MOLECULAR CHARACTERIZATION OF A GREEN ALGAE ISOLATE BY 16S rRNA IN IMPROVEMENT OF CAROTENOID PRODUCTION

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Abstrak

Sintesis karotenoid alami belum pernah melebihi produk sintetik pada skala komersial. Kurangnya pemahaman mengenai aspek mikrobiologis dan ekofisiologis isolat penghasil karotenoid seringkali menyebabkan terjadinya kesalahan penamaan spesies. Satu isolat lokal alga hijau dari Perairan Jepara yang digunakan sebagai pakan alami sumber karotenoid hewan-hewan perikanan, pada mulanya dianggap sebagai *Dunaliella*. Penamaan *Dunaliella* hanya dilakukan berdasarkan pengamatan mikrobiologis dan ekofisiologis yang kurang lengkap. Tujuan utama penelitian ini adalah menentukan spesies satu isolat lokal alga hijau secara molekuler menggunakan 16S rDNA untuk mendeteksi jalur biosintesis karotenoid yang digunakan.

Urutan basa 16SrRNA yang diperoleh dianalisis menggunakan *Multiple Alignment Analysis* dan analisis filogenetik melalui program *ClustalX, ClustalW, GeneDoc, Phylip* dan *NjPlot*. Hasil penelitian memperlihatkan bahwa Isolat alga hijau menunjukkan similaritas yang tinggi dengan anggota-anggota Sianobakteria. Keserupaan tertinggi dimiliki dengan *Cyanobacterium* sp. MBIC 1021 sebesar 99 %, diikuti *Synechocystis* PCC6308 sebesar 95 %. Hasil analisis similaritas dan filogenetik memperlihatkan peluang bahwa Isolat alga hijau mengikuti jalur baru non-mevalonat dalam biosintesis karotenoidnya.

Abstract

Carotenoids production levels are not yet competitive with carotenoid levels presently produced by fermentation, synthesis and isolation. An attempt to optimize carotenoid production from local isolate of green algae from BBAP Jepara has faced several problems, primarily related to the microbiological and eco-physiological characteristic which affecting growth that have not sufficiently been understood. A misnamed of species also have arisen due to wrong characterization. One local isolate of *Dunaliella* species from BBAP Jepara was found potentially useful as source of carotenoids in food additives or as food supplement in fish farming. The present study aimed to characterize the species of green algae isolate from Jepara waters based on molecular techniques using 16S rRNA approach to detect its carotenoid biosynthetic pathway.

Similarities analysis and phylogenetic relationship of 16S rRNA sequence was analyzing with *Multiple Alignment Analysis* by *ClustalX, ClustalW, GeneDoc, Phylip* and *NjPlot* Programs. Molecular analysis showed close relationship among isolate of green algae and Cyanobacteria with 99 % similarity with *Cyanobacterium* sp. MBIC 1021 and 95 % similarity with *Synechocystis* PCC6308. The result of this analysis indicated possibilities that a green algae isolate following the new non-mevalonate pathway for its catotenoid biosynthetics.

Keywords : A green algae isolate, *Dunaliella*, 16S rRNA, Cyanobacteria

INTRODUCTION

Carotenoids, some of which are provitamin A, have range of diverse biological function and actions, such as species specific coloration, photo protection, and light harvesting, and they serve as precursors of many hormones (Vershinin, 1999 *in* Lee and Schmidt-Dannert, 2002). Carotenoids are used commercially as food colorants, animal feed

supplements and, more recently, as nutraceuticals for cosmetic and pharmaceutical purposes. The demand and market for carotenoids is anticipated to change drastically with the discovery that carotenoids exhibit significant anti-carcinogenic activity and play an important role in the prevention of chronic diseases (Lee and Schmidt-Dannert, 2002).

