

Genetic characterization and phylogenetic study of Indonesian cuscuses from Maluku and Papua Island based on *16S rRNA* gene

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Abstract

Background and Aim: Indonesian cuscuses are now becoming scarce because of the reduction of habitat and poaching. Further, molecular characterization of Indonesian cuscuses is still very lacking. This study aimed to determine genetic markers and phylogenetic relationships of Indonesian cuscuses based on *16S rRNA* gene sequences.

Materials and Methods: This study used 21 cuscuses caught from two provinces and 16 islands: 13 from Maluku and eight from Papua. Cuscus samples were taken by biopsy following ethics guidelines for animals. The genome isolation was done using gSYNC DNA Mini Kit (Geneaid Biotech Ltd., Taiwan). The *16S rRNA* gene was amplified by primers (16SKUSAF and 16SKUSAR), and the polymerase chain reaction product obtained was 1875 base pair (bp). The analysis of genetic characterization and the phylogenetic relationship was performed using MEGA version X software (<https://www.megasoftware.net/>).

Results: *16S rRNA* gene sequencing attained 1598 bp for all samples. Based on the *16S rRNA* nucleotide sequences, cuscuses from Papua and Maluku belong to the genus *Phalanger* and *Spilocuscus*. *Phalanger* spp. and *Spilocuscus* spp. from Papua can be distinguished from *Phalanger* and *Spilocuscus* from Maluku, except *Spilocuscus* from Ternate has a very close relationship with cuscus from Sentani, Papua.

Conclusion: Indonesian cuscuses were derived into two clades based on *16S rRNA* gene sequence, one group to genus *Phalanger* and another group to *Spilocuscus*.

Keywords: Indonesian cuscuses, Maluku, Papua, *Phalanger*, phylogeny, *Spilocuscus*.

Introduction

Animal distribution studies are increasingly being conducted, so the characterization of each species needs to be carried out both morphologically and molecularly. One animal originating from the Wallace line is the small Sulawesi cuscus (*Strigocuscus celebensis*); another is Sulawesi bear cuscus (*Ailurops ursinus*). There are at least 20 additional species which belong to the family Phalangeridae [1-3]. *A. ursinus* is spread throughout Sulawesi and nearby islands in Togian, Peleng, Muna, Buton, and Lirung (Talaud Islands). *Spilocuscus maculatus*, described by Desmarest [4], has a range that extends from Queensland (Australia), through Papua, to Buru and Caram, and the small island of Selayar, at the tip of the Southeast Sulawesi. Peninsula cuscus is an Australian animal, marsupial mammal, belonging to the family

Phalangeridae, whose distribution is limited in East Indonesia (Sulawesi, Maluku, and Papua), Australia, and Papua New Guinea. From the five genus cuscus, four genera are found in Indonesia, namely, *Ailurops*, *Phalanger*, *Spilocuscus*, and *Strigocuscus*, and there are at least 24 species of cuscus spread across the three islands [5-7]. In Papua, two genera were found, namely, *Phalanger* (bottled cuscus) and *Spilocuscus* (spotted cuscus); in Maluku, there are two genera found, namely, *Phalanger* and *Spilocuscus*; and in Sulawesi, the genus *Spilocuscus* and *Ailurops* are found, which are endemic species in Sulawesi Island [8-10]. This research is significant because Indonesian cuscuses are now beginning to become scarce due to the decreasing habitat it occupies, many local people are hunting for consumption, trade, and, in some communities, ritual purposes [11,12].

Cuscus is one of the protected wildlife in Indonesia based on the decree of the Indonesian Ministry of Agriculture No. 247/KPTS/UM4/1979 and PP. No. 7 of 1999 concerning Preservation of Plants and Animals. Efforts to maintain the preservation of these animals need to be done *in situ* and *ex situ* conservation. The data from these animals, both morphologically and genetically, are lacking, so the molecular characteristics

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need to be studied more closely. Latinis [13] has researched hunting cuscus in Central Maluku. Fatem and Sawen [14] identified cuscus in the northern coastal region of Manokwari, Papua, based on its morphological characteristics. Molecular research on cuscus has been carried out by Munemasa *et al.* [15] using the mitochondrial genome but at the level of the Phalangeridae family. Molecular studies at the species level have never been carried out. According to the previous studies, the NADH dehydrogenase subunit 3 (ND3) gene sequence, the 4L NADH dehydrogenase subunit (ND4L) gene, and 12 rRNA gene can be used as genetic markers of *Tarsius bancanus* from Sumatra, Kalimantan with *Tarsius* from Sulawesi [16-18].

