Identification of Local Isolate of Microalgae *Chlorella Vulgaris* using Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Large Subunit (rbcL) Gene

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**Abstract.** Identification of microalgae has relied on microscopic observation which is time-consuming and high accuracy requiring. Molecular identification provides a solution to identify microalgae accurately and quickly. This study aims to identify locally isolated of *Chlorella vulgaris* by using rbcL gene. The study used exploration methods. The sample was *C. vulgaris* obtained from the local region in Situbondo, Indonesia. *C. vulgaris* DNA was isolated using the kit Zymo Research Plant. The *C. vulgaris* DNA was amplified by PCR using rbcL gene primer then the sequencing of the PCR product was conducted. Phylogenetic analysis was conducted using the MEGA6 software. Research shows that DNA sequences were located at 593bp. After compared with the Chlorella sequences data in the GenBank using BLAST methods, it shows similarities of 88-99% and E-value of 0.0. The phylogenetic reconstruction show that the local *C. vulgaris* with code STB01 was clade with another *C. vulgaris*, the population originates from a common ancestor. Although all population groups are separated by ecogeography. Each population was still genetically similar to short distance among populations. Molecular identification of local *C. vulgaris* was successfully carried out using primers rbcL and local isolate *C. vulgaris* have a genetic similarity of 99% with *C. vulgaris* in others region in the world.

1. **Introduction**

Microalgae are very large biodiversity of which about 40,000 have been described or analyzed [1]. Microalgae are divided into 10 (ten) divisions and eight of them are unicellular forms. Of the eight divisions, six have been used as natural feed for fish farming. Each division of algae not only has a special function that contributes to the character of the group but also represents species that also distinguish numbers from other species [2]. Chlorophyta was of ancient descent microalgae having diverse taxonomies and already be described around 8,000 species [3] and remain undescribed at least 5,000 species, in estimating value, especially in the tropics and subtropics [4].

The exploitation of algae is mostly limited to use as natural food for cultivated fish. However, microalgae can be used as a highly nutritious food supplement. Algae are a source of bioactive components that are beneficial to life. For example, *Chlorella sp.* is widely used as a supplement because it contains unsaturated fatty acids (omega 3, 6, and 9), fiber, vitamins, proteins, and minerals. *Chlorella sp.* and Spirulina can also be used as antioxidants [5] for the potential and properties of *N. oculata* microalgae show the feasibility as a raw material for biofuels [6].

One microalgae that have enormous potential to be utilized is *C. vulgaris*, which includes the following scientific classifications. The domain is Eukaryota; the kingdom is protista; the division is
Chlorophyta; the class is Trebouxiophyceae; the order is Chlorellales; the family is Chlorellaceae; the genus is Chlorella, and the species is *C. vulgaris*. Chlorella species, one of the most famous unicellular green algae, have been studied in the early research on photosynthesis and used as food and sources for biotechnology and commercial applications, such as feed, food additives, and bioenergy sources [7].

Green microalgae identification often difficult to be conducted and requires careful microscopic handling of observations by trained specialists [8]. DNA barcoding can provide a means for identifying green microalgae quickly and consistently, regardless of the life stage [9]. Potential targets of Chlorophyta DNA barcode include mitochondria (COI), chloroplasts (tufa, rbcL, and Cp23S) and nuclear genes (nuITS1, nuITS2, and 18S rDNA) [10]. DNA barcoding is a method applied for identifying a species based on the similarity of DNA sequences to species sequence databases described in priority [11].

The ribulose biphosphate carboxylase (rbcL) sequence method has been widely used in studies of phylogeny, evolution, population genetics, biogeography, and systematics because it can be easily repeated for related species. In many studies, the order of rbcL has been reported and it is clear that this primer has great potential and benefits for studying genetic variations of the natural population [12]. This study aims to identify green microalgae species isolated from Situbondo BPBAP Indonesia through the use of primary rbcL molecules as DNA barcoding.

