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Short Communication: Taxonomic status of Great Argus (*Argusianus argus*) Sumatra and Borneo based on cytochrome B gene

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Abstract. *Ericca C, Tjong DH, Roesma DI, Novarino W, Syafullah, Janra MN, Aandrean. 2022. Short Communication: Taxonomic status of Great Argus (*Argusianus argus*) Sumatra and Borneo based on cytochrome B gene. Biodiversitas 23: 4670-4676.* Great Argus (*Argusianus argus*) belongs to the bird family Phasianidae. Currently, there are two Great Argus subspecies: *A. argus argus* in Sumatra and the Malay Peninsula, and *A. argus grayi* in Borneo. Therefore, this study aims to determine the taxonomic status of Great Argus across its distribution region. Phylogenetic relationships between two Great Argus subspecies were inferred in this study using the mitochondrial cytochrome b gene. DNA was isolated, amplified, and sequenced from the feathers of Great Argus sampled from Kinantan Wildlife and Cultural Park, Bukittinggi, West Sumatra, Indonesia. Phylogenetic relationships were analyzed with Maximum Likelihood methods. The phylogenetic trees show monophyletic relationships between Sumatran Great Argus subspecies. Analysis result shows that Bornean Great Argus has a 3.26-3.80% genetic distance from the Sumatran Great Argus based on analysis using the 789 bp of cyt b gene. Based on the result, it also refers that Bornean Great Argus and Crested Argus (*Rheinardia ocellata*), have a close relationship, indicated by low genetic distances (5.87-6.73%). This study suggests that Bornean Great Argus is genetically different from Sumatran Great Argus.

Keywords: Cytochrome b gene, genetic distance, Great Argus, monophyletic, phylogenetic

INTRODUCTION

Indonesia is one of the countries with high bird diversity. There are 1812 bird species distributed over the Indonesian region, with 557 protected species, 532 endemic species, and 461 species with limited distribution (Fahmi 2021). Birds perform an essential role in the functioning of ecosystems. Birds help in seed dispersal, controlling pests, pollinating flowers, and scavenging carrion (Sekercioglu at al. 2016). The seed dispersal mechanism of birds plays a role in the distribution of numerous plant species and, therefore, in increasing biodiversity (Deng and Yimam 2020). One of the birds found on the island of Sumatra and used as the mascot of West Sumatra Province based on Ministry of Home Affairs No. 48 year 1989 is the Great Argus (Rafi et al. 2017).

The Great Argus (*Argusianus argus*) is the only species of the genus *Argusianus* of Phasianid birds. The Great Argus solely exists in Southeast Asia, distributed across Malay Peninsula, Sumatra, and Borneo (Mackinnon et al. 2000). The bird is mainly characterized by a large body, wing and tail feathers uniquely marked with ocelli-shaped patterns. The male Great Argus spreads its wings into a typically huge fan when displaying to females (Davison 1980). The female Great Argus has a smaller body size, shorter wings and tail feathers than the male, has a darker colour and doesn't have ocelli-shaped markings (Mackinnon et al. 2000). The Great Argus is protected under the Minister of Environment and Forestry's Regulation Number 106 Year 2018 and has been categorized as vulnerable species by the IUCN (2020). Its population continues to decline, resulting from habitat destruction, forest degradation, land conversion, and hunting (Winarno and Sugeng 2018).

The Great Argus was distributed in the part of the Sunda Shelf that encompasses the islands of Sumatra, Java, and Borneo. The significant capture of the Siam headwaters by the East Sunda Basin is over the last 30 kyr. This capture lasted until the Sunda Shelf completely flooded 10 kyr ago (Salles et al. 2021), and separated Sumatra, Borneo and Malay Peninsula. Previous research about phylogeography of vertebrates on the Sunda Shelf has shown that the most common biogeographical pattern identified based on the data was a close relationship between Sumatra and the Malay Peninsula, with Borneo being more divergent (Leonard et al. 2015).

