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Comparison of ITS RFLP patterns of *Gracilaria* (Rhodophyceae, Gracilariales) populations from Chile and New Zealand and an examination of interfertility of Chilean morphotypes

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Abstract

Restriction fragment length polymorphism (RFLP) patterns of the internal transcribed spacer (ITS) of the nuclear ribosomal cistron and crossability trials were used to characterize four morphotypes of *Gracilaria* from Lenga, Isla Santa María and Maullín, Chile, and two morphotypes from sites in New Zealand. PCR products from all Chilean morphotypes resulted in a major single band of ca. 1198 bp. ITS-RFLP profiles generated with the restriction enzymes Cla I, Hae III, Pst I, Hha I, Rsa I and Taq I, were identical in all cases. All crosses within, as well as between, morphotypes resulted in cystocarp differentiation, with the production of viable carpospores. Based upon these data, it is concluded that the four morphotypes from Chile correspond to a single species, *G. chilensis*, and that the ITS-RFLP pattern is a useful marker to predict genetic relatedness at the specific level in *Gracilaria*. A comparison of the ITS-RFLP patterns of the Chilean morphotypes with the patterns of two samples of *G. chilensis* from New Zealand revealed that the sample from Scorching Bay, Wellington, fits the Chilean ITS-RFLP patterns. The population from Blockhouse Bay, Auckland, appears to correspond to another species.

Introduction

Investigations at the DNA level have provided useful taxonomic tools for distinguishing organisms difficult to identify by more traditional means. In *Gracilaria*, in which some species share a common but variable morphology and consequently are often misidentified, plastid DNA RFLP patterns have served to identify geographically distant conspecific taxa, as well as to differentiate morphologically similar taxa (Goff & Coleman, 1988; Bird et al., 1990; Rice & Bird, 1990). Nucleotide sequence data from intergenic spacer regions have also been used to discriminate species in various algal genera (Bakker et al., 1992, 1995; Kooistra et al., 1992; van Oppen et al., 1993; Coleman et al., 1994; Coleman & Mai, 1997) including *Gracilaria* (Goff et al., 1994). Goff et al. (1994) indicate that characterization of the ITS region, either by sequencing or by RFLP should permit relatively rapid and accurate species identification within the Gracilariales.

In addition to molecular tools which have helped to solve many taxonomic problems within *Gracilaria* and in closely related taxa, a classical attribute for delimiting biological species is the test of interfertility. Although Burrows (1954) and Sanbonsuga and Neushul (1979) have shown successful interspecific and intergeneric crosses in brown algae, reproductive isolation is well documented for many species of

red algae including *Gracilaria* (Bird & McLachlan, 1982; Plastino & Oliveira, 1988; Yamamoto & Sasaki, 1988). Hybridization trials by Bird and McLachlan (1982) using *G. verrucosa*, *G. foliifera*, *G. tikvahiae*, *G. bursa-pastori*, and *Gracilaria* sp., led them to conclude that these species are genetically segregated, and unable to interbreed with the facility exhibited by some fucalean and other large brown algae. In a review of recent taxonomic concepts in the Gracilariaceae, Bird (1995) concluded that reproductive isolation is a fundamental species concept that has proven useful to *Gracilaria* taxonomy.

In central Chile at least 3 species of *Gracilaria* with cylindrical thalli and similar morphology have been recorded (Ramirez & Santelices, 1991). In spite of the great variability in thallus morphology, however, it appears that most of the populations of *Gracilaria* in Chile belong to only one species, *G. chilensis*. This conclusion is based on the spermatangial structure (Bird et al., 1986, 1990), on the RFLP pattern of the organellar genome (González et al., 1995, 1996) and on genomic RAPD analysis (Meneses, 1996). Bird et al. (1986) when describing *G. chilensis* reported that reciprocal crosses of populations from Bahia Herradura and Rio Maullín resulted in fertile tetrasporophytes with viable tetraspores.

As part of a revision of New Zealand species of *Gracilaria* (Nelson, 1987), a species found in estuarine and moderately sheltered areas was described as *G. sordida*. Almost simultaneously *G. chilensis* Bird, McLachlan & Oliveira was described from central Chile (Bird et al., 1986). Subsequently, a comparative study (Bird et al., 1990) was carried out using material from the type locality of *G. sordida* (Pauatahanui in the lower North Island, New Zealand) and *G. chilensis* from Bahia Herradura (paratype locality). This evaluation of the two species involved a comparison of growth in culture and interfertility, chromosome numbers, organellar DNA restriction profiles, and reproductive anatomy, and as a result *G. chilensis* and *G. sordida* were deemed to be conspecific.

