

**APPLYING AMPLIFIED FRAGMENT LENGTH
POLYMORPHISM (AFLP) METHODS TO RED ALGAE
WITH A PRELIMINARY INVESTIGATION
OF POPULATION STRUCTURE IN
CHONDRUS CRISPUS STACKHOUSE**

by

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ABSTRACT

A new molecular technique, Amplified Fragment Length Polymorphism (AFLP), was successfully applied to red algae. The AFLP technique is attractive as no prior sequence knowledge is necessary and only a minimal amount of DNA is required. The AFLP technique has been described as being reliable and reproducible, but contrary to previous studies, a lack of reproducibility of the AFLP technique was uncovered. It is suggested here that the use of more stringent DNA extraction and purification techniques may remedy the problem with reproducibility. The AFLP technique can be an excellent addition to the repertoire of fragment analysis methods, if caution is used in choosing DNA extraction and purification procedures. After successfully developing the AFLP technique for use with red algae, I endeavored to perform a small scale population study on the red alga *Chondrus crispus*. Thirteen populations of *Chondrus crispus* Stackhouse were analyzed with AFLP. Little population structure was uncovered, but the substantial genetic variation previously reported for *C. crispus* was confirmed. This genetic variation most likely accounts for the lack of population structure.

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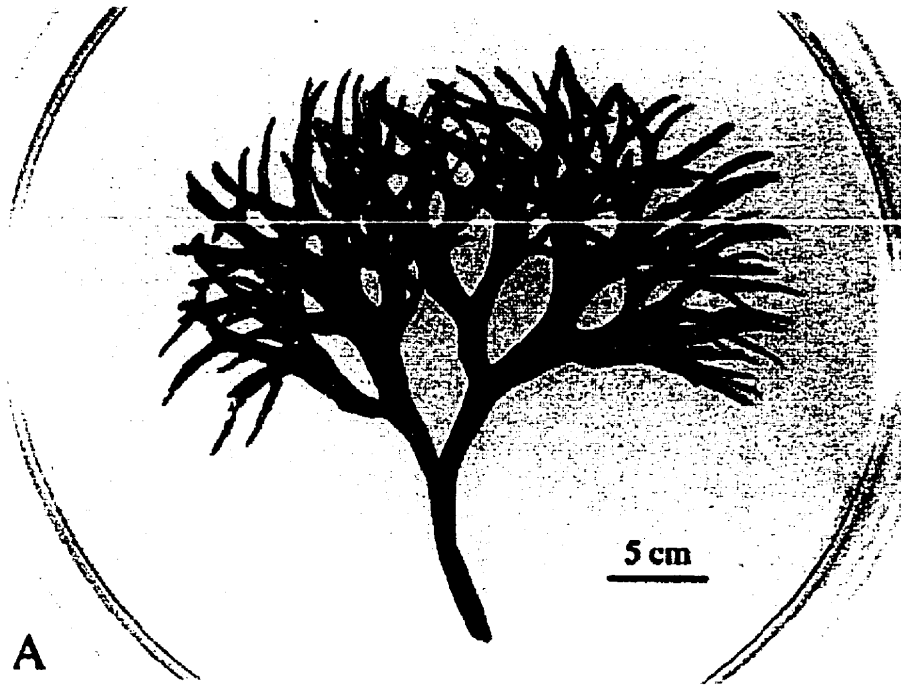
INTRODUCTION

Chondrus crispus Stackhouse

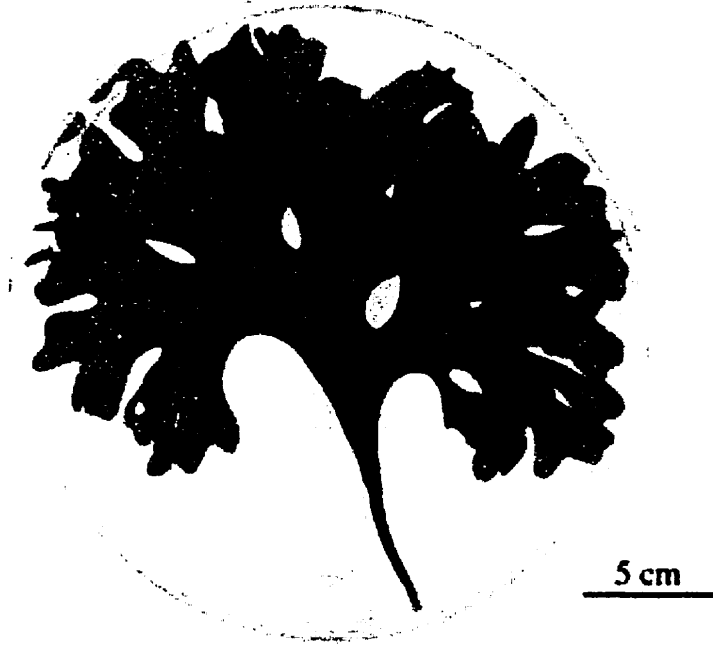
Chondrus crispus Stackhouse (Fig. 1), a macroscopic marine red alga, is found in most temperate Atlantic waters (MacFarlane 1968). *Chondrus crispus* is an epilithic species that can be found from near low water to 30 meters below mean low water (MLW) (Mathieson and Burns 1975). In New England waters, the stands of maximum density are found from between 3 meters below MLW and 6 meters below MLW (Mathieson and Burns 1975, Prince and Kingsbury 1973). In exceptional situations, when tidal amplitude is high or when persistent tide pools are present, *C. crispus* has been found several meters above MLW (Mathieson and Prince 1973).

Chondrus crispus displays an isomorphic, diplohaplontic life history (Chen and McLachlan 1972). A diploid tetrasporophyte releases haploid tetraspores that germinate into haploid male or female gametophytes. The female gametophyte produces a carpogonial branch that terminates in a carpogonium. The carpogonium develops an outstretched portion called the trichogyne, and the basal portion contains the nucleus. The male gametophyte releases non-flagellate haploid spermatia into the water that drift passively to the trichogyne. The spermatial nucleus then travels down the trichogyne to fuse with the carpogonial nucleus generating a diploid zygote. The zygote nucleus is transferred to the supporting cell of the carpogonial branch (functioning as the auxiliary cell), which subsequently undergoes mitosis and produces numerous carposporangia contained within a carposporophyte that develops hemiparasitically on the female gametophyte. The diploid carpospores are released and germinate into tetrasporophytes (Chen and McLachlan 1972).

Figure 1. Photographs of *Chondrus crispus*. A- Example of the narrow morphology. B- Example of the broad morphology.



A



B

Morphological Divergence

Chondrus crispus displays numerous dichotomously branched fronds growing from a discoid holdfast (Fig. 1). The morphological divergence of *C. crispus* isolates has been reported by several authors (Chen and Taylor 1980a, Cheney and Mathieson 1979, Chopin and Floc'h 1992, Chopin *et al.* 1996, Floc'h 1969, MacFarlane 1968, Newton 1931, Newton *et al.* 1959, Thomas 1938). In exposed habitats *C. crispus* isolates can have narrow fronds (Fig. 1a), whereas *C. crispus* growing in sheltered habitats can have larger and broader fronds (Fig. 1b) (Taylor and Chen 1973). There is, however, no agreement as to whether this morphological divergence should be assigned taxonomic status. Among authors who support taxonomic designations for the various morphotypes, there is a debate as to whether the morphologies should be described as forms or varieties (MacFarlane 1968, Newton *et al.* 1959, Thomas 1938). In a few cases, the authors' explanation of the source of the morphological divergence does not even agree with the original definition of the taxonomic designation they used (Thomas 1938). The term "variety" was originally defined as pertaining to differences induced by the environment. The term "form" has been used traditionally to recognize morphologically divergent isolates growing in close proximity to plants of the "typical" morphology. Such morphological divergence is generally assumed to have a genetic basis (Stuessy 1990).

The debate about the existence of forms and varieties in *C. crispus* has been ongoing for over a century. Harvey (1846) stated that there were too many morphologically different *C. crispus* isolates to describe and, therefore, recognized only the two most divergent forms: a narrow form found in the lower littoral ("exposed to the full 'dash' of the sea"): and, a broad form found in estuaries that he described as "much lobed and fringed". Thomas (1938) acknowledged 22 forms of which only three had been observed in North America. Thomas believed these forms arose due to a range of differing environmental conditions and, therefore, should have referred to his taxa as varieties, according to the original definitions of form and variety. Newton (1931) described nine

varieties also due to differing environmental conditions. Newton *et al.* (1959) observed a plot of *C. crispus* off the coast of France where they classified young isolates as one variety, but as the same plants aged they started to take on the appearance of a different variety. It was for this reason that Newton *et al.* (1959) recognized only six varieties in Great Britain and hypothesized that some of the varieties that had been recognized in the past were simply juveniles of other recognized varieties. MacFarlane (1968) suggested that the morphological differences of *C. crispus* be renamed “ecological forms” (this informal category obviating the more formal taxonomic designation of “variety”). Newton *et al.* (1959) also questioned the usage of the taxonomic term variety, but described nine varieties in the same paper.

Although the terms variety and form were never mentioned, Floc'h (1969) performed a reciprocal transplant experiment in France at one site where two morphologically divergent strains of *C. crispus* grow. Isolates with narrow fronds were found in the lower littoral and isolates with broad fronds grew in the upper littoral. Floc'h (1969) transplanted partially cut isolates from the lower to the upper littoral and *vice versa*, and observed their regrowth. Floc'h (1969) found no difference in morphology after seven months and concluded that if the environment does play a role in the morphology of *C. crispus* isolates, it is slow acting.

Chen and Taylor (1980a) described two strains of *C. crispus* found in the Maritime provinces of Canada, which they labeled N for narrow (Fig. 1a) and B for broad (Fig. 1b). Chen and Taylor used the term ‘strain’ in lieu of any taxonomic designations. They made observations on these two strains in the field, examining the environmental conditions at the sites where the divergent morphologies were found. Chen and Taylor (1980b) then examined isolates of these strains in the laboratory by performing culture studies. They subjected the isolates to differing culture conditions such as temperature, aeration and day length, and observed that the isolates did not alter their original morphology. They also cultured male isolates of the N strain with female isolates of the B strain and no

carposporophytes appeared on the female plant indicating no reproduction. They concluded that this lack of hybridization was strong evidence for genetic divergence of the two strains. Guitierrez and Fernández (1992) examined a site in Spain where many morphologically divergent isolates of *C. crispus* grow and performed a cluster analysis based on morphological variables. The cluster analysis indicated only two discrete morphologies - Chen's and Taylor's N and B strains. Guitierrez and Fernández indicated that the N strain was prevalent in exposed sites in the lower littoral, and the B strain was found in sheltered sites in the upper littoral. They determined that water movement had more of an effect on morphology than tidal level and hypothesized that the different morphologies could be due to water motion and emersion times, especially at high tide levels. Chopin and Floc'h (1992) observed a location in France where a broad morphology of *C. crispus* grew in the upper littoral, a narrow morphology grew in the lower littoral, and a range of intermediate morphologies were present within centimeters of each another. The wide range of intermediate morphologies growing in close proximity to one another would experience similar environmental conditions. Because of this, Chopin and Floc'h (1992) stated that the environment is not solely responsible for the morphological polymorphism of *C. crispus* and that there must be a genetic component. Nevertheless, Kübler and Dudgeon (1996) indicated that *C. crispus* populations are morphologically diverse and often that morphological diversity is due to environmental stress. They showed that temperature has an effect on the complexity of *C. crispus* fronds, but did not extend this to hypothesize about the existence of varieties of *C. crispus*.

Molecular Investigations

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 of several species of the red algal genus *Eucheuma* (Cheney and Babble 1978).

This implies genetic variability for *C. crispus* that is not present in other red algal species. Cheney and Mathieson (1979) briefly mentioned that the lower littoral populations of *C. crispus* were morphologically distinct from the upper littoral populations, but did not indicate whether the genetic differentiation was correlated with the morphological differences.

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 ITS1, 5.8S and ITS2 coding regions 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* f. *ocellatus*. 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% sequence divergence over the 780 bp of the ITS regions sequenced. The genetic diversity did not, however, correlate with the phenotype for the plants investigated. Although this level of ITS variation is high for intraspecific comparisons relative to other red algae (Goff *et al.* 1996; see Discussion), it was not substantial enough to warrant a wide-scale population genetic survey of *C. crispus* using the ITS technique. Chopin *et al.* (1996) suggested analyzing *C. crispus* populations using more sensitive molecular techniques in order to resolve a genetic basis for its morphological polymorphism.

Fragment Techniques

Sequencing analyses have obvious benefits, as the actual nucleotide sequence can be 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 sequencing specific regions of the genome. Fragment techniques, therefore, have some benefits as compared to sequencing analyses: little sequence knowledge is required for fragment analyses 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, the majority of fragment analysis techniques have shortcomings of various kinds themselves. Restriction 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. This is time consuming and relatively expensive. The RFLP markers, however, can have the benefit of being codominant, where homozygotes and heterozygotes are discernible by their banding pattern (requires *a priori* knowledge of the portion of genome under study). This allows for a more complete picture of the mode of inheritance to be established for the genetic markers in use. Randomly Amplified Polymorphic DNA (RAPD) analysis involves the polymerase chain reaction (PCR) amplification of random regions of the genome with short primers (Welsh and 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). The RAPDs are dominant markers, alleles of the same gene are not discernible from alleles of different genes. This can cause an overestimate of the variation present in the population being studied. For this reason, investigation into new molecular techniques has been ongoing. A new molecular technique that has been showing some promise in a range of fields is called Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.* 1995).

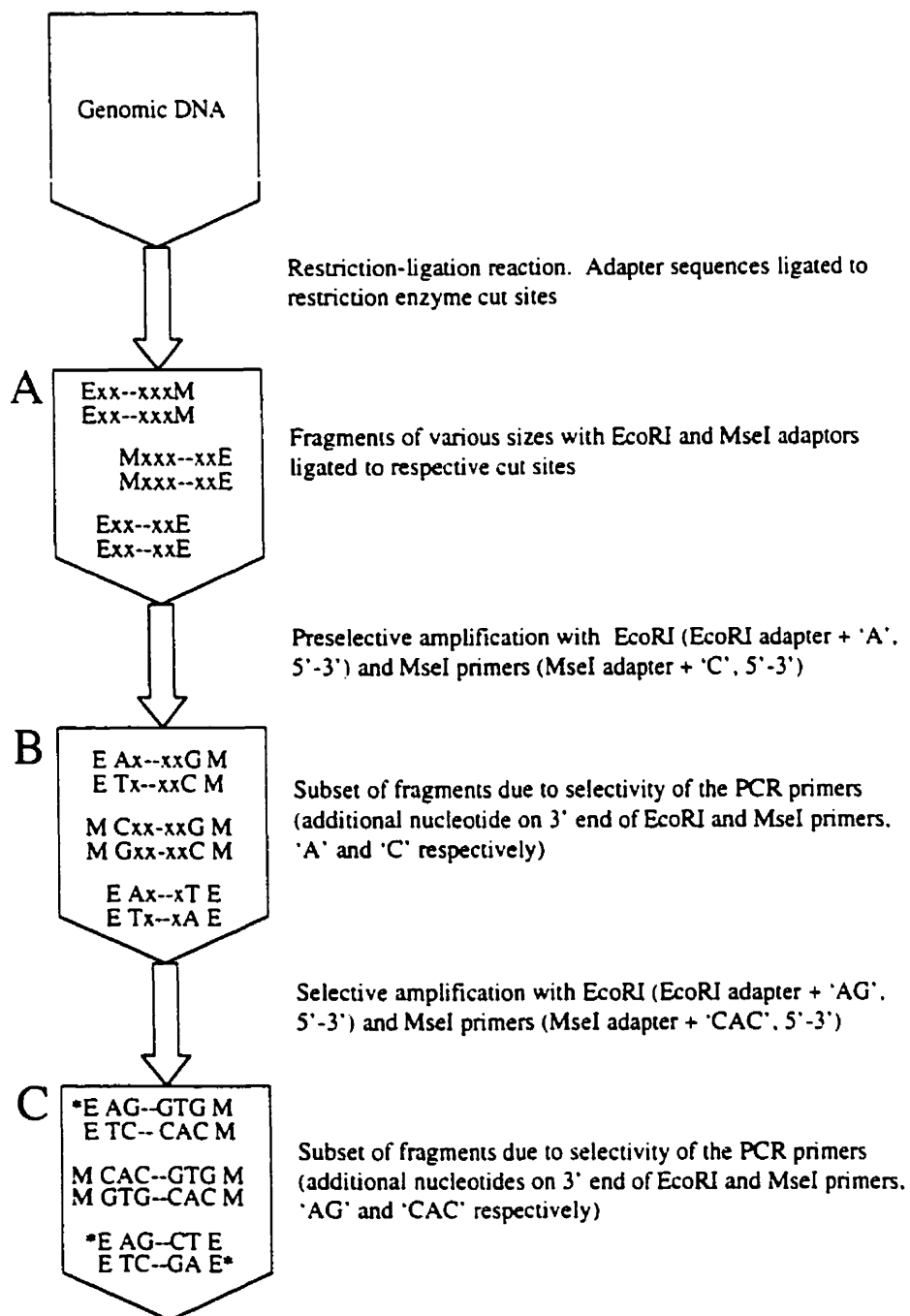
Amplified Fragment Length Polymorphism (AFLP)

The 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 determination of genetic diversity among populations of the endangered plant *Astragalus cremnophylax* (Travis *et al.* 1996), the genetic analysis of single fungal spores (Rosendahl and Taylor 1997), the biosystematics of the *Solanum* genus (Kardolus *et al.* 1998), the phylogenetics of chicory varieties (Koch and Jung 1997), and for the assessment of diversity in potato cyst nematode populations (Folkertsma *et al.* 1996).