Currently, there are two Great Argus subspecies acknowledged, *A. argus argus* in Sumatra and the Malay Peninsula, and *A. argus grayi* from Borneo (Lepage and Denis 2020). Morphological differences between the two subspecies are noticeable, especially on their chest, back, and ocelli (Eaton et al. 2016). The phylogenetic position of Phasianidae was recently updated and inferred a peacock cluster consisting of *Argusianus* and *Pavo* (Shen et al. 2014), but there is no valid information about the sample origin of the Great Argus (*Argusianus*) used in the study.

Previous research has examined the position of the Great Argus species in the Phasianidae group (Kimball et al. 1997; Shen et al. 2014), but there has been no research on the relationship between the two Great Argus subspecies. Molecular studies about the relationship of the Great Argus based on their distribution have rarely been attempted.

Comparative analysis to assess the relationship among organisms has often used the combination of morphological data and DNA sequence data. The use of molecular data for phylogenetic analysis is more convenient than using morphological data (Patwardhan et al. 2014). Furthermore, phylogenetic studies in birds and other vertebrate groups commonly use the cytochrome b gene (known as cyt b). The cyt b gene is among protein-coding genes in mitochondrial DNA that is conserved, with a high mutation and variation rate, as well as a fast evolutionary rate. Therefore, cyt b is widely used in studying phylogenetic relationships at various taxonomic levels.

Several studies have been carried out using cyt b, including analysis of the evolutionary history of peacocks and other taxa (Sun et al. 2014), identification of avian species (Awad et al. 2015), analysis of genetic variation and phylogenetics of Indian peafowl from different locations in Pakistan (Naseer et al. 2018), and analysis of genetic diversity of Japanese quail (Adimaka et al. 2019). Unfortunately, previous studies on great the argus rarely look at its molecular aspects. Thus, phylogenetic information regarding the Great Argus is still lacking, especially from molecular and systematic perspectives. Recalling that a long period of geographical separation between the two Great Argus subspecies could be a potential driving force for allopatric speciation, this study aims to determine the taxonomic status of Great Argus across its distribution region. This study was to learn more about the genetic diversity and phylogenetic relationships of Great Argus using a partial sequence of the mtDNA cyt b gene.

MATERIALS AND METHODS

Sample collection

The study was carried out from April to November 2021, involving the collection of feather samples from two Great Argus individuals caged in the Kinantan Wildlife and Cultural Park, Bukittinggi, West Sumatra. Each feather sample was stored in sterile zip-lock bags. Molecular work was performed at the Genetics and Biomolecular Laboratory, Department of Biology, Universitas Andalas.

DNA extraction

The calamus part of feather samples was frozen with liquid nitrogen, then diced and crushed to powder using a mortar. DNA extraction was carried out using the GeneAll ExgeneTM Genomic DNA kit. The DNA concentration was assessed using a nanodrop to evaluate DNA purity. The quality of isolated DNA was observed with electrophoresis using 1.2% agarose gel before being stored at -20°C.

Polymerase Chain Reaction (PCR)

The cyt b gene amplification process used the forward primer L15164 5' GCAAACGGCGCCTCATTCTT 3' (Kimball et al. 1999) and the reverse primer H16012 5' GTTGAGTATTTTGTTTTC 3' (Avise et al. 1994) with product length reaching 885 bp. The amplification was performed in a 25 µL reaction containing a mixture of 12.5 µL PCR supermix Bioline, 3.5 µL nuclease-free water, two µL each primer pair, and five µL DNA template. The amplification temperature was set at 94°C for 1 minute of predenaturation, 94°C for 1 minute of denaturation, 50°C for 1 minute of annealing, 72°C for 1 minute of elongation, and 72°C for 5 minutes of post-elongation; the process involved a total 35 cycles. The DNA was then visualized using electrophoresis on 2% agarose gel. The PCR products were sent to First BASE Company Singapore for sequencing.

Data analysis

The DNA sequences were edited and assembled using the Bioedit program. The sequences were then compared with other cyt b genes sequences using *Ortalis vetula* (Cracidae) as an outgroup on the NCBI website (https://pubmed.ncbi.nlm.nih.gov/) using BLAST (Basic Local Alignment Search Tool). The aligned sequences of cyt b gene were subsequently analyzed for genetic distance and comparison using Clustal W program in MEGA version 7 (Kumar et al. 2016). Analysis of polymorphic sequences was conducted with DNA Sequence Polymorphism 5.10 to see the variation of nucleotide bases at the polymorphic sites. Finally, the phylogenetic tree was reconstructed using the combination of Maximum Likelihood (ML) methods with a bootstrap value of 1000 in all cases.

