The amplification and analysis of cytochrome oxidase 1 (CO1) Gene of *Ornithoptera croesus* from Bacan Island

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Abstract. *Ornithoptera croesus* is one of the endemic butterflies in Bacan island. The results of previous studies stated that O. croesus has genetic variation based on morphological and molecular character of RAPD. In male and female butterflies there are variations in color and body size. The purpose of this study was to amplify the CO1 gene in *Ornithoptera croesus* butterflies collected from Mount Sibela in Bacan Island. The method used in this study is total DNA isolated from tissue using the ZymoBionic (Zymo Research DNA Extraction) Kit, DNA amplification using the CO1 gene, sequencing and BLASTn analysis. The results showed that the specimen was successfully amplified with an amplicon size of 500 bp. Furthermore, the results of the BLASTn analysis revealed that the sequence had a 99% similarity with O. croesus NCBI in both males and females, and based on phylogenetic analysis it had a closed position. The application of CO1 DNA barcodes is effective for identifying butterflies at species level.

1. Introduction

*Ornithoptera croesus* is one of the endemic butterfly species on the island of Bacan. The results of the study of Mas’ud et al. (2018) found that there is genetic diversity in O [1]. Croesus both based on morphological and molecular characters of RAPD. O. Croesus butterfly was first discovered by Wallace (1869) in the Mount Sibela [2] reserve on the Malay Archipelago expedition [3]. The color morphology of the wings in O. croesus females varies at various altitudes [4].

Based on the analysis of economic potential, and tourism O. croesus is a macrolepidoptera group that has a beautiful body color. This potential can be developed for the butterfly tourism sector which can increase regional income. In addition, the development of the O. croesus butterfly tourism sector can have an impact on conservation efforts. Furthermore, to carry out efforts to conserve endemic butterflies of Bacan island, identification and analysis of the existence of endemic butterflies are needed based on genetic diversity data. Correct naming and determination of species is very important for conducting bioecological studies and other studies. Along with the development of molecular biology, a new method for the identification of species based on DNA has been found, known as DNA Barcoding [5,6]
DNA barcoding provides speed and accuracy in species identification with the focus of analysis on small segments of mtDNA [7]. DNA barcoding can be a taxonomic crisis solution [8]. The genetic diversity studies of O. croesus that have been studied previously need to be clarified with a genetic approach using DNA barcoding methods. DNA barcoding is a technique for characterizing and identifying species using DNA sequences called DNA barcodes. The cytochrome c oxidase subunit 1 (CO1) gene is a coding protein in mitochondrial DNA that has been widely used as an identification tool for animal species. Segments near the 5’ end of CO1 along about 650 bases are areas that are widely used as DNA barcodes for fauna [6]. The effectiveness of CO1 has been validated for various groups of fauna and most types of fauna studied can be distinguished using DNA barcodes. This effectiveness is caused by low intraspecific variations, but interspecific variations are high especially in adjacent taxa [9,10].

This study aims to confirm the study of genetic identification and variation in O. croesus using amplification of DNA barcoding for the CO1 gene. Molecular characterization in this research is the first step to form DNA barcode species which belongs to the genus Ornithoptera spp in Bacan Island. The results of this study can be used as a means of identifying natural resources especially animals in the framework of classification/taxonomy, conservation and law enforcement, as well as breeding.

2. Method

2.1. DNA, PCR, and Sequence Preparation

DNA material was extracted from male and female O. croesus butterfly foot tissue obtained from the of Bacan island. DNA extraction was carried out using procedures from the Presto TM Mini DNA gDNA KIT (Geneid) kit. The amplification process uses MyTag Red Mix (Bioline). The CO1 gene amplification process using the CO1aF forward sequence sequence (5GA-CGAAAATGACTT TATTCACA -3’ ) and reverse sequences namely CO1aR (5’ -AGCAGTAATTCCAACAG CTC -3’ ). A total of 30 mL PCR reaction mixture (MyTag Red Mix, Primary, ddH2O, and DNA template). The PCR process was carried out under denaturation conditions at 950C, annealing at 550C, and extension at 720C and post extension at 720C. The product PCR was purified using Zimoclean TM DNA recovery KIT gel (Zimo research). Bidirectional sequencing uses the services of Malaysia's 1st Base

2.2. Phylogenetic Analysis

The data obtained were analyzed with the help of computer programs, including: analysis. DNA allignment with the MEGA 5 program, BLASTn DNA samples with DNA sequences in GenBank, DNA sequencing from GenBank, and phylogenetic Neighbor Joint (NJ) tree construction using the MEGA 5 program [11]

3. Results and Discussion

The results of genomic DNA isolation from O. croesus foot tissue samples detected using agarose gel are shown in Figure 1. These results indicate that the genomic DNA of all samples was successfully isolated, that is sample code A and code B
**Figure 1.** Visualization of whole genom and Morphology of *Ornithoptera croesus* Male and Female