González et al. (1995, 1996) studied 4 morphotypes of *Gracilaria* from Lenga (36°45′S) and Maullín (41°40′S) with each of the morphotypes present in both localities. The present account, deals with these same morphotypes, but also two morphotypes of *Gracilaria* from Isla Santa María (37°03′S) and two from New Zealand. The aim of this study was to examine whether all the Chilean morphotypes identified as *G. chilensis* are interfertile, and whether the ITS-RFLP pattern is an appropriate tool to predict genetic relatedness at the specific level within *Gracilaria*.

Materials and methods

Collection and culture of Gracilaria

Samples from Chile (Table 1) consisted of mature cystocarpic thalli which were collected from 3 localities and 2 different habitats. In the laboratory, thalli were carefully cleaned and briefly washed in tap water to eliminate epiphytes. Cultures were started from carpospores according to the method of Candia (1988).

Thalli of *G. chilensis* from Scorching Bay, Wellington (41°18′S; 174°50′E), and Blockhouse Bay, Auckland (36°51′S; 174°42′E) (New Zealand), were dried in silica gel. The thalli from Blockhouse Bay were fine and growing on cobbles on a protected, low intertidal shore, and the thalli from Scorching Bay were robust and growing on cobbles and on rocks in the low intertidal and upper subtidal zones on a moderately exposed beach. Nuclear DNA of *Gracilariopsis lemaneiformis* from Moss Landing, CA, USA (provided by L.J. Goff) was used for comparison.

Crossability tests

Interfertility tests within and between morphotypes were performed using gametophytic thalli grown from tetraspores obtained from laboratory-grown tetrasporophytes. Virgin female gametophytes were produced over a period of 3 months repeated subculturing of excised apices of female thalli. Hybridization experiments were performed by placing in a Petri dish (20 × 100 mm) one or two explants (1 cm long) of male thalli with four apical explants (2 cm long) of female thalli. All trials were performed in duplicate. Cultures were maintained at 15 °C ±2 °C, with a photoperiod of 16:8 (L:D) and under a photon flux density of 12 to 20 μmoles m^{-2} s^{-1}, in filtered enriched seawater (PES/2; Provasoli, 1968). In each case, duplicates of four female explants were maintained in isolation of male explants and under the same culture conditions as a control. The crossing experiments lasted for ca. 3 months during which time the medium was changed once a week. The criterion used to determine interfertility within and between morphotypes was the release of viable spores from fully developed cystocarps.

Table 1. Location, habitat and morphotype denomination of the Chilean morphotypes of *Gracilaria*

Locality	Habitat	Morphotype denomination
Lenga	Estuarine-intertidal; on rocks	L1
(36°45′S, 73°10′W)	Marine-subtidal; in sand	L2, L3, L4
Maullín	Estuarine-intertidal; on rocks	M1
(41°36′S, 73°38′W)	Marine-subtidal; in sand	M2, M3, M4
Isla Santa María	Marine-subtidal; on rocks	IS1, IS2
(37°3′S, 73°30′W)		

L1 = M1: Thalli of intermediate thickness, profusely branched, 10–30 cm length.
L2 = M2 = IS1: Thalli fine, profusely branched, 30–80 cm length.
L3 = M3 = IS2: Thalli coarse, 40–130 cm length.
L4 = M4: Thalli similar to M2 but olive-green.

DNA extraction and purification

The extraction and purification procedures employed to isolate DNA from the Chilean and the New Zealand morphotypes were those described by González et al. (1995). Approximately 1 to 2 g of thalli of the New Zealand samples were used.

PCR amplification and restriction endonuclease digestion of the ITS

PCR was completed carried out in a Perkin Elmer 280 thermocycler. The primers used to amplify the ITS1, 5.8S rRNA gene and the ITS2 of the nuclear ribosomal repeat were TW18 (5′ GGGATCCGTTTCCGTAGGTGAACCTGC, anneals to the 3′ of the 18S rDNA) and AB28 (GGGATCCATATGCT TAAGTTCAGCG GGT, anneals to the 5′ end of the 25S rDNA).

