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MOLECULAR CHARACTERIZATION OF *Dunaliella salina* AND *Chlorella vulgaris* FUSANT USING 18SrDNA GENE

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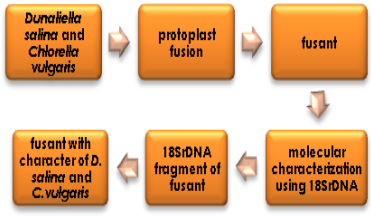
Graphical abstract



Abstract

Protoplast fusion was found to be an efficient method in improving carotenoid production from fusant of carotenogenic microalgae *D. salina* and *C. vulgaris*. Molecular characterization is needed for identifying the dominant parental genome in the fusant using ribosomal DNA sequences. The research was carried out by analyzing the gene encodes for 18S rDNA of fusant and determining relationship of fusant with *D. salina* and *C. vulgaris* species from GenBank. Quantitative analysis showed that *C. vulgaris* was not remarkably dominant in fusant with 84 % similarity compare to *D. salina* with 82 % similarity. The result indicated that the fusant gained both character from their parents.

Keywords: *Chlorellla*, *Dunaliella*, protoplast fusion, 18SrDNA

1.0 INTRODUCTION

Recent decades has shown remarkable developing of the biotechnology of microalgae. Valuable product for food and other applications will extend into broader area. Production of genetically improved strain by hybridization and somatic fusion on algae have been reported in algae. Protoplast fusion was done on intraspesific species green microalgae *Chlamydomonas reinhardii* (P. A. Dang, 1888) [1]. The protoplast fusion between two different phyla which sexually incompatible also has been conducted between the red alga *Porphyridium cruentum* (S.F. Gray) Nägeli, 1849) with *D. bardawil* (Ben-Amotz & Avron, 1982) or *D. salina* (Dunal) Teodoresco 1905 [2]. *D. salina* is the source of β-carotene and glycerol [3, 4]. *Chlorella* is widely used as a healthy food and feed supplement, as well as in the pharmaceutical and cosmetics industries [5]. Improvement of valuable metabolites from *Chlorella* and *Dunaliella* microalgae was done using biotechnological methods that allowed somatic hybridization by protoplast fusion [6]. This technique is required because crosses between two strains will not occur naturally and a diploid is desired. This intergeneric fusion also enables nuclear and cytoplasmic genomes to be combined, fully or partially, at the intergeneric levels. Carotenogenic microalgae which live in different environmental salinity, namely *Chlorella* and *Dunaliella* have produced stable hybrids possessing combined characteristics of the parents [6]. Their application as natural supplement for aquaculture animal was potential for synthetic feed substitution since it contains proteins, carbohydrates, lipids and vitamins, carotenoid as antioxidants, and trace elements [6–9]. Supplementation feeding on *Penaeus monodon* postlarvae with microalgae exhibited significant effect on growth, weight, survival related to microbial diseases resistance and immune response in high and low salinity [10–12]. In the present study, fusant

between *D. salina* and *C. vulgaris* were obtained. The purpose of this study was conducting molecular characterization on fusant using 18SrDNA to asses genetic exchange among parental strains during fusant formation. The methodologies were conducted by isolation and amplification of 18SrDNA region of fusant, followed by analysis on variable and conserved sequences that were contained in the 18SrDNAs of the organisms. The comparison of rDNA sequences between parental and progeny is a potencial tool for deducing dominancy and combination of each parental to improve their advantages in fusant. The present study expands on the use of DNA technology for the genotype within the fusant comparing to parental.

2.0 EXPERIMENTAL

2.1 *D. salina* and *C. vulgaris*

D. salina and *C. vulgaris* microalgae were obtained from Brackishwater Aquaculture Development Centre (BBPBAP) on Jepara, Indonesia. They were held in seawater tanks, recirculated and aerated, with the temperature set at 25 °C to 28 °C and salinity at 30 ‰ to 32 ‰. The tanks were cleaned daily. The microalgae were cultivated using sea water enriched with Walne media. A two–component gel, it is easy to modify the molecular structure of either of the two components.