RESULTS AND DISCUSSION

DNA amplification

A total 885 bp of cyt b gene was successfully amplified in this study. The length of the sequenced fragment from feather samples collected from two Great Argus individuals ranged from 831 to 860 bp, with 789 bp alignment sequences that are effective for further analysis.

BLAST and alignment analysis

The BLAST search returned with 97% and 99% similarity values Great Argus cyt b sequences stored in Genbank. BLAST analysis of 789 bp showed a high similarity value confirming that all samples used in this study belong to *A. argus.* The alignment of all sequences consisted of 789 bp that, proportionately composed of 505 bp (64.01%) conserved sites, 284 bp (35.99%) variable sites, 225 bp (27.37%) parsimony-informative sites, and 68 bp (8.61%) singleton sites. The nucleotide composition was 53.5% Adenine + Thymine (A+T) and 46.7% Guanine + Cytosine (G+C). The A+T composition in the complete mitochondrial genome is higher than the base content of G+C. This closely resembled that observed in *Francolinus pintadeanus*, another Phasianid, which was found to have

54.7% A+T and 45.3% G+C. Analysis of 20 Phasianid and cracid sequences in 789 bp of the cyt b gene produced 20 haplotypes. Each sample of *A. argus* had nucleotide base variations, so they constituted different haplotypes.

Phylogenetic analysis

Three Maximum Likelihood (ML) phylogenetic methods, along with Kimura 2-parameter model, were used to construct a phylogenetic tree. Phylogenetic analysis involved 18 sequences that were registered in GenBank (Table 1). The analysis indicated two main clusters of phasianids, as seen in Figure 1. The first cluster contains three sub-clusters. Sub-cluster 1 consisted of all *A. Argus* samples and *Rheinardia ocellata*, sub-cluster 2 contains *Polyplectron*, *Afropavo*, and *Pavo*, and sub-cluster 3 is formed by *Lophophorus* and *Lophura*. The second cluster is home for the four *Gallus* species sampled. The average genetic distance between the four clusters of Phasianidae is *13% (Argusianus-Gallus, Lophophorus-Gallus, Argusianus-Polyplectron). Argusianus* and *Rheinardia* were indicated to be a clade, supported by a 100% bootstrap value.

Table 2 shows that Sumatran Great Argus has genetic distances of <1% (0.0063-0.0076) to the Southeast Asian Great Argus and 3.26%-3.39% to the Great Argus of Malaysia (Borneo). The Southeast Asian Great Argus has a 3.80% genetic distance to the Bornean Great Argus. In comparison, the genetic distance between *Rheinardia* Great Argus ranges from 0.0587-0.0673 (6-7%).

Table 1. DNA sequences list and GenBank accession number of samples

Species	Accession number	Author
A. congensis	AF013760.1	Kimball et al. (1997)
A. argus grayi	EF620765.2	Davison et al. (2007) Unpublished
A. argus	KY411590.1	Wang et al. (2017)
A. argus	AF013761.1	Kimball et al. (1997)
R. ocellata	AF330060.1	Kimball et al. (2001)
G. gallus	HQ857212.1	Miao et al. (2010) Unpublished
G. lafayetii	AB044990.1	Nakaki et al. (2000) Unpublished
G. sonneratii	AB044989.1	Nakaki et al. (2000) Unpublished
G. varius	AB044988.1	Nakaki et al. (2000) Unpublished
L. impejanus	NC.040850.1	Chen et al. (2018)
L. sclateri	AY265310.1	Zhan and Zhang (2003)
L. bulweri	MN991594.1	Boyce et al. (2020) Unpublished
L. diardi	AF028797.1	Kimball et al. (1999)
P. cristatus	L08379.1	Kornegay et al. (1993)
P. muticus	AF013763.1	Kimball et al. (1999)
P. bicalcaratum	AF028799.1	Kimball et al. (1999)
P. chalcurum	AF330061.1	Kimball et al. (2001)
O. vetula	L08384.1	Kornegay et al. (1993)

