JSMARTech
Journal of Smart Bioprospecting and Technology **p-ISSN**: 2686-0805 **e-ISSN**: 2714-7894
April 2023, VOL 04 NO 01 **DOI**:https://doi.org/10.21776/ub.jsmartech.2023.004.01.25

Phylogenetic construction of Green Algae Based on the *rbc*L Gene

Aliarani RosyidianaPutri[1], Galuh Arkana[1], Haikal Ageng Maulana[1], Septhyanti Aprilia Kavitarna[1], Rayhan Yugo Nurul Maulana[1], Muhammad Wisam Wira Sakti[1], Turhadi Turhadi[1,*], and Fatchiyah Fatchiyah[1,2]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, East Java, Indonesia, 65145
[2]Research Center of Smart Molecule of Natural Genetics Resource, Brawijaya University, Malang, East Java, Indonesia, 65145

Submission: 26 March 2023; **Revised**: 21 April 2023; **Accepted**: 30 April 2023

*__Corresponding author__: Turhadi Turhadi; **e-mail**: turhadibiologi@ub.ac.id

ABSTRACT. *Chlorophyta (green algae) is one of the algae that can be found in (both oceans and freshwater lakes) as well as land with temperate to dry climates. Chlorophyta or green algae can be found in oceans, freshwater lakes, temperate land, and dry climate land. In addition, chlorophyta can also be found in semi-aquatic environments, such as rocks, moist soil, and moist tree bark. This study aims to analyze the phylogenetic relationship among chlorophyta species based on rbcL gene to classify the diversity of green algae in Indonesia. The green algae gene rbcL sequence with 20 species was obtained from NCBI. The sequences obtained were aligned with ClustalW and the mutation patterns were analyzed. The aligned sequences were made phylogenetic trees by MEGA-X with the Maximum Parsimony method and 1000 bootstrap. The total percentage of transversions that occurred was 10.75%, while the percentage of transitions was 7.85%. The construction of the phylogenetic tree shows that the chlorophyta species are grouped based on their habitat in the same clade. Clad 1 consists of genera Chlorella, Coccomyxa, and Volvox with freshwater habitats and a genus of Parachlorella with soil habitat. Clade 2 consists of genera Chlorococcum, Tetradesmus, Crucigenia, and Desmodesmus with freshwater habitats and a genus of Chlorococcum with soil habitats. Clad 3 consists of genera Ulva and Chlorella salina with marine habitat. The furthest pairwise distance analysis results were shown by Coccomyxa subellipsoidea and Sargassum schnetteri with a value of 1.18902. The kinship of the species of the Chlorophyta division based on the rbcL gene is divided into 3 clade separated by habitat. The phylogenetic tree in the Chlorophyta division shows that the rbcL gene is sensitive enough to separate among genera and can be utilized in phylogenetic studies.*

Keywords: *Chlorophyta, habitat, mutation, phylogenetic, rbcL*

INTRODUCTION

Development of potential biological resources in various fields is one of the things that has become a hot topic in recent decades. Macroalgae are low-level plants in the form of thallus which cannot be differentiated among the roots, stems and leaves. One group of macroalgae is green algae (Chlorophyta), which is a type of macroalgae that contains chlorophyll a and b so that it shows green pigments [1]. Chlorophyta can be found in waters (both oceans and freshwater lakes) as well as land with temperate to dry climates [2]. In addition, Chlorophyta can also be found in semi-aquatic environments, such as rocks, moist soil, and moist tree bark [3]. Green algae generally have thallus in the form of filaments with branches or unbranched and some have leaf-like forms. The optimal temperature for algae growth is 24-30 °C with an optimal pH of 8.1-8.2 and salinity around 30 ppt [4]. One of the methods to preserved is phylogenetic study, the analysis of kinship based on the characteristics of their ancestors [5,6,7].

A phylogenetic tree is a grouping of organisms to analyze their evolutionary relationships using molecular analysis with sequences from a single gene or single protein that are described through the form of branching [8]. Phylogenetic can be constructed with DNA sequences which have the advantage of being faster and better for determining the kinship of living things [7]. The nucleotide sequences used to construct a phylogenetic tree can be orthologous genes from various taxonomies. This is an alternative for identification through molecular data such as DNA or protein which can take a long time because it requires laboratories and other sophisticated equipment [9,10]. There are many construction methods are Maximum Parsimony (MP), Maximum Likelihood (ML), Neighbor-Joining (NJ), and Unweighted Pair Group Method with Arithmetic Mean (UPGMA). One of the methods widely used in phylogeny