2.2 Microalgae Media

Walne media for microalgae growth and cultivation consist of H_3BO_3 3.36 g L^{-1}, $NaNO_3$ 10 g L^{-1}, $FeCl_3$ 0.15 g L^{-1}, $MnCl_2.4H_2O$ 0.36 g L^{-1}, Na_2EDTA 45 mg L^{-1}, NaH_2PO_4 20 g L^{-1}, trace metal solution 1 mL L^{-1}, and distilled water. Trace metal solution was consist of H_3BO_3 2.86 g $\cdot L^{-1}$; $MnCl_2.4H_2O$ 1.81 g L^{-1}; $ZnSO_4.7H_2O$ 0.222 g L^{-1}; $NaMoO_4.5H_2O$ 0.39 g L^{-1}; $CuSO_4.5H_2O$ 0.079 g L^{-1}; $Co(NO_3)_2.6H_2O$ 0.0494 g L^{-1}; pH 6.8. The ingredients were dissolved in 200 mL of distilled water. The solution was boiled for 10 min while adjusting the pH to 7.6 with HCl or NaOH, filtered and bring to 1 L. Sterilization was done by autoclaving at 15 lb in^{-2} (103 kPa and 120 °C). The medium was using by adding 0.1 mL solution to each 10 mL of seawater [13, 14].

2.3 DNA Extraction

The DNA was extracted from 3 L media of the fusant in logarithmic phase of growth. The preparation of a DNA genomic of fusants was carried out by modification of CTAB methods [15, 16]. DNA was kept on -20 °C or used directly for PCR.

2.4 Amplification of 18SrDNA Microalgae

The 18SrDNA fragment was amplified using specific primers. Sequence of forward primer was 5'–GTAGTCATATGCTTGTCT–3', reverse primer was 5'–GCTGGCACCASACTTGCCCT–3' [17]. PCR was carried out in mixture containing 50 ng of genomic DNA,a 2.5 μL PCR buffer (KAPA), a 10 mM concentration of deoxynucleoside triphosphate mix (KAPA), 2.5 pmol of each primer, and 0.625 U (1 U=1/60 micro katal) of *Taq* Extra Hotstart DNA Polymerase and ddH_2O until volume 25 μL. PCR conditions were performed according to the PCR protocol using the manufacturer's instructions and protocols. To amplify the 18SrDNA, amplification reactions were performed on a T-Gradient thermocycler (Biometra GmbH, Gottingen, Germany). Aliquots (1 μL) of the reaction mixtures were analyzed by 0.8 % horizontal agarose gel electrophoresis to confirm the presence of product. The PCR products were purified using the Gel PCR Clean-Up System (Applied Biosystems, Foster, CA). Sequencing reactions were performed using a Dye Deoxy Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), and sequencing fragments were analyzed on a ABI Prism 377 DNA Sequencer.

2.5 Sequencing and Phylogenetic Analysis

The 18S rDNA partial fragment gene sequences from the fusant were searched against GenBank using BLAST as illustrated in Table 1. The nucleotides were aligned using the program ClustalX, respectively. Sequences containing fewer than 400 nucleotides or in excess of 1 000 nucleotides were removed, and sequences not belonging to *D. salina* and *C. vulgaris* microalgal species were discarded from this study. A phylogenetic tree was constructed using the neighbor–joining (NJ) algorithm [18] using Kimura's two–parameter model of sequence evolution, as implemented in the MEGA5 program package [19]. The bootstrap consensus tree inferred from 1 000 replicates [20] is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The evolutionary distances were computed using the Jukes-Cantor method [21] and are in the units of the number of base substitutions per site. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} . All positions containing gaps and missing data were eliminated.

Table 1 *Dunaliella* and *Chlorella* species rDNA sequence from Genbank used in this study