The AFLP technique 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 (Fig. 2). The most common fragments created by this restriction-ligation reaction are those with an MseI site on both ends, followed by those fragments bounded by both an EcoRI site and an MseI site. Finally, fragments created with two EcoRI cut sites would be uncommon as EcoRI fragments would be generally long, increasing the probability of an MseI cut site within the fragment.

An initial polymerase chain reaction amplification - preselective amplification - is completed using primers complimentary to the ligated adapter sequences with one additional nucleotide added to the 3' end (Fig. 2). 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 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 (Vos *et al.* 1995) so that EcoRI - MseI fragments can be detected. It might be expected that the majority of the

Figure 2. Schematic of the AFLP technique. The tubes illustrate the complement of fragments present at each step in the procedure. A- After the restriction ligation reaction. B- After the preselective PCR amplification. C- After the selective PCR amplification. -- refers to fragment specific lengths of nucleotides, x indicates any of the four nucleotides, E refers to the EcoRI adapter, M indicates the MseI adapter. * refers to a fluorescently labelled primer and fragment (only labelled fragments are visualized in the final analysis).



amplified product 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 and can be observed by electrophoresing the products. The products are scored as present or absent.

Objectives of this project

This study contains two elements. The first objective was to develop the AFLP methodology for use with red algae, as this technique has only recently been considered for use with algae. The objective of the second part was to perform a preliminary population survey on *Chondrus crispus* to lay the groundwork for more in-depth examinations of this species. Collection sites were selected to include areas along the entire coastline of Nova Scotia and New Brunswick [including the two sites where Chen and Taylor (1980a) collected the narrow (Fig. 1a) and broad (Fig. 1b) strains of *C. crispus*, Cheticamp NS and Cape D'Or NS, respectively], as well as part of Prince Edward Island. Ten study sites were selected (Fig. 3) and ten individuals from each of the sites were analyzed.

MATERIALS AND METHODS

Sample Collection

Ten isolates of *Chondrus crispus* were studied from each of ten study sites in the Maritime provinces of Canada (Fig. 3). Two individuals were analyzed from a population in Parsonage Point, New York, U.S.A., two individuals were analyzed from Cap Gris Nez, northern France, and one isolate was analyzed 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). Only

Figure 3. The Maritime provinces of Canada illustrating the 10 collection sites in this study.

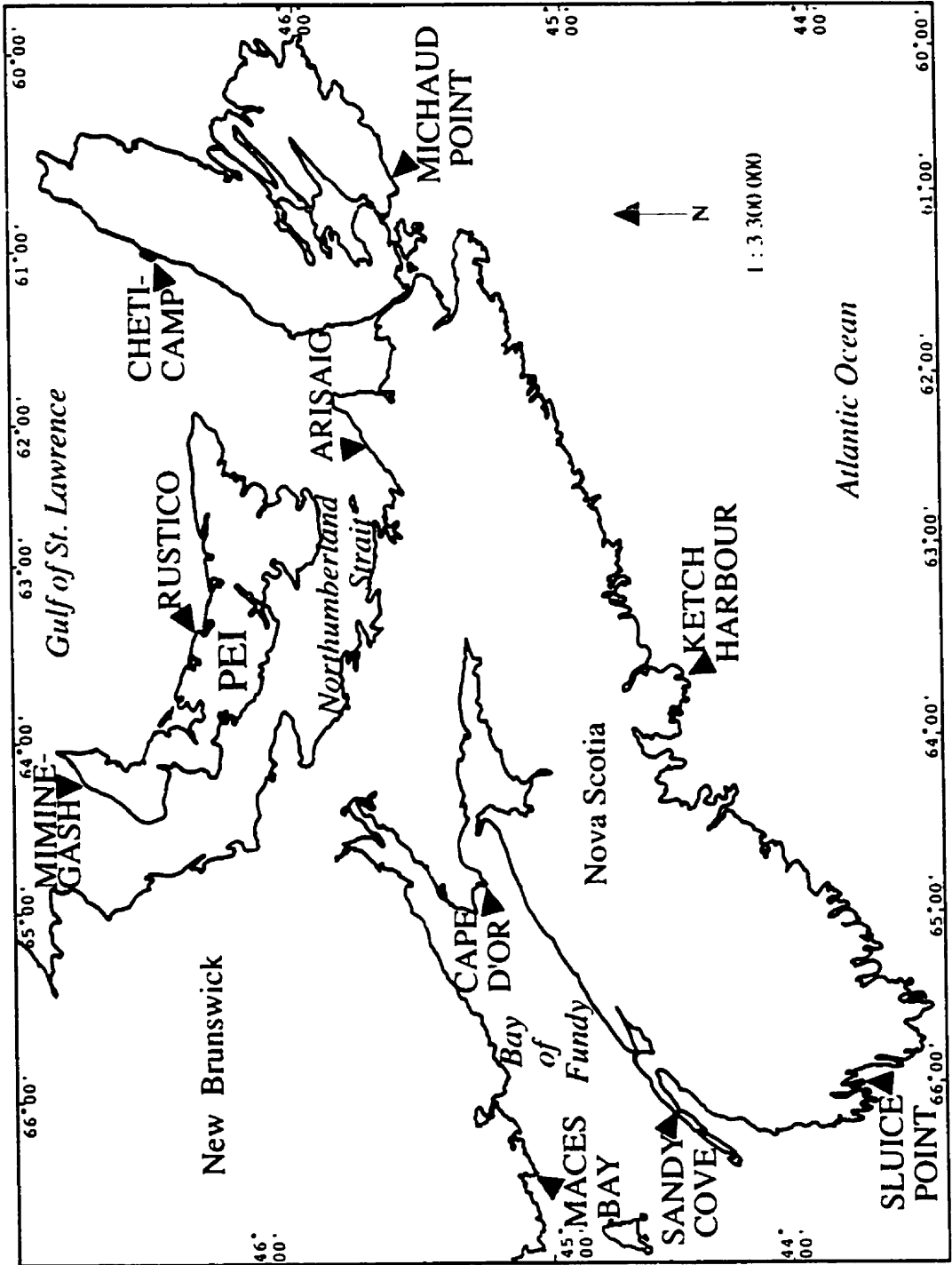


Table 1. List of sites where *Chondrus crispus* was collected, abbreviations of collection sites used in the text, collection dates and collectors.

Collection Site (Abbreviation)	Date of Collection	Collector
Arisaig, Nova Scotia (AR)	November 4 1997	T. Chopin
Cape D'Or, Nova Scotia (CD)	November 1 1997	T. Chopin
Cheticamp, Nova Scotia (CC)	November 3 1997	T. Chopin
Ketch Harbour, Nova Scotia (KH)	Summer 1998	S. Donaldson
Maces Bay, New Brunswick (MB)	Fall 1997	S. Donaldson
Michaud Point, Nova Scotia (MP)	November 2 1997	T. Chopin
Miminegash, Prince Edward Island (PV)	October 31 1997	G. Sharp
Rustico, Prince Edward Island (RU)	October 30 1997	G. Sharp
Sandy Cove, Nova Scotia (SC)	Summer 1998	S. Donaldson
Sluice Point, Nova Scotia (SP)	November 11 1997	S. Spinney
Outgroups		
Parsonage Point, New York (PP)	July 24 1997	C. Yarish
Ile de Ré, France (PB)	August 19 1997	T. Chopin
Cap Gris Nez, France (CG)	August 21 1997	G. Saunders

tetrasporophytes were used and were identified by eye or by the resorcinol procedure (Garbary and De Wreede 1988). The samples were collected haphazardly, in that only one isolate was collected from a patch of *C. crispus*. Several upright fronds can grow from the same holdfast to make up a patch or clump of *C. crispus*. Because of this, it was necessary to ensure that two isolates were not collected from the same patch, as they could be genetically identical and result in an underestimate of the genetic variation for a population. After the isolates were collected, all epiphytes were removed by gently rubbing plant surfaces either at the site or in the lab. The algae were dried immediately after collection and stored in silica. In order to remove epiphytes in the lab, 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 at -20°C.

DNA Isolation

During the initial stages of this project, DNA was extracted by scaling up the procedure of Saunders (1993). In the scaled up procedure, 500 mg of dry, ground algal material was used initially with 3 mL of lysis buffer (0.1 M Tris, 0.05 M Na₂EDTA, 0.2 M NaCl, 2.5 M potassium acetate, pH 8.0), 300 µL of 10% Tween 20 and 30 µL of proteinase K (20mg/mL). The entire mixture was incubated at room temperature for one hour with frequent gentle mixing. Polysaccharides were precipitated out of the mixture by cooling the extraction tubes on ice. The supernatant was mixed with phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v) and centrifuged to obtain the aqueous layer. The aqueous layer was agitated with phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v) and centrifuged. The aqueous layer was combined with chloroform : isoamyl alcohol (24 : 1, v/v) and centrifuged. Again, the aqueous layer was mixed with chloroform : isoamyl alcohol (24 : 1, v/v) and centrifuged. The DNA was precipitated out of the final aqueous layer by adding twice the volume of cold ethanol and cooling on ice for at least an hour. The

mixture was centrifuged to pellet the DNA. The pellet was dried and rehydrated in deionized, distilled water.

After the AFLP method was established in preliminary experiments, the original DNA extraction procedure (Saunders 1993) was used for the individuals from the thirteen study sites. The volumes of the lysis buffer, 10% Tween 20 and proteinase K were reduced to 600 μ L, 60 μ L and 6 μ L, respectively. The rest of the protocol was identical to the scaled up procedure. Total genomic DNA was gel purified using the procedure in Saunders (1993) as follows. Total cellular DNA was further purified by loading into the well of a 0.8% agarose gel and electrophoresing at 60 volts for approximately two hours. The genomic DNA band was cut from the gel and centrifuged at 10 000 rpm for 10 minutes through a column containing dimethyldichlorosilane (DMCS) coated glass wool. This caused the gel to collapse and forced the liquid present in the gel (including the DNA) through the column. The DNA was precipitated as above and rehydrated in deionized distilled water.

AFLP Procedure

AFLP procedures (Fig. 2) were performed following the manufacturer's protocol (Perkin Elmer) as follows. Approximately 50 ng of 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 nullify the restriction site, ensuring that re-digestion does not occur. This allows the restriction and ligation reactions to occur concurrently in a single tube, overnight at room temperature (approximately 15 to 24 hours), because the restriction enzymes and T4 DNA ligase (New England Biolabs) are active in a common buffer system (55 mM Tris-HCl, 11 mM MgCl₂, 11 mM DTT, 1.1 mM ATP, 605 μ g/mL BSA, 55 mM NaCl). The resulting product was diluted five fold and 4 μ L were used for PCR reactions with the preselective primers (sequences in Table 2) complementary to the EcoRI (plus A) and MseI

Table 2. Sequences of the restriction enzyme recognition sites, adapters and PCR primers used in the AFLP technique. Black arrows indicate where the restriction enzymes cut.

EcoRI recognition sequence	<pre> ↓ 5' GAATTC 3' CTTAAG ↑ </pre>
MseI recognition Sequence	<pre> ↓ 5' TTAA 3' AATT ↑ </pre>
EcoRI adapter	<pre> 5' CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA 3' </pre>
MseI adapter	<pre> 5' GACGATGAGTCCTGAG TACTCAGGACTCAT 3' </pre>
EcoRI preselective primer	5' GACTGCGTACCAATTCA 3'
MseI preselective primer	5' GATGAGTCCTGAGTAAC 3'
EcoRI selective primers	5' GACTGCGTACCAATTCA(C or G) 3'
MseI selective primers	5' GATGAGTCCTGAGTAAC(AA or AC or AT or TG or TT) 3'

(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 3 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 min at 72°C. Primers for the selective amplification were 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 1 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, which has the benefit of a four colour fluorescent dye system, allowing several samples to be run at once or 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 base pairs were scored. Reproducibility was tested by repeating the entire procedure for nine individuals, starting with the original ground algal samples.

Statistical Analyses

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

$$\text{Coincidence index} = 2h / a + b$$

Where: h = the number of shared presences in both a and b
 a = the number of fragments for individual a
 b = the number of fragments for individual b

The Dice similarity coefficient is unique as only shared presences are considered, not shared absences. This is beneficial for analyzing fragment data as there are several ways to lose a fragment and, therefore, it is easier to lose a fragment than to gain a fragment (Dowling *et al.* 1990). The Dice coefficient was used to construct a UPGMA (Unweighted Pair-Group Method with Arithmetic mean) cluster analysis in NTSYS-*pc* (Version 1.8; Rohlf 1993) to visualize the population structure. In addition, the distance metric of Nei and Li (1979) was calculated:

$$F = 2n_{XY} / (n_X + n_Y)$$

Where: F = the proportion of fragments shared by two individuals

n_X = the number of fragments for individual X

n_Y = the number of fragments for individual Y

n_{XY} = the number of shared presences and absences between X and Y

As opposed to the Dice coefficient, the distance metric of Nei and Li (1979) considers both the shared presence and the shared absence of a fragment to be a similarity. The distance metric of Nei and Li (1979) was used to calculate a neighbour-joining analysis in PAUP (paup4.0d65. Swofford 1999). As a test of support for the tree topology, 1000 bootstrap replicates (Felsenstein 1985) were performed on the neighbour-joining tree in PAUP.

RESULTS

First Objective: Application of the AFLP Technique to Red Algae

The first objective of this study was to develop the AFLP methodology for use with red algae. The data are visualized as electropherograms by the ABI genetic analyzer (Fig. 4). On first glance it is obvious that the banding patterns differ, with some individuals sharing similar banding patterns (Fig. 5), whereas others displayed quite different banding patterns (Fig. 6) for a specific primer pair. Different scoring procedures were attempted in the

Figure 4. Example of an electropherogram (primer pair: E – AC, M – CAC) for an individual from the Ketch Harbour population (KH07) in the format outputted by the 310 ABI prism genetic analyzer. Clear peaks represent the data, grey peaks represent the internal size standard. The horizontal scale denotes the size of the fragments in base pairs. The vertical scale indicates the fluorescence signal intensity.