This study aimed to obtain nucleotide sequences and measure diversity using *16S rRNA* mitochondrial gene in cuscus from Maluku and Papua. These mitochondrial DNA sequences are unique and can be used as genetic markers for species identification. The *16S rRNA* gene has been widely used for phylogenetic study and detection in other species [19-22]. It is expected that the nucleotide diversity of each cuscus species can be used as genetic markers and can determine the phylogenetic relationship of the cuscuses from Maluku and Papua. It is hoped that by identifying genetic markers will help conservation efforts, especially for animals that have lost their habitat and need to be conducted *ex situ*. The information gained from these efforts can apply to other endangered species that are threatened with extinction, preserving the biological wealth in Indonesia.

Materials and Methods

Ethical approval

This study was approved by the Animal Ethics Committee for using Animal and Scientific Procedures in the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia.

Sample collection, study period, and location

Cuscus samples were taken from their natural habitat, namely, Maluku Province (13 individuals) and Papua Province (8 individuals) (Table-1). All cuscus samples were identified based on morphological characteristics (Figure-1) and sample tissues were preserved in RNA lather buffer (Qiagen, Germany). The Indonesian cuscus samples in this study were unrelated genetically because they were taken individually from the habitat of each location. This research was conducted from January to June 2020, starting from sample collection to data analysis.

Genome isolation

Total DNA was extracted from tissue biopsy (30 mg). Isolation and purification of DNA were performed using a DNA Isolation Kit (Qiagen). The isolated DNA was detected after migration on a 1% agarose gel using a $1 \times$ TBE buffer in the Submarine Electrophoresis device (Hoefer, USA). We observed the isolates with the help of ultraviolet (UV) light

($\lambda = 260$ nm) after the gel was stained with Bioatlas (Genaid, Taiwan). The isolated DNA was then stored at -20°C before being used for the next step.

Primer design

The primers are designed using the Primer3 output program (http://www-genome.wi.mit.edu/cgi-bin/primer3.cgi/results_from-primer3) based on the mitochondrial sequences of *Phalanger vestitus* (Access number AB241057.1). The sequence of the primers for the amplification of the *16S rRNA* gene is presented in Table-2.

Amplification of the *16S rRNA* gene by polymerase chain reaction (PCR)

Genomic DNA was used as DNA template for the amplification of *16S rRNA* gene. DNA amplification by PCR in this study used an Infinigen PCR machine. Amplification of the *16S rRNA* gene each using a self-designed pair of primers based on *P. vestitus* mitochondrial genome sequence from GenBank

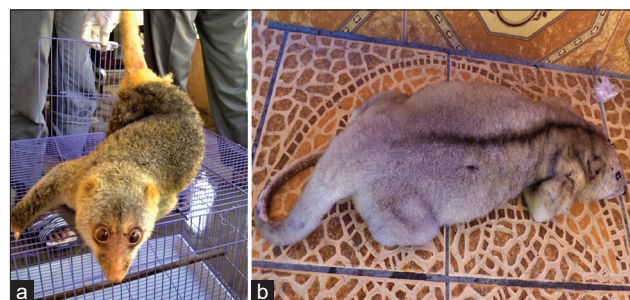


Figure-1: The cuscuses were from Maluku (a) and Papua (b).

Table-1: Origin of cuscus samples from Maluku and Papua Island.

Origin of cuscus	
Maluku Island	Papua Island
Allang P. Ambon	Moor Nabire Island
Gesor Island	Nabire Island
Gorom Island	Sentani Jayapura Island
Halmahera Island	Wanggar Nabire Island
Hatu Ambon Island	Yaro Nabire Island
Kariu Haruku Island	
Lakor Island	
Manipa Island	
North Seram Island	
South Seram Island	
Soya Ambon Island	

Table-2: Primer sequences for amplifying *16S rRNA* gene.

Primer	Sequence	Product size (bp)	Tm ($^{\circ}\text{C}$)
16SKUSAF	5' TTAGGAAGGCA ATTGCTAGG 3'	1875	51
16SKUSAR	5' CCGTCACCC TCCTCAATTA 3'		53
16SKUSBF	5' TTAGAAAAGCA ATTGCTAGG 3'	1875	54
16SKUSBR	5' CGTCACCCTC CTCAATTA 3'		53

Table-3: Estimates of evolutionary divergence over sequence pairs between groups.