For many years, it was accepted that carotenoid was synthesized through the well known acetate/mevalonate pathway. However, recent studies have demonstrated that the mevalonate-dependent pathway does not operate in all living organisms. More recently, photosynthetic organisms such as green algae, *Scenedesmus obliquus*, *Chlorella fusca*, *Chlamydomonas reinhardii* and higher plants use non mevalonate pathway known as deoxyxylulose 5-phosphate (DXP) pathway for their carotenoid biosynthesis. DXP Synthase gene (*DXS*) was catalyzes a limiting enzyme on DXP pathway. The exclusive occurrence of the non-MVA pathway for the biosynthesis of plastidic isoprenoids and of sterols might represent a general feature of many green algae (Lois *et al.*, 1998; Lichtenthaler, 1999). A combination of genes from different organisms that follow diferrent branches of the pathway from mevalonate and non-mevalonate for carotenoid productions make it possible to increase carotenogenesis. For example, levels of accumulated carotenoids were increased up to 10.8 times the level of not overexpression *dxs* gene from DXP pathway (Matthews & Wurtzel, 2000), over-exspression of *dxs* gene increased lycopene production 2-3 fold (Verdoes & van Ooyen, 1999, co-exspression *dxs* & *dxr* : lycopene 1.4 – 2 fold (Kim & Keasling, 2001) , over-exspression *idi* : lycopene 3.6-4.5 fold (Kajiwara *et al*, 1995) and astaxanthin 6 fold (Wang *et al*, 1999).

A local isolate of an algal species from BBAP Jepara, suspected as representing a strain of *Dunaliella*, was found potentially useful as source of carotenoids in food additives or as food supplement in fish farming. Thus, it was of great interest to know if this local isolate of algae would also follow the non-MVA pathway for carotenoid biosynthesis. It was therefore attempted to solve this problem through detection of a *DXS* gene, using a molecular approach. Previous research was done subsequently by using *Polymerase Chain Reaction* (PCR) techniques. Further research in detecting *DXS* gene from this "*Dunaliella*", has faced several problems that might be caused by misnamed of the species (Kusumaningrum *et al*., 2004; Kusumaningrum *et al*., 2006). It is apparent that microbial identifications based only on microbiological characterization have, until recently, failed to achieve the necessary requirements of prediction, stability and objectivity (Priest and Austin, 1993). Therefore, it is important to examine identification of species based on molecular technique using 16S rRNA sequence, for supporting microbiological and eco-physiological characterization. The present study aimed to investigate the species characterization of a green algae isolates from Jepara Waters based on 16S rRNA approach.

MATERIAL AND METHODS

1. Culture Media

The medium artificial sea water (ASW) used was modified from Johnstons (1963) and Quraishi and Spencer (1971). ASW media was enrichment solution for *Dunaliella primolecta*. ASW was consist of $MgCl_2.6H_2O$ 4.7 g/L, K_2HPO_4 1 g/L, $NaNO_3$ 10 g/L. $FeCl_3.6H_2O$ 1.25 mg/L, $MnCl_2.4H_2O$ 0.8 g/L, Na_2EDTA 50 mg/L, $NaHCO_3$ 0.18 g/L, distilled water. The ingredients were dissolved in 200 ml of distilled water. The solution was boiling for 10 min while adjusting the pH to 7.6 with HCl or NaOH, filtered and bring to 250 ml. Sterilization was done by autoclaving at 15 lb/in^2 (103 kPa and 120^oC). The medium was using by adding 0.1 ml solution to each 10 mL of seawater. For induction of β-carotene synthesis, cells was grown in a sulfate-free medium ($MgCl_2$ instead of $MgSO_4$). BBM (Bold basal Medium) was consist of: KH_2PO_4 17.5 g/l; $CaCl_2.2H_2O$ 2.5 g/l; $MgSO_4.7H_2O$ 7.5 g/l; $NaNO_3$ 25 g/l; K_2HPO_4 7.5 g/l; NaCl 2.5 g/l; Na_2EDTA 10 g/l; KOH 6.2 g/l; $FeSO_4.7H_2O$ 4.98 g/l; H_2SO_4 1 ml/l; larutan "Trace Metal" 1 ml/l (H_3BO_3 2.86 g/l; $MnCl_2.4H_2O$ 1.81 g/l; $ZnSO_4.7H_2O$ 0.222 g/l; $NaMoO_4.5H_2O$ 0.39 g/l; $CuSO_4.5H_2O$ 0.079 g/l; $Co(NO_3)_2.6H_2O$ 0.0494 g/l; H_3BO_3 11.5 g/l; agar 1.5 %; pH 6.8.

