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REVEALING GENETIC MARKERS IN *GELIDIUM VAGUM* (RHODOPHYTA) THROUGH THE RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) TECHNIQUE[1]

Mohsin U. Patwary,[2] *Ron M. MacKay, and John P. van der Meer*

Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia, Canada B3H 3Z1

ABSTRACT

The recently developed random amplified polymorphic DNA technique was evaluated as a method for characterizing isolates of the agarophyte Gelidium vagum *Okamura. Reaction conditions for single primer polymerase chain reaction were optimized to obtain a high degree of reproducibility of the amplified bands generated from purified* G. vagum *DNA. A total of 165 primers, including both (A + T)- and (G + C)-rich sequences, was screened for DNA amplification using template DNA from a single* Gelidium *isolate. None of the 45 (A + T)-rich primers was positive (i.e. band-producing). Of the (G + C)-rich primers, 47 were positive, generating a total of 322 prominent amplification products for DNA from 13 different* G. vagum *isolates. Polymorphic DNA loci were detected by 37 of the primers. Unweighted pair-group arithmetic average cluster analysis (UPGMA) of these loci was used to group the* G. vagum *isolates and thereby determine which were most similar.* G. latifolium, *used as an outgroup for the UPGMA analysis, showed a high degree of dissimilarity.*

Key index words: amplification fragment length polymorphism; DNA amplification fingerprinting; Gelidium vagum; *genetic marker; polymerase chain reaction (PCR); random amplified polymorphic DNA (RAPD); Rhodophyta*

Many marine red algae have similar haploid and diploid morphologies with both phases growing interspersed in the same habitat. There exists the possibility that the two phases compete with each other for resources. In such competitions, the diploid potentially would gain an advantage over the haploid through heterosis, i.e. from genetic heterozygosity that allows its gene products to produce a more "flexible" physiological response to environmental challenges. Although this suggestion is plausible, the existence of hybrid vigor is only presumed, based on analogy with other organisms. It has never been directly documented for a red alga. An attempt to demonstrate heterosis in diploid sporophytes of *Gracilaria tikvahiae* (Patwary and van der Meer 1983) was completely negative, suggesting the possibility that natural selection acting on the haploid gametophytes removes unfavored alleles that could otherwise lead to heterosis in heterozygous plants. The scope of the experiment, however, was much too limited to provide significant support for this suggestion.

Gelidium vagum, a monoecious red alga with isomorphic haploid and diploid thalli, provides an interesting opportunity to examine further the heterosis issue. This would be of interest, not only in

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[2] Address for reprint requests.

connection with intraspecific competition between phases, but also with respect to using hybrid vigor as a breeding strategy for obtaining more productive algal strains for aquaculture. Because *G. vagum* gametophytes are monoploid as well as monoecious, self-fertilization yields completely homozygous sporophytes, which could be compared with hybrid plants from crosses to measure the amount of heterosis generated by the heterozygous condition. One can eliminate the time-consuming rounds of sibling crosses needed to establish inbred (i.e. nearly homozygous) lines when dealing with dioecious species. Unfortunately, there is a small snag that needs to be overcome when using *G. vagum* in this fashion. Although the formation of homozygotes is a trivial exercise, self-fertilization cannot be prevented during crossing, and the identification of hybrid plants within mixed progenies depends on the use of genetic markers (van der Meer 1987). For studies on heterosis, any markers used to select the hybrids must be physiologically neutral so as not to compromise the outcome of the growth experiments.

Mutant phenotypes, isozyme polymorphisms, and DNA sequence variations are all commonly used as genetic markers. Each has its strengths and weaknesses in specific applications. Induced mutations have made excellent markers in some phycological applications (Patwary and van der Meer 1982, van der Meer 1987, van der Meer and Patwary 1991), although they require considerable time and effort to characterize. The nature of the observable mutation itself (loss of chlorophyll, excessive branching, etc.) or the possible background presence of undetected secondary mutations generated by the mutagenesis process severely limit the usefulness of visible mutants for heterosis experiments. Isozyme polymorphisms are potentially useful and relatively straightforward to detect; however, as genetic markers, they are generally limited to a small number of loci, many of which may be invariant within a particular population (Murphy et al. 1990).

