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Research Article

DNA Barcoding of Cuscuses (Marsupialia: Phalangeridae) from Maluku and Papua

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Abstract

Background and Objective: The DNA barcode can be used as a basis for determining the objectives and direction of development of conservation activities and can clarify differences and kinship relationships between species in the population. This study aimed to determine nucleotides sequences as DNA barcode for investigation of genetic diversity and phylogenetic from cuscuses in Maluku and Papua as well as other biogeography on *COX3* gene with sequencing method. **Materials and Methods:** The genome DNA from 25 samples, extracted according to the Qiamp DNA protocol is the Mini Blood Kit (Qiagen) and is used as a template for COX3 gene amplification using PCR method. **Results:** Nucleotides alignment of *Phalanger* members found 23 nucleotides distinguishing and *Spilocuscus* members found 8 nucleotides distinguishing, which can be used as barcode. The phylogram tree was using Neighbor joining classified cuscus from Maluku and Papua in clade A (*Phalanger*) and clade B (*Spilocuscus*), respectively. Clade A and clade B subdivided into clade A1 (*Phalanger* from Maluku), A2 (*Phalanger* from Papua) and clade B1 (*Spilocuscus* from Maluku), B2 (*Spilocuscus* from Papua). Clade B2 subdivided into one subclade B2' be under cuscus from Halmahera dan Sentani, Jayapura Islands. **Conclusion:** It was concluded that identification of *Spilocuscus* and *Phalanger* members from Maluku, Papua, Halmahera and Sentani could be distinguished by COX3 gene. *Spilocuscus maculatus* from Halmahera is derived from Papua, it introduced in the colonization of the Austronesian tribes on the Island. *S. maculatus* from Halmahera and Sentani Islands have one nucleotide distinguish, that is sites position 354 which distinguishes the cuscuses with cuscus from Papua and Maluku.

Key words: COX3, barcoding, cuscuses, mitochondrial, marsupialia, phalangeridae, Maluku, Papua

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mitochondrial DNA (mt-DNA) is the genetic material with a simple molecular structure, exists outside the nucleus in eukaryotic cells and have become a widely used tool for addressing phylogenetic relationships at various levels taxons¹⁻³. The importance of using comprehensive amount of mitochondrial sequence data for inferring phylogenetic relationships was demonstrated by Kunda *et al.*² and Widayanti *et al.*³. In fact, the rapid rate of change in the mt-DNA sequence results in differences between populations that have only been separated for long periods of time. In other words, sequence divergences are much larger among species than within species and thus mt-DNA genealogies generally capture the biological discontinuities recognized by taxonomists as species. Taking advantage of this fact, taxonomic revisions at the species level now regularly include analysis of mt-DNA divergences¹. Species revision of cuscuses have been defined, in part, on the basis of divergences in their mt-DNA¹⁻³.

Cuscuses are arboreal marsupials are divided into the 6 genera, *Ailurops*, *Phalanger*, *Spilocuscus*, *Strigocuscus*, *Trichosurus* and *Wyulda*. Based on morphological characters, there are 4 species of cuscuses in Maluku: *Spilocuscus maculatus* (spotted/white cuscus), *Spilocuscus rufoniger* (black spotted cuscus), *Phalanger orientalis* (northern common cuscus) and *Phalanger vestitus* (stein cuscus)^{2,3}. The local people sometimes seem to recognise more than two species but this is probably due to sexual and developmental dimorphism in coat colour⁴. Both species taken for food by the local people⁵.

In Papua, there are more than six species of cuscuses based on morphological characters: *Phalanger gymnotis* (ground cuscus), *P. orientalis* (northern common cuscus), *Spilocuscus maculatus* (spotted/white cuscus), *Spilocuscus rufoniger* (black spotted cuscus), *Spilocuscus papuensis* (Waigeo cuscus) is endemic species of Waigeo Island, West Papua Province³ and *Spilocuscus wilsoni* (Black spotted cuscus) is endemic species to the islands of Biak and Supiori in the Cenderawasih Bay, Papua Province⁶.

The spotted cuscuses are a group of colorful, medium sized, arboreal frugivore-folivores endemic to tropical forests in the Australo-Papuan region. They are found in primary and secondary tropical moist forest. They also have been recorded in mangrove forest⁷. The female thought to carry one single young only⁵. Among Phalangeridae genera, *Spilocuscus* characterized by a unique combination of traits, including sexual dichromatism in pelage coloration and pronounced sexual dimorphism, with females larger than males. It is powerfully built and covered in thick woolly fur and weighs

about 1-3 kg⁵. The males are usually spotted, but sometimes also completely white, whereas the larger females are usually non-spotted and grey-brown in colour. The young goes through a sequence of colour changes. Its solitary, nocturnal life style means that it hides in the 3 crowns or holes, or among masses of epiphytic vegetation during the day^{2,5}.

Recent molecular studies have included of the *Spilocuscus* and *Phalanger* genera members from Maluku and Papua^{1,3}, using 12S rRNA and NADH Dehydrogenase Sub-unit 1 (ND1) genes and using of Cyt b gene sequences for status affirmation of stein cuscus from Manipa Island². It was asserted that cuscus from Manipa Island has genetic relationship with *Spilocuscus maculatus* (94%) than *Phalanger* members².

If a short region of mt-DNA that consistently differentiated species can be found and accepted as a standard, a library of sequences linked to vouchered specimens would make this sequence an identifier for species, a "DNA barcode"^{8,9}. Recent work suggests that a 648 bp region of the mitochondrial gene, cytochrome oxidase I (COXI), might serve as a DNA barcode for the identification of animal species^{9,10}. The DNA barcoding usually consists of a fragment of the mitochondrial gene Cytochrome oxidase c subunit I (COX1) but other genes are also employed, sometimes with varying levels of success⁹. The method has many applications among which is an efficient means of identifying species because levels of divergence among individuals are usually much lower of the same species than between closely related species.

Past phylogenetic work often focused on mitochondrial genes encoding ribosomal (12S, 16S) DNA, but their use in broad taxonomic analyses is constrained by the prevalence of insertions and deletions (indels) that greatly complicate sequence alignments¹. Cytochrome c oxidase subunit III gene (COX3) has an important advantage, COX3 gene appears because possess a greater range of phylogenetic signal besides COX1 gene. Sequences of COX3 gene is more divergent than COX1 and Cyt b gene sequences¹¹. However, there is limited information on the suitability of the COX3 gene for the phylogenetic analysis within mammalian species. In fact, the evolution of these genes are slow enough to allow discrimination not only allied species, but also phylogeographic groups within a species¹². This research will provide phylogeny and useful information providing a clear understanding of population history, evolutionary mechanisms and can be used as a genetic marker (barcodes) of the same genus or species.

