Molecular identification and antioxidant test of Chaetoceros sp. from Gondol, Bali

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Abstract. Chaetoceros is one of the largest genera of microalgae that has more than 400 species and it is the primary producer in the marine ecosystem. Chaetoceros sp. has some of the pigment which is very important for their survival including chlorophyll and carotenoid pigments. This study aims to identify Chaetoceros sp. from Gondol Bali using ITS fragments, to understand its phylogenetic relationship between microalgae-based on their antioxidant activity. The results of ITS fragments identification and antioxidant activity of Chaetoceros sp. will be used to develop further research related to its potency. The method for microalgae DNA isolation was conducted using the Doyle and Doyle method following with amplification using ITS4 and ITS5 primer. Then, the sequences will be analyzed. The antioxidant activity will be performed by the DPPH method. The results of DNA isolation showed the concentration and purity for about 2842.1 ng / µl and1.97 respectively. PCR products from the amplification of the ITS fragment produced 882 bp. Phylogenetic analysis revealed that Chaetoceros sp. from Gondol Bali had a close relationship with C. muelleri KF 998567.1 and the antioxidant activity test showed IC50 values were about 72,386 ppm.

1. Introduction
In the vast ocean of Indonesia, there is an extraordinary biological diversity that lived including a microalga. From many types of microalga, Chaetoceros is one of the genera living and acting as a primary producer in the marine ecosystems. The existence of this microalga as a primary producer is due to the abundance of their living cells in the ocean [1].

C. gracilis has beneficial properties like antibacterial and antifungal compounds that effectively inhibited the growth of Vibrio harveyi and Staphylococcus aureus [2]. Moreover, some species of Chaetoceros have various pigments, such as chlorophyll and carotenoids. The other compounds, fucoxanthin, diatoxanthin, and diadinoxanthin were also discovered in the Chaetoceros in large amounts [3]. Several shreds of evidence proved that β-carotene and fucoxanthin pigment [4] were having antioxidant and anticancer properties, respectively. They also act as the protector of the chlorophyll from photooxidative reaction during photosynthesis activity [5]. In addition, the carotenoid pigment of Chaetoceros was contained high antioxidants against free radicals (DPPH). C. calcitrans unpredictably showed higher antioxidant activity compared with curcumin for inducing free radical inhibition. Furthermore, these advantages are important for the industrial application of Chaetoceros sp. such as cosmetics, pharmacy, and health products [6]. Therefore, investigation of those potential microalga using molecular identification is important to determine the potency of the species of Chaetoceros relating to their abundance of antioxidant types, especially carotenoid.
The molecular identification technique is used to identify the genetic relationship of this carotenoid producing microalgae accurately as well as to get the faster result [7]. The study about the relationship among species is related to the ribosomal gene or DNA ribosomal (rDNA). Generally, ribosomal DNA is known as the encoder region of the genome for ribosome RNA. This region contains several conservative areas such as rRNA 18S, 5,8S and 28S, respectively. Moreover, there are important variation regions among them known as ITS (Internal Transcribed Spacer).

*Chaetoceros* sp. has two regions of ITS inside it. Both of them, ITS-1 and ITS-2, present in different spaces. ITS-1 is located between the 18S gene and 5.8S gene while the ITS-2 is located between 5.8S dan 28S gene, respectively. ITS regions, intriguingly, they could have a behavior for mutating itself to be different and unique individual for each species. Therefore, those ITS methods are very useful for molecular identification to investigate *Chaetoceros* species. In addition, ITS was assessed to be the region with the highest varies of nucleotide among other genes, with the total amount of 85.84% which is suitable for phylogenetic study. ITS region is used to determine the species, indeed they were probably applied for other levels such as strain, as well [8]. Thus, this study is aimed to identify *Chaetoceros* sp. from Gondol Bali using ITS fragment as well as to quantify the antioxidant activity.

2. Material and methods

2.1. Sample preparation of *Chaetoceros* sp.

*Chaetoceros* sp. came from Gondol, Bali and they were cultivated using Walne media with illumination treatment at 500 lux for 24 hours. *Chaetoceros* sp isolate was incubated for 11 days in the controlled seawater as well as 30 ppt of salinity and 25°C of temperature condition. Pellet cell of microalgae was collected from 450 mL culture using a series of culture centrifugation, as a source for DNA isolation and antioxidant analysis. The process was continued until it obtained more than 0.3 grams of *Chaetoceros* sp. [9].

