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DNA Polymorphism of *Porphyra yezoensis* and *P. tenera*, and Its Application to Cultivar Discrimination

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Abstract The cultivar discrimination of the laver (*Porphyra yezoensis* and *P. tenera*) was examined with DNA fingerprinting and random amplified polymorphic DNA (RAPD) analysis using purified genomic DNA. DNA fingerprinting was carried out with three oligonucleotides, $(CAC)_6$, $(GT)_8$ and $(GGAT)_4$, as probes. When genomic DNA from six cultivars were digested with *Hae* III or *Afa* I, and hybridized with oligonucleotide probes, hybridization patterns appeared to be specific for individual cultivars. In addition, these fingerprints seemed to be similar during several years. RAPD analysis of laver genomic DNA was then attempted. A total of 91 primers were used for the preliminary screening of RAPD primers, 18 of which were found to generate polymorphic PCR products (RAPD markers) among six cultivars. Eight of the 18 produced RAPD markers that were specific for a single cultivar and the other ten primers produced RAPD markers that were shared by more than two cultivars. All six cultivars could be distinguished by using combinations of these RAPD markers. RAPD banding patterns were similar among three cultivars that were cultured and harvested in different years. These results indicate that cultivars of laver can be distinguished from each other on the basis of the polymorphic patterns of DNA fingerprinting and RAPD analysis.

Key words: DNA fingerprinting, repetitive DNA, cultivated laver, cultivar identification.

The red marine algae *Porphyra yezoensis* and *P. tenera*, usually called "laver (nori in Japanese)", are commercially important marine products. Laver is extensively cultured along coastal regions of Japan, and recently, laver production has been introduced to several countries in Eastern Asia. The value of cultivated laver is more than one thousand million dollars per year in Japan and, therefore, effort is being made to improve cultured stocks and culture techniques for laver.

Many cultivars or cultivated species are listed for the cultured stocks of these laver. However, since the morphological characteristics of laver are affected by growing conditions such as temperature, salinity, nutrient salt levels, ebb and flow and tidal current, it is difficult to identify and distinguish cultivars of laver on the basis of morphological characteristics only. Therefore, biochemical means are needed for the correct identification of the cultivars. Molecular markers are expected to provide the best means for the identification of laver cultivars.

Since the discovery of human minisatellite DNA (Jeffereys *et al.*, 1985a), repetitive DNA sequences have been used as DNA molecular markers to produce DNA fingerprints in a wide variety of organisms (Jeffereys *et al.*, 1985b; Jeffereys and Morton, 1987; Hillel *et al.*, 1989; Wirgin *et al.*, 1991; Thomas *et al.*, 1993). One class of DNA sequences is known as microsatellite or simple repetitive sequences (Litt and Luty, 1989; Tautz, 1989), which consist of a small repeat units generally less than four nucleotides long. A microsatellite is characterized by a high degree of polymorphism, most of which results from a different number of basic repeat units. These DNA sequences are, therefore, useful for

DNA fingerprinting to distinguish cultivars of many species.

On the other hand, Williams *et al.* (1990) and Welsh and McClelland (1990) reported a technique based on the amplification of random DNA sequences by polymerase chain reaction (PCR) with arbitrary primers. The amplified products, RAPD (random amplified polymorphic DNA) markers, segregate in Mendelian fashion (Williams *et al.*, 1990). Therefore, RAPD PCR is used as a tool in such tasks as gene mapping, population analysis, phylogenetic studies and strain identification.

The present paper describes the application of DNA fingerprinting and RAPD markers to the identification of laver cultivars.

Materials and Methods

Algae

The following cultivars were used, *Porphyra yezoensis* cultivars Noma, Saga-5, Narawa-susabimidorime (mutant strain with green color), Fukuoka-1, and *P. tenera* cultivars Ariake-1 and Sashiki. These algae were cultured using "nori-nets" at sea farms in Ariake Bay, Japan. When they were less than 6 cm in length, they were sun dried for about 3 h after being harvested and then stored at −20℃. Laboratory-cultured algae were not used in the present study since such algae were remarkably less efficient than those cultured at sea farms in the production of protoplasts from which nuclear DNA could be extracted (Mizukami *et al.*, 1992). In addition, procedures for DNA extraction directly from whole algae have not been successful due to the large contamination of soluble wall polysaccharides (Mejjad *et al.*, 1994).