2. Methods

2.1. Sample of *C. vulgaris*

*C. vulgaris* in the study was obtained from Balai Perikanan Budidaya Air Payau (BPBAP) Situbondo. Then, the sample was coded as *C. vulgaris* STB01.

2.2. Extraction of DNA and Total genomic DNA of *C. vulgaris*

Extraction of the genomic DNA was conducted using ZR Plants and Seed DNA MiniPrep™ Kit (Zymo Research). 150 mg of fine sample and 750 μl Lysis solution were filled into the tube. Next step was the centrifugation process in a microcentrifuge of ≥10,000 xg for 1 min. Then, the supernatant of 400 μl was filled into a Collection Tube and centrifuged of 7,000 xg for 1 min. DNA Binding Buffer (1,200 μl) was added to the filtrate in the Collection Tube and mixed it. The flow was discarded through from the Collection Tube before adding DNA Pre-Wash Buffer (200 μl) into a Collection Tube and centrifuged of 10,000 xg for 1 min. The next steps were to fill DNA Wash Buffer (500 μl) into the Column and centrifuged of 10,000 xg for 1 min then the Column was put into a microcentrifuge tube 1.5 ml. DNA Elution Buffer of 20 μl (10 μl minimum) was filled into the column matrix. The column was centrifuged of 10,000 xg for 30 s to elute the DNA before the eluted DNA was transferred in a microcentrifuge tube 1.5 ml and centrifuged of 8,000 xg for 1 min. The filtered DNA is now suitable for PCR analysis. Calculation of total genomic samples of *C. vulgaris* was done using Nanodrop (Nano Photometer™, Implen).

2.3. The rbcL Gene Amplification

Polymerase Chain Reaction (PCR) analysis was performed using Primer rbcL Forward: (AAA GC CCA ACA GAG ACT TCA ATG) and rbcL Reverse: (GTA AAA TCA AGT CCA CCR CG). PCR conditions were as follows: (1) initial denaturation for 1 cycle at 95°C for 3 min, (2) denaturation for 35 cycles at 98°C for 10s, (3) annealing at 53°C for 30s, and (4) extension 68°C for 45s. Agarose gel electrophoresis 1.4% with the EtBr staining was used to show the rbcL PCR products. The sequences would be compared with the BLAST program at the NCBI database, to confirm PCR targets.

2.4. DNA Sequencing and Phylogenetic Analysis

The phylogenetic tree and taxonomic affinity of each of the two algal strains were determined by rbcL gene sequencing. The sequence was set to at least two different amplicons using BigDye® Terminator v3.1 Cycle Sequencing Kit. Phylogenetic relationships between specimens of *C. vulgaris* were analyzed.
based on rbcL gene data using Maximum Likelihood (ML) to infer phylogenetic relationships in the MEGA software (version 6).

3. Findings and Discussion

3.1. Sequence Analysis of C. vulgaris

Results of PCR amplification of C. vulgaris using the rbcL gene was shown in Figure 1.

![Figure 1. Results of PCR amplification of C. vulgaris using the rbcL gene](image)

The DNA concentration can be measured from the absorbance value at λ 260 nm, while its purity is obtained by looking at the ratio A260 : A280. The results of genomic DNA isolation showed that each sample was pure and the DNA obtained was of good quality. The results of DNA isolation were obtained at a concentration of 18 ng/μl and the A260/280 ratio had a value of 1.8. The purity value of DNA influences the success of amplification because the good purity value is obtained when visualization is done under UV light, then the DNA band formed can be seen more clearly. The range of numbers has met the requirements needed in the further molecular analysis [13].

The PCR results of the C. vulgaris sample using the rbcL gene showed amplified results with a length of 593 bp (Table 1). DNA sequencing obtained was then analyzed using the BLAST (Basic Local Alignment Search Tool) analysis to see the test sample homology with C. vulgaris. The base sequence homology analysis sequencing shows homology of 99% of 589 bases (Figure 2) and E-value of 0.0. Based on these results, the difference and similarity of the C. vulgaris sample with other sequences in the GenBank data center can be seen. The results show that the test sample sequences have a high homology level. This is supported by previous research [14] that with a homology level of 99% to 100%, it can be said that species are identical and identified as the species. It suggests that DNA sequences can be said to have homology if the E-value is smaller than e-0.4 [15].

