Journal of Ecosystem & Ecography

Astuti et al., J Ecosys Ecograph 2016, 6:2
DOI: 10.4172/2157-7625.1000195

Research Article Open Access

Phylogenetic Position of *Psittacula* Parakeet Bird from Enggano Island, Based on Analyses of Mitochondrial Cytochrome B Gene

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Received date: June 03, 2016; **Accepted date:** July 02, 2016; **Published date:** July 08, 2016

Abstract

Enggano Island of Indonesia has *Psittacula* parakeet bird; namely *Psittacula longicauda modesta*. Phylogenetically, the position of the bird has not been studied yet. The present study used DNA sequences of mitochondrial cytochrome b (cyt b) gene to analyze phylogenetic relationships within *Psittacula* parakeet birds; especially to reveal the phylogenetic position of Psittacula longicauda modesta. Blood samples were collected from two *Psittacula* species; *Psittacula alexandri* from Jawa island, and *Psittacula longicauda* (*Psittacula longicauda modesta* from Enggano island and *P. l. defontainei* from Natuna island). Blood samples were taken from each bird and DNA was extracted from each blood sample. PCR was performed to amplify a single fragment of cyt b gene, by using a pair of nucleotide primer. The DNA targets were then be sequenced. Totally 868-bp of cyt b was used to calculate genetic divergence within and between *Psittacula* parakeet, and to construct phylogenetic trees. DNA sequence data from others *Psittacula* species were taken from GenBank. *Columba livia*, Accipiter, and Cacatua were used as outgroup species. The mean genetic divergence within *Psittacula longicauda* was 2.16% for *P. l. modesta* vs *P. l. defontainei*, 2.37% for *P. l. modesta* vs *P. l. longicauda*, and 1.51% for *P. l. defontainei* vs *P. l. longicauda*. The mean genetic divergences within *Psittacula* were 0.0512 ± 0.0051. Both Phylogenetic (NJ and ML) trees showed that *P. l. defontainea* (Natuna is.) and *P. l. longicauda* grouped together and to be sister group, while the position of *Psittacula* parakeet from Enggano island (*P. l. modesta*) was distant from and as a sister group of (*P. l. defontainei and P. l. longicauda*). *P. longicauda* and *P. alexandri* group together and appeared to be sister group.

Keywords: *Psittacula* parakeet; Enggano island; DNA sequence; DNA mitochondrial; Cyt b; Genetic divergence; Phylogenetic relationships

Introduction

Enggano Island is one of the outer islands of Indonesia, located in Indian Ocean, approximately 100 km South West of the mainland Sumatra Island, 5°17' - 5°31' S and 102° 05' - 102° 25' E. It is separated from Sumatra Island by marine basin with a depth of 2000 m. Biologically, it has high endemicity and a wealth of biodiversity. Based on Enggano expedition on 2015 there were many animal species that were found, including around 27 bird species. One of the birds is parrot bird; named *Psittacula longicauda modesta*; a subspecies of Psittacula longicauda and as an endemic bird in Enggano Island [1,2]

Other subspecies of *P. longicauda* are *P. l. tytlery*, *P. l. nicobarica*, and *P. l. longicauda* [3], *P. l. defontainei*, and *P. l. modesta* [1]. Each subspecies is distributed in the other islands and outside of Indonesia. In Indonesia, *P. l. longicauda* is occured in Sumatra, Borneo, Riau Islands, while P. l. defontainei is occured in Natuna Island [1].

Psittacula parakeet bird belongs to parrot bird (order: *Psittaciformes*). In the world, there are 15-16 species of *Psittacula* parakeets which are *Psittacula eupatria* , *P. wardi*, *P. krameri*, *P. echo*, *P. exsul*, *P. (himalayana) himalayana*, *P.* (*himalayana*) *finschii*, *P. intermedia*, *P. cyanocephala*, *P. roseata*, *P. columboides*, *P. calthropae*, *P. derbiana*, *P. alexandri*, *P. caniceps*, *and P. longicauda* [1-4].

Some previous authors have studied on relationships and the evolutions of *Psittacula* parakeet birds based on spot colors on the head [5] and molecular data [5,6]. However, there has been no philogeny research report that reveals the position of the parakeet from Enggano Island (*P. l. modesta*) either by genetical or morphological character.

Mitochondrial DNA is DNA markers that have been used and recognized to have characters that can reveal the problems of taxonomic species [7]. Mitochondrial DNA consisted of 22-protein-coding genes; one of them is cyt b gene. The gene was chosen in this study because it has been used in animal to reveal taxonomic problems in Parrot birds (e.g. Groombridge et al. [5]; Kundu et al. [6]; Schweizer et al. [8]), and ease to amplify the gene by PCR.