Cuscus	<i>S. maculatus</i>	<i>Phalanger</i>	Cuscus from Ambon	Cuscus from Papua
<i>S. maculatus</i> *		0.008	0.006	0.004
<i>Phalanger</i> *	0.091		0.006	0.006
Cuscus from Maluku	0.063	0.076		0.005
Cuscus from Papua	0.045	0.081	0.054	

*From GenBank data, *Spilocuscus maculatus* (KJ868160.1), *Phalanger vestitus* (access number AB241057.1)

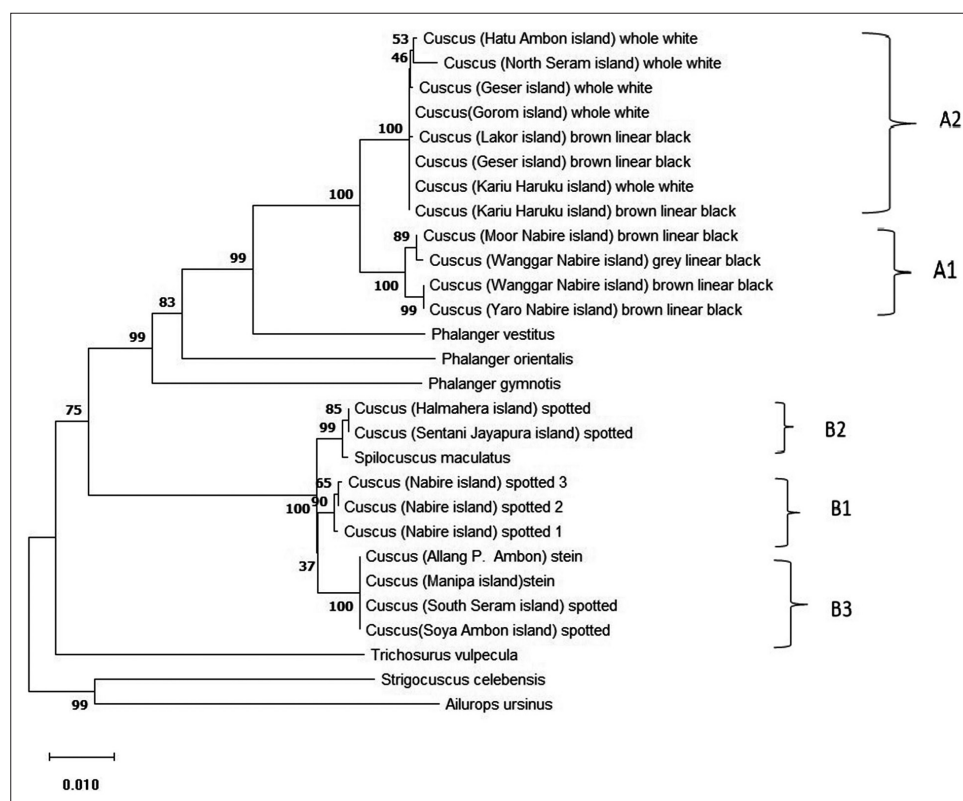


Figure-6: Evolutionary relationships of Indonesian cuscuses (with color of coat) and some cuscuses from GenBank database *Phalanger vestitus* (Access number AB241057.1), *Spilocuscus maculatus* (KJ868160.1), *Phalanger gymnotis* (KJ868142.1), *Phalanger orientalis* (AY228381.1), and *Trichosurus vulpecula* (AF357238.1).

Kei, Buru, Seram, Ambon, Selayar, Banda, Pandjang, Timor, and Cape York Peninsula, Australia. Prehistory and archeology of the cuscus evidenced that *P. orientalis* may have been actively introduced to Maluku, Seram, Buru, Sanana, and the Kai Islands as early as 6500 years ago. *P. vestitus* and *S. maculatus* were found in Papua and Maluku region [13,29]. The recent study on cuscus from Maluku, Kusumaningrum, and Abinawanto [30] studied cuscuses based on COI gene sequences and found that there were two types of cuscus, namely, *S. maculatus* and *P. vestitus*.

The results showed that, for the cuscus from Maluku and Papua, each origin included two genera, namely, the genus *Phalanger* and *Spilocuscus*. Widayanti *et al.* [8] studied molecular characterization of cuscus from Maluku and Papua Island based on the NADH dehydrogenase subunit 1 gene, grouping cuscus from Papua and Maluku became two genera *Phalanger* and *Spilocuscus* and classified into *Phalanger* spp. and *S. maculatus* species. The taxonomic review based on the biogeographic history of Phalangeridae is complicated because the geological

history of Southeast Asia is very complicated to describe. As a result, the historical interpretation of phalangerids biogeography provided can only be temporary [1-3]. The molecular phylogeny presented in this study is thought to reflect better the evolutionary relationships of Phalangeridae that differ from previous taxonomic determinations; the molecular phylogeny tree shows high suitability using statistical calculations [31].

