Genetic diversity of Indonesian protected eclectus parrot (*Eclectus roratus*) based on mitochondrial gene sequences

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**Abstract.** Eclectus Parrot (*Eclectus roratus*) Bird is included in the group of parrots, the Psittacidae family, consists of nine subspecies. The body size is 35-42 cm, and its weight is 355–615 g. Eclectus bird has nine subspecies. Their distribution is in the Solomon Islands, Sumba, New Guinea, and its nearby islands, northeastern Australia, and the Maluku Islands (Moluccas). Seven of these subspecies are in the territory of Indonesia. The wild population is in decline. The species has been included in CITES appendix II. Like other parrot birds, this bird has high economic value, so its existence needs to be conserved for use sustainably. Information about its genetic diversity might be useful for its conservation program. Therefore, this study aimed to assess the genetic diversity of these birds revealed based on 720 bp of DNA sequences of the mitochondrial cytB gene. This information might be needed for their conservation efforts. Blood samples of 0.1 mL were taken from 103 birds in the zoo and two captivities. The total genomic DNA was extracted from each blood sample. DNA fragments of mitochondrial cytB gene were amplified through a PCR process and then the nucleotide was sequenced. The DNA sequence data of the cytB gene were analyzed using ProSeq, DnaSP, and Mega7 specific software to obtain information about variations, genetic diversity, and grouping of individual birds. The cyt B sequences observed had no stop codons. Of the 720 cytB base pairs had no stop codons observed. There were eight variable sites starting at 212 to 616 position. Genetic distance among individual birds ranged from 0.00 to 0.012. The sequences had three haplotypes. Based on the comparison to *E. roratus* from Genbank data as well as NJ tree, it was suggested that the studied birds consisted of three subspecies. Haplotype diversity (Hd) value was 0.500 ± 0.136 and nucleotide diversity (Pi) value was 0.004431 ± 0.00140. Hence, the genetic diversity among the birds in this study was relatively low.

1. **Introduction**

Eclectus Parrot (*Eclectus roratus*) bird is included in the parrot group of the Psittacidae family. Its body sizes are 35-42 cm, and its weight is 355–615 g. Its sexual dimorphism is in color feather, which male is green, and female is red [1]. The Eclectus bird has nine subspecies, and each subspecies is distributed on a different island, i.e., Solomon Islands, Sumba, New Guinea, and its nearby islands, northeastern Australia, and the Maluku Islands (Moluccas). Seven of these subspecies are restricted to the Indonesian region [2]. According to current genetic analyses the populations of these subspecies, namely Sumba (*Eclectus cornelia*), Tanimbar Islands (*E. riedeli*), Moluccas (*E. roratus*), and New Guinea (*E. polychloros* incl. Aru Islands (Aru Islands) *E. aruensis*), and Solomon Island (*E. solomonensis*), are considerer as valid species [3]. This bird is recorded in the IUCN Red List with the
category Least Concern and included in the CITES Appendix II [4] and also as a protected bird in Indonesian by law PP No. 7, 1999, and No. 5, 1990.

Like the other parrots, this bird has a high economic value in the wildlife trade. The bird population in nature is declining, and this may cause inbreeding and decrease its genetic diversity [5]. This situation needs conservation efforts; both in-situ and ex-situ; hence, they can be used sustainably for humans.

Genetic diversity is one of the biodiversities. The level of genetic diversity can affect the conditions of resistance of animals, including climate change. Animals with high genetic diversity can adapt better to disease and drastic climate change. In contrast, those with low genetic diversity are more vulnerable to disease and climate change, causing further to death and population decline [6].

The study of genetic diversity using mitochondrial DNA markers has been carried out on several species of animals, including birds, e.g. [7], *Cacatua* [8], *Psittacula alexandri* [9], *Acridotheres melanopterus* [10]. The mitochondrial DNA has the following characteristics: an efficient marker for estimating the rate of evolution and speciation, haplotype diversity, and natal origin [11], molecular diversity [12], genetic diversity [13, 14].

