Journal of Applied Phycology **12:** 25–35, 2000.

An assessment of the AFLP method for investigating population structure in the red alga *Chondrus crispus* Stackhouse (Gigartinales, Florideophyceae)

Sylva L. Donaldson[1,2], Thierry Chopin[3] & Gary W. Saunders[1,*]
[1] *Department of Biology, University of New Brunswick, Fredericton, NB, Canada, E3B 5A3*
[2] *Current address: Botany Department, University of Toronto – Scarborough Campus, 1265 Military Trail, Scarborough, Ontario, Canada, M1C 1A4*
[3] *Centre for Coastal Studies and Aquaculture, University of New Brunswick, Saint John, NB, Canada, E2L 4L5*

(* Author for correspondence: E-mail: gws@unb.ca)

Received 4 January 2000; revised 8 January 2000; accepted 9 January 2000

Key words: AFLP, *Chondrus*, Florideophyceae, genetic markers, population, Rhodophyta

Abstract

The appropriateness of the Amplified Fragment Length Polymorphism (AFLP) technique for investigating *Chondrus crispus* Stackhouse populations in the Maritime Provinces of Canada was assessed. The AFLP procedure was first subjected to reproducibility testing and three shortcomings were noted: 1) failure to reproduce band intensity between replicate runs for the same individual and primer pair; 2) failure of some bands to replicate; 3) lack of reproducibility for complete replicate runs for some individuals and primer pairs. In the last-mentioned case, the lack of reproducibility resulted in characteristic electropherograms indicative of weak reactions. These weak runs can be attributed to poor restriction digest/ligation reactions and/or substandard PCR, these failures ultimately resulting from low and inconsistent DNA quality. We recommend that reproducibility testing should be completed routinely in studies using the AFLP technique. In the current work, only fragments and individuals that gave reproducible results were used in subsequent analyses.

The AFLP method resulted in highly variable markers within and between the populations of *C. crispus* included in this investigation, which prevented successful resolution of population structure. This situation could result from a lack of suitability for AFLP markers in population genetic studies, and/or too extensive genetic variation for *C. crispus* populations to be discerned by the AFLP technique. These two possible explanations are discussed.

Introduction

Molecular techniques are becoming employed more frequently to answer questions pertaining to the relatedness of organisms within and between species. The two types of molecular analyses used most often are sequencing and fragment analyses of DNA. Sequencing analyses have obvious benefits because the actual nucleotide sequence is obtained, providing extensive information about the genetic diversity for the organisms in question. However, sequencing is both expensive and time consuming, and projects are constrained by the need to develop primers for specific regions of the genome. Fragment techniques, therefore, offer some benefits as compared to sequencing analyses: little sequence knowledge is required for many fragment methods; and, a large number of samples can be analyzed relatively easily and affordably. As well, with fragment techniques it is easy to sample variable regions of the genome, regions for which sequencing primers have not necessarily been developed (Dowling et al., 1990). Unfortunately, fragment techniques also have their shortcomings. Restric-

tion Fragment Length Polymorphism (RFLP) analysis involves cutting DNA with restriction enzymes and comparing the banding patterns of individuals (Dowling et al., 1990). An RFLP analysis requires large amounts of DNA. This is problematic for the analysis of algal populations as often isolates must be pooled in order to obtain sufficient quantities of DNA (Chopin et al., 1996). In addition, where large genomes such as eukaryotic nuclear genomes are under investigation, Southern blotting and subsequent hybridization with 'known' DNA regions are required to observe banding patterns (Dowling et al., 1990). This is time consuming and relatively expensive. Randomly Amplified Polymorphic DNA (RAPD) analysis involves the polymerase chain reaction (PCR) amplification of random regions of the genome with short primers (Welsh & McClelland, 1990). This method is fast, easy and economical, producing a wealth of genetic markers. Unfortunately, RAPDs are often sensitive to differing reaction conditions, making reproducibility difficult (Jones et al., 1997). For this reason, investigation into new fragment techniques has been ongoing. A relatively new molecular technique showing promise in a variety of fields is Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995).

AFLP methodology was patented by Zabeau and Vos (1993) and since has been successfully applied to the molecular typing of bacteria (Lin et al., 1996), the assessment of diversity in potato cyst nematode populations (Folkertsma et al., 1996), the determination of genetic diversity among populations of the endangered plant *Astragalus cremnophylax* (Travis et al., 1996), the phylogenetics of chicory varieties (Koch & Jung, 1997), the genetic analysis of single fungal spores (Rosendahl & Taylor, 1997) and the biosystematics of *Solanum* (Kardolus et al., 1998).