construction with nucleotide sequences that have strong similarity is Maximum Parsimony (MP). MP is an approach or method that can be used to construct a phylogenetic tree by using a character in the form of a nucleotide or amino acid sequences and the closest distance that refers to the status of that character. In this method, calculations are performed to find all trees with the best potential and have a topology in the form of the smallest change that occurs (the smallest number of steps needed to produce a variation in the sequence) considered to explain the entire evolutionary process, so that the construction of the tree is the best to choose. If there is no homoplasy (repeated replacement) in the positions of the sequences being analyzed, then the resulting tree is close to the truth. The advantage of MP method is suitable for sequences that are similar and have short sequence lengths. Meanwhile, the disadvantages are that it takes quite a long time to run, construction can fail in different sequences (weak similarity), and does not provide consistent results in various evolutionary models, so adding data still has the possibility of being wrong [11,12].

Several genetic markers from specific regions within DNA sequences that can be used for phylogenetic analysis are Internal Transcribed Spacer (ITS), *psbA-trnH*, and *rbc*L. [13]. The *rbc*L gene is a gene that encodes the large subunit of the photosynthetic enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo) and is located in the chloroplast DNA (cpDNA). The location of the gene in the chloroplast allows it to be used as a DNA barcode. This is because this region has universality so that it can be analyzed easily. *rbc*L has many supporting characters to be studied phylogenetically. This is because rbcL has a nucleotide sequence around 1400 bp and has a low probability of mutation than other biological markers in cpDNA. The low probability of mutation in *rbc*L is due to high degree of similarity among species [14]. The major regulatory enzyme that catalyzes CO2 fixation and the dual function reaction of Ribulose diphosphate oxygenase is encoded by the *rbc*L gene. The RuBisCo holoenzyme consists of eight small subunits (4–15 kDa). This small subunit is encoded by the family nuclear gene (rbcS). Meanwhile, eight large subunits (52-kDa) are encoded by a single gene (*rbc*L) in the chloroplast genome. The *rbc*L gene is more conserved than rbcS. Therefore, the *rbc*L gene is more widely used for research, for example in previous studies such as breeding *Camellia oleifera* cultivars to increase oil biosynthesis by increasing photosynthetic efficiency. This is based on reducing power, ATP, and other metabolites that support the synthesis of high amounts of fatty acids [15]. However, variation in *rbc*L generally occurs at taxonomic levels above species and variations at the species level are rare. This makes *rbc*L bad for species discrimination [13].

This study aims to analyze the phylogenetic relationship among green algae (Chlorophyta) species with *rbc*L gene to classify the diversity of green algae in Indonesia. Furthermore, this knowledge can also be one of the initial studies to prevent reduced biodiversity wealth in Indonesia. In addition, this research can also be used as a first step in developing the potentials of macroalgae in various fields.

RESEARCH METHODS

Materials

The tools used in this research are NCBI (www.ncbi.nlm.nih.gov) for data mining from green algae nucleotide sequences and MEGA-X for sequence alignment, mutation analysis, and construct the phylogenetic tree. The data used are *rbc*L gene sequences from 20 different species, that consist in groups including *Chlorella vulgaris*, *Scenedesmus obliquus*, *Ulva fasciata*, *Ulva lactuca*, *Chlorella sorokiniana*, *Chlorella pyrenoidosa*, *Parachlorella kessleri*, *Tetradesmus dimorphus*, *Coccomyxa subellipsoidea*, *Crucigenia mucronata*, *Volvox dissipatrix*, *Volvox reticuliferus*, *Ulva rotundata*, *Chlorella* sp., *Crucigenia quadrata*, *Chlorella salina*, *Chlorococcum hypnosporum*, *Chlorococcum* sp., *Chlorococcum tetrense*, *Coccomyxa* sp., and *Sargassum schnetteri* was selected as an out group.

Multiple Sequence Alignment (MSA)

Sequences that have been obtained from data mining, 20 species of green algae and 1 species of out group, were aligned with Multiple Sequence Alignment in MEGA-X. Alignment was performed with ClustalW. After aligned, transition and transversion mutations were identified.