2. Isolation and purification of an algae isolate

Mix culture of algae were came from BBAP (Balai Budidaya Air Payau Jepara) from Jepara SeaWaters, Indonesia. An isolate of green algae which was suspected as representing a strain of *Dunaliella* was a dominant species. "*Dunaliella*" were isolated and purified by growing in ASW agar media and BBM agar media under high light intensities (1000 lux) treatment using Halogen lamps in the dark room. Single cell colony of "*Dunaliella*" were picked up and grown in 250 ml flasks with 100 ml BBM media under agitation and illumination (Rabbani *et al*., 1998).

3. DNA Extraction

Preparation of a DNA genomic algae isolate was carried out by modification of CTAB methods (Sambrook *et al.*, 1989; Ausubel *et al*, 1995). 15 ml Culture of algae were centrifugated 13.000 rpm for 3 minutes. Pellet were pulverized on cold mortar and pestled to a fine powder. 1 ml Warm CTAB extraction buffer [(2 % (w/v) CTAB, 100 mM Tris-HCl pH 8; 20 mM EDTA pH 8; and 1.4 M NaCl, 1 % (w/v) pre warmed on 65 °C] was added to the pulverized algae and mixed to wet thoroughly. 25 µl Lisozyme enzyme with concentration 25 mg/ml was added to suspension, homogenized and incubated in waterbath on 37 °C for 1 hour with occasional mixing. 750 ml of SDS 10% was added, incubated again in waterbath on 37 °C for 1 hour. The extraction was incubated in waterbath on 65 °C for 1 hour with occasional mixing. The homogenate was extracted with an equal volume of chloroform, and mixed well by inversion. The homogenate was centrifugated 5 min at 13.000 rpm in microcentrifuge. The top (aquaeous) phase was recovered and the supernatant was removed. The nucleic acid was precipitated by adding 0.6 vol isopropanol and 1/10 vol Sodium asetat 3 M. The suspension was incubated over night on -20 °C. The suspension was centrifugated 5 min at 13.000 rpm. The pellet was washed with 100 µl etanol 70 %, air dried and resuspended in 50 µl TE buffer(10 mM Tris pH 8; 1 mM EDTA pH 8). DNA was purified with RNaseA and incubated in waterbath on 37°C for 1 hours. DNA was kept on -20 °C or used directly for PCR.

4. Amplification of *Dunaliella* sp. 16S rRNA

The 16S rRNA fragment was amplified by PCR using spesific primers. Sequence of forward primer was 5'- AGAGTTTGATCMTGGCTCAG-3', reverse primer was 5'-TACGGYTACCTTGTTACGACTT-3' corresponding to base pairs 1541 respectively (Widada, 2005 - pers.comm.). PCR was carried out in mixture containing 50 ng of genomic DNA, 2.0 mM of $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate, 2.5 pmol of each primer, and 1.8 U of Taq Polymerase and ddH_2O until volume 25 µl.

PCR conditions were performed with *hot start* for 2 minutes at 94 °C, denaturation for 1 minutes at 94 °C, *annealing* for 1 minutes at 50 °, polimerization for 1 minutes at 72 °C, extra extention at 72°C for 2 minutes, with 30 cycles of PCR reactions. In this PCR, a single DNA fragmen of 1.5 kb was amplified.

5. Sequencing and Phylogenetic Analysis

The 16S rRNA fragment were sequenced on BPPT Jakarta. Sequencing process involves several steps. First step was purification of amplification product of PCR by Qiagen Purification Kit. The next step was *cycle seq* process. The reaction composition consist of DNA tempate, primers, buffer, ddH_2O and *big dye* (DNA polymerase enzyme, ddNTP, and dNTP). The last step was sequencing using ABI Prism 310 *sequencer*.