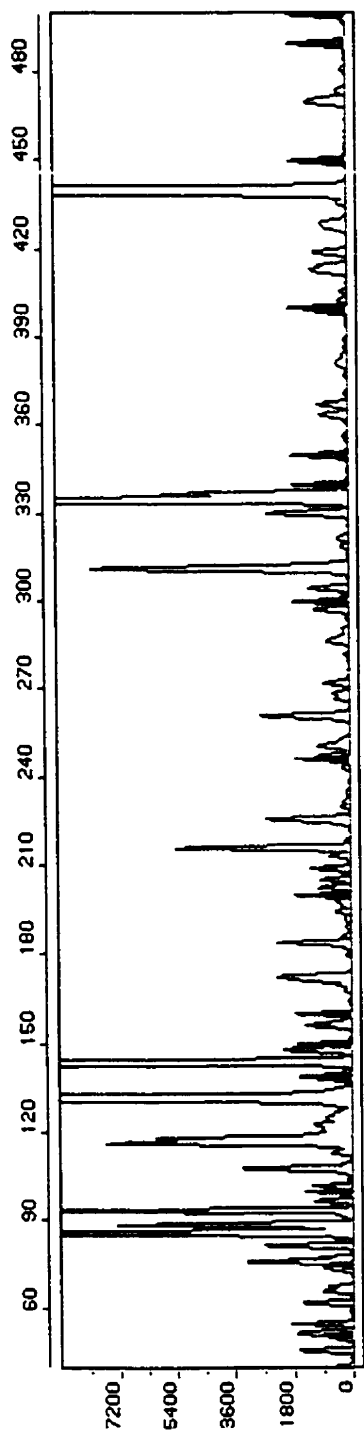


Figure 5. Example of electropherograms (primer pair: E – AG, M – CAT) showing similar banding patterns. The upper panel represents an individual from the Ketch Harbour population (KH07), the bottom panel represents individual KH08. Clear peaks represent the data, grey peaks represent the internal size standard. The horizontal scale denotes the size of the fragments in base pairs. The vertical scale indicates the fluorescence signal intensity.

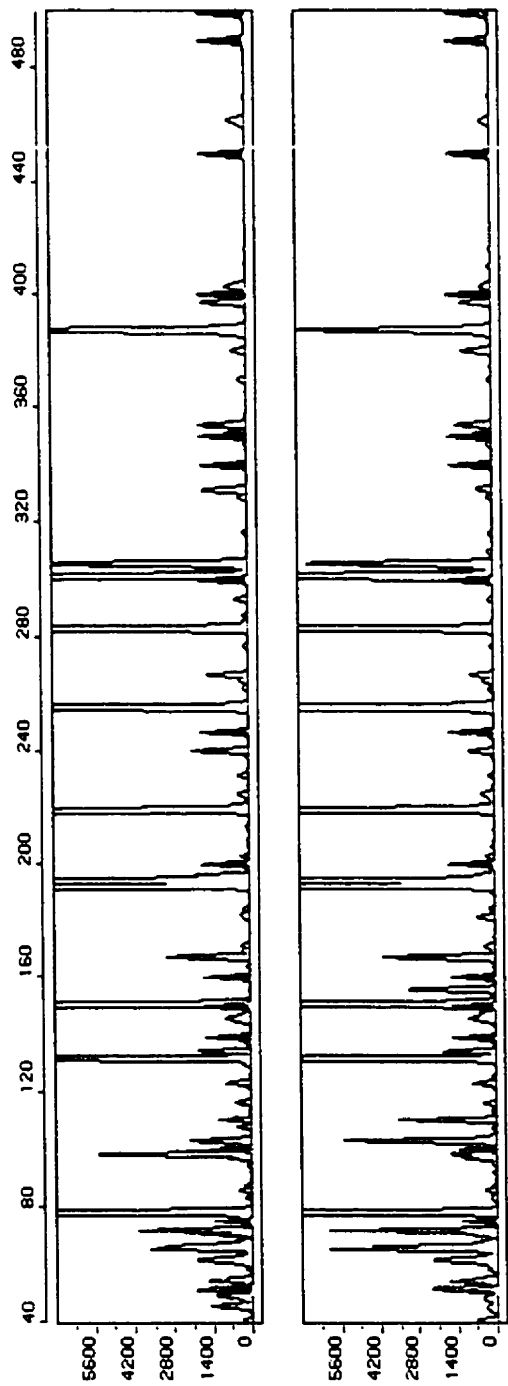
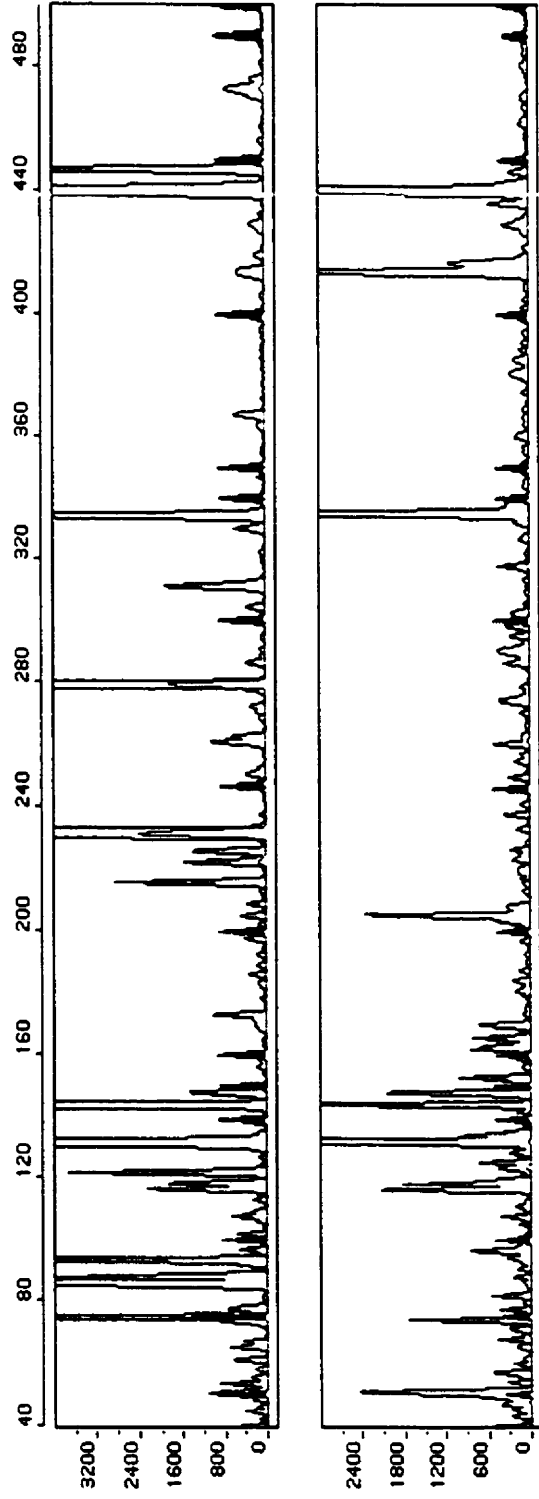


Figure 6. Example of electropherograms (primer pair: E – AG, M – CAC) showing dissimilar banding patterns. The upper panel represents an individual from the Ketch Harbour population (KH08), the bottom panel represents an individual from the population from Cap Gris Nez in France (CG02). Clear peaks represent the data, grey peaks represent the internal size standard. The horizontal scale denotes the size of the fragments in base pairs. The vertical scale indicates the fluorescence signal intensity.



beginning (see Discussion), it was finally decided to score all bands that were obviously not background noise, and to only score presence or absence.

Reproducibility of Data

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. It was discovered that not only were some individuals not reproducible, but some bands were not reproducible as well. Examples of good reproducibility (Fig. 7) and poor reproducibility (Fig. 8) are illustrated. There were different aspects to the reproducibility experiments. First, the intensity was often not reproduced within a sample. It was observed that a strong band in the original run could sometimes be barely discernible from background noise in the second 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 individuals was also noted. Sometimes a primer pair was not reproducible for an individual, and this was often because of a weak run. A weak run was defined as one in which the general intensity of fragments was low, and no fragments longer than 200 base pairs were observed (Fig. 8). This pattern was indicative of a poorly reproduced run. Because of this, 21 individuals displaying this pattern for any of the six primer pairs were subsequently removed from all analyses. Bands that were not reproducible, 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 was 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.

Figure 7. Example of good reproducibility for the primer pair E – AG, M - CAC. Each panel represents data for the same individual from the Cheticamp population (CC11); the DNA extraction, purification and AFLP procedures were run at different times. Clear peaks represent the data, grey peaks represent the internal size standard. The horizontal scale denotes the size of the fragments in base pairs. The vertical scale indicates the fluorescence signal intensity.

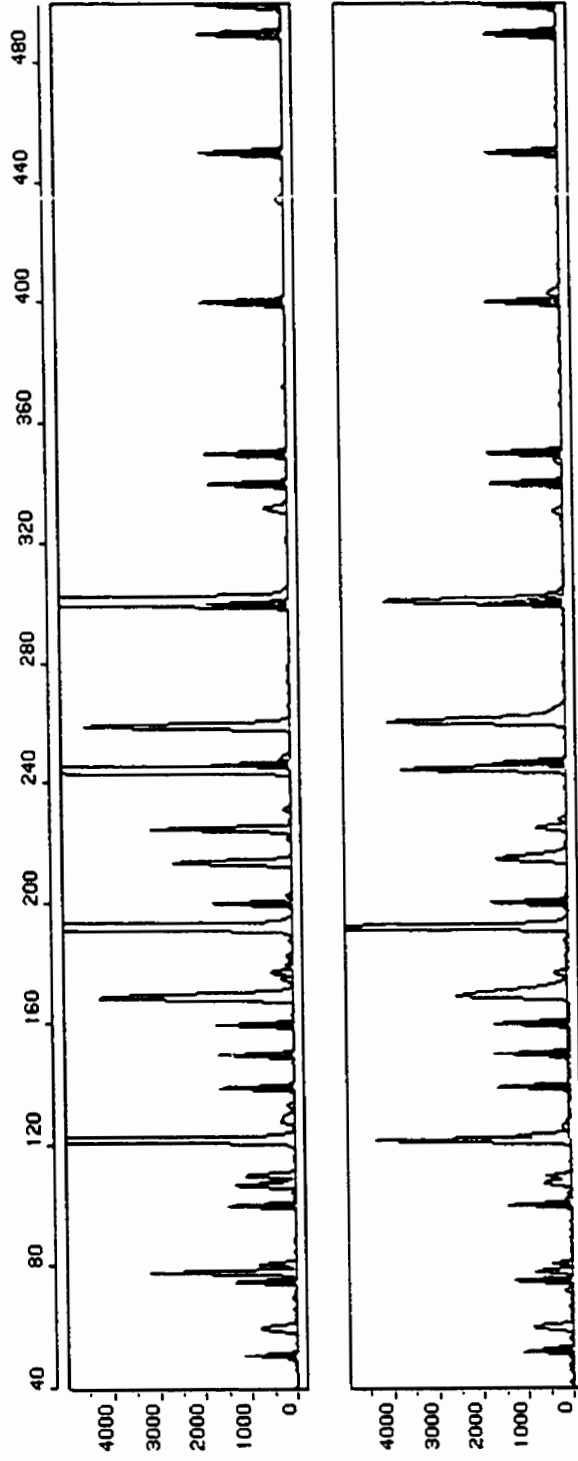
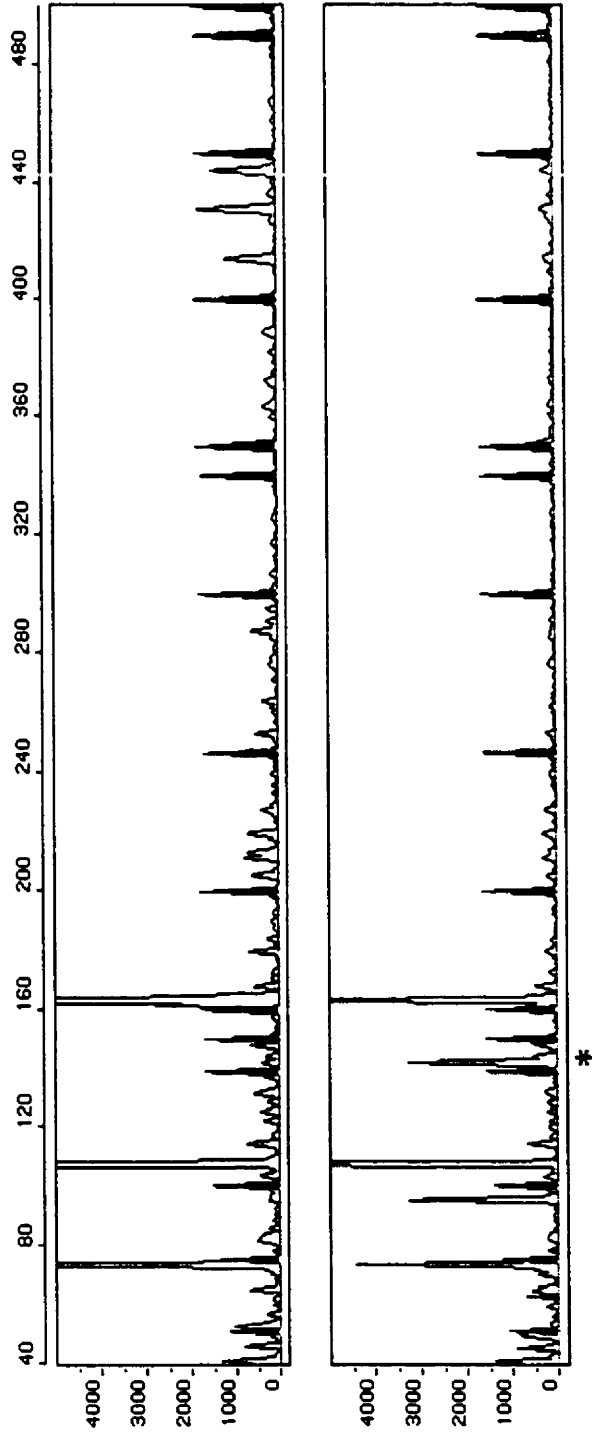


Figure 8. Example of poorly reproducible data for the primer pair E – AG, M - CAA. Each panel represents data for the same individual from the Sluice Point population (SP01); the DNA extraction, purification and AFLP procedures were run at different times. Clear peaks represent the data, grey peaks represent the internal size standard. The horizontal scale denotes the size of the fragments in base pairs. The vertical scale indicates the fluorescence signal intensity. The lower panel is an example of a “poor run”. * Note the additional peak at 142 bp and the general absence of fragments > 200 bp.



Second Objective: Preliminary Population Survey of C. crispus

The Dice similarity coefficients between all pairwise comparisons of individuals ranged from approximately 0.5 to 0.9 (Appendix 1), where the higher the value, the more similar the individuals. The UPGMA analysis using the Dice coefficient matrix (Fig. 9) showed that individuals from a population affiliate in a few loose clusters that failed to associate relative to other populations, with one notable exception - individuals from the Arisaig population were scattered throughout the tree. The Cape D'Or, Rustico, Miminegash and Sluice Point populations clustered together, the Maces Bay and Ketch Harbour populations clustered together, and the other three populations (Michaud Point, Cheticamp and Sandy Cove) clustered separately. In this analysis, the outgroups appeared in two distinct clusters, neither of which were basal to the tree.

