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A preliminary report of gene analysis of *Agarum oharaense* Yamada (Laminariaceae,Phaeophyta), with special reference to the Phylogeny

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Abstract The nuclear ribosomal DNA (18SrDNA) internal transcribed spacer (ITS-1 and ITS-2) sequences were determined for *Agarum oharaense* (Ohno-aname), *A. cribrosum* f. *cribrosum* (Aname) and *A. cribrosum* f. *rugosum* (Zara-aname), and RuBisCo-spacer sequence was also investigated. As a result, in sequence of total 296 ITS-2 nucleotides, there were 20 substitution, 36 insertion and 4 deletion in *A. oharaense* in comparing with other two taxa examined, and 21 substitution in sequences of total 426 RuBisCo-spacer nucleotides in *A. oharaense*. Gene analysis, especially on ITS-2 and RuBisCo-spacer, suggest that *A. oharaense* is different taxon from *A. cribrosum* f. *cribrosum* and *A. cribrosum* f. *rugosum*.

Key words: *Agarum oharaense*, *A. cribrosum* f. *cribrosum*, *A. cribrosum* f. *rugosum*,ITS-1, ITS-2, RuBisCo-spacer, taxonomy, Phylogeny

Introduction

The species of *Agarum* (Laminariacea, Phaeophyta) living in subtidal on the rock, have been described from northern Pacific Ocean and west coast of the northern Atlantic Ocean after establishment of genus *Agarum* by Dumortier (1822) after the presentation of Gmelin (1768) as *Fucus agarum*. Thereafter, Postels and Ruprecht (1840) distinguished three species, *A. gmelin*, *A. turuneri* and *A. pertusum*, based on the features of midrib, substances of blade and size of perforation, from Kamchatka, Alaska, Canada and Greenland. Later, Setchell and Gardner (1925) referred these three species to *A. cribrosum* established by Bory (1826). Furthermore, Harvey (1851) described *A. fimbriatum* from Esquimalt, Br. Columbia, Canada, by reason of stipe fimbriated on margins and blade with few perforations.

In Japan three species, *A. gmelin* (Okamura, 1902), *A. turuneri* (Miyabe, 1902; Okamura, 1925) and *A. cribrosum* (Tokida,1932; 1954; Miyabe and Nagai, 1933; Okamura, 1936), were described from northern Japan, Sakhalin, Kurile Islands, and Yamada (1961, 1962) distinguished new two endemic speices, *A. oharaense* based on flattened, twisty stipe with rhizoidal hapters from Ohara, Boso Peninsula, Honshu Island and *A. yakishiriense* on flattened stipe with many hapters from Rishiri Island. After that Yamada (1974) taxonomically reexamined the species of Agarum on the coast of Hokkaido and adjacent regions at the point of local populations. As a result, he proposed 4 forms in *A. cribrosum*. Now there are 5 taxon which consist of 2 species and 4 forms in genus *Agarum*, *A. oharaense* (Japanese: Ohno-aname) as endemicity and *A. cribrosum* f. *cribrosum* (Aname) and *A. cribrosum* f. *rugosum* (Zara-aname), *A. cribrosum* f. *rishiriense* (Rishiri-aname) , *A. cribrosum* f. *yakishiriense* (Teuri-aname) (Yoshida, 1998).

Under these circumstances, it seems to us that specific status is not enough by the very few morphological criteria influenced possibly by environmental factors such as temperature, light condition and wave action. This discrepancy is due to morphological plasticity which is possibly. It appears necessary to evaluate the phylogenetic affinities among the *Agarum* taxa

using characteristic independent of morphology as molecular data. Recently, the nuclear ribosomal DNA internal transcribed spacers, ITS-1 and ITS-2, have been indicated to provide good phylogenetic resolution at lower taxonomic levels, such as inter- and intraspecific taxa of Phaeophyta (Peters et al., 1997; Leclerc et al., 1998).

The purpose of this study is to reexamine the specific position of *A. oharaense* in genus *Agarum*, by nuclear ribosomal DNA (18SrDNA) ITS-1, ITS-2 and RuBisCo-spacer sequences. This study is a part of research projects, Seaweeds flora at the coast of Boso Peninsula, organized by Natural History Museum & Institute, Chiba.

