Botanica Marina Vol. 45, 2002, pp. 87–90

Short Communication

Type II DNA Topoisomerase (Top2) as Promising Molecular Marker for Phylogenetic Analysis in Rhodophyta

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Taxonomic uncertainty still exists in the Rhodophyta, and more suitable molecular markers for their phylogeny are required to establish conclusively their taxonomic relationship. In this paper, we describe sequencing, characterisation of partial nucleotide sequence of the *TOP2* and phylogenetic analysis of the Top2, which is translated from the *TOP2* in *Porphyra yezoensis*. The length of the partial nucleotide sequence of *TOP2* was 1,208 bp and the predicted amino acid sequence was 402 amino acids. The present sequences matched to known *TOP2* or Top2 sequences in the public databases and consensus sequences for the motifs [Top2 signature, ATP/GTP-binding site motif A (P-loop), etc.] are indicated in the amino acid sequences. In our phylogenetic trees, the Rhodophyta occupied an independent position within the crown eukaryotes. Thus, we can advocate that we succeeded in the determination of *TOP2* and Top2 sequences of *P. yezoensis* and Top2 may be a promising marker for phylogenetic analysis of the Rhodophyta. This is the first report to deal with Top2 from the Rhodophyta.

Introduction

Recently, phylogenetic studies using molecular markers, i. e. SSU rDNA, ITS, *rbc*L, *rbc*S and RUBISCO spacer have been conducted to resolve the phylogenetic relationships in the Rhodophyta. However, taxonomic uncertainty still exists, and more suitable molecular markers are required to establish their taxonomic relationship conclusively.

A few studies (Yamamoto and Harayama 1996, 1998) reported phylogenetic relationships of bacteria based on the nucleotide sequences of DNA gyrase subunit B gene (*gyrB*). They showed the following effectiveness of the genes for bacterial phylogenetic analysis; the features of the *gyrB* were that (i) it does not transmit horizontally, (ii) its molecular evolution rate is appropriate for phylogenetic analysis, and (iii) it is distributed universally among the members of bacteria. Eukaryotic type II DNA topoisomerase (Top2) has a common structure (Watt and Hickson 1994) and the N-terminal quarter of these proteins exhibiting ATP activity is highly homologous with the ATPase domain of the bacterial DNA gyrase (Lynn *et al.* 1986). We selected type II DNA topoisomerase gene (*TOP2*) that encodes type II DNA topoisomerase (Top2) as a new molecular marker for phylogenetic studies of the Rhodophyta. In the present paper, we described sequencing, characterisation of partial nucleotide sequence of the *TOP2* and phylogenetic analysis of the Top2, which is translated from the *TOP2* in *Porphyra yezoensis* Ueda.

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Material and Methods

Porphyra yezoensis Ueda (strain TU-1) was obtained from the culture collection at the Center for Advanced Technology, Tokai University. The DNA was extracted from gametophytic thalli according to the method described previously (Nakajima *et al.* 1999). The reaction volume (50 μL) for PCR amplifications consisted of 20–50 ng DNA template, 200 pmol primers, 5 μL 10 × PCR buffer, 8 μL dNTP mixture, 2.5 units Gene Taq NT (Nippon Gene, Japan) and sterile water. Degenerate PCR primers for the amplification of partial *TOP2* were designed from two conserved regions of the amino acid sequences of the following Eukaryotes: *Arabidopsis thaliana* (L.) Heynh. (GeneBank/EMBL/DDBJ accession number L21015), *Plasmodium falciparum* Welch (P41001) and *Caenorhabditis elegans* (Maupas) Dougherty (P34534). The two conserved amino acid sequences were reverse translated, and the 24-nucleotide N-terminal PCR primer and 22-nucleotide C-terminal PCR primer were synthesised (Table I). The PCR amplified DNAs were analysed by gel electrophoresis on SeaKem GTG agarose (FMC, USA) and SYNERGEL (Diversified Biotech, Italy) mixed gel (appropriate to 1.5% agarose gel). Putative *TOP2* fragments were purified using Gene Clean (Bio 101, USA) according to the manufacture's instructions. Purified fragments (about 1,300 bp in length) were cloned into pT7 Blue T Vector (Novergen, USA) using ligation kit ver. 2 (Takara, Japan) and introduced into the competent cells of *Escherichia coli* (Migula) Castellani *et* Chalmers JM109 (Takara, Japan). Sequences were performed using dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer, USA).