A neighbour-joining analysis (Saitou and Nei 1987) was performed in PAUP using the distance metric of Nei and Li (1979) (Fig. 10). The tree was rooted with the two individuals from the population from Cap Gris Nez in France. In this case individuals from the Arisaig population were not as widely scattered throughout the tree, but some isolates did group with other populations. The Rustico and Sluice Point populations continued to group together, as did the Ketch Harbour and Maces Bay populations. Some clusters were unique to the neighbour-joining analysis: the Cape D'Or and Cheticamp populations cluster, there is a cluster of Arisaig and Miminegash individuals, and two isolates from Sluice Point group with the individual from New York (Fig. 10).

One thousand bootstrap replicates were performed on the data matrix using the neighbour-joining algorithm (Fig. 11). Hillis and Bull (1993) considered a node with 80% support or higher to be an accurate estimate of the true relationship. A few of the resolved nodes received support (>80%) from this analysis. Those clusters with support were for a few individuals from within a population. Two individuals from Cape D'Or allied with 82% bootstrap support, and three other individuals from Cape D'Or group together with 91% support. A group of three Ketch Harbour isolates cluster together with 99% support,

Figure 9. UPGMA cluster analysis based on the Dice similarity coefficient. The horizontal scale represents the Dice similarity values.

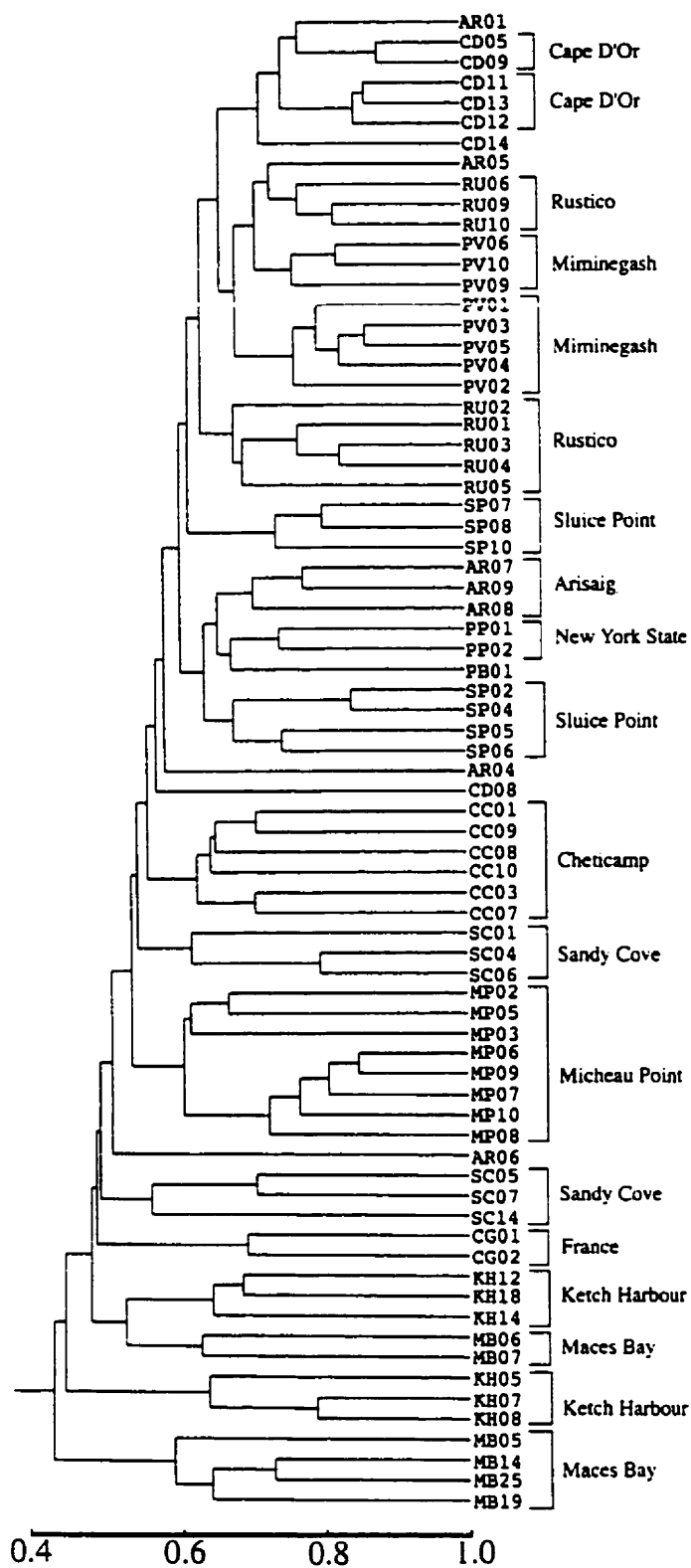


Figure 10. Neighbour-joining tree calculated using the Nei-Li distance metric.

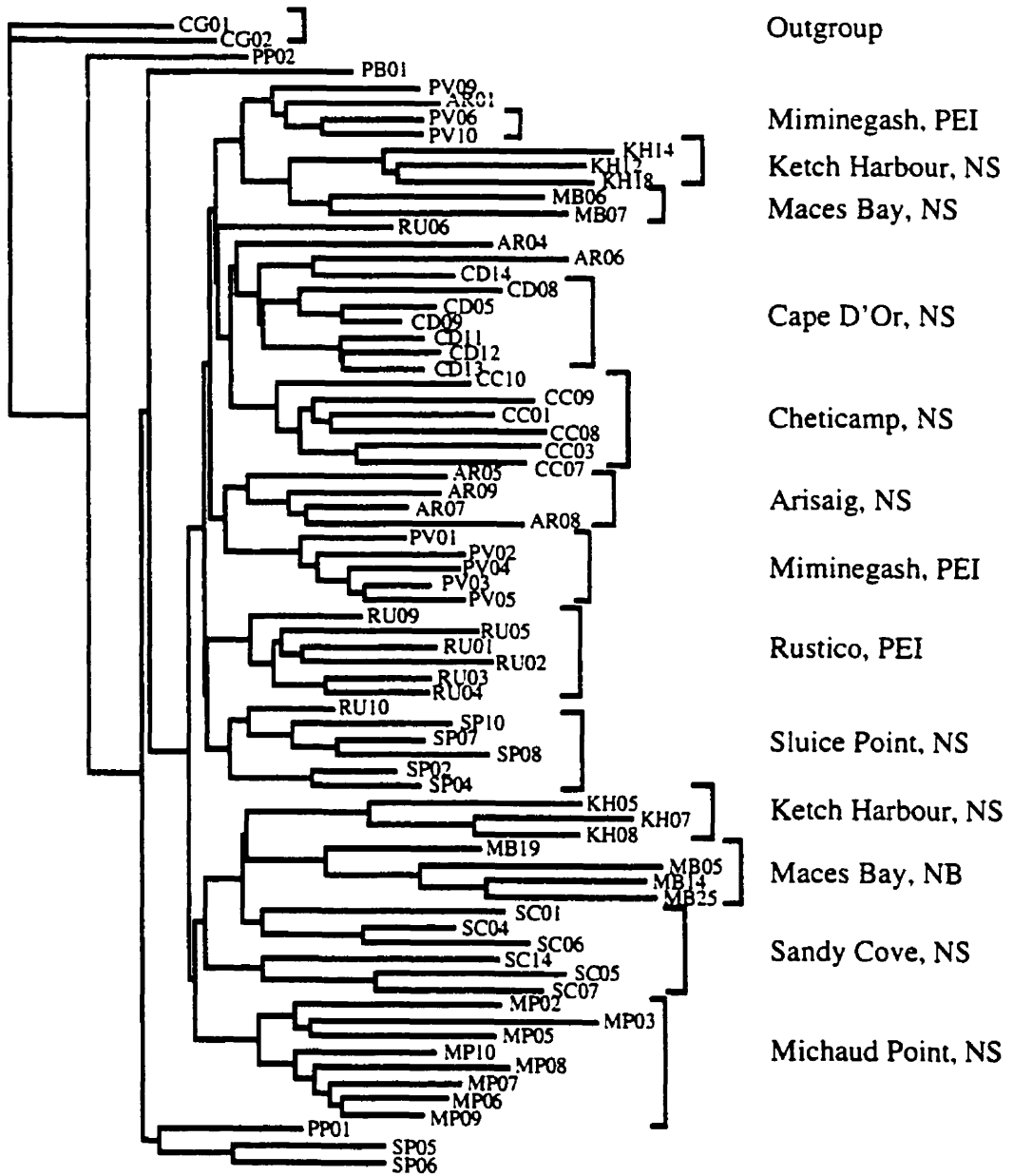
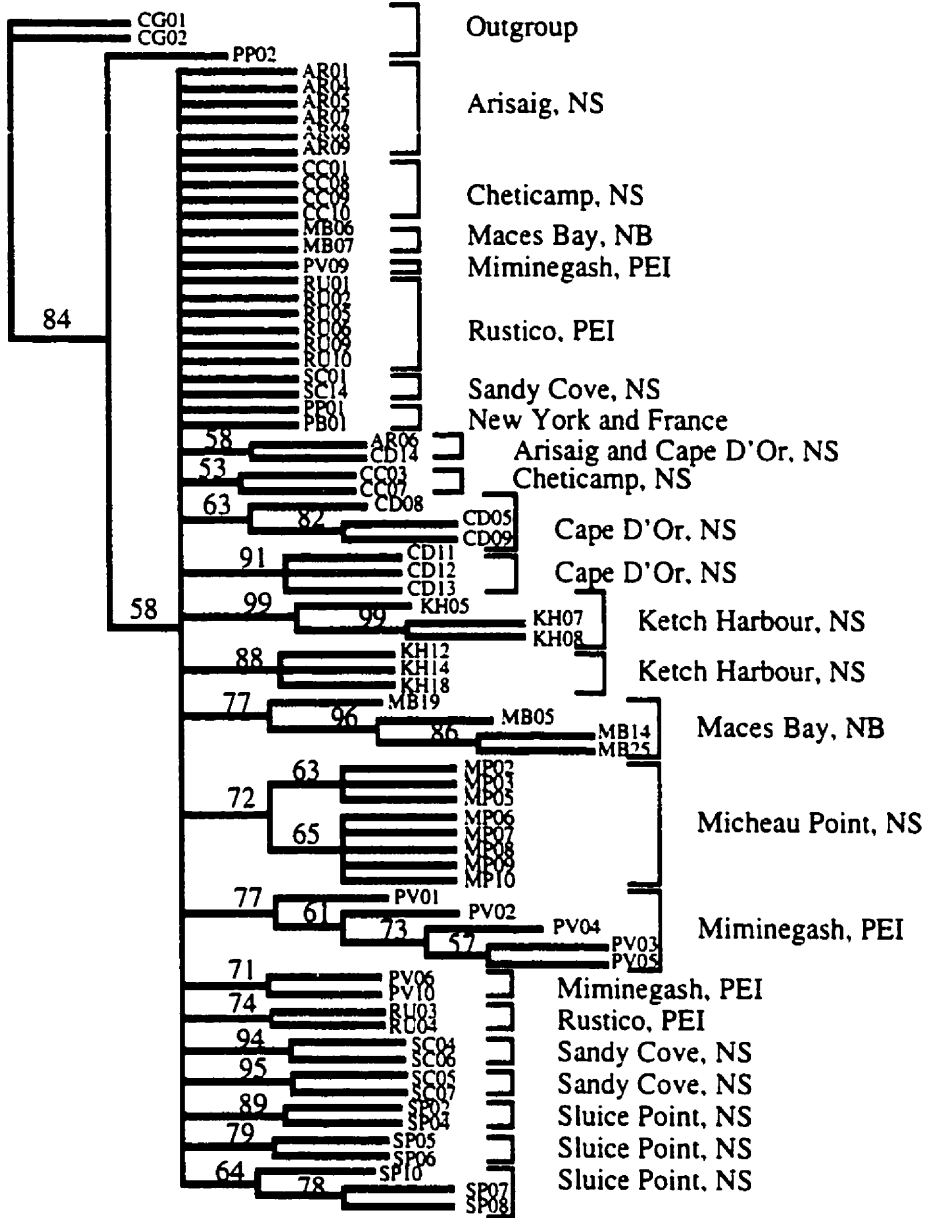


Figure 11. Neighbour-joining (bootstrap consensus) tree calculated using the Nei-Li distance metric. Numbers represent the percentage of 1000 bootstrap replicates that a particular node was resolved. All other nodes had less than 50% bootstrap support and are considered unresolved.



and three other Ketch Harbour individuals cluster with 88% support. Four Maces Bay isolates group together with 77% bootstrap support, and the support for relationships within that group is even higher at 96% and 86% (Fig. 11). All individuals from Michaud Point cluster together with 72% bootstrap support, although the relationships within that group are essentially unresolved. Five isolates from Miminegash cluster together with 77% support, and only one node within that group is resolved with 73% support. Two Rustico isolates group with 74% support. Two Sandy Cove individuals cluster with 94% bootstrap support, and two other isolates cluster with 95% support. Three pairs of Sluice Point isolates cluster separately with 89%, 79% and 78% support. Finally the two individuals from Cape Gris Nez in France cluster together with 84% bootstrap support. Relationships among populations are 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 (Fig. 11).

DISCUSSION

AFLP Technique

The first objective of this study has been met, the AFLP technique has been successfully applied to a red alga. The AFLP technique presents several advantages. No prior sequence knowledge is necessary to use the method making it easy to establish the protocol for use with new organisms and only small amounts of DNA are required [the AFLP technique was successfully applied to single fungal spores (Majer *et al.* 1996)], as opposed to the RFLP technique. Stringent reaction conditions should make AFLP banding patterns more reproducible than RAPD profiles. The number of AFLP markers obtained is directly proportional to the genome size of the organism (Vos *et al.* 1995). An organism with a large genome can produce upwards of 100 fragments per primer pair. The AFLP technique

is also flexible, the number of fragments obtained for each primer pair can be manipulated by changing the number (0 – 3) of selective nucleotides on the 3' end of the selective PCR primers (Vos *et al.* 1995). Selective PCR primers with two additional nucleotides on the 3' end will amplify more fragments than those with three nucleotides. Even though there is only one nucleotide difference, the shorter primer requires a less specific annealing site than the longer primer. Because of this, the shorter primer will anneal more often, amplifying more fragments. A combination of selective PCR primers can be chosen so as to obtain the desired number of fragments. As with all technique development, difficulties were encountered and are discussed below.

Scoring Bands

When originally scoring, no consideration was given to weak runs and all bands that were discernable from background noise were scored. It was soon realized that the intensity for a given band was not consistent across samples and it was decided to score the intensity of the bands. Fragments that were considered “weak” had less intensity than the internal size standard and fragments that were scored as “strong” were those whose intensity was more than the internal size standard. Little consistency in fluorescence intensity was found for a given band between individuals. A few bands were consistently weak, but the majority of fragments were a mix of intensities across individuals. The occurrence of several weak fragments in a run could be due to a weak reaction. With as many as 20 fragments amplified in one PCR tube there is only a small chance that all fragments get amplified to the same intensity across samples. The problem was compounded by the ABI genetic analyzer. Small differences in intensity are easily discernible in the outputted data. The intensity of bands has been used to score AFLP fragments as codominant (van Eck *et al.* 1995). An individual homozygous for an allele would have that allele present at approximately twice the intensity of an individual heterozygous for the same allele. The

sensitivity of the ABI genetic analyzer makes the scoring of band intensity too subjective. Therefore, it was decided to continue to score the AFLP data as dominant.

