



## Research Article

# Study of Genetic Marker of Cuscuses (Marsupialia: Phalangeridae) from Maluku and Papua Based on Cytochrome b Gene Sequences

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## Abstract

Cuscuses is marsupials animal (Phalangeridae) which has limited spread in eastern Indonesia (Sulawesi, Maluku, Papua and Timor islands), Australia and Papua New Guinea. The *ex-situ* and *in-situ* conservation of cuscuses under captivating condition is an alternative solution to protect from extinction. This study aimed to determine nucleotide sequences and genetic marker on cyt b gene with sequencing method of each species on two provinces. Whole genome DNA was extracted from 22 samples of cuscuses obtained from different habitats, Maluku (13 individuals) and Papua (8 individuals) according to the protocol of Qiamp DNA Blood Mini Kit (Qiagen) and then it was used as template for amplification of cyt b gene by using PCR method. The PCR product were then purified using column chromatography and were used as template for sequencing reaction. Results sequencing of cyt b gene were analyzed using MEGA program versions 6.0. The PCR product gives results nucleotides of 982 bp according to database GeneBank and sequencing product gives results nucleotides of 771 bp. Nucleotides alignment of Phalanger members was found 24 nucleotides distinguishing and Spilocuscus members was found 11 nucleotides distinguishing, which can be used as genetic marker between Phalanger and Spilocuscus members from Papua and Maluku.

**Key words:** Genetic marker, cytochrome b gene, conservation genetic, phalangeridae, mitochondria

**Received:** November 15, 2015

**Accepted:** January 25, 2016

**Published:** February 15, 2016

**Citation:** Rony Marsyal Kunda, Niken Satuti Nur Handayani, Hery Wijayanto and Rini Widayanti, 2016. Study of genetic marker of cuscuses (Marsupialia: Phalangeridae) from Maluku and Papua based on cytochrome b gene sequences. Pak. J. Biol. Sci., 19: 122-135.

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**Competing Interest:** The authors have declared that no competing interest exists.

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

## INTRODUCTION

Mitochondrial DNA (mt-DNA) is the genetic materials that exists outside the nucleus in eukaryotic cells. It has a simple molecular structure. It does not undergo recombination with nuclear DNA and has no identical sequence with nuclear DNA. It has multiple copies and a rapid evolutionary rate and follows maternal inheritance. Cytochrome b gene (Cyt b) is one of the genes that is coded by mt-DNA and its gene product plays an important role in electron transfer in the respiration chain. Cytochrome b gene has a moderate evolutionary rate and a clear evolutionary pattern that makes it suitable for the studies on the phylogenetic evolution at the intra-and interspecific levels (Kocher *et al.*, 1989; Irwin *et al.*, 1991; Orekhov *et al.*, 1999).

The family Phalangeridae comprises approximately two dozen extinct and extant species, including brushtailed possums (*Trichosurus*), scaly tailed possum (*Wyulda*) and cuscuses (*Phalanger*, *Strigocuscus*, *Spilococuscus* and *Ailurops*) (Springer *et al.*, 1990; Flannery, 1994; Hamilton and Springer, 1999; Osborne and Christidis, 2002). *Trichosurus* and *Wyulda* are found predominately in Australia whereas *Phalanger* and *Spilococuscus* are, for the most part, limited to New Guinea and surrounding islands. The 24 or more species of the family Phalangeridae that range from Tasmania to the Talaud island and from Sulawesi to the Solomon islands (Latinis, 1996).

There are four species of cuscuses in Maluku: *Spilococuscus maculatus* (spotted/white cuscus), *Spilococuscus rufoniger* (black spotted cuscus), *Phalanger orientalis* (northern common cuscus) and *Phalanger vestitus* (Stein cuscus) (Petocz, 1994) and more than six species of cuscuses in Papua: *Phalanger gymnotis* (ground cuscus), *P. orientalis* (northern common cuscus), *Spilococuscus maculatus* (spotted/white cuscus), *Spilococuscus rufoniger* (black spotted cuscus) and *Spilococuscus papuensis* (Waigeo cuscus) is endemic species of Waigeo island, West Papua Province (Menzies, 1991). *Spilococuscus wilsoni* (Biak spotted cuscus) is endemic species to the islands of Biak and Supiori in the Cenderawasih Bay, Papua Province (Aplin and Helgen, 2008). In addition, Flannery (1995), stated the bear cuscus (*Ailurops ursinus*) and pigmy cuscus (*Strigocuscus celebensis*) is endemic species and it is found only on Sulawesi. Moreover, Helgen and Flannery (2004), asserted *Phalanger* and *Spilococuscus* genera are endemic to tropical forest in the Australian-Papuan region.

In Indonesia, cuscus includes in species protected by the government regulation, Ministry of Agriculture Decree No. 247/Kpts/Um/4/1979, Government Decree No. 7 of 1999. The

reductions of habitat cover leading to the decrease of food availability are the most common threats that effect cuscus population and currently and according to the IUCN criterion cuscuses belongs to the group of animal with least concern and not included in extinction species (Baillie and Groombridge, 1996). In addition Norris (1999), reported that Phalangeridae is still considered to be vulnerable by virtue of restricted distribution. Previous studies only at the morphological level and there are less reports on some of the molecular level.

Recent molecular studies have not included of the *Spilococuscus* and *Phalanger* genera members from Maluku and Papua and limited to *Ailurops ursinus* and *Strigocuscus celebensis*. Kirsch and Wolman (2001), using DNA hybridization concluded that *Ailurops* may not be the sister taxon to all other phalangeridae. Ruedas and Morales (2005), examined phalangeridae relationships using mitochondrial 12S rRNA. Their results yielded support for an association of *Ailurops*, *Strigocuscus*, *Phalanger* and *Spilococuscus* to the exclusion of the trichosurin genera *Trichosurus* and *Wyulda*. Within the former clade, *Ailurops* grouped with *Strigocuscus* and *Phalanger* with *Spilococuscus*. Raterman *et al.* (2006), using nuclear sequences data from the breast cancer and ovarian cancer susceptibility gene 1 (BRCA1) to test previous mitochondrial DNA results and support *Ailurops* as sister taxon to *S. celebensis* and this clade as sister to *Phalangerini*.