Sequence of 16S rRNA of a green algae isolate was analyzing by similarity (homology). Sequence data was submitted to GeneBank website at www.ncbi.nlm.nih.gov and European Bioinformatics Services website at www.ebi.ac.uk . Setting up database search was using BLASTN Program. Database searches and phylogenetic analyses also performed with 16S rRNA sequence were retrieved from public and proprietary genomic sequence databases. Preliminary sequence data were also obtained from GeneBank. The nucleotides were aligned using the program ClustalX and CLUSTALW version 1.7) with the BLOSUM62 similarity matrix and gap opening and extension penalties of 10.0 and 0.05, respectively. Phylogenetic trees were constructed by neighbor-joining (NJ) methods with PHYLIP version 3.5c. for each set of alignments.

RESULT AND DISCUSSION

Using the archaebacteria primers, we are able to amplify a 1019-bp fragment from a green algae isolate. Electroferogram of a green algae isolate 16S rRNA amplification was showed one clear band as illustrated by Fig. 1. Sequencing result of base pairs from 16S rRNA from a green algae isolate was showed by Fig 2.

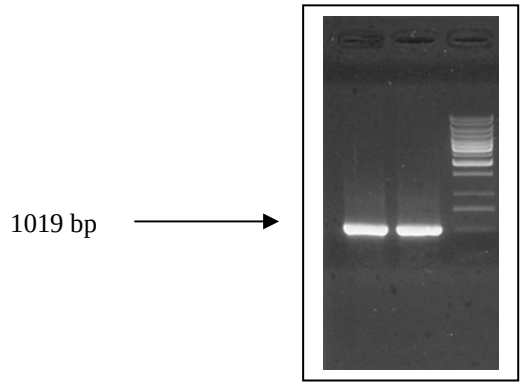

Figure 1. Electroferogram 16S rRNA of a green algae isolate

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           *         20          *         40          *         60
5′-CGTCCTTGCT TCCGACCTCC CCGAGGCTTT TCGCAGGTTG CTACGTCCTT CTTCGCCTCT
           *         80          *        100          *        120
   GTGTGCCTAG GTATCCACCA TCGGCCCTTC TTTTCTTGAC CTTTTTTTCC CTATTTTTCT
           *        140          *        160          *        180
   ATGCAGTTTT CAAGGTTCTC ACTGAACACT ACGTTCAGAA GTGGGGACAT CTCACCGGGC
           *        200          *        220          *        240
   TAAACTCGTT CTTCTGGAGG TAAGCGGACT CGAACCGCTG ACATCCTGCT TGCAAAGCAG
           *        260          *        280          *        300
    GCGCTCTACC AACTGAGCTA TACCCCCAAT GGGCCATCCT GGACTTGAAC CAGGGACCTC
           *        320          *        340          *        360
    ACCCTTATCA GGGGTGCGCT CTAACCACCT GAGCTAATAG CCCCAACACC TCTTTCTTTA
           *        380          *        400          *        420
    TTTTTCTAAC CATCCACCAT TCCTTGCTGA CTTCTTATCC GAAGTCTCCC TTAAAGGAGG
           *        440          *        460          *        480
    TGATCCAGCC ACACCTTCCG GTACGGCTAC CTTGTTACGA CTTCACCCCA GTCACTAGTC
           *        500          *        520          *        540
    CCACCTTCGG CGCCTCCCTC CATTACGGTT GAGATAACGA CTTCGGGCGT GACCAACTTC
           *        560          *        580          *        600
    CATGGTGTGA CGGGCGGTGT GTACAAGACC CGGGAACGGA TTCACCGCAG TATGCTGACC
           *        620          *        640          *        660
    TGCGATTACT AGCGATTCCT CCTTCATGCA GGCGAGTTTC AGCCTGCAAT CTGAACTGTG
           *        680          *        700          *        720
    GCTGGGTTTG ATGAGATTCG CTCCACCTCG CGGTTTGGAA TCTTCCGAAT TTCGATGAAA
           *        740          *        760          *        780
    GTTGAGAGTG CCTAAGGGAA CGCAGAGACA GGTGGTGCAT GGCTGTCGTC AGCTCGTGTC
           *        800          *        820          *        840
    GTGAGATGTT GGGTTAAGTC CCGCAACGAG CGCAACCCTC GTCCTTAGTT GCCAGCATTA
           *        860          *        900          *        920
    AGTTGGGGAC TCTAGGGAGA CCGCCGGGGA GAACTCGGAG GAAGGTGGGG ATGACGTCAA
           *        940          *        960          *        980
    GTCAGCATGC CCCTTACGTC TTGGGCTACA CACGTACTAC AATGGTTGGG ACAAAGGGGT
           *       1000          *       1010          *       1019
    GCGAAACCGC GAGGTGGAGC GAATCTCATC AAACCCAGCC ACAGTTCATA TTGCAGGCT-3′
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Figure 2. Full length Sequence of 16S rRNA a green algae isolate (A= Adenine, T= Thymine, C= Cytosine, G = Guanine)