The AFLP technique (Figure 1) involves the restriction endonuclease digestion of total cellular DNA with a relatively rare (EcoRI) and a relatively frequent (MseI) cutter. In the same reaction tube, restriction-endonuclease-site specific adapters are ligated to the ends of the cut fragments. The adapters are designed such that they void the restriction site to prevent digestion of ligated fragments. An initial polymerase chain reaction amplification – preselective amplification – is completed using primers complementary to the ligated adapter sequences with one additional nucleotide added to the 3' end (Figure 1). This functions to amplify a specific subset of the many fragments present. A second round of PCR amplification – selective amplification – is performed using the previous

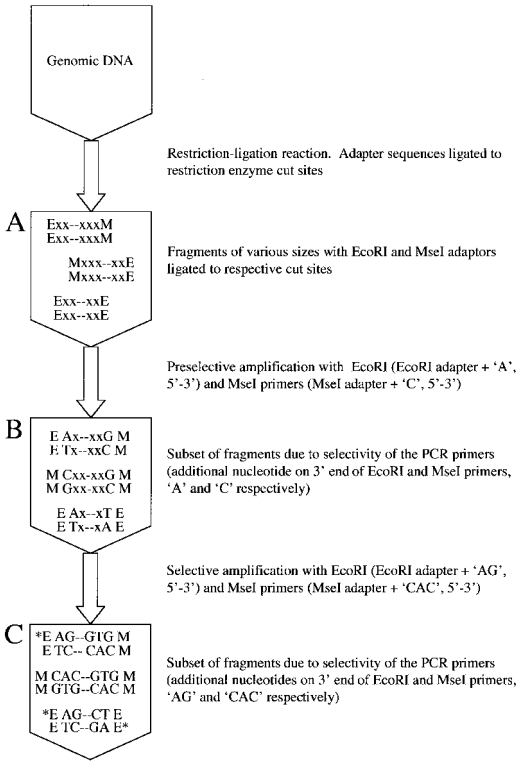

Figure 1. A schematic of the AFLP procedure. The tubes indicate the complement of fragments generated at each step. A) After the restriction-ligation reaction. B) After preselective PCR amplification. C) After selective PCR amplification. '–' refers to a fragment specific length of nucleotides. 'x' indicates any of the four nucleotides. 'E' and 'M' refer to the EcoRI and MseI adapters, respectively. ∗ indicates the presence of fluorescently labeled primers and fragments (only labeled fragments are visualized in the final analyses).

preselective primer sequence with one or two additional nucleotides added to the 3' end. This round of amplification selects a subset of all the fragments resulting from the preselective amplification, selectivity dependent on the nucleotides added to the 3' end of the preselective primers. In the selective PCR amplification, the EcoRI primer is labeled with a fluorescent dye or radioisotope (Vos et al., 1995) so that EcoRI – MseI fragments can be detected. It might be expected that the majority of the amplified products would be MseI–MseI fragments, however, in experiments where the MseI primer was labeled instead of the EcoRI primer, considerably fewer fragments were obtained (Vos et al., 1995). It was concluded that amplification of the MseI–MseI fragments is somehow inhibited. Discrete fragments result from the selective PCR, which can be size fractionated by electrophoresis and identified

by their label. Every fragment observed during the investigation is scored as present or absent for each individual studied.

Molecular investigations of Chondrus crispus

Cheney and Mathieson (1979) examined the isozyme patterns of eight populations of *C. crispus* from New Hampshire and the Maritime Provinces of Canada. The results indicated substantial genetic differentiation over short distances for *C. crispus*, relative to Florida populations for several species of the red algal genus *Eucheuma* (Cheney & Babble, 1978). This implies greater genetic variability for *C. crispus* than in some other red algal species. Chopin et al. (1996) examined several isolates of *C. crispus* including two that they considered the most morphologically divergent plants. A restriction enzyme digestion of the plastid genome and sequencing the first internal transcribed spacer (ITS1), 5.8S coding region and second internal transcribed spacer (ITS2) of the nuclear ribosomal cistron were performed. The *C. crispus* plastid DNA RFLP banding patterns were very similar for all isolates as compared to the plastid banding pattern of *C. ocellatus* Holmes. Because of this, Chopin et al. (1996) determined that the *C. crispus* isolates examined were all of the same species. There was as much as 2.18% sequence divergence over the 780 bp (base pairs) of the ITS regions sequenced – relatively high for within a species, but too low to justify a wide-scale population investigations using this system. The genetic diversity did not, however, correlate with the phenotype for the plants investigated or the geographic origin of the isolates. Chopin et al. (1996) suggested analyzing *C. crispus* populations using more sensitive molecular techniques in order to resolve the population structure relative to morphology and biogeography.

The objective of this study was to perform a preliminary population survey on *Chondrus crispus* to lay the groundwork for more in-depth examinations of this species, and to assess the suitability of AFLP markers for investigations at this level in red algae.

Materials and methods

Sample collection

Ten isolates of *Chondrus crispus* were studied from each of ten study sites in the Maritime Provinces of Canada (Figure 2). In addition, two individuals each were analyzed from a population in Parsonage Point, New York, USA, and from Cap Gris Nez, northern France, and one isolate from Ile de Ré (Phare de la Baleine), southwest France. These five samples were considered the outgroup to the ingroup of the ten Maritime populations (all collection information is provided in Table 1). The samples were collected haphazardly, in that only one isolate was collected from a patch of *C. crispus*. After the isolates were collected, all epiphytes were removed by gently rubbing plant surfaces either while in the field or later in the laboratory. The algae were dried immediately after collection and stored in silica. In order to remove epiphytes in the laboratory, some isolates were rehydrated in deionized water and epiphytes were removed by gentle rubbing. The algae were then re-dried at 40 °C and either stored in silica at room temperature or ground in liquid nitrogen and stored cold (–20 °C).