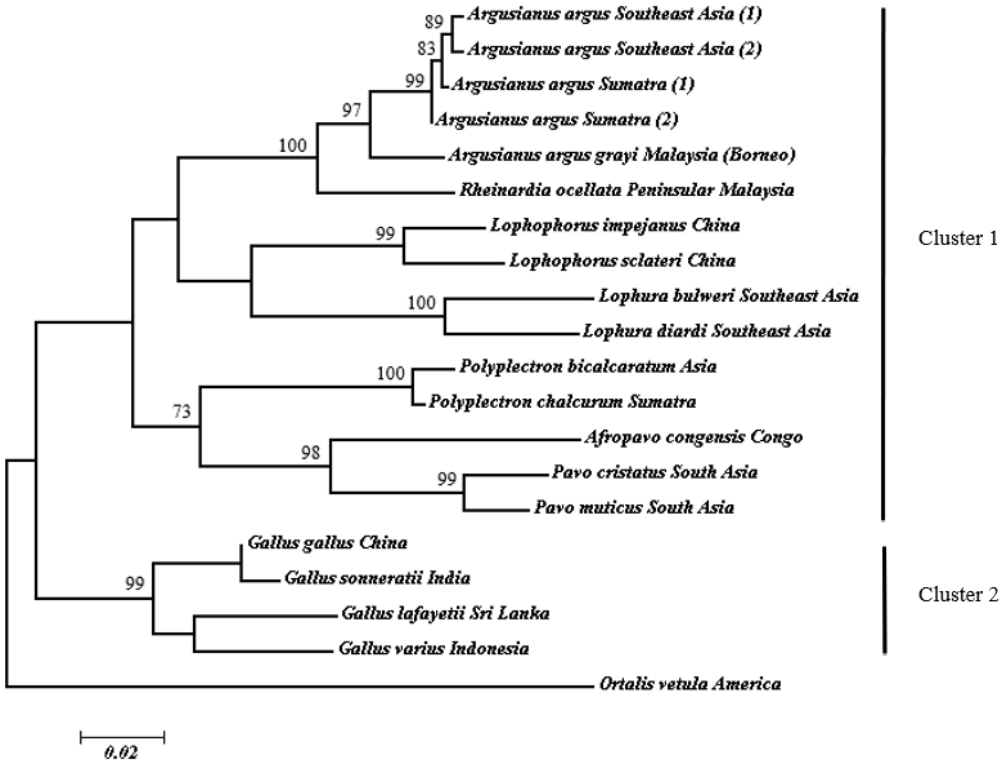

Figure 1. The ML phylogenetic tree of Phasianidae using cyt b gene with 1000 bootstrap values

Table 2. Genetic distance percentage (%) among the Phasianidae members based on cyt b gene