2.2. DNA Isolation of *Chaetoceros* sp

DNA isolation was performed using the Doyle and Doyle method [1][10].

2.3. Quantitative and qualitative assay of DNA

We measured the concentration and purity of the DNA sample using the Nanodrop 2000 spectrophotometer. Then, electrophoresis was carried out using gel agarose 2% to separate the DNA fragment obtained, using Electrical voltage at 100 V for 20 minutes. Finally, electrophoresis results were visualized by GelDoc BioRad XR+.

2.4. Molecular identification using an internal transcribed spacer

Molecular identification to acquire the ITS of DNA ribosomal gene of *Chaetoceros* sp microalga isolate was used PCR (Polymerase Chain Reaction) method. *Chaetoceros* sp amplification process was using ITS 4 and ITS 5 primers, consist of (5'- TCC TCC GCT TAT TGA TAT GC-3') and (5'-GGA AGT AAA AGT CGT AAC AAG G-3'), respectively. Furthermore, PCR reaction is proceeded by exceeding the hot start condition for 4 minutes at 94°C, continued by succeeding the 30 cycles, 1 minute of annealing process was performed for 3 minutes at 55°C, followed by chain elongation for 1 minute at 72°C. The last chain elongation step was conducted for 7 minutes at 72°C. The PCR mixture was consist of 12.5 µl of My Taq Red Mix (MgCl₂ 2.0 mM; dNTPs 0.2 mM; Taq1 Unit of DNA Polymerase), 3' µl of Genome DNA of *Chaetoceros* sp. 50 ng/µl as a template, 1.5 µl of Primer forward 10 pmol, and 1.5 µl of reverse primer 10 pmol, as well as ddH₂O 6.5 µl until reaching total amount 25 µl.
2.5. Sequecing
Gene sequencing process was performed at the laboratory of the 1st Base Malaysia lab.

2.6. Antioxidant activity test with DPPH (1,1-diphenyl-2-picrylhydrazyl) and carotenoid assays
The antioxidant activity and carotenoids assay with DPPH was measured according to another study [13].

2.7 Determination of antioxidant potential
The antioxidant analysis was carried out using 0.5 gr of Chaetoceros sp. precipitates which are dissolved into absolute methanol liquid 5 ml. Subsequently, it was homogenized softly by vortex for 1 minute followed by incubation for 15 minutes at 37°C. Then, it was centrifuged under 3000 rpm for 5 minutes continuously until the two layers of pellets-supernatant were formed. After that, the supernatant must be directly moved into the sterile tube to make 100.000 mg/L of concentration. In addition, the concentration series production would be conducted in 10.000 mg/L, 20.000 mg/L and 30.000 mg/L respectively, that was completed by dilution in 0,1 ml; 0,2 ml, and 0,3 ml of stock solutions into each tube. Finally, each tube was added by methanol continuously up to 0,5 ml of volume.

Each of sample extract was added with 1,5 ml of DPPH 0,1 mM (0,002 gr of DPPH, methanol 50ml) and incubated at 37°C for 30 minutes, then the absorbance specifically was measured using UV-Vis spectrophotometer at $\lambda = 517$ nm. The result obtained from each extract of concentration was calculated for antioxidant activity (%). In addition, the value of IC$_{50}$ (Inhibition concentration) was enumerated using the regression equality formula. The control used for the test was DPPH solutions over 0,1 mM of methanol p.a. [13].

3. Result and discussion

3.1. Growth of Chaetoceros sp.
Cells growth of Chaetoceros sp. was presented in Figure 1., showed that the culture of that microalga reached out to the top of the growth phase on the tenth days of incubation time. In addition, this result was signed by increasing of the living cells up to $99 \times 10^6$ cell/mL. Another study was exhibited that C. calcitrans culture grew as much as $21 \times 10^5$ cells/mL in total on the ninth days [14][15]. Increases in the growth rates of microalga depend on the nutritious source as well as a suitable environment for the optimal growth of the culture. Furthermore, investigation on C. social growth drastically achieved to the climax of the logarithmic phase on the seventh day [16]. Then, the growth rates of Chaetoceros would decrease drastically.