AFLP procedure

DNA was extracted and purified using the procedure of Saunders (1993). The AFLP technique was performed (Figure 1) following the manufacturer's protocol (PE Applied Biosystems) as follows. Approximately 50 ng total cellular DNA was double-digested with EcoRI and MseI (New England Biolabs) and adapters specific to EcoRI and MseI digested DNA were ligated to the restriction fragments (sequences in Table 2). When ligated, the adapters nullified the restriction site, ensuring that re-digestion did not occur. This allows the restriction and ligation reactions to occur concurrently in a single tube, the restriction enzymes and T4 DNA ligase (New England Biolabs) active in a common buffer system (55 mM Tris-HCl, 11 mM $MgCl_2$, 11 mM DTT, 1.1 mM ATP, 605 μg mL^{-1} BSA, 55 mM NaCl). The restriction-ligation reaction was incubated overnight (approximately 15 to 18 h) at room temperature. The resulting product was diluted five fold and four μL were used for PCR reactions with the preselective primers (sequences in Table 2) complementary to the EcoRI (plus A) and MseI (plus C) adapter sequences. The amplification parameters were: 2 min at 94 °C; 20 cycles of 1 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C; and a final 4 °C hold. The preselective amplification products were diluted five fold and three μL were used in selective PCR amplification reactions: 2 min at 94 °C; 9 cycles of 1 s at 94 °C, 30 s at 65 °C descending 1 °C each cycle, and 2 min at 72 °C; and, a final 23 cycles with 1 s at 94 °C, 30 s at 56 °C and 2

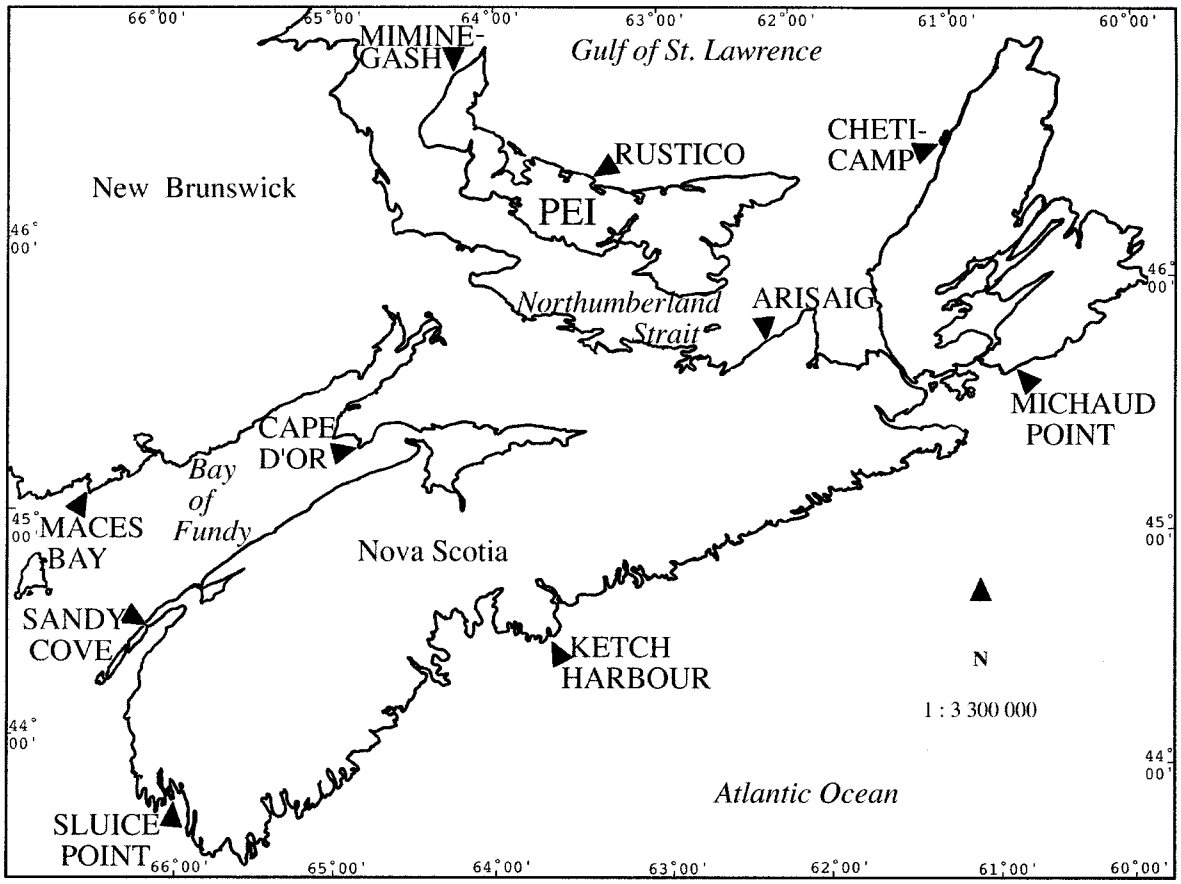

Figure 2. Map of collection locations in the Maritime Provinces of Canada.