However, these studies were missing one or both of *Ailurops* and *Strigocuscus pelengensis*. In contrast, previous morphological studies have suggested that *Ailurops* is basal to all other phalangerids (Flannery *et al.*, 1987; Crosby and Norris, 2003; Crosby *et al.*, 2004; Crosby, 2007) and that *Strigocuscus celebensis* and the *Trichosurus*+*Wyulda* clade are sister taxa (*Trichosurini*) (George, 1987; Flannery *et al.*, 1987; Crosby and Norris, 2003; Crosby *et al.*, 2004; Crosby, 2007). An association of *Trichosurus*, *Wyulda* and *S. celebensis* together based on morphology suggests that the periotic morphology exhibited by these taxa is homologous. However, our finding that trichosurini is diphyletic implies convergent evolution in periotic morphology between *Trichosurus*+*Wyulda* and *S. celebensis*.

Groves (1987), considered this taxon a close relative of *S. celebensis* but retained both species in the genera *Phalanger*. George (1987), recognized *Strigocuscus celebensis* as the only extant species in *Strigocuscus* and placed *pelengensis* in *Phalanger*. Flannery *et al.* (1987), suggested that *pelengensis* is basal to a clade containing both *Phalanger* and *Spilococuscus* or alternatively belongs within *Phalanger*. Our

finding that *S. pelengensis* is associated in a clade with Phalanger and Spilocuscus is generally consistent with Flannery *et al.* (1987) and George (1987), although with the caveat that Flannery *et al.* (1987), *Strigocuscus gymnotis* is instead *Phalanger gymnotis* (Springer *et al.*, 1990).

Previous molecular sequence analyses, limited that included Ailurops and Strigocuscus (Ruedas and Morales, 2005) and Ailurops, Spilocuscus and Phalanger members from Sulawesi, Maluku and Papua (Widayanti *et al.*, 2015), were based on a single mitochondrial gene, 12S rRNA but can not samples collected from many area in Maluku and Papua biogeography regions. Given that mitochondrial genes trees can conflict with another's genes trees (Flynn and Nedbal, 1998; Murphy *et al.*, 2001; Arnason *et al.*, 2002), it is important to test the conclusions of Widayanti *et al.* (2015), using sequences from 12S rRNA. Here, we examine the genetic marker of the species cuscuses from Maluku and Papua, that based on morphological characters refers to as *Spilocuscus maculatus* (spotted/white cuscus), *Spilocuscus rufoniger* (black spotted cuscus), *Phalanger orientalis* (northern common cuscus/brown-linear-black) and the *Phalanger vestitus* (stein cuscus) using sequences from cytochrome b gene (cyt b).

The purpose of this study was to investigate the genetic marker and relationships of Phalanger and Spilocuscus members from two population regions, Maluku and Papua. To provide basic recommendations for the development of conservation strategies, evaluated genetic diversity at population and species levels, examined genetic differentiation among populations and identified individuals seizure results and re-introduction cuscus by Nature Conservation Agency, Indonesia.

## MATERIALS AND METHODS

**Research times and samples collection:** This research project was conducted from starting date March 03, 2014 to ending date June 03, 2014. In this study twenty two tissue samples biopsy result were collected from two provinces which natural habitat, the Maluku and Papua (Fig. 1). In Papua collected eight samples (seven samples of Nabire island and one sample of the Sentani island, Jayapura), in Maluku collected 13 samples (four samples from the Ambon island, five samples of Seram island, two samples of the Haruku island, one sample of the Manipa island and one sample of the Lakor island). All individuals were identified on the basis of morphological