DNA extraction

Thallus samples (2–3 g of wet weight), consisting of approximately 450 to 700 thalli shorter than 6 cm in length, were treated with 2% papain (from Carica papaya, product of Merck) in sea water for 40 min, washed several times with sea water and digested with cell wall-lytic enzyme solution consisting of abalone acetone powder and "bacterial crude enzyme", as described previously (Mizukami *et al.*, 1992; Mizukami *et al.*, 1993). Total cells which consisted of protoplasts, cell aggregates or small cell clusters were washed three times by centrifugation at 800 $\times g$ for 10 min in 0.8 M mannitol in 10 mM Tris-HCl (pH 7.5). The resultant pellet was suspended in the lysis buffer (0.4 M sucrose, 0.5% Triton X-100, 80 mM KCl, 50 mM Tris-HCl, pH 7.5), kept on ice for 10 min and filtered through a 40 μm-mesh nylon gauze. The filtered cell lysate was centrifuged at 8,000 $\times g$ for 20 min, and the resultant nuclear-rich pellet was then suspended in the extraction buffer (0.1M NaCl, 5 mM EDTA, 20 mM Tris-HCl, pH 7.5), and 10% sarkosyl was added to a final concentration of 1.0%. After 5 min at room temperature, this solution was extracted twice with phenol/chloroform. The aqueous layer was dialyzed overnight against 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, and then treated with proteinase K and RNase as described previously (Mizukami *et al.*, 1983). The DNA in the extraction solution was precipitated in 2.5 volumes of cold ethanol, washed with 80% ethanol, vacuum-dried and dissolved in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA.

Southern blot analysis

DNA samples were loaded onto 1.2% agarose gels and electrophoresed at 1V/cm for 36 to 40 h with recirculating running buffer. Lambda DNA size markers were co-electrophoresed on the side of the DNA samples in identical gels. After electrophoresis, the gel was depurinated in 250 mM HCl for 15 min, denatured in 0.5 M NaOH/1.5 M NaCl for 20 min and neutralized in 0.5 M Tris-HCl (pH 7.5)/1.5 M NaCl for 20 min. The DNA in the gel was transferred to a nylon membrane (Amersham Co., Amersham, UK) and fixed to a membrane by UV cross-linking. The membrane was hybridized with synthetic oligonucleotide probes which were obtained from Kurabo Co. (Osaka, Japan) and labeled with fluorescein-11-dUTP using Amersham ECL-labeling system (Amersham). After hybridization, membranes were washed twice in 5× SSC−0.1% SDS for 5 min, and twice in 4× SSC−0.1% SDS for 5 min at room temperature. Membranes were then incubated in the solution consisted of 0.75 M NaCl, 0.1 M Tris-HCl (pH 7.5), 0.5% (W/V) bovine serum albumin and anti-fluorescein antibody conjugate

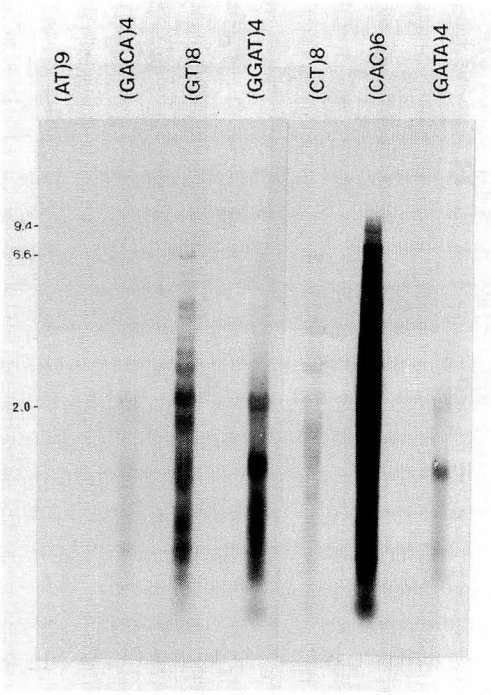

Fig. 1. Hybridization patterns of laver genomic DNA with synthetic oligonucleotide probes. *Hinf* I -digested genomic DNA of Saga -5 were hybridized with the oligonucleotide probes indicated above each lane. Size markers on the left are given in kilobases.