Table 1. List of sites where *Chondrus crispus* was collected, abbreviations of collection sites used in the text, collection dates, and collector

Collection Site	Date of Collection	Collector
Arisaig, Nova Scotia (AR)	4 November 1997	T. Chopin
Cape D'Or, Nova Scotia (CD)	1 November 1997	T. Chopin
Cheticamp, Nova Scotia (CC)	3 November 1997	T. Chopin
Ketch Harbour, Nova Scotia (KH)	12 July 1998	S. Donaldson
Maces Bay, New Brunswick (MB)	18 September 1997	S. Donaldson
Michaud Point, Nova Scotia (MP)	2 November 1997	T. Chopin
Miminegash, PEI (PV)	31 October 1997	G. Sharp
Rustico, PEI (RU)	30 October 1997	G. Sharp
Sandy Cove, Nova Scotia (SC)	14 July 1998	S. Donaldson
Sluice Point, Nova Scotia (SP)	November 1997	S. Spinney
Parsonage Point, New York (PP)	24 July 1997	C. Yarish
Ile de Ré, France (PB)	19 August 1997	T. Chopin
Cap Gris Nez, France (CG)	21 August 1997	G. Saunders

Table 2. Sequences of adapters and PCR primers used in the AFLP technique. [1] EcoRI primers with a terminal C or G were combined with MseI primers ending with AA, AC, TG, and TT, and with AC and AT, respectively, to give the six primer combinations used in this study

Name	Sequence
EcoRI recognition sequence	5′ GAATTC 3′
MseI recognition sequence	5′ TTAA 3′
EcoRI adapter	5′ CTCGTAGACTGCGTACC 3′ CATCTGACGCATGGTTAA
MseI adapter	5′ GACGATGAGTCCTGAG 3′ TACTCAGGACTCAT
EcoRI preselective primer	5′ GACTGCGTACCAATTCA 3′
MseI preselective primer	5′ GATGAGTCCTGAGTAAC 3′
EcoRI selective primers[1]	5′ GACTGCGTACCAATTCA(C or G) 3′
MseI selective primers	5′ GATGAGTCCTGAGTAAC(AA or AC or AT or TG or TT) 3′

min at 72 °C. Primers for the selective amplification were the same as the preselective primers with one additional nucleotide added to the 3' end of the EcoRI primer, and two nucleotides added on to the 3' end of the MseI primer (Table 2). Six different selective PCR primer combinations were used (Table 2). Deionized formamide, or template suppression reagent (PE Applied Biosystems), and GeneScan 500-Rox (PE Applied Biosystems) size standard were combined with one μL of the selective amplification product. This mixture was denatured at 94 °C for two minutes and immediately placed on ice for five minutes.

Samples were electrophoresed on an ABI Prism-310 genetic analyzer (PE Applied Biosystems), which has the benefit of a four-color fluorescent dye system, allowing several samples to be run at once (multiplexed). Multiplexing was employed in this study with two different samples co-electrophoresed during each run. This allows both increased cost-efficiency and time-efficiency. Only bands between 35 and 500 bp were scored.

The reproducibility of the AFLP technique was tested on nine individuals by starting with the original ground sample, re-extracting the DNA, and completing all procedures of the AFLP method for all six primer pairs.

Statistical analyses

Fragments were scored as present or absent, with no consideration given to intensity. The Dice similarity coefficient (Dice, 1945) was calculated using the following formula:

Coincidence index = 2h / a + b

where:

h = the number of fragments shared in a and b
a = the number of fragments for individual a
b = the number of fragments for individual b.

The Dice coefficient was used to generate a similarity matrix that was converted to a dendogram with UPGMA (Unweighted Pair-Group Method with Arithmetic mean) cluster analysis in NTSYS-pc (Version 1.8; Rohlf, 1993).

In addition, the distance metric of Nei and Li (1979) was applied to pairwise comparisons between all individuals to estimate nucleotide substitution from the AFLP fragment data. Although estimation of nucleotide substitution from fragment data relies on an assumption that is likely violated (fragment differences result from restriction site gain and loss, not insertion and deletion events), we reasoned that this estimation would provide a preferred distance matrix (relative to similarity values, which fail to consider in any way how restriction sites evolve) for tree construction. The proportion of fragments (F) shared between pairwise comparisons of all individuals were estimated using an equation similar to the Dice coefficient. These values were then used to estimate the number of nucleotide substitutions (d) per site between pairs of individuals (Nei & Li, 1979). The resulting distance matrix was converted to a dendogram with neighbour-joining as implemented in PAUP (paup4.0d65; Swofford, 1999). As a test of support for the tree topology, one thousand bootstrap replicates (Felsenstein, 1985) were performed with the neighbour-joining analyses in PAUP.