Therefore, through the analyses of mitochondrial cyt b gene sequences, this study was conducted to know 1) phylogenetically, where the position of *P. l. modesta* from Enggano island is, 2) genetically, whether *Psittacula* from Enggano is separated from others subspecies of *Psittacula longicauda*, 3) how far the differgencies (sequence divergences) between *Psittacula* from Enggano and others subspecies of *Psittacula longicauda* are, 4) how far the difergencies (sequence divergences) between species of *Psittacula* are, 5) genetically, whether *Psittacula* from Enggano is as subspecies of *P. longicauda* or become a new species.

Methods

Sampling

Genetic materials in form of bloods were collected from each live *Psittacula* parakeet bird in its origin locations. Totally 13 bird blood samples consisted of 5 samples of *Psittacula longicauda* defontainei

from Natuna Is., 4 samples of *P. l. modesta* from Enggano Is., and 4 samples of *P. alexandri* from Java Is. Each blood sample was preserved separately in the 96% of ethanol absolute in 2 ml tube.

DNA extraction

DNA was extracted from each blood sample using QIAGEN DNA Mini Kit by following manufacture protocol. Then, each DNA sample was visualized in the electrophoresis process and ultravioled photo. This process was performed to check the DNA content, quality, and quantity.

Gene fragment amplification

Polymerase Chain reaction (PCR) was performed to amplify a single DNA fragment target of cyt be gene by using a pair of oligonucleotide primers; L14841 AAAAAGCTTCCATCCAACATCTCAGCATGATG AA and H 15767 ATGAAGGGATGTTCTACTGGTTG[9]. PCR condit ions were: 95°C, 35 X (94°C- 30 sec., 52°C-30 sec, 72°C- 60 sec), 72°C-7 min. Each PCR product was containing the DNA fragment target, then to be purified and sequenced. DNA sequencing was done in Fist Base Company.

DNA sequence data analyses

All DNA sequence data's of each bird and gene were aligned using ProSEq software. DNA variations, substitutions, base compositions, etc. were analysed using MEGA5 version 4 software. Each sequence data was translated to the amino acid to check and examine the absence of pseudo gene and stop codons. DNA sequence data of others *Psittacula* parakeet and outgroup species (Columba, Accipiter, and Cacatua) were adopted from GenBank. The species name and accession numbers from Genbank are *P. columboides* (AY220108), *P. krameri* (AY220114.1), *Psittacula roseata* (KJ456438.1), *Psittacula cyanocephala* (GQ996508.1), Psittacula finschi (KJ456435.1), *Psittacula himalayana* (KJ456436.1), *Cacatua alba* (AB177973.1), *Cacatua galerita* (AB177977.1), *Columba livia* (KC811464.1), and *Accipiter bicolor* (AY987307).

Phylogenetic analyses

From around 1100-bp of DNA fragment targets of cyt b gene, in this study we decided to analyze only 868-bp to construct NJ and ML trees using MEGA5 version4 [10]. NJ tree was performed using 1000 replicates with Kimura 2-parameters distance, and calculated all codon positions. Model Test was analysed in Mega5 version 4 software models with the lowest BIC scores (Bayesian Information Criterion) was considered to describe the substitution pattern the best, and resulted HKY+G model [11] is the best model for cyt b data in constructing ML tree in this study.

Results

Profiles of DNA sequences in the cyt b gene of *Psittacula* Parakeets

DNA sequences of cyb be gene in this study were no insertions and deletions, no stop codons, and no pseudogenes. The sequence data were divided into three codon positions and consisted of 287 amino acids. Base compositions in the 868-bp of cyt b gene were presented in Table 1. Composition of C+G was 48.97% less than A+T (51.03%). Cytosine (C) was the highest (35.62%) and Guanine was the lowest (13.17%). Thymine was the highest at second codon position, C and A was the highest at third codon position, and Guanine (G) was the lowest at third codon position.

Codon position	Thymine (T)	Citosine (C)	Adenin (A)	Guanine (G)	Total base
	%	%	%	%	
1st position	23.55	29.67	24.66	22.11	290
2nd position	37.23	28.50	20.24	14.03	289
3rd positions	10.32	48.66	37.65	3.37	289
All positions	23.68	35.62	27.52	13.17	868

Table 1: Mean base composition in the cyt b gene sequences of *Psittacula* parakeets examined in the current study.