For each amplification, a 50 μL aliquot of a reaction mix was used. The composition of the aliquot was: 1× reaction buffer (Tris HCl 10 mM pH 8.3, KCl 50 mM, Triton X-100 0.1%), 1.5 mM $MgCl_2$ (or Mg SO_4), 400 μM of each dNTP mixture, 0.4 μM of each primer and 5 U Taq polymerase to which 50 ng of nuclear DNA was added. To prevent evaporation of the reaction mixture during the PCR, each sample was overlaid with one drop of sterile mineral oil. In each case a negative control (lacking DNA) was included. The amplification parameters (provided by A.W. Coleman, Brown University, RI, USA) were as follows: step 1, 5 min at 95 °C; step 2, 1 min at 90 °C; step 3, 2 min at 50 °C (steps 2 and 3 were repeated 5 times); step 4, 1 min at 72 °C; step 5, 1 min at 90 °C; step 6, 1 min at 60 °C; step 7, 1 min at 72 °C (steps 5 to 7 were repeated 30 times); step 8, 10 min at 72 °C (terminal extension). After completion of the amplification the samples were stored at 4 °C.

To test for positive DNA amplification and to determine the size of the product, 5 μL of the reaction mixture were added to 2 μL of the tracking dye and loaded into wells of 2% agarose in TAE 1X buffer at 50 V for 90 min. Phage Lambda DNA digested with Hae III was used as a standard molecular marker. The gel was stained in 0.5 μg mL^{-1} ethidium bromide and photographed with a Polaroid MP4 camera. All of the amplifications were completed in triplicate. For RFLP analyses the three replicates were mixed and used as a single sample. For each morphotype, 3–10 μL (ca. 200 ng μL^{-1}) of the amplified DNA product (ITS) were digested with 5 U of the following restriction endonucleases: Cla I, Hae III, Pst I (6 bp-cutters), Hha I, Rsa I, Taq I, Dpn II, Hpa II and Sty I (4 bp-cutters) (New England BioLabs, Inc., USA) following the protocol of the supplier. The reaction mixtures were incubated at 37 °C (except for Taq I – 56 °C) for 3 h. To stop reactions, 0.2 volume of the track dye solution was added. DNA fragments were separated by electrophoresis for 3 h at 50 V on 4% horizontal agarose gels in TAE 1x buffer and stained and photographed as above.

Results

Crossability test

Mature tetrasporophytes were observed 5 to 7 months after initiation of the carpospore cultures. Differentiation of mature female and male gametophytes occurred 4 to 6 months after tetraspores germin-

ated. All crosses, within as well as between the Chilean morphotypes, resulted in cystocarp differentiation with the production of viable carpospores. Early recognition of crossing success was enabled by the appearance of swellings 3 to 4 weeks after male and female gametophytes had been placed into common culture; cystocarp maturation required 2 additional weeks. Explants of the female gametophytes incubated in the absence of male counterparts failed to differentiate cystocarps.

PCR amplification and restriction endonuclease digestions of the ITS

In all morphotypes tested, PCR amplification of the ITS (ITS1, ITS2) and 5.8S rRNA gene resulted in a single band of ca. 1198 bp. For the two samples of *Gracilaria* from New Zealand, the amplified fragment was slightly shorter (ca. 1181 bp). The same DNA region was amplified for *Gracilariopsis lemaneiformis* as a control, and had a single amplification product of ca. 950 bp (Figures 1, 4). Restriction profiles obtained from the PCR-amplified ITS fragments of the 4 morphotypes from Lenga digested with 6 bp-cutter endonucleases (Cla I, Hae III, Pst I) were identical (Figure 2). Digestion of the PCR product from *Gracilariopsis lemaneiformis* with Cla I resulted in a single cut site being resolved (Figure 2A), whereas Hae III or Pst I (Figure 2B, C) failed to cut this fragment. ITS fragments of the different morphotypes from Lenga, Maullín and Isla Santa María, digested with 4 bp-cutter endonucleases (Hha I, Rsa I, Taq I), showed the same restriction patterns (Figure 3: data from Santa María, not shown). As shown in Figure 3, the three enzymes recognized 2 restriction sites in each morphotype, generating in each case three bands of different length depending on the enzyme. On the other hand, the ITS region of *Gracilariopsis lemaneiformis* gave a complete different pattern, and no evidence of digestion was observed when this amplified DNA was incubated with Rsa I.