With the advent of new molecular biological techniques, there has been an increasing emphasis on the use of DNA characteristics as genetic markers. Naturally occurring variations in DNA can be detected in a number of ways. The use of restriction fragment length polymorphisms (RFLPs) is well established, and DNA-fingerprinting techniques, using either general or specific probes to detect hypervariable loci, are also widely applied (Nybom 1991). Unfortunately, these techniques are expensive and time-consuming and generally require substantial amounts of DNA for the analysis (Olsen 1990, Baird et al. 1992, Horn and Rafalski 1992). Such techniques could be used to identify DNA markers in a small number of algal strains, but they would be demanding as a screening method for the examination of a large number of plants (e.g. for detecting hybrid plants in a mixed population of *G. vagum* sporelings).

Recently, a new technique based on the polymerase chain reaction (PCR) (Saiki et al. 1988, Erlich et al. 1991) was reported almost simultaneously by three groups [Williams et al. 1990, as random amplified polymorphic DNA (RAPD); Welsh and McClelland 1990, as arbitrary primed PCR; and Caetano-Anollés et al. 1991a, as DNA amplification fingerprinting]. With this technique, single, short, oligonucleotide primers of arbitrary sequence are used to amplify anonymous regions of genomic DNA, after which the amplification products are resolved by gel electrophoresis. Although binding of the primer and initiation of DNA synthesis may occur in many places throughout the genome, amplification will occur only for regions of DNA that are appropriately flanked by sequences complementary to the primer, i.e. when the flanking sequences are on opposite strands and reasonably near one another (see Bassam et al. 1991, Caetano-Anollés et al. 1991b). Caetano-Anollés et al. (1991b) suggested that a genetic marker revealed by this technique be referred to as an amplification fragment length polymorphism (AFLP).

Compared to other DNA techniques, detection and use of AFLP markers is relatively fast, easy, and inexpensive. Being a PCR technique, only a very small amount of target DNA is needed in each reaction, many reactions can be amplified simultaneously in commercially available thermal cyclers, and the reaction products can be resolved and documented quickly using gel electrophoresis and photography. AFLPs have already been used, *inter alia*, for mapping higher plant genomes (Weeden 1990, Williams et al. 1990, Halward et al. 1992), identifying strains of bacteria (Welsh and McClelland 1990, 1991) and fungal pathogens of plants (Goodwin and Annis 1991), identifying inter- and intraspecific somatic hybrids in tomato (Baird et al. 1992), genetic fingerprinting of cocoa clones (Wilde et al. 1992), nondestructive genetic diagnosis of microspore-derived *Brassica* embryos (Horn and Rafalski 1992), and finding markers linked to a *Pseudomonas*-resistance gene in tomato (Martin et al. 1991). Genetic segregation of AFLP markers has been examined in diploid alfalfa (Echt et al. 1992). Considering its strengths and past successful applications, the RAPD technique appeared to offer a solution for obtaining the "neutral"markers needed to characterize genetically parental isolates and identify hybrids of *G. vagum* in preparation for the proposed heterosis experiments.