The purposes of this study as preliminary study on mitochondrial genome based on COX3 sequences that declared as a DNA barcode for investigation of genetic

diversity and phylogenetic from cuscuses in Maluku and Papua as well as other biogeography. Such tests require the analysis of phalangeridae members (*Spilocuscus* and *Phalanger*) from Maluku and Papua that have been before studied intensively so that declared COX3 gene as a DNA barcode. Cuscus conservation strategies will be more organized and useful if genetic marker can be known with certainty. It is hoped that the COX 3 gene sequences may act as a specific genetic marker (barcode) between species of cuscuses in the populations. Data from this study can be used to assist in the determination of conservation units as well as the determination of conservation strategies against these endemic protected animal.

MATERIALS AND METHODS

Samples collection: Twenty-five tissue biopsy samples were collected from two cuscuses of native habitat in Maluku (16 samples), Papua (9 samples) and, Halmahera Island (1 individual). All individuals were identified on the basis of morphological characteristics and a sample tissues collected from each species and preserved in RNA Lateral buffer (*Qiagen*) for the purpose of DNA isolation.

Molecular techniques and data analysis: Genomic DNA isolated from the tissues using DNA isolation kit (*Qiagen*) according to protocol method in combination with RNase treatment. The primer used to amplify COX3 gene was as follows: The PCR protocol was: The DNA template 1 µL, Kappa 2G Ready Mix 25 µL, each forward and reverse primer 1 µL (10 pmol) and added H₂O in a total of 50 µL. The PCR amplification was conducted using a Infigen Thermal Cycler according to the program, an initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min and 30 sec. A final

extension at 72°C for 5 min was performed to completely extend the amplified product. The DNA quality assessed by electrophoresis on 1.5% agarose gel (3 µL PCR products added with 1 µL of loading dye). Electrophoresis was run on 100 Volt condition for 30 sec and amplification result could be seen on the UV light. The purified PCR products were sequenced directly by 1st BASE Sequencing INT (Singapore).

The DNA sequences of COX3 gene were initially automatically alignments using Clustal W, followed by manual editing. Phylogenetic analysis of the aligned sequences was performed with the Molecular Evolutionary Genetics Analysis (MEGA) program version. 6.0. Distance analysis was conducted using the Neighbor-Joining (NJ) option employing the Kimura-2-parameter method with gap data treated as pair wise deletions. Molecular phylogeny analysis was conducted using heuristic searches, 1000 bootstrap replicates.

RESULTS

A total of 25 DNA samples have been isolated, then serves as a template for COX3 gene amplification by PCR method. The results PCR products 919 base pair (bp) after electrophoresis on a 1% agarose gel with a 1 kilo base pair (kbp) DNA Ladder (1st base) are shown in (Fig. 1).

The PCR product size is 919 bp obtained after the primers specific that will be used as alignment using the BLAST program against mitochondrial genome *P. vestitus* (access numbers AB241057.1). In this study, 8 species were used for comparison to determine the genus and species of cuscuses from Maluku and Papua Island. The nucleotide sequences data were determined for the present study deposited in *Genebank* under accession numbers, NC_008137 (*P. vestitus*), NC_003039 (*T. vulpecula*), NC_868096 (*A. ursinus*), NC_868161 (*S. celebensis*), NC_868160 (*S. maculatus*), KJ_868165

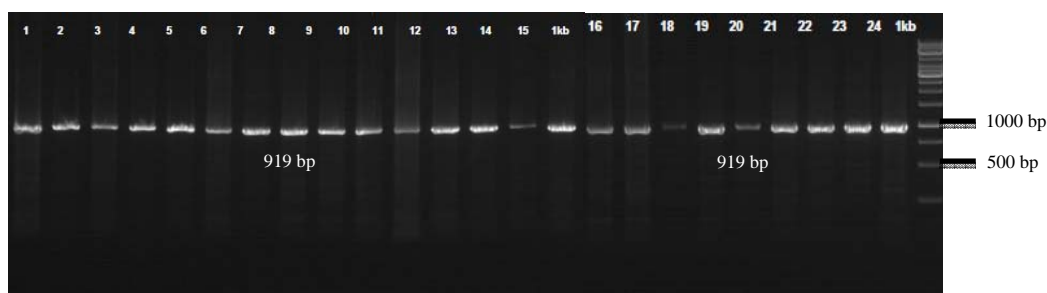


Fig. 1: PCR Product COX3 gene of cuscuses on a 1.5% agarose gel, Description: 1-25: PCR product of cuscuses samples (919 base pair), *1 kilo base pair (kbp) = 1000 base pair (bp)

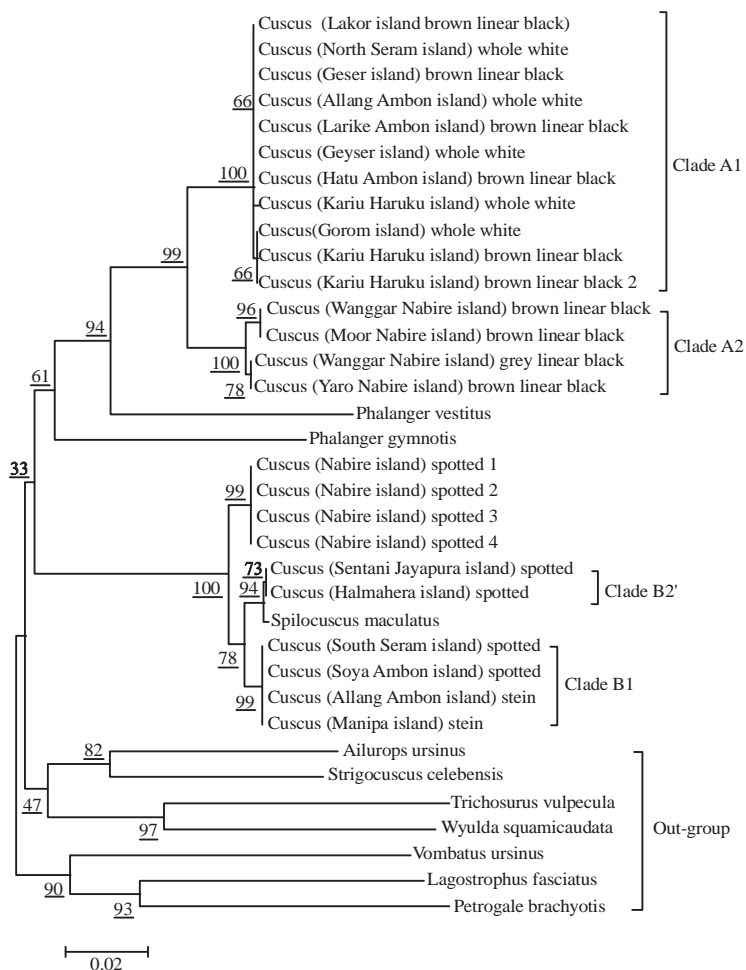


Fig. 2: Phylogenetic relationship of cuscuses (*Phalanger* and *Spilocuscus* genera) from Maluku and Papua based on mitochondrial COX3 gene sequences

(*Wyulda squamicaudata*), NC_003322 (*Vombatus ursinus*), KJ_868140 (*Petrogale brachyotis*). The result alignment COX3 gene was studied cuscuses obtained 783 nucleotides for subsequent analysis. There are differences in the nucleotides of 0-86 when inter cuscuses studied and 1-104 with cuscuses from *Genebank* (Table 1).