Table I. PCR primers used in this study.

Sense	CGA	RAT	HII	IGT	NAA	YGC	NGC	NGA
Antisense	GIS	WIC	CRT	CII	IRT	CYT	GRT	C

N = A, G, T, C
R= A, G
S = C, G
W = A, T
Y = C, T
I = inosine

The determined sequences of *TOP2* were then compared with sequences in the DNA and protein databases using the basic local alignment search tool programs (Altschul *et al.* 1990) and MOTIF program for amino acid consensus similarities. The resulting sequences were aligned with other organisms [*Arabidopsis thaliana* GeneBank/EMBL/DDBJ accession number L21015; *Candida albicans* (Robin) Berkhaut Y10377; *Crithidia fasciculata* Léger P27570; *Dictyostelium discoideum* Raper D82024; *Emericella nidulans* (Eidam) Vuill. AB014886; *Escherichia coli* D65172; *Leishmania chagasi* (Cunha *et* Chagas) Nicoli AF; *Penicillium chrysogenum* Thom AB029613; *Pisum sativum* L. Y14559; *Plasmodium falciparum* P41001; *Pseudomonas putida* Migula X54631; *Saccharomyces cerevisiae* Hansen M13814; *Schizosaccharomyces pombe* Lindner X04326; *Synechocystis* sp. D90908; *Trypanosoma cruzi* Chagas M91165] using CLUSTAL W (Thompson *et al.* 1994) and the terminal 5' and 3' ends of the putative *TOP2* sequence corresponding to the PCR primers were excluded from the alignment. Phylogenetic relationships among 15 eukaryotes and 3 prokaryotes were shown by neighbour-joining (N-J; Saitou and Nei 1987), most parsimony (MP; Swofford 1993) and maximum likelihood (ML; Felsenstein 1981) methods. In the constructed trees, eukaryotic taxa were rooted with bacterial *gyrB* sequences as outgroup taxa. The N-J tree was constructed using the computer program package of PHYLIP (Felsenstein 1989, gaps treated as missing data). The ML analysis was performed using PUZZLE program (Strimmer and von Haeseler 1996). The MP analysis was completed using heuristic search of phylogenetic analysis using parsimony (PAUP; Swofford 1993). Bootstrap re-samplings (1,000 replicates) were completed for each neighbour-joining and parsimony analysis to estimate the robustness of internal branches (Felsenstein 1985). Puzzling steps (1,000 replicates) were also completed for likelihood analysis.

Results and Discussion

Our PCR strategy amplified not only *TOP2* fragments but also a few additional fragments from *Porphyra yezoensis* DNA (Fig. 1). Thus the putative *TOP2* fragments were purified and cloned into plasmid vectors