Sequence analysis of 16S rRNA fragmen nucleic acid in GeneBank and European Bioinformatics shows similar result. Analysis by multiple alignment methods revealed a close relationship of a green algae isolate with some member of Cyanobacteria as illustrated from Treeview in Fig. 3.

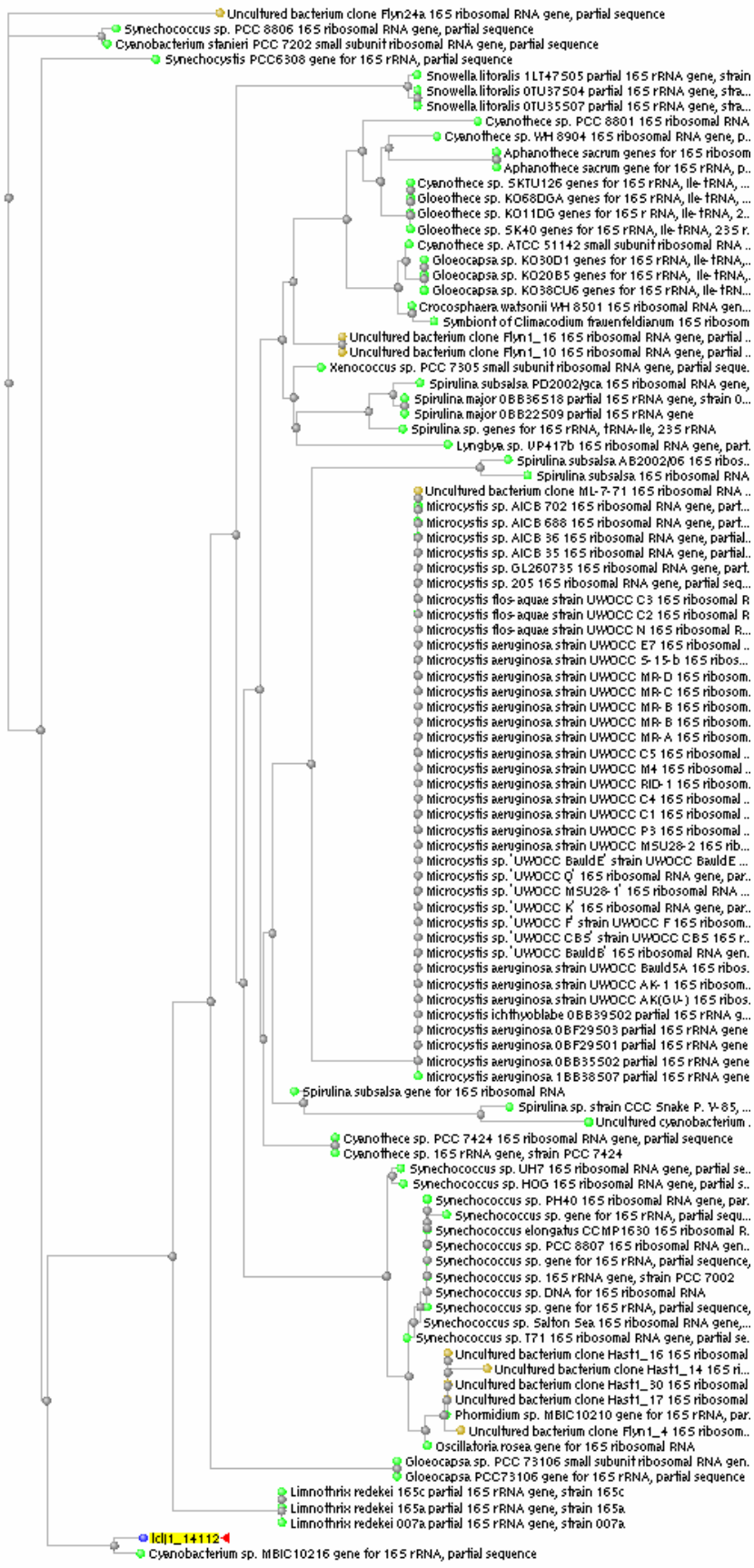

Figure 3. Phylogenetic Tree of a green algae isolate and some Cyanobacteria using Treeview Program (Idi1_14097 = a green algae isolate)

Homology analysis with Cyanobacteria members retrieved from GeneBank and European Bioinformatics demonstrates close similarities between a green algae isolates with those of *Cyanobacterium* sp. MBIC 120 with 99% homology and *Synechocystis* PCC6308 with 95% homology. Eight species sequence with most close homologies were used to construct phylogenetic tree by the neighbour-joining methods using 16S rRNA chloroplast of *Dunaliella salina* as an outgroup. As illustrated in Fig. 4. , a green algae isolate conformed homology analysis in forming a distinct clade with *Cyanobacterium* sp. MBIC 10216 and *Synechocystis* PCC6308 with 50% bootstrap confidence value. This clade was clustered also with *Cyanobacterium stanieri* PCC7202 and *Synechococcus sp.* PCC8806. Intraspecies homologies ranged from 99% homologies to 100%. However, an isolate can be readily assigned to one species when its homology with one of the sequence classes is higher that 99.1% Kusumaningrum (1999) as previous research that work with 50 species member of genus *Bacillus*.

