990; Bird *et al*, 1992; Goff, 1993 and Goff *et al*, 1994). The first nucleic acids research on *G. chilensis* was done by Bird *et al* (1990b). The authors made a critical comparison of this taxon, from culture material from Bahía La Herradura, with *G. sordida* from New Zealand. Based on restriction fragment analysis of organellar DNA plus other taxonomical criteria, they concluded that the two species were conspecific.

In this paper, the sequence polymorphism of the organellar DNA of four morphotypes recognized by Candia and Reyes (1993) in a population of Lenga (VIIIth Region) will be characterized.

MATERIALS AND METHODS

Collection of Gracilaria

Thalli corresponding to each morphotype were collected either by scuba diving from different areas of the subtidal bed located in Caleta Lenga, San Vicente Bay, VIIIth Region (36°45'S; 73° 10'W) or directly from the estuary, located in the same place (Fig 1). According to differences in thalli length, width, color and provenance, Candia and Reyes (1993) nominated them as the following: estuarine (L1), thin (L2), thick (L3) and green (L4) (Fig 2). Fresh vegetative and/or reproductive thalli devoid of contaminants were used in each case.

Nucleid acids extraction

A hundred grams of fresh weight thalli were used for each morphotype. The DNA extraction technique was as in Rice and Bird (1990) with some modifications. The tissue grinding was done in liquid nitrogen in a prechilled mortar. Cellular lysis was obtained by adding 200 ml of lysis buffer solution (4% SDS, 0.2 M NaCl, 0.05 M Tris pH 8.0, 0.1 M EDTA) and gently mixed at 0°C for 60 min. The lysate was passed through a sterile double layer of cheesecloth; the residue was reground in liquid nitrogen, dissolved in 100 ml of lysis buffer and strained again through cheesecloth. Both lysates were kept at –70°C overnight. The lysates were then defrosted at room temperature and centrifuged at 2000 rev min^{-1} for 10 min at 4°C. Proteins were removed from the supernatant (200 ml for each morphotype) by incubation with proteinase K (50 μg m^{-1}) in a water bath at 37°C for 30 min and the supernatant was then extracted three times: twice with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1), and once with an equal volume of chloroform: isoamyl alcohol (24:1). After each extraction, the aqueous phase (which has the nucleic acids) was separated from the organic phase by centrifugation at 5000 rev min-1 for 10 min at 4°C. The aqueous phase was removed using a wide bore pipette. Nucleic acid precipitation was performed by adding 0.1 volume of 3 M sodium acetate

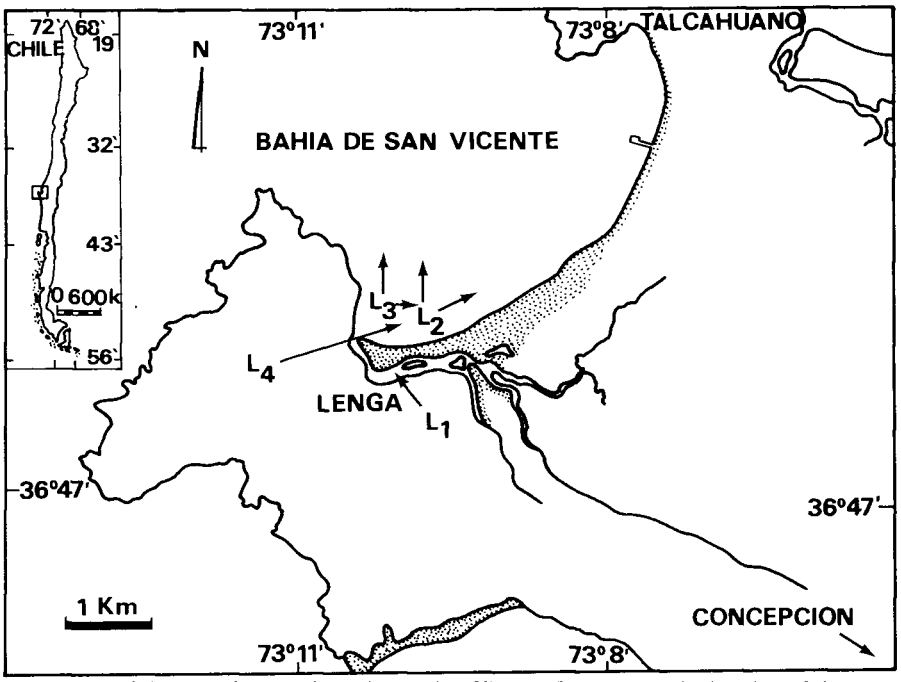

Fig 1. Map of the site of the study, Bahía de San Vicente (Lenga), and the location of the morphotypes sampling stations.