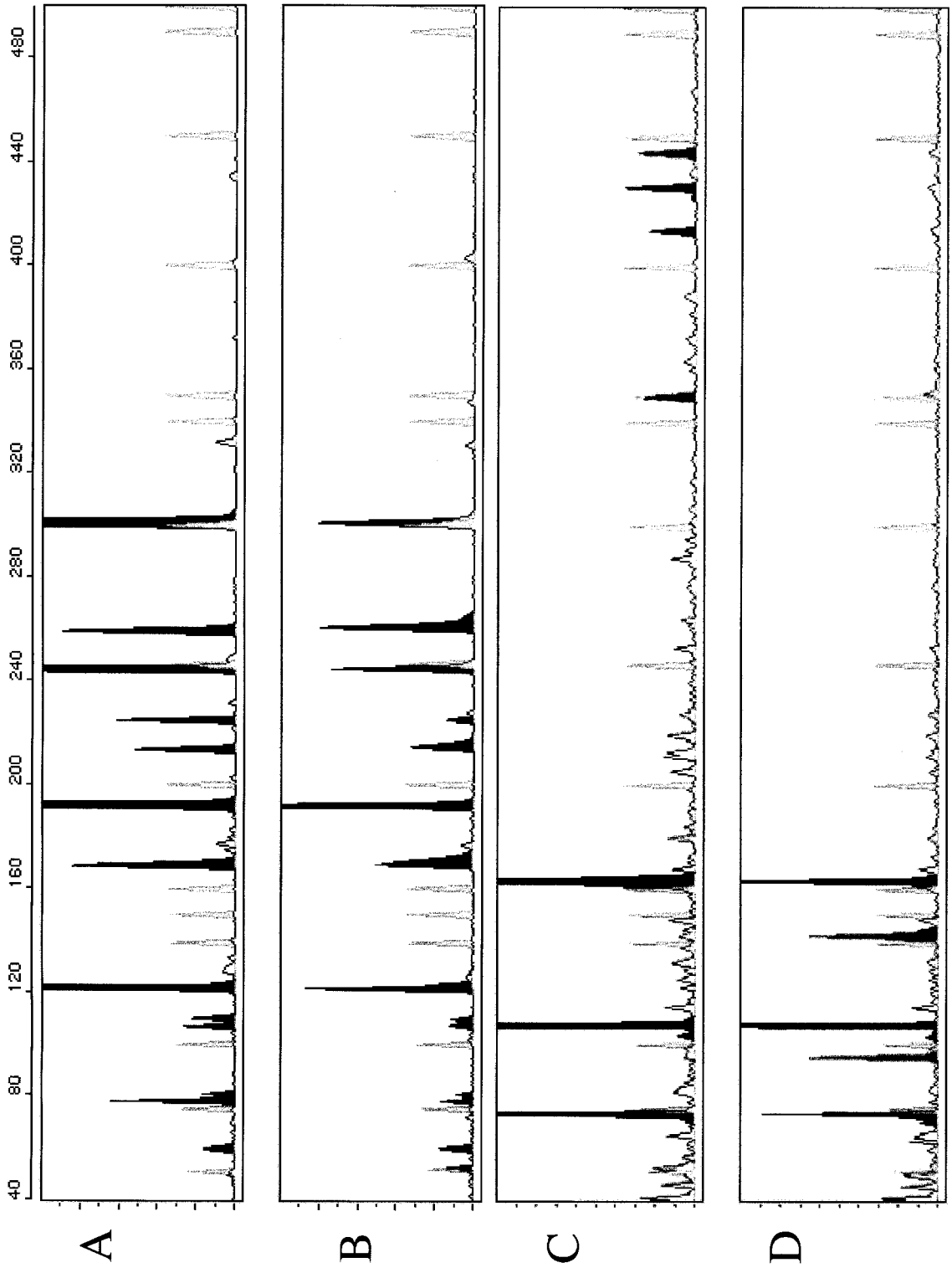

Figure 3. Panels A and B are examples of good reproducibility for the primer pair E-AG/M-CAC with the isolate CC11. Panels C and D are examples of poor reproducibility for the primer pair E-AC/M-CAA with the isolate SP01. The DNA extraction and AFLP procedures were performed at different times. Black peaks represent the data (specific fragments for that primer pair and that isolate), whereas the grey peaks represent the internal size standard. The horizontal scale indicates the size of fragments in bp, while the vertical scale indicates the fluorescence intensity of a fragment in a run.

Results

Reproducibility of data

Examples of good and poor reproducibility (Figure 3) are illustrated. There were different issues regarding the reproducibility experiments. First, the intensity of fragments was often not reproduced between replicates. It was observed that a strong band in the original run could sometimes be barely discernible from background noise in the replicate run and vice versa. Intensity was also not reproduced across samples and it was rare that a band was consistently strong or weak in all samples.