Cuscuses of Papua and Maluku belonging to the genus *Phalanger* (Figure-6) showed that the two cuscus groups are separated in different sub-branches and show that *Phalanger* from Papua and Maluku can be distinguished [8,9]. Usmany *et al.* [11] reported that four types of cuscus lived in Seram (Maluku), namely, brown cuscus (*P. orientalis*), gray cuscus (*P. vestitus*), white cuscus (*Phalanger ursinus*), and spotted cuscus (*S. maculatus*). These four types of cuscus showed similarities in morphology. The spotted cuscus and gray cuscus have similarities of the earlobe, which are covered with hair. Still, white cuscus and brown cuscus are similar in that the earlobe is not covered with

hair [14]. Likewise, for Papua and Maluku cuscuses belonging to the genus *Spilocuscus* are also in different sub-branches. However, except for Tobelo (ternate) cuscus, the cuscus has historically been brought along with the migration of people from Papua to Maluku (Ternate) so that cuscus from Ternate has a very close relationship with those from P. Sentani, Papua [13,29].

Conclusion

Cuscuses from Papua and Maluku based on the *16S rRNA* gene nucleotide sequence are classified in the genus *Phalanger* and *Spilocuscus*. *Phalanger* spp. and *Spilocuscus* from Papua can be distinguished from *Phalanger* and *Spilocuscus* from Maluku, except *Spilocuscus* from Ternate has the closest relationship with cuscus from Sentani, Papua. The *16S rRNA* gene can be used to identify genetic markers and differentiate cuscus species from Maluku and Papua. Genetic characterization information, especially genetic markers, is needed to carry out the conservation and breeding of each species.

Authors' Contributions

RW and SP designed this research and collected cuscus samples for this study. SP, RABP, and RMK conducted research in the laboratory. RW and SP analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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References

- Colgan, D.J., Flannery, T.F., Trimble, J. and Aplin, K.P. (1993) Electrophoretic and morphological analysis of the systematics of the *Phalanger orientalis* (*Marsupialia*) species complex in Papua-New-Guinea and the Solomon-Islands. *Aust. J. Zool.*, 41(4): 355-378.
- Norris, C.A. and Musser, G.G. (2001) Systematic revision within the *Phalanger orientalis* complex (*Diprotodontia*, *Phalangeridae*): A third species of lowland gray cuscus from New Guinea and Australia. *Am. Mus. Nov.*, 1(3356): 1-20.
- Molloy, S.W., Davis, R.A. and van Etten, E.J. (2016) Incorporating field studies into species distribution and climate change modelling: A case study of the koomal *Trichosurus vulpecula hypoleucus* (*Phalangeridae*). *PLoS*