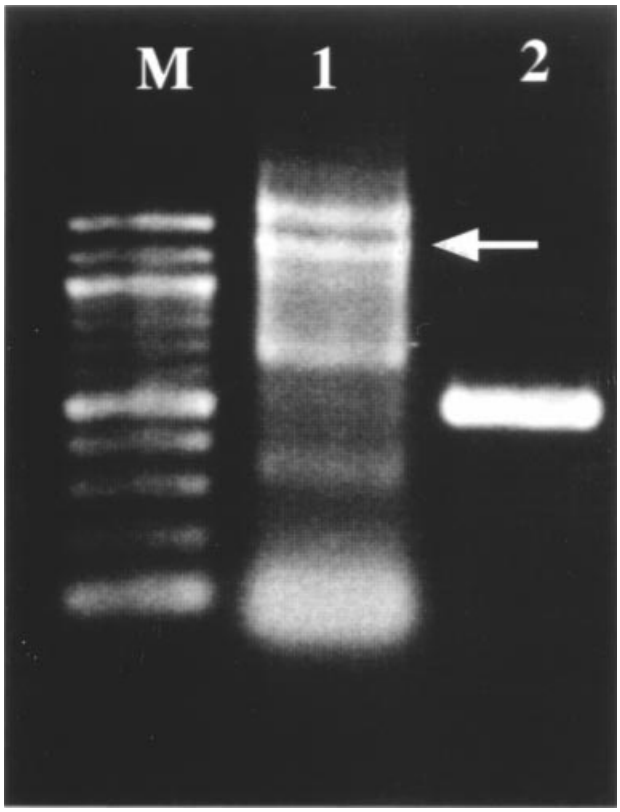

Fig. 1. Gel electrophoresis of PCR products amplified with degenerate primers (Primers A and B). Possible *TOP2* is indicated as an arrow. Lane M, molecular size marker (100 bp DNA ladder); lane 1, amplified fragments of DNA of *Porphyra yezoensis*; lane 2, positive control (λ-DNA).

to introduce into *E. coli* competent cells. Two clones containing putative *TOP2* were successfully obtained, and partial sequences of the clone fragment were determined. The length of the partial nucleotide sequence of *TOP2* was 1,208 bp and the predicted amino acid sequence was 402 amino acids. The present sequences matched to known *TOP2* or Top2 sequences in the public databases. Some motifs were identified by using the MOTIF program. Consensus sequences for the motifs [Top2 signature, ATP/GTP-binding site motif A (P-loop), etc.] are indicated in the amino acid sequences (Fig. 2). Generally, the motifs were located on well-conserved regions.

The multiple alignment was converted to phyloge-

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1   NNGAGIPVAMHAKEGVYVPELIFGHLLTSSNYDDGEKKVVGGRNGYGAKLANIFSTRFVV  60
61  ETADASSGKTYKQTFKRNMMDRGTPAIRASAKPRDTWTRITFEPDLAKFGMDRLNEDAVA 120
121 VLQMRVVDMAGVVPGIAVYLNGAKLKINSFKQYVDLHLADAPDAPRIHDATSGRWEVVVS 180
181 LSDGQLTQTSFVNSIATTKGGTHVNHVADQLVSRIQEHIAKKHKTLKVKPFQIKSQLSIY 240
241 VKCLVENPAFDSQTKEMLTSRPASFGSKWVCPDRMAKDLIKSGVVESVLSLAESRQVKEL 300
301 AKTDGGKRARVSGIPKLDDANLAGTRDSAKCTLILTEGDSAKALAISGLSVVGRDYYGVF 360
361 PLRGKLLNVREATHKQIMDNAEITNLKKIIGLQHG
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Fig. 2. An amino acid sequence of Top2 from *Porphyra yezoensis*. Underlines indicate putative motifs [1: ATP/GTP-binding site motif A (P-loop); 2: Top2 signature]. Amino acid sequence was compared with protein sequence patterns in sequence databases (SWISS-PROT, PDBSTR, PIR, PRF and Genes) using MOTIF program for amino acid consensus similarities.

