博士（水産科学）　朴　　恩　貞

学 位 論 文 題 名

Genetic polymorphism within *Porphyra yezoensis* - related species (Bangiales, Rhodophyta) from Japan and Korea detected by CAPS analysis

（CAPS 法による日本と韓国のスサビノリ近縁種の多型解析）

学位論文内容の要旨

The red alga *Porphyra yezoensis* Ueda is an economically important marine species cultivated extensively in Japan, Korea and China. It has recently received great attention as a candidate for the most viable macroalga for investigation at genome level in marine plants. In an effort to develop *P. yezoensis* as a model system for genetic studies and several applied fields, it is desirable to construct a high density genetic map based on DNA markers. Although DNA sequencing is a straightforward approach for identifying variations at certain loci, it is expensive and laborious. Moreover, since phenotypes are not always rigid criteria for identifying general variability or specific heterozygosity, molecular markers are now recognized as convenient tools not only for genotyping and variability analysis, but also for mapping various traits, cloning important genes and identifying appropriate sources for gene introgression. Various types of molecular marker have, therefore, been developed in the past few years to evaluate DNA sequence polymorphism.

In our laboratory, establishment of *P. yezoensis* as a model organism for marine plant has been set up as an important theme of research for over a decade. As a part of the ongoing efforts, I undertook a subject for my thesis with the following objectives:

1. To identify the 10 pure lines of *P. yezoensis*-related species for confirmation of species recognition based on detailed morphological observation as well as molecular biological analysis.
2. To examine the utility of a molecular marker (CAPS: cleaved amplified polymorphic sequence) for assessing genetic variation in the 10 strains of *P. yezoensis*-related species and for selection of suitable combination of parents for cross-fertilization.
3. To exploit molecular tools for screening and confirmation of hybid performance, namely, determining their utility in selection of cross-fertilized heterozygotes.

Important research findings of my work are:

1. Understanding of species boundaries in two closely related species, *P. yezoensis* and *P. tenera*

based on detailed morphological studies in conjunction with molecular biology techniques.

All 10 *P. yezoensis*-related species used in this work were successfully established as pure lines. The phylogenetic relationship among the 10 strains was examined to confirm their identity based on detailed morphological observations and their SSU rDNA sequences. All 10 thalli were entire margin, monostromatic and monoecious. The division formula was 64 (a/4, b/4, c/4) for spermatangium and 4 (a/2, b/1, c/2) for carposporangium. In comparison with the other strains, the three Korean strains, KTY1, KTY2 and KTY3 were characterized by long spindle-shaped carpogonium having conspicuous tricogynes while the other seven ones were characterized by elliptical or ovate carpogonium having inconspicuous tricogynes. From the detailed observations, it was confirmed that these 10 strains are extremely similar in their features; therefore, these morphological criteria are lacking reliability for confirmation of species recognition.

The SSU rDNA structures were categorized into three types by differences in the number of intron(s) and its position; (1) TU-1, TU-2, TUH-25, JHU and KPH had a 516 intron ranging from 510- to 517 bp inserted in an upstream location. (2) KTY1, KTY2, KTY3 and KGJ had a 1506 intron ranging from 521- to 524 bp inserted in a downstream one. (3) JHS had both the 516 and 1506 introns in size of 513- and 525 bp at the nucleotide positions of 569 and 2,318, respectively. From the BLAST search of the GenBank nucleotide database, all the five Japanese strains, TU-1, TU-2, TUH-25, JHS and JHU, and two Korean strains, KGJ and KPH, were identified as *P. yezoensis* while the other three Korean strains, KTY1, KTY2, and KTY3 were identified as *P. tenera,* although the number of intron(s) and its position were quite various among the examined strains. DNA sequence analysis of nuclear SSU rDNA would be a useful and reliable tool to assess discrimination of the specimens for overcoming some of the limitations associated with the classical morphological approach.

2. Demonstration of utility of CAPS polymorphism in assessing genetic variation within 10 strains of *P. yezoensis*-related species and in selection of suitable parents for cross-fertilization.

In Chapter III, polymorphisms of the three strains TU-1 (wild type), TU-2 (green type) and TUH-25 (red type) in *P. yezoensis* were investigated by CAPS analysis with 71 primer pairs based on the sequences of EST clones. All of 71 primer pairs were initially screened by PCR using their corresponding cDNAs and genomic DNAs as templates, and 19 ones were found to be able to amplify the target sequences. However, with the screened 19 primer pairs and 22 restriction endonucleases (REs), no length polymorphism in the restriction fragments was detected among the three strains. These results suggest that the three strains may be too close genetically, and not applicable to a linkage analysis of *P. yezoensis* using CAPS markers employed herein.