- One*, 11(4): 154161.
- Desmarest, A.G. (1817) Crustacés fossiles. In: *Nouveau Dictionnaire d'Histoire Naturelle, Appliquée aux Arts, à L'agriculture, à L'économie Rurale et Domestique, à La Médecine*. 2nd ed. Deterville, Paris. p495-519.
- Menzies, J. (2011) A Handbook of New Guinea's Marsupials and Monotremes. University of Papua New Guinea Press, Papua New Guinea.
- Petocz, R.G. (1994) *Mamalia Darat Irian Jaya*. Grafitipers, Jakarta.
- Flannery, T.F. (1994) *Possums of the World: A Monograph of the Phalangeroidea*. GEO, Sydney, New South Wales, Australia.
- Widayanti, R., Agustianti, T.S., Kunda, R.M. Pakpahan, S. (2016) Phylogenetic relationship of cuscuses (*Marsupialia: Phalangeridae*) from Papua and Maluku based on mitochondrial sequences of NADH dehydrogenase subunit 1 gene. *Biotechnology*, 15(1-2): 17-25.
- Kunda, R.M., Handayani, N.S.N., Wijayanto, H. and Widayanti, R. (2017) DNA barcoding of cuscuses (*Marsupialia: Phalangeridae*) from Maluku and Papua. *Asian J. Anim. Vet. Adv.*, 12(5): 227-238.
- Nugraha, R. and Mustari, A.H. (2017) Habitat characteristics and diet of bear cuscus (*Ailurops ursinus*) in Tanjung Peropa wildlife reserve, Southeast Sulawesi. *J. Wasian*, 4(2): 55-68.
- Usmany, M., Tuaputty, H. and Kakisina, P. (2015) Cuscus (Family *Phalangeridae*) phenotype study in Lumoli Rural District Breeding Farm, Piru, Maluku. *J. Sain Vet.*, 33(2): 180-189.
- Sawen, D. and Sinery, A.S. (2020) The feed plants species of cuscus *Phalanger orientalis* in Yamma Island, Sarmi Regency. *World J. Adv. Res. Rev.*, 6(1): 31-39.
- Latinis, K. (1996) Hunting the cuscus in Western Seram: The role of the *Phalanger* in subsistence economies in Central Maluku. *Cakalele*, 7(1): 17-32.
- Fatem, S. and Sawen, D. (2007) The species of cuscus in Northern coastal areas of Manokwari, Papua. *Biodivers. J. Biol. Divers.*, 8(3): 86-91.
- Munemasa, M., Nikaido, M., Donnellan, S., Austin, C.C., Okada, N. and Hasegawa, M. (2006) Phylogenetic analysis of *Diprotodontian marsupials* based on complete mitochondrial genomes. *Genes Genet. Syst.*, 81(3): 181-191.
- Widayanti, R., Handayani, N.S.N. and Budiarsa, I. (2011) Keragaman genetik gen penyandi dehidrogenase Sub-unit 3 mitokondria pada monyet hantu (*Tarsius* sp.). *J. Vet.*, 12(1): 26-33.
- Widayanti, R. and dan Susmiati, T. (2012) Studi keragaman genetik *Tarsius* sp. Asal Kalimantan, Sumatera, dan Sulawesi berdasarkan sekuen gen NADH dehidrogenase sub-unit 4L (ND4L). *J. Kedokt. Hewan*, 6(2): 105-111.
- Widayanti, R., Wijayanto, H., Wendo, W.D. and Kunda, R.M. (2015) Identification of genetic diversity 12Sr RNA genes as genetic marker for determining species cuscus. *J. Vet.*, 16(2): 227-235.
- Saikia, D.P., Kalita, D.J., Borah, P., Sarma, S., Barman, N.N. and Dutta, R. (2015) Differentiation of sheep and goat species by PCR-RFLP of mitochondrial *16S rRNA* gene. *J. Anim. Res.*, 5(2): 213-217.
- Abdullah, H.H., El-Molla, A., Salib, F.A., Allam, N.A., Ghazy, A.A. and Abdel-Shafy, S. (2016) Morphological and molecular identification of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* (*Acari: Ixodidae*) vectors of rickettsioses in Egypt. *Vet. World*, 9(10): 1087.
- Aphale, D. and Kulkarni, A. (2018) Modifications and optimization of manual methods for polymerase chain reaction and *16S rRNA* gene sequencing quality community DNA extraction from goat rumen digesta. *Vet. World*, 11(7): 990-1000.
- Wang, Z.L., Yang, X.Q., Wang, T.Z. and Yu, X. (2018) Assessing the effectiveness of mitochondrial COI and *16S*

- rRNA* genes for DNA barcoding of farmland spiders in China. *Mitochondrial DNA A DNA Mapp. Seq. Anal.*, 29(5): 695-702.
23. Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, 22(22): 4673-4680.
 24. Kumar, S., Stecher, G. and Tamura, K. (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.*, 33(7): 1870-1874.
 25. Kumar, S., Nei, M., Dudley, J. and Tamura, K. (2008) MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief. Bioinform.*, 9(4): 299-306.
 26. Saitou, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4(4): 406-425.
 27. Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39(4): 783-791.
 28. Tamura, K. and Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.*, 10(3): 512-526.
 29. Flannery, T. (1995) Mammals of the South-West Pacific and Moluccan Islands. Reed Books, Sydney.
 30. Kusumaningrum, E.N. and Abinawanto, A. (2018) Implementation DNA Barcoding for Genetic Identification of Cuscus from Ambon Island. In: AIP Conference Proceedings. Vol. 2023. AIP Publishing LLC, Melville, NY. p020123.
 31. Raterman, D., Meredith, R.W., Ruedas, L.A. and Springer, M.S. (2006) Phylogenetic relationships of the cuscuses and brushtail possums (*Marsupialia: Phalangeridae*) using the nuclear gene BRCA1. *Aust. J. Zool.*, 54(5): 353-361.