Analysis of the nucleotides matrix (Table 1), shows that cuscuses from Papua (*Phalanger* members) on the Yaro, Nabire (brown linear black), Moor Nabire (brown linear black) and Wanggar Nabire (Gray linear black) does not have different nucleotides (0 nucleotide). This also happens to cuscuses from Maluku (*Phalanger* members) on the Gorom island (whole white), Geser Island (whole white and brown linear black), North Seram (whole white), Lakor Island (brown linear black), Kariu village, Haruku Island (all samples) and Allang village, Ambon Island (whole white).

In this study, cuscuses from Papua (*Spilocuscus* members) on Nabire Island (total of four samples) does not

have different nucleotides (0 nucleotide). The same thing seen in cuscuses from Maluku (*Spilocuscus* members) on the South Seram Island (spotted cuscus), Manipa Island (stein cuscus), Soya and Allan village, Ambon Island (spotted and stein cuscuses) does not have different nucleotides (0 nucleotide). Cuscuses collected from Halmahera Island (spotted cuscus) have 8 nucleotides (8 nucleotides) distinguish with Maluku samples, although being in one biogeographic regions, but it same as the *Spilocuscus* members from Papua, has no nucleotide differences (0 nucleotide) (Table. 1). The NJ phylogenetic tree showed no conflicts between molecular and morphological identifications but the conflict is based on biogeography region (Fig. 2). Twenty three nucleotides distinguish can be used as a barcode among *Phalanger* members from Maluku and Papua and 4 nucleotides distinguish can be used as a barcode among *Spilocuscus* members from Maluku and Papua (Fig. 3).

Table 1: Number of differences (783 nucleotides) of mitochondrial COX3 gene of cuscuses with sequences comparison from GeneBank database (basepair., bp)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Phalanger vestitus (genebank)																											
Cuscus (Wanggar Nabire island) brown linear black	66																										
Cuscus (Wanggar Nabire island) grey linear black	66	4																									
Cuscus (Yaro Nabire island) brown linear black	66	4	0																								
Cuscus (Moor Nabire island) brown linear black	68	0	4																								
Cuscus (Hatu Ambon island) brown linear black	68	26	24	24	26																						
Cuscus (Geser island) whole white	68	26	24	24	26	0																					
Cuscus (Larika Ambon island) brown linear black	68	26	24	24	26	0	0																				
Cuscus (Allang Ambon island) whole white	68	26	24	24	26	0	0	0																			
Cuscus (Geyser island) brown linear black	68	26	24	24	26	0	0	0	0																		
Cuscus (Lakor island) brown linear black	68	26	24	24	26	0	0	0	0	0																	
Cuscus (North Seram island) whole white	68	26	24	24	26	0	0	0	0	0																	
Cuscus (Kariu Haruku island) whole white	69	27	25	25	27	1	1	1	1	1	1	1	1	1	1												
Cuscus (Gorom island) whole white	68	27	25	25	27	1	1	1	1	1	1	1	2														
Cuscus (Kariu Haruku island) brown linear black	68	27	25	25	27	1	1	1	1	1	1	2	0														
Cuscus (Kariu Haruku island) brown linear black 2	68	27	25	25	27	1	1	1	1	1	1	2	0	0													
Phalanger gymnotis (genebank)	93	81	81	81	81	77	77	77	77	77	77	77	78	78	78	78	86										
Cuscus (Nabire island) spotted 4	93	79	77	77	79	76	76	76	76	76	76	76	75	76	76	76	86										
Cuscus (Nabire island) spotted 3	93	79	77	77	79	76	76	76	76	76	76	76	75	76	76	76	86	0									
Cuscus (Nabire island) spotted 1	93	79	77	77	79	76	76	76	76	76	76	76	75	76	76	76	86	0									
Cuscus (Nabire island) spotted 2	93	79	77	77	79	76	76	76	76	76	76	76	75	76	76	76	86	0	0								
Cuscus (SouthSeram island) spotted	91	83	81	81	83	78	78	78	78	78	78	78	79	78	78	78	81	10	10	10	10	10	10	10	10	10	10
Cuscus (Soya Ambon island) spotted	91	83	81	81	83	78	78	78	78	78	78	78	79	78	78	78	81	10	10	10	10	10	10	10	10	10	10
Cuscus (Allang Ambon island) stein	91	83	81	81	83	78	78	78	78	78	78	78	79	78	78	78	81	10	10	10	10	10	10	10	10	10	10
Cuscus (Manipa island) stein	91	83	81	81	83	78	78	78	78	78	78	78	79	78	78	78	81	10	10	10	10	10	10	10	10	10	10
Cuscus (Sentani Jayapura island) spotted	93	83	81	81	83	78	78	78	78	78	78	78	77	78	78	78	84	12	12	12	12	12	12	12	12	12	12
Cuscus (Halimaheira island) spotted	93	83	81	81	83	78	78	78	78	78	78	78	77	78	78	78	84	12	12	12	12	12	12	12	12	12	12
Spilociscus maculatus (genebank)	94	84	82	82	84	79	79	79	79	79	79	79	78	79	79	79	83	11	11	11	11	11	11	11	11	11	11