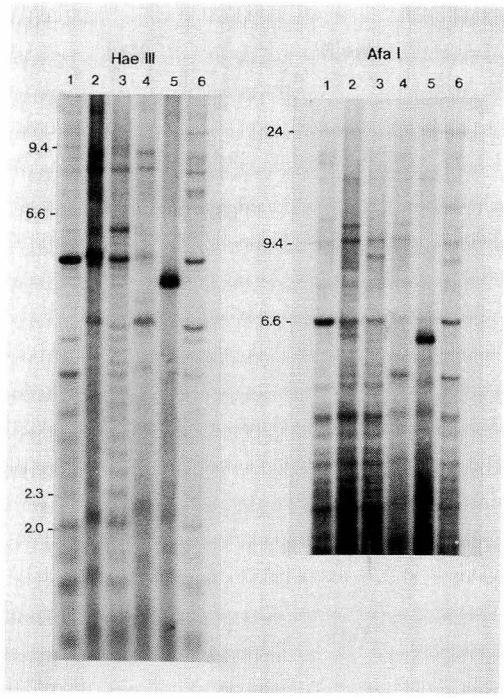

Fig. 2. Hybridization patterns detected by oligonucleotide probe $(CAC)_6$ in *Hae*III and in *Afa*I digests of genomic DNA from six cultivars. Lane 1, cultivar Saga-5; lane 2, Ariake-1; lane 3, Noma; lane 4, Fukuoka-1; lane 5, Narawasusabimidorime; lane 6, Sashiki.

(Amersham) for 30 min at room temperature, washed three times with 0.75 M NaCl-0.1 M Tris-HCl and exposed to X-ray film.

DNA amplification

Ten and twelve-base oligonucleotide primers purchased from Operon Technologies (Alameda, California) and Wako Junyaku (Tokyo, Japan) were used for PCR amplification following the protocol reported by Williams *et al* (1990). After preliminary screening with a total of 91 primers, 31 primers were selected for the RAPD analyses of six cultivars. The sequences of the primers which are described in this paper are as follows: A-2, TGCCGAGCTG; A-3, AGTCAGCCAC; B-13, TTCCCCCGCT; C-2, GTGAGGCGTC; C-14, TGCGTGCTTG; C-16, CACACTCCAG; D-8, GTGTGCCCCA; D-12, CACCGTATCC; D-14, CTTCCCCAAG. The reaction components were 0.2 μM dNTP, 0.2 μM primer, 1 unit of Taq polymerase, and 25 ng of genomic DNA in Taq polymerase buffer. The final volume per tube for the amplification reaction was 25 μl. The Taq polymerase, Taq polymerase buffer and dNTP were purchased from Takarashuzo (Kyoto, Japan). Amplification was performed in a Perkin Elmer Cetus DNA thermal cycler programmed for 45 cycles at 92℃ for 1 min, 35℃ for 1 min and 72℃ for 2 min. Reaction products were loaded on 1.2% agarose gels and stained with EtBr. *Hind*III-cut λ DNA was used as a molecular standard.

Results and Discussion

DNA fingerprinting with oligonucleotide probes

Genomic DNA of the cultivated laver (*Porphyra yezoensis* and *P. tenera*) were hybridized with seven oligonucleotide probes: $(AT)_9$, $(GACA)_4$, $(GT)_8$, $(GGAT)_4$, $(CT)_8$, $(CAC)_6$ and $(GATA)_4$, that are simple sequence repeti-