A comparison of the RFLP patterns of the morphotypes of *G. chilensis* from Chile with the samples from New Zealand revealed that only the sample from Scorching Bay fitted the Chilean pattern. The ITS-RFLP pattern of *G. chilensis* from Blockhouse Bay was completely different (Figure 4).

Discussion

The crossability tests, as well as the ITS-RFLP analyses, confirm the genetic identity of the Chilean morphotypes of *Gracilaria* studied. All correspond to *G. chilensis*. Bird (1995) strongly argued for the use of culture studies and interfertility analyses in systematic studies of *Gracilaria*, although noting that laboratory crossings are time consuming and in consequence not routinely performed. In *Gracilaria* there are further difficulties because of spontaneous bisexual thallus formation, the difficulty in recognizing male thalli in natural populations, and the production of non-functional cystocarps (Bird & Rice, 1990). However, studies of interfertility in various species of *Gracilaria* have permitted the determination of compatibility, and clarified and/or enabled the identification of taxa (Plastino & Oliveira, 1988; Yamamoto & Sakaki, 1988; Bird et al., 1990; Bird & Rice, 1990). The latter authors observed that populations of *Gracilaria verrucosa* from Argentina, France and Norway were interfertile and also exhibited similar plastid DNA restriction patterns.

The demonstration of *in vitro* interfertility in the four Chilean morphotypes studied suggests the feasibility of genetic flow in the wild. Morphological variations observed within and between localities could possibly be the expression of phenological adaptation to varying habitats. This is clearly evident within the same locality for estuarine and subtidal morphotypes.

The occurrence of olive-green thalli in Lenga and Maullín is probably due to spontaneous gene mutation, as reported by van der Meer and Bird (1977). Zhang and van der Meer (1987) working with both spontaneous and induced color mutants have shown that in *Gracilaria* there are two different transmission patterns for this type of mutation: recessive nuclear and maternal transmission. Unfortunately, in our studies of crossability, we did not follow the progeny of the crossings involving the color mutant.

Length variations of the ITS region (containing the 5.8S rRNA gene) have been observed at interspecific as well as intraspecific levels in various eukaryotes (Furlong & Maden, 1983; Chambers et al., 1986; O'Donnell, 1992). In algae, Steane et al. (1991) analyzed the ITS length variation in Gigartinales (Rhodophyceae) and found this region too polymorphic to be used as a taxonomic marker at the species level and above. However, more recently ITS sequence analyses in green (Coleman et al., 1994; Coleman & Mai, 1997), in brown (Bakker et al., 1992, 1995; Kooistra

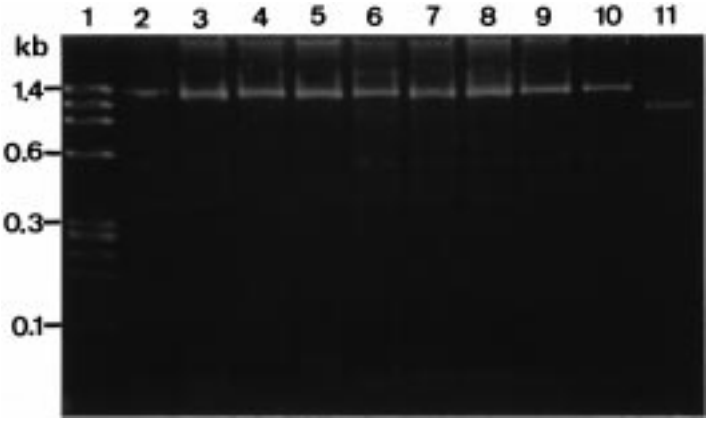

Figure 1. Amplified products of the ITS region (ITS1 + 5.8S + ITS2) from morphotypes of *Gracilaria* from Chile (lanes 2–10) and *Gracilariopsis lemaneiformis* (lane 11). Lanes 2–4, morphotypes from Lenga; lanes 5–8, morphotypes from Maullín; lane 10, morphotype from Isla Santa María. Lane 1, ϕ X digested with Hae III.