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 within a given individual. This supported the decision to score the AFLP bands as dominant. It was also observed that fragments were not reproducible across the samples. Often a fragment that was obviously present in the first replicate was not present in the reproduced replicate. In a few cases, complete runs were poorly reproduced. There was a characteristic pattern observed, where the general intensity of the fragments was low, and no fragments longer than 200 base pairs were present (Fig. 8, lower panel). It was hypothesized that this was due to a weak PCR, where the level of amplification was low for all fragments in the sample. After observing the lack of fragments longer than 200 base pairs during the reproducibility testing, the raw data for all primer pairs and all individuals were reexamined. When no fragments of longer than 200 base pairs were present in any sample, that individual was removed from subsequent population analyses. After undertaking the reproducibility experiments a total of 31 individuals (out of 105) and 65 fragments (out of 434) were removed from the dataset.

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 weak runs, which they eliminated from their analyses. They did not specify how far back in their procedure they went to perform these reproducibility experiments. 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) examining the reproducibility of several fragment techniques. In the Jones *et al.* study identical extracted DNA samples and the necessary protocols were sent to various laboratories across Europe to test the reproducibility of the AFLP technique and it was concluded that the AFLP technique was highly reproducible. However, Jones *et al.* (1997) used the same original DNA sample, which does not constitute a true 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, the DNA from the original ground algal sample was re-extracted for the replicate runs. 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.

It can be hypothesized that the lack of reproducibility of the AFLP technique is due to inconsistency in the quality of the DNA. The DNA extraction procedure used 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 the soluble polysaccharides found in red algae and RNA 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 (laver). They found that only DNA purified by CsCl gradient could create reproducible RAPD patterns.

Problems with the reproducibility of the AFLP technique may be minimized by using more stringent DNA extraction and purification procedures. Complete reproducibility experiments (*i.e.* back to 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 (Fig. 11) indicate almost no resolution within populations and no resolution among populations of *Chondrus crispus* in the Maritime provinces. Unfortunately, this does not clarify the genetic relationship of Chen's and Taylor's (1980a) narrow (Fig. 1a) and broad (Fig. 1b) strains. 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. Previous research indicates the latter is most likely. Firstly, the AFLP technique has been used to distinguish among populations of *Populus nigra* subsp. *betulifolia* (black poplar) (Winfield *et al.* 1998) and *Astragalus cremnophylax* var. *cremnophylax* (the sentry milk-vetch plant) (Travis *et al.* 1996). Although neither study performed bootstrap analysis, their cluster analyses indicated reasonable population structure, contrary to the present study (Fig. 9). 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. They calculated the phenotypic identity as a measure of similarity, which is based on the genotypic identity of Hedrick (1971). The phenotypic identity takes into consideration the number of different isozyme banding patterns (phenotypes) in the populations and compares the frequency of banding patterns in two populations. The phenotypic identity among these populations of *C. crispus* ranged from 0.851 to 0.951, where 1 indicates complete similarity and 0 indicates no similarity. A previous study on species of the red algal genus *Eucheuma* (Cheney and Babbel 1978) showed that the mean phenotypic identity for *E. isiforme* (C. Agardh) J. Agardh was 0.985. The mean phenotypic identity for *C. crispus* was 0.901. In comparing the phenotypic identity values in these two studies, Cheney and Mathieson (1979) 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. Chopin *et al.* (1996) concluded that there was too little ITS sequence divergence among *C. crispus* isolates for a detailed population study, and that a more variable technique might possibly uncover more variation. 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 relatively high in *C. crispus* in relation to other red algae.

It seems most likely 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 certain point of dissimilarity the occurrence of homoplasy, or noise, overwhelms genetic signal (Dowling *et al.* 1990). Homoplasy occurs when two fragments of the same size do not correspond to the same region of the genome. The exact point at which homoplasy overwhelms phylogenetic signal in simple fragment comparisons is debatable. Upholt (1977) believed that comparisons should not be made between samples whose banding patterns differ by more than 15%, whereas Kessler and Avise (1985) suggested that 25% is a more appropriate cut off point. Extensive divergence is reflected in the distance metrics used in this study. The highest distance value obtained with the Nei and Li (1979) metric was 0.13 (or 13%) and the lowest Dice similarity value was 0.5 (or 50%). These values are not, however, true representations of evolutionary distance. Band gain is more evolutionarily conservative than band loss, as there are several ways to lose a fragment, such as a point mutation in any of the bases in the recognition sequence of the restriction enzyme. In order to gain a band, a specific point mutation must occur to create a new recognition sequence (Dowling *et al.* 1990). This implies that band gain deserves more weight than band loss when calculating evolutionary distance. The Nei and Li distance metric considers both the shared presence and absence of a fragment equally. The more divergent two populations are relative to one another, the greater the chance of independent loss of homologous bands in each lineage, entering homoplasy into the data and resulting in an underestimation of evolutionary divergence. The Dice similarity coefficient only considers the shared presence of a fragment and, therefore, overestimates divergence by ignoring homologous band absence (signal rather than noise). This indicates that the maximum Nei and Li distance value in my dataset of 13% is an underestimate of true evolutionary divergence, and in fact, many pairwise comparisons in my dataset have

reached the level of saturation outlined by Upholt (1977) and possibly even that outlined by Kessler and Avise (1985). Extensive divergence is also reflected in the amount of monomorphic bands observed here. Folkertsma *et al.* (1996) observed only 15.8% polymorphic bands for potato cyst nematode populations, and Keim *et al.* (1997) noted only 3% polymorphic bands for strains of *Bacillus anthracis*, whereas 97% polymorphic bands were observed in my study. This increased divergence is inevitably accompanied by increased homoplasy. Extensive homoplasy is also illustrated by the lack of resolution among populations in the bootstrap analysis (Fig. 11). This does not indicate that the AFLP technique is not useful for some studies, but that *Chondrus crispus* is too genetically variable to be analyzed by this technique.

One consequence of *Chondrus crispus* populations in the Maritime provinces of Canada being too genetically variable to be analyzed by the AFLP technique is that there is no resolution among populations in my study. As such, I am not able to state whether or not stands in the Maritime provinces have genetic structure. If an absence of genetic structure is ultimately uncovered for *Chondrus crispus* in the Maritime provinces, then a few probable contributing factors are evident. As gamete dispersal increases among populations, the genetic population structure decreases (Avise 1994). That is, as the gametes or individuals of a population become more dispersed among neighbouring populations, it will become more difficult to distinguish among individuals from those populations genetically. There are two possible mechanisms for increased dispersal among *C. crispus* populations in the Maritime provinces of Canada. Approximately 13 000 years ago the Northumberland Strait may have been continuous with the Bay of Fundy (Scott *et al.* 1987), allowing gene flow between the Bay of Fundy and the Northumberland Strait that may have caused a decrease in population structure (Fig. 12). As well, extensive aquaculture farming of *C. crispus* occurs in the Maritime provinces and isolates have been transplanted from one body of water to another. Figure 13 illustrates a few examples of transplantations that are thought to have occurred. For example, there are aquaculture sites

Figure 12. Map of the Maritime provinces of Canada showing the possible geology approximately 13 000 years ago (Scott *et al.* 1987). Note the lack of land bridge in between Nova Scotia and New Brunswick.

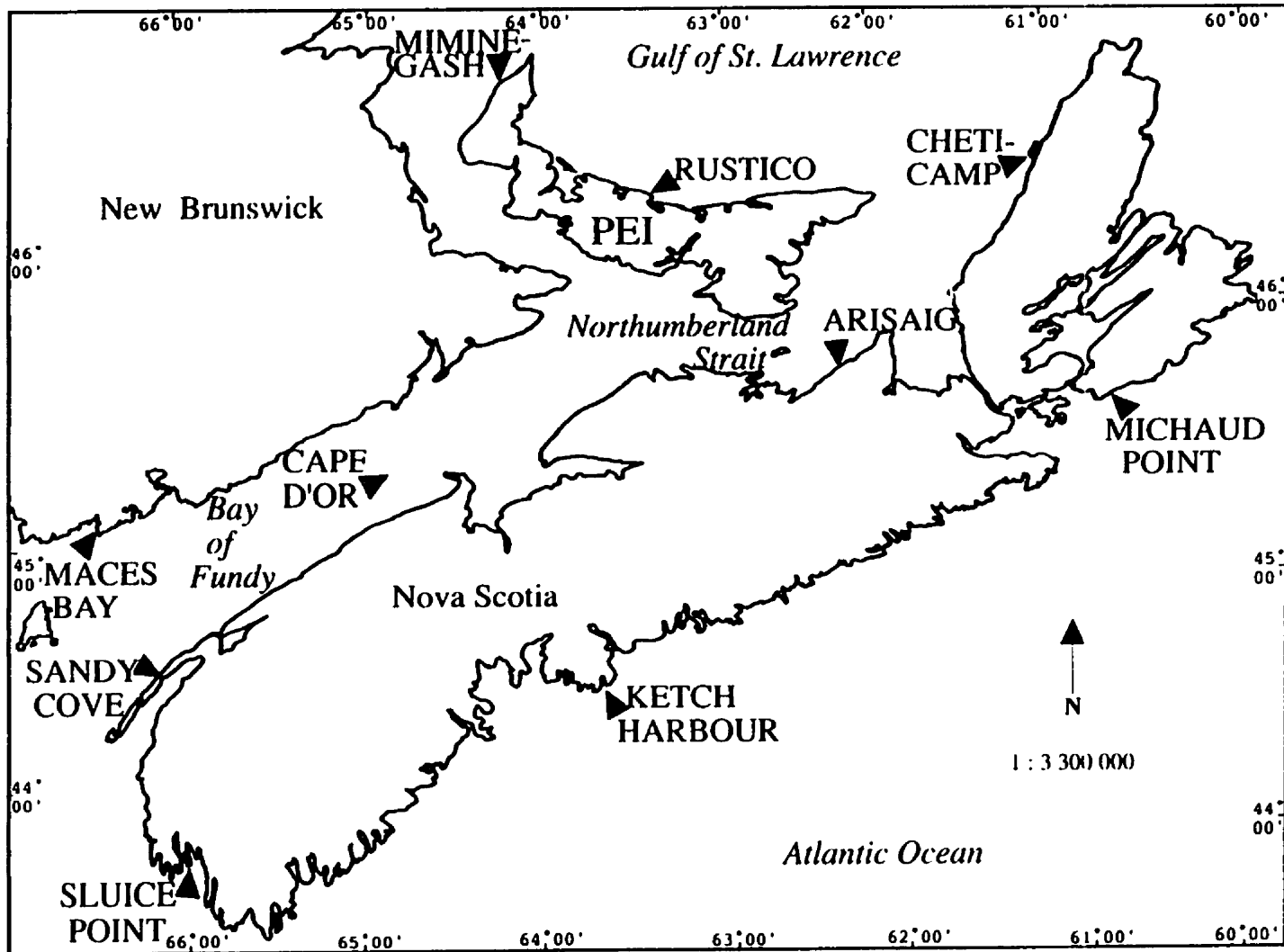
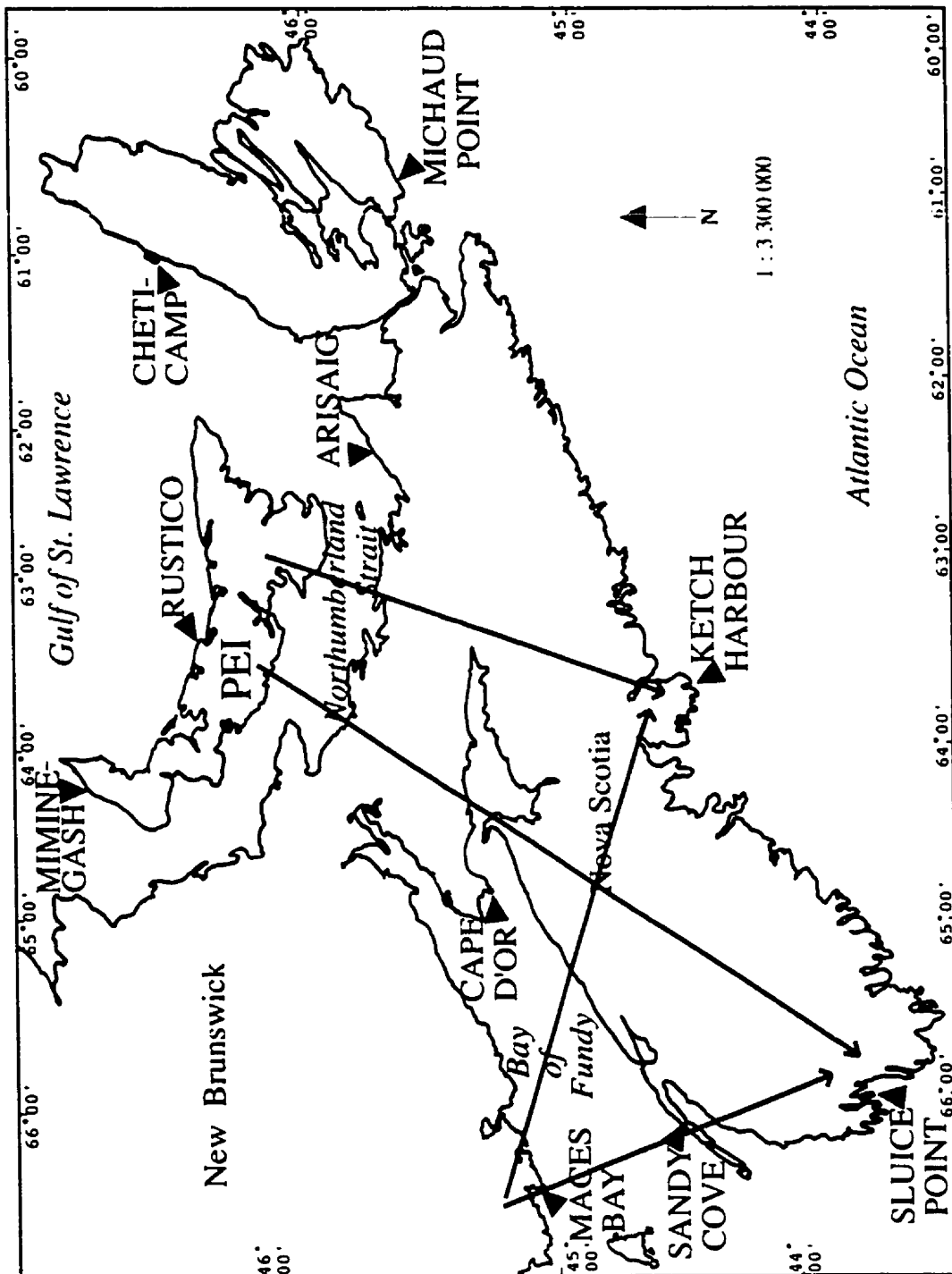


Figure 13. Map of the Maritime provinces of Canada showing the hypothesized movement of *Chondrus crispus* for aquacultural purposes.



near my Ketch Harbour and Sluice Point collecting sites where *C. crispus* from PEI is regularly farmed. Such transplants would not only reduce population structure for *Chondrus crispus* in the Maritime provinces, but could also increase the variability within the populations over the short term.

The present study supports the protein isozyme data of Cheney and Mathieson (1979) indicating that *Chondrus crispus* displays extreme genetic variation across its range. The ITS sequencing data of Chopin *et al.* (1996) also indicated substantial genetic variation of *C. crispus* relative to other red algal species. In both the study by Chopin *et al.* (1996) and the present study, geographic location could not be associated with genetic similarity.

Conclusions

The primary objective of this study was to develop the AFLP technique for use with red algae and this was achieved. As a result of this study, one must caution that the AFLP technique may only be reproducible when stringent DNA extraction and purification techniques are employed. The secondary objective of performing a preliminary population study suggests substantial genetic variation of *Chondrus crispus* populations.