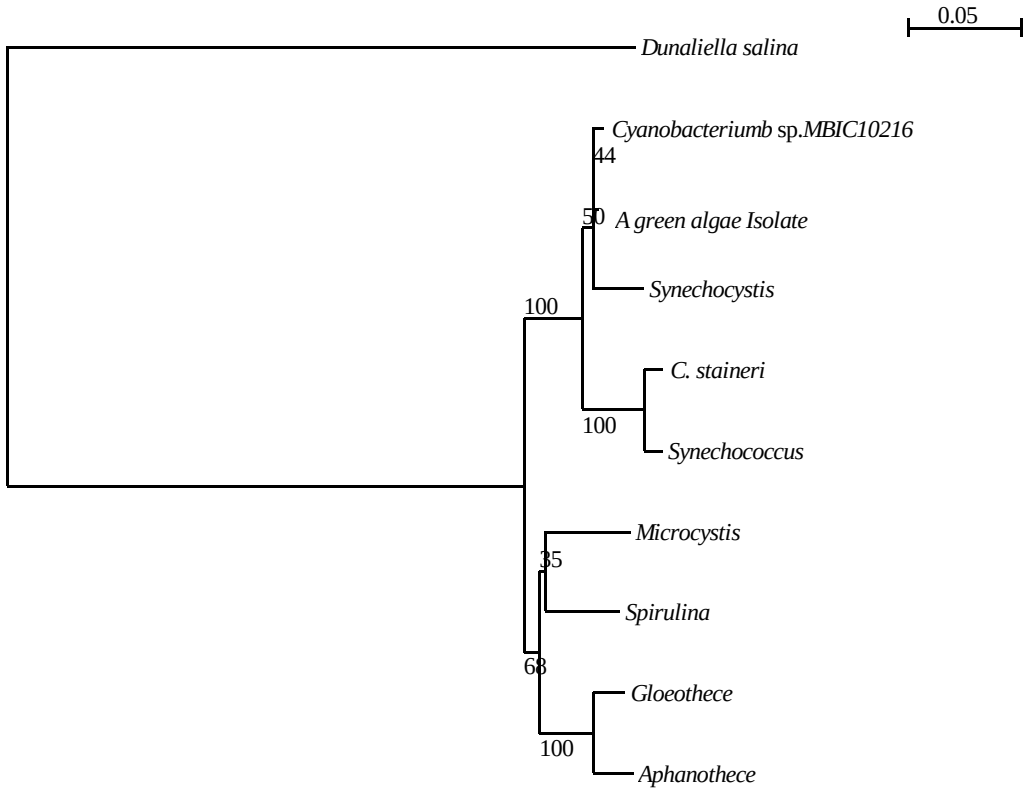

Figure 4. Phylogenetic tree among eight species having most close relationship with a green algae isolate

The microbiological characteristics of a green algae isolate from the previous research (Kusumaningrum *et al.*, 2006a) also confirmed this molecular analysis result, in exhibited a unique characteristic that was very different with all of the Cyanobacteria characteristics. We have no explanation for this discrepancy yet, it still need further experiment in improving possibilities that a green algae isolate might be one of the Prochloron member. Prochloron was a unicellular prokaryotic algae which was not a cyanobacterium. It resembeles cyanobacteria in its cell structure and biochemistry, but its photosynthetic pigments are similar to green algae (Tze, 1993).

	Cyanobacterium		*Synechocystis*		*Aphanothece*		*Microcystis*		
	Synechococcus		*IsolatCyanobacteriumsp.MBIC10216*				*Gloeothece*		*Spirulina*
	1	2	3	4	5	6	7	8	9
1	0								
2	0.152	0							
3	0.479	0.358	0						
4	0.155	0.242	0.454	0					
5	0.489	0.368	0.10	0.459	0				
6	0.375	0.476	0.664	0.351	0.659	0			
7	0.329	0.445	0.627	0.305	0.622	0.122	0		
8	0.400	0.516	0.665	0.346	0.660	0.190	0.217	0	
9	0.297	0.413	0.630	0.318	0.625	0.219	0.179	209	0
	1	2	3	4	5	6	7	8	9

Figure 5. Genetic distance matrix among among eight species having most close relationship with a green algae isolate

Genetic distance matrices constructed based on PCR product by GeneDoc Program apresented in Fig. 5. These matrices show the genetic distances ranging from 93% to 99%. This mens no high genetic variation among a green algae isolate with other species member of Cyanobacteria.

The result of all this analysis was a valuable parameter for the interpretation of observation on the identiyng the species of green algae isolate from Jepara Waters. From this analysis, it can be assumed that the species of green algae isolate was close to the member of Cyanobacteria. It means that a green algae isolate was prokaryotic algae, whereas *Dunaliella* was an eucaryotic green algae.

The result of this analysis also indicated possibilities that a green algae isolate following the new non-mevalonate pathway for its catotenoid biosynthetics as several other Cyanobacteria member especially *Synechocystis* and *Synechococcus.*

Conclusion

The results obtained through 16SrRNA based molecular characterization indicated that a green algae isolate showed close relationship with most member of Cyanobacteria with *Cyanobacterium* sp. MBIC 1021 especially with 99 % similarity and 95 % similarity with *Synechocystis* PCC6308. The result of this analysis indicated possibilities that a green algae isolate following the new non-mevalonate pathway for its catotenoid biosynthetics.

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