Fig 2. Morphotypes of *Gracilaria* from Lenga. (A) L1 (estuarine morphotype), (B) L2 (thin morphotype), (C) L3 (thick morphotype), and (D) L4 (dark green morphotype).

and 2.5 volume of –20°C ethanol 95%. After incubation in an ice bath, the precipitate was collected by spooling and/or centrifugation at 8000 rev min-1 for 30 min at 4°C. The pellet was washed in 70% ethanol, air dried and dissolved in *ca.* 30 ml of sterile TE (0.01 M Tris, pH 8.0 and 0.001 M EDTA).

DNA purification

Organelle and nuclear DNA fractions were separated from residual contaminants (carbohydrates and any denatured proteins) and RNA by ultracentrifugation in a Hoechst 33258 cesium chloride gradient (1.198 g ml^{-1}) at 39000 rev min^{-1} for 40 h at 18°C. Both fractions were removed separately from the gradient, and after the Hoechst was removed using a NaCl water-saturated 2-propanol (3-4 rinses), they were precipitated by the addition of 2 volumes of sterile deionized water and then 2 volumes of 95% ethanol at 20°C. After setting at 4°C overnight, the samples were centrifuged at 8000 rev min^{-1} for 30 min at 4°C, washed in 70% ethanol and subsequently recentrifuged at 8000 rev min^{-1} for 5 min at 4°C, to obtain the pellet. The pellet was resuspended in 500 (organelle DNA) and 1000 µl (nuclear DNA) TE and reprecipitated by the addition of 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of –20°C ethanol 95%. After precipitation and centrifugation, the pellets were washed in cold 70% ethanol, air dried and

resuspended in 500 or 1000 µl of TE to give a final concentration of 10 ng DNA µl^{-1}. The nuclear DNA of each morphotype was saved to be further analyzed by the random amplified polymorphic DNA (RAPD) technique.

To determine the concentration of the organelle DNA fractions, 10 µl of each was electrophoresed in 0.8 % agarose using 100, 150 and 200 µg of lambda phage DNA as a control.

Restriction endonuclease digestions and electrophoresis

Ten µl organelle DNA aliquots from each morphotype (20 ng µl^{-1}) were digested with 2 or 10 U of the following endonucleases: Bam H I, Bgl II, Cla I, EcoR I, Hae III, Hind III, Kpn I, Not I, Pst I, Pvu I, Sal I, Xba I and Xho I (New England BioLabs, Inc, USA), following the protocol suggested by the supplier. Lambda phage DNA, cut with endonucleases, was used to make lanes of molecular weight standards. Restriction endonuclease-cut aliquots containing *ca.* 200 ng DNA were placed in wells of 0.5, 0.7 and 1.0 % agarose horizontal gels. Electrophoresis was carried out in 1X TAE buffer (Sambrook *et al*, 1989) at 20-30 V for 16 h. The gels were stained with ethidium bromide (0.5 µg ml^{-1}), rinsed with water, and examined and photographed on a UV transilluminator. A Polaroid MP4 camera with a Wratten 23A filter and Polaroid Type 665 film was used to photograph the gels.

RESULTS AND DISCUSSION

The method of total DNA isolation, followed by fractioning on Hoechst cesium chloride gradients, was effective in separating organellar from nuclear DNA of the four morphotypes studied (Fig 3). The certainty that the upper fraction corresponds to the organellar DNA is based on the different buoyant density the organellar DNA has in relation to nuclear DNA. Hoechst binds preferentially to DNA with a high A+T:G+C ratio which is typical of organellar DNA, thus decreasing the density of this DNA more than it does that of the DNA with lower ratio (nuclear DNA) (Roell and Morse, 1991). Moreover, the same fractionation pattern has been found in *Gracilaria*, and in other red algae, by Goff and Coleman (1988), Carroll (1989), Bird and Rice (1990), Rice and Bird (1990), Parsons *et al* (1990) and Maggs *et al* (1992).