In Chapter IV, in order to search an appropriate crossing partner for use in various genetic analyses, I investigated the genetic variation among 10 strains of *P. yezoensis*-related species by CAPS analysis with a total of eight primer pairs designed based on the five gene loci. Those specific primer pairs for PCR amplification were targeted at three gene regions with intron, *EF-1α* including 5'-UTR, *PyARP4* and *V-ATPase,* and five gene regions without intron, two discrete regions of *ß-tubulin,* ORF and 3'-UTR

of *EF-1α* and *TOP2*. The PCR products were digested with 34 REs. Genetic polymorphisms between *P. yezoensis* strains and *P. tenera* ones were detected from the examined all eight gene regions with 18 kinds of RE. On the other hand, genetic polymorphisms between Japanese and Korean strains of *P. yezoensis* were detected from five gene regions, ORF and 5'-UTR of *EF-1α*, *PyARP4*, *TOP2* and *V-ATPase*, with 14 kinds of RE. The level of intraspecific polymorphism for *V-ATPase* with intron was much higher than that for *TOP2* without intron. These CAPS profiles showing codominant inheritance will be useful for confirmation of intra- or interspecific cross-fertilization products between Japanese and Korean *P. yezoensis* or *P. yezoensis* and *P. tenera*. Two Korean *P. yezoensis* strains, KGJ and KPH were recommended as a more suitable candidate for cross experiment with Japanese *P. yezoensis* strain than three Korean *P. tenera* strains, KTY1, KTY2, and KTY3, because higher possibility of cross-fertilization is expected. This is the first report to examine the genetic diversity in *P. yezoensis*-related species from Japan and Korea using CAPS analysis, and the markers developed in this study will be useful as a ready source for cross experiments to initiate linkage analysis.

3. Verification of utility of CAPS markers based on genetic distance in cross-fertilization and acquisition of heterozygotes [TU-2 female × KGJ male] for linkage analysis.

Based on the results mentioned above, the cross experiments [TU-2 female × KGJ male] were performed for the first step in linkage analysis. A total of 42 and 186 wild-type colored conchocelis colonies were randomly chosen from the controlled- and random-crossing experiments, respectively. The first screening of heterozygotes was conducted using a codominant CAPS marker in *EF-1α* (ORF) region with *Mse* I digestion. Screened heterozygotes were reconfirmed by CAPS profiles of *V-ATPase* region with *BamH* I digestion. A total of 49 heterozygotes were obtained from six (14 %) out of 42 colonies in controlled- and 43 (23 %) out of 186 colonies in random-crossing experiments. DNA fragments of cross-fertilized heterozygotes showed both CAPS patterns of TU-2 and KGJ. CAPS analysis, being codominant markers, would be an extremely useful method for preliminary screening for rapid identification of heterozygotes with genetic variation.

In this study, as a part of construction of *P. yezoensis* genetic linkage map, 10 strains of *P. yezoensis*-related species were established as pure lines and examined genetic polymorphisms using CAPS analysis. Heterozygotic sporophytes from the cross-fertilization [TU-2 female × KGJ male] were confirmed and separated by the obtained discriminate two CAPS profiles. It is expected that these results will serve as a ready source for initiate linkage analysis within the *P. yezoensis*-related species. In conclusion, construction of a linkage map in *P. yezoensis* should facilitate further refinement of this species as a new model organism in combination with recent success in establishing pure lines and cryopreservation systems and in generating ESTs.

学位論文審査の要旨

主　査　教　授　嵯　峨　直　恆
副　査　教　授　荒　井　克　俊
副　査　教　授　阿　部　周　一
副　査　助教授　北　出　幸　広

学位論文題名

Genetic polymorphism within *Porphyra yezoensis* - related species (Bangiales, Rhodophyta) from Japan and Korea detected by CAPS analysis

(CAPS法による日本と韓国のスサビノリ近縁種の多型解析)