Phalanger vestitus (Genebank)	ATG	ACT	CAC	CAA	ACA	CAC	GCA	TAT	CAC	ATA	GTT	AAC	CCA	AGC	CCA	[45]
Cuscus (Wanggar Nabire Island) brown linear blackCC	[45]
Cuscus (Wanggar Nabire Island) grey linear blackCC	[45]
Cuscus (Yaro Nabire Island) brown linear blackCC	[45]
Cuscus (Moor Nabire Island) brown linear blackCC	[45]
Cuscus (Hatu Ambon Island) brown linear blackCC	[45]
Cuscus (Geyser Island) whole whiteCC	[45]
Cuscus (Geyser Island) brown linear blackCC	[45]
Cuscus (Gorom Island) whole whiteAC	[45]
Cuscus (Lakor Island) brown linear blackCC	[45]
Cuscus (Kariu Haruku Island) brown linear blackAC	[45]
Cuscus (Kariu Haruku Island) whole whiteCTC	[45]
Cuscus (Kariu Haruku Island) brown linear black 2AC	[45]
Cuscus (Larike Ambon Island) brown linear blackCC	[45]
Cuscus (Allang Ambon Island) whole whiteCC	[45]
Cuscus (North Seram Island) whole whiteCC	[45]
Phalanger gymnotis (Genebank)	ATG	ACC	CAC	CAA	ACG	CAC	GCT	TAT	CAC	ATA	GTT	AAT	CCA	AGT	CCA	[45]
Phalanger vestitus (Genebank)	TGA	CCA	CTA	ACA	GGA	GCC	CTA	TCA	GCC	CTA	CTG	CTT	ACA	TCA	GGC	[90]
Cuscus (Wanggar Nabire Island) brown linear black	T..A	..CC	[90]
Cuscus (Wanggar Nabire Island) grey linear blackA	..CC	[90]
Cuscus (Yaro Nabire Island) brown linear blackA	..CC	[90]
Cuscus (Moor Nabire Island) brown linear black	T..A	..CC	[90]
Cuscus (Hatu Ambon Island) brown linear blackA	..CC	[90]
Cuscus (Geyser Island) whole whiteA	..CC	[90]
Cuscus (Geyser Island) brown linear blackA	..CC	[90]
Cuscus (Gorom Island) whole whiteA	..CC	[90]
Cuscus (Lakor Island) brown linear blackA	..CC	[90]
Cuscus (Kariu Haruku Island) brown linear blackA	..CC	[90]
Cuscus (Kariu Haruku Island) whole whiteA	..CC	[90]
Cuscus (Kariu Haruku Island) brown linear black 2A	..CC	[90]
Cuscus (Larike Ambon Island) brown linear blackA	..CC	[90]
Cuscus (Allang Ambon Island) whole whiteA	..CC	[90]
Cuscus (North Seram Island) whole whiteA	..CC	[90]
Phalanger gymnotis (Genebank)	TGG	CCA	TTA	ACA	GGA	GCT	CTA	TCA	GCC	CTA	CTA	CTC	ACA	TCA	GGC	[90]
Phalanger vestitus (Genebank)	CTC	ATC	ATA	TGG	TTC	CAC	TTC	AAC	TCC	CCA	CTC	TTA	GTA	CTA	ATC	[135]
Cuscus (Wanggar Nabire Island) brown linear blackAT	T.C	A..TC	[135]
Cuscus (Wanggar Nabire Island) grey linear blackAT	T.C	A..TC	[135]
Cuscus (Yaro Nabire Island) brown linear blackAT	T.C	A..TC	[135]
Cuscus (Moor Nabire Island) brown linear blackAT	T.C	A..TC	[135]
Cuscus (Hatu Ambon Island) brown linear blackAT	T.C	A..TC	[135]
Cuscus (Geyser Island) whole whiteAT	T.C	A..TC	[135]
Cuscus (Geyser Island) brown linear blackAT	T.C	A..TC	[135]
Cuscus (Gorom Island) whole whiteAT	T.C	A..TC	[135]
Cuscus (Lakor Island) brown linear blackAT	T.C	A..TC	[135]
Cuscus (Kariu Haruku Island) brown linear blackAT	T.C	A..TC	[135]
Cuscus (Kariu Haruku Island) whole whiteAT	T.C	A..TC	[135]
Cuscus (Kariu Haruku Island) brown linear black 2AT	T.C	A..TC	[135]
Cuscus (Larike Ambon Island) brown linear blackAT	T.C	A..TC	[135]
Cuscus (Allang Ambon Island) whole whiteAT	T.C	A..TC	[135]
Cuscus (North Seram Island) whole whiteAT	T.C	A..TC	[135]
Phalanger gymnotis (Genebank)	CTC	ATC	ATA	TGA	TTC	CAC	TTC	AAC	TCC	TCT	ACA	TTA	GTA	CTA	ATC	[135]
Phalanger vestitus (Genebank)	GCA	TTC	TTT	TTT	CTA	GGG	TTT	TTC	TGA	GCC	TTT	TAT	CAC	TCG	AGC	[315]
Cuscus (Wanggar Nabire Island) brown linear black	..TCT	..CTA	...	[315]
Cuscus (Wanggar Nabire Island) grey linear black	..TCT	..CTA	...	[315]
Cuscus (Yaro Nabire Island) brown linear black	..TCT	..CTA	...	[315]
Cuscus (Moor Nabire Island) brown linear black	..TCT	..CTA	...	[315]
Cuscus (Hatu Ambon Island) brown linear black	..TCT	..CTA	...	[315]
Cuscus (Geyser Island) whole white	..TCT	..CTA	...	[315]
Cuscus (Geyser Island) brown linear black	..TCT	..CTA	...	[315]
Cuscus (Gorom Island) whole white	..TCT	..CTA	...	[315]
Cuscus (Lakor Island) brown linear black	..TCT	..CTA	...	[315]
Cuscus (Kariu Haruku Island) brown linear black	..TCT	..CTA	...	[315]
Cuscus (Kariu Haruku Island) whole white	..TCT	..CTA	...	[315]
Cuscus (Kariu Haruku Island) brown linear black 2	..TCT	..CTA	...	[315]
Cuscus (Larike Ambon Island) brown linear black	..TCT	..CTA	...	[315]
Cuscus (Allang Ambon Island) whole white	..TCT	..CTA	...	[315]
Cuscus (North Seram Island) whole white	..TCT	..CTA	...	[315]
Phalanger gymnotis (Genebank)	GTA	TTC	TTC	TTC	CTA	GGA	TTC	TTC	TGA	GCC	TTC	TAC	CAT	TCA	AGC	[315]