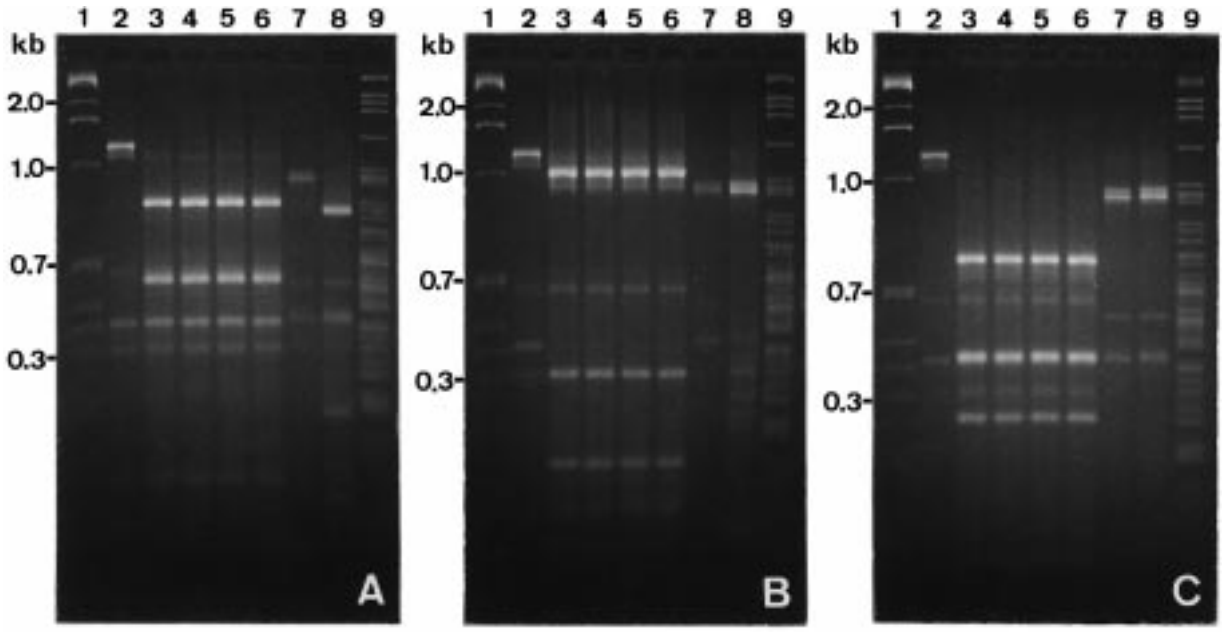

Figure 2. Restriction fragment patterns of the ITS region of the four morphotypes from Lenga (lanes 3–6) and *Gracilariopsis lemaneiformis* (lane 8) digested with (A) Cla I, (B) Hae III and (C) Pst I. Lanes 2 and 7 are non-digested ITS region of *Gracilaria* and *Gracilariopsis*, respectively. Lane 1 is 1kb ladder marker and lane 9 is lambda DNA digested with Hae III.

et al., 1992; van Oppen et al., 1993) and in red algae (Goff et al., 1994; Patwary et al., 1998) have proved that this region is very conservative at the species and, in some cases, at the generic level. Goff et al. (1994) found that although the size of the ITS region may be variable between species of *Gracilaria* and *Gracilariopsis*, the sequence in this region is conserved enough to permit alignment of closely related species. According to these authors, the most valuable use of the ITS region in these taxa is its ability to clearly delineate species. Analyses of the amplified ITS region of the morphotypes from Lenga (4), from Maullín (4) and Isla Santa María (2), on agarose gels showed that their lengths were comparable (ca. 1198 bp) to the corresponding ITS regions of other species of *Gracilaria* (*G. pacifica*: 1263 bp, *G. robusta*: 1167 bp, *G. verrucosa*: 1125 bp, *G. tikvahiae*: 1167 bp) (Goff et al., 1994). Also, a slight difference exists between the ITS region from *G. chilensis* from Chile and *G. chilensis* from New Zealand (1198 versus 1127, this paper).