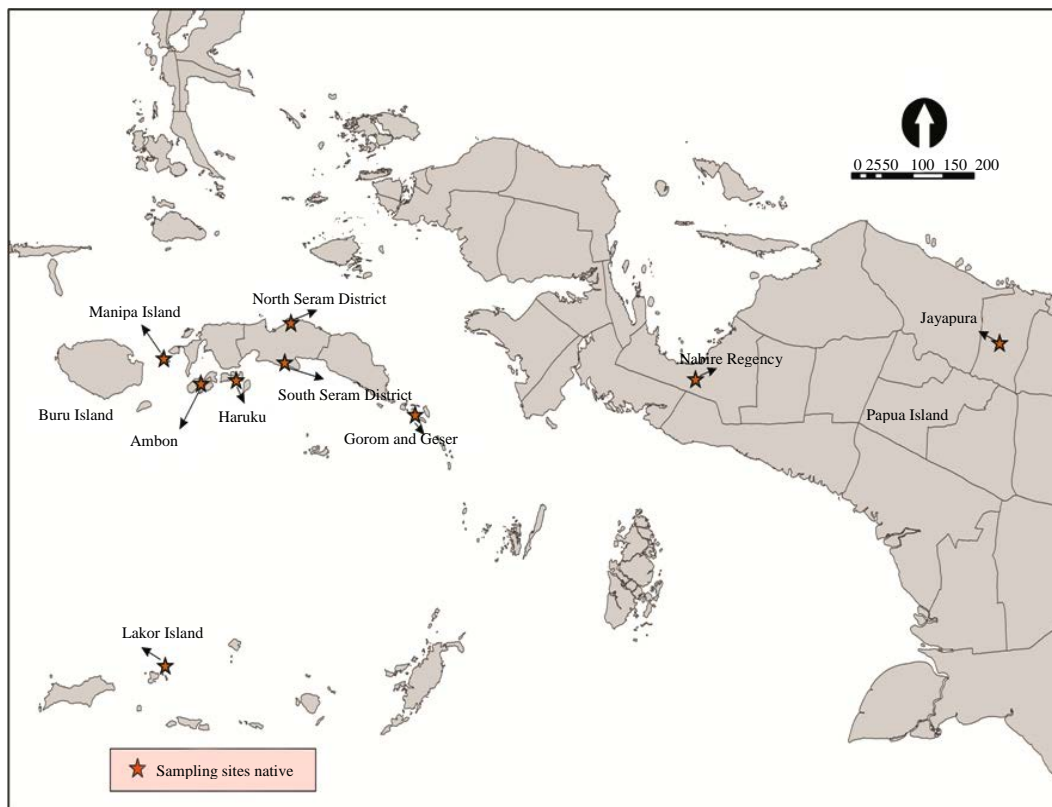


Fig. 1: Map of the localities of the Maluku and Papua cuscus samples

characteristics and a sample tissues was collected from each species and preserved in RNA Lateral buffer (Qiagen) for the purpose of DNA isolation.

**Isolation DNA:** Genomic DNA was isolated from the tissues using DNA isolation kit (Qiagen) according to protocol method in combination with RNase treatment. The DNA quality was assessed by running samples on 1% agarose gel using 1xTBE buffer and visualizing by UV-transilluminator. The DNA concentration was measured with the NanoDrop 2000 Spectrophotometer (Thermo Scientific) using the absorption at 260 nm. The DNA was diluted to a final concentration of 50 ng mL<sup>-1</sup> for PCR amplification.

**Amplification cyt b gene and DNA sequencing:** Amplification cyt b gene (approximately 982 bp), using a pairs of primer cytb1 (5'-GAGGAAGTATCATTCTGGTT-3') and cytb2 (5'-GAGGAAGTATCATTCTGGTT-3') were used for this study. The PCR protocol was carried out in a 25 µL reaction, containing 5 µL dNTP (1 mM), 2.5 µL (10×) reaction buffer, 1 µL (25 mM) MgCl<sub>2</sub>, 1 µL (10 mM) each primer, 1 µL total DNA (50 ng mL<sup>-1</sup>) and 1 U Taq DNA polymerase. The PCR cycles for amplifications were performed using the following conditions: 35 cycles of predenaturasi at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 46°C for 45 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 was assessed by electrophoretic on 1% agarose gel. After the electrophoretic run, DNA molecules were visualized under UV light and analyzed. Purifying PCR products were sequenced with the both PCR primer, due to the fragments length by the following protocol of Development Kit Methods (Beckman Coulter)

and the ethanol precipitation. Samples were sequenced with 8 capillaries CEQ 8000 Beckman Coulter analyser.

**Data analysis:** The DNA sequences of cyt b gene were initially automatically alignments using Clustal W (Thompson *et al.*, 1994), followed by manual editing. Phylogenetic analysis of the aligned sequences was performed with the Molecular Evolutionary Genetics Analysis (MEGA) program version 6.0 (Kumar *et al.*, 2001). Distance analysis was conducted using the Neighbor-Joining (NJ) option employing the Kimura-2-parameter with gap data treated as pairwise deletions. Molecular phylogeny analysis was conducted using heuristic searches, 1000 bootstrap replicates. The nucleotides sequences data determined for the present paper was deposited in GeneBank under accession numbers, AB241057.1 (*P. vestitus*), AF357238.1 (*T. vulpecula*), KJ868163.1 (*Strigocuscus celebensis*), KJ868096.1 (*A. ursinus*), KJ868113.1 (*Dorcopsulus vanheumi*), AF108222.1 (*S. maculatus*), KJ868156.1 (*Setonix brachyurus*), AF027986.2 (*Macropus agilis*), AF027996.2 (*Lagorchestes hirsutus*), KJ868164.1 (*Wallabia bicolor*) and AF027991.2 (*Thylogale stigmatica*).

## RESULTS

A total of 22 DNA samples that have been isolated, then serves as a template for cyt b gene amplification by PCR method. The results PCR product size 982 basepair (bp) after electrophoresis on a 1% agarose gel with a 1 kb DNA Ladder (1st base) (Fig. 2).

The PCR product size was 982 bp obtained after the primers spesific that will be used to be alignment using the BLAST program against mitochondrial genome *P. vestitus* (access numbers AB241057.1). The results alignment cyt b

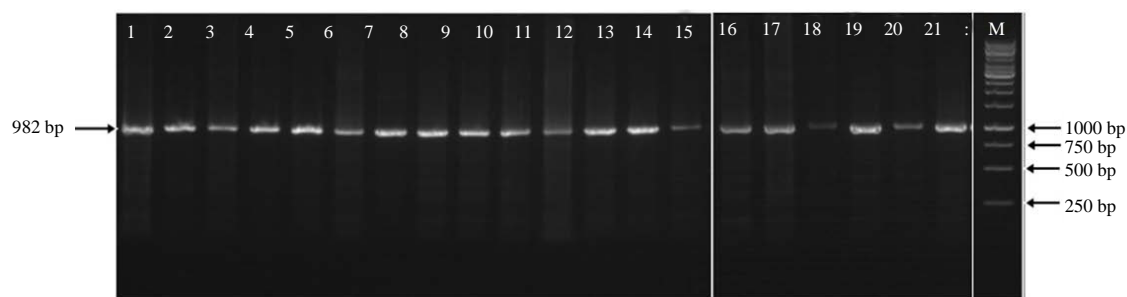


Fig. 2: PCR Product cyt b gene of cuscuses on a 1% agarose gel, Description: 1 kb: DNA ladder 1000 bp, 1-22: PCR product of cuscuses samples