Fig. 3: Continue

Phalanger vestitus (Genebank)	CTA	GCC	CCA	ACC	CA	GAA	TTA	GGA	GGC	TGC	TGA	CCC	CCT	ACC	GGA	[360]
Cuscus (Wanggar Nabire Island) brown linear black	A	..C	[360]
Cuscus (Wanggar Nabire Island) grey linear black	C.	A	..C	[360]
Cuscus (Yaro Nabire Island) brown linear black	C.	A	..C	[360]
Cuscus (Moor Nabire Island) brown linear black	A	..C	[360]
Cuscus (Hatu Ambon Island) brown linear black	C.C	[360]
Cuscus (Geyser Island) whole white	C.C	[360]
Cuscus (Geyser Island) brown linear black	C.C	[360]
Cuscus (Gorom Island) whole white	C.C	[360]
Cuscus (Lakor Island) brown linear black	C.C	[360]
Cuscus (Kariu Haruku Island) brown linear black	C.C	[360]
Cuscus (Kariu Haruku Island) whole white	C.C	[360]
Cuscus (Kariu Haruku Island) brown linear black 2	C.C	[360]
Cuscus (Larike Ambon Island) brown linear black	C.C	[360]
Cuscus (Allang Ambon Island) whole white	C.C	[360]
Cuscus (North Seram Island) whole white	C.C	[360]
Phalanger gymnotis (Genebank)	CTA	GCC	CCA	ACC	CAC	GAA	CTA	GGA	GGC	TGT	TGA	CCA	CCA	ACC	GGA	[360]
Phalanger vestitus (Genebank)	ATC	CAC	CCA	TTA	AAC	CCA	CTC	GAA	GTC	CCA	CTA	CTC	AAC	ACA	TCC	[405]
Cuscus (Wanggar Nabire Island) brown linear blackTT	[405]
Cuscus (Wanggar Nabire Island) grey linear blackTT	[405]
Cuscus (Yaro Nabire Island) brown linear blackTT	[405]
Cuscus (Moor Nabire Island) brown linear blackTT	[405]
Cuscus (Hatu Ambon Island) brown linear blackTT	[405]
Cuscus (Geyser Island) whole whiteTT	[405]
Cuscus (Geyser Island) brown linear blackTT	[405]
Cuscus (Gorom Island) whole whiteTT	[405]
Cuscus (Lakor Island) brown linear blackTT	[405]
Cuscus (Kariu Haruku Island) brown linear blackTT	[405]
Cuscus (Kariu Haruku Island) whole whiteTT	[405]
Cuscus (Kariu Haruku Island) brown linear black 2TT	[405]
Cuscus (Larike Ambon Island) brown linear blackTT	[405]
Cuscus (Allang Ambon Island) whole whiteTT	[405]
Cuscus (North Seram Island) whole whiteTT	[405]
Phalanger gymnotis (Genebank)	ATT	CAT	CCA	TTA	AAT	CCA	CTT	GAA	GTT	CCA	CTA	CTT	AAC	ACA	TCA	[405]
Phalanger vestitus (Genebank)	ATT	CTA	CTA	GCC	TCC	GGA	GTA	TCA	ATC	ACA	TGA	GCC	CAC	CAC	AG	[450]
Cuscus (Wanggar Nabire Island) brown linear black	T.GT	..GG	[450]
Cuscus (Wanggar Nabire Island) grey linear black	T.GT	..GG	[450]
Cuscus (Yaro Nabire Island) brown linear black	T.GT	..GG	[450]
Cuscus (Moor Nabire Island) brown linear black	T.GT	..GG	[450]
Cuscus (Hatu Ambon Island) brown linear black	T.GT	..GG	[450]
Cuscus (Geyser Island) whole white	T.GT	..GG	[450]
Cuscus (Geyser Island) brown linear black	T.GT	..GG	[450]
Cuscus (Gorom Island) whole white	T.GT	..GG	[450]
Cuscus (Lakor Island) brown linear black	T.GT	..GG	[450]
Cuscus (Kariu Haruku Island) brown linear black	T.GT	..GG	[450]
Cuscus (Kariu Haruku Island) whole white	T.GT	..GG	[450]
Cuscus (Kariu Haruku Island) brown linear black 2	T.GT	..GG	[450]
Cuscus (Larike Ambon Island) brown linear black	T.GT	..GG	[450]
Cuscus (Allang Ambon Island) whole white	T.GT	..GG	[450]
Cuscus (North Seram Island) whole white	T.GT	..GG	[450]
Phalanger gymnotis (Genebank)	ATC	TTA	TTG	GCT	TCC	GGA	GTA	TCA	ATT	ACA	TGG	GCT	CAC	CAC	AGC	[450]
Phalanger vestitus (Genebank)	CTA	ATA	GAA	GGC	AGC	CGA	GAA	CAA	ATA	ATT	CAA	GCA	CTA	TCA	ATT	[495]
Cuscus (Wanggar Nabire Island) brown linear blackAC	...	A.C	[495]
Cuscus (Wanggar Nabire Island) grey linear black	GAC	...	A.C	[495]
Cuscus (Yaro Nabire Island) brown linear black	GAC	...	A.C	[495]
Cuscus (Moor Nabire Island) brown linear blackAC	...	A.C	[495]
Cuscus (Hatu Ambon Island) brown linear blackA	...	A.C	[495]
Cuscus (Geyser Island) whole whiteA	...	A.C	[495]
Cuscus (Geyser Island) brown linear blackA	...	A.C	[495]
Cuscus (Gorom Island) whole whiteA	...	A.C	[495]
Cuscus (Lakor Island) brown linear blackA	...	A.C	[495]
Cuscus (Kariu Haruku Island) brown linear blackA	...	A.C	[495]
Cuscus (Kariu Haruku Island) whole whiteA	...	A.C	[495]
Cuscus (Kariu Haruku Island) brown linear black 2A	...	A.C	[495]
Cuscus (Larike Ambon Island) brown linear blackA	...	A.C	[495]
Cuscus (Allang Ambon Island) whole whiteA	...	A.C	[495]
Cuscus (North Seram Island) whole whiteA	...	A.C	[495]
Phalanger gymnotis (Genebank)	CTA	ATA	GAA	GGA	AAC	CGA	AAA	CAA	ATA	ATT	CAA	GCA	CTA	TCA	ATT	[495]