Table 2 presented that nucleotide substitutions were occurred in the sequence data of cytb gene in *Psittacula* parakeets that were examined in the present study. The nucleotide substitutions were the highest in the third codon position (34 pairs) followed by first (5 pairs) and second codon positions (3 pairs). Among to the 868-bp, the numbers of transitional substitutions (37 pairs) were more abundant than transitional substitutions (5 pairs), and transitional: transversional ratio (R) was 7.40.

Codon position	Identical pairs (ii)	Transitional pairs (si)	Transversional pairs (sv)	si/sv ratio (R)
1st position	285,00	4,00	1,00	5,03
2nd position	286,00	2,00	1,00	3,11
3rd positions	256,00	31,00	3,00	10,09
All positions	827,00	37,00	5,00	7,40

Table 2: Nucleotide substitution pairs in the 868-bp of cyt b sequences of *Psittacula.*

Nucleotide substitution between thymine (T) and cytosine (C) (T ←----→ C) were the highest (26 bases) followed by A↔G (11 bases). But, there were no substitutions between T ←--→ A, C ↔G, and T ↔ G (Table 3). Among to the 868-bp of cyt b, the number of invariable was 580 sites, and variable was 228 sites which 183 sites were phylogenetically informative.

Codon position	Base pair								
	TT	GG	CC	AA	TC	CA	AG	TA	CG
1st position	67,00	63,00	84,00	71,00	3,00	0,00	1,00	0,00	0,00
2nd position	107,00	40,00	81,00	58,00	1,00	1,00	1,00	0,00	0,00
3rd positions	19,00	5,00	129,00	103,00	22,00	3,00	9,00	0,00	0,00
All positions	193,00	109,00	294,00	232,00	26,00	4,00	11,00	0,00	0,00

Table 3: Base Pairwise substitution in the 868-bp of cyt b gene sequences of *Psittacula*.

Intraspecific variations were found in the cyt b sequence data of *Psittacula*. Low intraspecific variation resulted very low intraspecific genetic distances/genetic divergence in the *Psittacula,* which ranged from 0.00% (*P. l. modesta*) to 0.40% (*P. a. alexandri*), while for *P. l. defontainea* was 0.2% (Table 4). The intraspecific differences in *Psittacula* not affected to the profiles of both phylogenetic NJ and ML trees, because every birds of the same species were grouped together into same clade that supported by 100% bootstrap values (Figures 1 and 2).

Overall, mean distance between species of Psittacula based on cyt b gene was 0.0512 ± 0.0051, from the lowest which is around 0.0380 ± 0.00567 (*P. finschii vs P. himalayana*) to the highest which is around 0.1003 ± 0.0123 (*P. himalayana vs P. alexandri*), whereas mean distance within *P. longicauda* was around 0.0151 ± 0,0085 (*P. l. longicauda* vs *P. l. defontainei*), 0,0216 ± 0,0080 (*P. l. modesta* vs *P.l. defontainei*), and 0,0237 ± 0,0058 (*P. l. modesta* vs *P. l. longicauda*) (Table 5).

S No.	Species	Distance
1	*P. l. defontainea*	0,0023 ± 0,0012
2	*P. l. modesta*	0.0000 ± 0.0000
3	*P. alexandri alexandri*	0,0026 ± 0.0016

Table 4: Intraspecific distance calculated from cyt b gene sequences.

S No.	Species name	1	2	3	4	5	6	7	8	9	10
1	*P. columboides*		0,0122	0,0121	0,0095	0,0096	0,0108	0,0096	0,0096	0,0096	0,0095
2	*P. finschii*	0,0926		0,0057	0,0078	0,0119	0,0106	0,0106	0,0099	0,0105	0,0103
3	*P. himalayana*	0,0937	**0,0380**		0,0092	0,0111	0,0109	0,0123	0,0107	0,0116	0,0116
4	*P. cyanochepala*	0,0749	0,0437	0,0651		0,0098	0,0105	0,0109	0,0107	0,0103	0,0104
5	*P. krameri*	0,0595	0,0855	0,0764	**0,0767**		0,0121	0,0112	0,0105	0,0102	0,0111
6	*P. roseata*	0,0808	0,0780	0,0851	0,0665	0,0825		0,0112	0,0106	0,0122	0,0114
7	*P. alexandri alexandri*	0,0661	0,0900	**0,1003**	0,0888	0,0712	0,0825		0,0081	0,0047	0,0033
8	*P. longicaoda defontainei*	0,0640	0,0866	0,0911	0,0878	0,0665	0,0712	0,0615		0,0080	0,0085
9	*P. longicaoda modesta*	0,0652	0,0844	0,0896	0,0832	0,0667	0,0665	0,0547	**0,0216**		0,0058
10	*P. longicauda longicauda*	0,0665	0,0886	0,0978	0,0874	0,0738	0,0852	0,0684	**0,0151**	**0,0237**	

Table 5: Genetic distances (below diagonal) and standrat error (above diagonal) between species of *Psittacula*, based on 868-bp of cyt b sequences.