We examined the applicability of the RAPD technique for *G. vagum*. First, a series of RAPD reactions, using a variety of primers, were conducted to establish the characteristics of primers that initiated successful amplification and to determine whether or not any of these identified polymorphic DNA loci in *G. vagum*. Next, the limited number of *G. vagum* isolates in our culture collection, all from a few small populations on the west coast of Canada, were ex-

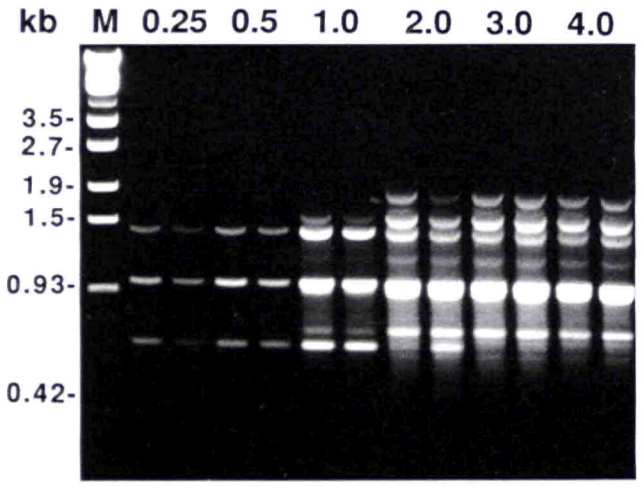

FIG. 1. The effect of *Taq* polymerase concentration on RAPD amplification of *G. vagum*. Amplification reactions included 25 ng of DNA from isolate No. 12 and primer No. 100 (ATCGGGTCCG) at 0.2 μM. Products from duplicate reactions were resolved by electrophoresis through 1.4% agarose gels and stained with ethidium bromide. The numerals above the lanes indicate the units of *Taq* polymerase present in the respective reactions. The lane marked M is restriction enzyme *Sty*I-digested bacteriophage λ DNA. The lane marked kb indicates the sizes (in kilobase pairs) of the λ DNA fragments.

amined extensively to assess whether or not they encompassed sufficient genetic variability to act as starting plants for a heterosis study and, if so, to identify the most divergent lines available for crossing.

MATERIALS AND METHODS

Independent isolates of *Gelidium vagum* Okamura were obtained from populations that are limited to Denman and Hornby islands in the Strait of Georgia, British Columbia, Canada (Renfrew et al. 1989). Thalli were collected from various sites; 5-mm tips were thoroughly cleaned and treated repeatedly in 0.25% Javex-12 (Colgate-Palmolive Canada Inc., Toronto, Ontario) in distilled water, often followed by antibiotic treatment, until plants were found to be free of contaminating organisms under a stereomicroscope. The tips were grown in unbuffered sterile seawater media (van der Meer and Patwary 1991) in the laboratory. A total of 13 independent homozygous tetrasporophytic lines of *G. vagum* (obtained by selfing individual haploid gametophytes) and a clone of *G. latifolium* (collected from northern Spain) were used in this study.

DNA was extracted from 5 g laboratory-grown fresh samples by a method similar to Doyle and Doyle (1990). The sample was ground to a powder in liquid nitrogen and immediately mixed with 25–30 mL 100 mM Tris-HCl, pH 8, 20 mM EDTA, 1.4 M NaCl, 3% cetyl trimethyl ammonium bromide, and 0.2% (v/v) β-mercaptoethanol. The mixture was frozen at −70° C for 30 min, thawed, and incubated at 60° C for 30 min with occasional gentle swirling. The sample was extracted with an equal volume of chloroform–isoamyl alcohol (24:1) for 10 min and centrifuged. The nucleic acids in the aqueous phase were precipitated with ⅔ volume isopropanol, pelleted by centrifugation, washed in 70% ethyl alcohol, vacuum-dried, and redissolved in 10 mM Tris-HCl pH 8 and 1 mM EDTA. The sample was incubated at 37° C for 10–15 min and then centrifuged. The solution, containing mostly nucleic acids and some low molecular weight polysaccharides, was removed from the tube by pipette, leaving behind a pellet composed largely of polysaccharides with some trapped nucleic acids. The DNA was further purified by CsCl gradient centrifugation (Rice and Bird 1990).