Fig. 3: Continue

Phalanger vestitus (Genebank)	TAC TAC GAA TCA TCC TTT ACA ATC TCA GAA GGC ATC TAC GGA TCC [585]
Cuscus (Wanggar Nabire Island) brown linear black	..T ..TCT ..T ..G ..A [585]
Cuscus (Wanggar Nabire Island) grey linear black	..T ..TCT ..T ..G ..A [585]
Cuscus (Yaro Nabire Island) brown linear black	..T ..TCT ..T ..G ..A [585]
Cuscus (Moor Nabire Island) brown linear black	..T ..TCT ..T ..G ..A [585]
Cuscus (Hatu Ambon Island) brown linear black	..T ..TCT ..T ..G ..A [585]
Cuscus (Geyser Island) whole white	..T ..TCT ..T ..G ..A [585]
Cuscus (Geyser Island) brown linear black	..T ..TCT ..T ..G ..A [585]
Cuscus (Gorom Island) whole white	..T ..TCT ..T ..G ..A [585]
Cuscus (Lakor Island) brown linear black	..T ..TCT ..T ..G ..A [585]
Cuscus (Kariu Haruku Island) brown linear black	..T ..TCT ..T ..G ..A [585]
Cuscus (Kariu Haruku Island) whole white	..T ..TCT ..T ..G ..A [585]
Cuscus (Kariu Haruku Island) brown linear black 2	..T ..TCT ..T ..G ..A [585]
Cuscus (Larike Ambon Island) brown linear black	..T ..TCT ..T ..G ..A [585]
Cuscus (Allang Ambon Island) whole white	..T ..TCT ..T ..G ..A [585]
Cuscus (North Seram Island) whole white	..T ..TCT ..T ..G ..A [585]
Phalanger gymnotis (Genebank)	TAC TAT GAA TCA TCT TTC ACC ATT TCA GAC GGC ATC TAC GGA TCA [585]
Phalanger vestitus (Genebank)	CAC TTC ACA TCC ACT CAC CAC TTT GGA TTT GAA GCA GCT GCC TGA [720]
Cuscus (Wanggar Nabire Island) brown linear blackTATG [720]
Cuscus (Wanggar Nabire Island) grey linear blackTATG [720]
Cuscus (Yaro Nabire Island) brown linear blackTATG [720]
Cuscus (Moor Nabire Island) brown linear blackTATG [720]
Cuscus (Hatu Ambon Island) brown linear blackTATG [720]
Cuscus (Geyser Island) whole whiteTATG [720]
Cuscus (Geyser Island) brown linear blackTATG [720]
Cuscus (Gorom Island) whole whiteTATG [720]
Cuscus (Lakor Island) brown linear blackTATG [720]
Cuscus (Kariu Haruku Island) brown linear blackTATG [720]
Cuscus (Kariu Haruku Island) whole whiteTATG [720]
Cuscus (Kariu Haruku Island) brown linear black 2TATG [720]
Cuscus (Larike Ambon Island) brown linear blackTATG [720]
Cuscus (Allang Ambon Island) whole whiteTATG [720]
Cuscus (North Seram Island) whole whiteTATG [720]
Phalanger gymnotis (Genebank)	CAC TTT ACA TCC ACT CAC CAC TTC GGA TTT GAA GCA GCT GCC TGA [720]
Phalanger vestitus (Genebank)	TAT TGA CAC TTC GTA GAT GTA GTA TGA CTC TTC TTA TAC GTA TCA [765]
Cuscus (Wanggar Nabire Island) brown linear blackTACG [765]
Cuscus (Wanggar Nabire Island) grey linear blackTACG [765]
Cuscus (Yaro Nabire Island) brown linear blackTACG [765]
Cuscus (Moor Nabire Island) brown linear blackTACG [765]
Cuscus (Hatu Ambon Island) brown linear blackTACG [765]
Cuscus (Geyser Island) whole whiteTACG [765]
Cuscus (Geyser Island) brown linear blackTACG [765]
Cuscus (Gorom Island) whole whiteTACG [765]
Cuscus (Lakor Island) brown linear blackTACG [765]
Cuscus (Kariu Haruku Island) brown linear blackTACG [765]
Cuscus (Kariu Haruku Island) whole whiteTACG [765]
Cuscus (Kariu Haruku Island) brown linear black 2TACG [765]
Cuscus (Larike Ambon Island) brown linear blackTACG [765]
Cuscus (Allang Ambon Island) whole whiteTACG [765]
Cuscus (North Seram Island) whole whiteTACG [765]
Phalanger gymnotis (Genebank)	TAC TGA CAC TTC GTG GAC GTA GTA TGA CTC TTC CTA TAT GTG TCA [765]
Phalanger vestitus (Genebank)	ATT TAC TGA TGA GGT TCG [783]
Cuscus (Wanggar Nabire Island) brown linear blackC ..A [783]
Cuscus (Wanggar Nabire Island) grey linear blackC ..A [783]
Cuscus (Yaro Nabire Island) brown linear blackC ..A [783]
Cuscus (Moor Nabire Island) brown linear blackC ..A [783]
Cuscus (Hatu Ambon Island) brown linear blackC ..A [783]
Cuscus (Geyser Island) whole whiteC ..A [783]
Cuscus (Geyser Island) brown linear blackC ..A [783]
Cuscus (Gorom Island) whole whiteC ..A [783]
Cuscus (Lakor Island) brown linear blackC ..A [783]
Cuscus (Kariu Haruku Island) brown linear blackC ..A [783]
Cuscus (Kariu Haruku Island) whole whiteC ..A [783]
Cuscus (Kariu Haruku Island) brown linear black 2C ..A [783]
Cuscus (Larike Ambon Island) brown linear blackC ..A [783]
Cuscus (Allang Ambon Island) whole whiteC ..A [783]
Cuscus (North Seram Island) whole whiteC ..A [783]
Phalanger gymnotis (Genebank)	ATT TAC TGA TGA GGC TCA [783]