![Figure 1. The growth rates of Chaetoceros sp. cells.](image-url)
Meanwhile, other researchers reported that the concentration of *C. calcitrans* cells increased significantly on the 20th days [18]. Moreover, it was explained that the growth rate of *Chaetoceros* sp generally increased up to the maximum rate of specific growth, subsequently, and tends to decreased slowly due to quality and quantity reduction of nutrition sources, and other abiotic factors as well. Furthermore, specific rates of growth were also generally affected by inoculum concentrates [19].

3.2. Measurement of *Chaetoceros* sp. DNA
The DNA isolation from the amount of cell for about 287 x 10^4 cell/ml, measuring by nanodrop showed the purity was 1.97 and concentration number about 2842.1 ng/µl, respectively, as showed on Table 1. In addition, another study exhibited *C. debilis* isolation was obtained 1.99 for purity and 1989 ng/µl for concentration as well [16]. Moreover, the DNA is categorized as pure, if it represents the numbers of $A_{260}/A_{280}$ for about 1.8 until 2.0 [20]. Less than 1.8 showed isophenol or protein contaminant in the DNA extraction process. The purity of DNA more than two was indicated RNA contamination.

### Table 1. DNA Quantities and quality result of *Chaetoceros* sp.isolate

| Sample       | Concentration (ng/µl) | $A_{260}$ | $A_{280}$ | $A_{260}/A_{280}$ |
|--------------|-----------------------|-----------|-----------|-------------------|
| *Chaetoceros* sp. | 2842.1                | 56.841    | 28.851    | 1.97              |

3.3. *Chaetoceros* sp DNA amplification
The DNA amplification result used ITS5 and ITS4 primer that was visualized in Figure 2. showed a band with a size of about 882 bp. This result was supported by another researcher who gaining the same result using the same primer was able to get ITS1 and ITS2 region [21]. Moreover, the result is also similar to the study on *C. tenuissimus* Meunier in obtaining the DNA band of this microalga species for about 641 bp [1]. Although, the difference in results between *Chaetoceros* sp. and *C. tenuissimus* Meunier is due to the difference in the sequence of ITS regions of each species. These results also supported research which found that ITS is the potential marker that can be used to found microalgae species distinction comparing with another region [22][23][24]. Furthermore, it was proved that the success of gene amplification is affected by the quality and quantity of the DNA genetic material [25].

![Figure 2. Visualization of PCR Product of ITS of *Chaetoceros* sp. (M. DNA Ladder 100 bp; A. *Chaetoceros* sp.)](image-url)
3.4. Sequences analysis of the ITS region of *Chaetoceros* sp.

The BLAST result showed that *Chaetoceros* sp. intriguingly have the same base as *C. muelleri* KF998567.1 for about 873 from 878 with a percentage of over 99% (Figure 3). Moreover, the alignment result also presented no gap.

![Sequence alignment](sequence_alignment.png)

**Figure 3.** Homology analysis result; nucleotide sequences of *Chaetoceros* sp. was compared with *C. muelleri* KF998567.1.
Based on this research, if there is a gap in the result in the ITS region, it must be doing mutation either deletion or insertion, in the evolution process, like the other [26]. Subsequently, deletion and insertion potentially present the behavior characteristics of the species on the analysis [27]. Bases alignment between Chaetoceros sp. and C. muelleri KF998567.1 also elucidated the location of similarities as well as base differences. In addition, the difference between Chaetoceros sp. with C. muelleri KF998567.1 indicated by base substitution in Chaetoceros sp. and guanine (G) becomes adenine (A) in C. muelleri KF998567.1 as well. That substitution was the alternation between the same base pairs (purines). Furthermore, there was also an alteration in the pyrimidine base pair, thymine (T) turn to be cytosine (C). The alteration of those bases could affect the organism being evolved [2]. Based on the result, it can be concluded that Chaetoceros sp. exhibit similar characteristics with C. muelleri KF998567.1.