Oligodeoxyribonucleotides of 9 and 10 bases were used to prime PCR reactions. Sixty 9-base primers (0970-01 to 0970-30 and 0955-01 to 0955-30) were obtained from Dr. Roland Brousseau, Biotechnology Research Institute, National Research Council of Canada. Four 10-base primers (CTGATGCTAC, GCAAGTAGCT, TGGTCACTGA, ACGGTACACT) were synthesized on a Milligen Biosearch DNA synthesizer, and another 101 primers (1-100 and Ap-4) were obtained from Dr. John E. Carlson, Biotechnology Laboratory, University of British Columbia, Canada.

Typical amplifications were performed in 25-μL volumes containing *Taq* DNA polymerase buffer (BioCan), 100 μM each deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and deoxyribosyl thymine triphosphate (Pharmacia), 0.2 μM primer, 25 ng genomic DNA, and 1.0 unit *Taq* DNA polymerase (BioCan). The amplifications were performed in an Ericomp Twinblock temperature cycler programmed for 40 cycles of 1 min at 94° C, 1 min at 30° C (when screening primers), or 36° C (in all other experiments), and 2 min at 72° C. Reaction products were separated by electrophoresis through 1.4% agarose gels and stained with ethidium bromide (Maniatis et al. 1982).

A data matrix was prepared based on the presence or absence of individual prominent bands generated by each primer, and a similarity matrix for all pairwise comparisons was calculated (Table 2) using the widely adopted Jaccard's coefficient, $Jij = Cij/(ni + nj - Cij)$, where Cij is the number of common bands for a pair of isolates, and ni and nj are the numbers of bands in i and j members of the pair, respectively (Sneath and Sokal 1973). The coefficient values of 1 and 0 indicate an identical match and complete dissimilarity, respectively. A dendrogram was constructed based on the similarity matrix data by applying UPGMA cluster analysis (Nei 1987) using the BMDP program (Engelman et al. 1979).

RESULTS

The first experiments were conducted to optimize PCR conditions for our preparations of *Gelidium vagum* DNA. Magnesium concentrations below 1.5 and above 3.0 mM, primer concentrations below 0.1 μM, and individual dNTP concentrations below 50 μM failed to yield sufficient amplification. Fewer than 30 thermal cycles provided insufficient amplification, while more than 40 cycles did not significantly increase yield.

Amplification of target DNA loci occurred at all tested *Taq* DNA polymerase concentrations (Fig. 1). The yield and complexity of amplified products were relatively low at 0.25 and 0.5 units of polymerase per 25-μL reaction. As the quantity of enzyme was increased to 4.0 units per reaction, many additional bands, both prominent and faint, became evident. This result suggests a competition between loci for a limiting amount of enzyme. Presumably, some loci are bounded by one or two sites that are less perfectly complementary to the primer than are other sites. Loci bounded by these former sites are at a disadvantage when polymerase is limiting and are efficiently amplified only with an excess of enzyme.

If secondary bands are produced under a condition of enzyme excess, then the addition of sufficient DNA should overcome this condition. When 1.0 unit of polymerase and varying quantities of DNA were used in standard amplifications (Fig. 2), the

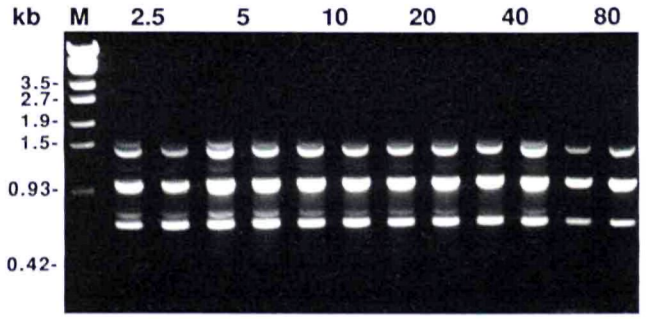

FIG. 2. The effect of DNA concentration on RAPD amplification of *G. vagum* DNA. Duplicate amplification reactions included 1.0 unit of *Taq* polymerase. The numerals above the lanes indicate the nanograms of DNA present in the respective reactions. Other methods and notations are explained in the legend to Figure 1.