Fig. 3: Continue

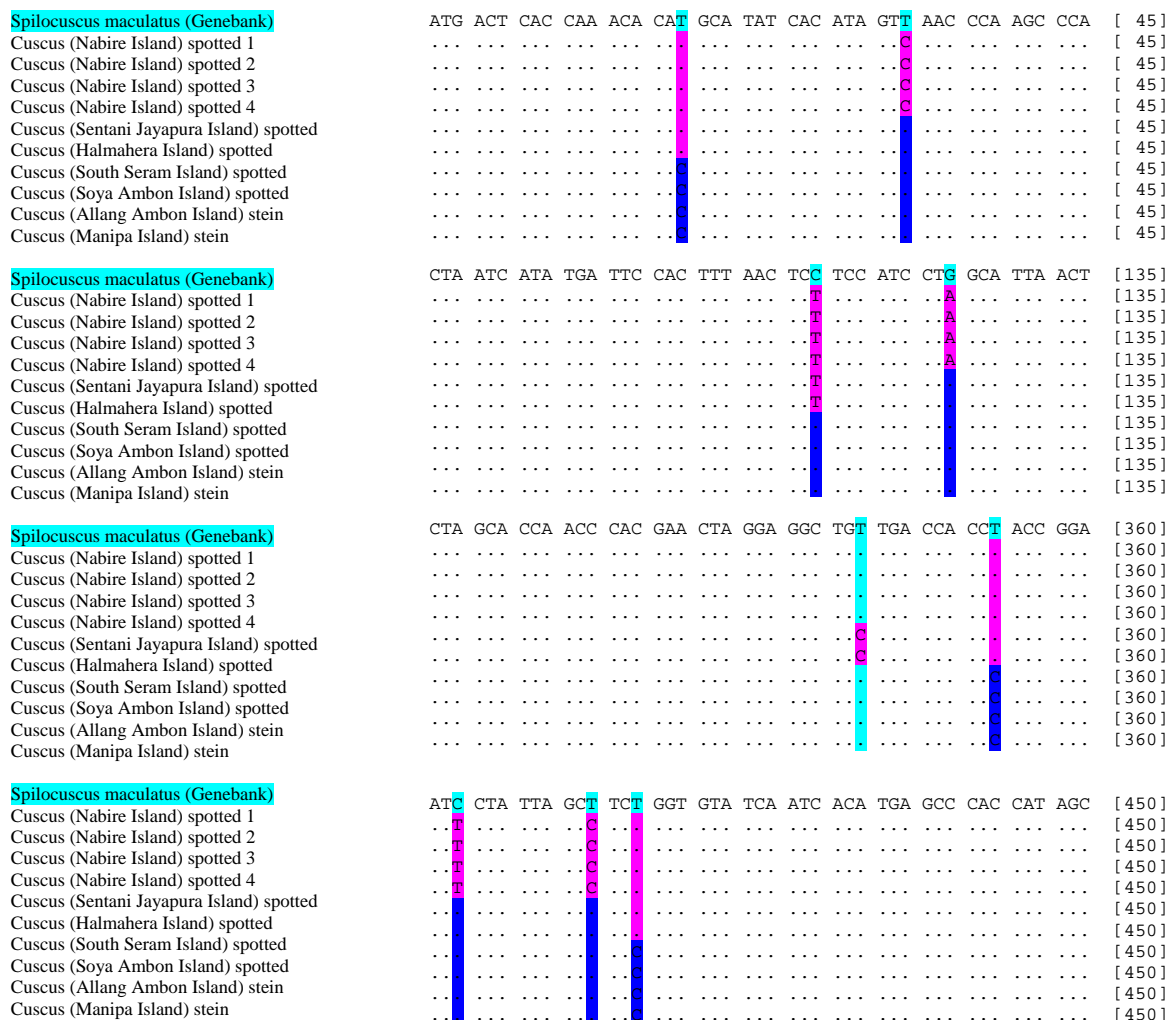


Fig. 3: Sites nucleotides barcode of the COX3 gene of *Phalanger* and *Spiloglossus* genera from Maluku and Papua. Descriptions: *Yellow is *Phalanger* genera members from Papua, *Red is *Phalanger* genera members from Maluku, *Pink is *Spiloglossus* genera members from Papua and Halmahera Islands, *Dark Blue is *Spiloglossus* genera members from Maluku, *Green, Grey and Light Blue is Species compare from Genebank

The occurrence of individuals groupings of Halmahera and Sentani populations Islands, in this study is possible due to the spread of cuscus from other locations causing the flow of genes between populations occurs regularly. Another possibility is that small populations around the site function as an intermediate population, so that sharp barriers such as mountains and seas that act as dispersal limiting factors do not play a role or inhibit gene flow. The *Phalanger* genera is composed of 2 separate clades of A1 (Maluku) supported by a bootstrap values of 100% and clade A2 (Papua) is supported by a 100% bootstrap values. *Spiloglossus* genera consists of 2 separate clades, B1 (Maluku) is supported by 99% bootstrap values and B2 (Papua) is supported by 100% bootstrap values.

Two individuals from the Halmahera and Sentani Islands form a separate 1 sub-clade as part of the clade B2, that is clade B2' (Fig. 3).

DISCUSSION

Sites barcodes and biogeography of cuscuses from maluku and Papua: This study provides an input 23 new sequences (sites nucleotides) of *Phalanger* species from Maluku and Papua for the DNA barcode database and 4 new sites nucleotides of *Spiloglossus* species from Maluku and Papua (Fig. 3). Twenty three nucleotides distinguish can be used as a barcodes among *Phalanger* species from Maluku and Papua,

that is sites positions 15-(G/A), 33-(T/C), 48-(G/A), 90-(T/C), 114-(T/C), 123-(C/T), 124-(C/T), 282-(C/T), 288-(A/G), 306-(C/T), 330-(C/T), 351-(C/A), 370-(C/T), 399-(C/T), 450-(C/T), 462-(T/C), 465-(T/C), 570-(C/T), 573-(C/T), 579-(C/T), 705-(T/C), 741-(G/A) and site 768-(C/T). Twenty three sites barcode is the results alignments of *Phalanger* members between the 2 populations (Maluku and Papua) (Fig. 3). The DNA barcode technique is a rapid way to identify and discover new species. Its success depends on the reciprocal monophyly of species and on the strength of the establishment of a "barcode gap", i.e., a clear delimitation between intra- and inter-specific sequence variability for genes sequences are studied¹³.

Results nucleotides alignment of *Spilocuscus* members from Maluku and Papua, were found 8 nucleotides distinguish between the two populations (Fig. 3), that is sites positions 18-(C/T), 33-(T/C), 117-(C/T), 126-(A/G), 354-(C/T), 408-(T/C), 417-(C/T) and site 420-(C/T). Eight nucleotides distinguish can be using as a barcodes among *Spilocuscus* members from Maluku and Papua. The results of alignment showed that there is substitution (transversion: Transition) in each genus of cuscuses (*Phalanger* and *Spilocuscus*). The majority of nucleotide substitutions that occur at the species level transition occurs, while at the genus level transversions occur. Substitution ratio differences at the level of species and genus can be caused by different biogeographic and metabolic rate¹⁴. In addition Kunda *et al.*², it is reported that differences in nucleotide substitution ratio of a gene, can be attributed to differences in reproduction, life history, or the process of evolution and adaptation. The level of substitution of a population is interesting to study because it may vary in different organisms or genes in various organisms. Differences in nucleotide substitutions high very influential in percentage composition of nucleotides, which is in turn has implications for the phylogenetic analysis¹⁵. In addition Vali *et al.*¹⁵, is asserted that the scale of the difference deviation is so high nucleotide composition that will be a solid foundation and is instrumental in phylogenetic analyzes.

Changes in the composition of nucleotide substitution caused by both transversion and transition can be used as a molecular marker (barcode) to study natural populations¹⁻³. Nucleotide substitution either transversion or transition can provide a clear understanding of the geographical evolutionary history of the population and can be used as a genetic marker (barcode)^{2,3}. Not all of the genes can be used in the study of genetic markers and genes only with certain requirements which can be used as a barcode⁸.

The phylogram (Fig. 2) generated through NJ method using K2P distance was highly reliable as the out group used

(*Strigocuscus celebensis* and *Ailurops ursinus*) segregated in separate clade as expected. The ability of COX3 gene in distinguishing 2 different genera was proved through the phylogram as 2 different clades (Clades A and B) distinguishable. Clade A as the *Phalanger* and clade B as the *Spilocuscus*. These results are in accordance with the declaration of Widayanti *et al.*^{1,3} and Fatem and Sawen⁴ that genus cuscus in Papua and Maluku is *Phalanger* and *Spilocuscus*. The results of studies using COX3 loci prove that *S. maculatus* origin Halmahera Island has a strong phylogeny with *S. maculatus* from Sentani Island. Mitochondrial sequences data from the ND1 gene supported the genetic association between *S. maculatus* species from Halmahera and Sentani Islands, but the study conducted by Widayanti *et al.*³, did not explain the correlation of genetic relationship between *S. maculatus* species from the 2 Islands based on biogeography.