Species	Accs number	Species	Accs number
C. vulgaris	AB080308.1	*D. salina* strain KU13	KF825552.1
C. vulgaris isolate UMT-M1	KJ561358.1	*D. salina* strain KU07	KF825551.1
C. vulgaris isolate KS-MA2	KJ561357.1	*D. salina* strain KU11	KF825550.1
C. vulgaris	KF574391.1	*D. salina* strain KU07	KF825551.1
C. vulgaris cc CCAP 211/79	FR865683.1	*D. salina* isolate B32	HQ735296.1
C. vulgaris strain KTP2	KF746940.1	*D. salina* isolate I3	HQ735295.1
C. vulgaris strain nm27	JQ256478.1 |	*D. salina* strain B34	JF900404.1
C. vulgaris isolate YL-2	KP341004.1	*D. salina* strain B24	JF831044.1
C. vulgaris strain A1-65	KF661335.1	*D. salina*	EF195157.1
C. vulgaris strain CCAP211/21A	KJ756823.1	*D. salina* strain CCAP 19/12	KJ756842.1
C. vulgaris strain CCAP211/75	KJ756813.1	*D. salina* str BuriRam KU01	JN052202.1
C. vulgaris strain LC9	KF569735.1	*D. salina* strain I2	JF831045.1
C. vulgaris strain LC8	KF569734.1	*D. salina* strain CCAP 19/18	EF473745.1
C. vulgaris strain LF5	KF569724.1	*D. salina* strain SAG 19-3	EF473739.1
C. vulgaris strain AG-35_ZF1	AB699112.1	*D. salina* strain Dsge	EF473731.1
C. vulgaris cc CCAP211/11P	FR865658.1	*D. salina* strain KMMCC 1428	JQ315781.1
C. vulgaris strain CCAP211/110	FN298918.1	*D. salina*	M84320.1
C. vulgaris strain CCAP211/109	FN298917.1	*D. salina* strain CCC	HQ843776.1
C. vulgaris strain CCAP211/82	FM205855.1	*D. salina* strain JR102	EU589200.1
C. vulgaris strain CCAP211/81	FM205854.1	*D. salina*	EU239363.1
C. vulgaris strain CCAP211/80	AM231734.1	*D. salina*	AF506698.1
C. vulgaris strain CCAP 211/11F	AY591515.1	*D. salina*	M84320.1
C. vulgaris strain CCAP 211/63	FR865681.1	*D. salina* strain CCC	HQ843776.1
C. vulgaris strain SAG 211-11b	FM205832.1	*D. salina* strain JR102	EU589200.1
C. vulgaris strain KMMCC FC-15	HQ702287.1	*D. salina*	EU239363.1
C. vulgaris strain KMMCC FC-12	HQ702286.1	*D. salina*	AF506698.1
C. vulgaris strain KMMCC FC-12	HQ702286.1	*D. salina*	M84320.1
C. vulgaris strain KMMCC C-111	GQ122346.1	*D. salina* strain CCC	HQ843776.1
C. vulgaris strain KMMCCFC-16	HQ702294.1	*D. salina* strain JR102	EU589200.1
C. vulgaris strain KMMCCC-117	HQ702318.1	*D. salina* strain B33	JF831042.1
C. vulgaris strain KMMCCC-119	HQ702309.1	*D. salina* strain UTEX LB 200	DQ009779.1
C. vulgaris strain KMMCCFC-42	HQ702285.1	*Dunaliella* sp. BBPBAP	KC875350
C. vulgaris strain KMMCCEC-5	HQ702321.1	*D. salina* isolate NIOT-28(ANCOST-17)	KC470060.1
C. vulgaris strain KMMCCFC-33	HQ702295.1	*D. salina* strain CCAP 19/30	DQ447648.1
C. vulgaris cc KMMCC FC-1	GQ122369.1	*C. vulgaris* cc KMMCC C-27	GQ122334.1
C. vulgaris strain NIES-1269	AB488579.1	*C. vulgaris* var vulgaris strNIES-642	AB488577.1
C. vulgaris strain: PS-2670	AB488582.1	*C. vulgaris* var vulgaris strNIES-227	AB488575.1
Chlorella sp. *WO10-1*	FJ946886.1	*C. vulgaris* var vulgaris str NIES-641	AB488576.1
C. vulgaris strain KMMCCEC-10	HQ702292.1	*C. vulgaris* var vulgaris str NIES-686	AB488578.1
C. vulgaris strain KMMCCEC-3	HQ702293.1	*C.vulgaris* isolate D2	JX185298.1
C. vulgaris strain KMMCCFC-16	HQ702294.1	*C. vulgaris* strain IAM C-27	AJ242757.1
C. vulgaris strain KMMCCC-119	HQ702309.1	*C. vulgaris* strain KMMCCC-111	GQ122346.1
C. vulgaris strain KMMCCFC-42	HQ702285.1	*C. vulgaris* cc KMMCC C-88	GQ122340.1

Note: cc = culture collection; v= varian; accs = accession

3.0 RESULT AND DISCUSSION

3.1 Amplification of 18SrDNA fusant

The products of the 18SrDNA fusant amplification are shown in Figure 1. Several annealing temperature were applied due to anticipation of genetic diversity inside fusant genome. Almost all temperature annealing showed positive bands with 55.2 °C exhibited the best result. Those data confirmed that primer design was suitable to conserved region of fusant.