One of the protein-coding genes in mitochondrial DNA is the cytB gene, which has a size of 1140 bp [15]. This gene is believed to be used as a DNA marker for identification of animal species or groups [16], molecular phylogeny in birds [17-19], molecular forensic in animals [20, 21], genetic diversity of Red-Backed Shrikes *Lanius collurio* [22], the distribution and pattern of genetic variation and divergence of the wild population [23].

This study used DNA sequence data from a partial mitochondrial cytB gene 720-bp to reveal the character and genetic diversity of birds Eclectus parrot (*Eclectus roratus*), and to estimate the origin and subspecies of *E. roratus*. The result of this study is expected to be used for a breeding program for this ex-situ conservation of birds in Indonesia.

2. Materials and methods

2.1. Sampling

Blood samples for genetic materials were obtained from living birds in the Bali Bird Park and animal captivity in Sukabumi, West Java. Each collected sample was preserved in absolute alcohol in a two cc Eppendorf tube and stored in room temperature or refrigerator. Only ten blood samples were collected in this study.

2.2. DNA extraction, PCR amplification, and DNA sequencing

0.001 ml of blood fluid or about 0.5 mg of dried blood from 10 birds was used, and DNA was extracted from each blood sample using the phenol-chloroform method. The obtained DNA solution was then checked in 1% agarose gel run electrophoretically for 15-20 min using 110 Volt/50 Ampere.

The cyt B gene fragments were amplified through the PCR process with 25 mL as the final volume. The PCR process was carried out under PCR conditions of 95 °C - 5 min, 35 X cycles of (95 °C - 1 min, 52 °C - 1 min, 72 °C - 1.5 min), and 72 °C - 10 min. The oligonucleotide primers used in the PCR process were L1484 - H1667 [24]. The PCR product of the cytB gene target was then checked by electrophoresis on agarose gel 1.5% for 20 min. The DNA sequencing of the PCR product was performed using the services of FirstBase Company in Malaysia.

2.3. DNA sequence data analysis

The DNA sequence data from ten studied birds were analyzed and aligned together with cyt B DNA sequence data of several *E. roratus* from the Genbank NCBI. The alignment process of all DNA sequence data was done using Mega7 software. The primers sequences were removed and not used in further analyzes. Therefore, only 720 bp were analyzed. Furthermore, the data were analyzed to get information about DNA characters, including base composition, number of variable/polymorphic sites, number of invariable sites, genetic distance, and individual grouping. The parameters of genetic diversity, including haplotype sequences (h), haplotype diversity (hd), nucleotide diversity (Pi) were carried out using DnaSP 4.0 software.
2.4. Neighbor-joining (NJ) analysis

NJ analysis was done to perform the filogenetic tree to determine the grouping of \textit{E. roratus}. DNA sequence data, both from ten birds collected in this study and from Genbank (AB177965.1, MH645644.1, MG429727.1, KM372496.1, KM372497.1, KM372500.1, KM372506.1, KM372507.1, KM372508.1, KM372509.1, NCO27842.1, KM611469.1, KM372498.1, KM372499.1, KM372503.1, KM372501.1, KM372502.1), were used. DNA sequence data of the outgroup species, \textit{Psittacula roseate} obtained from Genbank (MH645642.1, were used.

3. Results and discussion

Analysis of all DNA sequence data of cyt B gene of 103 \textit{E. rorartus} birds showed that there were no stop codons. The total percentage of nucleotide compositions and each codon position is presented in Table 1. The percentage of cytosine (C) was the highest, and guanine (G) was the lowest. The positions of first and second codons were dominated by C, while the third codon position was dominated by thymine (T). The content of GC (47.6%) was smaller than AT (52.4%). These characters were similar to DNA sequence data from the cyt B gene in other animals, such as fish [23] and parrots [24]. Thus, the target fragments obtained from the PCR process were the cytB gene.