I suggest that future research entail analyzing other red algal populations with the AFLP technique to determine if in fact *C. crispus* populations are genetically diverse as compared to other red algal species.

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APPENDIX I. Table of Dice pairwise similarity values

	AR01	AR04	AR05	AR06	AR07	AR08	AR09	CC01	CC03	CC07	CC08	CC09	CC10	CD05	CD08	CD09	CD11
AR01	1.00																
AR04	0.69	1.00															
AR05	0.75	0.69	1.00														
AR06	0.64	0.63	0.60	1.00													
AR07	0.70	0.68	0.74	0.64	1.00												
AR08	0.61	0.64	0.65	0.52	0.77	1.00											
AR09	0.72	0.67	0.76	0.62	0.80	0.72	1.00										
CC01	0.72	0.69	0.69	0.63	0.68	0.60	0.65	1.00									
CC03	0.66	0.62	0.64	0.64	0.63	0.51	0.62	0.71	1.00								
CC07	0.69	0.65	0.68	0.62	0.70	0.60	0.67	0.69	0.74	1.00							
CC08	0.66	0.61	0.63	0.63	0.65	0.56	0.62	0.73	0.73	0.88	1.00						
CC09	0.63	0.61	0.65	0.60	0.64	0.53	0.65	0.74	0.68	0.64	0.68	1.00					
CC10	0.71	0.68	0.66	0.62	0.71	0.60	0.68	0.72	0.72	0.66	0.69	0.69	1.00				
CD05	0.80	0.89	0.89	0.88	0.71	0.62	0.73	0.89	0.70	0.71	0.65	0.65	0.66	1.00			
CD08	0.68	0.63	0.64	0.63	0.67	0.59	0.70	0.62	0.63	0.60	0.63	0.65	0.75	1.00			
CD09	0.79	0.73	0.70	0.65	0.69	0.64	0.71	0.75	0.71	0.69	0.69	0.66	0.69	0.69	0.79	1.00	
CD11	0.76	0.71	0.71	0.66	0.73	0.64	0.70	0.68	0.65	0.64	0.67	0.63	0.70	0.77	0.71	0.80	1.00
CD12	0.80	0.67	0.74	0.65	0.72	0.65	0.67	0.66	0.63	0.64	0.65	0.65	0.68	0.74	0.67	0.75	0.86
CD13	0.76	0.68	0.72	0.71	0.73	0.63	0.71	0.67	0.68	0.68	0.71	0.65	0.69	0.82	0.69	0.80	0.87
CD14	0.69	0.68	0.68	0.72	0.68	0.61	0.67	0.63	0.68	0.64	0.60	0.64	0.66	0.77	0.67	0.75	0.77
KH05	0.56	0.58	0.61	0.56	0.62	0.55	0.58	0.59	0.55	0.55	0.54	0.56	0.58	0.59	0.60	0.60	0.56
KH07	0.51	0.58	0.55	0.53	0.56	0.52	0.55	0.55	0.57	0.54	0.56	0.57	0.57	0.53	0.59	0.56	0.53
KH08	0.55	0.61	0.57	0.57	0.57	0.52	0.54	0.55	0.58	0.53	0.54	0.55	0.58	0.60	0.62	0.61	0.58
KH12	0.64	0.57	0.59	0.50	0.63	0.61	0.61	0.62	0.56	0.54	0.53	0.55	0.62	0.59	0.60	0.65	0.58
KH14	0.62	0.58	0.60	0.54	0.64	0.59	0.63	0.58	0.56	0.55	0.58	0.53	0.62	0.56	0.55	0.60	0.66
KH18	0.63	0.55	0.59	0.54	0.59	0.62	0.64	0.58	0.54	0.52	0.54	0.55	0.63	0.57	0.58	0.63	0.58
MB05	0.52	0.55	0.50	0.52	0.55	0.51	0.52	0.51	0.57	0.53	0.56	0.52	0.54	0.55	0.58	0.58	0.56
MB06	0.75	0.62	0.58	0.57	0.62	0.58	0.64	0.62	0.59	0.60	0.58	0.53	0.65	0.66	0.61	0.69	0.67
MB07	0.65	0.55	0.62	0.60	0.62	0.55	0.60	0.65	0.59	0.63	0.59	0.53	0.63	0.61	0.59	0.62	0.62
MB14	0.50	0.52	0.49	0.49	0.55	0.52	0.53	0.53	0.55	0.49	0.54	0.58	0.54	0.54	0.57	0.55	0.52
MB19	0.60	0.62	0.59	0.58	0.68	0.62	0.64	0.64	0.61	0.61	0.59	0.61	0.61	0.64	0.64	0.66	0.62
MB25	0.53	0.56	0.48	0.52	0.55	0.53	0.54	0.52	0.51	0.46	0.53	0.55	0.55	0.57	0.60	0.60	0.56
MP02	0.56	0.56	0.58	0.60	0.61	0.57	0.55	0.55	0.53	0.54	0.56	0.51	0.58	0.64	0.58	0.63	0.65
MP03	0.59	0.59	0.60	0.51	0.56	0.58	0.58	0.55	0.49	0.58	0.53	0.55	0.59	0.57	0.52	0.57	0.59
MP05	0.66	0.62	0.62	0.60	0.63	0.63	0.56	0.63	0.57	0.56	0.56	0.59	0.63	0.62	0.58	0.67	0.63
MP06	0.64	0.65	0.62	0.58	0.66	0.62	0.64	0.60	0.60	0.63	0.59	0.59	0.63	0.67	0.63	0.69	0.68
MP07	0.62	0.60	0.64	0.57	0.68	0.62	0.67	0.59	0.58	0.59	0.61	0.57	0.64	0.61	0.61	0.65	0.65
MP08	0.63	0.61	0.66	0.53	0.66	0.58	0.64	0.58	0.54	0.58	0.57	0.58	0.62	0.61	0.59	0.61	0.63
MP09	0.66	0.66	0.66	0.59	0.73	0.69	0.69	0.64	0.59	0.64	0.62	0.59	0.68	0.66	0.65	0.68	0.69
MP10	0.64	0.67	0.64	0.59	0.72	0.63	0.66	0.68	0.65	0.63	0.66	0.63	0.64	0.64	0.60	0.67	0.65
PV01	0.71	0.65	0.75	0.61	0.74	0.72	0.73	0.71	0.66	0.71	0.63	0.64	0.68	0.72	0.62	0.73	0.74
PV02	0.70	0.64	0.65	0.59	0.67	0.61	0.66	0.63	0.59	0.68	0.54	0.60	0.70	0.67	0.62	0.66	0.67
PV03	0.69	0.68	0.72	0.60	0.73	0.67	0.70	0.65	0.60	0.73	0.57	0.60	0.67	0.70	0.64	0.68	0.69
PV04	0.65	0.65	0.70	0.56	0.65	0.64	0.67	0.64	0.61	0.66	0.56	0.59	0.66	0.69	0.59	0.68	0.69
PV05	0.64	0.66	0.72	0.56	0.70	0.68	0.69	0.65	0.61	0.71	0.57	0.61	0.63	0.68	0.61	0.67	0.68
PV06	0.79	0.63	0.71	0.60	0.73	0.65	0.65	0.67	0.64	0.71	0.61	0.63	0.69	0.69	0.64	0.72	0.70
PV09	0.75	0.71	0.73	0.60	0.76	0.68	0.70	0.66	0.65	0.71	0.64	0.61	0.72	0.71	0.63	0.71	0.70
PV10	0.78	0.67	0.70	0.63	0.72	0.61	0.66	0.70	0.65	0.70	0.64	0.66	0.67	0.74	0.60	0.75	0.72
RU01	0.67	0.66	0.64	0.61	0.70	0.62	0.68	0.64	0.63	0.59	0.61	0.65	0.67	0.68	0.67	0.72	0.75
RU02	0.65	0.63	0.64	0.58	0.64	0.62	0.69	0.59	0.57	0.58	0.60	0.61	0.62	0.68	0.64	0.70	0.71
RU03	0.69	0.66	0.66	0.63	0.66	0.62	0.69	0.64	0.63	0.59	0.62	0.60	0.67	0.70	0.64	0.71	0.73
RU04	0.73	0.66	0.69	0.63	0.65	0.59	0.65	0.67	0.65	0.60	0.63	0.65	0.70	0.66	0.64	0.70	0.73
RU05	0.66	0.58	0.69	0.52	0.65	0.62	0.65	0.61	0.60	0.58	0.56	0.64	0.61	0.65	0.60	0.69	0.68
RU06	0.72	0.71	0.76	0.69	0.74	0.63	0.72	0.68	0.69	0.74	0.62	0.70	0.68	0.70	0.69	0.72	0.69
RU09	0.77	0.70	0.77	0.67	0.73	0.62	0.70	0.70	0.68	0.69	0.68	0.68	0.71	0.71	0.68	0.72	0.74
RU10	0.76	0.71	0.77	0.68	0.73	0.65	0.69	0.74	0.68	0.70	0.65	0.72	0.70	0.77	0.68	0.81	0.78
SC01	0.62	0.63	0.67	0.63	0.62	0.53	0.64	0.62	0.65	0.60	0.61	0.63	0.64	0.67	0.62	0.69	0.64
SC04	0.61	0.62	0.62	0.61	0.67	0.60	0.65	0.58	0.62	0.58	0.63	0.60	0.65	0.69	0.63	0.71	0.70
SC05	0.56	0.53	0.57	0.58	0.59	0.52	0.61	0.54	0.54	0.55	0.56	0.61	0.60	0.63	0.59	0.62	0.64
SC06	0.57	0.57	0.56	0.60	0.59	0.52	0.58	0.62	0.63	0.58	0.59	0.62	0.61	0.65	0.60	0.67	0.62
SC07	0.58	0.52	0.53	0.61	0.60	0.55	0.60	0.56	0.55	0.52	0.53	0.60	0.55	0.61	0.58	0.62	0.60
SC14	0.63	0.53	0.62	0.55	0.66	0.58	0.63	0.59	0.55	0.55	0.52	0.56	0.57	0.58	0.58	0.59	0.62
SP02	0.68	0.64	0.67	0.62	0.71	0.65	0.67	0.64	0.62	0.61	0.63	0.65	0.68	0.73	0.70	0.76	0.74
SP04	0.66	0.60	0.69	0.58	0.69	0.70	0.72	0.61	0.59	0.61	0.62	0.60	0.62	0.70	0.68	0.73	0.71
SP05	0.59	0.62	0.65	0.56	0.72	0.70	0.67	0.55	0.53	0.56	0.59	0.58	0.65	0.64	0.63	0.63	0.67
SP06	0.60	0.61	0.61	0.54	0.74	0.70	0.68	0.58	0.54	0.57	0.57	0.59	0.61	0.64	0.59	0.63	0.63
SP07	0.68	0.66	0.68	0.72	0.70	0.59	0.68	0.64	0.67	0.70	0.68	0.67	0.65	0.71	0.63	0.70	0.68
SP08	0.61	0.58	0.60	0.64	0.61	0.57	0.63	0.62	0.60	0.59	0.59	0.62	0.57	0.65	0.64	0.68	0.68
SP10	0.69	0.61	0.63	0.63	0.65	0.63	0.62	0.63	0.60	0.61	0.57	0.61	0.61	0.66	0.63	0.68	0.71
PP01	0.71	0.68	0.73	0.59	0.75	0.70	0.69	0.64	0.59	0.69	0.62	0.62	0.68	0.71	0.64	0.72	0.67
PP02	0.66	0.66	0.66	0.56	0.75	0.67	0.71	0.65	0.59	0.63	0.60	0.63	0.64	0.67	0.62	0.70	0.62
CG01	0.58	0.55	0.61	0.51	0.67	0.57	0.62	0.58	0.56	0.55	0.58	0.62	0.58	0.59	0.56	0.58	0.58
CG02	0.54	0.55	0.61	0.50	0.65	0.59	0.63	0.57	0.49	0.55	0.59	0.59	0.56	0.56	0.56	0.59	0.59
PB01	0.63	0.63	0.68	0.59	0.74	0.70	0.65	0.70	0.62	0.65	0.65	0.69	0.65	0.64	0.61	0.67	0.68