Electrophoresis of intact organellar DNA on 0.7 % agarose gel demonstrated the absence of RNA in the samples and the integrity of the DNA molecule as no significant breakage occurred during the purification process (Fig 4A).

Digestion of the upper band DNA from the density gradients of each morphotype with twelve restriction endonucleases, and the subsequent electrophoretic separation generated the same restriction pattern for all of them (Figs 4B-D, 5A-C). The only 8-cutter endonuclease used, Not I, did not cut the organellar DNA of any of the morphotypes.

Plastid and/or organellar DNA restriction fragment patterns have been a useful tool to differentiate red algal genera and species (Parsons *et al*, 1990; Maggs *et al*, 1992) including *Gracilaria* (Goff and Coleman, 1988; Bird and Rice, 1990; Rice and Bird, 1990; Bird *et al*, 1990). Bird *et al* (1990a)

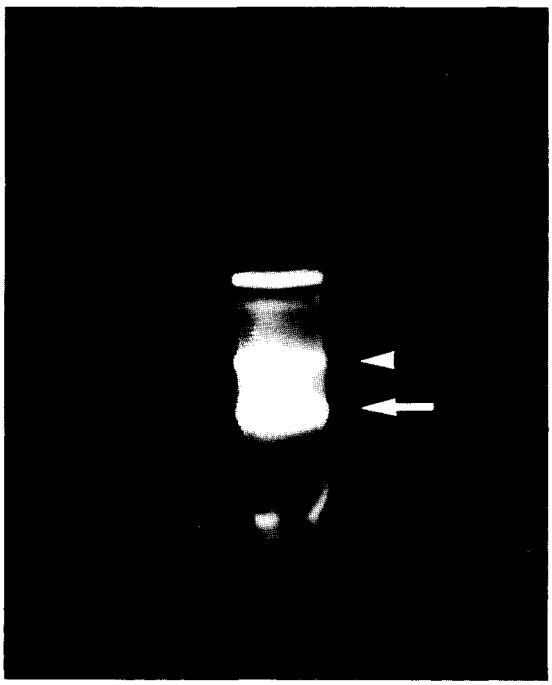

Fig 3. Hoechst CsCl gradients of one of the *Gracilaria* morphotype DNA. Arrowhead indicates organellar DNA band, and arrow indicates nuclear DNA band.