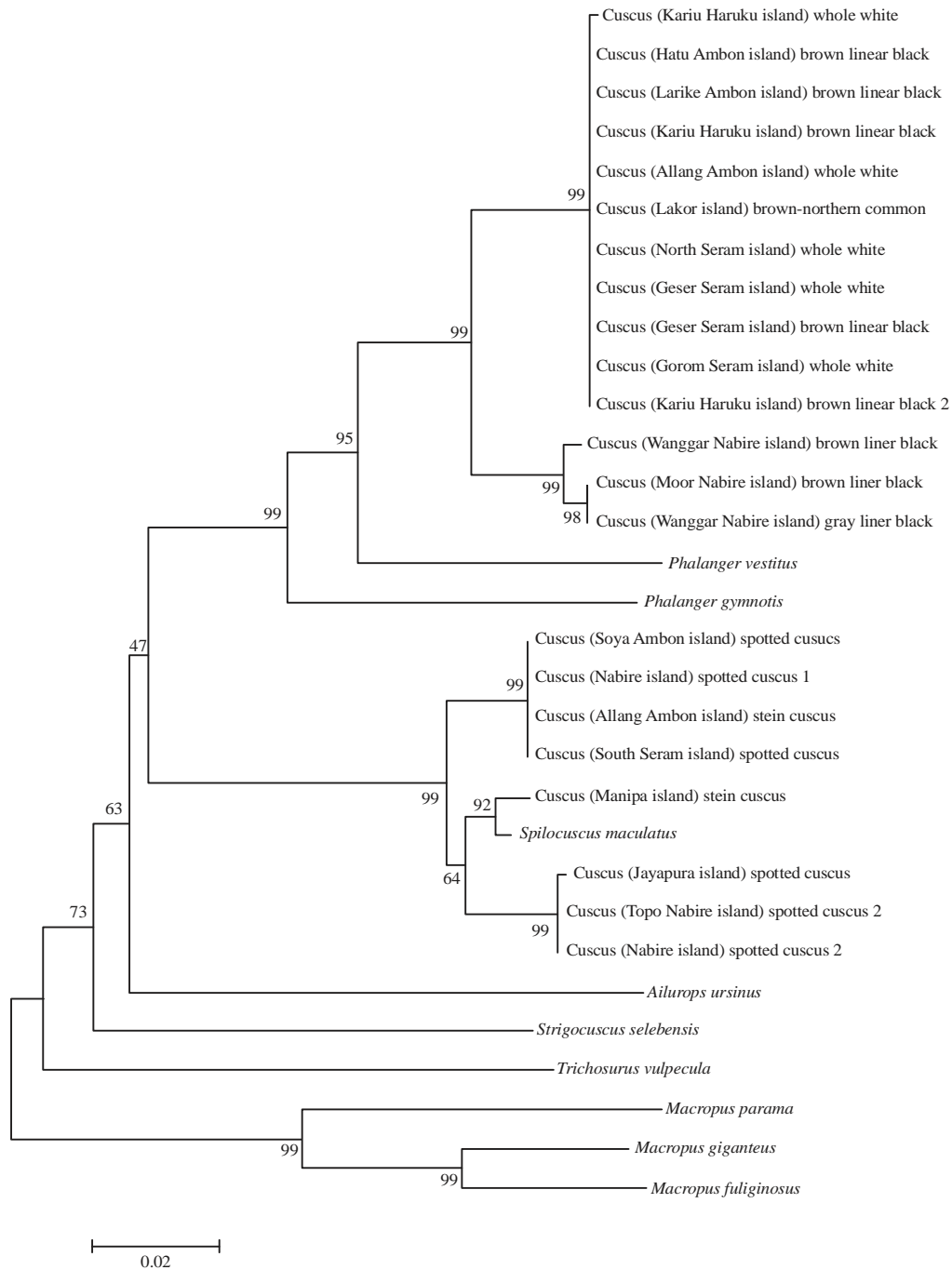


Fig.3: Phylogenetic relationship of cuscuses (Phalanger and Spilocusculus genera) from Papua and Maluku based on mitochondrial cyt b sequences

gene studied cuscuses obtained 771 nucleotides for subsequent analysis. There are differences in the nucleotides of 0-96 when inter cuscuses studied and 6-104 with cuscuses from GeneBank (Table 1). Table 1 shows, cuscuses from Papua on the Moor Nabire (brown linear black) and Wanggar Nabire (Gray linear black) does not have different nucleotides (0 nt), as well cuscuses from Maluku on the Gorom island (whole

white), Geser island (whole white and brown linear black), north Seram (whole white), Lakor island (brown linear black) and Allang village, Ambon island (whole white). Phylogenetic relationship designed using neighbor joining the bootstrap 1000 replicates used to indicate the position of kinship cuscuses in research with comparative *Phalanger* spp. from GeneBank (Fig. 3).

Table 1: Number of differences (771 nucleotides) of mitochondrial cyt b gene of cuscuses (Phalanger and Spilocuscus) with sequences from GeneBank database