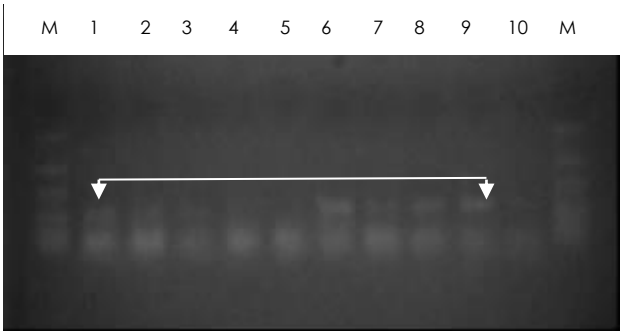


Figure 1 Amplification of 18SrDNA fusant with several annealing temperature using gradient PCR. Lane 1: molecular weight marker. Lanes 2 to 10: amplification with annealing temperature 45 °C; 46.6 °C; 48.4 °C; 50.9 °C; 52.9 °C; 55.2 °C; 57.2 °C; 59.9 °C; 61.7 °C; and 63 °C, respectively

Furthermore, the primer also can be used to amplify fusant and also obtained similar band from parental *D. salina* confirmed that primers are highly specific to *D. salina*. On the other hand, by applying primer on parental *C. vulgaris* single band was obtained but the sequences were not as good as *D. salina* and fusant.

This result indicated that the primer was not in the conserved region of *C. vulgaris* in amplifying specific band. Implication of this result also showed the divergence of sequences in *C. vulgaris* comparing with *D. salina*. However, this result has not interfered with the homology analysis of fusant sequences since *C. vulgaris* showed conserved region among them.

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Fusant     19  ATTGTACTCATTCCGATT-GCAGAACCCGAAGGGCTCCGCATCGTTATTTATTGTCACTACCTCCCTG  85
               |||||||||||||| |||  ||| | |  ||  || | | || ||||||||||||||||||||||| |
D.salina  469  ATTGTACTCATTCCAATTACCAG-A-CAAAAATGCCCGGTATTGTTATTTATTGTCACTACCTCCCCG  402

Fusant     86  TGTTAGGATTGGGTAATTTACGCGCCTGCTGCCTTCCTTAGATGTGGTAGCCGTTTCTCAGGCTCCCT  153
               |||| |||||||||||||| ||||||||||||||||||| ||||||||||||||||||||||||||||
D.salina  401  TGTTGGGATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCT  334

Fusant    154  CTCCGGAATCGAACCCTAATTCTCCGTTACCCGTTAACGCCACGGTAGGCCAATACCCTACCGTCGAA  221
               |||||||||||||||||||| |||||| |||||||| | ||| ||||||||  || |||||| |||||
D.salina  333  CTCCGGAATCGAACCCTAATCCTCCGTCACCCGTTACCACCATGGTAGGCCTCTATCCTACCATCGAA  266

Fusant    222  AGCTGATAGGGCAGAAACTTGAATGAACCATCGT-GCCG-AA-GCA---CGATTCGCTTAGTTATTAT  283
               || |||||||||||||| ||||||||| |||||  ||  || ||    |||||||   |||||| |||
D.salina  265  AGTTGATAGGGCAGAAATTTGAATGAAACATCGCCGGCATAAAGCCGTGCGATTCGTGAAGTTATCAT  198

Fusant    284  GACTCACCA-G-G--G---ATT---G-CTGG--TTGTATCTAATAAATACACCTCTTGC-GAGGTTGG  337
               || || ||| | |  |   |     | ||||  || |||||||||||||  | ||| | || || ||
D.salina  197  GATTCGCCAAGAGTCGGGCAAGCCCGGCTGGCCTTTTATCTAATAAATACGTCCCTTCCAGAAGTCGG  130

Fusant    338  -----ACGCATGTATTAGCTCTAGAATTACTACGGTTATCCAAGTAGTAGGGGACTATCAAATAAACT  400
               |||||||||||||||||||||||||||||||||||| ||||  |||  || ||||||||||||
D.salina  131  GATTTACGCACGTATTAGCTCTAGAATTACTACGGTTATCCGAGTAA-AGGT-ACCATCAAATAAACT  66

Fusant    401  ATAACTGATATAATGAGCCATTCGCAGTTTCACCGTATAA-AGGCTTATACTTAG-ACATGCA  461
               |||||||| |||||||||||||||||||||| ||||||   | |||||||||| |||||||
D.salina   65  ATAACTGATTTAATGAGCCATTCGCAGTTTCACAGTATAAGCAGTTTATACTTAGGACATGCA  3
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Figure 2 Similarity among fusant and *D. Salina*

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Fusant       1 CATGTCT-AGTAT-AGC--CTTTATACGGTG-AACTGCGAATGGCTCATTATATCAGTTATAGTTTAT  63
               ||||||| ||||| | |  |||||||| ||| ||||||||||||||||||| ||||||||||||||||
C.vulgaris    53 CATGTCTAAGTATAAACTGCTTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTAT  120