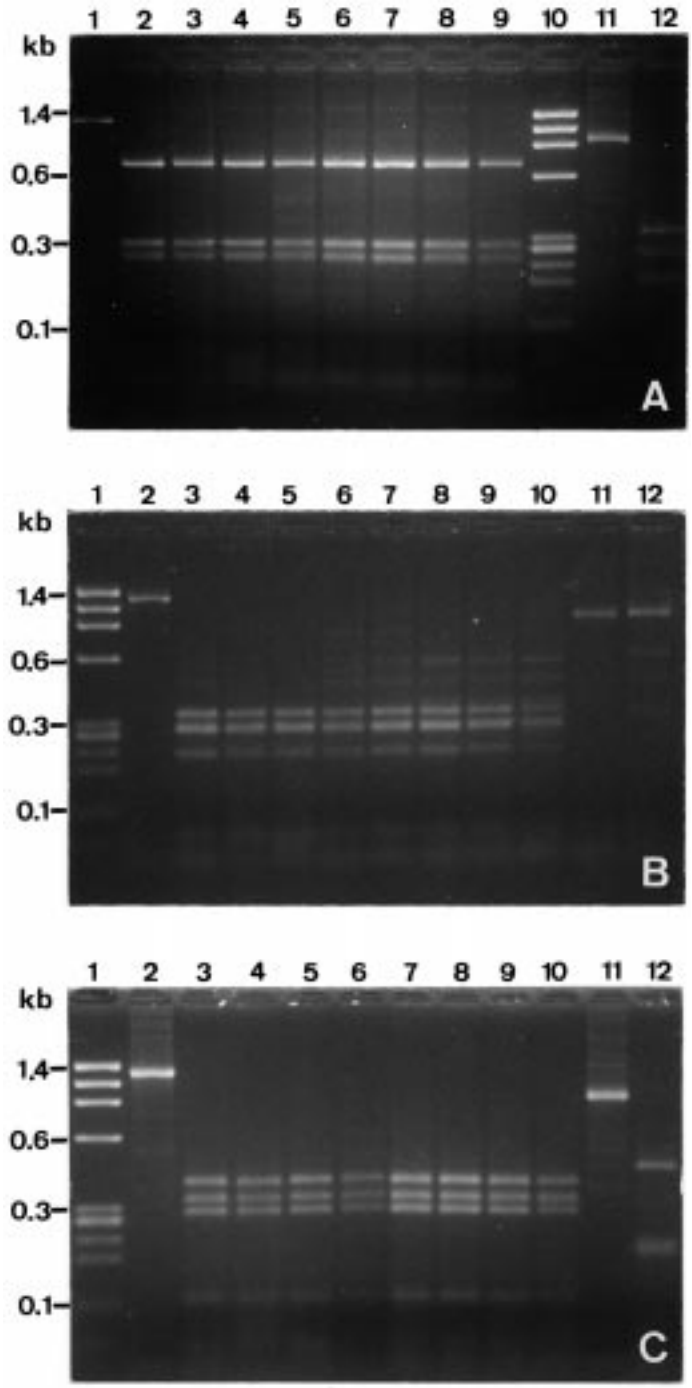

Figure 3. Restriction fragment patterns of the ITS region of the morphotypes of *Gracilaria* from Lenga (lanes 2–5 in A; lanes 3–6 in B and C) and Maullín (lanes 6–9 in A; lanes 7–10 in B and C) and of *Gracilariopsis lemaneiformis* (lane 12) digested with Hha I (A), Rsa I (B) and Taq I (C). Lanes 1 (in A), 2 and 11 (in B and C) are non-digested ITS region of *Gracilaria* and *Gracilariopsis*, respectively. The standard marker is ϕ X digested with Hae III.