No.	Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	<i>Phalanger vestitus</i>																								
2	Cuscus (Moor, Nabire island) brown, linear black	57																							
3	Cuscus (Wanggar, Nabire island) gray, linear black	57	0																						
4	Cuscus (Wanggar, Nabire island) brown, linear black	58	5	5																					
5	Cuscus (Gorom, Seram island) whole white	60	27	27	26																				
6	Cuscus (Geser, Seram island) brown, linear black	60	27	27	26	0																			
7	Cuscus (Geser, Seram island) whole white	60	27	27	26	0	0																		
8	Cuscus (North, Seram island) whole white	60	27	27	26	0	0	0																	
9	Cuscus (Lakor island) brown-northern common	60	27	27	26	0	0	0	0																
10	Cuscus (Allang, Ambon island) whole white	60	27	27	26	0	0	0	0	0															
11	Cuscus (Kariu, Haruku island) whole white	61	28	28	27	1	1	1	1	1															
12	Cuscus (Kariu, Haruku island) brown, linear black	60	27	27	26	0	0	0	0	0	0	1													
13	Cuscus (Larike, Ambon island) brown, linear black	60	27	27	26	0	0	0	0	0	0	1	0												
14	Cuscus (Hatu, Ambon island) brown, linear black	60	27	27	26	0	0	0	0	0	0	1	0	0											
15	Cuscus (Kariu, Haruku island) brown, linear black 2	60	27	27	26	0	0	0	0	0	0	1	0	0	0										
16	Cuscus (Manipa island) Stein cuscus	103	92	92	94	88	88	88	88	88	88	89	88	88	88	88									
17	Cuscus (South, Seram island) spotted cuscus	101	90	90	92	88	88	88	88	88	88	89	88	88	88	88	18								
18	Cuscus (Allang, Ambon island) Stein cuscus	101	90	90	92	88	88	88	88	88	88	89	88	88	88	88	18	0							
19	Cuscus (Soya, Ambon island) spotted cuscus	101	90	90	92	88	88	88	88	88	88	89	88	88	88	88	18	0	0						
20	Cuscus (Nabire island) spotted cuscus1	101	90	90	92	88	88	88	88	88	88	89	88	88	88	88	18	0	0	0					
21	Cuscus (Topo, Nabire island) spotted cuscus	103	94	94	96	91	91	91	91	91	91	92	91	91	91	91	18	22	22	22	22				
22	Cuscus (Nabire island) spotted cuscus 2	103	94	94	96	91	91	91	91	91	91	92	91	91	91	91	18	22	22	22	22	0			
23	Cuscus (Jayapura island) spotted cuscus	104	95	95	97	92	92	92	92	92	93	92	92	92	92	19	23	23	23	23	1	1			
24	<i>Spilocuscus maculatus</i>	101	88	88	90	86	86	86	86	86	87	86	86	86	86	6	18	18	18	18	16	16	17		

## DISCUSSION

**Phylogenetic relationships within Phalangeridae:** The cuscuses (the Phalanger and Spilocuscus genera) comprise a group of colorful, medium sized, arboreal frugivore-folivores endemic to tropical forests in the Australia-Papuan region (Helgen and Flannery, 2004). The distribution of the genera includes the lowlands of New Guinea, tropical northeastern Australia and many Melanesian islands, including several New Guinean land bridge islands (Yapen, Misool, Salawati and the Aru Islands) and several oceanic islands near New Guinea, including the Bismarck Archipelago, Waigeo and Batanta, Biak-Supiori, Numfoor and the Kai Islands (Flannery, 1994). The range of one species of the genera (*Spilocuscus maculatus chrysorrhos*) also extends to the island of Salayer south of Sulawesi as a result of human sponsored introduction (George, 1987) and to the Central Moluccas (Seram, Ambon, Buru and the Banda Islands) where it might be either native or introduced (Flannery, 1995; Helgen, 2003).

Evolutionary relationships among Phalangeridae have been studied using both morphological characters and molecular techniques (Tate, 1945; George, 1982, 1987; Flannery *et al.*, 1987; Springer *et al.*, 1990). Flannery *et al.* (1987), notes the separated Phalangeridae into two subfamilies. The subfamily Phalangerinae was separated into two tribes: Trichosurini (Trichosurus, Wyulda and Strigocuscus) and Phalangerini (Phalanger and Spilocuscus). *Ailurops*

*ursinus* was placed into its own subfamily, Ailuropinae. However, the relationships of the Phalangeridae members to these two clades has been debated (Flannery *et al.*, 1987; Springer *et al.*, 1990; Norris, 1994).

The results showed cuscuses from Maluku and Papua are grouped into two groups, namely is Phalanger and Spilocuscus (Fig. 3). In the Phalanger genera, it appears that the cuscuses from Maluku and Papua has the closer genetic relationship with *P. vestitus* (strong support by bootstrap value 99%) from GeneBank. There are differences 26-28 nucleotides in Phalanger members from Maluku and Papua (Table 1), which in a different sub-group. Allegedly, that members of the Phalanger from Maluku and Papua still have a close genetic relationship or are derived from the same parent, although it has variations in cyt b gene sequences. Nucleotides variations in cyt b gene between Phalanger members from Maluku and Papua are the results of natural selection and adaptation due to changing environmental conditions due to the mutations (Brum *et al.*, 1994). This event allows the occurrence of spontaneous changes in DNA sequence at random in relation to the individual response to changing environmental conditions (Starr, 1990).

We find that spotted/white cuscus (*S. maculatus*) have different phenotype with black striped brown cuscus (*P. orientalis* northern common cuscus) but this study shows that the two are not including different species but the same species as in genotype was not found nucleotide diversity

(0 nt). According to the observation and conversation with respondents at the sampling locations, show that the qualitative character of young spotted/white cuscus (*S. maculatus*) equal to *P. orientalis* overall hair colors is brown with a black stripe from the head to the base of the tail but *S. maculatus* have change hairs color at maturity phase towards the predominantly white color while *P. orientalis* remains brown. Sexual dimorphism is clearly visible on spotted cuscus (McKay and Winter, 1989). Isaac and Johnson (2003), asserted *T. vulpecula* is a members Phalangeridae that can be used as a model to study the interaction between sexual dimorphism, the mating season, as well population density.

Steroid hormone plays an important role in the mechanism of sexual differentiation in the reproductive glands and the central nervous system of brushtail possum. Structural dimorphism responsible influence reproductive behavior of brushtail possum in adulthood (Gilmore, 2002). However, higher levels of aromatase activity during early development in male opossums have been reported. Sex differences have been identified in the localization of cholecystokinin-immunoreactive structures in the marsupial brain indicating that the expression of this neuropeptide is differentially regulated in each sex (Gilmore, 2002).