Fusant       64 TTGATAGTCCCCTACTACTTGGATAACCGTAGTAATTCTAGAGCTAATACATGCGT-----CCAACCT  131
               ||||| ||  ||||||||| ||||| ||||||||| ||||||||||||||| |||||      || || |
C.vulgaris   121 TTGATGGT-ACCTACTACTCGGATACCCGTAGTAAATCTAGAGCTAATACGTGCGTAAATCCCGACTT  187

Fusant     132  C-GCAAGAGGTGTATTTATTAGATACAA--CC-A----GCAAT--CC--CT---GGTGAGTCATAATA  185
               | | ||| |  |||||||||||||| ||  || |     ||  |  ||  ||   ||||| |||| |||
C.vulgaris   172 CTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGGGCTCTGCCCGACTCGCGGTGAATCATGATA  255

Fusant     186 ACTAAGCGAATCG--T-G-CTT-CGGC-ACGATGGTTCATTCAAGTTTCTGCCCTATCAGCTTTCGAC  241
               |||   |||||||  | | ||| || |  ||||| ||||||||| ||||||||||||||| |||||||
C.vulgaris   256 ACTTCACGAATCGCATGGCCTTGCGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGAT  323

Fusant     242 GGTAGGGTATTGGCCTACCGTGGCGTTAACGGGTAACGGAGAATTAGGGTTCGATTCCGGAGAGGGAG  309
               |||||| ||  |||||||| ||| | |||||||| |||||| |||||||||||||||||||||||||
C.vulgaris   324 GGTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGGGAG  391

Fusant     270 CCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTAACACAGGGA  377
               |||||||||||||||||||||| |||||||||||||||||| ||||||||||||||| |||||||||
C.vulgaris   352 CCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGA  459

Fusant     330 GGTAGTGACAATAAATAACGATGCGGAGCCCTT-CGGGTTCTGC-AATCGGAATGAGTACAATTTAAA  443
               |||||||||||||||||| || | | ||| || | ||| |||  ||| ||||||||||||| ||||
C.vulgaris   412 GGTAGTGACAATAAATAACAATACTGGGCCTTTTCAGGT-CTGGTAATTGGAATGAGTACAATCTAAA  525

Fusant     390 CCCCTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAG  487
               ||||||||||||||| |||||||||||||||||||||||||||
C.vulgaris   472 CCCCTTAACGAGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAG  570
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Figure 3 Similarity among fusant and *C. vulgaris*

3.2 Sequence Analysis of Fusant

Sequence analysis of 18S rDNA fragmen nucleic acid in GenBank GenBank and European Bioinformatics shows close relationship between fusant and *D. salina* strain BBPBAP and *C. vulgaris*. Analysis of homology between fusan and *D. salina* showed 82 % homology (Figure 2). Analysis of homology between fusan and *C. vulgaris* showed 84 % homology (Figure 3). Identities founded 320 in 435 with gaps 6 %.

Homology analysis inside the 18SrDNA sequences of fusant and parental identified 77 % similarity in the region as illustrated in Figure 4. Both *D. salina* and *C. vulgaris* exhibited conservation region between sequences and the similarity among them was 94 % in the 18SrDNA region which suggested that this sequence was well conserved between species of *Dunaliella* and *Chlorella*. Analysis and comparison of sequences on fusant and parental detected 77 % similarity with 33 SNPs and 24 deletion in the 18SrDNA region (Figure 4). A number of base were inherited between two parents into the fusant exhibiting by several substitution. Combination of bases from two parents into the fusant made differences from both parent but *C. vulgaris* tend to inherited more dominant comparing with *D. salina*.