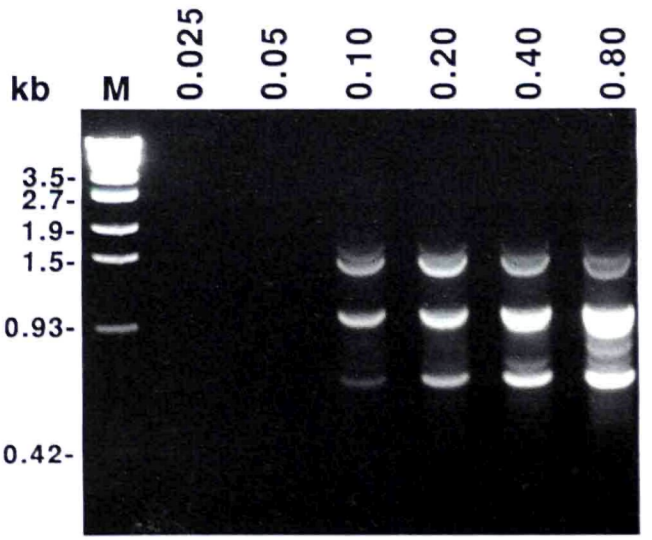

FIG. 3. The effect of primer concentration on RAPD amplification of *G. vagum* DNA. The numerals above the lanes indicate the primer concentration (μM) in the amplification reactions. Other methods and notations are described in the legend to Figure 1.

secondary band formation observed with 2.5–40 ng of DNA was suppressed with 80 ng. Primer concentrations above 0.20 μM enhanced the production of some bands (Fig. 3) but did not improve the discrimination of major bands from the background signal.

A total of 165 different arbitrary primers was screened to identify positive (band-producing) primers with the DNA from a selected isolate of *G. vagum* (Table 1). None of the predominantly (A + T)-rich primers produced any amplified products from *G. vagum* DNA. In contrast, the (G + C)-rich (≥50%) primers were generally positive, each yielding from 1 to 12 bands (see examples in Fig. 4). There was a positive correlation between the (G + C) content of a primer and the likelihood that the primer would be positive for band generation (Table 1).

The 57 positive primers identified during the first screening were used to compare the banding patterns of 13 *G. vagum* isolates and to further compare these with results for *G. latifolium*. Ten of the primers gave poor band resolution; thus, results for these primers were not included in the analysis. Of the remaining 47 primers, 37 yielded polymorphisms among the *G. vagum* isolates (322 loci were revealed, of which 117 were polymorphic; see examples in Fig. 4), while the others yielded monomorphic banding patterns. Banding patterns obtained for *G. vagum* and *G. latifolium* were different for nearly every primer tested. These results (Table 2) are summarized in a cluster analysis dendrogram (Fig. 5). In this analysis, the distance between *G. vagum* isolates varied from 0.10 to 0.31. Isolates sharing greatest similarity grouped into clusters, (5, 13, 12, 7, 128, 8, 16, 127) and (131, 132), while others remained as individual lineages (4, 130, and 129). As expected from the raw data, *G. latifolium* was only very distantly connected to the *G. vagum* isolates.

DISCUSSION

When appropriate precautions were taken in conducting the RAPD procedure, the major banding pattern for a particular combination of primer and homozygous *G. vagum* DNA was remarkably reproducible, for replicates both in an experiment and between experiments. Although none of the tested (A + T)-rich primers yielded any amplification products, suggesting that these are inappropriate choices for use with *G. vagum* DNA, analysis of *G. vagum* isolates using (G + C)-rich primers yielded a large number of prominent, often polymorphic bands. Clearly, the RAPD technique yields reproducible banding patterns for *G. vagum* DNA. Moreover, the high frequency of polymorphic bands suggests that the isolates of *G. vagum* represented in our collection have sufficient genetic diversity for conducting a valid heterosis experiment.