APPENDIX I. Table of Dice pairwise similarity values

	CD12	CD13	CD14	KH05	KH07	KH08	KH12	KH14	KH18	MB05	MB06	MB07	MB14	MB19	MB25	MP02	MP03
AR01																	
AR04																	
AR05																	
AR06																	
AR07																	
AR08																	
AR09																	
CC01																	
CC03																	
CC07																	
CC08																	
CC09																	
CC10																	
CD05																	
CD08																	
CD09																	
CD11																	
CD12	1.00																
CD13	0.86	1.00															
CD14	0.75	0.76	1.00														
KH05	0.60	0.58	0.59	1.00													
KH07	0.54	0.52	0.59	0.70	1.00												
KH08	0.58	0.57	0.63	0.69	0.81	1.00											
KH12	0.63	0.56	0.59	0.60	0.50	0.53	1.00										
KH14	0.63	0.59	0.58	0.57	0.45	0.47	0.71	1.00									
KH18	0.62	0.59	0.58	0.60	0.51	0.57	0.73	0.69	1.00								
MB05	0.54	0.56	0.54	0.52	0.53	0.54	0.49	0.46	0.49	1.00							
MB06	0.65	0.65	0.62	0.48	0.50	0.54	0.57	0.62	0.63	0.56	1.00						
MB07	0.63	0.67	0.57	0.56	0.48	0.53	0.62	0.65	0.66	0.57	0.69	1.00					
MB14	0.53	0.55	0.51	0.55	0.56	0.55	0.50	0.47	0.47	0.70	0.57	0.56	1.00				
MB19	0.58	0.63	0.63	0.59	0.59	0.61	0.57	0.54	0.56	0.63	0.67	0.67	0.74	1.00			
MB25	0.53	0.55	0.54	0.55	0.53	0.54	0.47	0.46	0.47	0.66	0.57	0.53	0.76	0.65	1.00		
MP02	0.60	0.63	0.62	0.54	0.53	0.57	0.56	0.51	0.52	0.55	0.53	0.54	0.51	0.55	0.53	1.00	
MP03	0.58	0.61	0.55	0.49	0.50	0.53	0.53	0.49	0.51	0.56	0.52	0.52	0.50	0.49	0.55	0.68	1.00
MP05	0.64	0.61	0.64	0.52	0.53	0.58	0.59	0.57	0.58	0.58	0.66	0.56	0.58	0.62	0.59	0.72	0.68
MP06	0.65	0.64	0.68	0.58	0.59	0.62	0.61	0.57	0.57	0.59	0.60	0.60	0.57	0.62	0.58	0.68	0.69
MP07	0.61	0.61	0.63	0.60	0.60	0.61	0.62	0.56	0.57	0.61	0.56	0.57	0.60	0.60	0.57	0.66	0.66
MP08	0.61	0.58	0.61	0.58	0.57	0.59	0.57	0.55	0.54	0.55	0.54	0.55	0.53	0.57	0.53	0.61	0.61
MP09	0.67	0.66	0.66	0.60	0.61	0.60	0.66	0.61	0.60	0.59	0.58	0.60	0.55	0.61	0.57	0.70	0.70
MP10	0.62	0.63	0.65	0.59	0.60	0.62	0.65	0.58	0.59	0.58	0.60	0.58	0.54	0.65	0.53	0.69	0.63
PV01	0.70	0.72	0.70	0.60	0.56	0.56	0.63	0.60	0.58	0.51	0.64	0.60	0.54	0.65	0.52	0.63	0.60
PV02	0.66	0.65	0.68	0.55	0.54	0.59	0.63	0.58	0.61	0.53	0.67	0.57	0.51	0.61	0.48	0.58	0.61
PV03	0.66	0.66	0.72	0.59	0.56	0.61	0.59	0.56	0.54	0.55	0.60	0.59	0.58	0.68	0.55	0.64	0.64
PV04	0.68	0.69	0.73	0.55	0.56	0.60	0.59	0.57	0.56	0.54	0.62	0.55	0.56	0.62	0.51	0.59	0.61
PV05	0.68	0.70	0.65	0.55	0.52	0.56	0.58	0.55	0.52	0.52	0.60	0.58	0.57	0.67	0.52	0.61	0.61
PV06	0.71	0.69	0.66	0.62	0.54	0.58	0.69	0.65	0.67	0.50	0.69	0.64	0.52	0.62	0.50	0.62	0.56
PV09	0.69	0.68	0.68	0.56	0.54	0.59	0.61	0.60	0.58	0.51	0.70	0.63	0.52	0.64	0.51	0.64	0.62
PV10	0.71	0.74	0.74	0.56	0.55	0.59	0.63	0.65	0.61	0.49	0.72	0.62	0.52	0.65	0.50	0.61	0.57
RU01	0.73	0.71	0.67	0.61	0.56	0.58	0.61	0.59	0.59	0.56	0.62	0.56	0.57	0.63	0.58	0.60	0.56
RU02	0.69	0.68	0.64	0.55	0.54	0.55	0.57	0.60	0.57	0.54	0.59	0.55	0.52	0.59	0.54	0.58	0.56
RU03	0.69	0.70	0.73	0.57	0.55	0.61	0.64	0.61	0.62	0.55	0.64	0.60	0.53	0.61	0.52	0.64	0.60
RU04	0.70	0.67	0.70	0.59	0.56	0.64	0.65	0.62	0.62	0.54	0.63	0.62	0.54	0.63	0.52	0.61	0.58
RU05	0.70	0.65	0.64	0.55	0.53	0.58	0.63	0.58	0.61	0.52	0.57	0.55	0.58	0.61	0.50	0.56	0.51
RU06	0.70	0.73	0.71	0.68	0.62	0.63	0.59	0.59	0.62	0.59	0.67	0.68	0.61	0.69	0.56	0.60	0.60
RU09	0.73	0.71	0.73	0.63	0.62	0.69	0.65	0.62	0.65	0.58	0.64	0.65	0.55	0.64	0.52	0.63	0.61
RU10	0.79	0.78	0.76	0.67	0.62	0.67	0.65	0.61	0.64	0.54	0.65	0.68	0.60	0.69	0.58	0.61	0.62
SC01	0.68	0.65	0.63	0.60	0.56	0.60	0.57	0.53	0.60	0.57	0.56	0.57	0.56	0.60	0.61	0.55	0.54
SC04	0.70	0.71	0.68	0.61	0.59	0.58	0.58	0.59	0.63	0.61	0.57	0.59	0.58	0.63	0.60	0.60	0.55
SC05	0.61	0.70	0.62	0.55	0.52	0.52	0.52	0.54	0.54	0.49	0.51	0.52	0.52	0.57	0.53	0.56	0.52
SC06	0.60	0.63	0.61	0.61	0.59	0.60	0.53	0.51	0.55	0.64	0.57	0.57	0.61	0.67	0.63	0.55	0.53
SC07	0.59	0.64	0.64	0.53	0.53	0.54	0.49	0.51	0.53	0.53	0.57	0.55	0.57	0.64	0.55	0.55	0.52
SC14	0.61	0.58	0.62	0.57	0.50	0.53	0.60	0.60	0.60	0.49	0.61	0.59	0.55	0.61	0.54	0.57	0.54
SP02	0.74	0.73	0.73	0.60	0.58	0.63	0.62	0.62	0.62	0.55	0.63	0.62	0.56	0.66	0.62	0.62	0.59
SP04	0.71	0.72	0.68	0.56	0.55	0.61	0.61	0.62	0.64	0.57	0.63	0.61	0.57	0.67	0.60	0.62	0.57
SP05	0.67	0.69	0.63	0.58	0.57	0.55	0.56	0.55	0.59	0.53	0.57	0.54	0.61	0.65	0.59	0.63	0.57
SP06	0.61	0.66	0.60	0.55	0.55	0.55	0.53	0.55	0.58	0.55	0.57	0.59	0.61	0.68	0.62	0.58	0.55
SP07	0.67	0.71	0.76	0.61	0.65	0.66	0.53	0.55	0.59	0.55	0.64	0.62	0.57	0.66	0.54	0.61	0.57
SP08	0.63	0.64	0.71	0.59	0.61	0.64	0.57	0.55	0.59	0.54	0.63	0.61	0.59	0.66	0.58	0.54	0.53
SP10	0.70	0.68	0.71	0.60	0.55	0.58	0.57	0.59	0.62	0.53	0.64	0.63	0.55	0.64	0.55	0.59	0.57
PP01	0.68	0.66	0.66	0.64	0.57	0.61	0.64	0.59	0.65	0.53	0.59	0.64	0.54	0.66	0.52	0.62	0.59
PP02	0.62	0.64	0.60	0.59	0.54	0.57	0.67	0.62	0.65	0.49	0.58	0.62	0.54	0.64	0.53	0.58	0.57
CG01	0.57	0.58	0.58	0.56	0.52	0.53	0.51	0.53	0.51	0.51	0.53	0.53	0.54	0.60	0.53	0.59	0.57
CG02	0.55	0.61	0.55	0.51	0.50	0.51	0.51	0.52	0.50	0.44	0.47	0.52	0.50	0.58	0.49	0.53	0.54
PB01	0.68	0.70	0.65	0.59	0.56	0.60	0.56	0.55	0.56	0.56	0.59	0.60	0.60	0.69	0.55	0.59	0.60

APPENDIX I. Table of Dice pairwise similarity values

	MP05	MP06	MP07	MP08	MP09	MP10	PV01	PV02	PV03	PV04	PV05	PV06	PV09	PV10	RU01	RU02	RU03
AR01																	
AR04																	
AR05																	
AR06																	
AR07																	
AR08																	
AR09																	
CC01																	
CC03																	
CC07																	
CC08																	
CC09																	
CC10																	
CD05																	
CD06																	
CD09																	
CD11																	
CD12																	
CD13																	
CD14																	
KH05																	
KH07																	
KH08																	
KH12																	
KH14																	
KH18																	
MB05																	
MB06																	
MB07																	
MB14																	
MB19																	
MB25																	
MP02																	
MP03																	
MP05	1.00																
MP06	0.75	1.00															
MP07	0.69	0.83	1.00														
MP08	0.62	0.77	0.73	1.00													
MP09	0.72	0.88	0.82	0.80	1.00												
MP10	0.69	0.80	0.76	0.74	0.82	1.00											
PV01	0.65	0.64	0.67	0.64	0.67	0.70	1.00										
PV02	0.64	0.64	0.65	0.63	0.65	0.69	0.78	1.00									
PV03	0.64	0.67	0.66	0.67	0.72	0.67	0.80	0.81	1.00								
PV04	0.61	0.64	0.60	0.61	0.66	0.66	0.81	0.79	0.85	1.00							
PV05	0.61	0.61	0.60	0.59	0.65	0.67	0.64	0.78	0.87	0.84	1.00						
PV06	0.64	0.67	0.68	0.63	0.70	0.67	0.74	0.74	0.70	0.68	0.69	1.00					
PV09	0.69	0.70	0.68	0.65	0.69	0.70	0.77	0.73	0.74	0.71	0.77	0.78	1.00				
PV10	0.67	0.68	0.65	0.60	0.67	0.68	0.76	0.72	0.72	0.73	0.71	0.64	0.80	1.00			
RU01	0.61	0.63	0.66	0.60	0.65	0.67	0.71	0.68	0.68	0.68	0.67	0.67	0.85	0.87	1.00		
RU02	0.58	0.64	0.63	0.61	0.64	0.61	0.63	0.63	0.65	0.66	0.65	0.63	0.84	0.63	0.78	1.00	
RU03	0.64	0.65	0.70	0.61	0.66	0.67	0.74	0.74	0.71	0.72	0.69	0.67	0.72	0.70	0.80	0.73	1.00
RU04	0.63	0.67	0.67	0.63	0.68	0.69	0.69	0.70	0.69	0.68	0.67	0.72	0.73	0.70	0.79	0.71	0.84
RU05	0.57	0.58	0.62	0.62	0.60	0.61	0.72	0.68	0.67	0.71	0.67	0.68	0.65	0.70	0.76	0.70	0.71
RU06	0.62	0.69	0.66	0.66	0.68	0.72	0.74	0.72	0.73	0.70	0.72	0.74	0.72	0.72	0.70	0.66	0.69
RU09	0.64	0.69	0.73	0.69	0.72	0.73	0.74	0.74	0.74	0.72	0.69	0.79	0.74	0.77	0.70	0.79	
RU10	0.65	0.69	0.68	0.67	0.69	0.69	0.79	0.73	0.72	0.74	0.73	0.79	0.78	0.80	0.75	0.73	0.75
SC01	0.55	0.58	0.60	0.54	0.58	0.61	0.62	0.60	0.60	0.59	0.59	0.64	0.59	0.60	0.65	0.64	0.66
SC04	0.63	0.65	0.66	0.59	0.64	0.64	0.68	0.63	0.62	0.63	0.61	0.62	0.64	0.64	0.70	0.69	0.66
SC05	0.57	0.59	0.61	0.56	0.60	0.59	0.60	0.57	0.58	0.59	0.57	0.57	0.59	0.61	0.57	0.56	0.57
SC06	0.58	0.60	0.60	0.53	0.56	0.62	0.66	0.63	0.61	0.63	0.62	0.60	0.60	0.60	0.62	0.61	0.59
SC07	0.59	0.64	0.63	0.58	0.58	0.59	0.63	0.63	0.63	0.60	0.60	0.58	0.58	0.63	0.62	0.56	0.61
SC14	0.59	0.65	0.63	0.63	0.64	0.68	0.67	0.68	0.67	0.63	0.65	0.67	0.66	0.66	0.64	0.59	0.65
SP02	0.66	0.71	0.68	0.63	0.69	0.65	0.70	0.66	0.69	0.69	0.67	0.68	0.74	0.72	0.70	0.68	0.72
SP04	0.63	0.68	0.67	0.61	0.69	0.66	0.69	0.64	0.71	0.70	0.70	0.68	0.71	0.70	0.70	0.71	0.71
SP05	0.58	0.59	0.59	0.60	0.64	0.61	0.69	0.61	0.68	0.67	0.67	0.60	0.65	0.61	0.67	0.64	0.66
SP06	0.61	0.65	0.63	0.64	0.69	0.66	0.64	0.55	0.65	0.60	0.64	0.58	0.69	0.63	0.64	0.60	0.60
SP07	0.61	0.67	0.67	0.63	0.66	0.67	0.70	0.67	0.69	0.67	0.65	0.70	0.73	0.77	0.68	0.63	0.69
SP08	0.60	0.64	0.64	0.60	0.62	0.62	0.63	0.60	0.64	0.66	0.62	0.64	0.65	0.69	0.64	0.63	0.66
SP10	0.64	0.69	0.64	0.66	0.67	0.63	0.71	0.68	0.64	0.68	0.63	0.69	0.69	0.71	0.68	0.64	0.68
PP01	0.61	0.67	0.69	0.71	0.73	0.67	0.71	0.69	0.73	0.69	0.67	0.77	0.72	0.70	0.66	0.66	0.68
PP02	0.63	0.65	0.64	0.67	0.68	0.69	0.68	0.65	0.64	0.63	0.64	0.69	0.68	0.68	0.64	0.61	0.61
CG01	0.58	0.59	0.64	0.65	0.64	0.63	0.65	0.62	0.66	0.63	0.69	0.63	0.63	0.64	0.62	0.61	0.62
CG02	0.58	0.59	0.61	0.63	0.64	0.61	0.63	0.58	0.59	0.57	0.61	0.57	0.60	0.59	0.61	0.57	0.60
PB01	0.63	0.64	0.64	0.65	0.64	0.65	0.72	0.64	0.70	0.66	0.69	0.65	0.67	0.66	0.67	0.62	0.63

APPENDIX I. Table of Dice pairwise similarity values

	RU04	RU05	RU06	RU09	RU10	SC01	SC04	SC05	SC06	SC07	SC14	SP02	SP04	SP05	SP06	SP07	SP08
AR01																	
AR04																	
AR05																	
AR06																	
AR07																	
AR08																	
AR09																	
CC01																	
CC03																	
CC07																	
CC08																	
CC09																	
CC10																	
CD05																	
CD08																	
CD09																	
CD11																	
CD12																	
CD13																	
CD14																	
KH05																	
KH07																	
KH08																	
KH12																	
KH14																	
KH18																	
MB05																	
MB06																	
MB07																	
MB14																	
MB19																	
MB25																	
MP02																	
MP03																	
MP05																	
MP06																	
MP07																	
MP08																	
MP09																	
MP10																	
PV01																	
PV02																	
PV03																	
PV04																	
PV05																	
PV06																	
PV09																	
PV10																	
RU01																	
RU02																	
RU03																	
RU04	1.00																
RU05	0.74	1.00															
RU06	0.68	0.67	1.00														
RU09	0.84	0.75	0.78	1.00													
RU10	0.75	0.76	0.81	0.84	1.00												
SC01	0.68	0.61	0.65	0.69	0.71	1.00											
SC04	0.64	0.67	0.66	0.68	0.73	0.68	1.00										
SC05	0.60	0.59	0.62	0.60	0.62	0.62	0.67	1.00									
SC06	0.60	0.63	0.66	0.64	0.67	0.67	0.81	0.62	1.00								
SC07	0.60	0.61	0.66	0.57	0.63	0.54	0.63	0.74	0.63	1.00							
SC14	0.66	0.65	0.66	0.68	0.66	0.62	0.62	0.61	0.62	0.68	1.00						
SP02	0.70	0.67	0.66	0.73	0.81	0.67	0.71	0.63	0.63	0.63	0.65	1.00					
SP04	0.71	0.66	0.66	0.72	0.75	0.65	0.67	0.65	0.60	0.63	0.69	0.65	1.00				
SP05	0.61	0.61	0.64	0.66	0.70	0.61	0.65	0.59	0.57	0.56	0.59	0.72	0.73	1.00			
SP06	0.61	0.64	0.65	0.67	0.68	0.57	0.66	0.59	0.56	0.59	0.60	0.73	0.71	0.78	1.00		
SP07	0.67	0.66	0.77	0.77	0.79	0.65	0.66	0.60	0.64	0.64	0.62	0.75	0.66	0.64	0.66	1.00	
SP08	0.62	0.63	0.68	0.70	0.78	0.62	0.64	0.56	0.61	0.63	0.63	0.77	0.71	0.60	0.64	0.62	1.00
SP10	0.67	0.66	0.70	0.70	0.79	0.59	0.66	0.58	0.57	0.66	0.68	0.75	0.68	0.63	0.69	0.76	0.78
PP01	0.70	0.69	0.74	0.79	0.78	0.67	0.65	0.58	0.57	0.59	0.63	0.72	0.70	0.75	0.74	0.70	0.62
PP02	0.63	0.66	0.71	0.68	0.69	0.61	0.62	0.60	0.58	0.63	0.66	0.66	0.66	0.63	0.74	0.63	0.62
CG01	0.61	0.64	0.65	0.64	0.65	0.54	0.58	0.57	0.58	0.62	0.66	0.61	0.61	0.60	0.62	0.61	0.58
CG02	0.59	0.63	0.61	0.61	0.65	0.53	0.59	0.55	0.51	0.55	0.57	0.64	0.58	0.62	0.67	0.62	0.59
PB01	0.66	0.66	0.72	0.70	0.74	0.59	0.65	0.59	0.64	0.63	0.62	0.69	0.67	0.67	0.65	0.66	0.61