Phylogenetic relationships

Based on cyt b gene data, the filogenetic analyses using Neigborn-joining (NJ) and Maximum-Likelihood (ML) revealed the same topologies of both NJ and ML trees. All birds from the same species were clustered in the same clade and supported by 100% bootstrap value. For instance, four individuals of *P. longicauda defontainea* were clustered together in the same clade, four individuals of *P. l. modesta* were grouped together in the same clade, this was also occurred for *P. alexandri*.

In both NJ and ML trees, *P. l. longicauda* and *P. l. defontainea* were grouped together in the same clade and supported by bootstrap values 100% for NJ and 93% for ML. Whereas, *P. l. modesta* appeared to be distant from and to be sister group of *P. l. longicauda* and *P. l. Defontainea* that supported by bootstrap values 100% (NJ) and 100% (ML). *P. alexandri* appeared to be a sister group of P. longicauda with bootstrap values 88% (NJ) and 81% (ML).

Others species of Psittacula from outside of Indonesia (*P. columboides*, *P. krameri*, *P. roseata*, *P. cyanocephala*, *P. finschii*, *P. himalayana*) were grouped in different clade. In ML tree, they were

very clearly separated from Psittacula of Indonesia (*P. longicauda* and *P.alexandri*) with bootstrap value was 100%. In both NJ and ML trees *P. columboides* and *P. krameri* were grouped together in same clade with 74% (NJ) and 68% (ML) bootstrap values. Other clade consisted of *P. roseata*, *P. cyanocephala*, *P. finschii*, and *P. himalayana* with 94% (NJ) and 77% (ML) bootstrap values, respectively. Relationship between clade consisted of (*P. columboides* and *P. krameri*) and clade consisted of (*P. columboides*, *P. krameri*, *P. roseata*, *P. cyanocephala*, *P. finschii*, *P. himalayana*) was not stable. The NJ tree clade of (*P. roseata*, *P. cyanocephala*, *P. finschii*, and *P. himalayana*) was separated from clade of *P. columboides* and *P. krameri*) by 99% bootstrap value. However according to ML tree, they appeared to be a sister group though only supported by 51% bootstrap value.

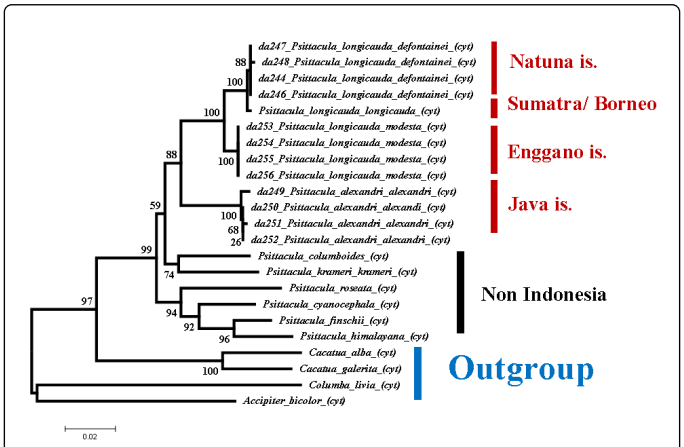

Figure 1: NJ tree Psittacula based on 869-bp of cyt b sequences. The numbers above are bootstrap values.

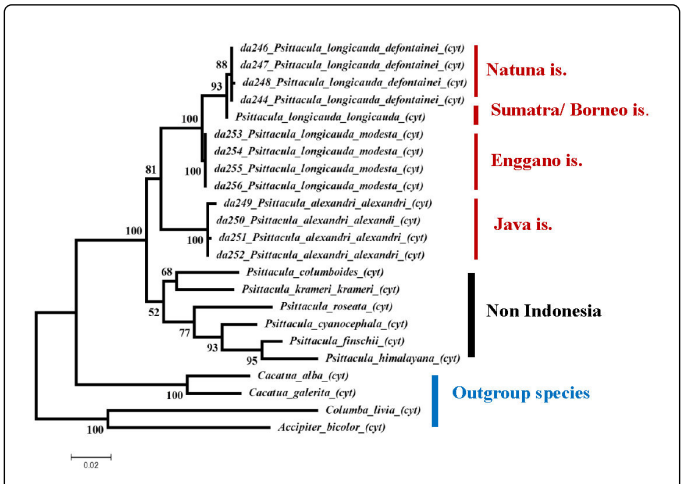

Figure 2: ML tree of Psittacula based on 869-bp of cyt b sequences. The numbers above are bootstrap values.