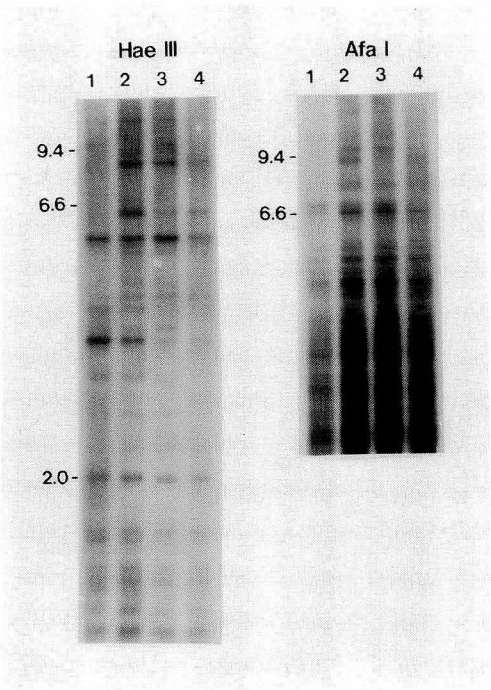

Fig. 3. Reproducibility of $(CAC)_6$-detected DNA fragments in cultivar Noma. DNA was extracted from thallus samples of cultivar Noma which were cultured in seafarms in 1992, 1993 or 1994, and stored at −20℃. DNA was extracted from these stocked thalli, digested with *Hae*III or *Afa*I, and Southern-hybridized with oligonuclotide probe $(CAC)_6$. Lane 1, Saga-5 (control); lanes 2-4, Noma cultured in 1992, 1993 or1994, respectively.

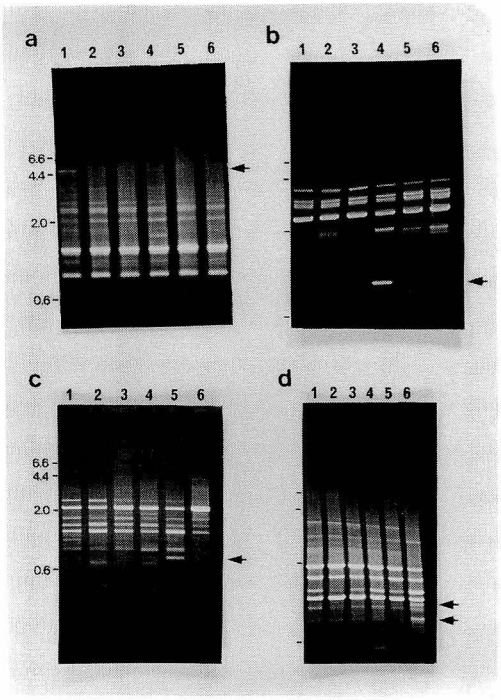

Fig. 4. RAPD banding patterns of laver DNAs showing cultivar specific DNA markers. The numbers above the lanes indicate DNAs from cultivars Saga-5 (lane 1), Noma (lane 2), Fukuoka-1 (lane 3), Narawasusabi-midorime (lane 4), Ariake-1 (lane 5) and Sashiki (lane 6). RAPD reactions were performed with primers either A-3 (a), C-14 (b), D-14 (c) or C-16 (d). The arrows indicate cultivar specific DNA bands. The size of DNA standard are indicated in kilobase pairs on the left sides.

tions previously found in other eukaryotic genomes (Tautz and Renz, 1984; Epplen, 1988; Akkaya *et al.*, 1992; Beyerman *et al.*, 1992; Thomas *et al.*, 1993). As shown in Fig. 1, hybridization signals were obtained with six out of the seven probes utilized. The hybridization patterns were highly reproducible. A dark smear over a wide molecular weight range was observed with $(CAC)_6$ probe, indicating that trinucleotide repeat $(CAC)_6$ was the most abundant in and widely dispersed through the laver genome. Highly abundant simple sequence repeats have been used as probes to fingerprint and to isolate microsatellite markers in both animals (Cornall *et al.*, 1991; Hazan *et al.*, 1992) and plants (Zhao and Kochert, 1992). Several bands superimposed on a smear were observed using $(GT)_8$, $(CT)_8$ and $(GGAT)_4$, which suggested that these simple repetitive sequences were also dispersed in the genome and can be applied to fingerprinting. A faint smear or no hybridization signal was observed with $(GACA)_4$ and $(AT)_9$, suggesting that these sequences were rare in the laver genome.