アマノリ属植物は海苔（ノリ）として日本をはじめ，アジア諸国において水産業の重要な一角を占めている．近年，アマノリ属植物の 1 つであるスサビノリ（*Porphyra yezoensis*）は，実験生物としても注目を集めている．これまで，スサビノリを実験生物として確立するため，形質転換，遺伝地図，変異株作出，生活環の制御，cDNA ライブラリー，EST（Expressed Sequence Tag）解析などの基盤技術に関する研究が行われている．そのうち遺伝地図は種々の環境で正常に発育し，かつ高品質のノリを作出するための有用形質固定において重要な指標となる．DNA マーカーは多くのモデル生物で遺伝地図作成の際のランドマークとして利用されており，効率的な遺伝地図作成のため，DNA マーカーの研究開発が強く望まれる．共優性マーカーの 1 つである CAPS（Cleaved Amplified Polymorphic Sequence）マーカーは特定の遺伝子配列を PCR によって増幅し，制限酵素で消化後，個体間で多型の検出を行う方法である．本研究では，海産紅藻スサビノリの遺伝地図作成の一環として，染色体上のマーカーを作出する際に用いることのできる適度な遺伝距離があり，かつ交配可能な株を CAPS マーカーを使って探索することを目的とした．

本論文は，7 つの章より構成され，第 1 章では，全体における緒言を示し，第 2 章において純系株を作出し，形態学的および分子生物学的方法による同定を行なった．第 3 章および第 4 章では，純系 10 株において CAPS マーカーによる多型解析を行なった．第 5 章では，純系 2 株の交配実験を行い，得られた糸状体が交配株（異型接合体）かどうかを CAPS マーカーを用いて調べた．第 6 章では，研究の総括を行い，将来の遺伝地図作成における今後の課題ならびに研究方針について論じた．第 7 章では，本研究のまとめと結論について論じた．以下に 2-5 章の詳細を記す。

第2章においては，日本と韓国のスサビノリ近縁種10株の純系株を作出を行い，形態学的観察とSSU rDNA配列の分析により種の同定を行った．その結果，7つの株がスサビノリ（日本：TU-1, TU-2, TUH-25, JHS, JHU，韓国：KGJ, KPH ）として，また3つの株（韓国：KTY1, KTY2, KTY3）がアサクサノリ(*P. tenera*)として同定された．

第3章においては，日本のスサビノリTU-1，TU-2，TUH-25株を用い，スサビノリのEST情報を元に設計された71プライマーペアと22種類の制限酵素を用いてCAPS法によるDNAの多型解析を行なった．その結果，これらの3株間において多型は検出されず，お互いに遺伝距離が近すぎることが示唆された．

第4章においては，作出された純系10株を対照に8種類のプライマーペアを用いて特定の遺伝子を増幅させ，34種類の制限酵素で切断後，多型性を調べた．その結果，スサビノリとアサクサノリの間では18種類の制限酵素により8種類の全ての遺伝子領域で多型が検出された．一方，日本と韓国のスサビノリの間では14種類の制限酵素により5種類の遺伝子領域で多型が検出された．この結果から，CAPS分析はスサビノリ近縁種において遺伝的多様性の検索に有用であることが示された．2章と4章の結果から日本のスサビノリとの交配実験の適切な株として，同種内で多型性を有する韓国のKGJとKPHの2つの株が選ばれた．

第5章においては，日本と韓国のスサビノリ葉状体[♀:TU-2 (green type) × ♂:KGJ (wild type)]を用いて交配実験（Controlled- and Random-crossing experiments）を行った．その後，得られた糸状体の色彩およびCAPSマーカーによるバンドパターンから交配株(異型接合体)の単離を行なった．Controlled-crossing experimentから得られた49コロニーのうち6個(14%), Random-crossing experimentから得られた186コロニーのうち43個(23%)が両親由来のバンドパターンを表し，交配株であることが確認された．以上の結果から，共優性であるCAPSマーカーは種内または種間の交配において交配株の確認に有効である事が明らかになった．本研究で開発されたCAPSマーカーは連鎖解析に必要である交配実験において有用な基礎情報となり，遺伝地図の作成を促進するものと期待される．

主論文は平成18年1月26日 13時から14時まで第二研究棟特別講義室において，審査員および関連教官14名および一般聴講27名出席のもと発表された．一般聴講においては，交配実験に関して，CAPSマーカーによりヘテロと判定された糸状体からその後キメラ葉状体が得られているかどうかと，TU-2 と KGJ 以外の組み合わせ，例えばTU-1とKGJでも可能かどうかについて質疑・応答がなされた．また，審査員および関連教官においては，今回研究開発された分子マーカーの連鎖解析以外の利活用や遺伝的多型と地域性との関係について質疑・応答がなされた．また，JHS株がゲノム重複の可能性があり興味深い，今後の研究の展開として集団遺伝学的な手法の導入が考えられるなどのコメントがなされた．これらの研究で開発されたCAPSマーカーは，大型藻類では唯一のものであり，スサビノリの遺伝学の発展に大いに貢献すると判断し，博士（水産科学）の学位を授与される資格のあるものと判定した．