Second, the reproducibility of the results for the six primer pairs (Table 2) for nine individuals was considered. Of the 54 replicate electrophoresis runs (six primer pairs for each of nine individuals), 13 were not reproducible, however, seven displayed a common pattern associated with weak reactions. A weak run was defined as one in which spurious fragments were common, and fragments longer than ca. 200 bp were generally absent (Figure 3). This pattern of poorly reproduced runs was observed for 56 of the initial 630 electropherograms (six primers for each of 105 individuals) and 31 individuals (weak run for one or more primer pairs) were removed from all subsequent analyses.

Finally, it was discovered that not only were some results for a given primer pair of an individual not reproducible, but some bands were not reproducible as well. Bands that were not reproducible (65 from a total of 434 bands), even in good runs, were also removed from all analyses.

In the end, the six selective PCR primer pairs (Table 2) produced a total of 369 reproducible AFLP fragments for 74 individuals from 13 populations. A total of 13 monomorphic fragments were observed, ranging from 0 to 6 monomorphic fragments per primer pair. A fragment was considered monomorphic if it was present in 99% of the individuals.

Preliminary population survey of Chondrus crispus

The Dice similarity coefficients between all pairwise comparisons of individuals ranged from approximately 0.5 to 0.9 (the higher the value, the more similar the individuals). The UPGMA analysis using the Dice coefficient matrix (not shown) resulted in individuals from each population affiliating into a few loose clusters that failed to associate relative to clusters from other populations, with one notable exception – individuals from the Arisaig population were scattered throughout the dendogram. In this analysis, which generates rooted topologies, the outgroups appeared in two distinct clusters, neither of which was basal to the tree.

One thousand bootstrap replicates were performed on the data matrix for Nei and Li corrected distances using the neighbour-joining algorithm (Saitou & Nei, 1987) (Figure 4). Hillis and Bull (1993) considered a node with 80% support or higher to be an accurate estimate of true relationship. A few of the resolved nodes received this level of support ($>$ 80%) in our analysis (Figure 4) and were confined to clusters of a few individuals from within populations. Two individuals from Cape D'Or allied with 82% bootstrap support, and three other individuals from Cape D'Or grouped together with 91% support. A group of three Ketch Harbour isolates clustered together with 99% support, and three other Ketch Harbour individuals allied with 88% support. Four Maces Bay isolates grouped together with 77% bootstrap support, and the resolution within that group was even higher at 96% and 86% (Figure 4). All individuals from Michaud Point clustered together with 72% bootstrap support, although the relationships within that group were essentially unresolved. Five isolates from Miminegash clustered together with 77% support, and only one node within that group was resolved (73% support). Two Rustico isolates grouped with 74% support. Two Sandy Cove individuals clustered with 94% bootstrap support, and two other Sandy Cove isolates allied with 95% support. Three pairs of Sluice Point isolates clustered separately with 89%, 79% and 78% support. Finally, the two individuals from Cap Gris Nez in France associated with 84% bootstrap support. Relationships among populations were completely unresolved. The relationships within the Arisaig and Cheticamp populations were completely unresolved, whereas every other population showed support for at least two individuals clustering together (Figure 4).

Discussion

Reproducibility testing

Reproducibility experiments were performed on nine individuals by re-extracting the DNA from the original ground algal sample. Fragment intensity was often not reproduced for bands of all primer pairs when replicated for an individual. It was also observed that

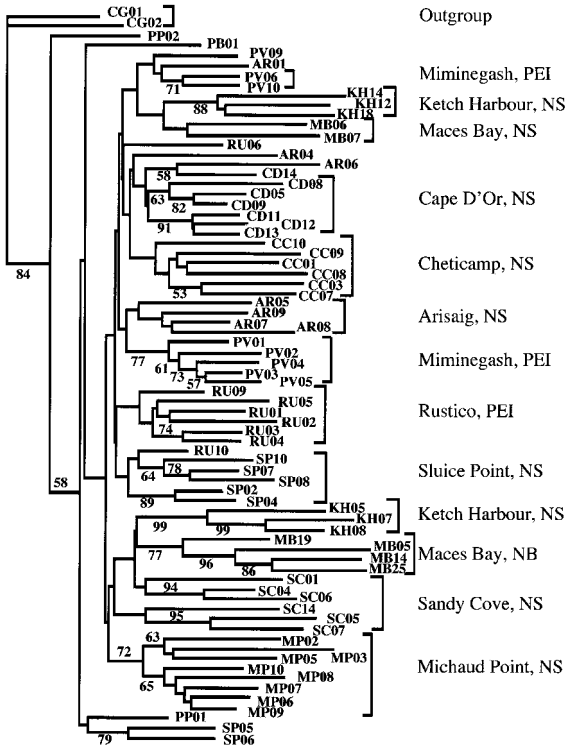

Figure 4. Neighbor-joining tree calculated using the Nei-Li distance metric. Numbers represent the percentage of 1000 bootstrap replicates that a particular node was resolved. Branches lacking values received less than 50% support from the bootstrap analysis.