| Codon position | Nucleotide composition | Total |
|----------------|------------------------|-------|
|                | T (%)                  | C (%) | A (%) | G (%) |       |
| Total basa     | 24.1                   | 35.4  | 28.4  | 12.2  | 720   |
| 1st codon      | 9.3                    | 51.1  | 38.1  | 1.5   | 240   |
| 2nd codon      | 25.4                   | 27.4  | 26.1  | 21.1  | 240   |
| 3rd codon      | 37.1                   | 27.7  | 21.1  | 14.1  | 240   |

Nucleotide variables occurred at eight sites, started from site 212, and ended at site 616. The number of invariable sites was 712. Hence, only a small number of nucleotide sites were substituted. Of the eight variable sites, there were three sequence haplotypes (Table 2). The Hap-1cytbEr was owned by nine birds; the hap-2cytbEr was owned by one bird, and the hap-1cytbEr was owned by three birds (Table 2).

| No  | Sample code  | Species name | Variable sites in the cyt B gene of \textit{E. roratus} | Haplotype Code |
|-----|--------------|--------------|----------------------------------------------------------|----------------|
| 1   | KM611469.1   | \textit{E. roratus} | 2 2 2 2 3 4 5 6 6 | hap-1cytbEr |
| 2   | NC 027842.1  | \textit{E. roratus} | . . . . . . . . . | hap-1cytbEr |
| 3   | da173        | \textit{E. roratus} | . . . . . . . . . | hap-1cytbEr |
| 4   | da174        | \textit{E. roratus} | . . . . . . . . . | hap-1cytbEr |
| 5   | da175        | \textit{E.roratus} | . . . . . . . . . | hap-1cytbEr |
| 6   | da176        | \textit{E. roratus} | . . . . . . . . . | hap-1cytbEr |
| 7   | da177        | \textit{E. roratus} | . . . . . . . . . | hap-1cytbEr |
| 8   | da178        | \textit{E. roratus} | . . . . . . . . . | hap-1cytbEr |
| 9   | da185        | \textit{E.roratus} | T C G A T G A T | hap-2cytbEr |
| 10  | da186        | \textit{E.roratus} | . . . . . . . . . | hap-1cytbEr |
| 11  | da187        | \textit{E. roratus} | T C A G A T | hap-3cytbEr |
| 12  | da188        | \textit{E. roratus} | T C .16 A G A T | hap-3cytbEr |
| 13  | MH645644.1   | \textit{E. roratus} | T C . A G A T | hap-3cytbEr |
The haplotype diversity (hd) value was 0.500 ± 0.136, and the nucleotide diversity (Pi) value was of 0.004431 ± 0.00140 (Table 3). The value of haplotypes and nucleotide diversities were relatively low. Those indicated that the genetic diversity of birds in this study was low. The low genetic diversity probably has several reasons: 1) the rate of evolution or basic substitution in this bird was slow, 2) the effect of inbreeding, and 3) small number of individuals. However, the small number of populations may not be the cause of the low genetic diversity of *E. roratus*. In general, the relationship between mitochondrial DNA diversity and population size is difficult to evaluate because of the scarcity of population size estimates for wild species [25]. The number of individual birds or population size does not positively correlate with low diversity [26]. *Larus dominicanus* (Charadriiformes, Laridae) had 0.273 of haplotype diversity and 0.00083 of nucleotide diversity [27], but the Phaethontiformes White-tailed tropicbird with a small population had a high genetic diversity value [28].

The pattern of birds’ genetic diversity depends on the effectiveness of the evolutionary population, the size of the population, and the rate of molecular evolution [13]. Increasing the risk of extinction and conservation policies are needed to minimize the loss of diversity [14].