APPENDIX I. Table of Dice pairwise similarity values

	SP10	PP01	PP02	CG01	CG02	PB01
AR01						
AR04						
AR05						
AR06						
AR07						
AR08						
AR09						
CC01						
CC03						
CC07						
CC08						
CC09						
CC10						
CD05						
CD08						
CD09						
CD11						
CD12						
CD13						
CD14						
KH05						
KH07						
KH08						
KH12						
KH14						
KH18						
MB05						
MB06						
MB07						
MB14						
MB19						
MB25						
MP02						
MP03						
MP05						
MP06						
MP07						
MP08						
MP09						
MP10						
PV01						
PV02						
PV03						
PV04						
PV05						
PV06						
PV09						
PV10						
RU01						
RU02						
RU03						
RU04						
RU05						
RU06						
RU09						
RU10						
SC01						
SC04						
SC05						
SC06						
SC07						
SC14						
SP02						
SP04						
SP05						
SP06						
SP07						
SP08						
SP10	1.00					
PP01	0.68	1.00				
PP02	0.65	0.77	1.00			
CG01	0.62	0.67	0.68	1.00		
CG02	0.59	0.66	0.74	0.73	1.00	
PB01	0.66	0.74	0.70	0.69	0.65	1.00

APPENDIX II. Raw data indicating presence (1) or absence (0) of AFLP fragments. Sizes of the fragments in base pairs are at the top, and individuals down the left side of the page

Primer Combination: E - AC, M - CAA	34	38	40	48	51	56	60	69	72	73	75	81	83	91	95	98	105	107	116	119
AR01	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
AR04	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
AR05	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
AR06	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	1
AR07	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
AR08	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
AR09	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
CC01	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
CC03	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
CC07	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	1	0	0
CC08	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
CC09	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
CC10	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
CD05	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
CD08	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
CD09	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
CD11	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
CD12	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
CD13	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
CD14	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0
KH05	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0
KH07	1	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0
KH08	1	0	1	0	1	0	0	1	0	1	1	1	0	0	1	1	0	1	0	1
KH12	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0
KH14	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0
KH18	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
MB05	1	0	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	1	1	0
MB06	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
MB07	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0
MB14	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0
MB19	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	1
MB25	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0
MP02	1	0	0	0	1	0	0	1	0	1	1	0	1	0	0	0	0	1	0	0
MP03	1	0	1	1	0	0	0	0	0	1	0	0	1	1	0	0	0	1	0	0
MP05	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0
MP06	1	0	0	0	1	0	0	0	0	1	1	0	0	1	0	0	0	1	0	0
MP07	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
MP08	1	0	0	0	1	1	0	0	0	1	0	1	0	1	0	0	0	1	0	0
MP09	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
MP10	1	0	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0
PV01	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
PV02	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0
PV03	1	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	1	0	0
PV04	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0
PV05	1	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	1	0	0
PV06	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
PV09	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
PV10	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
RU01	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
RU02	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0
RU03	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
RU04	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0
RU05	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0
RU06	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
RU09	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
RU10	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
SC01	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
SC04	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
SC05	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
SC06	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1
SC07	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	1
SC14	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	1	0	1
SP02	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
SP04	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
SP05	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
SP06	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
SP07	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
SP08	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1
SP10	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
PP01	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
PP02	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
CG01	1	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	0
CG02	1	0	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	0
PB01	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0

Primer Combination: E - AC, M - CAA

	125	129	143	147	159	163	164	167	172	175	182	190	205	211	217	226
AR01	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
AR04	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1
AR05	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
AP76	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0
AR07	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0
AR08	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
AR09	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
CC01	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
CC03	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
CC07	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CC08	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
CC09	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CC10	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CD05	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CD08	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CD09	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CD11	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
CD12	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CD13	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CD14	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
KH05	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
KH07	1	1	1	0	0	0	1	0	0	1	0	0	0	0	0	1
KH08	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
KH12	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
KH14	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
KH18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MB05	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0
MB06	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
MB07	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
MB14	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
MB19	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
MB25	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
MP02	0	0	0	0	1	0	1	0	0	1	0	1	1	0	0	0
MP03	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
MP05	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
MP06	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
MP07	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
MP08	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1
MP09	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
MP10	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0
PV01	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0
PV02	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
PV03	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1
PV04	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1
PV05	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
PV06	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
PV09	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
PV10	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
RU01	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0
RU02	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
RU03	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
RU04	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
RU05	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0
RU06	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
RU09	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0
RU10	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
SC01	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
SC04	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
SC05	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
SC06	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
SC07	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
SC14	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
SP02	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
SP04	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
SP05	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	0
SP06	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0
SP07	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1
SP08	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1
SP10	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
PP01	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
PP02	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
CG01	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0
CG02	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
P801	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0

Primer Combination: E - AC, M - CAA

	236	248	253	266	274	280	284	287	289	292	320	350	359	368	372	392
AR01	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
AR04	0	0	0	1	1	0	0	1	0	0	1	1	0	0	0	0
AR05	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	1
AR06	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
AR07	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0
AR08	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
AR09	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
CC01	1	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0
CC03	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0
CC07	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
CC08	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
CC09	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0
CC10	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
CD05	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
CD08	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0
CD09	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
CD11	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
CD12	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
CD13	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
CD14	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
KH05	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	1
KH07	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	1
KH08	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	1
KH12	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KH14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KH18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MB05	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0
MB06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MB07	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MB14	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
MB19	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
MB25	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0
MP02	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
MP03	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
MP05	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
MP06	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
MP07	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
MP08	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
MP09	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
MP10	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
PV01	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
PV02	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
PV03	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
PV04	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
PV05	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0
PV06	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
PV09	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
PV10	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
RU01	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
RU02	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
RU03	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
RU04	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
RU05	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
RU06	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
RU09	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
RU10	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
SC01	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1
SC04	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1
SC05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SC06	0	0	1	0	0	0	1	1	0	1	0	1	0	1	1	0
SC07	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
SC14	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
SP02	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
SP04	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
SP05	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0
SP06	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
SP07	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
SP08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP10	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
PP01	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
PP02	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
CG01	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0
CG02	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0
FB01	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0

Primer Combination: E - AC, M - CAA

	394	409	414	430	444	449	457	482	489
AR01	0	0	1	0	1	0	0	1	0
AR04	0	0	1	1	0	0	0	0	0
AR05	1	0	1	1	0	0	0	0	0
AR06	0	0	0	0	1	0	0	0	0
AR07	1	0	1	0	0	0	0	1	0
AR08	0	0	1	0	0	0	0	1	0
AR09	0	0	1	1	0	0	0	1	0
CC01	0	0	1	1	0	1	0	1	0
CC03	0	0	1	1	0	0	0	0	0
CC07	1	0	1	1	0	0	0	1	0
CC08	0	0	1	1	1	1	0	0	0
CC09	0	0	1	1	1	0	0	0	0
CC10	0	0	1	1	0	0	0	1	0
CD05	0	0	1	0	1	0	0	0	0
CD08	0	0	1	0	1	0	0	0	0
CD09	0	0	1	1	1	0	0	0	0
CD11	0	0	1	0	0	0	0	0	0
CD12	0	0	1	0	1	0	0	0	0
CD13	0	0	1	0	1	0	0	0	0
CD14	0	0	1	0	0	0	0	0	0
KH05	0	0	1	1	0	0	0	0	0
KH07	0	0	1	1	0	0	0	0	0
KH08	0	0	1	1	0	0	0	0	0
KH12	0	0	0	1	0	0	0	1	0
KH14	0	0	0	1	0	0	0	0	0
KH18	0	0	0	1	0	0	0	0	0
MB05	1	0	1	1	1	0	0	0	0
MB06	0	0	0	1	0	0	0	0	0
MB07	0	0	0	1	0	0	0	1	0
MB14	0	0	1	1	1	0	0	0	0
MB19	0	0	1	1	0	0	0	0	0
MB25	0	0	1	0	1	0	0	0	0
MP02	0	0	1	1	0	0	1	0	1
MP03	0	0	1	1	1	0	0	1	1
MP05	0	0	1	1	1	0	0	0	1
MP06	0	0	1	1	0	0	0	0	1
MP07	0	0	1	1	0	0	0	1	0
MP08	0	0	1	1	0	0	0	0	0
MP09	0	0	1	1	0	0	0	1	0
MP10	0	0	1	1	0	0	0	0	0
PV01	0	0	1	1	0	0	0	1	0
PV02	0	0	1	1	0	0	0	1	0
PV03	1	0	1	1	0	0	0	1	0
PV04	1	0	1	1	0	0	0	0	0
PV05	0	0	1	1	0	0	0	1	0
PV06	0	0	1	1	0	0	0	1	0
PV09	0	0	1	1	0	0	0	1	0
PV10	0	0	1	1	0	0	0	0	0
RU01	1	0	1	1	0	0	0	0	0
RU02	0	0	1	1	0	0	0	0	0
RU03	0	0	1	1	0	0	0	1	0
RU04	0	0	1	1	0	0	0	1	0
RU05	1	0	1	1	0	0	0	0	0
RU06	1	0	1	1	0	0	0	0	0
RU09	1	0	1	1	0	0	0	1	0
RU10	0	0	1	1	0	0	0	1	0
SC01	0	1	1	1	0	0	0	0	0
SC04	0	0	1	1	1	0	0	0	0
SC05	0	0	1	0	1	0	0	0	0
SC06	0	1	1	1	1	0	0	0	0
SC07	0	0	1	0	1	0	0	0	0
SC14	0	0	0	1	0	0	0	0	0
SP02	0	0	1	1	1	0	0	0	0
SP04	0	0	1	1	0	0	0	0	0
SP05	1	0	1	0	0	0	0	0	0
SP06	1	0	1	0	0	0	0	0	0
SP07	1	0	1	1	0	0	0	0	0
SP08	0	0	1	1	0	0	0	0	0
SP10	0	0	1	0	0	0	0	0	0
PP01	1	0	1	1	0	0	0	1	0
PP02	0	0	1	1	0	0	0	0	0
CG01	0	0	1	1	0	0	0	0	0
CG02	0	0	1	1	0	0	0	0	0
PB01	0	0	1	1	0	0	0	1	0

Primer Combination: E - AC, M - CTT

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	413
AR01	0
AR04	0
AR05	0
AR06	0
AR07	0
AR08	0
AR09	0
CC01	0
CC03	0
CC07	0
CC08	0
CC09	1
CC10	0
CD05	0
CD08	0
CD09	0
CD11	0
CD12	0
CD13	0
CD14	0
KH05	0
KH07	0
KH08	0
KH12	0
KH14	0
KH18	0
MB05	0
MB06	0
MB07	0
MB14	0
MB19	0
MB25	0
MP02	0
MP03	0
MP05	0
MP06	0
MP07	0
MP08	0
MP09	0
MP10	0
PV01	0
PV02	0
PV03	0
PV04	0
PV05	0
PV06	0
PV09	0
PV10	0
RU01	0
RU02	0
RU03	0
RU04	0
RU05	0
RU06	0
RU09	0
RU10	0
SC01	0
SC04	0
SC05	0
SC06	0
SC07	0
SC14	0
SP02	0
SP04	0
SP05	0
SP06	0
SP07	0
SP08	0
SP10	0
PP01	0
PP02	0
CG01	1
CG02	1
PS01	1

Primer Combination: E - AG, M - CAC

	331	334	344	387	392	434	440
AR01	0	0	0	0	0	0	0
AR04	0	0	0	0	0	0	0
AR05	0	0	0	0	0	0	0
AR06	1	0	0	0	0	0	0
AR07	0	0	0	0	0	0	0
AR08	0	0	0	0	0	0	0
AR09	0	0	0	0	0	0	0
CC01	1	0	0	0	0	0	0
CC03	1	0	0	0	0	0	0
CC07	0	0	0	0	0	0	0
CC08	1	0	0	0	0	0	0
CC09	1	0	0	0	0	0	0
CC10	0	0	0	0	0	0	0
CD05	0	0	0	0	0	0	0
CD08	0	0	0	0	0	0	0
CD09	0	0	0	0	0	0	0
CD11	0	0	0	0	0	0	0
CD12	0	0	0	0	0	0	0
CD13	0	0	0	0	0	0	0
CD14	0	0	0	0	0	0	0
KH05	1	0	0	0	0	0	0
KH07	1	0	0	1	0	0	0
KH08	1	0	1	1	0	0	0
KH12	0	0	0	0	0	0	0
KH14	0	0	0	0	0	1	0
KH18	1	0	1	0	1	1	0
MB05	1	0	0	0	0	0	0
MB06	0	0	0	0	0	0	0
MB07	1	0	0	0	0	1	0
MB14	1	0	0	0	0	0	0
MB19	1	0	0	1	0	0	0
MB25	1	0	0	0	0	0	0
MP02	0	0	0	0	0	0	0
MP03	0	0	0	0	0	0	0
MP05	0	0	0	0	0	0	0
MP06	0	0	0	0	0	0	0
MP07	1	0	0	0	0	0	0
MP08	0	0	0	0	0	0	0
MP09	0	0	0	0	0	0	0
MP10	0	0	0	0	0	0	0
PV01	0	0	0	0	0	0	0
PV02	0	0	0	0	0	0	0
PV03	0	0	0	0	0	0	0
PV04	0	0	0	0	0	0	0
PV05	0	0	0	0	0	0	0
PV06	0	0	0	0	0	0	0
PV09	0	0	0	0	0	0	0
PV10	0	0	0	0	0	0	0
RU01	1	0	0	0	0	0	0
RU02	1	0	0	0	0	0	0
RU03	1	0	0	0	0	0	0
RU04	1	0	1	0	0	0	0
RU05	0	0	1	0	0	0	0
RU06	0	0	0	0	0	0	0
RU09	1	0	1	0	0	0	0
RU10	1	0	0	0	0	0	0
SC01	1	0	0	0	0	0	0
SC04	1	0	0	0	0	1	1
SC05	0	0	0	0	0	0	0
SC06	1	0	0	0	0	0	0
SC07	1	0	0	0	0	0	0
SC14	0	0	0	0	0	0	0
SP02	1	1	0	0	0	0	0
SP04	1	1	0	0	0	0	0
SP05	1	0	0	0	0	1	0
SP06	1	0	0	0	0	1	0
SP07	1	0	0	0	0	0	0
SP08	1	0	0	0	0	0	0
SP10	1	0	0	0	0	0	0
PP01	1	0	0	0	0	1	0
PP02	0	0	0	0	0	1	0
CG01	1	0	0	0	0	0	0
CG02	0	0	0	0	0	1	0
PB01	1	0	0	0	0	0	0

Primer Combination: E - AG, M - CAT

	460
AR01	0
AR04	0
AR05	0
AR06	0
AR07	0
AR08	0
AR09	0
CC01	0
CC03	0
CC07	0
CC08	0
CC09	0
CC10	0
CD05	0
CD06	0
CD09	0
CD11	0
CD12	0
CD13	0
CD14	0
KH05	0
KH07	0
KH08	0
KH12	0
KH14	0
KH18	0
MB05	0
MB06	0
MB07	0
MB14	0
MB19	0
MB25	0
MP02	0
MP03	0
MP05	0
MP06	0
MP07	1
MP08	0
MP09	0
MP10	0
PV01	0
PV02	0
PV03	0
PV04	0
PV05	0
PV06	0
PV09	0
PV10	0
RU01	0
RU02	0
RU03	0
RU04	0
RU05	0
RU06	0
RU09	0
RU10	0
SC01	0
SC04	0
SC05	0
SC06	1
SC07	0
SC14	0
SP02	0
SP04	0
SP05	0
SP06	0
SP07	0
SP08	0
SP10	0
PP01	0
PP02	0
CG01	0
CG02	0
PB01	0