Phylogenetic Tree Construction with Maximum Parsimony (MP) Method

Phylogenetic tree was constructed using data from MSA result of *rbc*L gene. Prior to construction, trimming was carried out on the DNA sequences resulting from MSA at the 3' and 5' ends, so that the total nucleotide sequence used was 344 bp. Trimming was done to remove uninformative site cause by substitution or deletion [26]. Then, phylogenetic tree construction was carried out using the Maximum Parsimony (MP) method with bootstrap 1000 times. Genetic distance (pairwise distance) is made with MEGA-X, then the results are saved in

excel format (xlsx). Next, green algae kinship were analyzed based on the result of phylogenetic tree.

rbcL Gene Mutation Pattern

Mutation patterns of the *rbc*L gene in green algae sequences from MSA were analyzed using the nucleotide pair frequencies approach in the "Statistics" menu on MEGA-X. Directional (16 pairs) was choose then the results are saved in MS Excel Workbook format (xlsx). The result of the mutation pattern analysis are converted into percent units and presented in table.

RESULTS AND DISCUSSIONS

Green Algae Polymorphisms

Visualization of the Multiple Sequence Alignment (MSA) result (Figure 1) shows that there are differences in nucleotide sequences in 20 green algae sequences and 1 brown algae. Those differences are caused by transitions, transversions, insertions, or deletions in the nucleotide sequences of the species. The occurrence of these changes may be called mutations or Single Nucleotide Polymorphisms (SNP) depending on the total percentage of nucleotides that change from the entire nucleotide sequence.

Table 1. Nucleotide mutation patterns (%), Transitions(1), Transversions(2)

	A	T	G	C
A	**24,13**	3,20(1)	1,45(2)	0,87(2)
T	2,91(1)	**23,26**	0,87(2)	2,33(2)
G	1,45(2)	1,16(2)	**16,57**	0,58(1)
C	0,58(2)	2,62(2)	0,58(1)	**15,70**

Based on the results of the MSA, transitions and transversions are the main factors for differences in nucleotide sequences. Transition is nucleotide change from one type of purine to another purine (A > G, G > A), as well as in pyrimidine (C > T, T > C). While the transversion is a change in purine base to pyrimidine or vice versa (A > C, A > T, G > C, G > T, C > A, C > G, T > A, T > G) (Vallejos-Vidal et al., 2020). The MSA result shows that the number of conserved sites was 103 sites or 29.94%, while the changed sites (variable sites) were 236 sites or 68.60% (Table 1). These results indicates that the species have mutated.

The percentage of mutations patterns on the nucleotides indicates that transversions are higher than transitions. The total percentage of transversions that occurred were 10.75% of the entire sequence. Meanwhile, the percentage of transitions in the sequence were 7.85%. In addition, areas that have neither transitions nor transitions are 79.66%.

Transitions and transversions in mutations are also called microlesion mutations. Microlesion mutations are closely related to the presence of chemical mutagens as triggering factors [16]. This is associated to the macroalgae habitats, that are in waters and soils. where chemical factors are the cause of mutagens in the species.

Phylogenetic Analysis

The phylogenetic tree of the *rbc*L gene of green algae (chlorophyta) was constructed using phylogenetic tree with Maximum Parsimony. The construction of the phylogenetic tree aims to see the kinship of species of organisms based on their evolutionary relationships. The organism sequences were obtained from mining at the NCBI site. The sequences obtained can be seen along with the accession numbers from NCBI in Table 2.

A phylogenetic tree has lines that show the evolutionary distance between species and common ancestor. The evolutionary distance can be seen in the short length of the line. The longer the line, the further the evolutionary distance and it can be said that the species has more advanced character. Meanwhile, the shorter the line, indicates a species has more primitive character [17]. The results of the phylogenetic tree construction with Maximum Parsimony (MP) from green algae (Chlorophyta) are presented in Figure 2.

Bootstrap values shown were inferred based on 1000 replicates to represent the evolutionary history of the taxa analyzed. The bootstrap value itself is a value to test the appropriate level of the model data set used. A clade in phylogenetics can be trusted if it has a bootstrap value more than 90% and cannot be trusted if the bootstrap value is 25%. Meanwhile, if the bootstrap value is more than or equal to 70%, then the results appear to be reliable or valid. Meanwhile, organisms with branches that are separate and located at the bottom are called out groups . In reconstructing phylogenetics, out groups are needed in order to polarize characteristics, both apomorphic and plesiomorphic. Apomorphic characters are characteristics that experience changes and are inherited from the in group , while plesiomorphic characters are characteristics that are primitive in the out group [18,19].