Based on biogeography, *S. maculatus* from Halmahera Island, must have a kinship with *S. maculatus* from Maluku, but it raises a conflict, relations with the biogeographic distribution of species. The biogeographic conflict is clearly explained that about 6,500 years ago, human colonization occurred by the Austronesian tribes on the Halmahera Island. In that period they brought food crops and pets one of them *S. maculatus*. Large Island in Maluku, namely is Halmahera has ideal conditions and natural way to detect the translocation of animals, where irian act as the area of origin of the animals. This study proves that the results published by Widayanti *et al.*³, empirically proven have been demonstrated use different loci, but still maintain the stability of the phylogenetic tree between ND1 and COX3 loci. Phylogenetic signals are evident in the clade A the *Phalanger* of Papua segregated from the *Phalanger* of Maluku with few other related species (strong support by bootstrap value 100%). Similarly for phylogenetic signals are evident in the clade B the *Spilocuscus* of Papua segregated from the *Spilocuscus* of Maluku (strong support by bootstrap value 100%).

This study asserted that *S. maculatus* from Sentani and Halmahera Islands have a genetic kinship (94%) close to *S. maculatus* from Nabire. These phylogenetic 3 yields are consistent with the research reported by Widayanti *et al.*³ and supporting monophyly species from the Phalangeridae group. Ternate Island is far from Papua biogeographic regions, but both *Spilocuscus* has a very close relationship. Evidence suggests that cuscus species were transported to New Ireland (Papua New Guinea) as early as 10,000-20,000 years ago. Some species were introduced to the Solomons as early as 6600 years ago and to Timor as early as 6500 years ago. *Phalanger orientalis* may have been actively introduced to

Seram, Buru, Sanana and Kei Islands but the initial introductory evidence is not known with certainty. The period when the spotted cuscus (*S. maculatus*) reached northern Maluku, so are the ornate cuscus (*Phalanger ornatus*) reached Northern Maluku remains enigmatic as well, though they likely arrived in Maluku well in advance of other large terrestrial mammalian species.

Historically, species-level systematics of *Spilocuscus* have been highly labile^{2,3}. Within the last decade, however, taxonomy within the genus has stabilized. Recent research asserted that cuscus origin Manipa Island that is previously based on morphological characters classified into *Phalanger* group members, but based on genetic analysis (Cytb gene) sequences more appropriately classified into *Spilocuscus* group members⁵.

Study reported by Widayanti *et al.*¹, examined phalangeridae relationships using mitochondrial 12S rRNA. Their results yield support for an association of *Ailurops*, *Phalanger* and *Spilocuscus*. Research by Raterman *et al.*¹⁶, asserted strong phylogenetic support found for both the *S. celebensis* sister taxon to *Ailurops* and the *S. celebensis*+*Ailurops* sister to Phalangerini relationships. First authors to include 3 genera (*Ailurops* from Sulawesi and *Spilocuscus* and *Phalanger* from Maluku and Papua islands) in a molecular study, used mitochondrial 12S rRNA gene¹.

Results nucleotides alignment were found 3 sites and 13 sites that can be used as genetic markers between *Spilocuscus* and *Phalanger* members from Papua and Maluku¹. Three sites positions of *Spilocuscus* genera that can be used a barcodes are 127-(G/A), 481-(C/T), 885-(T/C) and 13 sites positions of *Phalanger* genera are 67-(A/G), ke-89 (G/C), ke-137 (T/C), ke-285 (G/A), ke-468 (T/C), ke-595 (T/C), ke-598 (T/C), ke-647 (T/C), ke-654 (G/A), ke-665 (T/C), ke-769 (C/T), ke-874 (C/T), ke-876 (A/G)¹. Added³ that cuscus from Manipa Island have genetic relationship with *Spilocuscus maculatus* (94%) than *Phalanger* members. Research conducted by Kunda *et al.*² asserted that Stein cuscus from Manipa Island is closer to *Spilocuscus maculatus* than to *Phalanger* genera members. Previous molecular studies have not include specimens from Halmahera Island¹. Research by Meredith *et al.*¹⁷ considered this taxon a close relative of *S. celebensis*, but retained both species in the genus *Phalanger*.

Research by Kunda *et al.*², asserted that the colors and patterns of the hair can not be used as a basis for determining taxonomic cuscuses. Cuscuses from clade A are very varied from the colors and patterns of hairs (brown linear black, gray linear black and whole white) but the views of the nucleotides sequences from COX3 gene showed that all of

them are the same species (*Phalanger* sp.) and have phylogenetic relationship with *P. vestitus* (100%) from *genebank*. The same thing happened at group B. They are many variations in colors and patterns of hair (spotted and stein) but it genetically the same species is *Spilocuscus maculatus*. The results of this study are different from the conventional taxonomy⁴. The availability of sequences in public reference database enables the effectiveness of DNA-based identification of species¹⁸, overcoming morphological identification difficulties.

This way, as the first broad data set of the COX3 barcoding for cuscuses species, it can potentially become an alternative or a complementary methodology to morphological identification. Accurate molecular identification to species-level can be a potential and valuable alternative to morphological identification, including phylogenetic relationships among species in the population. So, the COX3 barcoding sequences provided here can be helpful for processing phylogenetic traces from cuscuses. The molecular phylogenies presented in this study are considered more reflective on the true evolutionary relationships of Phalangeridae than previous morphological analyses, as the molecular phylogenies show that the high levels of congruence between them versus has been seen in the morphologically based on phylogenies.

CONCLUSIONS

The sequence of mt-DNA COX3 gene can be used as a DNA barcode for cuscuses from Maluku and Papua and found 23 nucleotides distinguish of *Phalanger* genera and 4 nucleotides distinguish of *Spilocuscus* genera from 2 populations (Maluku and Papua). It was proved that Spotted cuscus from Halmahera Island has the closest genetic relationship with *S. maculatus* from Papua (Sentani, Jayapura island) which is a species introduced from Papua when the human colonization of Austronesian tribes. Present result revealed that COX3 gene grouping cuscuses from Papua and Maluku become 2 clade A and clade B. Clade A is *Phalanger* genera and clade B is *Spilocuscus* genera and entry into *Phalanger vestitus* (and *Spilocuscus maculatus* species).

SIGNIFICANCE STATEMENT

This study discovers the possible *S. maculatus* from Halmahera have the closest genetic relationship with *S. maculatus* from Sentani, Jayapura Island which is a specie introduced from Papua when the human colonization of Austronesian tribes. This study will help researchers to uncover

Phylogenetic Species Concept (PSC) that will provide an accurate understanding of the identity of a species in taxon and is an important framework for understanding biodiversity. Thus, a new theory on Phylogenetic species concept is very relevant to design a policy on what to protect, how to protect and how to facilitate biodiversity on an continuous basis.

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