Furthermore, quantitative analysis of DNA concentration and purity, as in table 1 below:

| No. | Sample name | Conc (ng/mL) | A260/280 | A260/230 | Volume (μL) |
|-----|-------------|--------------|----------|----------|-------------|
| 1   | Male *O. croesus* (A) | 53,3         | 1,47     | 0,16     | 30          |
| 2   | Female *O. croesus* (B) | 51,2         | 1,82     | 1,29     | 30          |

The results of the analysis of the purity and concentration of DNA showed that the genome *O. croesus* DNA had good concentration and purity

### 3.1 Amplification of CO1 gene fragments

Visualization of the results of CO1 gene amplification from *O. croesus* samples analyzed by electrophoresis as in Figure 2

**Figure 2.** Electrophorogram results of the CO1 gene amplification *O. croesus* from Bacan Island.

Amplification of the CO1 gene shows a DNA band with a size of ± 500 bp. Samples A and B with CO1 primers were well amplified. Furthermore, an analysis of BLASTn (NCBI genebank) search was made known that *O. croesus* from Bacan island is identical to *Ornithoptera euphorion*, *Ornithoptera aesacus* and *Ornithoptera priamus* with an identical value of 95%. Data similarity analysis results (Blastn) from samples A and B as in Figure 3 and 4 below.
Figure 3. The analysis of BLAST CO1 sequence of *O. croesus* male

Figure 4. The analysis of BLAST CO1 sequence of *O. croesus* female

Figure 5. The analysis of BLAST CO1 sequence of *O. croesus* male
3.2 Phylogenetic Analysis

To identify the phylogenetic analysis of the selected samples compared to data from BLAST’s genebank search results on NCBI. From this phylogenetic analysis it can be seen the position of the taxon from the *O. croesus* endemic sample of Bacan Island. Data from the analysis of kinship (phylogenetic) as in Figure 7 and 8 below.

![Figure 6. The analysis of BLAST CO1 sequence of *O. croesus* female](image)

![Figure 7. Phylogenetic analysis from a sample (A) *O. croesus* male](image)
Figure 8. Phylogenetic analysis from a sample (B) O. croesus female

The results of phylogenetic analysis by the Neighbor Join (NJ) method note that the CO1 gene can be used to determine the taxon position in the identification of O. croesus. A specimen from different regions can be together in the same cluster [12]. Phylogenetic trees show species relationships based on genetic similarity. Sample O. croesus Bacan islands (A) and (B) are located close to O. aesacus, O. priamus and O. euphoria, all of which are genus Ornithoptera spp with distribution in Eastern Indonesia (Fig. 7 and 8).

The results showed that the identification of O. croesus endemic of Bacan island was quite effective. In the endemic O. croesus sample of Bacan island both male and female are identical to O. euphorion, O. aesacus and O. priamus with 99% similarity value with NCBI database. Technically the CO1 gene can be amplified with a high success rate with one or two kinds of universal primers. It was further stated that when compared to other barcode gene candidates, the CO1 gene has a high success rate of bidirectional sequencing (two-way sequencing with forward and reverse primers) [13].

The COI gene is a gene in the mitochondria that can be used in the study of genetic characters and phylogeny from O. croesus. The size and structure of the cytochrome oxidase subunit 1 (COI) gene has become the focus of animal group analysis and evolution studies as shown in the study of genetic characters and evolutionary patterns in T. helena [14], in ducks in the Philippines [15] COI is a standard gene that is often used as a marker gene in animal identification [6]. The advantages of the COI gene include: 1) the universal primer of this gene is very sturdy, so it can recognize the 5’ end of most animal groups; 2) COI genes have the highest molecular evolution compared to genes in other mitochondria, so they have low intraspecific variations, but high interspecific divergence between adjacent taxa [10].

In this study a 95% similarity value was obtained that O. croesus is endemic to Bacan Island with the NCBI database. It can be said that the use of CO1 primers requires confirmation with other primers, for example 16S rRNA. The 16S rRNA gene is another marker gene that is useful in species identification and for describing phylogenetic relationships between marine organisms [14]. The 16S rRNA gene is used as a standard marker of DNA barcoding to supplement COI [15]. Some researchers
use a combination of COI and 16S rRNA gene sequences to infer kinship relationships through phylogenetic trees in groups of animals that have high cryptic levels, such as the Mollusk group [16]. The findings and recommendations of the results of this study are to obtain the results of molecular dentification with COI barcoding DNA in *O. croesus* butterflies can also be combined with 16SrRNA.

4. Conclusion

*O. croesus* endemic butterfly Bacan Island has been successfully amplified with an amplicon size of 500 bp. Furthermore, the results of the BLASTn analysis revealed that the sequence had a similarity of 95% *O. euphorion*, *O. aescucus* and *O. priamus* NCBI database

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