Phylogenetic analysis using the Maximum Parsimony (MP) method using MEGA-X showed a consistency index (0.433409), a retention index (0.563478), and a composite index (0.316218) and an index value of (0.244216) for all informative site in Parsimony. Percentage of phylogenetic tree replication i.e. taxa grouped together in a bootstrap test of 1000 x replicates. The MP tree

was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with a search level of 1, namely the initial tree was obtained by adding sequences randomly (10 replicates). Phylogenetic analysis using the MP method involved 21 nucleotide sequences with the codon positions found in this analysis, namely 1st+2nd+3rd+Noncoding. There were a total of 344 positions in the final dataset used in the phylogenetic analysis [20,21,22].

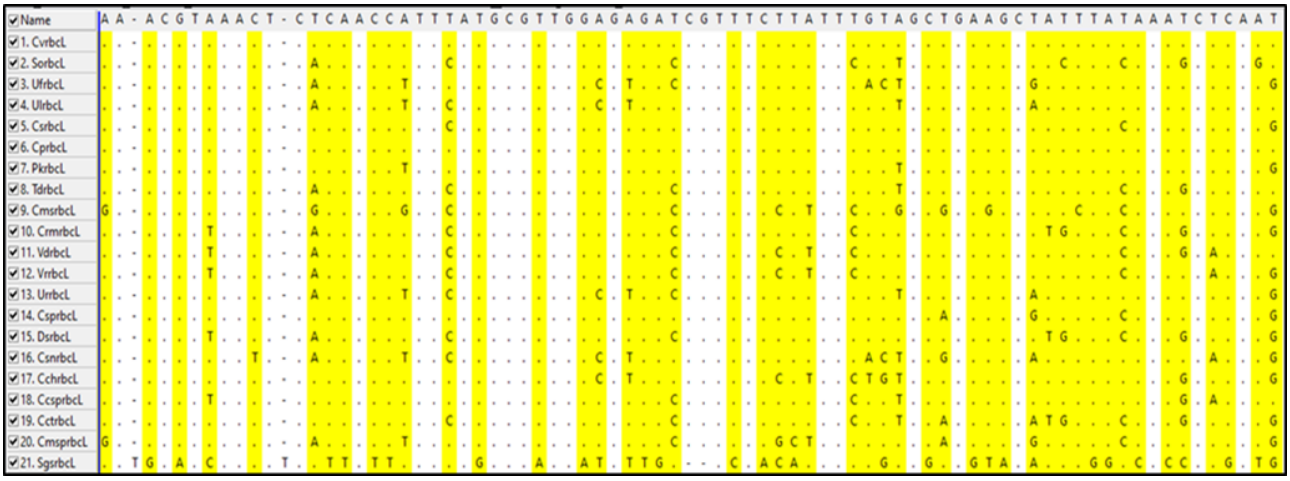

Figure 1. Visualization of differences in nucleotide sequences based on the results of Multiple Sequence Alignment (MSA) on 20 sequences of green algae + 1 brown algae.

Table 2. Sequences of organisms from NCBI data mining with accession number

Species name	Code	*Base pair* length (bp)	NCBI accession number	Habitats
Chlorella vulgaris	Cv*rbc*L	1428	MK295221.1	Freshwater
Chlorella pyrenoidosa	Cp*rbc*L	1428	EU038283.1	Freshwater
Chlorella sp.	Csp*rbc*L	1428	MK295222.1	Freshwater
Chlorella sorokiniana	Cs*rbc*L	654	MT647139.1	Freshwater
Parachlorella kessleri	Pk*rbc*L	676	KX021358.1	Soil
Coccomyxa subellipsoidea	cms*rbc*L	773	JF502548.1	Freshwater
Coccomyxa sp.	Cmsp*rbc*L	718	MT316600.1	Freshwater
Volvox dissipatrix	Vd*rbc*L	1128	LC427120.1	Freshwater
Volvox reticuliferus	Vr*rbc*L	1128	LC090154.1	Freshwater
Chlorococcum hypnosporum	Ch*rbc*L	772	LT594545.1	Soil
Chlorococcum sp.	Ccsp*rbc*L	773	KP698025.1	Freshwater
Chlorococcum tetrense	cct*rbc*L	703	LT594550.1	Freshwater
Scenedesmus obliquus	Sor*bc*L	852	JQ713934.1	Freshwater
Tetradesmus dimorphus	Td*rbc*L	889	KT777957.1	Freshwater
Crucigenia mucronata	Crm*rbc*L	1127	MW191598.1	Freshwater
Desmodesmus sp.	Ds*rbc*L	1084	GU192434.1	Freshwater
Chlorella salina	Csn*rbc*L	1382	MN519695.1	Sea
Ulva rotundata	Ur*rbc*L	1302	EU484404.1	Sea
Ulva fasciata	UfrbcL	1342	EU484418.1	Sea
Ulva lactuca	Ul*rbc*L	1300	EU484413.1	Sea
Sargassum schnetteri	Sgs*rbc*L	594	KF437962.1	Sea