Materials and methods

Mature sporohytes of *Agarum oharaense* Yamada (Japanese: Ohno-aname) were collected at depth 25m, on October 10, 2001, at Ohara, Chiba Prefecture. *A. cribrosum* f. *cribrosum* I. Yamada (Aname) and *A. cribrosum* f. *rugosum* (Zara-aname). I. Yamada were from Muroran and Nemuro, respectively, on July to October, 2001. Total DNA was extracted from sporohytes. DNA extraction, Coordination of Oligonucleotide primers, PCR amplification, Sequencing of ITS-1, ITS-2 and RuBisCo-spacer, Sequence alignment and tree rooting were based on the methods of Yotsukura et al. (1999).

Results and Discussion

The total of 296 ITS-2 nucleotides are indicated in Fig.1. The length of ITS-2 was 259 b.p. in *A. cribrosum* f. *rugosum* (agcrib-mu) and *A. cribrosum* f. *cribrosum* (agcrib-ne) for 296 b.p. in *A. oharaense* (agoharaen).The sequences were identical within two form of *A. cribrosum*. And in *A. oharaense* in sequences of 296 ITS-2 nucleotides, there were 20 substitution, 36 insertion and 4 deletion.

The total of 426 RuBisCo-spacer nucleotides are shown in Fig.2. The length of RuBisCo-spacer was completely identical in three taxa examined. For *A. cribrosum* f. *rugosum* and *A. cribrosum* f. *cribrosum*, RuBisCo-spacer was almost identical, except for one nucleotide substitution from G to C at position 128, and in *A. oharaense* there were 21 substitution in sequences of nucleotides.Phylogenetic relationships by means of maximum parsimony (MP) and distance matrix methods are now under review.

The ITS regions appear to be very useful for

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agcrib-mu       GACACCACTCGCCCCCCT----------TCTCTCCCCCCTG-------TAACAGGGG---
agcrib-ne       GACACCACTCGCCCCCCT----------TCTCTCCCCCCTG-------TAACAGGGG---
agoharaen       GACACCACTCGCCCCCCTCTCTCTCTCTTCTCCCCCCTCCGGTCCTGTTAACAGGGGTCG
                ******************          **** **** * *       *********

agcrib-mu       -CGGGG--------GGGATCGCGGGG-CGGACTCTGAGTGTTCCGGAGTTCCCATGCTCC
agcrib-ne       -CGGGG--------GGGATCGCGGGG-CGGACTCTGAGTGTTCCGGAGTTCCCATGCTCC
agoharaen       TCGGGTCGGGTGTCGGGATCGCGGGGGCGGACTCTGAGTGTTCCGGAGTTTTC----TCC
                 ****          ************ **********************  *    ***

agcrib-mu       GAGTGCACCTAATCTCGTGAACGAAGCCTCTCGCGCCCTGCC-GCACAGAGTTGTTGACG
agcrib-ne       GAGTGCACCTAATCTCGTGAACGAAGCCTCTCGCGCCCTGCC-GCACAGAGTTGTTGACG
agoharaen       GAGTGCACCTAATCTCGTGAACGAAGCCTCTCGCGCCCTGCCCGCGCAGAGTTGTTGACG
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agcrib-mu       GCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAACGTGCGCAGGCTGCGG-GCTTCTTC
agcrib-ne       GCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAACGTGCGCAGGCTGCGG-GCTTCTTC
agoharaen       GCGCTCGCTTCGGCGGCGACTCTCGGCTCACCAAACGTGCGCAGGCTGCGGAGCTTCTTC
                ************************ ************************ ********

agcrib-mu       CGGCGC--TCCAGAAGATCTCTCTCGAG-ACCTTTTGGAAA-CCGTACCACTTTCG
agcrib-ne       CGGCGC--TCCAGAAGATCTCTCTCGAG-ACCTTTTGGAAA-CCGTACCACTTTCG
agoharaen       CGGCGCGCTCCATGACATGTCTCTAAAACGTGTGATGGAAATCCGTACCACTTTCG
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Fig. 1. Alignment of internal transcribed spacer ITS-2 sequence data for *A. cribrosum* f. *rugosum* (agcrib-mu) from Muroran, Hokkaido, *A. cribrosum* f. *cribrosum* (agcrib-ne) from Nemuro, Hokkaido and *A. oharaense* (agoharaen) from Ohhara, Chiba Pref..