fragment intensities were not reproducible across the individuals for a given primer pair. Caution is recommended when using fragment intensity as an indicator of biological (e.g., homozygote versus heterozygote) or phylogenetic (viz., scoring bands by intensity rather than presence versus absence) significance. In addition, some fragments obviously present in the initial electropherograms were not observed in replicates, and vice versa, and these fragments must be identified prior to any analyses. In a few cases complete runs were poorly reproduced. There was a characteristic pattern observed in which fragments longer than ca. 200 bp were generally absent (Figure 3). It was hypothesized that this was due to poor restriction digest/ligation reactions and/or weak PCR. When fragments greater than ca. 200 bp were absent for any of the six primer pairs from an individual, that individual was removed from subsequent analyses. After undertaking the reproducibility experiments a total of 31 individuals (out of 105) and 65 fragments (out of 434) were removed from the data set.

This general lack of reproducibility is not unique to this AFLP study. In their investigation into the genetic variation of *Astragalus cremnophylax*, Travis et al. (1996) observed runs with poor amplification, which they did not include in their analyses. In contrast, Hongtrakul et al. (1997) performed reproducibility experiments when determining the genetic diversity among inbred sunflower lines and found no scoring discrepancies between replicate runs. Hongtrakul et al. (1997) used the same DNA samples in their replicate runs as they did in their original runs and, therefore, did not perform complete replicates. An extensive study was performed by Jones et al. (1997) whereby identical extracted DNA samples and the necessary protocols were sent to various laboratories across Europe to test reproducibility and it was concluded that the AFLP technique was highly reproducible. However, Jones et al. (1997) used the same original DNA sample, which also does not constitute a complete replicate. As well, in earlier investigations by Donaldson et al. (1998), substantial reproducibility was achieved when the same original purified DNA was used for replicate runs. In the present study, DNA from the original ground algal sample was re-extracted for the replicate runs and a lack of reproducibility was observed. Consequently, the assurance of reproducibility of the AFLP technique in the literature (Hongtrakul et al., 1997; Jones et al., 1997; Donaldson et al., 1998) is based on incomplete replicates and should, therefore, be accepted with caution.

We hypothesize that the lack of reproducibility of the AFLP technique is due to inconsistency in the quality of the DNA. The DNA extraction procedure used here is a simplified method that does not remove all contaminants from the samples. These contaminants can affect the restriction enzyme digestion and subsequent PCR. Specifically, EcoRI can have star activity, cleaving the DNA at sequences not corresponding to its recognition sequence (Maniatis et al., 1982). Star activity occurs under adverse conditions such as high salt concentration, high glycerol concentration, non-optimal temperature and prolonged incubation. Vos and Kuiper (1997) stressed that contaminants are often co-purified with DNA, but it is only when the concentration of DNA is low that the contaminants interfere with the restriction digestion. They stressed that DNA preparations of poor quality are most common for organisms with a small genome, such as *Arabidopsis*. The genome size of *C. crispus* is approximately 100 Mb (B. Metz, pers. comm.), comparable to that of *Arabidopsis*. Mizukami et al.

(1998) indicated that both RNA and the soluble polysaccharides found in red algae are often co-extracted with DNA. These components can interfere with PCR, specifically for Random Amplified Polymorphic DNA (RAPD) analysis. Because of this, Mizukami et al. (1998) investigated the reproducibility of RAPD patterns using five different extraction procedures for *Porphyra yezoensis* Ueda. They found that only DNA purified by CsCl gradient could generate reproducible RAPD patterns.

Using more stringent DNA extraction and purification procedures may minimize problems with the reproducibility of the AFLP technique. Complete reproducibility experiments (i.e., DNA extraction from the original field sample) should be undertaken in every AFLP study so that the utmost confidence can be placed in the data. It may be necessary to extract every individual twice and run each primer pair twice, using only reproducible bands in the final analysis.

Chondrus crispus population structure

The bootstrap values on the neighbour-joining tree (Figure 4) indicate almost no resolution within populations and no resolution among populations of *Chondrus crispus* in the Maritime Provinces of Canada. The lack of resolution could be interpreted two ways: either data generated by the AFLP technique are too variable to be useful at the population level, or *C. crispus* populations have extensive genetic variation and can not be investigated with this technique. Previous research indicates that either could be possible. Firstly, the AFLP technique has been used to distinguish among populations of *Astragalus cremnophylax* var. *cremnophylax* (Travis et al., 1996) and *Populus nigra* subsp. *betulifolia* (Winfield et al., 1998). Although neither study performed bootstrap analysis, their cluster analyses indicated reasonable population structure, contrary to the present study. However, in both studies the genetic diversity of the populations in question was substantially lower than would be expected in 'typical' natural populations. *Astragalus cremnophylax* var. *cremnophylax* is a critically endangered plant that has undergone a bottleneck, decreasing the genetic diversity of the populations (Travis et al., 1996). The population of *Populus nigra* subsp. *betulifolia* has also been decreasing. At most, 5% of the current population of *Populus nigra* subsp. *betulifolia* in Great Britain is female (Winfield et al., 1998). This would decrease the genetic diversity of the population. The utility of AFLP for population level investigations is, therefore, still equivocal.