Phylogenetic tree construction with Maximum Parsimony (MP) shows green algae (Chlorophyta) species grouped based on habitat in the same clade. Clade 1 consists of 9 species, *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Chlorella sp*., *Chlorella sorokiana*, *Coccomyxa subellipsoidea*, *Coccomyxa sp*., *Volvox dissipatrix*, *Volvox reticuliferus* with habitat taken from fresh water and one species of *Parachlorella kessleri* originating from soil habitat. *Volvox dissipatrix* and *Volvox reticuliferus* are paraphyletic, forming 2 branching points. Green algae in clade 1 are grouped based on the habitat when they were found, fresh water. However, there is a species originating from the soil, Parachlorella kessleri which is grouped into a freshwater habitat which is related to the species Chlorella sorokiana with a bootstrap value of 49. According to Nawkarkar et al. [23], Parachlorella kessleri was isolated from the Indian Ocean and also in fresh water with the CCALA 225 strain. Phylogenetic analysis showed that it had freshwater ancestor. Green algae in clade one are grouped based on the habitat when they were found, fresh water. However, there is a species originating from the soil, *Parachlorella kessleri* which is grouped into a freshwater habitat

which is related to the species *Chlorella sorokiana* with a bootstrap value of 49. According to Nawkarkar et al. [23], *Parachlorella kessleri* was isolated from the Indian Ocean and also in fresh water with the CCALA 225 strain. Phylogenetic analysis showed that it had freshwater ancestor.

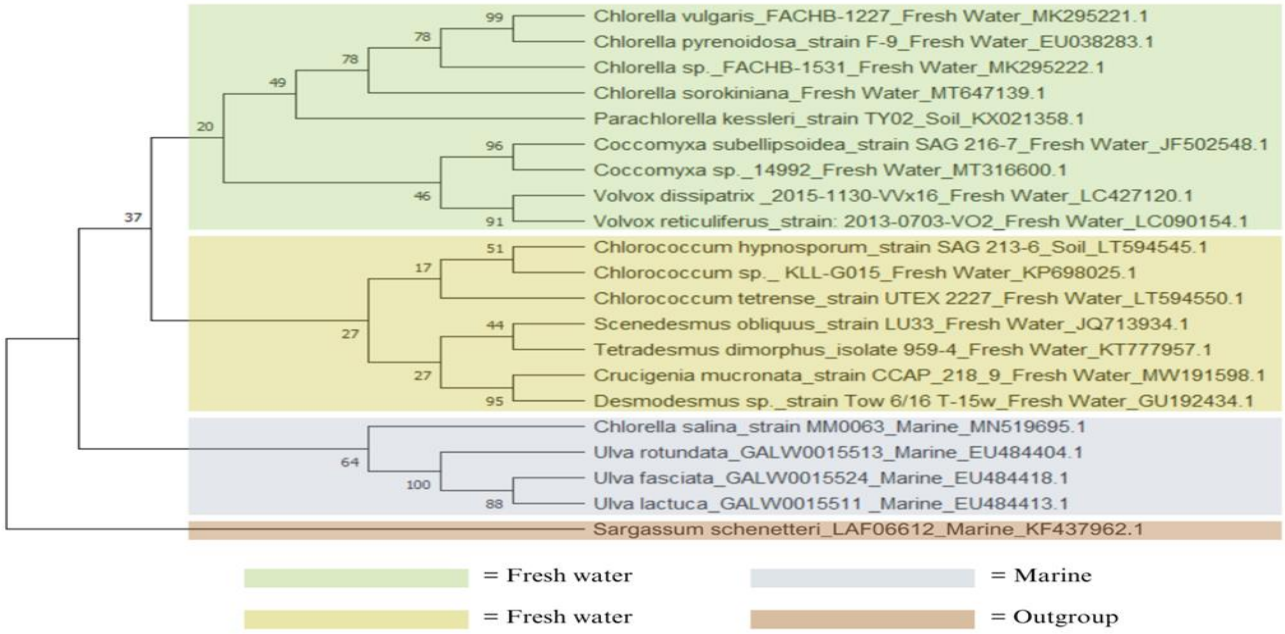

Figure 2. Phylogenetic tree of green algae (Chlorophyta) *rbc*L gene using Maximum Parsimony (MP) method.