Taxonomically, by referring to the results of BLAST and BOLD System v4 analysis, the species has the following characteristics. The phylum is Chlorophyta; the class is Chlorophyceae; the order is Chlorococcales; the family is Oocystaceae; the genus is Chlorella, and the species is C. vulgaris.
Table 1. Results of the sequence of *C. vulgaris* using Primary rbcL

| Assembly of sequences 593 bp | Sequences |
|----------------------------|-----------|
| 1 GTTACCCACAAA AAGCAGGTGC GCAGATTTAAA AAGATTATCG |
| 61 TTTAAACTAC ATTACTCTCTG ATTATCAACC AAGAAACTA CAGCAATTTCG |
| 121 TAGTACACCA CACCGAGTGT TACAGCAGA AGAAGCTTGG CTGCTGTGTG CTCAGAACATC |
| 181 TTTACAACTGT ACTCTGACCA CTGATATGAG AGAGGTTTCTA ACTGTTTGA ATPGATTAACAA |
| 241 AAGTCGCTGGT TTAGATATG TAGCAGTACC AAGCAGGAA GATACCATAT TTTGGATAT |
| 301 TGCTTTACCTT TTAGACCTTT TTTAGAAGAGG TTTGCATAGC AATTATATTA CTGCTATG |
| 361 AGTTAACACGT TTTGCTTTCG AAGCCTTCTG TGGATTACTG CTGAGATAGC TTCGATTC |
| 421 ACCAGATAT GTCAGAGCTT TCCAGAGAC TCCAGCAAGG AGTACGGATG AAAGGT |
| 481 ACTAAAGAAA TACGGCTGTT CCGTTTTAGG TGTGACTATT AAAAAAACAA TTTGGATTTTC |
| 541 TCCGAAAACAC TATGCGCTTG CAGTTTATGA GTGTATTCGG CGTGGATTTAT |

Figure 2. Results of BLAST analysis of *C. vulgaris* sample

3.2. Phylogenetic Analysis.

Based on the phylogenetic analysis of the research sequence with the code of *C. vulgaris* STB01 and the same genus sequence in the GenBank data, it was found that the species in this study were closer to *C. vulgaris* and *C. pyrenoidosa* (Figure 3). The phylogenetic tree describes the evolutionary lineage of species, organisms, or from one distinct ancestor. Based on the phylogenetic tree made, genetic relations between species in one population and between populations can be known. The phylogeny is also useful
for combining knowledge of biological diversity for structural classifications and for generating insight into events that occur during evolution [16].

The relationship between *C. vulgaris* STB01 and other species as the comparison is because they are in the same division, namely the Chlorophyta group. In addition, the similarity of *C. vulgaris* STB01 with *C. pyrenoidosa* may be because both have the same nature based on habitat. The phylogenetic tree obtained illustrates the changes that occur in the marker genes of each species. The longer a branch, the more changes that occur in the marker gene will be, and, as a result, the species in that branch can be said to be more advanced. The length of each branch represents the number of changes that occur in the character used, that very similar characters will be close to each other in the branching. The phylogenetic tree provides information about population classification based on evolutionary relationships because, in the reconstruction of phylogenetic trees, molecular data is more widely used and is considered more stable in the evolutionary process than morphological data.

![Phylogenetic Tree of C. vulgaris STB01](image)

**Figure 3.** Phylogenetic Tree of *C. vulgaris* STB01
4. Conclusion

Molecular identification of *C. vulgaris* was successfully carried out using rbcl primers with a sequence of 593 bp. The *C. vulgaris* isolate microalgae used has a similarity of 99% genetically with other *C. vulgaris* in the world.

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