Discussion

The present study was using blood, tissue samples, and analysing mitochondrial DNA. Occasionally, blood samples mitochondrial DNA was contaminated by nuclear pseudogene, consequently DNA sequences of the cyt b gene were also contained pseudogene. Whereas, mitochondrial DNA from tissue samples relatively had low density of copy of mt DNA. In the present study, there was no indication of pseudogene because there were no stop codons. At the moment we translated DNA sequences to the amino acid, each of three nucleotides constructed an amino acid. Cyt b gene data of the present study had C +G 48.97% of C+G lest than A+T (51.03%) and low in Guanine. It was typically or often occurred in the mitochondrial DNA [12] as well as the mitogenome of *A. fasciata* and *B. lagopus* A + T content of 54.2% and 55.0%, respectively [13].

There is no intraspecific variation in the cyt b of *P. l. modesta* from Enggano Island which indicated that the four birds of *P. l. modesta* were identical sequences. It was assumed that it might be caused by inbreeding value [14], small population [15], habitat fragmentation [16], effect of endemism [17], or isolated population [18].

P. l. longicauda and *P. l. defontainea* were grouped in the same clade by 1.51% genetic divergence. It was relevant to the genetic divergence of cyt b from two subspecies of *Pyrrhula pyrrhula* (1.0 % to 1.5% of genetic divergence). It was indicated that the status of *P. l. longicauda* and *P. l. defontainea* were comfortable as subspecies of *P. longicauda*. In case of *P. l. modesta*, its genetic divergence from *P. l. defontainea* was 0.216% and 0.237% from *P. l. longicauda*. It was relatively higher than genetic divergence between *P. l. longicauda* + *P. l. defontainea*. In Pyrruhula, genetic divergences between species were range from 2.6% to 7.5% [19]. Even though genetic divergence between *P. l. modesta* and *P. l. defontanea* or *P. l. longicauda* was more than 1.5 % but it was less than 2.6% to 7.5% [19], 10.1% to 12.8% (*Catharthes aura* vs *C. burrovianus*) [20], and also less than 5.12% (3.80% to 10.03%) of mean genetic distance/divergence between others species of *Psittacula* that this study examined. Based on the genetic/sequence divergence resulted in the present study, we assumed that *P. l. modesta* from Enggano Island was still as a subspecies of *P. longicauda*.

According to Wink [21] the genetic divergence might be useful in some instances to discuss the taxonomic position of the species. *P. alexandri* appeared as a sister group of *P. longicauda* with genetic divergence range from 5.47% to 6.84%. While, subspecies of *P. longicauda* were grouped together with genetic devergence range from 1.51% to 2.37%. If we follow Shields and Wilson [22] and Avise [23] that 2% sequence divergence is equivalent to 1 MY or sequence divergence 2% per MY, we assumed that might be *P. alexandri* from Java island separated from *P. longicauda* around 3 – 3.5 MYA, and *P. l. modesta* (from Enggano Island) was separated from others species of *P. longicauda* around 2 MYA [24-26].

Conclusion

The present study concluded that 1) Phylogenetically, the position of *Psittacula* from Enggano Island (*Psittacula longicauda modesta*) was distant from and as a sister group of others subspecies of *Psittacula longicauda* (*P. l. longicauda* and *P. l. defontainea*), 2) sequence divergence within *Psittacula longicauda* were 2.37% *for P. l. modesta* vs *P. l. longicauda*, 2.16% for *P. l. modesta* vs *P. l. defontainea*, 1.51% for *P. l. longicauda* vs *P. l. defontainea*. 3) The mean sequence divergence between species of *Psittacula* was 5.12% ranged from 3.80% (*P. finschii* vs *P. himalayana*) to 10.03% (*P. himalayana* vs *P. alexandri*). Based on sequence divergence, we conclude that *Psittacula* from Enggano Island (*P. l. modesta*) is a subspecies of *P. longicauda* or is not a new species of *Psittacula*.

Acknowledgment

We would like to thank to staff members of the Zoological Division, Research Centre for Biology, and The Indonesian Institute of Sciences for their kind help in doing DNA analyses for the present study. Especial thank to Dr. Amir Hamidy (Coordinator of Enggano Island Expedition 2015) for his suggestions and financial supports. This research was supported by Enggano Expedition Project, R.C. for Biology, Indonesian Institute for Sciences.

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