No	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*A. congensis*_Congo																			
2	*A. a grayi*_M. Peninsula	13.88																		
3	*A. argus 1*_Southeast Asia	14.56	3.78																	
4	*A. argus 1*_Sumatra	14.26	3.39	0.64																
5	*A argus 2*_Southeast Asia	14.24	3.78	0.51	0.64															
6	*A. argus 2*_Sumatra	13.92	3.25	0.76	0.38	0.76														
7	*R. ocellata*_M. Peninsula	14.34	6.24	6.39	6.26	6.67	5.84													
8	*G. gallus*_China	15.41	12.54	12.73	12.43	12.73	12.10	12.52												
9	*G. lafayetii*_Sri Lanka	16.42	13.65	14.65	14.34	14.65	14.01	13.48	6.27											
10	*G. sonneratii*_India	16.51	13.72	13.92	13.62	13.92	13.28	13.70	0.90	6.99										
11	*G. varius*_Indonesia	16.65	13.04	14.02	13.72	14.02	13.39	13.34	6.01	6.71	7.04									
12	*L. impejanus*_China	15.73	12.20	12.85	12.71	12.85	12.23	11.11	12.13	14.52	13.31	13.26								
13	*L. sclateri*_China	15.27	11.29	11.78	11.64	11.78	11.32	11.58	12.62	14.70	13.81	13.44	4.31							
14	*L. bulweri*_Southeast Asia	17.27	13.82	14.14	13.84	13.51	13.67	15.33	14.86	14.96	16.11	14.70	12.78	13.23						
15	*L. diardi*_ Southeast Asia	16.67	12.52	12.52	12.23	12.52	12.06	13.67	14.20	14.82	15.43	15.04	12.99	12.50	6.83					
16	*P. cristatus*_South Asia	10.62	13.16	13.83	13.21	13.51	12.88	14.12	13.67	14.80	14.72	15.51	14.23	13.93	14.86	14.23				
17	*P. muticus* Southeast Asia	10.33	13.63	13.99	13.69	13.67	13.35	13.81	13.32	13.81	14.52	15.14	15.06	14.12	15.38	14.59	3.51			
18	*P. bicalcaratum*_Asia	12.94	13.24	13.74	13.60	14.06	13.11	13.22	13.89	14.87	15.11	14.92	14.05	14.07	14.60	14.66	12.58	12.89		
19	*P. chalcurum*_Sumatra	12.46	12.14	12.63	12.49	12.94	12.01	11.97	13.25	14.53	14.44	14.75	12.78	12.80	14.27	13.84	11.95	12.57	1.28	
20	*O. vetula*_America	22.51	21.91	23.88	23.34	23.88	23.11	21.69	20.37	20.53	21.26	20.61	21.53	19.86	24.64	23.41	21.54	22.47	21.69	20.92

Discussion

Sumatran, Southeast Asian, and Bornean Great Argus form a monophyletic group. The *Argusianus* groups and *Rheinardia* groups are monophyletic and incorporated into subcluster 1 (Figure 1). Subcluster 1 and subcluster 2 are separated by 11-15% genetic distance, similarly between subcluster 2 and 3 with 12-17% genetic distance. The genetic distance between Sumatran Great Argus and Southeast Asian Great Argus is 1% (0.0063-0.0076). The average genetic distance between distinct vertebrate species, as marked with the cyt b gene, is 1.38% ± 0.30; however, smaller values of genetic distance may apply to bird taxa since they have lower rates of nucleotide substitution than other vertebrate taxa.

Furthermore, Sumatran Great Argus and Bornean Great Argus in subcluster 1 have a 3.26% to 3.39% genetic distance and a 3.80% genetic distance between Southeast Asian and Bornean Great Argus (Table 2). Table 2 also details the genetic distances between some other species in the same genus, such as between *Lophophorus impejanus* and *Lophophorus sclateri* (4.36%) or *Polyplectron bicalcaratum* and *Polyplectron chalcurum* (1.28%). The genetic distance between species in *Pavo* is 3.1%, in *Lophura* they range between 2.5 and 4.5%, in *Tragopan* they range between 4.6% and 8.7%, and in *Chrysolophus* it is 2.5% (Wen and Liu 2010). The average genetic distance between sister phasianid species is 3.7% (Thomson et al. 2014). Bornean Great Argus from Sumatran Great Argus is genetically different. Based on the genetic distance value between the two populations of Great Argus that showed in this study, were suggests they are different species.

Males Sumatran and Bornean Great Argus are slightly different in morphology, specifically in the coloration of the chest, back, and ocelli. Bornean Great Argus have lighter orange on the chest, darker upper backs, and white spotted ocelli, while Sumatran Great Argus have reddish-brown chests, brown backs, and light yellowish-brown spotted ocelli (Eaton et al. 2016; Figure 2). These differences are not reported for females.

In addition to the slight morphological differences, Sumatran and Bornean Great Argus inhabit different elevations. Sumatran Great Argus inhabits up to 1000 m elevation, while its Bornean counterpart can be found up to 1800 m elevation (Eaton et al. 2016). Tree diameter and canopy cover within the habitat influence their existence (Nijman 1998). The different topographies between Sumatra and Borneo also affect the condition of the mating ring, which is used by male Great Argus to dance while courting females. In Sumatra, the mating ring tends to be established on flat terrain surfaces (Rafi et al. 2017), while in Kalimantan and Malaysia, most mating rings are on hill or ridge landscapes (Winarni 2002).