By comparing many polymorphic loci through cluster analysis, we could not only discern the general presence of genetic diversity among the isolates but also identify specifically which plants were most or least similar. The dendrogram summarizing the UPGMA analysis identifies isolates 4, 129, and 130 as the genetically most divergent *G. vagum* isolates in relation to isolates in the core cluster. These results are a useful guide for the selection of divergent lineages needed to generate highly heterozygous plants in crosses used to search for heterosis.

TABLE 1. *Screening primers for AFLP markers in* G. vagum.

Primer size	Number of G or C	Number of primers	Positive primers	% Positive
9	1–4	45	0	0
9	5–6	15	13	87
10	5	7	3	45
10	6	35	6	17
10	7	35	15	43
10	8	27	19	70
10	9	1	1	100

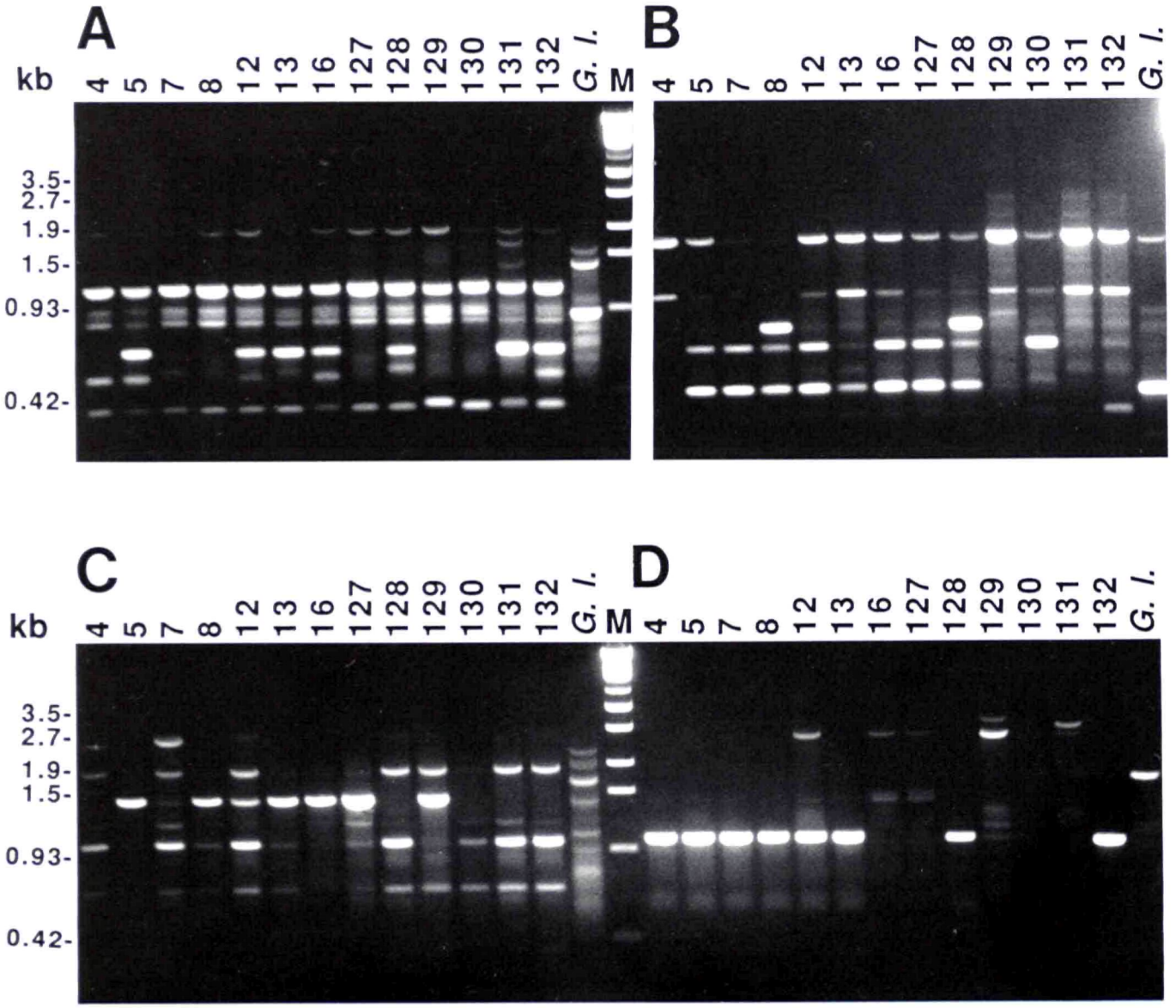

FIG. 4. RAPD amplification of *G. vagum* DNAs. The numerals above the lanes identify the *G. vagum* isolates used in this study. The lanes marked *G. l.* contain the products of amplification with *G. latifolium* DNA. Reactions were performed with either primer A) No. 17 (CCTGGGCCTC), B) No. 30 (CCGGCCTTAG), C) No. 50 (TTCCCCGCGC), or D) No. 61 (TTCCCCGACC). Other methods and notations are explained in the legend to Figure 1.

TABLE 2. *The similarity matrix based on Jaccard's equation. The total number of amplified products revealed in each isolate is shown in the diagonal.*

Isolates	4	5	7	8	12	13	16	127	128	129	130	131	132	*G. latif.*
4	172													
5	0.74	187												
7	0.78	0.81	189											
8	0.76	0.83	0.90	187										
12	0.78	0.80	0.83	0.81	174									
13	0.75	0.83	0.80	0.82	0.81	178								
16	0.75	0.88	0.86	0.87	0.82	0.83	186							
127	0.70	0.80	0.87	0.88	0.79	0.80	0.89	180						