3.5. Phylogenetic analysis of Chaetoceros sp.
The phylogenetic tree analysis exhibited the position of Chaetoceros sp. on the same branch with the sequences of C. muelleri KF998567.1 that came from China. Therefore, it signified that Chaetoceros sp. was closely related to C. muelleri KF998567.1 as well as originated from the same ancestor to be the monophyletic category. Based on other studies, members of the monophyletic group were assumed to carry the same character or genetic code as well as biochemistry compounds [28]. Moreover, it is also described that the monophyletic organism was an area group whose members belong to one ancestor [29]. However, the phylogenetic tree was prepared using the Neighbor-Joining Tree method. The result of the tree was illustrated in Figure 4.

**Figure 4.** Phylogenetic tree based on the ITS gene of Chaetoceros sp.

The value of the bootstrap exhibited by the phylogenetic tree was about 100. Those values explained that the construction of the phylogenetic tree gained over 100 of percentage, the branching of the phylogenetic tree was not going to change. In addition, the study exposed that the clade over 95% or
more than 90% were categorized as stable construction of the phylogenetic tree [30]. Although, the branch length value among Chaetoceros sp. and C. muelleri KF998567.1 over 0.03 indicated that every 100 bases there were 3 bases substituted. This result showed any differences in sequences of Chaetoceros sp. with C. muelleri KF998567.1, and yet it had a very close relationship. Additionally, the construction of the phylogenetic tree put Chlorella pyrenoidosa EU038290.1 as an outgroup. Yet the outgroup was noticed importantly as a part of the phylogenetic tree construction. Subsequently, related to the study described here, the outgroup exhibited more information about related sequences [31]. The result of phylogenetic analysis figured out that Chaetoceros distribution was originated from Asia and mostly from China as well. It was evidenced entirely by the observation of any close relationship detected between Chaetoceros sp. with C. simplex CCAP DQ358115.1 strain, C. gracilis UTEX DQ358113.1 strain well as C. gracilis UTEX DQ358112.1 strain that was suspected from China.

3.6. Antioxidant activity of Chaetoceros sp.
The result of the antioxidant activity of Chaetoceros sp. microalgae was exhibited in Table 2. The result showed that methanol used in the study was a suitable solvent to extract microalgae so well, supported by another study using the same solvent[6]. It was able to produce more carotenoids comparing with another solvent such as acetone and ethanol indeed. Furthermore, another researcher elucidated the strong effect of the extract in the inhibition of free radicals as showed by IC50 value [15]. The inhibition percentage value was obtained as illustrated by the chart with an R2 value of over 0.993 along with y=0.0007x - 0.6702. Thus, it can be figured that Chaetoceros sp. strongly produced a high activity of antioxidant.

Table 2. Antioxidant activity of Chaetoceros sp. based on IC50 values and inhibition

| Concentration (mg/L) | (% Inhibition) | IC50 Value |
|----------------------|---------------|------------|
| 0                    | 0             |            |
| 10.000               | 5.78          |            |
| 20.000               | 13.01         | 72,386 ppm |
| 30.000               | 21.42         |            |

Figure 5. The antioxidant and inhibition activity of Chaetoceros sp.

Several studies have shown the results of the antioxidant activity of IC50 from Chaetoceros sp. reached the highest level compared to other microalgae, even reaching more than 39.22 ppm [16] [32]. The chemical compound was categorized strongly producing a high level of antioxidant if the value of IC50 were less than 50 ppm [20]. The high-level value of the antioxidant was over 50-100 ppm, the
medium level was over 100-150 ppm, and at the low level was about 151-200 ppm. Hence, the lower the IC50 value it had, the higher the antioxidant activity produced. Since Chaetoceros sp. has a high IC50 value over 72,386 ppm, it is offering a potency to be improved further on large scale for industrial purposes.

4. Conclusion
The identification of Chaetoceros sp. used ITS5 dan ITS4 primer showed that this microalgae molecularly had a close relationship with C. muelleri KF998567.1 species from China with 99% of similarity. Intriguingly, Chaetoceros sp. has a high IC50 value over 72,386 ppm indicating the ability of their antioxidants activity.

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