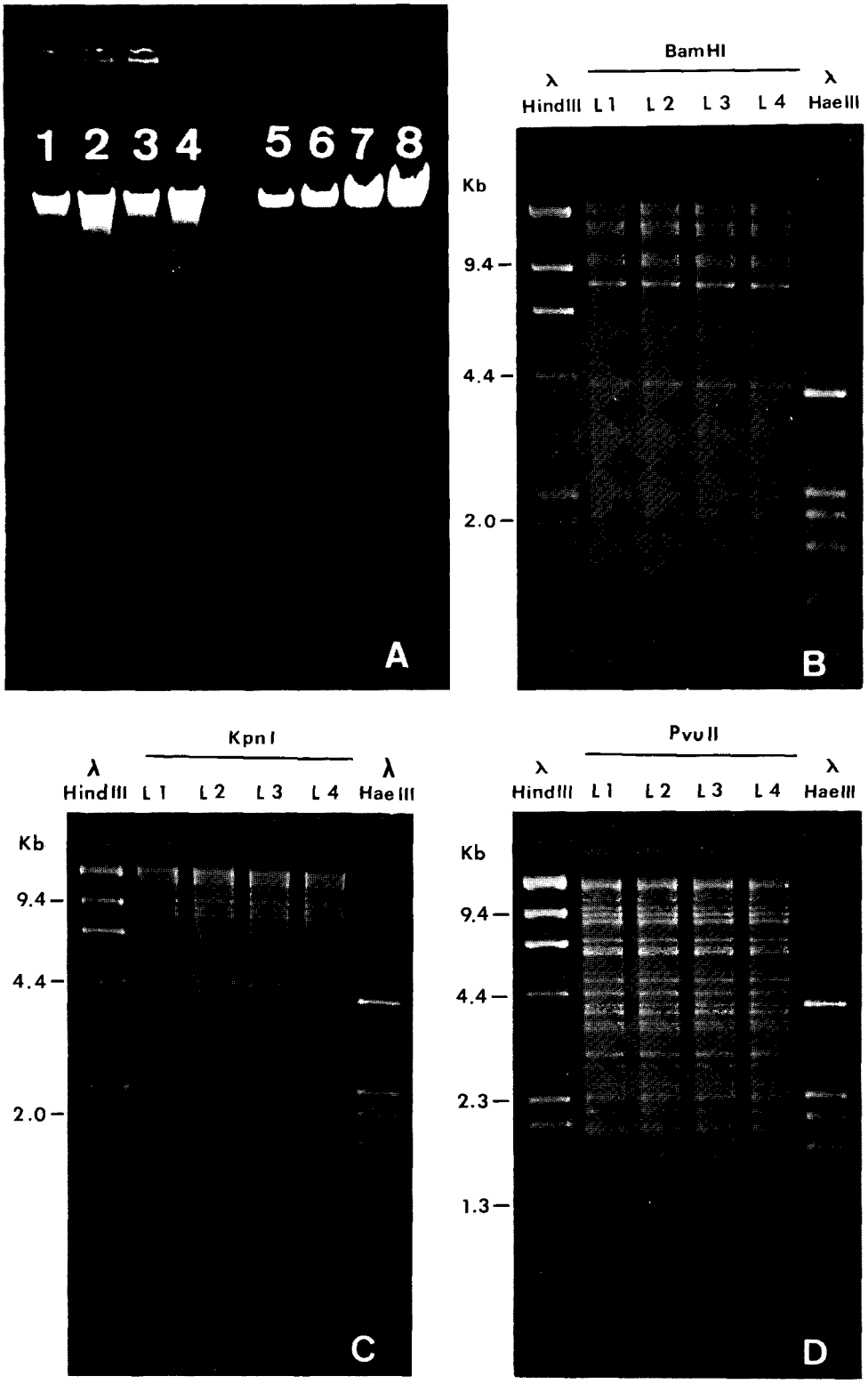

Fig 4. A. Agarose gel electrophoresis of standard lambda DNA and organellar DNA of the four morphotypes. Lanes 1-4: 10 µl of organellar DNA from L2, L1, L3 and L4. Lanes 5-8: 50, 100,150 and 200 µg of lambda DNA. **B-D.** Endonucleases patterns of organellar DNA of the four morphotypes digested with BamH I (B), Kpn I (C) and Pvu II (D). Marker is lambda digested with Hind III and Hae III.