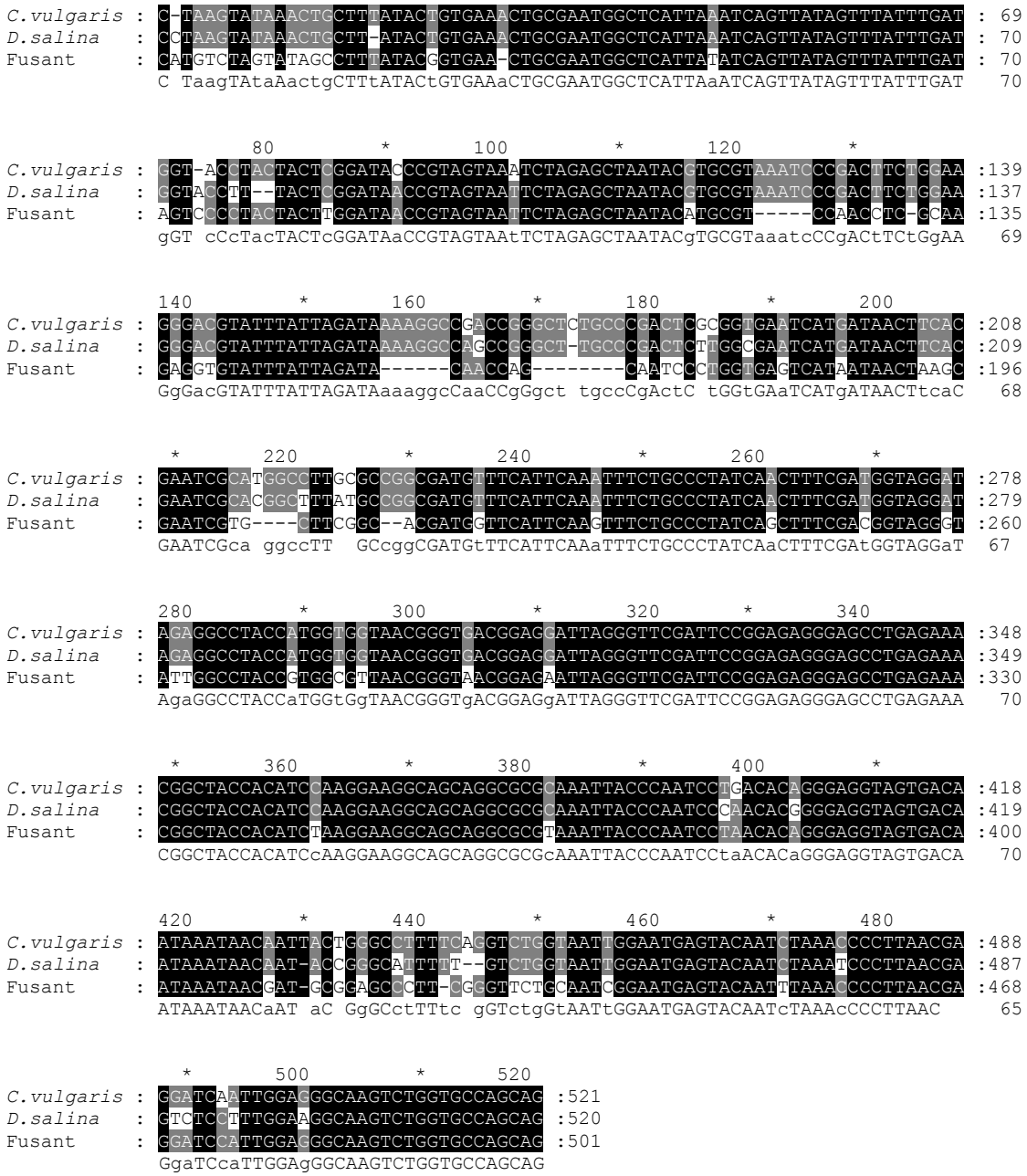

Figure 4 Homology region among fusant, *D. salina* and *C. vulgaris*

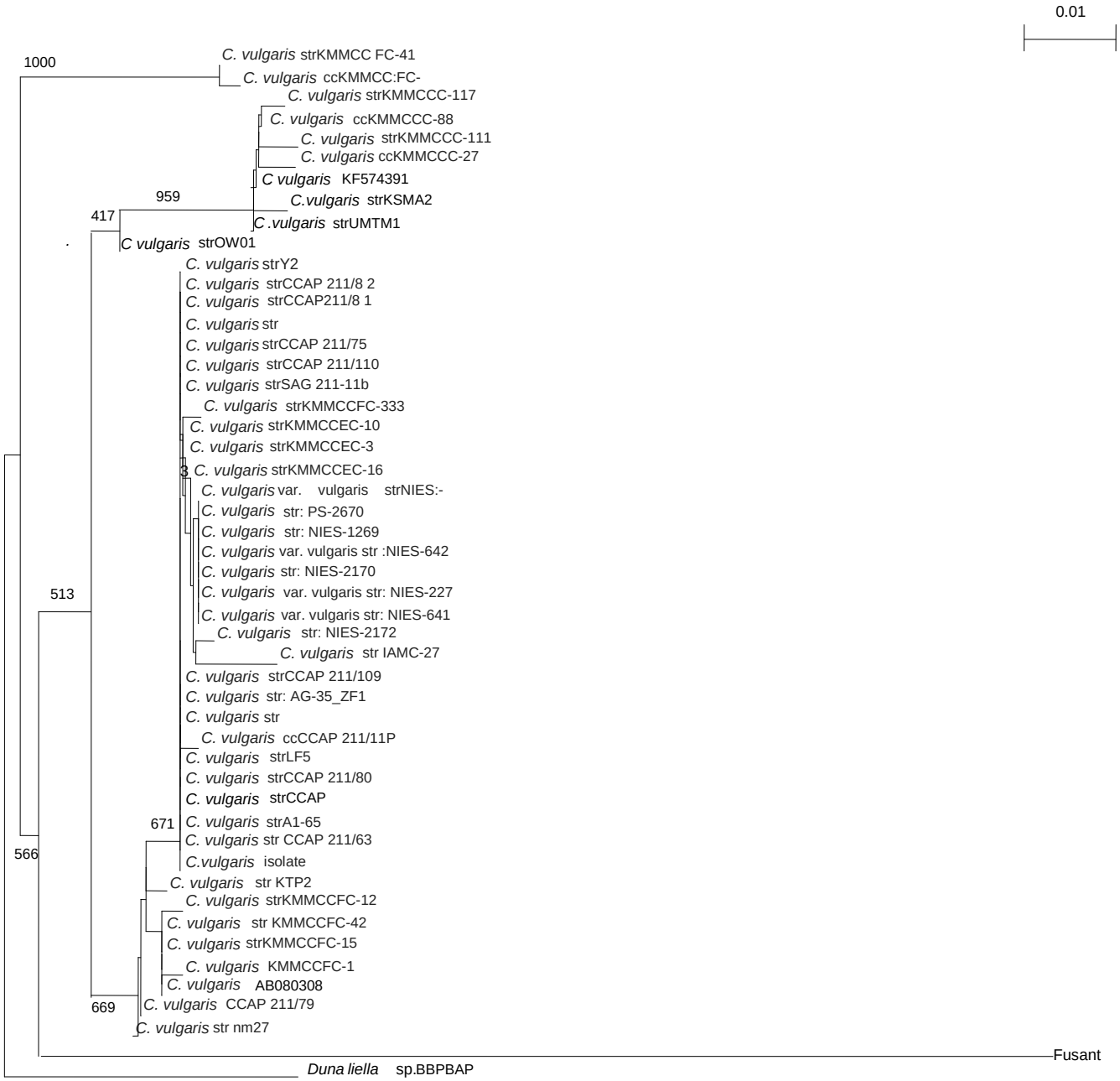

Figure 5 Phylogenetic evolutionary tree displaying the evolutionary relationship of fusant within a lineage shared by the *C. vulgaris* species with *Dunaliella* sp. BBPBAP as an outgroup (str = strain)

Selection on related similarity of fusant with other *D. salina* and *C. vulgaris* species with the genus *Dunaliella*, exhibited the 100 and 102 most related species. Alignment result from selected species showed that all the species had quite high percentage of similarity throughout the sequences (81 % to 85 %), presenting such differences inside the sequences. Analysis by multiple alignment methods revealed a close relationship of fusant with member of *D. salina* and *C. vulgaris* as illustrated from consensus tree using phylogenetic evolutionary analisis in Figure 5 and Figure 6. The reliability of the tree topology was estimated by bootstrapping. The 80 % bootstrap proportion consensus Neighbor Joining tree for 18SrDNA sequences is shown in Figure 5 and Figure 6.

Homology analysis with *C. vulgaris* species retrieved from GenBank is illustrated in Figure 5. which shows close similarities between a green algae isolates with those of *C. vulgaris* strain KMMCC FC-41 and *C. vulgaris* strain nm27. According to the phylogenetic tree, the studied fusant appeared as individual entity separated from the rest cluster. It was clearly shown

that fusant, instead of having almost equal similarities with all member of other *C. vulgaris*, it also had close relationship with *Dunaliella* sp. BBPAP as the other parental.