For the selection of restriction enzyme-probe combinations, we tested 12 restriction enzymes using DNA from Saga-5 and Ariake-1 (data not shown). The combinations of *Hae*III/$(CAC)_6$, *Afa*I/$(CAC)_6$, *Afa*I/$(GT)_8$ and *Afa*I/$(GGAT)_4$ were used for fingerprinting analyses because these combinations produced high numbers of identifiable DNA fragments and comparatively high degrees of polymorphisms. Figure 2 shows the hybrid-

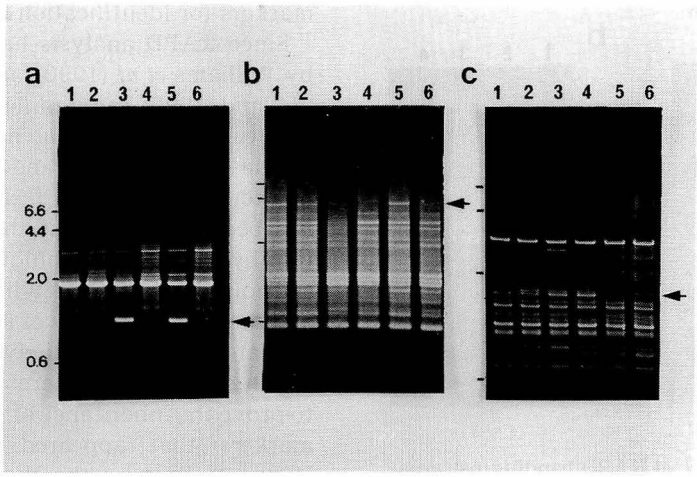

Fig. 5. RAPD banding patterns of laver DNAs showing the polymorphic DNA bands shared among cultivars. The numbers above the lanes refer to the cultivars described in the legend of Fig. 4. DNAs were amplified with primers either D-12 (a), A-2 (b) or D-8 (c). The arrows indicate polymorphic DNA bands. The size of the DNA standard is indicated in kb pairs on the left side.

ization patterns of *Hae*III and *Afa*I digested genomic DNA from six laver cultivars, detected by probe $(CAC)_6$. Each lane in the figure of *Hae*III-digest contained many distinguishable fragments, some of which did not coincide with fragments in the other lanes. The relative intensity of hybridization signal differed between fragments in different lanes. Since thallus samples of each cultivar consisted of 450 to 700 individuals of short thalli, the differences of hybridization patterns in Fig. 2 were considered to show intercultivar or cultivar specific variations of the genomic DNA and not intracultivar or individual variations of it. These hybridization patterns using synthetic oligonucleotides probes are, therefore, useful to discriminate cultivars, producing characteristic fingerprints.

To extract the genomic DNA, we used more than 2.0 g wet weight thalli of each cultivar which was cultured on respective "nori-nets" separated from each other at the coastal farms. However, there arises some doubts whether the "nori-nets" are not contaminated with conchospores of other cultivars during the outdoor-culture of thalli and whether the resultant thalli samples do not contain thalli of more than one cultivar. To solve these doubts and to examine the reproducibility of fingerprints of cultivated laver, hybridization patterns were compared between DNA samples. These DNA samples were from Noma which were cultured and harvested in 1992, 1993 or 1994, respectively. Figure 3 shows that the fingerprints appear to be similar among these three DNA samples although slight differences are detected in the region of more than 9.0 kb. On the other hand, distinct differences of fingerprints occur between DNAs of Noma and the control cultivar Saga-5. These results suggested that a little contamination from other cultivars occurred on the "nori-net" during the outdoor-culture of laver but the cultivar specificity of the fingerprint is stable and reproducible over several generations.

Cultivar identification by RAPD markers

A total of 91 different arbitrary primers were preliminarily screened for production of polymorphic bands among six laver cultivars, and 31 primers were found to produce more than three distinct DNA bands. Eighteen out of these 31 primers showed polymorphic patterns while the remaining 13 primers showed identical banding patterns among the six cultivars. Eight out of 18 primers produced polymorphic bands specific for a single cultivar. Figure 4 shows that cultivars Saga-5, Narawasusabimidorime, Ariake-1 and Sashiki could be separated by specific