A considerable genetic distance between Sumatran and Bornean Great Argus, as seen in this study, is presumably due to the geographic isolation between the subspecies populations that limits gene flow between them, resulting in the accumulation of differences in nucleotide bases. The Great Argus is more of a terrestrial bird that prefers running than flying in its evasive maneuvers, as it is a poor flyer (Davison 1980). Hence, the exchange of individuals between Sumatran and Bornean populations is next to impossible. This situation may have led to allopatric speciation, in which geographically separated populations become reproductively isolated (Tobias et al. 2020). Sumatra, Borneo and Malay Peninsula may have been an efficient barrier to gene flow that has promoted genetic divergences within forest-dependent taxa (Leonard et al. 2015).

The phylogenetic tree also infers that Crested Argus is sister species to a clade composed of Great Argus with a genetic distance ranging from 5.84-6.67%. This study established an average of 13% genetic distance value for distinct Phasianid species. This value is somewhat in line with what was established in previous studies, such as 9.8% to 18.6% genetic distance for Corvidae species (Ericson et al. 2005) or 13% genetic distance for Japanese quails of genus *Coturnix* (Adimaka et al. 2019); it is quite higher than 6.45% genetic distance stated for species in two hornbill genus (Jarulis et al. 2019).

Figure 2. The appearances of Sumatran Great Argus *Argusianus argus argus* (A) and Bornean Great Argus *Argusianus argus grayi* (B) (Source: https://ebird.org)

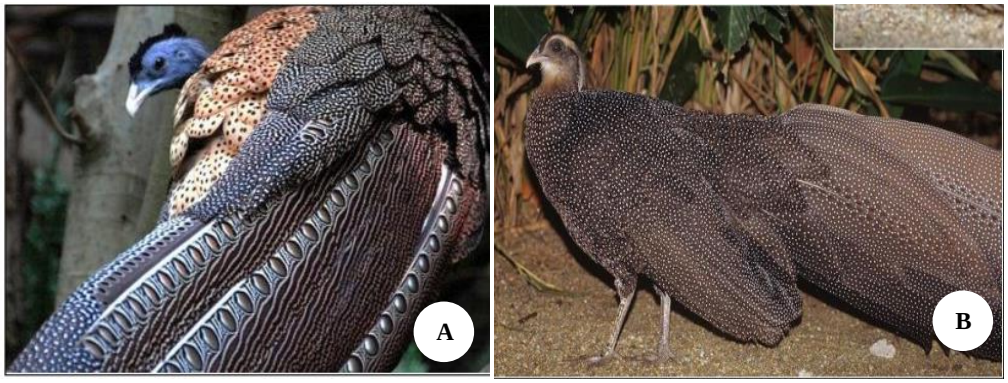

Figure 3. Morphological appearance of male Great Argus *Argusianus argus* (A) and male Crested argus *Rheinardia ocellata* (B) (Source: Trail 2012)

Great Argus and Crested Argus possess some morphological similarities (Figure 3). Male Great Argus has light brown on the wing feathers, secondary feathers, and long mid-tail feathers that are shaded with milky brown and patterned with dark brown spots (ocelli). These dark brown and black eye patterns on the tail and wing become more prominent when the bird poses in a stance that creates a huge fan-like display (Davison 1982; Eaton et al. 2016). Male Crested Argus also has considerably long tail feathers, rust-brown on the inner side and its dark gray ring-like fringe spotted with white; body and wing feathers dotted with white (Davison et al. 2020).

This research provides a baseline to consider the taxonomic status of Great Argus across its distribution range and serves as basic information for furthering conservation efforts. Therefore, further investigation is needed to confirm especially the taxonomic status of the Bornean and Sumatran Great Argus. The phylogenetic analysis on 789 bp cyt b gene in this study indicates that Sumatran and Malay Peninsula Great Argus is monophyletic to the other Great Argus population. This study also provides the foundation to propose Bornean Great Argus *A. argus grayi* as a distinct species, as well as the possibility to merge Crested Argus *Rheinardia* into *Argusianus.*

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