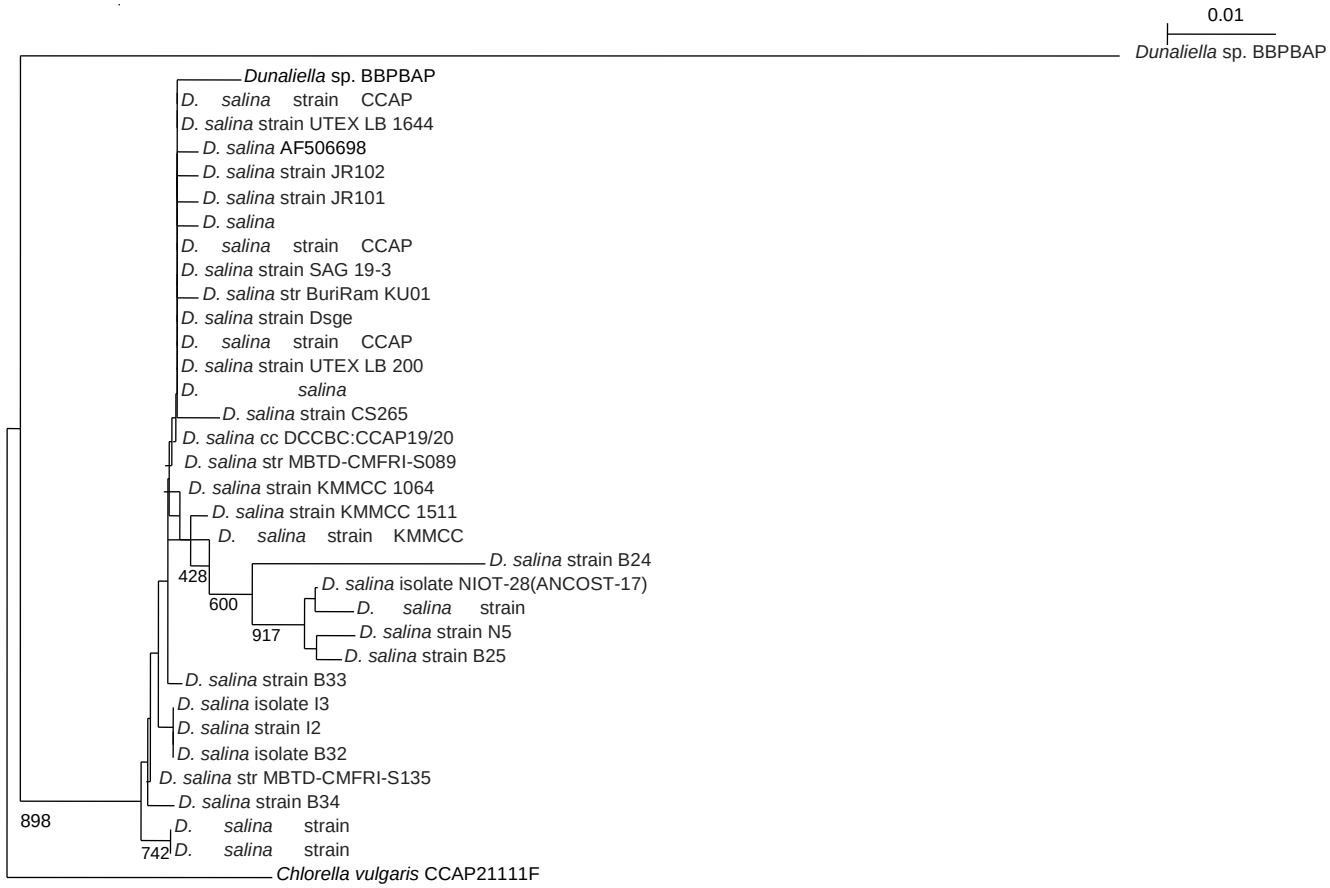

Figure 6 Phylogenetic evolutionary tree displaying the evolutionary relationship of fusant within a lineage shared by the *D. Salina* species. *C. vulgaris* is used as an outgroup sequence

Homology analysis with *D. salina* species in Figure 6. showed closest similarities between a green algae isolates with those of *D. salina* KU 13. However, phylogenetic evolutionary tree showed the position of fusant outside of the cluster of parental. These results suggested that fusant gained different character with parental species. In this sense, *C. vulgaris* seemed to be more related to *D. salina*, which was consistent with homology analysis result. Almost all of the fusant obtained from protoplast fusion process suggested higher total carotenoid production after the process compared with the parental strain (data not shown).

Although the two profiles of fusant and parental shared similarity, but the position of fusant in different cluster with parental indicating that there was indeed a difference in the cell of the two algal strains. On the basis of the gained results we could conclude that the dominant genotype in fusants between *D. salina* and *C. vulgaris* appertain to *C. vulgaris*. The difference in the bases profiles is further evidence that there is indeed a change at the genomic level. The result also showed that 18SrDNA gene can be used to calculate dominant genotype in fusant resulted from protopast fusion process.

4.0 CONCLUSION

Molecular analysis showed that *C. vulgaris* was more dominant in fusant comparing with *D. salina* based on homology analysis of 18SrDNA sequences. The result also indicated that the fusant gained both character from their parents due to conserved sequence of 18SrDNA in parental and progenitor. The research showed possibilities in potential acquisition of genomic combination of both parents.

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