Secondly, the literature on *C. crispus* suggests relatively substantial diversity for this species at the genetic level. Cheney and Mathieson (1979) performed a protein isozyme study on eight individuals of *C. crispus* from different locations in New Hampshire and the Maritime Provinces and concluded that *C. crispus* may exhibit 'considerable genetic differentiation' across short distances. Chopin et al. (1996) examined seven different isolates of *C. crispus* from different locations across both the Maritime Provinces and Europe by sequencing the internal transcribed spacer (ITS) region of the ribosomal cistron. Isolates were chosen based on divergent morphologies and examined to determine a genetic basis for morphological differences. They found from 0 to 2.18% sequence divergence among these isolates, where the two isolates with identical ITS coding regions were from France and New Brunswick, and the two isolates with 2.18% divergence were from Nova Scotia and PEI. Interestingly, the French isolates had more sequence similarity to some Maritime isolates than the latter did to other Maritime isolates. Goff et al. (1996) sequenced the ITS coding region of various red algal taxa and the intraspecific ITS sequence divergence ranged from 0.1% for two isolates of *Faucheocolax attenuata* Setchell to 3% for two isolates of *Sarcodiotheca gaudichaudii* (Montagne) Gabrielson. The average intraspecific sequence divergence for five different genera was 1.3%. Compared to these data, 2.18% intraspecific sequence divergence for *C. crispus* indicates that intraspecific variation is high in *C. crispus* relative to other red algae.

It is possible that the genetic variation of *Chondrus crispus* is too extensive to be discerned by the AFLP technique. One drawback to fragment techniques is that at a relatively low level of dissimilarity the occurrence of homoplasy, or noise, overwhelms signal (Dowling et al., 1990). Homoplasy occurs when two fragments of the same size do not correspond to the same region of the genome, as well as when fragments resulting from the same pair of flanking restriction sites resolve as different sized fragments owing to insertion and deletion events. The exact point at which homoplasy overwhelms phylogenetic signal in simple fragment comparisons is debated. Upholt (1977) considered that comparisons should not be made between samples whose divergence exceeds 15%, whereas Kessler and Avise (1985) suggested that 25% is a more appropriate cut off. Nei (1987), while discussing

the Nei and Li distance, considered that an accurate estimate of nucleotide substitution (d) could only be obtained for closely related individuals ($d < 0.05$) and that this equation underestimates nucleotide substitution for values of d greater than 0.1 (10%). The highest distance value obtained with the Nei anf Li (1979) metric in this study was 0.13 (or 13%). This indicates that the maximum Nei and Li distance value in our dataset of 13% is an underestimate of true evolutionary divergence, and in fact, many pairwise comparisons in our data set exceedd the levels of divergence acceptable for fragment analyses (Upholt, 1977; Nei, 1987). Extensive divergence is also reflected in the number of polymorphic bands observed in the current study. Folkertsma et al. (1996) observed only 15.8% polymorphic bands for potato cyst nematode populations (i.e. 84.2% of bands were monomorphic, or conserved in all individuals), and Keim et al. (1997) noted only 3% polymorphic bands for strains of *Bacillus anthracis*, whereas 97% polymorphic bands were observed in our study. This substantial divergence is inevitably accompanied by increased homoplasy. Extensive homoplasy is also illustrated by the lack of resoolution among populations in the bootstrap analysis (Figure 4). This does not indicate that the AFLP technique is not useful for some studies, but that *Chondrus crispus* is probably too genetically variable to be analyzed by this technique.

In summary, we caution that reproducibility of the AFLP technique must be monitored during every investigation, and that this is best accomplished by replicating all steps of the procedure including DNA extraction from the original sample. The lack of reproducibility for the AFLP technique observed in our study may be remedied by the use of stringent DNA extraction and/or purification procedures. Some RAPD studies have found that purifying the DNA with a CsCl gradient produces a reproducible banding pattern (Mizukami et al., 1998), and this also may be necessary for the AFLP technique. In any case, studies using AFLP should carry out routine and regular reproducibility testing.

Furthermore, our preliminary results indicate that AFLP may not be appropriate for population level investigations in *Chondrus* and other methods must be tried. This lack of useful molecular markers from AFLP may extend to other seaweed species in the field. Additional evidence is provided that *Chondrus crispus* may harbor unusually high genetic diversity relative to other species. This is an aspect worthy of future investigation.

Acknowledgements

We thank D. Barsi and N. MacAfee for technical assistance, G. Sharp, S. Spinney and C. Yarish for providing some of the isolates used in this study, P. Bentzen for discussion on data analysis, and R. Smith for assistance with computer generation of figures. This work was supported by Natural Sciences and Engineering Research Council of Canada Grants to TC and GWS. This paper is contribution No. 51 from the Centre for Coastal Studies and Aquaculture.

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