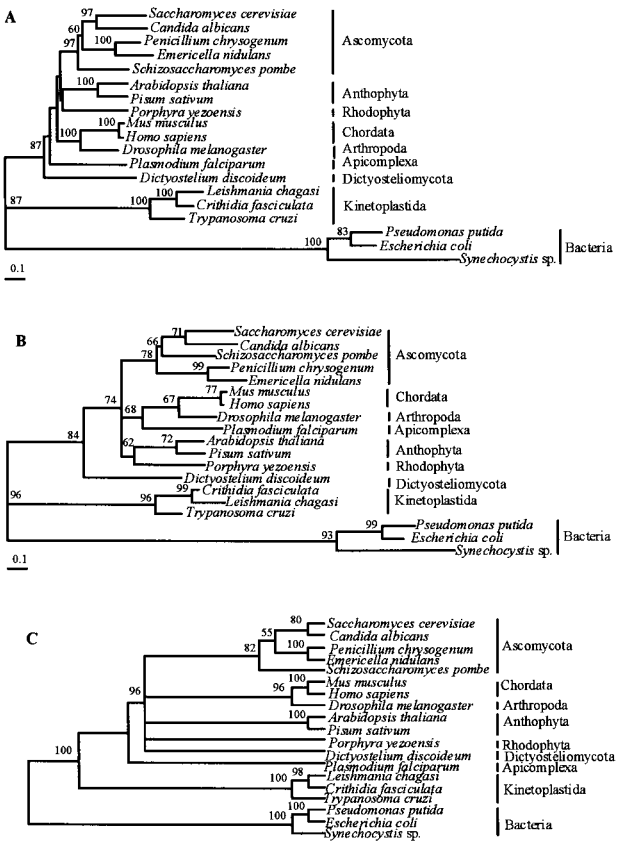

Fig. 3. Phylogenetic trees, which include Anthophyta, Apicomplexa, Arthropoda, Ascomycota, Bacteria, Chordata, Dictyosteliomycota, Kinetoplastida and Rhodophyta, based on the type II DNA topoisomerase amino acid sequences. Trees were artificially rooted on the branch leading to Bacteria. A: neighbour-joining tree. Distances were estimated using the Dayhoff PAM matrix. B: maximum likelihood tree. Model of amino acid substitutions was the JTT model. C: most parsimonious tree.

netic trees using the N-J, ML and MP methods (Fig. 3). The ingroup taxa were essentially resolved in seven higher-level lineages. Kinetoplastida (*Crithidia fasciculata, Leishmania chagasi, Trypanosoma cruzi*) formed a sister lineage, and branched off the main lineage as the earliest divergent group. The remaining ingroup taxa contained the representatives of the Eukaryotes, Anthophyta (*Arabidopsis thaliana, Pisum sativum*), Apicomplexa (*Plasmodium falciparum*), Arthropoda (*Drosophila melanogaster* Meigen), Chordata (*Homo sapiens* L., *Mus musculus* L.), Ascomycota (*Candida albicans, Emericella nidulans, Penicillium chrysogenum, Saccharomyces cerevisiae, Schizosaccharomyces pombe*), Dictyosteliomycota (*Dictyostelium discoideum*), and Rhodophyta (*Porphyra yezoensis,* examined in this study). In the N-J and ML trees, *Dictyostelium discoideum* was resolved as a group diverged prior to the higher eukaryote lineages of Anthophyta, Apicomplexa, Arthropoda, Ascomycota, Chordata and Rhodophyta. In contrast, the MP analysis yielded an unresolved polychotomy at the base of the higher eukaryotic group. In each tree, *Plasmodium falciparum* occupied a different position. The N-J and ML analyses placed *Porphyra yezoensis* as a sister group to Anthophyta supported by a weak bootstrap value, and the parsimony analysis placed it within a basal polychotomy.

In our trees, Rhodophyta occupied an independent position within the crown eukaryotes. The Anthophyta, Arthropoda, Ascomycota and Chordata clearly formed monophyletic groups with each other. Branchings of Anthophyta, Arthropoda, Ascomycota and Chordata were supported by high level of statistical values. However, the clusters joining Anthophyta, Arthropoda, Ascomycota, Chordata and Rhodophyta were supported by low statistical values. Thus, we can advocate that Top2 may be a promising marker for phylogenetic analysis on inter-relationships among divisions or phyla rather than intra-relationships of them. The isolation and characterisation of Top2 from *Porphyra yezoensis* were performed in the present study. This is the first report to deal with Top2 from the Rhodophyta and suggests the possibility of Top2 as a molecular marker for phylogenetic study in eukaryotes.

Acknowledgements

The authors are grateful to Drs S. Miyachi and Y. Kitade for their suggestions on and review of the present paper. This study was supported in part by the New Technology Research and Development Project from the Ministry of Education, Science, Sports and Culture, Japan; Mutal Aid Corporation for Private Schools, Japan; Tokai University, and a Grant-in-Aid for Scientific Research from the New Energy and Industrial Technology Development Organization (NEDO).

Accepted 18 September 2001.

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