128	0.75	0.80	0.91	0.88	0.83	0.80	0.85	0.84	184					
129	0.72	0.66	0.71	0.70	0.70	0.71	0.68	0.68	0.69	170				
130	0.72	0.72	0.78	0.75	0.74	0.68	0.73	0.72	0.76	0.59	156			
131	0.70	0.67	0.71	0.69	0.73	0.72	0.71	0.68	0.71	0.68	0.66	172		
132	0.79	0.74	0.78	0.77	0.78	0.78	0.75	0.71	0.77	0.72	0.70	0.81	174	
G. latif.	0.14	0.14	0.15	0.14	0.13	0.15	0.14	0.15	0.14	0.15	0.13	0.13	0.15	124

The results also suggest that the RAPD method may be useful in discriminating algal species as well as in determining relationships within species. Very few similarities between the banding patterns of *G. vagum* and *G. latifolium* were observed for any of the primers. Some primers yielded simple monomorphic patterns for all of the *G. vagum* isolates, which were different than the corresponding *G. latifolium* patterns. Thus, it is possible that specific subsets of primers could be identified that would unequivocally identify various *Gelidium* species. Such analyses might also be useful for discriminating between other difficult taxa in the Rhodophyta generally.

The large number of differences among isolates revealed by the RAPD technique indicates that it would be possible to establish a unique "fingerprint" for individual plants based on the combined results generated from a small collection of primers. Such a fingerprint might be desirable as the basis for protecting intellectual property rights associated with a specific strain of alga used in aquaculture. Although this is not currently an important issue, it might become increasingly important in the future as genetically improved algae are introduced into cultivation, and methods of protecting against strain theft need to be developed.