Clade 2 consists of 7 species, namely with one species of *Chlorococcum hypnosporum* originating from soil habitats and six species which are *Chlorococcum* sp., *Chlorococcum tetrense*, *Scenedesmus obliquus*, *Tetradesmus dimorphus*, *Crucigenia mucronata*, *Desmodesmus* sp. originating from freshwater habitats. The results of clade 2 show that algae are grouped based on their freshwater habitat. There are algae from soil habitats that are grouped with freshwater habitats, namely *Chlorococcum hypnosporum* and *Chlorococcum* sp. with a bootstrap value of 51. Green algae species with different habitats are grouped into the same genus so that the two species are still closely related. According to Allaguvatova et al. [24], *Chlorococcum* is a genus commonly found in terrestrial ecosystems. *Chlorococcum hypnosporum* is found in the forest steppes of Ukraine. *Chlorococcum hypnosporum* belongs to a species of soil algae found in volcanic soils on volcanoes. grouped with freshwater habitats, namely *Chlorococcum hypnosporum* and *Chlorococcum* sp. with a bootstrap value of 51. Green algae species with different habitats are grouped into the same genus so that the two species are still closely related. According to Allaguvatova et al. [24], *Chlorococcum* is a genus commonly found in terrestrial ecosystems. *Chlorococcum hypnosporum* is found in the forest steppes of Ukraine. *Chlorococcum hypnosporum* belongs to a species of soil algae found in volcanic soils on volcanoes.

Clade 3 consists of 4 species that are *Chlorella salina*, *Ulva fasciata*, *Ulva lactuca*, *Ulva rotundata* which come from marine habitats. Below clade 3 is an out group species, *Sargassum schnetteri* as a comparison species. Clade 3 is dominated by the genus *Ulva*, but there is one species of *Chlorella* genus, *Chlorella salina*, which found in marine. Based on Figure 2, *Chlorella salina* is known to be related to *Ulva rotundata* with a bootstrap value of 64. Based on the formed phylogenetic tree, 3 clusters separate based on habitat in the kinship in Chlorophyta. Separation between genera shows the ability of *rbc*L to discriminate between species well. Species in the phylogenetic tree construction come from different genera. According to Kang et al. [13], variations in *rbc*L generally occur at the taxonomic level above species and variations at the species level are rare. The sensitivity of *rbc*L in Chlorophyta proves that *rbc*L has variation at the taxonomic level over species and makes it possible to utilize *rbc*L in phylogenetic studies.

Genetic Distance Analysis (Pairwise Distance)

Phylogenetic analysis was strengthened by the calculation of genetic distance values (pairwise distance). The results of calculating the pairwise distance of green algae (Chlorophyta) are shown in Table 3.

Table 3. Genetic distance of green algae (Chlorophyta) *rbc*L gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
CvrbcL																					
SorbcL	0.143021																				
UfrbcL	0.222489	0.179757																			
UlrbcL	0.222511	0.17215	0.036417																		
CsrbcL	0.055145	0.121585	0.222715	0.226275																	
CprbcL	0	0.143021	0.222489	0.222511	0.055145																
PkrbcL	0.114694	0.132755	0.210541	0.206446	0.104514	0.114694															
TdrbcL	0.142756	0.039441	0.172282	0.172103	0.107821	0.142756	0.13607														
CmsrbcL	0.236557	0.231906	0.282364	0.294357	0.211844	0.236557	0.267299	0.223411													
CmrbcL	0.154049	0.074423	0.194784	0.190857	0.139316	0.154049	0.164728	0.08099	0.23183												
VdrbcL	0.168394	0.142706	0.214729	0.214748	0.142712	0.168394	0.179741	0.135579	0.227838	0.153703											
VrrbcL	0.172049	0.128554	0.191487	0.203083	0.132086	0.172049	0.17597	0.121618	0.19262	0.153588	0.055121										
UrrbcL	0.214335	0.16457	0.036574	0.030167	0.210275	0.214335	0.198585	0.157202	0.271786	0.175719	0.210341	0.183381									
CsprbcL	0.055113	0.142868	0.222465	0.234294	0.058402	0.055113	0.125258	0.142702	0.223993	0.153647	0.172002	0.168281	0.222158								
DsrbcL	0.15036	0.101031	0.190945	0.190892	0.142816	0.15036	0.16829	0.101017	0.24562	0.058352	0.146566	0.139316	0.179472	0.146358							
CsrrbcL	0.194919	0.210662	0.199359	0.195391	0.218288	0.194919	0.254957	0.202607	0.305203	0.234515	0.218408	0.203011	0.175894	0.222407	0.227012						
CchrbcL	0.150009	0.139409	0.206428	0.210353	0.175772	0.150009	0.175915	0.153843	0.267745	0.15036	0.206327	0.20238	0.214206	0.172009	0.179861	0.206356					
CcsprbcL	0.146375	0.081051	0.190898	0.18711	0.135633	0.146375	0.143058	0.087628	0.250816	0.118147	0.146351	0.139265	0.194724	0.149922	0.150008	0.230188	0.118245				
CctrbcL	0.183501	0.107787	0.202394	0.202376	0.153555	0.183501	0.187572	0.114733	0.246892	0.121613	0.160773	0.146282	0.186982	0.160964	0.142947	0.23435	0.175697	0.11811			
CmsprbcL	0.202965	0.230858	0.260673	0.277396	0.191544	0.202965	0.219194	0.202847	0.184889	0.226686	0.227031	0.211091	0.255814	0.187526	0.231127	0.258133	0.276801	0.246872	0.272528		
SgsrbcL	0.936766	1.11673	1.000137	0.970204	0.965601	0.936766	0.91599	1.06251	1.18902	1.060682	1.059676	1.085226	0.996854	0.994151	1.005193	1.070342	0.944677	1.030757	1.058164	1.12248	