Results nucleotides alignments of Phalanger members from Maluku and Papua, was found 24 nucleotides distinguishing between the two populations (Fig. 4). Twenty four nucleotides distinguish can be used as a genetic marker among Phalanger members from Maluku and Papua, that is sites positions 28-(T/C), 49-(A/G), 57-(C/T), 123-(A/G), 126-(A/C), 175-(T/A), 189-(G/A), 265-(C/T), 312-(T/C), 351-(A/G), 396-(A/C), 399-(T/C), 429-(C/T), 453-(T/C), 525-(A/G), 544-(C/T), 618-(C/T), 640-(A/G), 677-(T/C), 685-(A/G), 701-(T/C), 704-(A/G) 711-(C/T) and 720-(C/T).

Phylogenetic tree (Fig. 3), asserted that the group Spilocuscus from Maluku and Papua have a genetic relationship with *Spilocuscus maculatus* (strong support by bootstrap value 99%) species from GeneBank. There are differences 18-23 nucleotides in members Spilocuscus from Maluku and Papua which is a factor distinguishing the two populations (Table 1), which in a different sub-group. Results alignments nucleotides of Spilocuscus members asserted that gray cuscus (Stein cuscus) origin Manipa island, has a high genetic difference when compared with Spilocuscus members from Maluku and Papua. The Stein cuscus origin Manipa have four sites position which is a factor distinguishing between two populations. Results nucleotides alignment of Spilocuscus members from Manipa compared with Maluku and Papua populations, was found four nucleotides distinguishing

between the two populations (Fig. 5), that is sites positions 186-(C/T), 444-(T/C), 640-(G/A) and site 699-(C/T). This event caused the reduction of genetic diversity in the population, one of the factors responsible are genetic drift. Genetic drift results a change in the genotype character of gene pool, due process of selective segregation frequencies of alleles in the gametes that are hereditary (Brum *et al.*, 1994; Jones, 1997).

Results nucleotides alignment of Spilocuscus members from Maluku and Papua, was found 11 nucleotides distinguish between the two populations (Fig. 5) that is sites positions 30-(A/C), 39-(C/T), 62-(C/T), 108-(T/A), 114-(C/T), 265-(C/T), 294-(C/A), 461-(T/C), 492-(T/C), 502-(A/C) and site 690-(G/A). Eleven nucleotides distinguish can be using as a genetic marker among Spilocuscus members from Maluku and Papua.

The ground cuscus was originally classified in one of several species groups within the Phalanger genera. Tate (1945) grouped *P. gymnotis* (ground cuscus), with *P. orientalis* or *P. lullulae*, *P. vestitus* and *P. celebensis*. George (1982) placed *P. gymnotis* in a species group with *P. celebensis* and *P. ornatus*, split from the *P. orientalis* or *P. lullulae* group. George (1982, 1987) used the generic names Spilocuscus and Strigocuscus to split species groups from the Phalanger genera. George (1987), asserted *Strigocuscus celebensis* was separated at the generic level from *Phalanger gymnotis*. Flannery *et al.* (1987), revised the systematics of the Phalangeridae based analysis of 35 morphological characters as grouped *P. gymnotis* with Trichosurini. On the strength of their analysis, Flannery *et al.* (1987), asserted reclassification of the ground cuscus as *Strigocuscus gymnotis*, placing it with *Strigocuscus celebensis*, *S. ornatus* and *S. mimicus* in tribe Trichosurini. Springer *et al.* (1990), supported the grouping of *P. gymnotis* with *P. orientalis* and *P. vestitus*, to the exclusion of Spilocuscus genera. Kirsch *et al.* (1997), placed *P. gymnotis* within the Phalangerini clade. Flannery (1994, 1995), returned the *P. gymnotis* to the Phalanger genera in agreement with Springer *et al.* (1990). Hamilton and Springer (1999), asserted (bootstrap value of 99%) for the grouping of *P. gymnotis* with the (tribe Phalangerini including *Phalanger orientalis*, *P. lullulae*, *Spilocuscus maculatus* and *S. rufoninger*) to the exclusion of Trichosurus and the outgroups. The research results of Hamilton and Springer (1999), asserted there was also strong support (93%), *P. gymnotis* including monophyly of Spilocuscus genera.

Ruedas and Morales (2005), examined phalangerid relationships using mitochondrial 12S rRNA. Their results yielded support for an association of Ailurops, Strigocuscus, Phalanger and Spilocuscus to the exclusion of the trichosurin genera Trichosurus and Wyulda. Within the former clade, Ailurops grouped with Strigocuscus and Phalanger with