We have found that the RAPD technique requires careful attention to detail in execution and cautious interpretation until sufficient replication has been performed. The plant material should be clean and as free as possible of contaminating organisms. Extreme care must be taken during the preparation of reagents and amplification reaction mixtures to prevent contamination or DNA carryover from one vessel to another so as to reduce the possibility of false positives or false negatives (Kwok 1989). Variation in efficiency of amplification among heating block wells in the temperature cycler can lead to variable results (Linz 1990). We found it necessary to determine, in preliminary experiments, which of the wells in our temperature cycler provided reproducible results, between wells in an experiment and from experiment to experiment for each well (improvements in newer models of temperature cyclers are reducing this problem). Optimization of the reaction conditions and purification of the DNA template by CsCl gradient centrifugation also reduced variability, but the latter may not always be possible in practice.

Minor bands produced by the RAPD analysis (Fig. 4) were much more variable in expression and thus harder to score. It is probably better to ignore such weak bands and use only the most prominent ones as genetic markers or as data for calculation of similarity coefficients. Since some of the minor bands may be products of nonspecific priming, the number of such bands may be reduced by using more stringent reaction conditions and/or through the use of formamide (Sarkar et al. 1990) or tetramethylammonium chloride (Hung et al. 1990). Minor bands can also be reduced somewhat by maintaining an appropriate ratio of template DNA to *Taq* polymerase, so that the enzyme is present in limiting amounts and thus less likely to prime loci that are not perfectly complementary to the primer.

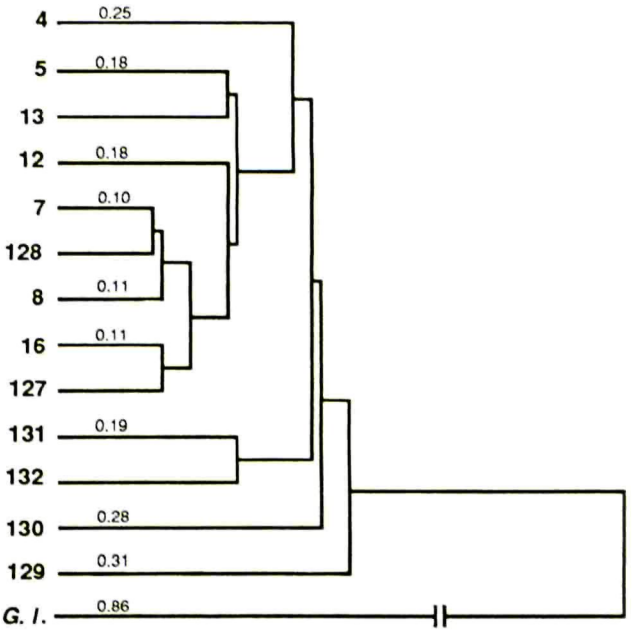

FIG. 5. Clustering *G. vagum* isolates and *G. latifolium* (G. *l.*) by UPGMA analysis of similarity matrix data. *G. vagum* isolates fell in closely related subclusters (5, 13, 12, 7, 128, 8, 16, 127) and (131, 132), and individual lineages (4, 130, and 129). Value above the lines are distances between isolates or groups of isolates. Note that *G. latifolium* is only distantly connected (0.86) to the *G. vagum* isolates.

Although the results we obtained in this study demonstrate that the RAPD methodology is very powerful for the discrimination of *G. vagum* isolates, the more general utility of these DNA polymorphisms as reliable genetic markers remains to be determined in crosses. We have already noticed that gametophytes and sporophytes derived by selfing these gametophytes do not always yield identical banding patterns (data not shown, experiments in progress). We do not yet know what this means. Further experiments are needed to determine whether such differences derive from an artifact in the methodology, contamination in the DNA sampies, or some underlying biological phenomenon related to differences (e.g. methylation) in the DNA of gametophytes and sporophytes. We mention it at this time only as a caution to other phycologists who may be interested in using the RAPD technique in their studies. This research is among the first to test the application of RAPD in phycology, and there is still much to learn.

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