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agcrib-mu       GTTGGAGAAGGTCCTGAAATCTTACGTAACGCAGCGGCTACTTGTGGTCCTTTAAAAGCA
agcrib-ne       GTTGGAGAAGGTCCTGAAATCTTACGTAACGCAGCGGCTACTTGTGGTCCTTTAAAAGCA
agoharaen       GTTGGAGAAGGTCCTGAAATCTTACGTAACGCAGCGGCTACTTGTGGTCCTCTTAAAGCA
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agcrib-mu       GCGTTAGATTTATGGAAAGATATTACTTTCGAGTACACTTCAACAGATACACCTGATTTC
agcrib-ne       GCGTTAGATTTATGGAAAGATATTACTTTCGAGTACACTTCAACAGATACACCTGATTTC
agoharaen       GCGTTAGATTTATGGAAAGACATTACTTTCGAGTACACTTCAACAGATACACCTGATTTC
                ******************** ************************************

agcrib-mu       GTTGAGAGTACAACTGAAAGCAACTAGTATACTGAAACTAAATTTATAAGAGATTAATTC
agcrib-ne       GTTGAGACTACAACTGAAAGCAACTAGTATACTGAAACTAAATTTATAAGAGATTAATTC
agoharaen       GTTGAGCTTCCTACTGAAAGCAAATAGTATACTGAAACCAAATTTATAAGAGATTAATCC
                ******  * * ********** ************** ******************** *

agcrib-mu       TAACTAATTCTAGCTATCTTTAGACAGCTAAAAAATACATGTAATAATTTTATTTACTTT
agcrib-ne       TAACTAATTCTAGCTATCTTTAGACAGCTAAAAAATACATGTAATAATTTTATTTACTTT
agoharaen       TAAATAATTCTAGCTATCTTTAGACAGCTAGAAAATACATGTAATAATCTTATTTACTTT
                *** ************************* ***************** ***********

agcrib-mu       ATTCTTAAAGTTATATTGATTATTTTGTTAAAAAATTTAGATTTGACCTTAAATTTGTTA
agcrib-ne       ATTCTTAAAGTTATATTGATTATTTTGTTAAAAAATTTAGATTTGACCTTAAATTTGTTA
agoharaen       ATTCTTAAAGTTATATTTATTATTTTGTTCTAAAATTTTGATTTGACCTTAAATTTGTTA
                ***************** ***********  ******* **********************

agcrib-mu       TAACTTCAGAAAAAATACTTAATACCCCATATTATTTTAAGCGAAAGTAGAGAGCAAATA
agcrib-ne       TAACTTCAGAAAAAATACTTAATACCCCATATTATTTTAAGCGAAAGTAGAGAGCAAATA
agoharaen       TAACCTTAGAAAAAATACTTAATACCCCATATTATTTTAAGCGAAAGTAGAGAGCAAATA
                **** * ************************************************

agcrib-mu       AAAATTTTAGTATATAACTAAAAATAAAATTTCTATAATTTATCTTTAAGGAATATTTGA
agcrib-ne       AAAATTTTAGTATATAACTAAAAATAAAATTTCTATAATTTATCTTTAAGGAATATTTGA
agoharaen       AAAATTTTAGTATATGACTAAGAATAAAATTTCTATAATTTATCTTTAAGGAATATTTGA
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agcrib-mu       ATAGTG
agcrib-ne       ATAGTG
agoharaen       ATAGTG
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Fig. 2. Alignment of RuBisCo-spacer sequence data for *A. cribrosum* f. *rugosum* (agcrib-mu) from Muroran, Hokkaido, *A. cribrosum* f. *cribrosum* (agcrib-ne) from Nemuro, Hokkaido and *A. oharaense* (agoharaen) from Ohhara, Chiba Pref..