Cuscus (Moor, Nabire island) brown,linear black	ATG	TAC	AAC	CTA	CGC	AAA	ACT	CAC	CCA	CTC	ATA	AAA	ATC	ATC	AAC	[ 45]
Cuscus (Wanggar, Nabire island) gray,linear black	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Wanggar, Nabire island)brown, linear black	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Gorom, Seram island) whole white	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Geser, Seram island) brown, linear black	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Geser, Seram island)whole white	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (North, Seram island)whole white	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Lakor island) brown-northern common	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Allang, Ambon island) whole white	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Kariu, Haruku island) whole white	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Kariu, Haruku island) brown, linear black	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Larike, Ambon island) brown, linear black	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Hatu, Ambon island) brown, lineablack	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Kariu, Haruku island)brown, linear black 2	...	...	...	...	...	...	...	...	...	T	...	...	...	...	...	[ 45]
Cuscus (Moor, Nabire island) brown,linear black	AAA	GCC	TTT	ATT	GAC	CTA	CCA	ACA	CCA	TCC	AAC	ATC	TCT	GCC	TGA	[ 90]
Cuscus (Wanggar, Nabire island) gray,linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Wanggar, Nabire island)brown, linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Gorom, Seram island) whole white	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Geser, Seram island) brown, linear black	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Geser, Seram island)whole white	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (North, Seram island)whole white	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Lakor island) brown-northern common	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Allang, Ambon island) whole white	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Kariu, Haruku island) whole white	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Kariu, Haruku island) brown, linear black	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Larike, Ambon island) brown, linear black	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Hatu, Ambon island) brown, lineablack	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Kariu, Haruku island)brown, linear black 2	...	A	...	C	...	...	...	...	...	...	...	...	...	...	...	[ 90]
Cuscus (Moor, Nabire island) brown,linear black	TGA	AAC	TTC	GGT	TCA	CTA	CTA	GGC	TTC	TGC	CTG	ACC	ATC	CAA	ATC	[135]
Cuscus (Wanggar, Nabire island) gray,linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	[135]
Cuscus (Wanggar, Nabire island)brown, linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	[135]
Cuscus (Gorom, Seram island) whole white	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (Geser, Seram island) brown, linear black	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (Geser, Seram island)whole white	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (North, Seram island)whole white	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (Lakor island) brown-northern common	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (Allang, Ambon island) whole white	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (Kariu, Haruku island) whole white	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (Kariu, Haruku island) brown, linear black	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (Larike, Ambon island) brown, linear black	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (Hatu, Ambon island) brown, lineablack	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (Kariu, Haruku island)brown, linear black 2	...	...	...	...	...	...	...	...	...	...	...	A	A	...	...	[135]
Cuscus (Moor, Nabire island) brown,linear black	CTG	ACA	GGC	CTA	TTT	CTA	GCA	ATA	CAT	TAC	ACT	TCC	GAC	ACA	CTA	[180]
Cuscus (Wanggar, Nabire island) gray,linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Wanggar, Nabire island)brown, linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Gorom, Seram island) whole white	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Geser, Seram island) brown, linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Geser, Seram island)whole white	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (North, Seram island)whole white	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Lakor island) brown-northern common	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Allang, Ambon island) whole white	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Kariu, Haruku island) whole white	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Kariu, Haruku island) brown, linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Larike, Ambon island) brown, linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Hatu, Ambon island) brown, lineablack	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Kariu, Haruku island)brown, linear black 2	...	...	...	...	...	...	...	...	...	...	...	...	...	T	...	[180]
Cuscus (Moor, Nabire island) brown,linear black	GGC	TGA	CTA	ATC	CGC	AAC	CTA	CAC	GCC	AAC	GGG	GCC	TCC	TTA	TTC	[270]
Cuscus (Wanggar, Nabire island) gray,linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Wanggar, Nabire island)brown, linear black	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Gorom, Seram island) whole white	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Geser, Seram island) brown, linear black	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Geser, Seram island)whole white	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (North, Seram island)whole white	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Lakor island) brown-northern common	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Allang, Ambon island) whole white	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Kariu, Haruku island) whole white	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Kariu, Haruku island) brown, linear black	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Larike, Ambon island) brown, linear black	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Hatu, Ambon island) brown, lineablack	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]
Cuscus (Kariu, Haruku island)brown, linear black 2	...	...	G	...	...	...	...	...	...	...	...	...	...	...	...	[270]

Fig. 4: Continue





Cuscus (Moor, Nabire island) brown,linear black	GGA GGC TTC TCC GTC GAC AAA GCC ACA CTG ACC CGA TTC TTC GCC	[540]
Cuscus (Wanggar, Nabire island) gray,linear black	...	[540]
Cuscus (Wanggar, Nabire island)brown, linear black	...	[540]
Cuscus (Gorom, Seram island) whole white	...	[540]
Cuscus (Geser, Seram island) brown, linear black	...	[540]
Cuscus (Geser, Seram island)whole white	...	[540]
Cuscus (North, Seram island)whole white	...	[540]
Cuscus (Lakor island) brown-northern common	...	[540]
Cuscus (Allang, Ambon island) whole white	...	[540]
Cuscus (Kariu, Haruku island) whole white	...	[540]
Cuscus (Kariu, Haruku island) brown, linear black	...	[540]
Cuscus (Larike, Ambon island) brown, linear black	...	[540]
Cuscus (Hatu, Ambon island) brown, linearblack	...	[540]
Cuscus (Kariu, Haruku island)brown, linear black 2	...	[540]
Cuscus (Moor, Nabire island) brown,linear black	CAC TTA CTC TTC CTA CAC GAA ACA GGA TCC AAT AAC CCC CTA GGA	[630]
Cuscus (Wanggar, Nabire island) gray,linear black	...	[630]
Cuscus (Wanggar, Nabire island)brown, linear black	...	[630]
Cuscus (Gorom, Seram island) whole white	...	[630]
Cuscus (Geser, Seram island) brown, linear black	...	[630]
Cuscus (Geser, Seram island)whole white	...	[630]
Cuscus (North, Seram island)whole white	...	[630]
Cuscus (Lakor island) brown-northern common	...	[630]
Cuscus (Allang, Ambon island) whole white	...	[630]
Cuscus (Kariu, Haruku island) whole white	...	[630]
Cuscus (Kariu, Haruku island) brown, linear black	...	[630]
Cuscus (Larike, Ambon island) brown, linear black	...	[630]
Cuscus (Hatu, Ambon island) brown, linearblack	...	[630]
Cuscus (Kariu, Haruku island)brown, linear black 2	...	[630]
Cuscus (Moor, Nabire island) brown,linear black	ATC AAC CCA GAC TCA GAC AAA ATC CCA TTC CAC CCA TAC TAC ACA	[675]
Cuscus (Wanggar, Nabire island) gray,linear black	...	[675]
Cuscus (Wanggar, Nabire island)brown, linear black	...	[675]
Cuscus (Gorom, Seram island) whole white	...	[675]
Cuscus (Geser, Seram island) brown, linear black	...	[675]
Cuscus (Geser, Seram island)whole white	...	[675]
Cuscus (North, Seram island)whole white	...	[675]
Cuscus (Lakor island) brown-northern common	...	[675]
Cuscus (Allang, Ambon island) whole white	...	[675]
Cuscus (Kariu, Haruku island) whole white	...	[675]
Cuscus (Kariu, Haruku island) brown, linear black	...	[675]
Cuscus (Larike, Ambon island) brown, linear black	...	[675]
Cuscus (Hatu, Ambon island) brown, linearblack	...	[675]
Cuscus (Kariu, Haruku island)brown, linear black 2	...	[675]
Cuscus (Moor, Nabire island) brown,linear black	ACC AAA GAC GCC CTA GGA CTA ATC CTC ATG CTA CTT ACC CTA CTT	[720]
Cuscus (Wanggar, Nabire island) gray,linear black	...	[720]
Cuscus (Wanggar, Nabire island)brown, linear black	...	[720]
Cuscus (Gorom, Seram island) whole white	...	[720]
Cuscus (Geser, Seram island) brown, linear black	...	[720]
Cuscus (Geser, Seram island)whole white	...	[720]
Cuscus (North, Seram island)whole white	...	[720]
Cuscus (Lakor island) brown-northern common	...	[720]
Cuscus (Allang, Ambon island) whole white	...	[720]
Cuscus (Kariu, Haruku island) whole white	...	[720]
Cuscus (Kariu, Haruku island) brown, linear black	...	[720]
Cuscus (Larike, Ambon island) brown, linear black	...	[720]
Cuscus (Hatu, Ambon island) brown, linearblack	...	[720]
Cuscus (Kariu, Haruku island)brown, linear black 2	...	[720]