Genetic distance between individual birds ranged from 0.000 to 0.012. It indicates that the individual bird studied belonged to one species. The intraspecific genetic distance of others *E. roratus* ranged from 0.00 to 0.012 [3] and 0.74% of other birds [16]. Meanwhile, the genetic distance between the subspecies of *E. roratus* ranged from 0.000 (*E. r. aruensis vs. E. r. polychloros*) to 0.029 (*E. roratus vs. E. riedeli*) [3].

| Table 3. Genetic characters and diversity of *E. roratus* based on cyt-b gene sequences. |
|----------------------------------------|------------------|
| **Parameter**                         | **Value**        |
| Number of birds                       | 13               |
| Number of the nucleotide of CytB      | 720 base pair    |
| Number of variable sites              | 8 sites          |
| Number of invariable sites            | 712 sites        |
| Nucleotide composition (%):           |                  |
| T                                     | 24.1             |
| C                                     | 35.4             |
| A                                     | 28.4             |
| G                                     | 12.2             |
| Genetic distance                      | 0.000 - 0.012    |
| Numbers of haplotype (h)              | 3 haplotypes     |
| Haplotype diversity (hd)              | 0.500 ± 0.136    |
| Nucleotide diversity (Pi)             | 0.004431 ± 0.00140 |
| Average number of nucleotide differences (k) | 2.615            |
| Tjima test (D)                        | 0.60470, Not significant P > 0.10 |

In detail, the genetic characters and diversity parameters are shown in Table 3. The average number of nucleotide differences (k) was 2.615. Tajima analysis showed no significant difference (P> 1).

NJ trees were performed to determine the grouping of individuals of *E. roratus* birds (Figure 1). The bird (da185) was clustered together with *E. r. aruensis*, and *E. polychloros*. The possibility was that the bird (da185) was *E. r. aruensis* from Aru Island or *E. polychloros* from New Guinea. Seven birds (da173, da174, da175, da176, da177, da178, and da186) were grouped with the birds (NC 027842.1 and KM611469.1) from Sumba. The *E.roratus* that occurs in Sumba is *E. r. cornelia* [24]. Therefore, the seven birds above might be *E. r. cornelia* from Sumba. Two birds (da187 and da188) were grouped with AB177965, MH645644, MG429727, and KM372510 as *E. r. roratus* from Moluccas (3).

*E. roratus* has nine subspecies. However, the individual birds studied were not identified as their origin and the subspecies name. From the sequence haplotypes (Table 2) and NJ tree (Fig 1), the birds studied might consist of three subspecies: *E.r. aruensis* from Aru Island or E.r. polychloros from New Guinea, *E. r. cornelia* from Sumba, and *E. r. roratus* from the Moluccas.
Figure 1. Neighbor-joining (NJ) tree among *E. roratus* based on 607-bp of cyt B gene sequences. *Psittacula roseata* was an outgroup species.

4. Conclusion
The haplotype and nucleotide diversities in this study were relatively low, indicating that the genetic diversity of the *E. roratus* studied based on cytB sequences was relatively low. The birds have three sequence haplotypes, which may consist of three subspecies of *E. roratus*. 

AB177965.1 *E. roratus*
MH645644.1 *E. roratus*
da187 *E. roratus*
da188 *E. roratus*
MG429727.1 *E. roratus*
KM372510.1 *E. roratus*
da185 *E. roratus*
KM372496.1 *E. r. aruensis*
KM372497.1 *E. r. aruensis*
KM372500.1 *E. r. polychloros*
KM372506.1 *E. r. solomonensis*
KM372507.1 *E. r. solomonensis*
KM372508.1 *E. r. solomonensis*
KM372509.1 *E. r. solomonensis*
da186 *E. roratus*
NC 027842.1 *E. roratus*
KM611469.1 *E. roratus*
da173 *E. roratus*
da175 *E. roratus*
da174 *E. roratus*
da176 *E. roratus*
da177 *E. roratus*
da178 *E. roratus*
KM372498.1 *E. r. cornelia*
KM372499.1 *E. r. cornelia*
KM372503.1 *E. r. riedeli*
KM372501.1 *E. r. riedeli*
KM372502.1 *E. r. riedeli*
MH645642.1 *Psittacula roseata*
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