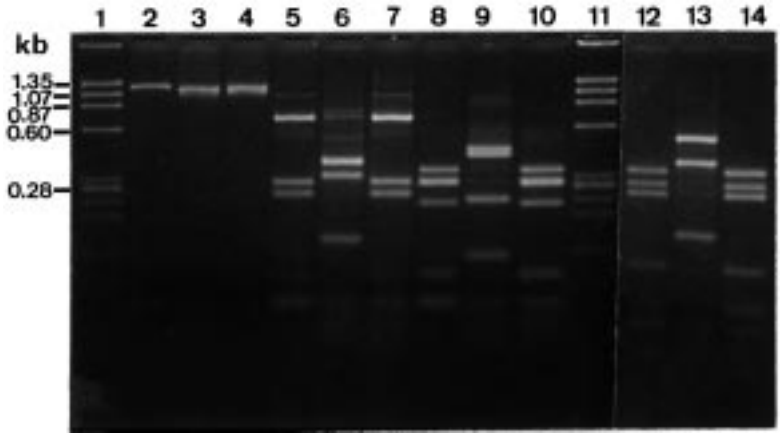

Figure 4. Restriction fragment patterns of the ITS region of morphotype L2 of *Gracilaria chilensis* from Chile (lanes 5, 8, 12) and two samples of *G. chilensis* from New Zealand (lanes 6, 9 and 13 from Blockhouse Bay, Auckland; lanes 7, 10 and 14 from Scorching Bay, Wellington) digested with Hha I (lanes 5–7), Rsa I (lanes 8–10) and Taq I (lanes 12–14). Lanes 2–4 are non-digested ITS region of *G. chilensis* from Chile (lane 2) and from New Zealand (lane 3: Auckland, lane 4: Wellington). Lanes 1 and 11 are ϕ X digested with Hae III.

Goff et al. (1994) give a very similar value for the ITS from *G. chilensis* from New Zealand (1119 bp).

In spite of the slight difference in length of the ITS region between *G. chilensis* from New Zealand and Chile, the digestion of this region in the Chilean morphotypes and in the sample from Scorching Bay (Wellington) gave the same RFLP patterns with all endonucleases tested. However, the RFLP analysis of this region from *G. chilensis* from Blockhouse Bay (Auckland) was completely different to either *G. chilensis* from Scorching Bay or to any of the morphotypes of *G. chilensis* from Chile. Additional concurrent results have been obtained through total genome RAPD analysis of these same morphotypes (Chilean + New Zealand) (manuscript in preparation).

Coleman et al. (1994) and Coleman and Mai (1997) working with Volvocales (Chlorophyceae) found that within interfertile groups (irrespective of geographical origin) there was a similar degree of ITS sequences (less than 5% difference), while different breeding groups had very distinctive ITS sequences (10–30% difference). According to this finding and from our results, even though we did not perform crossing experiments between the New Zealand and Chilean morphotypes, it appears that the population from Blockhouse Bay would correspond to another species. In a previous study (Bird et al., 1990) interfertility of New Zealand *G. chilensis* (from Pauatahanui, Wellington) and Chilean *G. chilensis* (from Bahía Herradura) was established, and fertile F_1 gametophytes were produced in culture. Additionally, the *G. chilensis* from Pauatahanui (fine morphotype, Bird et al., 1990) and from Scorching Bay (coarse morphotype – this study) have been shown by two different molecular techniques to be conspecific with Chilean populations of *G. chilensis*. This study has clearly shown the value of molecular approaches in the systematics of *Gracilaria*, and has unequivocally established the presence of a further, apparently undescribed species of *Gracilaria* in New Zealand. Voucher material of the sample from Blockhouse Bay is sterile and preliminary study reveals no clear morphological characters by which to distinguish the 'Blockhouse Bay' species. A further study is required to understand the distribution of the undescribed species and to identify characters by which it can be distinguished from other species of *Gracilaria* in New Zealand.

If the present results for RFLP of the ITS region are compared with the analyses of Scholfield et al. (1991) of different populations of *Gracilaria verrucosa* and *Gracilariopsis longissima*, but using the 18S DNA region, it can be concluded that the ITS region is more informative at the genus and species level. As shown in this paper, there exist length and sequence polymorphisms of the ITS region for both *Gracilaria* and *Gracilariopsis*. However, Scholfield et al. (1991) did not find length polymorphism for these genera in the amplified 18S DNA gene. They reported that digestion with only 3 out of 12 endonucleases of the 18S DNA gene gave informative restriction fragment length polymorphism.

It is concluded that the internal transcribed spacer (ITS) of the ribosomal DNA is a better region than the 18S rDNA to assess species limits in *Gracilaria*, and

specifically that the ITS-RFLP comparisons provide an appropriate tool for predicting genetic relatedness at the specific level in *Gracilaria*. It is also suggested that the RFLP of the ITS region be put to the test with other algae, particularly in those cases where many taxa need to be analyzed, and no information exists on breeding data.

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