The results of the genetic distance analysis (pairwise distance) found that the closest genetic distance is *Chlorella pyrenoidosa* and *Chlorella vulgaris* with 0 pairwise distance values. This shows that these two species are very closely related and have a resemblance to each other. Meanwhile, the farthest distance is *Coccomyxa subellipsoidea* and *Sargassum schnetteri* with pairwise distance value of 1.18902. According to Huber et al. [25], the distance in phylogenetic construction is defined as a pair of taxa indicating the number of character changes to the DNA nucleotide base or a genetic distance between the taxa pair. Genetic distance generally obtained from Multiple Sequence Alignment (MSA).

CONCLUSION

The genetic relationship of Chlorophyta species based on the *rbc*L gene is divided into 3 clusters separated by habitat. The cluster includes the first and second clusters which from fresh water, and the third cluster is from marine. The phylogenetic tree shows that the *rbc*L gene is sensitive enough to separate among genera and can be utilized in phylogenetic studies. Further research needs to use sequence repetition at the level of genera to confirm the specification of the *rbc*L gene ability limit to a certain taxonomic level and marker other than *rbc*L is needed to use to confirm the relationship formed from the phylogenetic tree in this study.

ACKNOWLEDGMENT

We are thank to lecturers and assistants of bioinformatics Brawijaya University for assistance, correction and writing guidance.

REFERENCES

[1] Kepel, R. C. & D. M. H. Mantiri. 2019. Biodiversitas makroalga di perairan pesisir Kora-kora, Kecamatan Lembean Timur, Kabupaten Minahasa. *Jurnal Ilmiah Platax*. 7(2): 383-393.

[2] Purbani, D. C., D. R. Noerdjito., I. Purnaningsih., Y. Yuliani, & D. A. Prabowo. 2021. Analisis Morfologi Dan Filogenetik Molekuler Alga Hijau Coccoid Yang Diisolasi Dari Pulau Enggano. *Berita Biologi*. 21(3): 301-312.

[3] Fauziah, S. M. & A. N. Laily. 2015. Identifikasi mikroalga dari divisi chlorophyta di waduk sumber air jaya dusun krebet Kecamatan Bululawang Kabupaten Malang. *Bioedukasi: Jurnal Pendidikan Biologi*. 8(1): 20-22.

[4] Meriam, W. P. M., R. C. Kepel. & L. J. Lumingas. 2016. Inventarisasi Makroalga Di Perairan Pesisir Pulau Mantehage Kecamatan Wori, Kabupaten Minahasa Utara, Provinsi Sulawesi Utara. *Jurnal Ilmiah Platax*. 4(2): 2302-3589.

[5] Palallo, A. 2013. Distribusi Makroalga Pada Ekonomis Lamun Dan Terumbu Karang Di Pulau Bonebatang Kecamatan Ujung Tanah Kelurahan Barrang Lompo. Thesis. Hasanuddin Makassar University. Marine Science Department.

[6] Izza, F. R., A. Retnoningsih, & K. K. Pukan. 2019. Pengembangan kunci determinasi tumbuhan hasil eksplorasi hutan wisata guci kabupaten tegal untuk sekolah menengah atas. *Indonesian Journal of Conservation*. 7(1): 119-130.