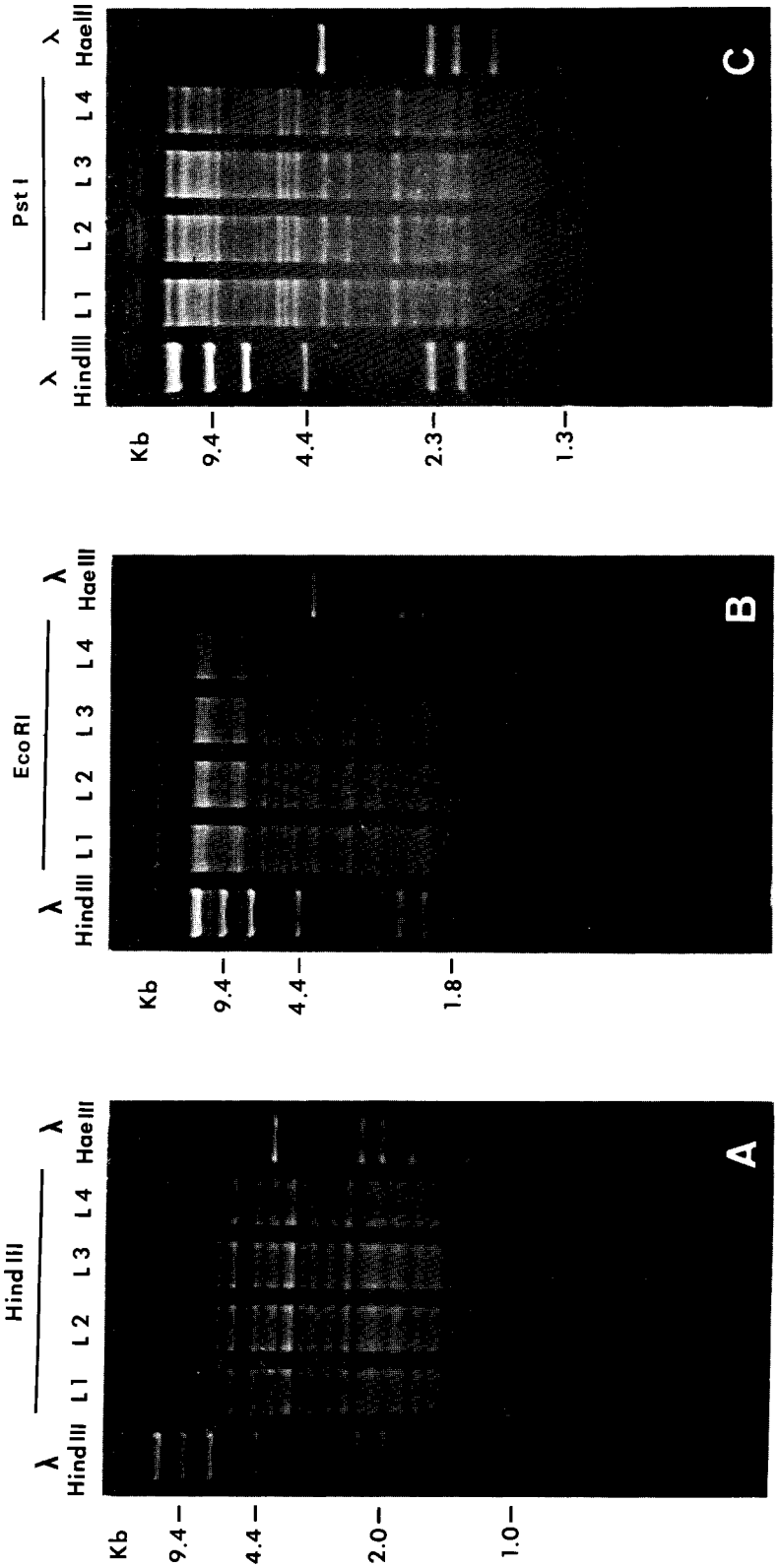

Fig 5. Hind III (A), EcoR I (B) and Pst I (C) digestion patterns of organellar DNA of the four morphotypes of *Gracilaria*. Marker as in Fig 4 (B-C).

compared the organellar restriction profiles of *Gracilaria chilensis* from Chile (Bahía La Herradura, IVth Region) with those of *G. sordida* from New Zealand, and found that both species shared the same restriction fragment pattern. Based on the DNA data and on reproductive anatomy, chromosome number and interfertility, they concluded that the species were conspecific.

The restriction fragment analyses of the organellar DNA of the morphotypes reported in this paper and that of *G. chilensis* reported by Bird *et al* (1990a, fig 6A, p 379), are identical when digested with the same endonucleases (Bam HI and XpnI). On the contrary, they differ from the organellar RFLP of *G. verrucosa* (Bird *et al*, 1990a, fig 6A) a species which is distinguished from *G. chilensis* by the presence of *verrucosa*-type spermatangial conceptacles.

Concurrently, unpublished preliminary data from hybridization trials (Candia, unpublished observations) among the morphotypes revealed that the four were interfertile (L1, L2, L3 and L4). This result plus the DNA data reported herein, and anatomical studies of the reproductive structures (Candia, 1988; Candia and Reyes, 1993) led us to conclude that all four of the morphotypes correspond to *Gracilaria chilensis*. Presently, we are continuing our studies, concentrating on the nuclear genome, by means of RAPDs in an attempt to unravel the nature of the differences of these morphotypes at the DNA level. It is well known right now (Weising *et al*, 1995), that the polymorphisms generated by RAPDs function as genetic markers and can have multiple uses such as construction of genetic maps, for plant and animal breeding applications, and for DNA fingerprinting with particular utility for studies of population genetics (Williams *et al*, 1990). Adams and Demeke (1993) have shown that RAPDs can also be utilized to discriminate taxa (*Juniperus*, a genus of Conifer) at sectional, species and varietal taxonomic level. In algae, this technique has been used in *Gelidium* to detect genetic markers in populations of *G. vagum* Okamura (Patwary *et al*, 1993). Due to the economic importance of *G. chilensis* in Chile, it appears obvious to us to undertake a search for DNA polymorphism at the population level. Although agar yield and gel strength have not been tested in the Lenga's morphotypes, this approach therefore would facilitate a pre-selection of strains with particular genetic traits relevant for the optimization of the exploitation of this research.

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