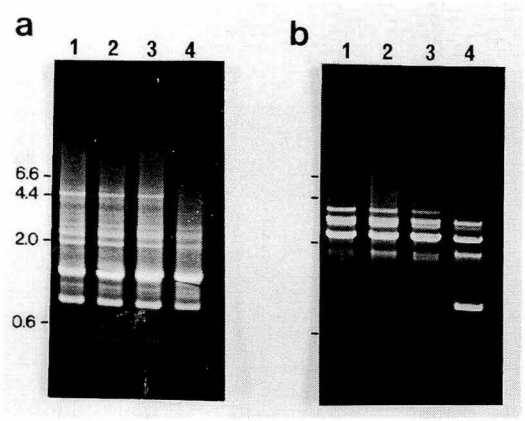

Fig. 6. Similarity of RAPD banding patterns among thallus samples cultured in different years. Thalli of cultivar Saga-5 were cultured and harvested in 1992 (lane 1), 1993 (lane 2) and 1994 (lane 3) using common stocks of the free conchocelis. DNAs from these thallus samples were PCR-amplified with primers A-3 (a) or C-14 (b), and the amplified products were analyzed on agarose gels. DNAs from cultivar Noma (a, lane 4) and Narawasusabi- midorime (b, lane 4) were also amplified using the same primers as described above and the products were co-electrophoresed with those of Saga-5 for the comparison of banding profiles. Note that primers A-3 and C-14 produced polymorphic DNA bands, as shown in Fig. 4 and 5. The size of the DNA standard are shown on the left sides (kb).

profiles resulting from primers A-3, C-14, C-16 and D-14, respectively. The other 10 primers yielded polymorphic bands which were shared among more than two cultivars. For example, Fig. 5 shows that primer A-2, D-12 and D-8 produced polymorphic bands which were shared among two, three or four cultivars, respectively. These primers could also serve to identify cultivars. Cultivar Fukuoka-1 could be distinguished by the combination of banding patterns generated by primers D-12 and D-14. Similarly, cultivar Noma could be identified by the comparison of banding patterns among primers A-2, A-3 and D-14. Thus, all six cultivars could be distinguished from each other by the combination of banding profiles produced by these primers, and the polymorphic bands produced by these primers could be used as genetic markers for identification of laver cultivars.

Since RAPD analysis has been developed by Williams *et al* (1990). and Welsh and McClelland (1990), the significance and reproducibility of this technique has been confirmed by the segregation of random amplified DNA markers in Mendelian fashion. However, it has been reported that the DNA amplification by the RAPD technique produces a few spurious DNA bands (Willams *et al.*, 1990), and the reproducibility of RAPD markers can appear problematic (Riedy, *et al.*, 1992; Ellsworth *et al.*, 1993). Therefore, it is necessary to prove the inheritance of polymorphic DNA markers that appeared in Fig. 4 and 5. However, because the thalli of *P. yezoensis* and *P. tenera* are monoecious and maturating gametes easily self-fertilized, it is somewhat difficult to produce F1 progeny of specific parents by artificial fertilization. Instead, we attempted to compare the RAPD profiles among laver samples which were managed as a single cultivar but cultured and harvested in different years. Figure 6 shows the RAPD analysis of DNAs from cultivar Saga-5 which were cultured and harvested either in 1992, 1993 or 1994. The banding profiles were very similar between all samples and cultivar specific markers were found to be in common with all samples. Similar experiments were carried out with Fukuoka-1 (harvested in either 1993 or 1994) and Noma (either 1993 or 1994) DNAs, respectively, and the same results as those described above were obtained (data not shown). These results suggest that the polymorphic DNA bands found in the specific cultivars were stable over generations and, therefore, provided a genetic basis for the cultivar specific markers. Thus, the analysis of RAPD profiles readily allows the discrimination of laver cultivars.

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アマノリの DNA 多型と養殖種の品種判別への応用

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$(CAC)_6$ 等のオリゴヌクレオチドと，制限酵素で処理されたアマノリ核 DNA との複合体形成実験にお

いて多数のDNA複合体が検出された。また，これらオリゴヌクレオチドをプローブにして，養殖アマノリ核DNAをフィンガープリント解析した結果，6品種間で互いに異なったDNAフィンガープリントが観察された。アマノリの生産年度が異なってもこれらのフィンガープリントに違いが見られなかった。一方，91種類のプライマーを用いてランダム増幅多型DNA（RAPD）法によるアマノリの品種判別を検討した。18種類のプライマーによって単一品種，または，複数品種共通に多型性を示すDNAバンド（RAPDマーカー）が検出され，さらに，生産年度の異なったアマノリにおいてもこれらのRAPDマーカーに変化がみられなかった。このような結果から，アマノリ核DNAには単純反復配列が散在し，それらを利用したフィンガープリント法によって養殖アマノリの品種判別が可能なこと，また，単一のRAPDマーカー，あるいは，それらの組み合わせが品種判別の遺伝的指標になり得ることが示唆された。