[7] Fietri, W. A., A. Razak, & Y. Ahda. 2021. Analisis filogenetik ikan tuna (*Thunnus* spp) di perairan Maluku utara menggunakan COI (*Cytochrome Oxydase* I). *BIOMA: Jurnal Biologi Makassar*. 6(2): 31-39.

[8] Kaya, G., C. Ezekannagha, D. Heider & G. Hattab. 2022. Context-Aware Phylogenetic Trees for Phylogeny-Based Taxonomy Visualization. *Frontiers in Genetics*. 13: 1-16.

[9] Erickson, D. L., & Driskell, A. C. 2012. Construction and analysis of phylogenetic trees using DNA barcode data. *DNA Barcodes: Methods and Protocols*. 395- 408.

[10] Rangkuti, A. B., A. Mawarni & R. R. Rangkuti. 2022. Phylogenetic reconstruction of tree species on the University of North Sumatra Campus, based on the *rbc*L gene. *IOP Conference Series: Earth and Environmental Science*. 1-5.

[11] Arbi, U. Y. 2016. Analisis Kladistik Berdasarkan Karakter Morfologi untuk Studi Filogeni: Contoh Kasus pada Conidae (Gastropoda: Mollusca). *Oseana*. 16(3):54-69.

[12] Hu, Y., S. Tiwari, K. K. Mishra, & M. C. Trivedi. 2019. Ambient Communications and Computer Systems. Springer. London.

[13] Kang, Y., Deng, Z., Zang, R., & Long, W. 2017. DNA barcoding analysis and phylogenetic relationships of tree species in tropical cloud forests. *Scientific report*. 7(1): 12564.

[14] Nurhasanah, Sundari, & N. Papuangan. 2019. Amplification and Analysis of *rbc*L Gene (Ribulose-1,5- Bisphosphate Carboxylase) of Clove in Ternate Island. *IOP Conf. Series: Earth and Environmental Science.* 276:012061.

[15] Chen, Y., B. Wang, J. Chen, X. Wang, R. Wang, S. Peng, L. Chen, L. Ma & J. Luo. 2015. Identification of Rubisco *rbcL* and *rbcS* in *Camellia oleifera* and their potential as molecular markers for selection of high tea oil cultivars. *Front Plant Sci.* 6(189):1-11.

[16] Lestari, E. G. 2016. **Pemuliaan tanaman melalui induksi mutasi dan kultur *in vitro***. IAARD Press. Bogor.

[17] Oktavia, R. E. & B. Badruzsaufari. 2021. Analisis Filogenetik Garcinia Spp. Berdasarkan Sekuens Gen Rrna. *Ziraa'ah Majalah Ilmiah Pertanian*. 46(2): 259-264.

[18] Russo, C. A. M. & A. P. Selvatti. 2018. Bootstrap and Rogue Identification Tests for Phylogenetic Analyses. *Mol. Biol. Evol*. 35(9):2327–2333.

[19] Pangestika, Y., A. Budiharjo. & H. P. Kusumaningrum. 2015. Analisis filogenetik Curcuma zedoaria (temu putih) berdasarkan gen Internal Transcribed Spacer (ITS). *Jurnal Akademika Biologi*. 4(4): 8-13.

[20] Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.

[21] Nei M. & S. Kumar. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

[22] Kumar S., G. Stecher., M. Li., C. Knyaz. & K. Tamura. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution*. 35:1547-1549.

[23] Nawkarkar, P., S. Chugh., S. Sharma., M. Jain., S. Kajla. & S. Kumar. 2020. Characterization of the chloroplast genome facilitated the transformation of parachlorella kessleri-I, a potential marine alga for biofuel production. *Current Genomics*. 21(8): 610-623.

[24] Allaguvatova, R. Z., A. Y. Nikulin., V. Y. Nikulin., V. B. Bagmet. & L. A. Gaysina. 2022. Study of biodiversity of algae and cyanobacteria of Mutnovsky and Gorely volcanoes soils (Kamchatka Peninsula) using a polyphasic approach. *Diversity*. 14(5): 375.

[25] Huber, K. T., L. V. Lersel, R. Janssen, M. Jones, V. Moulton & Y. Murakami. 2022. Level-2 networks from shortest and longest distances. *Discrete Applied Mathematics*. 306: 138-165.

[26] Steenwyk J. L., Buida T. J. III, Y. Li, X-X. Shen, & A. Rokas. 2020. ClipKIT: A multiple sequence alignment trimming software for accurate phylogenomic inference. *PLoS Biol*. 18(12): e3001007.