Fig. 4: Alignments of nucleotides sequences of the cyt b gene of Phalanger genera from Maluku and Papua

Spilocuscus. Raterman *et al.* (2006), asserted strong phylogenetic support was found for both the *S. celebensis* sister taxon to *Ailurops* and the *S. celebensis*+*Ailurops* sister to Phalangerini relationships. Widayanti *et al.* (2015), the first authors to include three genera (*Ailurops* from Sulawesi and *Spilocuscus* and *Phalanger* from Maluku and Papua islands) in a molecular study, used mitochondrial 12S rRNA gene. Results nucleotides alignment was found 3 sites and 13 sites that can be used as genetic markers between *Spilocuscus* and

*Phalanger* members from Papua and Maluku and Widayanti *et al.* (2015), asserted three sites positions of *Spilocuscus* genera is 127-(G/A), 481-(C/T), 885-(T/C) and 13 sites positions of *Phalanger* genera is 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) and ke-876 (A/G). Widayanti *et al.* (2015), asserted cuscus from Manipa island have genetic relationship with *Spilocuscus maculatus* (94%) than *Phalanger* members.



**Species variation within population Phalangeridae and biogeography:**

Genetic drift occurs in all population size but it is affecting a small population, because small genetic composition that has a real impact on the gene pool (Jones, 1997; Brum *et al.*, 1994). Species variation within a population caused to geographical factors, natural selection, mutation, environmental conditions and or recombination of genes (Jones, 1997). Stilling (1996), asserted that the species variation in the population caused to geographical factors, generally show the same phenotype character but the genotype character of members in the population may change. Species variation in the population is strongly influenced spatial and temporal patterns, due to an impact on the percentage of genetic material that flow (gene flow), the type of distribution characteristics of the environment, as well as the level of natural selection against members of the population (Karr, 1990; Laurance, 1991).

Geologically, the Manipa island derived from the Antarctic plate which split from Gondwana continent so as to bring Australia, Papua, East Sulawesi, East Seram and Tanimbar to the North. In that period, has formed a primitive mammals, birds and plants Spermatophyta (Primack *et al.*, 1998). During the period of geological, Australia moved to the South so it is very close to a fragment of Southeast Asia, resulting in the displacement of mammals and plants Spermatophyta between them. This events led to typical mammalian Gondwana fragments carried by Southeast Asia and then spread into Laurasia, resulting in East Sulawesi, East Seram and Tanimbar of mixing various marsupials which are typical Gondwana (Darlington, 1966). Hall (1998), asserted reconstructing the biogeographical history of the Phalangeridae is complicated by the very intricate and poorly understood geological history of Southeast Asia. As a result, the interpretation of the biogeographic history of phalangerids that we provide can only be considered tentative. The molecular phylogenies presented in this paper are considered more reflective of the true evolutionary relationships of Phalangeridae than previous morphological analyses, as the molecular phylogenies show high levels of congruence between them versus what has been seen in the morphologically based phylogenies. Flannery *et al.* (1987), asserted, Phalanger and Spilocuscus diversified in the newly formed northern parts of New Guinea at early pliocene. Glover (1986), notes that cuscus species were transported to Timor islands as early as 6500 years ago. Flannery (1995), asserted that *Phalanger orientalis* may have been actively introduced to Seram, Buru, Sanana and the Kei Islands but the date of initial introduction remains unknown. The period when the spotted cuscus (*Spilocuscus maculatus*) reached

central Maluku and 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. Latinis (1996), implies that cuscus remains are present in archaeological contexts throughout eastern Indonesia and the New Guinea region, indicating their contribution to early subsistence diets.

## CONCLUSION

Sequent of mt-DNA cyt b gene can be used as a genetic marker for cuscuses from Maluku and Papua. Be found twenty four nucleotides distinguish of Phalanger genera and 11 nucleotides distinguish of Spilocuscus genera. We prove that Stein cuscus (*P. vestitus*) from Manipa closer to Spilocuscus maculatus than is Phalanger genera members.

## ACKNOWLEDGMENTS

The authors would like to give a thank you remark to the Directorate General of Higher Education (DGHE) Indonesia No. LPPM-UGM/366/LIT/2014, March 03, 2014 that has given financial support to execute this study and also to all people involved in the Livestock Department.

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