resolving taxonomical problems and/or phylogenetic relationships among specific and/or intraspecific levels because of the diverging more rapidly, and among genera in Laminariacea genera such as Castro, Malaria and Chord (Yotsukura et al., 1999) , in contrast to a report that SUE Rena sequences was too conservative among morphologically distinct three families, Allures, Laminariaceae and Lousiness (Saunders and Duel, 1992). And Anderson and Stasovski(1992) reported that although ITS regions were not informative, intergeneric spacer (IGS) region between 26S RNA and 5S RNA of the nuclear ribosomal gene was informative in discriminating closely related species of the Basidomycetes, Armillaria. Practically, ITS region might not be good enough for analyzing these rapidly evolving taxa in Laminariaceae at specific and intraspecific levels.And this possibility has to be tested by extensive interfertility experiments, along with additional molecular approaches, using populations markers as random amplified polymorphic DNA, microsatellite and IGS.

In this preliminary report of gene analysis of *Agarum oharaense* Yamada (Laminariaceae, Phaeophyta), the gene analysis in ITS-2 and RuBisCo-spacer regions, with only unique morphological characters (Gmelin, 1768; Bory, 1826), twisty and flattened stipes with rhizoidal

hapters (Yamada, 1961), suggest that A. oharaense is different taxon from *A. cribrosum*. And furthermore, it is necessary to study ITS-2 and RuBisCo-spacer on other three taxa, A. *cribrosum* f. *rishiriense*, *A. cribrosum* f. *yaki-shiriense* (Yamada, 1961; Yamada, 1974) and *A. fimbriatum* living at the coast of North America (Harvey, 1851; Setchell,1925; Taylor,1937; Abbott and Hollenberg, 1976), to focus the phylogenetic relationships among the species of Genus *Agarum*.

We have small valuable informations of divergences in ribosomal DNA ITS-2 and RuBisCo-spacer sequences exists between three taxa in genus *Agarum*. The systematic ideas based on the phylogenetic relationships inferred from this molecular data set have some commonality with the morphological informations stored for long time (Yamada, 1961; 1962; Yamada, 1974; Duel et al., 1988; Yoshida, 1998).

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オオノアナメ *Agarum oharaense* Yamada の遺伝子解析と系統・予報

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褐藻コンブ科アナメ属はヨーロッパ大西洋沿岸を除く北大平洋沿岸に3種4品種，6分類群が分布する。日本列島及び周辺海域には，オオノアナメ *Agarum oharaense*，*A. cribrosum* の2種が分布し，後種に4品種（アナメ，ザラアナメ，テウリアナ，リシリアナメ）を認めて，2種4品種，5分類群が分布する。特にオオノアナメは千葉県大原海域に隔離分布する日本産固有種である。茎部が扁平でねじれ，根性体を有することで区別する，しかし，*A. cribrosum* の4品種と共有する形態的な特徴がある。また，北アメリカ大平洋沿岸には葉状部に穴が少なく，扁平な茎部の縁辺に突起のある *A. fimbriatum* が分布する。そこで，オオノアナメの種としての独立性を再検討し，6分類群の系統関係を推定するための予備的な研究としてオオノアナメ，アナメ，ザラアナメの3分類群の遺伝子解析をおこなった。その結果，18SrDNA の296塩基からなるITS-2領域において36塩基の挿入，4塩基の欠損，20塩基の置換を認め，426塩基からなる RuBisCo スペイサー領域においては，同じ塩基長でありながらアナメとザラアナメの間ではわずか1塩基の置換に対して，オオノアナメは両品種と比較して21塩基の置換を認めた。テウリアナメ，リシリアナメ，*A. fimbriatum* との比較が必要であるが，オオノアナメが *A. cribrosum* の品種であるアナメとザラアナメと異なる分類群であることを示唆した。本稿では，*A. cribrosum* 4品種の命名規約上の変更がおこなわれていない現状において，*A. cribrosum* Bory の *A. clathratum* Dumortier への変更を採用しない（Silva, 1992）。

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